Development and applications of murine asthma models induced by grass pollen allergens: Modulation of the immune response by a toll-like receptor agonist

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Abstract

Allergic asthma is a chronic inflammatory disease, in which sensitisation occurs via the respiratory tract. It involves T-helper(Th)2-lymphocytes, mast cells and eosinophils, leading to physiological and structural changes in the lung, i.e. airway hyper responsiveness, restricted airflow and airway remodelling. The hygiene hypothesis points out that the absence of Th1-favouring signals, e.g. bacterial pathogen associated-molecular patterns (PAMPs), promotes allergic sensitisation. PAMPs are sensed by a variety of receptors of the innate and adaptive immune response, e.g. toll-like receptors (TLR). TLR2 is expressed on dendritic cells, which are believed to be one of the key players of initiating and maintaining immune responses. Furthermore, dendritic cells modulate immunity either to Th1- or Th2-responses, or induction of tolerance. *Mycoplasma*-induced immune responses are mediated via TLR2-heterodimers and prevent a subsequent allergic sensitisation.

In this thesis, mouse models of sensitisation towards Timothy grass pollen allergens were developed, which reflect the acute, sub-chronic and chronic phase of allergic asthma. Mechanisms were clarified which led to prevention or augmentation of allergic inflammation, airway hyper responsiveness and airway remodelling when the *Mycoplasma*-derived TLR-agonist BPPcysMPEG was administered locally. Furthermore, synergistic effects of a combination of the TLR-agonist and the Th1-cytokine interferon (IFN)-γ were investigated and compared to the standard therapeutic dexamethasone.

The experimental models developed in this thesis resemble clinical signs of human allergic asthma, the grass pollen allergen used is of clinical relevance, and chronic sensitisation via the respiratory tract and structural changes in the lung could be reflected. Additionally, dexamethasone treatment showed comparable effects as observed in patients.

It could be demonstrated, that acute-phase allergic asthma is prevented and therapeutically intervened by BPPcysMPEG/IFN-γ-treatment. Also a therapeutic intervention of the sub-chronic phase was sufficient to reduce asthma symptoms. In a model of chronic respiratory sensitisation, prevention of allergic airway inflammation and airway remodelling could be achieved by the combinatory treatment as well as solely with the TLR2-agonist.

These beneficial effects on allergic asthma seem to be unrelated to a Th2/Th1-shift or induction of tolerance, but rather to impacts on general T-cell function. This overall effect makes this pharmacological approach promising for the prevention and treatment of diseases related to disturbed T-cell function, as asthma and inflammatory bowel disease.

Keywords: Toll-like receptor agonists, murine asthma models, grass pollen allergens

Zusammenfassung

Das allergische Asthma bronchiale ist eine chronische entzündliche Erkrankung der Lunge, in der die Sensibilisierung über die Atemwege erfolgt. Das Zusammenspiel von T-Helfer-Typ-2(Th2)-Lymphozyten, eosinophilen Granulozyten und Mastzellen verursacht physiologische und strukturelle Veränderungen der Lunge, wie Atemwegshyperreagibilität, eingeschränkten Atemfluss und Umbau der Atemwege. Die Hygienehypothese bringt die allergische Sensibilisierung mit verminderten Th1-induzierenden Signalen, wie zum Beispiel bakteriellen pathogen-assoziierten molekularen Mustern (PAMP,) in Verbindung. Bakterielle PAMPs werden von einer Vielzahl von Rezeptoren der angeborenen und adaptiven Immunantwort erkannt, wie z.B. Toll-like Rezeptoren (TLR). Der TLR2 ist auf Dendritischen Zellen exprimiert, denen eine grundlegende Rolle in der Einleitung und Aufrechterhaltung von Immunantworten beigemessen wird. Desweiteren modulieren Dendritische Zellen die Immunantwort in Richtung Th1, Th2 oder Toleranz. Durch *Mycoplasmen* ausgelöste Immunantworten werden über den TLR2 vermittelt und wirken präventiv auf eine nachfolgende Sensibilisierung.

In dieser Arbeit wurden Wiesen-Lieschgras-Allergen-induzierte Mausmodelle entwickelt, die sowohl die akute, die sub-chronische als auch die chronische Phase des Asthmas nachbilden. Wirkmechanismen einer lokalen präventiven oder therapeutischen Behandlung mit dem aus *Mycoplasma*-generierten TLR-Agonisten BPPcysMPEG auf die Erkrankung wurden beschrieben, synergistische Effekte der kombinatorischen Behandlung mit dem TLR-Agonisten und dem Th1-Zytokin Interferon(IFN)-γ untersucht und mit dem Standardtherapeutikum Dexamethason verglichen.

Die in dieser Arbeit entwickelten Mausmodelle weisen eine hohe Vergleichbarkeit mit dem allergischen Asthma des Menschen auf. Die Behandlung mit Dexamethason zeigte vergleichbare Wirkung wie im Patienten. Das verwendete Gräserpollenallergen besitzt hohe klinische Relevanz, und auch eine Sensibilisierung über die Atemwege konnte nachgestellt werden. Es konnte gezeigt werden, dass das allergische Asthma präventiv und therapeutisch mit BPPcysMPEG/IFN-γ beeinflusst werden kann.

Es gab klare Hinweise darauf, dass die positive Beeinflussung des allergischen Asthmas dabei nicht durch eine Verschiebung der Th2-Antwort in Richtung Th1 oder die Induktion von Toleranz verursacht werden, sondern eher durch Beeinflussung der T-Zell-Funktion. Der generelle Effekt weist die Behandlung als einen vielversprechenden neuen Ansatz der Prävention und Therapie von Erkrankungen aus, die mit fehlgeleiteter T-Zell-Funktion in Verbindung stehen, wie allergisches Asthma oder entzündliche Darmerkrankungen.

Schlagworte: Toll-like Rezeptoragonisten, murine Asthmamodelle, Graspollenallergene

Contents

1 Introduction

1.1 Allergic asthma bronchiale

If urgent action is not taken, the threat of deaths caused by asthma might increase by almost 20% in the next ten years [1]. Therefore, potent pharmaceuticals are needed. According to the World Health Organization, approximately 300 million humans worldwide suffer from allergic asthma. For the year 2005 255,000 deaths by asthma are estimated in WHO member states [1]. By now only few causative therapies exist. Most patients need a lifelong medicamentation, which influences the quality of life and causes enormous costs. Basically, an allergic vs. a non-allergic genesis of asthma is distinguished. In allergic asthma, one or several allergens can be verified, which cause inflammation of the airways. This is promoted by risk factors as atopy, existing allergic rhinitis or cigarette smoking [2].

In allergic asthma different clinical signs can be observed in patients, e.g. persistent eosinophilic inflammation, airway hyper reactivity towards allergens and unspecific stimuli, such as cold air or cigarette smoke, and structural changes in the airway architecture.

1.1.1 The hygiene hypothesis

The hygiene hypothesis relates the increasing prevalence and incidence of allergic diseases in the so called Western world with a cease in Th1-immune responses inducing triggers, e.g. bacterial infections [3, 4, 5]. Bacterial infections are reduced due to a less frequent direct confrontation to bacteria, but also indirectly due to a decrease in the number of siblings [6, 7]. Interestingly, farmers´children are much less likely to develop an allergic disorder during life than children from the same geographical region. This is again correlated to Th1-triggers in the direct environment [6].

The onset of allergic asthma is believed to be related to a perturbation in the Th1/Th2-balance. As a newborn, children possess a more Th2-characterised pattern of immune responses, and subsequent Th1-triggering might balance the immune system. Th1- and Th2-responses differ in their cytokine pattern: the characteristic cytokine in Th1-mediated responses, e.g. bacterial infections, is Interferon-γ (IFN-γ). The Th2-response is characterised by Interleukin(IL)-4, IL-5, and IL-13, leading to allergic inflammation [8].

1.2 Pathogenesis of allergic asthma - lessons from animal models

The clinical symptoms of allergic asthma are thoroughly investigated in patients, however, fundamental questions, e.g. about the disease onset and the role of cells and mediators involved, are mainly addressed in rodent models. These investigations broadened the current knowledge about the patho-mechanisms that result in allergic asthma.

1.2.1 Sensitisation

Dendritic cells (DCs) are understood to be key players in immune responses as allergic asthma, both in sensitisation as well as in maintenance of the disease [9]. As a prerequisite, DCs need to be located at the site of the first contact to the allergen, i.e. in the bronchial mucosa [10]. At this site, the allergen is incorporated, and afterwards processed by DCs for initiating the sensitisation cascade.

After migration to the draining lymph nodes, the peptides of allergens are presented via DC major histocompatibility complex (MHC) II to allergen specific T- and B-lymphocytes. At this point, the DC importantly influences the immune response by the second signal provided to the lymphocytes. Lung DCs seem to favour a Th2 response supposably by delivering CD80/CD86-signals [11, 12], however, a simultaneous secretion of e.g. IL-12p70 by DCs will favour a Th1-response. This secretion of IL-12p70 by DCs can be induced e.g. by toll-like receptor ligands [13].

After activation and proliferation, the allergen-specific lymphocytes invade the lung. Allergic responses of the immune system are related to T helper cells type 2 (Th2)-

lymphocytes. These cells can be found in elevated numbers in the airways and lung tissue [14] and play a fundamental role in allergic asthma as underlined by depletion experiments [15]. Activated Th2-lymphocytes secrete cytokines as IL-4, IL-5 and IL-13. These mediators play a role in an altered neuronal control of glands in the submucosa leading to a highly increased mucus production and thereby airway obstruction [16]. IL-13 is also thought to play a role in the development of airway hyper responsiveness [17]. Importantly, due to the release of IL-4 by Th2-cells, an isotype switch towards IgE is induced in antibody-secreting B-lymphocytes (plasma cells). The allergen specific IgE produced by these cells subsequently binds to Fcε-receptors on mast cells in the lung. This process finalises the sensitisation and fulfils the prerequisite for allergic responses.

1.2.2 The effector phase of allergic asthma

In sensitised individuals, cross-linking of receptor-bound IgE after recontact to the allergen causes activation and degranulation of mast cells very rapidly. Mediators released, e.g. histamine and heparin, are responsible for clinical signs such as oedema of the bronchial mucosa and constriction of the airway musculature. Moreover, leukotrienes lead to an increased mucus production, smooth muscle contraction and invasion of inflammatory cells into the lung [18].
Furthermore, mast cells secrete a variety of cytokines:
IL-5 and granulocytes and macrophages colony-stimulating factor (GM-CSF) influence eosinophils, both during maturation in bone marrow and activation [19]. The role of IL-5 is controversially discussed: At least in mice, IL-5 plays a central role in asthma [20]. In patients, therapies directed against IL-5 in fact reduced allergic inflammation, however, airway hyper responsiveness was only moderately ameliorated [21, 22].
Other mast-cell released cytokines, e.g. IL-4 and IL-13, amplify the Th2-reaction and the expression of cell adhesion molecules (VCAM-1) [23], thereby promoting the migration of leukocytes into the lung tissue.

Three to six hours after re-contact with the allergen and lasting up to several days without treatment, narrowing and hyper reactivity of the airways, eosinophilia of blood, and profound eosinophilic inflammatory infiltrates in the lung tissue occur. This makes

eosinophils to a main effector cell in allergic asthma. Eosinophils are attracted to the lung due to chemokines as e.g. eotaxins [24]. They show proinflammatory effects through a multitude of pre-synthesised mediators such as major basic protein (MBP), eosinophilic cationic protein (ECP) and eosinophilic peroxidase (EPX). These mediators trigger the release of histamine by mast cells, and moreover, they are toxic for parasites as well as mammalian cells. Eosinophilic degranulation therefore causes severe tissue damage [25]. Additionally, eosinophil-mediators contribute to the constriction of the airways [16].

In summary, DCs play an important role in initiation and maintenance of the allergic response, while eosinophils and Th2-lymphocytes are main effector cells of allergic asthma.

1.2.3 Chronic allergic asthma

In addition to the patho-mechanisms for acute-phase allergic asthma, the chronic phase of allergic asthma is characterised by structural changes of the airway architecture, the so called airway remodelling. Moreover, several cells and mediators emerge or change their pattern of contribution to pathology.

These observations were made in chronic asthma models. Basically, these models use ovalbumin for intraperitoneal or subcutaneous sensitisation with or without adjuvant as for example alum to induce allergen-specific Th2-driven immune responses, followed by a period of up to six months of either intranasal [26] or aerosolised [27, 28] allergen challenge to provoke experimental asthma. But also transgenic mouse models exist: The induction of Th2-responses and OVA-specific T cells is circumvented in mice overexpressing the Th2-responses related transcription factor GATA3 in addition to T cells carrying an OVA-specific receptor [29].

Remodelling

As an important characteristic of chronic asthma, the so called airway remodelling can be reflected in animal models. Airway remodelling includes features as goblet-cell hyperplasia [30, 31, 32], epithelial shedding, mucus hypersecretion [33], subepithelial fibrosis [34], changes in the extracellular matrix, e.g. deposition of collagen and fibronectin, smooth muscle hypertrophy [32], but also remodelling of large pulmonary vessels [35]. Due to

anatomical differences to humans, remodelling is in mice more prominent in the main bronchus [32, 26, 36]. These structural changes even persist after cessation of allergen challenges, uncoupled from inflammation [36, 34, 32]. Additionally, mucins [31] and mucin genes, e.g. Muc5a [37], are found, contributing to airway obstruction.

The mechanisms inducing all this structural alterations are not clearly understood yet, although there are hints that several mediators contribute to these changes. Direct and indirect contribution of IL-13, IL-10 and especially transforming growth factor (TGF)-β to remodelling processes could be demonstrated [38, 32, 39, 37, 40, 41]. During chronification, elevated TGF-β levels can be measured in bronchoalveolar lavage (BALF) [42]. Under healthy conditions as well as in low-dose allergen models, epithelial cells are the main source for TGF-β [43], but in allergic asthma invading eosinophils become more important. TGF-β mediates pro-fibrotic [44] as well as anti-inflammatory effects, while on the one hand inducing proliferation of fibroblasts and myofibroblasts and synthesis of extracellular matrix proteins [45] and on the other hand suppressing lymphocytes [46].

Additionally, a class of endopeptidases, i.e. matrix metalloproteinases (MMPs) and their inhibitors named tissue inhibitors of matrix metalloproteinases (TIMP), plays an important role in the equilibrium of degradation and deposition of a variety of extracellular matrix components, e.g. collagens and gelatines. A perturbation of the MMP/TIMP ratio is believed to be responsible for a dysfunction of this equilibrium and thereby promotion of remodelling [47, 48].

Cells and mediators in chronic asthma

The known main effector cells of acute-phase allergic responses also contribute to the chronic phase, as CD4+ lymphocytes [49], eosinophils and mast cells.
Increasing numbers of mast cells can be found in chronically challenged mice [50, 31].

IL-5 and eotaxin play a pivotal role as anti-apoptotic and chemo-attractive mediators in eosinophilic inflammation. The role of IL-5 in remodelling is not clarified yet: on the one hand, studies demonstrate an induction of eosinophilia and remodelling [51] and enhancement of airway reactivity related to IL-5. On the other hand subepithelial fibrosis and epithelial hypertrophy seem to be unrelated to IL-5, thereby implicating a dissociation

of remodelling and reduction of lung function [52].

Eotaxin expression shows differences when acute and chronic states were compared. Solely eotaxin was not sufficient to attract eosinophils to the airways, but may modulate other attractants [39].

Eosinophils are a main source of fibrogenic mediators as for example MMPs and TGF-β and therefore contribute to remodelling [53, 54]. Eosinophil-deficient mice are protected from both, an increase in collagen deposition as well as in smooth muscle mass in their lungs [55].

Bleedings of chronically inflamed tissue can also promote fibrotic processes: Red blood cells influence fibroblasts proliferation and apoptosis, and induce TGF-β and MMP secretion [56, 57].

A generally inflammation-related cytokine, IL-6, can be found in chronic asthmatic states, where it amplifies subepithelial fibrosis, but plays on the other hand a dual role by its capacity to down-regulate chronic inflammation [58].

Airway hyper responsiveness (AHR)

Importantly, physiological parameters of chronic asthma can be induced and determined in mice, which reflect an airway hyper responsiveness (AHR) to unspecific stimuli [26, 31, 59]. Fibrotic processes, e.g. observed during remodelling, are supposed to contribute to reduced lung function [44]. Interestingly, during the chronic phase elevated levels of a classically Th1-related cytokine, i.e. IFN-γ, can be measured [32], which may contribute to chronic AHR [43]. For the occurrence of AHR in animal models maintained allergen challenges seem to be necessary.
Studies showed an (alleviated) perpetuated airway inflammation, while AHR was absent after cessation of challenges [27, 32]. Although AHR was also absent in animals chronically challenged with low-level allergen doses, it could rapidly be induced, when a single moderate dose was administered [28].

1.3 Pharmacological intervention

For the treatment of asthma mainly symptom-alleviating pharmaceutics are applied. As early as in 1881, phosphodiesterase inhibitors were discovered, and are nowadays one of the most frequently applied asthma pharmaceutics [60]. Inhibition of phosphodiesterase blocks the degradation of cyclic adenosine-monophosphate (cAMP), which results in relaxation of airway smooth muscle. Via enhancement of intracellular cAMP-levels, phosphodiesterase-inhibitors consequently act bronchodilatory [61]. Additionally, they antagonise the bronchoconstrictor adenosine and bear anti-inflammatory potential, especially synergistically with steroids [62].

The most important drugs in asthma therapy are β2-mimetics. Biologically, these compounds are related to stress mediators, i.e. adrenalin. As in fight-and-flight reactions, these substances show strong bronchodilatory effects by activating β2-adrenergic receptors on airway muscle, thereby causing a relaxation and facilitation of deep breathing. β2-mimetics are under suspicion to be responsible for an enhancement of airway hyper reactivity, however, this finding seems to be of minor clinical relevance [63].

Leukotrien receptor antagonists show anti-inflammatory as well as a broncho-dilatory effects. These are mediated via a replacement of the physiological cys-leukotrien (LT) 1-receptor ligands LTC4, LTE4 and LTD4. Especially LTD4 bears broncho-constrictive actions. The anti-inflammatory effects are related to effects on granulocyte migration [47, 64, 65, 66].

Corticosteroids, as dexamethasone, are prescribed frequently in severe allergic asthma. This is due to their widespread beneficial effects on a variety of patho-mechanisms. Dexamethasone is effective by inhibition of chemokine and Th2-cytokine transcription and also inhibits phospholipase A, which is the catalyst for the first step in arachadionic acid metabolism. As a result leukotriene and prostagladine synthesis is blocked, partly explaining the anti-inflammatory effects of dexamethasone.
Additionally, the accumulation of leukocytes in the lung tissue can be suppressed by administration of dexamethasone. [67, 68]. Also the clinical symptom of airway hyper responsiveness can be attenuated, this effect is achieved by applying higher doses, though [69].

The interest in new and, more importantly, causative treatments is huge because of the number of patients which are contraindicated or steroid non-responders. Especially the class of steroids lost their acceptance because of side effects which for example occurred in the treatment of skin diseases. Moreover, recent data implied that steroid treatment not only augments inflammation and hyperreactivity, but also suppresses beneficial regulatory immune responses as induction of regulatory T-cells [70].

The only causative treatment nowadays, the specific immune therapy (SIT), acts via this induction of regulatory T-cells. Here, continued confrontation to the allergen is performed, either by subcutaneous injections or as allergen extracts or tablets sublingually (sublingual immune therapy, SLIT). The specific immune therapy modulates the immune response: The allergen is incorporated by dendritic cells, which migrate to the draining lymph node. Here, epitopes are presented to allergen-specific lymphocytes, and a regulatory T-cell response is induced, characterised by IL-10 and TGF-β [71, 72].

However, not every allergy can be cured safely using SIT by now, although currently the missing allergens are intensively investigated. Moreover, polysensitisations might recommend a more general immuno-modulation. Importantly, such general immuno-modulators, as for example toll-like receptor agonists, could also be effective drugs for asthma and allergy prevention.

1.4 Agonisation of toll-like receptors as an innovative pharmacological approach

Since a human homologue to drosophila toll-receptor had been firstly described [73], several members of the TLR-family has been discovered. Toll-like receptors play an important role in innate and adaptive immunity and in balancing immune responses with tolerance. Different pathogens are recognised by specific patterns (pathogen-associated molecular patterns, PAMPs), e.g. the TLR-5 ligand flagellin [74], lipopolysaccharide (LPS) of gram-negative bacteria detected by TLR-4 [75], or CpG-unmethylated DNA oligodesoxynucleotides binding to TLR-9 [76].

Immunostimulatory oligonucleotides as TLR9-ligating CpGs are promising innovative

approaches in the therapy of asthma. They showed strong immunomodulatory capacity in mice and are currently investigated in humans [77, 78]. But also TLR2 is related to protection against allergies and allergic asthma by sensing PAMPs as mycobacterial lipoproteins and lipopeptides. Interaction of host cells, preferentially antigen presenting cells like dendritic cells (DC), with these ligands basically bears the potential to modulate immune responses.

1.4.1 TLR2 in prevention of allergic asthma

Formulation of the hygiene hypothesis pointed out an inverse association of microbial load and Th2 disorders [79, 6]. Additionally, genetic variations in TLR2, but not in TLR4, which senses endotoxins [80], seem to be responsible for an observed protection of farmers' children from allergy and asthma [4]. It might be of special importance to start these protective actions already during pregnancy, when prenatal exposure to farm stables upregulates TLR expression of neonatal cells [81]. Moreover, smoking during pregnancy attenuates TLR-mediated immune responses, possibly increasing the risk for the offspring to develop allergies and asthma [82].

1.4.2 Cellular TLR2 expression

TLR2 is expressed on a variety of cells, both structural as well as immune cells, in humans and rodents as there are neutrophils [83], small airway epithelial cells as well as airway smooth muscle cells [84, 85], tracheal muscle layer [86], monocytes [87], macrophages [88], murine bone-marrow derived mast cells [89], and B cells [90, 91]. Its expression is inducible by TNF-α and IFN-γ. Very importantly, TLR2 is also expressed on DCs, which are due to their importance in immunity thought to be good target cells for modulating immune responses [13, 92, 93].

TLR2 in general senses lipopeptides and lipoproteins, whereby different heterodimers recognise different structures: diacylated lipopeptides, e.g. macrophage-activating lipopeptide, 2kD (MALP-2) [94], require TLR2/6 [83, 95], whereas triacylated lipopeptides, e.g. Pam_3CysSK_4, are recognised by TLR2/1 [83] and lipoproteins by TLR2/4 [96]. Although

effects of TLR2 agonisation dependent on the age of the experimental animal, such a correlation is not observed in humans so far [88, 97].

1.4.3 Immune modulation in allergy and asthma mediated via TLR2

TLR2 agonisation bears the potential to both inhibit and promote development of immune responses and is therefore manifold in its implementation. Different examples demonstrate the various effects of TLR2 agonisation, either for shifting Th2 towards Th1 [98, 99], aggravating Th2 [100] or induction of tolerance [101].

Its high immunomodulatory capacity as an adjuvant is further emphasised in experimental vaccination against HIV and measles [102, 103, 104, 105]. This makes TLR2 agonisation a promising approach for pharmaceutical intervention. TLR2 expression and function is influenced by administration of steroids, e.g. dexamethasone [84]. Dexamethasone suppresses receptor expression, however, receptor-upregulation by cytokines as IFN-γ and TNF-α is amplified [84].

Mycoplasma infections prevent asthma, an effect which is partly dependent on the TLR2-IFN-γ-pathway [106]. This finding led to the development of small *Mycoplasma*-derived compounds for potential pharmacological intervention of allergic diseases.

1.4.4 BPPcysMPEG, a *Mycoplasma fermentans*-derived TLR2 agonist

A prerequisite for therapeutic purposes is the use of specific, pure and standardised PAMP-receptor agonists. One of the few PAMP-agonists today available that fulfil these criteria is the MALP-2 (*Mycoplasma fermentans*-related macrophage-activating lipopeptide, 2kD) derived bisacyloxypropylcystein-conjugate (BPPcysMPEG, patent-number WO 2004/009125 A2 Mühlradt/Morr).
BPPcysMPEG is a lipopeptide conjugate of the pathogen *Mycoplasma fermentans*, which binds a double fatty acid substituted cystein via a carboxyl group to a polymeric conjugate rest showing a high water solubility (fig. 1.1). Moreover, this conjugate rest is physiological compatible and not immunogenic. A contamination of BPPcysMPEG with endotoxins

is excluded to almost 100% due to its synthetic production, which means the risk for sepsis is negligible. For its precursor molecule MALP-2 the immunomodulatory effect of a treatment in combination with IFN-γ has previously been shown in an *in vitro* allergy model [107]. In addition, efficacy of MALP-2 was proven in an ovalbumin mouse model of acute-phase allergic asthma [98].

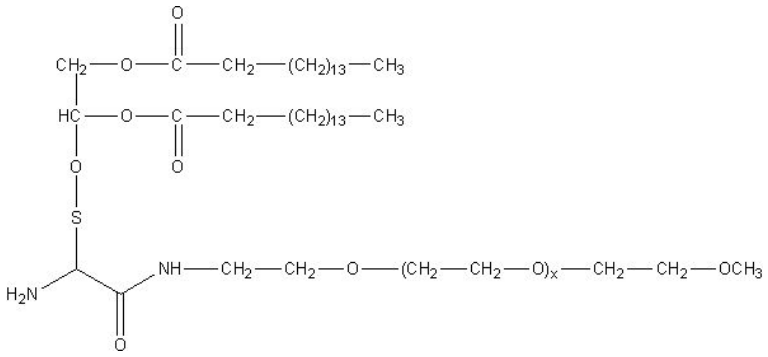

Figure 1.1: Structure of the toll-like receptor agonist BPPcysMPEG.

1.5 Improved mouse models of allergic asthma

1.5.1 Limitations of chronic models

Mouse models are useful tools to investigate the principal mechanisms involved in the development of allergic asthma and provide useful hints for the understanding of the disease and thereby possible intervention strategies. While studying acute-phase models, the contribution of a variety of cells and mediators to allergic asthma could be described, improving our current knowledge of the processes and mechanisms leading to this life threatening disorder. But at the same time differences to the observed symptoms in patients become more and more prominent, emphasising that allergic asthma is in fact a chronic disease.

Chronic models are useful tools in asthma research; however, they show some limitations when compared to human disease, which need to be improved.

One problem chronic models are facing is the resolution of airway eosinophilia [27, 43, 108], despite the fact that viability prolonging mediators for eosinophils are increasing

throughout the duration of the protocols. For example, synthesis of neurotrophins, which protect eosinophils of asthmatic patients from apoptosis [109], is upregulated by Th2-cytokines, IL-1β and TNF-α [110]. Additionally, T-lymphocytes seem to play a regulatory role in eosinophil viability. There is evidence that eosinophil apoptosis is mediated by Fas, and Fas-deficiency on T cells is sufficient to cause a long term allergic inflammation [111].

Another obstacle is the induction of tolerance and immunosuppression [112]. Tolerance in mice can be induced easily, which complicates to set up a model that replicates human pathology. Although tolerance is a naturally occurring and beneficial process in allergic diseases, the question remains what makes the immune system of allergic patients unable to cope with the disease.

1.5.2 Getting closer to human pathology

Currently little is known about differences in the immunologic reaction when the provocation route is altered, although there are some evidences from animal models that the pace of sensitisation is changed (topic vs. respiratory sensitisation in BN rats, reviewed in [113]). By the use of an adjuvant a rapid and intense immune response is induced, shortening the time of sensitisation and leading to severely inflamed tissues. Intraperitoneal sensitisations with an adjuvant might on the one hand amplify and quicken the sensitisation, but remain on the other hand incomparable to the human situation at all. In some models, the use of an adjuvant can influence the investigation strongly [114]. Recently, first models were described in which human relevant allergens could induce allergy symptoms by intranasal administration [115].

Moreover, a milder course of the disease might reflect the human pathology more closely. Besides the ability of developing characteristics of chronic asthma such as airway remodelling, an improved asthma model should provide similarity to the onset of the disease in humans including relevant allergens. Several groups worked on new models which use human relevant allergens either as recombinant proteins or extract preparations for induction of allergic inflammation. For example, allergens from cockroach [116], *Aspergillus fumigatus* [117] and house dust mite [30] in combination with adjuvant are under investigation. In these new and optimised animal models very distinct questions

can exclusively be investigated, for example from the field of desensitisation or specific immunotherapy.

1.5.3 Grass pollen allergens

In addition to moulds and faeces enzymes of house dust mite, a main source for allergens are grass pollen, e.g. of timothy grass *Phleum pratense*. Its major allergen 5 (Phl p5) is located cytoplasmic and on the so-called pollen starch granules (PSG) [118], which represent inspirable allergen carrying particles. Major allergen groups 1 and 5 are estimated to represent the most important allergen groups, whereby "almost 90% of grass pollen-allergic patients are sensitized against group 5 grass pollen allergens" [119]. These allergens show high immunogenicity in mice as well [120]. Meanwhile, recombinant Phl p5 as well as highly standardised allergen extract preparations can be purchased from commercial distributors, which allows the establishment of reliable and robust experimental protocols.

1.6 Aim of this work

Subject of this work was the development and validation of an optimised murine model of allergic asthma induced by Timothy grass pollen allergens, the characterisation of asthma parameters in this model and assessment of the effects of a potential new pharmacological intervention. The work includes the following steps:

1. Identification of susceptible mouse inbred lines
2. Establishment of a protocol to induce
   - acute allergic asthma
   - chronic allergic asthma
3. Assessment of effects of pharmaceutical interventions and immune modulation
   - in a *preventive* regime
   - in a *therapeutic* regime

   with the standard therapeutic dexamethasone and the TLR2-agonist BPPcysMPEG with/without the Th1-cytokine IFN-γ.

2 Material and methods

2.1 Standard techniques

2.1.1 Housing of experimental animals

Mice were housed under a LD12-12 cycle, constant humidity and temperature in groups of four to twelve mice per cage with *ad libitum* access to food and water. Microbiological and virological health monitoring was performed routinely according to FELASA guidelines. All animal experiments were performed with the permission of the local governmental representation according to the animal welfare law (TierSchG) of Lower Saxony. A minimum period of one week was allowed to acclimatise after transport from the breeders´ facility.

2.1.2 Intraperitoneal sensitisation towards recombinant Timothy grass pollen allergen

Materials:

Recombinant Phl p5	Biomay, Vienna, Austria
NaCl solution (0.9%)	
NaCl	Merck, Darmstadt, Germany
Aqua bidest	
ImjectAlum	Pierce (via Perbio Science, Bonn, Germany)
Timothy grass pollen extract	ALK Abello, Hørsholm, Denmark
Clean Bench	Integra Bioscience, Chur, Switzerland
Three-way valve	UNOPlast, (via VWR, Darmstadt, Germany)
Syringes (1 ml, 5 ml)	Braun, Melsungen, Germany

Cannulas	Braun, Melsungen, Germany
Pipette	Labsystem, Vantaa, Finland
Pipette tips	Biozym, Hess. Oldendorf, Germany
Inbred mice	Charles River, Sulzfeld, Germany
	Harlan Winkelmann, Borchen, Germany

Sensitisation was performed intraperitoneally with 5 μg recombinant Timothy grass pollen major allergen 5 (rPhl p5) and 1 mg of Alum as adjuvant in 200 μl sterile saline. The allergen was absorbed to the adjuvant by forcing the solution through a three-way valve for at least 5´. Sham sensitised animals received NaCl/Alum. Intraperitoneal sensitisations were performed on day 1 and repeated on day 14 and 21.

2.1.3 Chronic respiratory sensitisation towards standardised Timothy grass pollen allergen extract

Materials:

Timothy grass pollen extract	ALK Abello, Hørsholm, Denmark
PBS	Lonza Cambrex, Visp, Switzerland
Halothane (2-bromo-2-chloro-1,1,1-trifluoro-ethane)	Sigma Aldrich, Taufkirchen, Germany
Vapour system	Draeger, Hamburg, Germany
Pipette	Labsystem, Vantaa, Finland
Pipette tips	Biozym, Hess. Oldendorf, Germany
BALB/cAnCrl	Charles River, Sulzfeld, Germany

Female BALB/c aged 6-8 weeks were obtained from Charles River. For intranasal sensitisation, 50 μl Timothy grass pollen extract containing a dose of 9 μg major allergen was administered under inhalative 2-bromo-3-chloro-trifluoran narcosis in a vapour system twice a week on consecutive days. Negative controls received saline. Groups of animals were assessed at different time points.

2.1.4 Measurement of airway hyper responsiveness

Materials:

NaCl solution (0.9%)	
NaCl	Merck, Darmstadt, Germany
Aqua bidest	
MCh	Sigma Aldrich, Taufkirchen, Germany
Body for assessment of lung function	Medical School Hannover, Hannover, Germany
Pressure Transducer	Validyne Engineering Corp., Northridge, USA
Dry Cal DC-2	Bios International Corp., Butler, USA
Pneumotachographs	Hugo Sachs Electronic, March, Germany
Amplifier	Hugo Sachs Elektronik, March, Germany
Scantainer	Scanbur LTD, Karlslunde, Denmark
Parimaster aerosol generator	Pari (via Dr. Beckmann, Seefeld, Germany)
Pipette	Labsystem, Vantaa, Finland
Pipette tips	Biozym, Hess. Oldendorf, Germany
Latex "Dental Dam" collars	Roeko, Langenau, Germany
Hem 3.4 software	Notocord, Crossy-Sur-Seine, France

Airway hyper responsiveness to methacholine (MCh) was determined in non-anaesthetised spontaneously breathing animals using head-out bodyplethysmography 24 hours after the last allergen provocation. Baseline measurements were followed by provocation with increasing doses of MCh diluted in 0.9% NaCl. MCh aerosols were generated by a Pari Master aerosol generator. The respiratory signals were calculated from a pneumotachograph linked to a DP45-14 differential pressure transducer and amplified through a Carrier Frequency Bridge Amplifier. Flow dependent respiratory signals were transduced into lung function parameters and continuously registered. For determination of bronchoconstriction, the midexpiratory airflow (EF_{50}), i.e. the expiratory airflow (ml/s) at 50% tidal volume, was measured [121]. Respiratory signals were analysed using the Hem 3.4 system (figs. 2.1, 2.2).

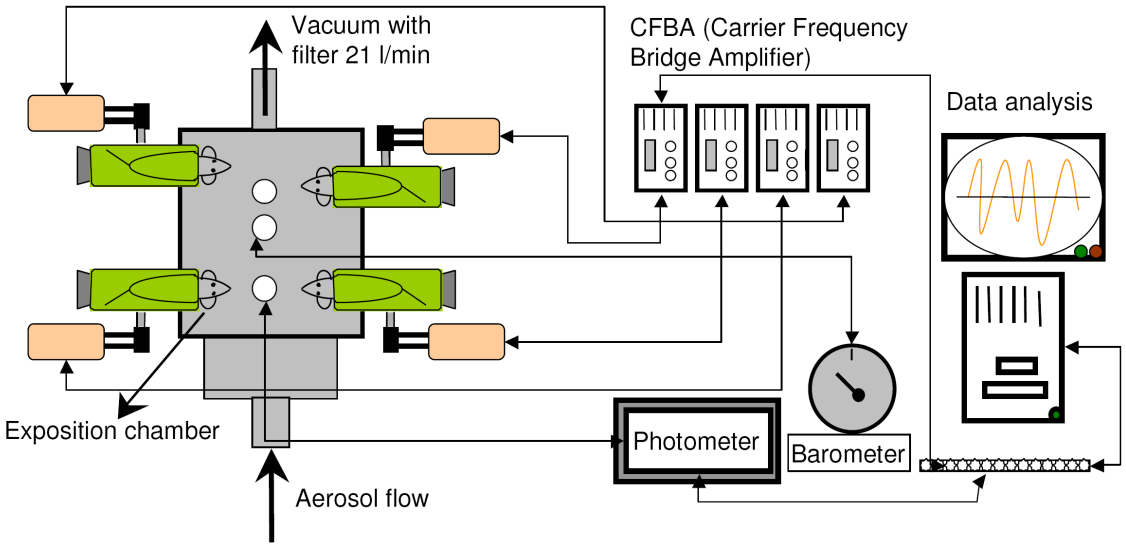

Figure 2.1: Schematic drawing of the head-out body plethysmography device.

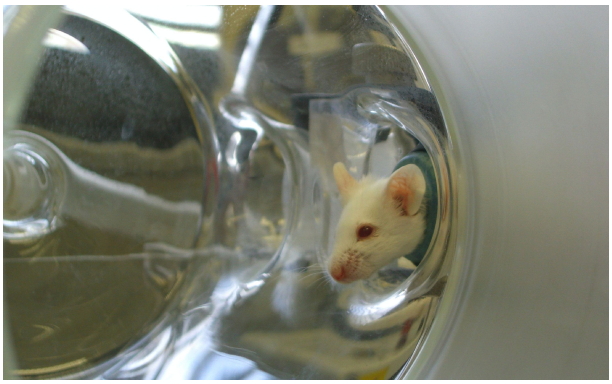

Figure 2.2: Photograph of mouse placed in head-out body plethysmography device.

2.1.5 Obtaining bronchoalveolar lavage fluid (BALF)

Materials:

PBS	Lonza Cambrex, Visp, Switzerland
Narcoren	Merial, Hallbergmoos, Germany
Pins and Scissors	VWR, Darmstadt, Germany
Permanent venous catheter (Abbocath)	Abbot GmbH & Co KG, Wiesbaden Germany
5 ml PP-tube	BD Falcon (via VWR, Darmstadt, Germany)
1 ml syringes	Braun, Melsungen, Germany
Cannulas	Braun, Melsungen, Germany
Ice bath	

Mice were sacrificed by injecting an overdose of Narcoren (1/5 diluted in PBS, 250 μl per mouse) and bronchoalveolar lavage (BAL) was performed.

The trachea was cannulated and the lung floated with 0.8 ml ice-cold PBS twice, followed by thoroughly winding up the syringe. BALF was pooled in 5 ml polypropylene tubes and immediately placed on ice until further processing.

2.1.6 Processing of BALF: Preparation of cytospins, differential cytology, preservation of supernatant

Materials:

Giemsa staining solution	Sigma Aldrich, Taufkirchen, Germany
May Grünwald staining solution	Sigma Aldrich, Taufkirchen, Germany
PBS	Lonza Cambrex, Visp, Switzerland
Cytofuge	Shandon GmbH, Frankfurt a.M., Germany
Centrifuge	Kendro, Langenselbold, Germany
ACT8 haematocytometer	Beckmann Coulter, München, Germany
Neubauer chamber	via VWR, Darmstadt, Germany
Microscope AxioVision2 plus	Zeiss, Jena, Germany
Pipette	Labsystem, Vantaa, Finland
Pipette tips	Biozym, Hess. Oldendorf, Germany
Glass slides	Menzel-Glaser, Braunschweig, Germany
Multitube storage box	Greiner, Nürtingen, Germany

BALF was centrifuged. Aliquots of the supernatant were stored at -70°C for protein quantification in ELISA. The cell pellet was resuspended in PBS and total cell counts were determined with either Neubauer chamber or ACT haematocytometer. Approximately 2×10^5 cells were cytospun on glass slides using a cytofuge. To quantify and qualify parameters of allergic inflammation cytospins were stained according to Pappenheim: 5´May-Grünwald, 2´ rinsed in PBS, 20´ in Giemsa (1/20 diluted in desalted water), rinsed in tab water and air dried. Differential cell counts of 500 cells / slide were performed on a microscope at 400-630x magnification due to morphological criteria (fig. 2.3). Eosinophils, neutrophils, lymphocytes and macrophages were assessed.

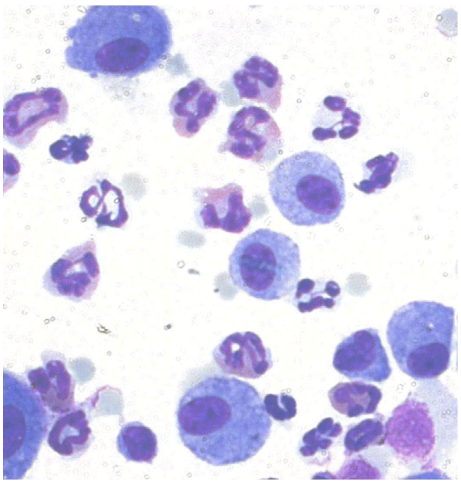

Figure 2.3: Example of a Pappenheim stained cytospin of BALF cells (allergic inflamed mouse). Four different cell types can be easily distinguished: macrophages (blue cytoplasm and nucleus, huge), lymphocytes (blue nucleus, only little blue cytoplasm, relatively small in size), neutrophils (polymorphic, segmented blue nucleus, clear to slightly blue cytoplasm), eosinophils (polymorphic, segmented nucleus, granula in the cytoplasm are stained red).

2.1.7 Plasma sampling

Materials:

EDTA 0.5 M	Lonza Cambrex, Visp, Switzerland
Centrifuge	Kendro, Langenselbold, Germany
1 ml syringes	Braun, Melsungen, Germany
Cannulas	Braun, Melsungen, Germany
Eppendorff tubes (2 ml)	Eppendorff, Hamburg, Germany

Mice were bled via the vena cava. EDTA-plasma was prepared by centrifugation at 15,000 g for 10´ and preserved for determination of total IgG_1 at -20°C.

2.1.8 FACS measurements of spleenocytes and lung draining lymph node cells

Materials:

RPMI + 25 mM HEPES	Gibco Invitrogen, Karlsruhe, Germany
FACS buffer	
5% FCS	Sigma Aldrich, Taufkirchen, Germany
5 mM EDTA	Merck, Darmstadt, Germany
PBS	Lonza Cambrex, Visp, Switzerland
FACS block buffer	
Normal goat serum	Dianova, Hamburg, Germany
1/25 in PBS	Lonza Cambrex, Visp, Switzerland
Haemolysis buffer (pH 7.3)	
8.29 g NH_4Cl	Merck, Darmstadt, Germany
0.037 g Na_2EDTA	Merck, Darmstadt, Germany
0.839 g $NaHCO_3$	Merck, Darmstadt, Germany
ad 1000 ml Aqua bidest	
Flowcheck	Beckmann Coulter, München, Germany
Antibodies	
CD45-FITC, foxp3-APC	eBioscience Natutec, Frankfurt a.M., Germany
B220-FITC	BD Bioscience, Heidelberg, Germany
CD11c-APC, CD4-PE	BD Pharmingen, Heidelberg, Germany
CD45-FITC	eBioscience Natutec, Frankfurt a.M., Germany
Rat $IgG2_a$-PE, Rat $IgG2_a$-FITC	Becton Dickinson Pharmingen, Heidelberg, Germany

Rat IgG2$_a$-APC	R&D bioscience, Wiesbaden-Nordenstadt, Germany
Armenian Hamster IgG-APC	Becton Dickinson Pharmingen, Heidelberg, Germany
7AAD	Beckman Coulter, München, Germany
Pipette	Labsystem, Vantaa, Finland
Pipette tips	Biozym, Hess. Oldendorf, Germany
Glass homogenator	via VWR, Darmstadt, Germany
Centrifuge	Kendro, Langenselbold, Germany
Cytomics FC500 flow cytometer	Beckman Coulter, München, Germany
5 ml PP-tube	BD Falcon (via VWR, Darmstadt, Germany)
ice bath	

Spleens were removed and rinsed with RPMI 1640 + 25 mM HEPES. Erythrocytes were lysed from the obtained cells suspension. All following steps were performed at 4°C or on ice, respectively. Approximately 10^5 spleenocytes as well as mediastinal lymph nodes cells obtained in a 2 ml glass homogenator of each individual were washed twice by adding 1 ml FACS buffer and subsequent centrifugation. Unspecific binding of antibodies was avoided by incubation with FACS block buffer for 20´. Cells were stained with the following antibodies and reagents: CD45-FITC / 7AAD for determination of vital leukocytes in the probe, B220-FITC and CD11c-APC to characterise professional antigen presenting cells (e.g. B-lymphocytes and DCs), and CD4-PE followed by intracellular staining of foxp3-APC for detection of regulatory T-lymphocytes, and appropriate isotype controls. After one additional washing step, cells were analysed on Cytomics FC500. Before measurement was started, the correct calibration of the FACS machine was controlled with FlowCheck according to the distributor´s instructions.

2.1.9 Histology and quantification of inflammatory and remodelling processes in the lung

Materials:

OCT kryotec	Sakura Finetek, Zoeterwoude, The Netherlands
PBS	Lonza Cambrex, Visp, Switzerland
Aqua bidest	
Liquid nitrogen	Linde, München, Germany
Giemsa staining solution	Sigma Aldrich, Taufkirchen, Germany
Azan novum according to Geidies	
Nuclear fast red	Merck, Darmstadt, Germany
China Blue	Merck, Darmstadt, Germany
Acid Orange 52	Sigma Aldrich, Taufkirchen, Germany
Wolframato phosphoric acid	Merck, Darmstadt, Germany
Aluminium sulphate solution	Fluka (via Sigma Aldrich, Taufkirchen, Germany)
Acetic acid	Roth, Karlsruhe, Germany
Isopropanol	Roth, Karlsruhe, Germany
Methanol	Merck, Darmstadt, Germany
Ethanol	Merck, Darmstadt, Germany
Xylol	Roth, Karlsruhe, Germany
Sircol Soluble Collagen Quantification Kit	Biocolor Ltd, Newtownabbey, UK
Permanent venous catheter (Abbocath)	Abbot GmbH & Co KG, Wiesbaden Germany
Eppendorff tubes (1.5 ml)	Eppendorff, Hamburg, Germany
Glass slides	Menzel-Glaser, Braunschweig, Germany
Cover slides	Menzel-Glaser, Braunschweig, Germany
1 ml syringes	Braun, Melsungen, Germany
Pipette	Labsystem, Vantaa, Finland
Pipette tips	Biozym, Hess. Oldendorf, Germany
Microscope AxioVision2 plus	Zeiss, Jena, Germany
AxioVision Digital Camera and software	Zeiss, Jena, Germany
Kryostat	Leica, Bensheim, Germany

Right lung lobes were filled with a 1/4 dilution of OCT kryotek in PBS instilled through the trachea. The lungs were removed and bedded in OCT to be immediately frozen in liquid nitrogen. 5 or 14 μm sagital sections displaying the main bronchus were prepared on a Kryostat and either Giemsa or Azan novum stained. To detect red dyed eosinophils in

tissue, Giemsa stained was performed by shortly dipping the 5 μm thick sample into water, followed by 1´May Grünwald , 1´ washing in PBS and 7´ in Giemsa 1/20 in distilled water solution. Afterwards, the slides were differentiated in methanol and ethanol / acetic acid, dehydrated 1´ in isopropanol and 3´xylol and covered with a cover slide. In Giemsa stained sections, the field of view (FOV) in 400x magnification was adjusted to the perivascular and peribronchial space. Eosinophils in this area were counted in three FOV per animal (n=3 per group) in the proximal, middle and distal part of the main bronchus.

For visualising remodelling processes, 14 μm sagital sections were Azan novum stained according to Geidies. This method stains collagen and reticular connective tissue intensively blue, mucous light blue, nuclei and musculature red and cell granula red to yellow. The solutions were prepared as follows:
0.1 - 0.2 g nuclear fast red was solved in 100 ml 5% aqueous aluminium sulphate solution by short boiling. Afterwards the solution was simmered 5´ and filtrated after cooling down. China Blue / Acid orange 52 mix: 0.5 g China Blue, 2 g Acid Orange 52 were solved in 100 ml Aqua bidest. Eight ml acetic acid were added, the solution shortly boiled and filtrated after cooling.

After 30´ in Nucleus fast red solution slices were washed in Aqua bidest. Pickling was performed for 10´ in 5% aqueous wolframato phosphoric acid, afterwards the slides were rinsed in Aqua bidest. Before additional washing, slides were stained 2´ in China blue / Acetic Orange 52 solution, dehydrated in isopropanol and xylol, and covered.

Photographs were taken at the distal part of the main bronchus with a digital camera connected to a microscope.

Scoring of remodelling and inflammation in lung tissue

Evaluation of histo-morphologic alterations was performed blind to the experimental groups. Lesions seen in the Giemsa-stained sections were scored for the overall severity from 0 to 3 (0 = no alterations, 1 = mild, 2 = moderate, 3 = severe lesions). The amount of inflammatory infiltrates was graded from 0 to 3 using the following criteria:

Very low amounts of inflammatory infiltrates near the primary bronchus were considered as background lesions and graded as 0, as these infiltrations are most common in older

mice. 1 = moderate amounts of inflammatory infiltrates surrounding the primary bronchus. 2 = moderate amounts of inflammatory infiltrates surrounding the primary bronchus accompanied by moderate amounts of inflammatory infiltrates surrounding medium sized bronchi and low amounts of inflammatory infiltrates surrounding small bronchi and vasculature. 3 = massive inflammatory infiltrates surrounding the main bronchus accompanied by massive amounts of inflammatory infiltrates surrounding medium and small bronchi and vasculature.

For the grading of the hyperplasia of the bronchial epithelium from 0 to 3, the following criteria were applied: 0 = normal bronchial epithelium. 1 = mild mucosal hyperplasia restricted to the primary bronchus. 2 = moderate mucosal hyperplasia affecting the primary bronchus as well as a few medium sized bronchi. 3 = severe hyperplasia affecting the main bronchus as well as the majority of medium sized bronchi.

Collagen quantification using Sircol Soluble Collagen Assay Kit

Preparation of lung samples started one day before measurement. Tissue was chopped in small pieces, transferred to a weighed Eppendorff reaction tube and protease inhibitor was added. Afterwards, the sample weight was measured. Samples were 1/10 diluted in pepsin extraction solution and incubated overnight at 4°C under agitation. The next day, samples were centrifuged 60´ at 15,000 g and dye solution was added. Collagen standard samples were prepared. Samples, standards and dye solution was incubated at room temperature for half an hour and centrifuged at 21,500 g. Supernatant was discarded and the pellet was resolved in alkali solution. Duplicates of samples, standards and blank were transferred to a 96-well plate and photometrically measured at 540 nm. Collagen content was calculated, expressed as promille wet weight. The kit contains the required solutions.

2.1.10 Enzyme-linked immuno sorbent assay (ELISA)

Materials:

2 N H_2SO_4	Sigma Aldrich, Taufkirchen, Germany
TMB One (enzyme substrate)	Biotrend, Cologne, Germany
Block Buffer	
FCS	Sigma Aldrich, Taufkirchen, Germany
BSA	Sigma Aldrich, Taufkirchen, Germany
Succrose	Merck, Darmstadt, Germany
PBS	Lonza Cambrex, Visp, Switzerland
Sodium nitrite (NaN_3)	Sigma Aldrich, Taufkirchen, Germany
ELISA Wash buffer	
PBS-Tween tablets	Medicago, Uppsala, Sweden
Aqua bidest	
MicroWell Plates Maxisorp	NUNC GmbH & Co KG, Wiesbaden, Germany
Adhesive seals	NUNC GmbH & Co KG, Wiesbaden, Germany
Combitips	Eppendorff, Hamburg, Germany
ELISA reader	Dynatech, Deisendorf, Germany
Pipette	Labsystem, Vantaa, Finland
Pipette tips	Biozym, Hess. Oldendorf, Germany
IgG_1-Quantification Kit	Bethyl Diagnostics Natutec, Frankfurt a.M., Germany
IL-5 and Eotaxin Duoset	R&D bioscience, Wiesbaden-Nordenstadt, Germany

ELISA is a standard method to quantify proteins. Basically, a target specific capture antibody is immobilised on a 96-well flat bottom plate and unspecific binding sites are blocked by proteins as FCS or BSA. Afterwards, samples as well as standards are incubated, followed by detection with a biotinylated detection antibody conjugated to horse-radish peroxidase. Enzymatic reactions transform the substrate TMB, the reaction is stopped by adding acid, the optical density is assessed photometrically and the protein concentration of the sample is calculated. In this work, ELISA kits were used following the distributors´ instructions. Measured values of duplicates showing a coefficient of variation below 0.2 were believed to be valid.

For determination of IgG_1 serum dilutions were incubated with capture antibody and detected with a 1/75,000 dilution of the secondary detection antibody. Enzyme reaction was stopped after 15´ by adding 2 N H_2SO_4. Both antibodies were taken from IgG_1

quantification kit.

2.1.11 Bioplex-array for bead-based protein quantification

Materials:

10-plex bead-array Kit (IL-2,-4, -5, -6, -10, -12p40, -12p70, TNF-α, IFN-γ, RANTES)	Biorad, München, Germany
Bioplex 200 system	Biorad, München, Germany
Pipette	Labsystem, Vantaa, Finland
Pipette tips	Biozym, Hess. Oldendorf, Germany

For detection and quantification of ten cytokines and chemokines using fluorescence-labelled micro beads, 10-plex bead-array was performed in BALF samples. Targets were IL-2, -4, -5, -6, -10, -12p40, -12p70, TNF-α, IFN-γ and RANTES. A minimum of 100 beads complexed with cytokines were assessed on Bioplex 200 System: the red laser identifies the bead and the green laser reads the fluorescence intensity of the reporter molecule. Afterwards, intensities are compared to a standard curve and proteins concentrations in the sample are calculated. The 10-plex bead-array was performed according to the manufacturer´s manual.

2.1.12 Statistical analysis

Materials:

Prism 4.03	Graph Pad Software Inc., San Diego, USA

To detect significant differences between groups, One-way ANOVA followed by Dunnet´s multi comparison test and Student´s t-test was applied using the Graph Pad Prism 4 software. Results of $p < 0.05\,(*)$, $p < 0.01\,(**)$ and $p < 0.001\,(***)$ were observed as statistically different.

2.2 Animal experiments

2.2.1 Development of a mouse model of acute-phase allergic asthma induced by grass-pollen allergens of Timothy grass

Materials:

Aqua bidest	
PBS	Lonza Cambrex, Visp, Switzerland
Timothy grass pollen extract	ALK Abello, Hørsholm, Denmark
Recombinant Phl p5	Biomay, Vienna, Austria
Timothy grass pollen	Allergon, Ängelholm, Sweden
Ketanest	Pfizer Pharma GmbH, Karlsruhe, Germany
Rompun	Bayer Healthcare AG, Monheim, Germany
Centrifuge	Kendro, Langenselbold, Germany
Clean Bench	Integra Bioscience, Chur, Switzerland
Analysis balance	Sartorius, Göttingen, Germany
Neubauer chamber	via VWR, Darmstadt, Germany
Microsprayer	Penn Centrury, Philadelphia, USA
Pipette	Labsystem, Vantaa, Finland
Pipette tips	Biozym, Hess. Oldendorf, Germany
Nylon mesh	via VWR, Darmstadt, Germany
For additional materials see	2.1.2, 2.1.5, 2.1.6.

Determination of the mouse strain

Table 2.1: Overview of the different mouse strains tested for susceptibility for sensitisation towards Timothy grass pollen allergens.

	Breeder	NEG n=	POS n=
A/JOlaHsd	Harlan, D	2	5
BALB/cAnCrl	Charles River, D	2	5
CBA/J	Charles River, D	2	5
C57/BL6	Charles River, D	2	5

To investigate whether inbred mice can be sensitised towards Timothy grass pollen allergens, a comparative study was performed. Mice of different inbred strains (tab. 2.1),

aged 6-8 weeks, were either sham-sensitised (n = 2) or sensitised (n = 5) on days 1, 14, and 21. Intranasal rPhl p5 provocations were performed with 10 μg protein in 50 μl sterile PBS on days 28 and 29. Monitoring of cellular inflammation in the lung was done by bronchoalveolar lavage on day 30 and differential cytology.

Determination of the allergen source and the mode of administration

To investigate which allergen source and which route of administration for provocation leads to allergic inflammation in the lung, a study was performed in which female A/JOlaHsd mice aged 6-8 weeks were sensitised or sham-sensitised towards rPhl p5 as mentioned above. Provocations were performed either with rPhl p5 i.n. or intratracheally administered as an aerosol using microsprayer technique, freshly isolated pollen starch granules of Timothy grass, or freshly prepared pollen extract from timothy grass pollen both administered intranasally under ketamine / rompun anaesthesia.

Isolation of pollen starch granules and extract was performed as follows: PSG were isolated under sterile conditions. Pollen grains were lysed by osmotic shock in Aqua bidest. Debris was separated by centrifugation and supernatant (i.e. extract) was filtered through a 3 μm mesh to obtain PSG. Purity of PSG was controlled using a scanning electron microscope (fig. 2.4). PSG were counted and adjusted to 8×10^8/ml in PBS to obtain an administered dose of 4×10^7 PSG in 50 μl PBS per confrontation.

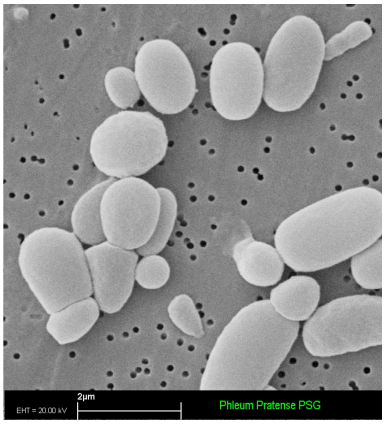

Figure 2.4: Electron scanning microscopic image of pollen starch granules obtained from pollen grains. (Friendly provided by Andrea Westendorff, Fraunhofer ITEM)

Confirmation of the protocol to induce acute-phase allergic asthma

Female 8 week old BALB/cAnNCrl were obtained from Charles River and sensitised towards rPhl p5/Alum (positive control, n = 10). Sham sensitised animals received NaCl/Alum (negative control, n = 10) (fig. 2.5). Sensitisation was repeated twice on days 14 and 21. To evoke allergic inflammation in the lung, mice were provoked intranasally on two consecutive days with standardised *P. pratense* extract containing 9 µg of major allergen on day 27 and 28. On day 29, airway hyper responsiveness was measured in head-out bodyplethysmography. Forty-eight hours after the last allergen challenge, mice were sacrificed and bronchoalveolar lavage (BAL) and differential cell counts were performed. BAL fluid was preserved for detection of cytokines using ELISA technique.

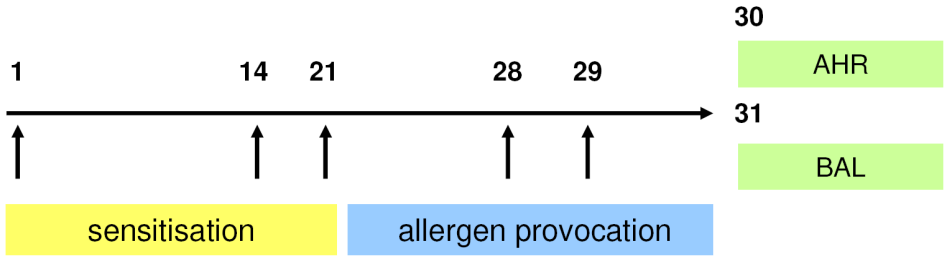

Figure 2.5: Protocol of acute-phase *P. pratense* induced allergic asthma. BALB/c were sensitised to rPhl p5 or NaCl/Alum as control. Allergic inflammation in the lungs was induced via i.n. challenges with *P. pratense* extract. Airway hyper reactivity (AHR) to methacholine was measured using head-out bodyplethysmography. BAL was performed 48 h after the last allergen challenge.

2.2.2 Intervention of acute-phase allergic asthma by a TLR2-agonist

Materials:

PBS	Lonza Cambrex, Visp, Switzerland
BPPcysMPEG, TLR-agonist	friendly provided by CA Guzman, Helmholtz Centre for Infection Research, Braunschweig, Germany
Murine IFN-γ	Strathmann Biotech, Hamburg, Germany
Dexamethasone	Ratiopharm, Ulm, Germany
Halothane (2-bromo-2-chloro-1,1,1-trifluoro-ethane)	Sigma Aldrich, Taufkirchen, Germany

Ketanest	Pfizer Pharma GmbH, Karlsruhe, Germany
Rompun	Bayer Healthcare AG, Monheim, Germany
Vapour system	Draeger, Hamburg, Germany
1 ml syringes	Braun, Melsungen, Germany
Cannulas	Braun, Melsungen, Germany
Pipette	Labsystem, Vantaa, Finland
Pipette tips	Biozym, Hess. Oldendorf, Germany
For additional materials see	2.1.2, 2.1.4, 2.1.5, 2.1.6.

Determination of TLR2-agonist BPPcysMPEG and IFN-γ treatment dose

To determine treatment doses of BPPcysMPEG and IFN-γ, dose finding studies were performed. Four groups of mice (BALB/cAnCrl, n = 5) were treated i.n. thrice with a pause of one week with either 20 ng ($\approx 6.67 \times 10^{19}$ mol), 10 ng ($\approx 3.33 \times 10^{19}$ mol), 4 ng ($\approx 1.67 \times 10^{19}$ mol) or 2 ng ($\approx 6.67 \times 10^{18}$ mol) BPPcysMPEG, respectively. Their condition and their body weight were monitored during the whole period. Twenty-four hours after the last treatment, animals were sacrificed and BALF was obtained. Cytospin preparations and differential cell counts were performed to investigate the inflammatory capacity of the treatment, i.e. induction of neutrophilia.

Prevention of acute-phase allergic asthma

To investigate asthma preventive effects of BPPcysMPEG/IFN-γ treatment, groups of 4 week old female BALB/cAnNCrl (n = 8) were treated thrice before the first and additionally before every recurrence of sensitisation leading to five times of treatment as follows: vehicle control (negative treatment control) i.n., dexamethasone i.p. (positive treatment control, 0.5 mg/kg body weight), 2 ng BPPcysMPEG i.n., 5×10^3 U IFN-γ i.n., 2 ng BPPcysPEG/5×10^3 U IFN-γ i.n (fig. 2.6). All treatments were performed under ketamine / rompun anaesthesia. Standard acute *P. pratense* model was performed as described earlier. Read-out, i.e. measurement of airway hyper responsiveness to methacholine using head-out bodyplethysmography and monitoring of cellular inflammation by performing a bronchoalveolar lavage was done 24 h after the last allergen provocation.

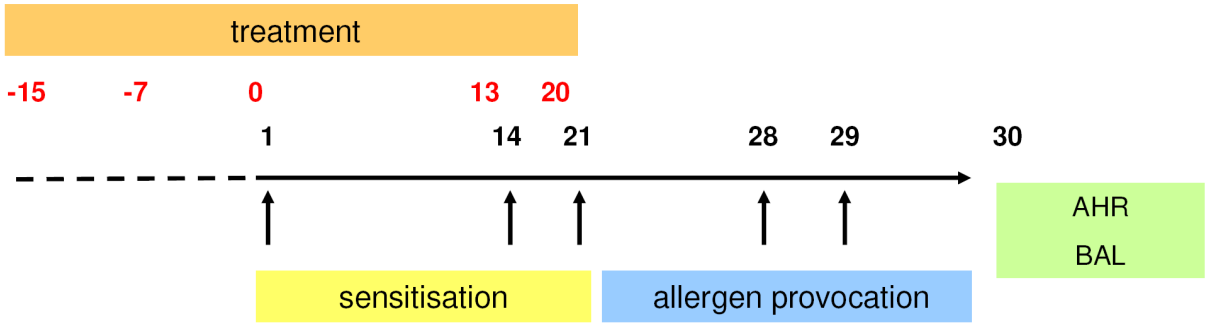

Figure 2.6: Preventive asthma treatment protocol in the acute *P. pratense* model. Groups of n = 8 BALB/c mice were treated with vehicle, dexamethasone, BPPcysMPEG, IFN-γ or BPPcysMPEG/IFN-γ, respectively, at indicated time points (red). All animals were sensitised against rPhl p5.

Therapeutic treatment of established acute-phase allergic asthma

To investigate therapeutic effects of BPPcysMPEG/IFN-γ treatment, groups of 6-8 week old female BALB/cAnNCrl (n = 8) were sensitised towards rPhl p5 and treated in a therapeutical setting as follows: vehicle control (negative treatment control) i.n., dexamethasone i.p. (positive treatment control, 0.5 mg/kg body weight), 2 ng BPPcysMPEG i.n., 5×10^3 U IFN-γ i.n., 2 ng BPPcysMPEG/5×10^3 U IFN-γ i.n (fig. 2.7). All treatments were performed under ketamine / rompun anaesthesia. Measurement of airway hyper responsiveness to methacholine using head-out bodyplethysmography and assessment of cellular inflammation by performing a bronchoalveolar lavage was done 24 h after the last allergen provocation. Supernatant of BALF was preserved for quantification of IL-5.

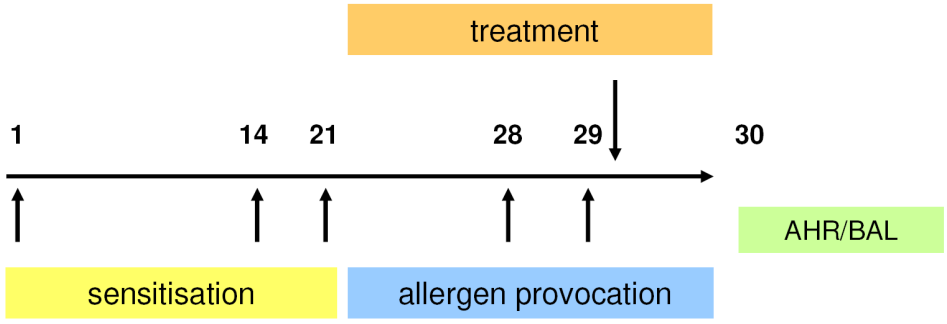

Figure 2.7: Therapeutic asthma treatment protocol in the acute *P. pratense* model.

Preventive treatment of sensitised animals before allergen provocation

This study was performed analogue to that of Weigt et al. [98]. To investigate whether allergic inflammation could be prevented by BPPcysMPEG/IFN-γ treatment, groups of 8 week old female BALB/cAnNCrl (n = 12) were treated before the first allergen provocation as follows: vehicle control (negative treatment control) i.n., dexamethasone i.p. (positive treatment control, 0.5 mg/kg body weight), 2 ng BPPcysMPEG i.n., 5×10^3 U IFN-γ i.n., 2 ng BPPcysMPEG/5×10^3 U IFN-γ i.n. (fig. 2.8). Treatment was performed under ketamine / rompun anaesthesia. Standard acute *P. pratense* model was performed as described earlier. Read-out, i.e. measurement of airway hyperresponsiveness to methacholine using head-out bodyplethysmography and monitoring of cellular inflammation by performing a bronchoalveolar lavage, was done 24 h after the last allergen provocation.

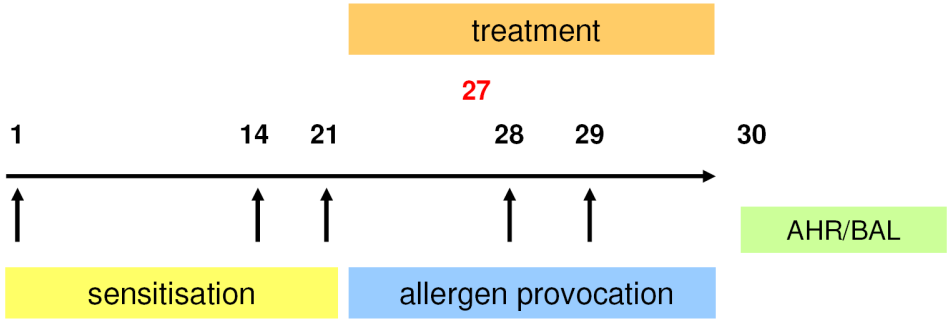

Figure 2.8: Preventive treatment protocol of allergic asthma in the acute *P. pratense* model. Groups of 12 mice were sensitised against rPhl p5 and treated with vehicle, dexamethasone, BPPcysMPEG, IFN-γ or BPPcysMPEG/IFN-γ, respectively, at day 27 (red).

2.2.3 Therapeutic intervention in sub-chronic asthma

The study included the groups summarised in tab. 2.2. Sampling was performed at three different time points (i.e., after 2, 10 and 22 allergen provocations). For the two earlier time points the group size was n = 8 each, for the latest time point 14 animals per group were assessed.

Table 2.2: Scheme on the different study groups.

	i.p. sensitisation	allergen provocation	Treatment
NEG	NaCl/Alum	NaCl	NaCl i.p.
POS	rPhl p5/Alum	*P. pratense* extract	NaCl i.p. & i.n.
DEX	rPhl p5/Alum	*P. pratense* extract	Dexamethasone i.p. & i.n.
B 2/I	rPhl p5/Alum	*P. pratense* extract	BPPcysMPEG/IFN-γ i.n.

To sensitise mice intraperitoneally towards grass pollen allergen, BALB/cAnNCrl aged 8 weeks were treated according to the above mentioned standard protocol (2.9). From day 28 on, allergen provocations were performed in all groups twice a week on two consecutive days using *P. pratense* extract (9 μg major allergen per mouse/treatment) under halothane narcosis. Negative control group received saline. Fifteen and two hours before every provocation, a group of 8-14 mice was treated with 0.5 mg/kg dexamethasone i.p., while positive and negative control group received saline injections. B 2/I-group received 2 ng BPPcysMPEG/5×10^3 IFN-γ one day after the second allergen provocation per week. Read-out parameters, i.e. measurement of AHR and sampling (preservation of BALF and serum and fixation of lung in formalin solution, Sigma Aldrich, Taufkirchen) were assessed after 2, 10 and 22 allergen provocations.

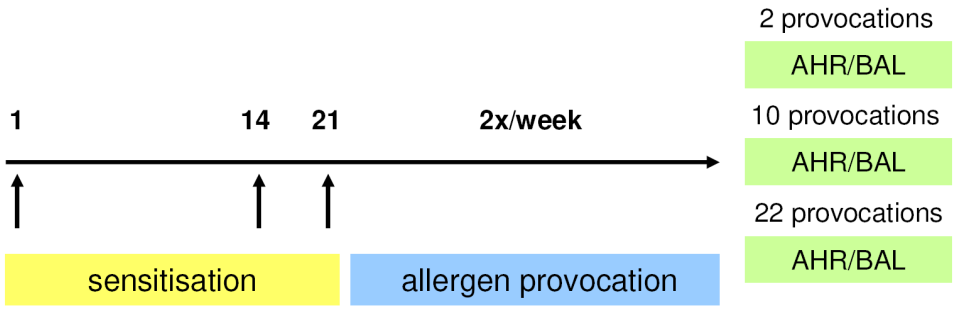

Figure 2.9: Protocol of sub-chronic *P. pratense* induced allergic asthma. BALB/c were i.p.-sensitised to rPhl p5 or NaCl/Alum as control. Dexamethasone treatment was performed 15 and 2 h before every allergen provocation. Airway hyperreactivity to methacholine was measured using head-out bodyplethysmography. BAL was performed 24 h after the last allergen challenge.

2.2.4 Chronic intranasal sensitisation to Timothy grass extract and modulation with a toll-like receptor agonist and/or IFN-γ

Materials:

For materials see 2.1.3, 2.1.4, 2.1.9, 2.1.10, 2.1.11, 2.2.2

Preventive intervention of chronic respiratory sensitisation towards Timothy grass pollen extract

Female BALB/c aged 6-8 weeks were obtained from Charles River and applied for the standard model of chronic respiratory sensitisation (fig. 2.10, section 2.1.3). Unless indicated, groups of n = 10 animals per treatment group were assessed after 30 intranasal provocations. To monitor the course of sensitisation and inflammatory responses, satellite groups of n = 10 were sacrificed after 10 and 22 provocations. Read-out parameters were measured, i.e. in BALF (cellular composition and cytokines/chemokines in supernatant using ELISA and Bioplex array), lung tissue (histology and quantification of inflammatory and remodelling processes), mediastinal lymph node and in spleen cells (FACS analysis).

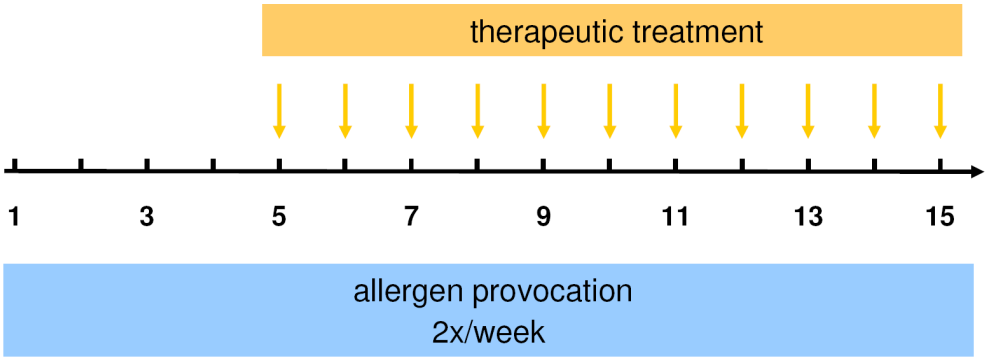

Figure 2.10: Preventive treatment protocol of in the model of chronic respiratory sensitisation.

Treatment

Treatments were performed during the phase of respiratory sensitisation, 8 h after the second weekly provocation intranasally starting after the tenth provocation. Sham-

treatment with saline was performed in negative and positive controls, while one group received 2 ng BBPcysMPEG/5 $\times$ 10^3 U IFN-γ (B2/I), 4 ng BBPcysMPEG/5 $\times$ 10^3 U IFN-γ (B4/I), 5 $\times$ 10^3 U IFN-γ (I), or 4 ng BPPcysMPEG (B4) in 50 μl saline, respectively. Positive treatment control received 0.5 mg/kg BW dexamethasone in 200 μl sterile saline intraperitoneally 18 and 2 h before each provocation.

3 Results

3.1 Development of an acute-phase asthma model induced by Timothy grass pollen allergens

3.1.1 Acute allergic inflammatory responses can be induced in different inbred mice strains

When applied to the standard intraperitoneal sensitisation protocol (fig. 2.5) and challenged with the recombinant major allergen 5 of Timothy grass (rPhl p5), the four tested inbred mice strains A/J, BALB/c, C57/BL6 and CBA/J showed elevated total cell counts, as well as eosinophil and lymphocyte numbers in BALF. At the same time, only weak neutrophilia was observed (fig. 3.1). Differential cytology on BALF cells therefore clearly demonstrates an allergic inflammation.

Two things were taken under advisement to conclude which inbred strain would be most suitable. Looking at the cellular composition of BALF, not only high eosinophil counts, but also elevated lymphocytes and an only light neutrophilia were believed to reflect human asthma closer. When the ratio of the different cell types towards each other was taken into consideration, susceptibility to the onset of allergic inflammation among the different strains was most prominent in A/J mice, followed by BALB/c, C57/BL6 and CBA/J. Additionally, for the development of a chronic model A/J and BALB/c seem to be the most suitable due to their ability of developing perpetual cellular inflammation [122]. Because the aim of this work was to establish both, an acute-phase and a chronic model of allergic inflammation, in the following experiments A/J and BALB/c mice were set into the focus of investigation.

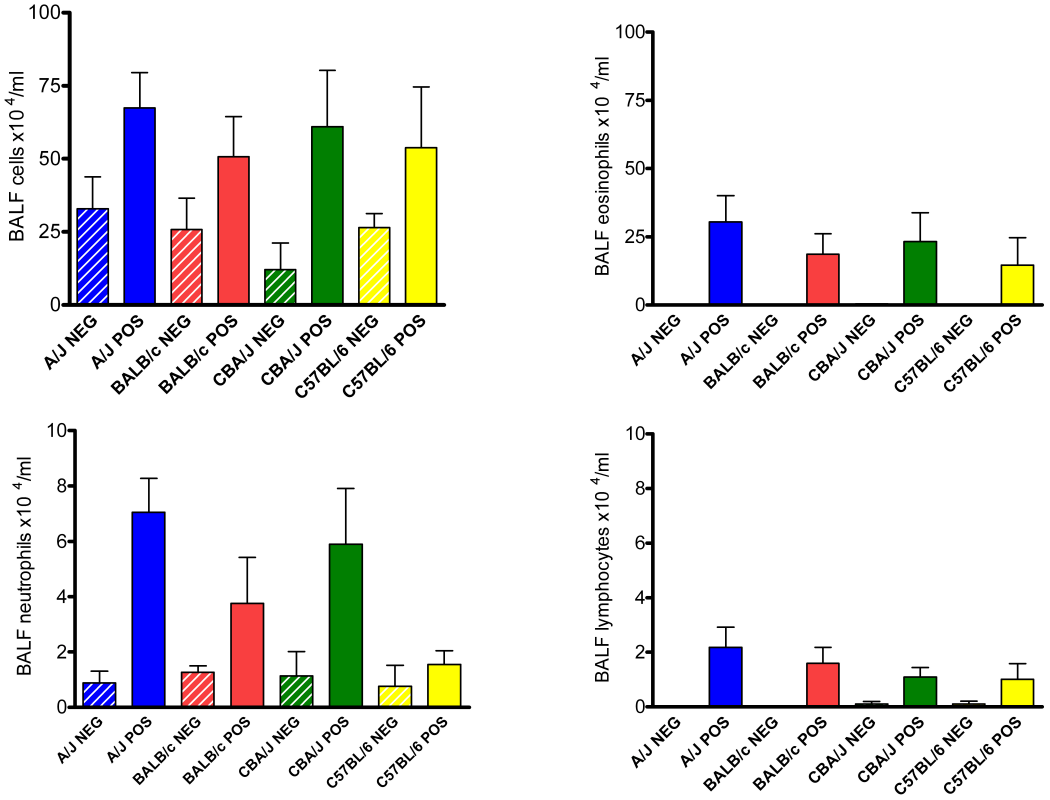

Figure 3.1: Differential cell counts in BALF in different inbred mouse strains. Mean and SEM are shown. Four different mouse strains were either sham-sensitised (n = 2) or sensitised (n = 5) towards rPhl p5/Alum and provocated with recombinant protein intranasally. Due to the small group sizes, no statistical significances could be detected.

3.1.2 Acute allergic inflammatory responses develop in sensitised mice after provocation with natural occurring allergen sources

To achieve a controlled sensitisation towards Timothy grass allergens, A/J mice were immunised by three rPhl p5/Alum injections within 21 days. Provocations with rPhl p5 were performed either intranasally or intratracheally as an aerosol. To resemble human pathology more closely regarding the induction of effector mechanisms of allergic asthma, sensitised mice were provocated with naturally occurring allergen sources, i.e. pollen starch granules and whole pollen grain extract containing proteins and lipids.

PSGs are believed to be the allergen carrying particle actually responsible for induction of allergic asthma in patients, because they are small enough to be inhaled into the deep

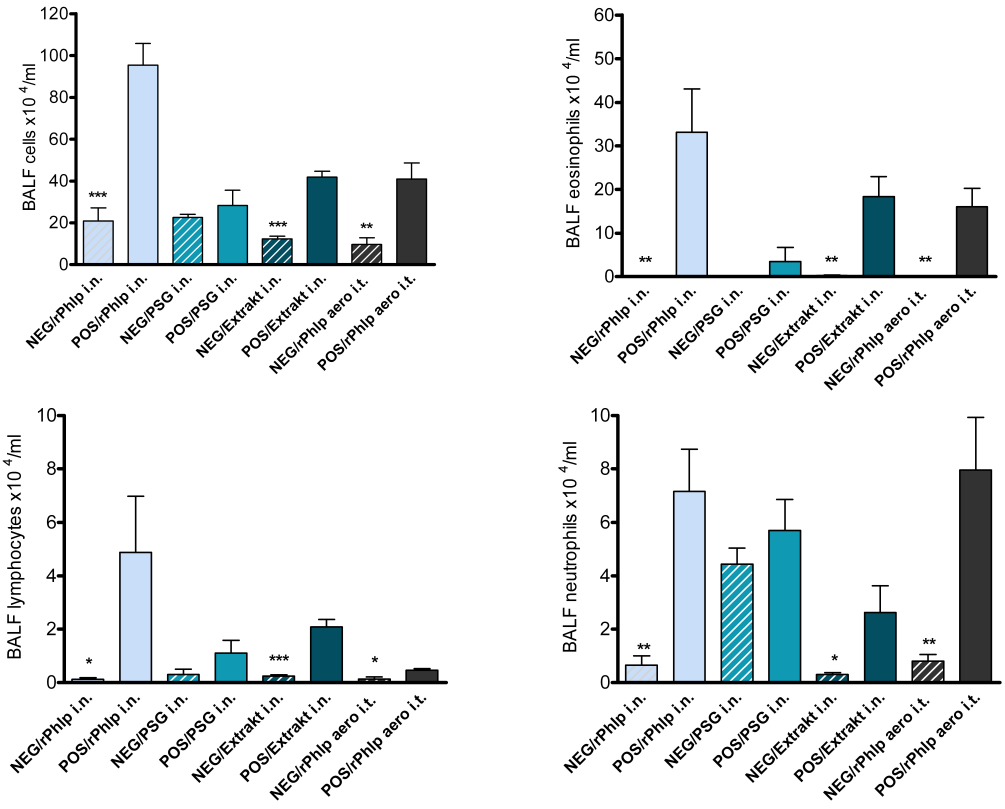

Figure 3.2: Differential cell counts in BALF after challenge with different allergen sources. Mean and SEM are shown. Two different application modi, i.e. intranasal and intratracheal administered aerosol, as well as three different Timothy grass pollen allergen sources, i.e. recombinant allergen rPhl p5, pollen starch granules (PSG) as well as pollen extract, were tested for provocation of acute allergic inflammation in A/J mice (NEG n = 5, POS n = 5).

airways. Also in experimental asthma, an intranasal PSG-provocation in sensitised mice increases eosinophil counts. However, soluble mediators of PSG preparations, supposedly endotoxins, seem to cause neutrophilia in sensitised, but also in sham-sensitised control animals. This excludes the use of PSGs in experimental models.

A clear induction of eosinophils in sensitised mice provocated with either the recombinant allergen or pollen extract could be detected (fig. 3.2). Interestingly, although intranasally administered recombinant protein was the most efficient to induce allergic inflammation, the BALF clearly showed elevated neutrophil counts, either when i.n. or i.t. administered. However, the induction of eosinophils in intratracheally aerosol challenged mice circumvents

the nose, and therefore excludes a limitation of the allergic processes to the upper parts of the airways.

An allergen confrontation does normally not happen with a single protein, but with a cocktail of substances. To improve the protocol e.g. towards standard ovalbumin-induced models with respect to resembling human pathogenesis, extract challenges seemed to be more advantageous. Moreover, in the following studies self-prepared extract could be substituted by highly standardised commercially distributed extract used for provocation diagnosis in humans.
However, measurement of airway hyper responsiveness proved to be unpractical due to abnormal reaction of A/J mice towards the methacholine aerosol. To enable assessment of lung function, BALB/c mice were chosen for all following experiments.

3.1.3 Acute-phase allergic asthma reflecting inflammatory as well as physiological parameters can be induced in mice

In mice sensitised to rPhl p5 and challenged with standardised extract intranasally (*see* 2.5), allergic inflammation as well as airway hyper responsiveness could be induced. Absolute cell counts in BAL fluid revealed significant higher numbers in sensitised BALB/c mice compared to sham sensitised animals (fig. 3.3). The counts of main effector cells in allergic response, i.e. eosinophils, were significantly elevated in sensitised animals, as were lymphocytes and to a moderate extent neutrophils. Measurement of IL-5 in BAL fluid showed significantly higher levels in sensitised mice compared to sham-sensitised mice (fig. 3.3).

Determination of airway hyper responsiveness was performed using head-out bodyplethysmography. The effective dose of methacholine (MCh) which caused a 50% decline in midexpiratory flow (EF_{50}) was calculated ($ED_{50}EF_{50}$) after sigmoid curve fitting of dose response curves for each individual. The positive control group reacted with a decline in airflow at lower doses of MCh compared to negative control, indicating a clear hyper reactivity of their lungs (fig. 3.4).

This new model of acute-phase allergic asthma induced by Timothy grass pollen allergens reflects important characteristics of human asthma. Also the allergen source used is of

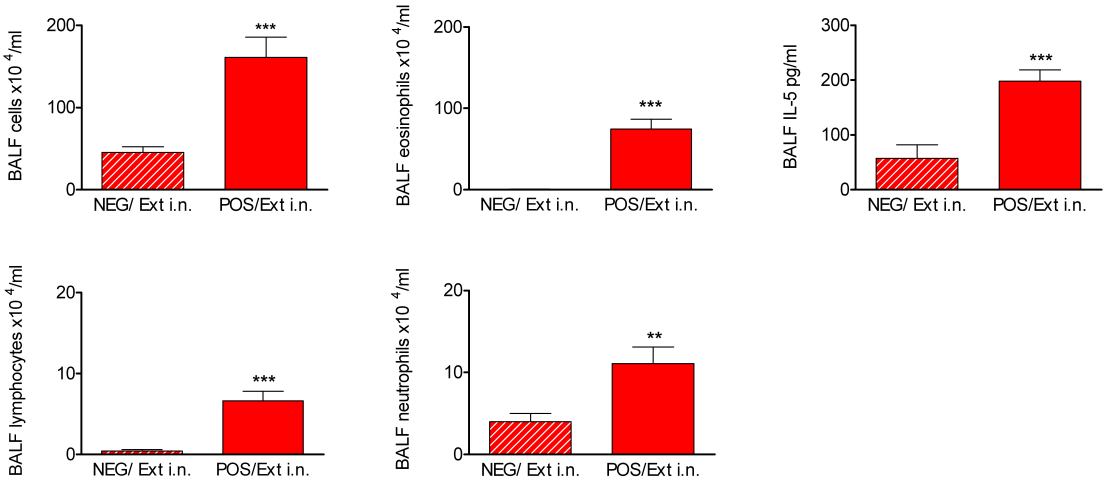

Figure 3.3: Differential cell counts and IL-5 in BALF in the acute model. Mean and SEM are shown. BALB/c were sensitised to rPhl p5 and challenged intranasally with standardised Timothy grass pollen allergen extract (n = 10 per group) according to the standard protocol.

important clinical relevance. Moreover, biological variances in allergenicity of the allergen preparations used are minimised as far as possible. Therefore, the reliable model can now be applied for efficacy tests of new pharmaceuticals, as e.g. TLR-ligands.

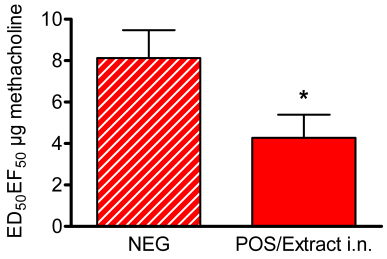

Figure 3.4: Effective dose of methacholine that decreases midexpiratory flow to 50% (determination of airway hyper responsiveness) in the acute model. Mean and SEM are shown. Positive control reacted at significant lower doses of methacholine with a decline of midexpiratory flow, clearly indicating an existing hyper responsiveness compared to negative control (n = 10 per group).

3.2 Development of a chronic model of allergic inflammation induced by Timothy grass pollen allergens

3.2.1 Intraperitoneal sensitisation followed by chronic allergen provocations causes transient severe allergic inflammation

Intraperitoneal sensitisation towards rPhl p5 followed by a prolonged period of repeated intranasal extract challenges (*see* 2.9) rapidly results in severe allergic inflammation. After eleven weeks of challenge (22 provocations) the allergic inflammation remains statistically significant elevated, although it is alleviated in BALF (fig. 3.5). Due to this reduction of cellular inflammation in positive controls at the last time point assessed, therapeutic effects of dexamethasone could not be detected after 22 provocations, although eosinophil counts in dexamethasone-treated animals remained stable over time.

Additionally, measurement of airway hyper responsiveness resulted in a surprising decline in $ED_{50}EF_{50}$ in sham-sensitised and -challenged controls (fig. 3.6). This might be due to chronic administrations of liquid into the lung, or, possibly more likely, a phenomenon of the ongoing aging of the animals. However, measurement of airway hyper responsiveness revealed no reliable differences between the groups at any time-point and could therefore not be established as a read-out parameter for this mouse model.
Remodelling processes are self-evidently occurring at the time point after 22 provocations (fig. 3.7), underlining that this important feature of the chronic phase of allergic asthma could be reflected.

However, the fact that a clear abrogation of eosinophilic inflammation by dexamethasone treatment could not be demonstrated at the latest time point, limits this model to reflecting a sub-chronic phase, i.e. ten provocations, in pharmacological intervention. At this time point, clearly elevated eosinophil and lymphocyte counts could be measured. Further on, dexamethasone showed the expected beneficial effects.

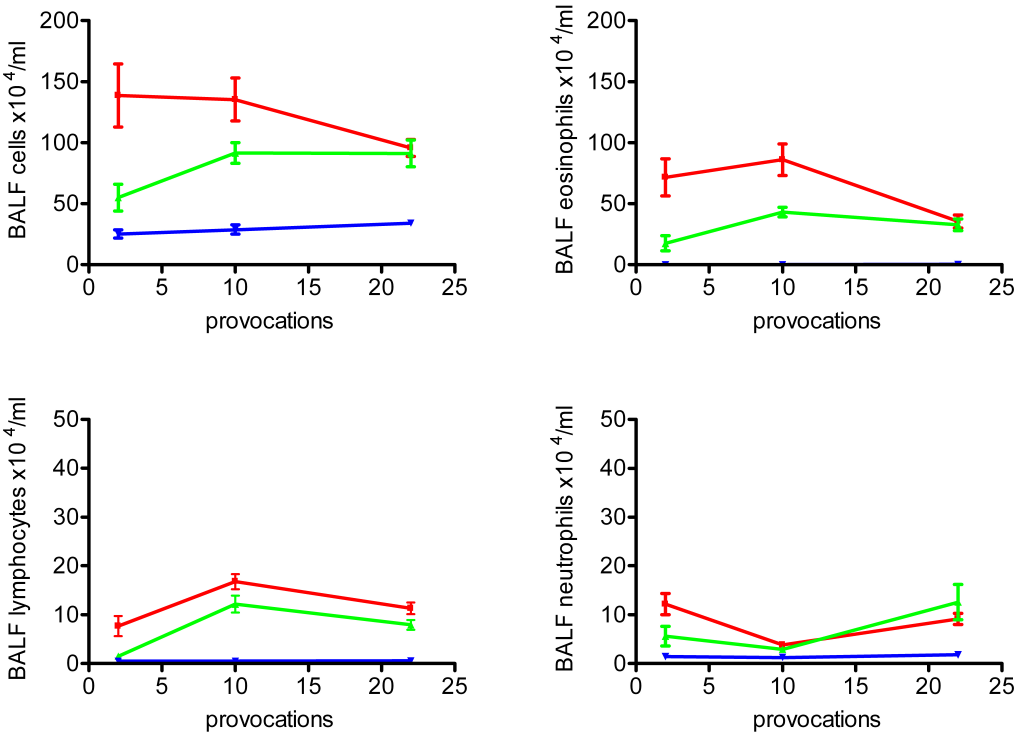

Figure 3.5: Differential cell counts in BAL fluid in the sub-chronic model. Time course over the duration of the study. Mean and SEM are displayed. Negative control group (NEG, blue line), i.p.-sensitised positive control group (POS, red line), i.p.-sensitised dexamethasone treated group (DEX, green line). Every group was assessed after 2 (n = 8), 10 (n = 8) and 22 (n = 14) provocations. Some data on this graph is also shown in figs. 3.15, 3.17.

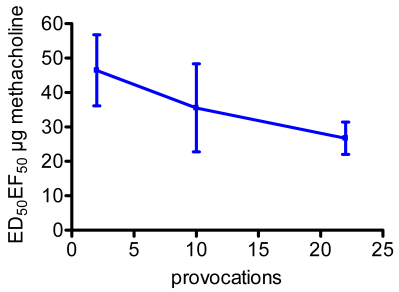

Figure 3.6: $ED_{50}EF_{50}$ (MCh) of i.p.-sensitised negative controls during continuation of the experiment. Mean/SEM of the effective dose of methacholine in μg, which causes a decrease of the midexpiratory airflow to 50% are depicted.

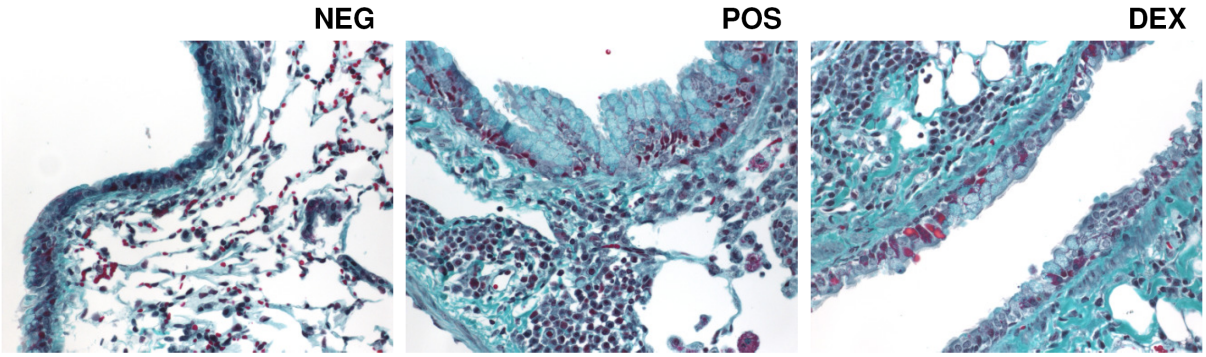

Figure 3.7: Histology on lung sections (Masson Goldner) of the chronic model using i.p.-sensitisations. Every group was assessed after 22 provocations. Masson Goldner stained sections on formalin-fixed paraffin-embedded lungs were provided by the Department of Pathology, Dr. Susanne Rittinghausen, Fraunhofer ITEM. Photographs were taken at 400x magnification at the distal part of the main bronchus. The results are as follows: Nuclei are stained brown-black, cytoplasm red, erythrocytes orange-yellow, connective tissue and sour mucus green.

3.2.2 Respiratory sensitisation to Timothy grass pollen allergens results in perpetuated allergic inflammation

When mice underwent the protocol of respiratory sensitisation (*see* 2.10), the results were as follows:

Throughout the protocol a prominent increasing allergic inflammation was induced, displayed by exaggerated total cell counts, eosinophil and lymphocyte numbers, and only moderate neutrophilia (fig. 3.8). This inflammation can be reduced to normal levels by dexamethasone treatment, as observed in patients. As in intraperitoneally sensitised mice, measurement of airway hyper responsiveness failed to result in a significant difference between intranasally sensitised and sham-sensitised animals (fig.3.9). However, the model of chronic respiratory sensitisation resembles human allergic airway inflammation closely regarding the allergen used, the route of sensitisation and the route of recurrent allergen confrontations, and in responses to standard treatments. Therefore, and because of the ability to reflect chronic eosinophilia, it is an important complement to common chronic asthma models.

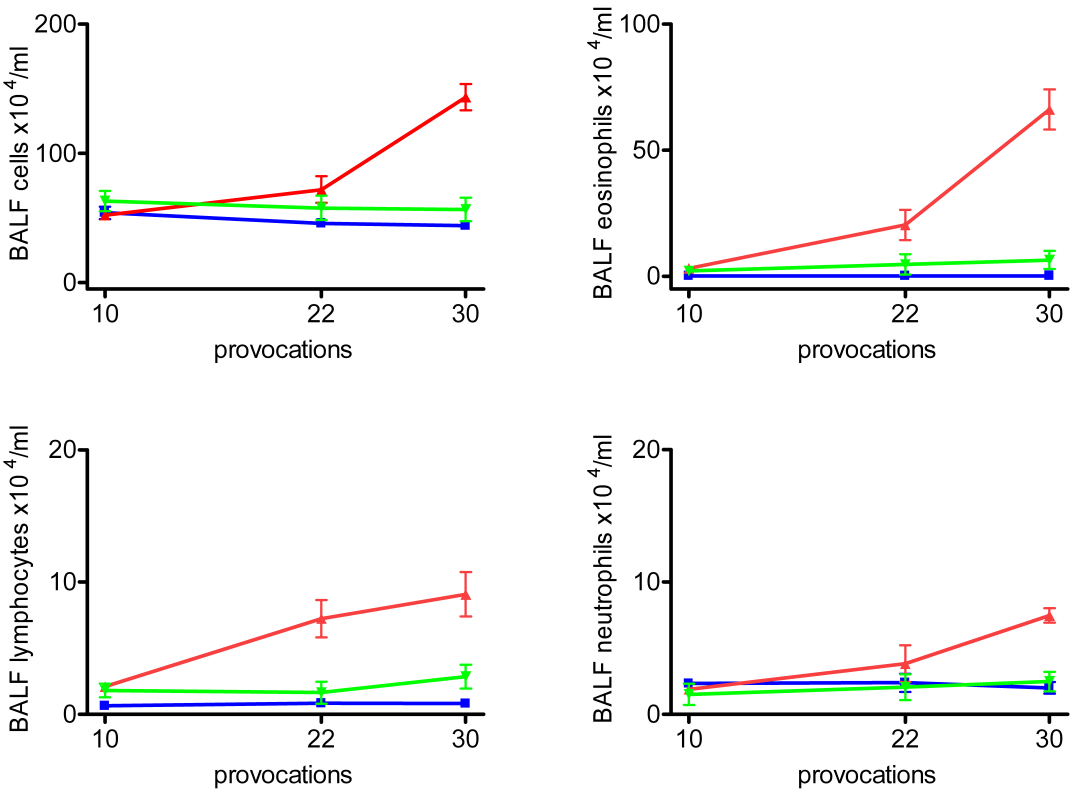

Figure 3.8: Differential cell counts in BALF in the model of chronic respiratory sensitisation. Time course over the duration of the study. Mean and SEM are displayed. Negative control group (blue line), sensitised positive control group (red line), sensitised dexamethasone treated group (green line). Every group was assessed after 10, 22 and 30 provocations. Some data on this graph is also shown in fig. 3.19.

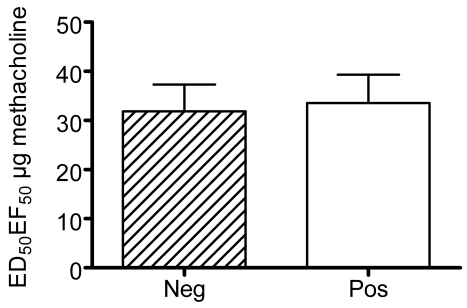

Figure 3.9: $ED_{50}EF_{50}$ methacholine in the model of chronic respiratory sensitisation. After 30 extract provocations, no difference between sham-sensitised and sensitised animals (n = 12, each) could be observed.

3.3 TLR2-agonists in intervention of allergic asthma

3.3.1 Titration of BPPcysMPEG and IFN-γ dose

Before BPPcysMPEG and IFN-γ could be administered for treatment of allergic asthma, dose finding studies were performed. First, different doses of IFN-γ were titrated against a constant dose of BPPcysMPEG, adapted from studies using the precursor molecule MALP-2 (fig.3.10). In a second step, the dose of BPPcysMPEG was titrated. Neither a decline in body weight, nor an influence on their general condition was observed in any treatment group. It has to be remarked that in the first set of dose finding-experiments, the groups receiving 10 ng and 20 ng of BPPcysMPEG/IFN-γ showed side effects in behaviour in terms of exaggerated locomotor and aggressive behaviour. When the experiment was repeated, these side effects did not occur any longer; the causative relation of this observation to the treatment could not be proven. However, the work was continued with the two lower doses, keeping in mind that repeated administrations would be needed in the sub-chronic and chronic model.

Tolerable doses, i.e. doses not resulting in an increase in total cell counts, mild neutrophilia of the BAL fluid cells and no behavioural side effects, were used for further experiments. Titration revealed a dose of 5×10^3 U IFN-γ and 2 ng or 4 ng BPPcysMPEG for further studies.

3.3.2 Prevention of acute allergic asthma can be achieved by TLR2-agonist/IFN-γ-treatment

Whether synthetically produced *Mycoplasma*-lipopeptide derived compounds as BPPcysMPEG can bear the same asthma preventive effects as observed for prior-to-sensitisation *Mycoplasma*-infections, was investigated in a preventive protocol (*see* 2.6).

In this preventive protocol TLR2-agonisation started before sensitisation and continued throughout this phase. When allergic inflammation was provoked by intranasal extract challenges, no significant effect on absolute cell counts was observed. Importantly, eosinophil counts in BAL fluid were significantly diminished in dexamethasone and

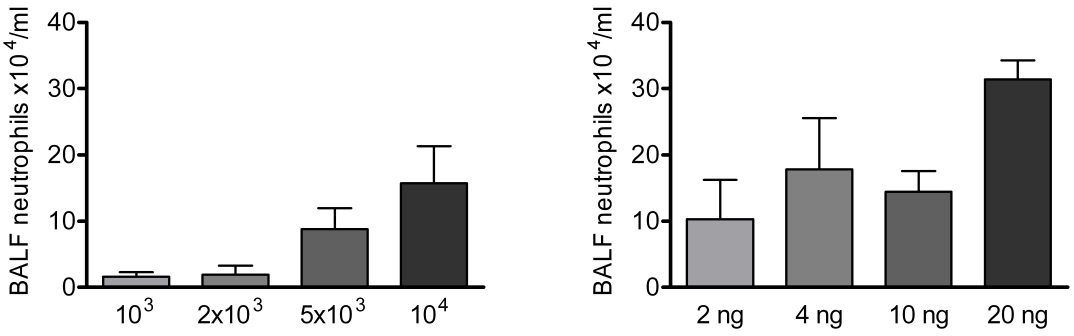

Figure 3.10: BPPcysMPEG and IFN-γ dose titration. Neutrophil counts in BALF. Mean and SEM are shown. Left: after treatment with 20 ng BPPcysMPEG and titrated doses of IFN-γ. Right: after treatment with 5×10^3 U IFN-γ and titrated doses of BPPcysMPEG. For further studies a dose of 5×10^3 U IFN-γ and 2 ng or 4 ng BPPcysMPEG was chosen.

BPPcysMPEG/IFN-γ treated mice. No treatment group showed increased neutrophil counts in BAL fluid (fig. 3.11). This proves the concept of a possible TLR2-mediated prevention of allergic inflammation and underlines the pharmacological potential of BPPcysMPEG.

Measurement of airway hyperresponsiveness showed that significantly elevated doses of methacholine were necessary to reduce midexpiratory flow to 50% in the dexamethasone treated group. Although calculation of statistical power revealed that this measurement actually requires larger group sizes, BPPcysMPEG/IFN-γ-treated animals showed a clear tendency to improved lung function ($p = 0.08$) (fig. 3.12).

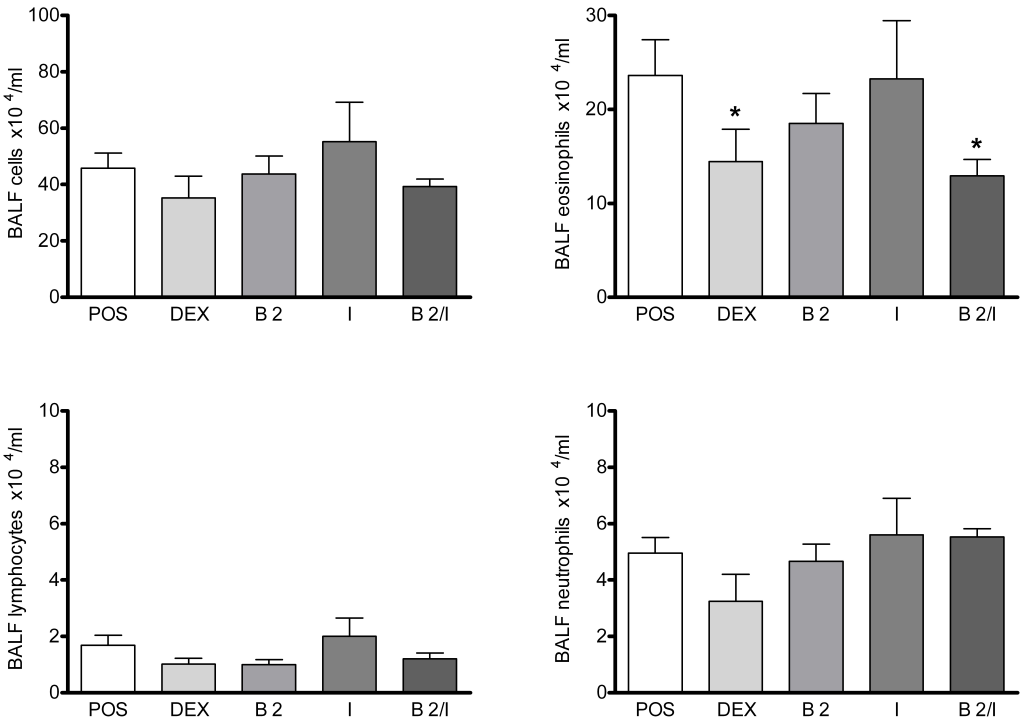

Figure 3.11: Differential cell counts in BALF in the preventive intervention of acute-phase asthma. Mice were treated in a preventive treatment regime, i.e. before and during the phase of sensitisation (n = 8 per group). POS = sensitised vehicle control, DEX = dexamethasone treated group, B 2 = 2 ng BPPcysMPEG, I = IFN-γ, B 2/I = 2 ng BPPcysMPEG/IFN-γ.

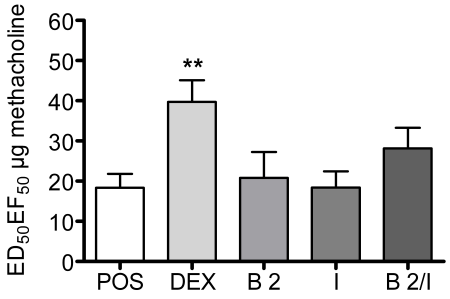

Figure 3.12: Effective dose of methacholine that decreases midexpiratory flow to 50% (airway hyperresponsiveness) in preventively intervened acute asthma. Mice were treated before and during sensitisation (n = 8). Mean and SEM are shown. POS= sensitised vehicle control, DEX = dexamethasone, B 2 = 2 ng BPPcysMPEG, I = IFN-γ, B 2/I = 2 ng BPPcysMPEG/IFN-γ. A statistically significant improvement of lung function was observed in dexamethasone-treatment group, however, B 2/I reached p = 0.08.

3.4 Intervention of acute-phase allergic asthma by a TLR2-ligand

3.4.1 A single preventive administration of TLR2-agonist/IFN-γ ameliorates lung function in sensitised mice

To test asthma preventive effects in already sensitised mice, treatment was performed one day before allergic inflammation was induced in the lung by extract provocation (*see* 2.8). A single preventive treatment turned out to be not sufficient to influence the allergic inflammation, however, it ameliorated lung function significantly. No treatment effect was observed in any group when looking at total BAL fluid cell counts, eosinophil or neutrophil numbers (fig. 3.13). Measurement of airway hyper responsiveness to methacholine revealed a significantly improved lung function in BPPcysMPEG/IFN-γ-treated mice (fig. 3.14).

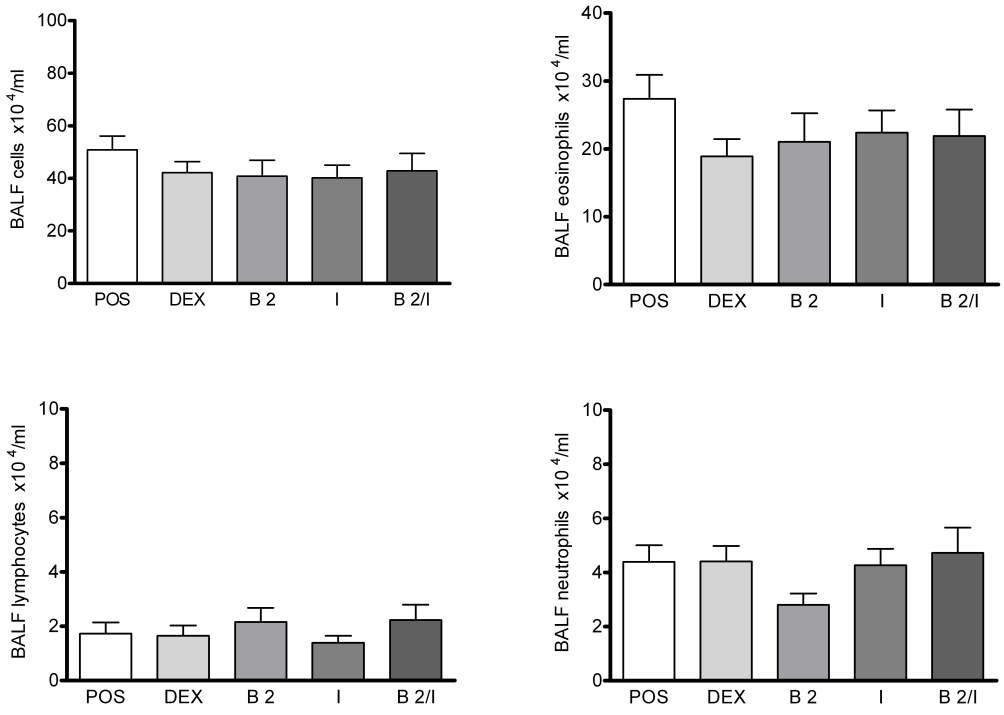

Figure 3.13: Differential cell counts in BALF in preventive intervention of acute asthma in sensitised mice. Mice were treated one day before the first extract provocation (d 27, n = 12). POS = sensitised vehicle control, DEX = dexamethasone, B 2 = 2 ng BPPcysMPEG, I = IFN-γ, B 2/I = 2 ng BPPcysMPEG/5 $\times$ 10^3 U IFN-γ.

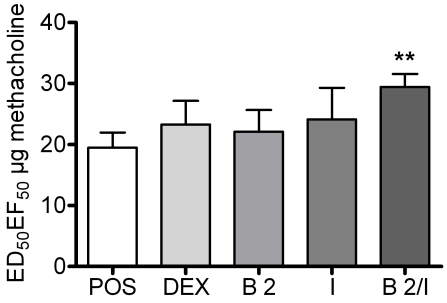

Figure 3.14: Effective dose of methacholine that decreases midexpiratory flow to 50% (airway hyper responsiveness) in preventive intervention of acute asthma in sensitised mice. Mice were treated at day 27, one day before the first extract provocation (n = 12 per group). POS = sensitised vehicle control, DEX = dexamethasone, B 2 = 2 ng BPPcysMPEG, I = IFN-γ, B 2/I = 2 ng BPPcysMPEG/IFN-γ. B 2/I-group showed improved lung function compared to the vehicle treated positive control.

3.4.2 A single therapeutic administration of TLR2-agonist/IFN-γ ameliorates allergic inflammation in sensitised mice

A single administration of the TLR2-agonist in combination with IFN-γ one day after the last allergen challenge (*see* 2.7) showed a significant amelioration of cellular inflammation (fig. 3.15).

Although absolute cell counts were not altered, eosinophil counts as well as neutrophils were diminished comparable to dexamethasone treated controls. Therefore, a therapeutic treatment with BPPcysMPEG and IFN-γ demonstrated to have strong beneficial influence on allergic inflammation.

Augmentation of lymphocytes did not reach a statistical significance, as did measurement of airway hyper responsiveness (fig. 3.16).

From the studies performed in sensitised mice it becomes obviously that the time point of pharmacological intervention influences amelioration of disease-related symptoms noticeably. While effects on lung function parameters seem to depend on an early start of treatment in a preventive manner, inflammation-related symptoms can effectively be alleviated after allergen confrontation.

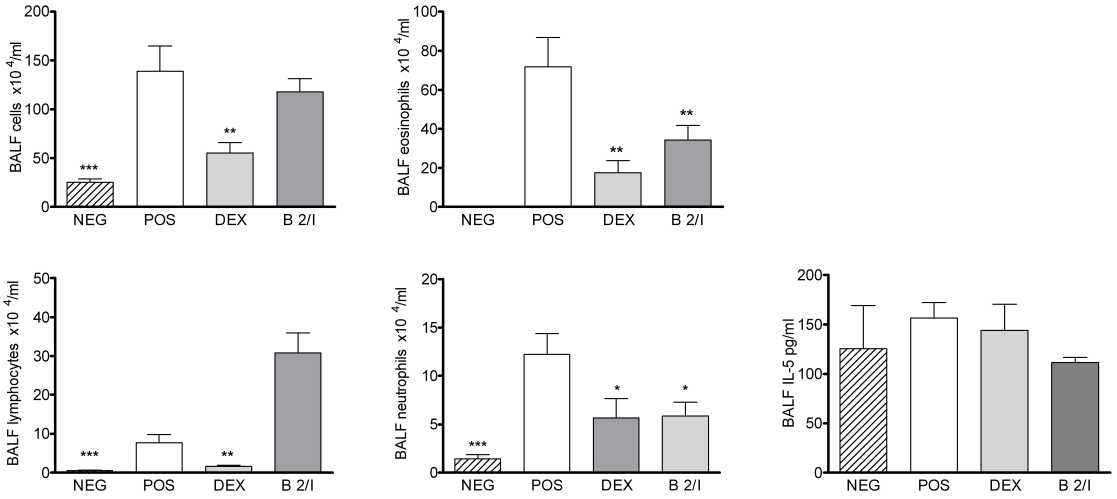

Figure 3.15: Differential cell counts and IL-5 in BALF in therapeutic intervention of acute-phase asthma. Mice were treated at day 29, one day after the last provocation, in a therapeutic protocol (n = 8 per group). NEG = sham-sensitised negative control, POS = sensitised vehicle control, DEX = dexamethasone, B 2/I = 2 ng BPPcysMPEG/5 × 10^3 U IFN-γ.

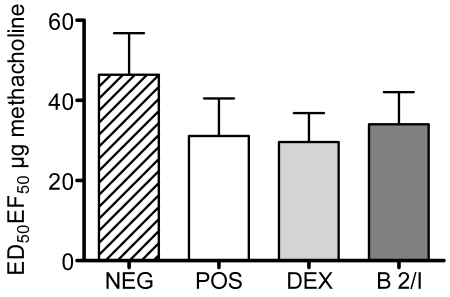

Figure 3.16: Effective dose of methacholine that decreases midexpiratory flow to 50% (airway hyper responsiveness) in therapeutic intervention of acute asthma. Mean and SEM are shown. Mice were treated at day 29, one day after the last provocation (n = 8 per group), in a therapeutic protocol. NEG = sham-sensitised negative control, POS = sensitised vehicle control, DEX = dexamethasone, B 2/I = 2 ng BPPcysMPEG/IFN-γ. No significant differences could be detected among the groups, indicating no improvement of lung function compared to positive controls.

3.5 Intervention of chronic allergic asthma by a TLR2-ligand and IFN-γ

3.5.1 Sub-chronic allergic inflammation in intraperitoneal sensitised mice is influenced by dexamethasone and therapeutic TLR2-agonisation in combination with IFN-γ

To investigate the effects TLR-agonisation could have on the sub-chronic phase of allergic asthma, the protocol was applied as shown in 2.9. Treatment was performed one day after the second allergen provocation per week.

In a sub-chronic phase, i.e. after ten extract provocations, eosinophils as well as neutrophils were significantly reduced in therapeutically BPPcysMPEG/IFN-γ treated mice compared to untreated positive controls.
Dexamethasone showed a generally inflammation-dampening character: total cell counts in BALF, eosinophils and lymphocytes were reduced. Neutrophils were not affected by dexamethasone treatment (fig. 3.17).

For a chronic phase of allergic asthma and also due to the closer resemblance to human pathology, focus was set to the investigations in the model of chronic respiratory sensitisation.

3.5.2 Prevention of chronic allergic asthma by dexamethasone, a TLR2-agonist and IFN-γ in a model of respiratory sensitisation

To resemble human pathology more closely, the model of chronic respiratory sensitisation towards grass pollen allergens was used. Intranasal treatment was performed 8 h after the second extract confrontation (*see* 2.10). Dexamethasone-treatment was performed intraperitoneally 18 h and 2 h before each provocation.

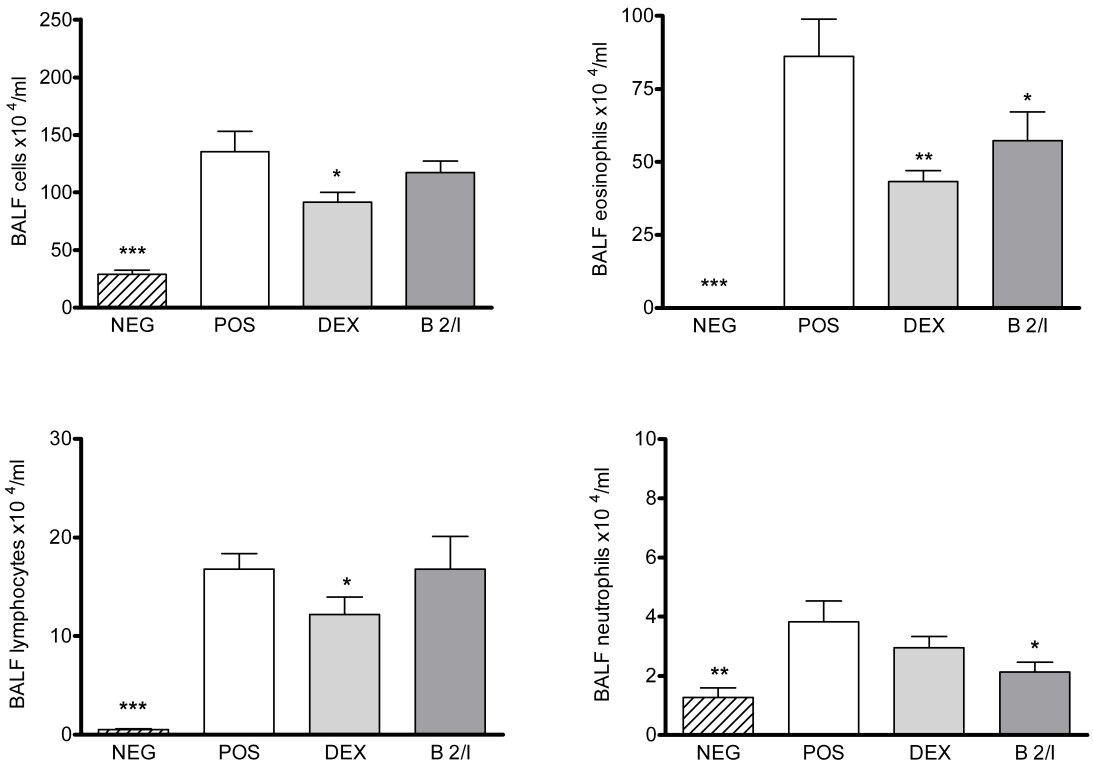

Figure 3.17: Differential cell counts in BAL fluid in intraperitoneal sensitised mice after 10 extract provocations (sub-chronic phase). Mean and SEM are displayed. Negative control group (NEG), i.p.-sensitised positive control group (POS), i.p.-sensitised dexamethasone treated group (DEX), 2 ng BPPcysMPEG/IFN-γ treated group (B 2/I). Every group consisted of (n = 8). Some data on this graph is identical with that displayed in fig. 3.5.

Intranasal administration of Timothy grass pollen allergens causes IgG_1 mediated sensitisation, which can be prevented by dexamethasone treatment

As shown before, sensitising mechanisms to Timothy grass pollen allergens in mice are mainly mediated via IgG_1 instead of IgE [123]. In previous studies, it could be proven that this is true for respiratory sensitisation as well (data not shown). IgG_1 concentration in plasma increased throughout the duration of allergen challenges and could only be effectively reduced by dexamethasone treatment. Additionally, the number of B220+ cells in mediastinal lymph nodes was reduced in this treatment group, implicating a restrained appearance of antibody-secreting plasma cells (fig.3.18).

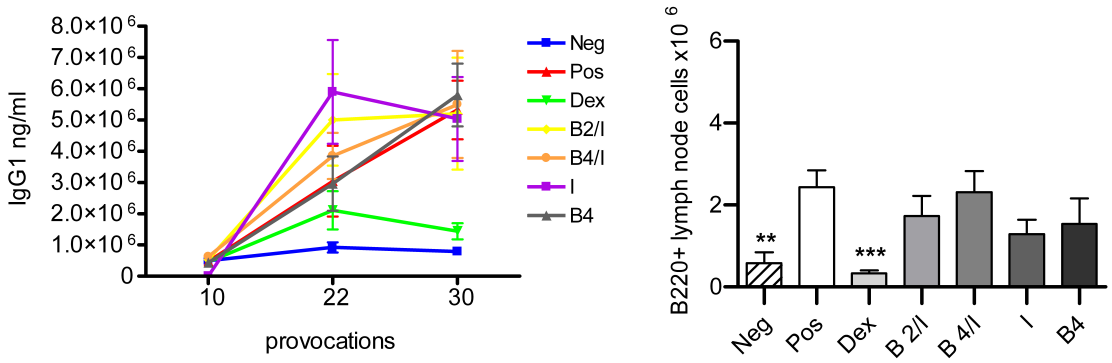

Figure 3.18: Plasma IgG_1 and B220+ lymph node cells in the model of chronic respiratory sensitisation. Course of plasma IgG_1 levels when Timothy grass pollen allergens were intranasally administered twice a week on two consecutive days, and B220+ mediastinal lymph node cells after 30 provocations. IgG_1 levels were significantly reduced in negative control and dexamethasone-treated group compared to positive control after 30 allergen provocations ($p < 0.001$, each).

Chronic respiratory sensitisation promotes the development of allergic inflammation

In allergen challenged mice, a prominent eosinophilic inflammation was induced, displayed as exaggerated total cell counts, granulocytes and lymphocytes as well as Th2-cytokines in BALF (fig. 3.19). Furthermore, eosinophils massively infiltrated the peribronchial and perivascular space. These exaggerations were not observed in dexamethasone treated mice (fig. 3.20).

The pharmacological intervention with BPPcysMPEG started during the phase of sensitisation. Treatment with the TLR-agonist in combination with IFN-γ, but also administered alone, resulted in strong reduction of BALF eosinophils and Th2-cytokine levels, while lymphocyte numbers were not affected (fig. 3.19). Although eosinophil counts in BALF were markedly reduced in all TLR-agonist treated groups, this reduction was observed in tissue after B4 treatment only (fig. 3.20).

Despite the induction of a profound allergic airway inflammation, measurement of airway hyper responsiveness to methacholine in head-out bodyplethysmography revealed no significant differences among the study groups (data not shown).

Interestingly, all treatments showed a clear reduction of CC-chemokines, either eotaxin (CCL11) or RANTES (CCL5) (fig. 3.21).

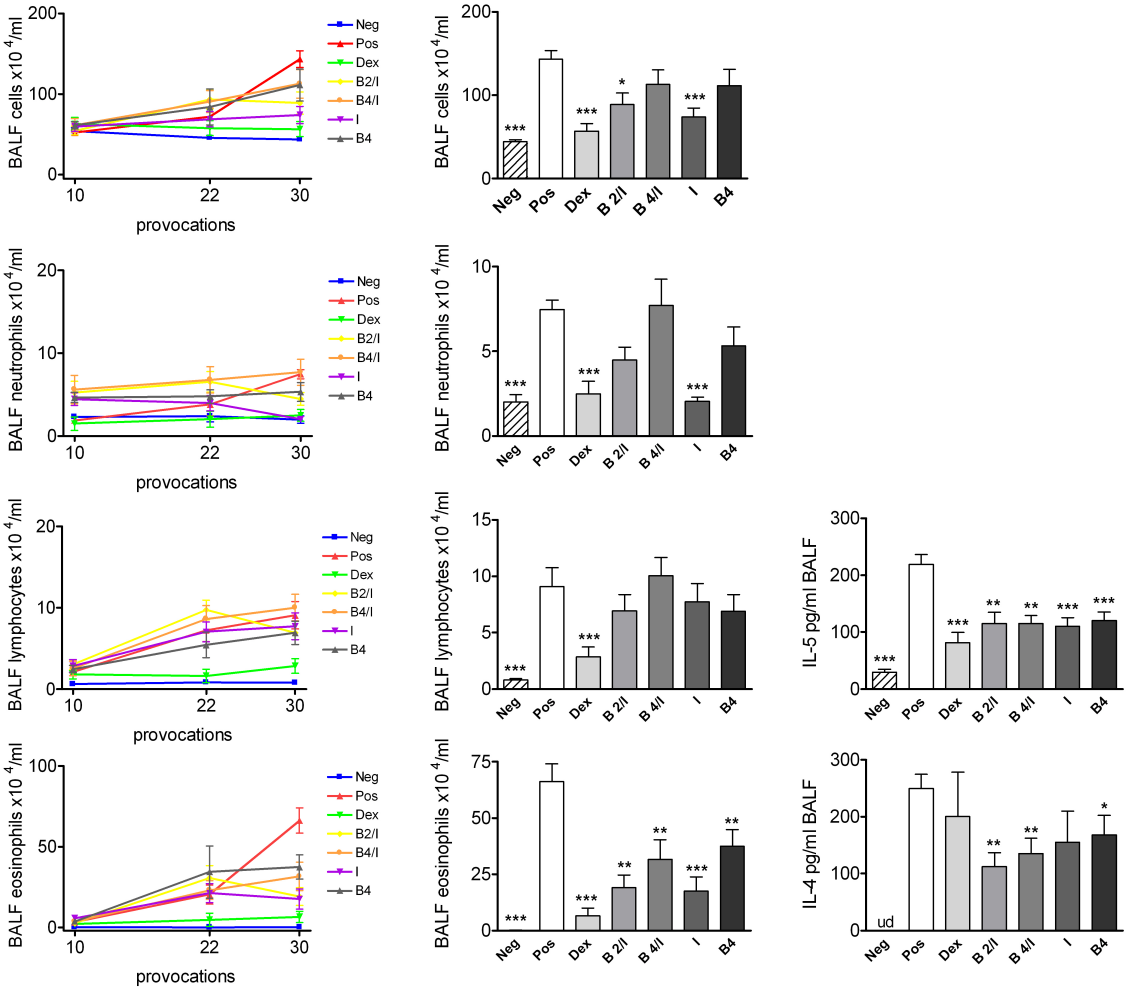

Figure 3.19: Cellular inflammation in BALF in the model of chronic respiratory sensitisation. Left panel displays the course of the contribution of lymphocytes, neutrophils and eosinophils to the total cell counts in BALF, middle panel the cellular composition after 30 Timothy grass pollen extract, which is equivalent to 10 intranasal TLR-agonist and /or IFN-γ instillations. Right: IL-4 and IL-5 levels in BALF after 30 provocations (ud= under detection limit).

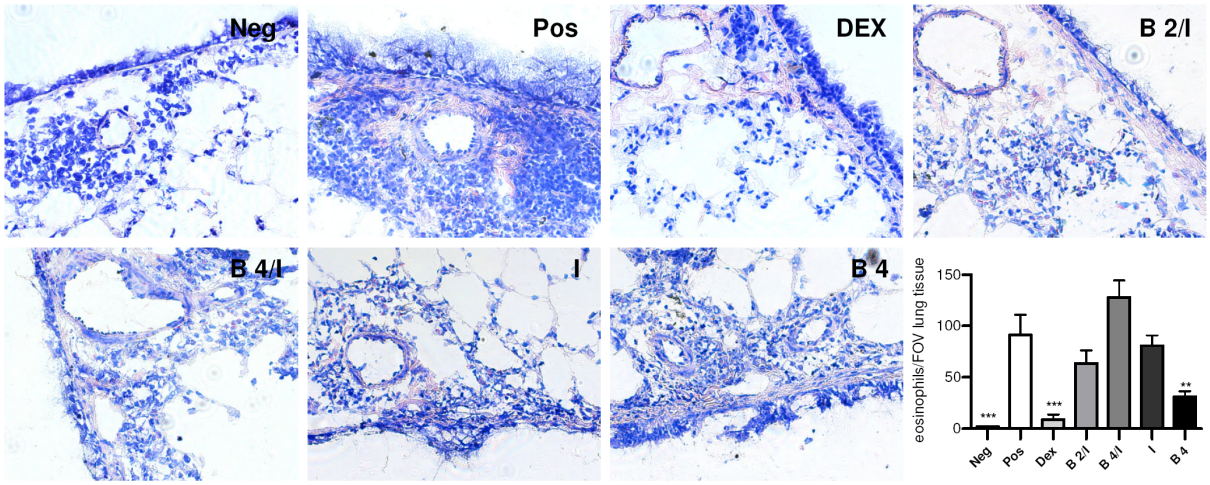

Figure 3.20: Histology of the lung (Giemsa) and eosinophils in tissue in the intervention of chronic respiratory sensitisation. Giemsa stained kryosections of the distal part of the main bronchus after 30 intranasal Timothy grass pollen extract instillations / 10 intranasal treatments with either 2 or 4 ng TLR-agonist BPPcysMPEG in combination with 5×10^3 U IFN-γ (B 2/I or B 4/I, respectively), the same dose IFN-γ without TLR-agonist, and 4 ng BPPcysMPEG (B 4). Dexamethasone (Dex) treatments were performed i.p. 18 h and 2 h before each extract provocation. All treatments started after 10 provocations. Photographs were taken at 400x magnification. To assess allergic inflammation in lung tissue red-stained eosinophils were counted (n=3 / group) in three fields of view (FOV) per animal.

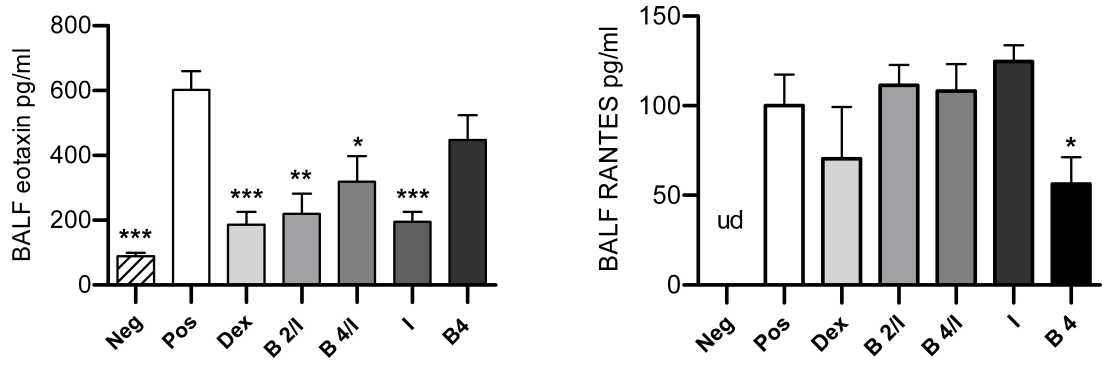

Figure 3.21: Eotaxin and RANTES in BALF in preventively intervened chronic respiratory sensitisation. Both CC-chemokines were assessed after 30 intranasal extract administrations. Significantly reduced amounts of chemotactic factors for eosinophils and Th2-lymphocytes were found in every treatment group. (Ud = under detection limit)

Remodelling

Massively infiltrated lung tissue as well as hyperplasia of the respiratory epithelium was prominent in sensitised animals (figs. 3.20, 3.22, 3.23). Quantitative assessment of collagen in whole lung homogenates did not reveal statistical differences, nor did scoring of epithelial basement membrane thickening (data not shown).

Evaluation of histo-morphologic alterations was performed blind to the experimental groups by an experienced pathologist (Marina Greweling, Helmholtz Centre for Infection Research, Braunschweig, Germany) as described in section 2.1.9. Scoring of Giemsa stained sections revealed varying amounts of peribronchial and perivascular infiltrates of inflammatory cells. The infiltrates contained eosinophils. Mast cells were found mainly within the epithelium. Furthermore, bronchial epithelial hyperplasia was frequently seen.

The overall severity of lesions was reduced in groups of mice treated with B 2/I or B 4, as was the score of infiltrates in B 2/I (fig. 3.23). Epithelial hyperplasia was reduced when mice received B 4. Summing up the different scores results in the total score of remodelling, this total score was reduced in mice treated with either B 2/I or B 4.

In summary, positive controls showed clear signs of remodelling processes. Dexamethasone, IFN-γ and 4 ng BPPcysMPEG/IFN-γ had no effect on remodelling. Such effects could be observed in B 2 I- and B 4-treatment groups.

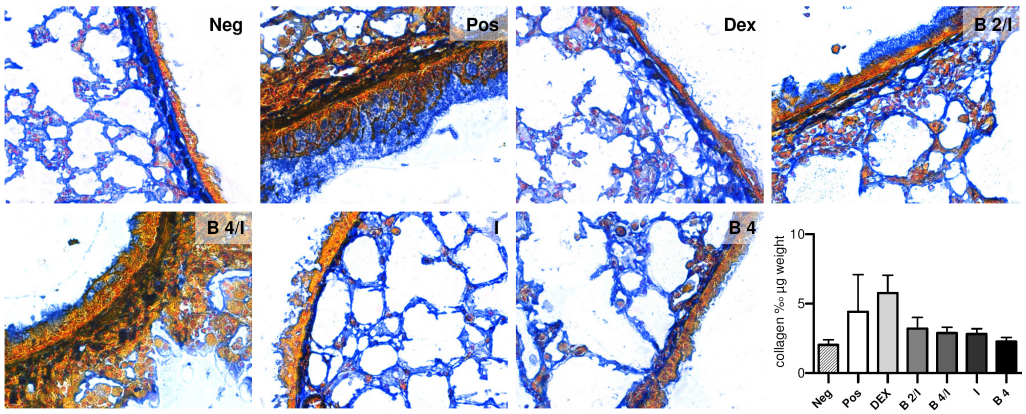

Figure 3.22: Localisation and quantification of collagen in lung tissue in preventive intervention of chronic respiratory sensitisation. Collagen deposition in lung kryosections was visualised by performing Azan novum stain. Covering the epithelium of the distal part of the main bronchus, mucus (light blue) can be identified. Muscles and reticular connective tissue is stained orange, nuclei of infiltrating inflammatory cells brownish. Collagen appears dark blue. A thickening of the basement membrane could not be proven. Collagen quantification of whole lung homogenates revealed no significant differences among the groups.

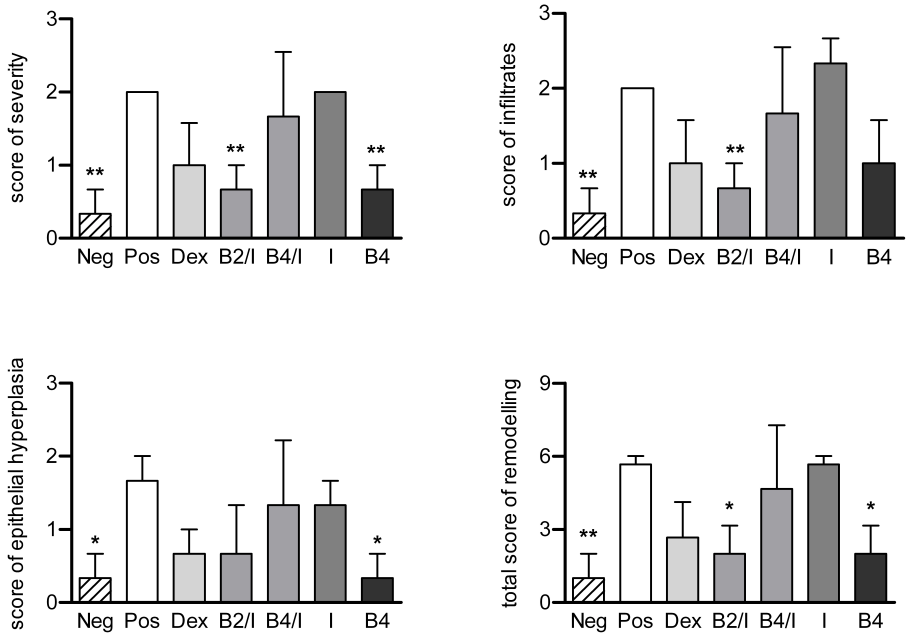

Figure 3.23: Score of remodelling in the intervention of chronic respiratory sensitisation. Giemsa-stained sections were scored for the overall severity, the amount of inflammatory infiltrates and the grading of bronchial epithelial hyperplasia from 0 to 3, following the criteria described in Material and Methods.

Dexamethasone reduces CD11c+ antigen presenting cells in lung draining mediastinal lymph nodes

In lung draining lymph nodes, an increase of CD11c+ cells could exclusively be diminished by dexamethasone treatment (fig. 3.24). Dexamethasone additionally reduced normal occurrence CD11c+ cells in spleen, underlining its systemic suppressive effects.

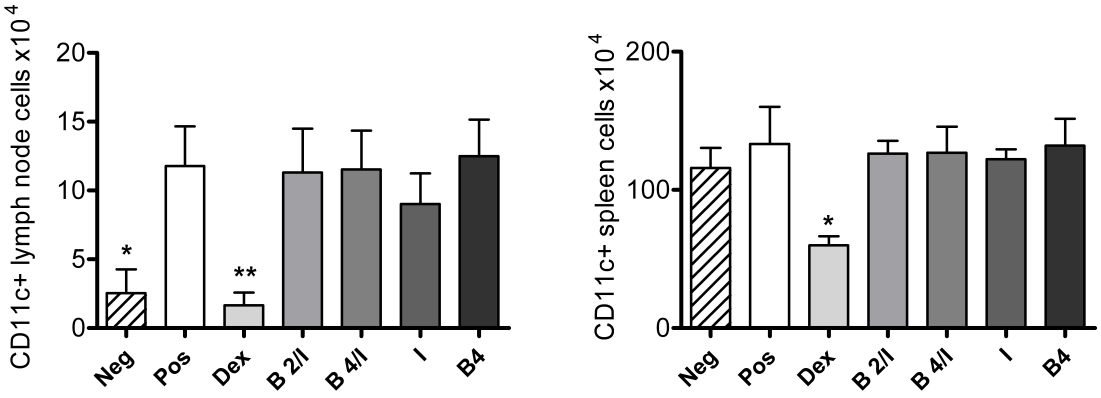

Figure 3.24: CD11c+ cells in mediastinal lymph nodes and spleen in preventively intervened chronic respiratory sensitisation. Cells were quantified after FACS analysis. In lymph nodes, CD11c+ cell counts were significantly increased, an effect not observed in spleen. Treatment with dexamethasone reduced CD11c+ cells in both organs.

TLR-agonist, IFN-γ or both in combination influence T-cell mediated immune responses, which leads to a reduction of allergic inflammation

Although lymphocyte counts in BALF were not altered compared to positive control, differences in T-cell mediators could be detected. Besides the already mentioned effects on Th2-cytokine and chemokine release into the bronchoalveolar space, IL-10 levels were raised in untreated sensitised Pos group. No significantly different occurrence of CD4+foxp3+ lymph node cells were observed among the groups (fig. 3.25). Therefore, it is concluded that the beneficial effects of dexamethasone, TLR-agonist and/or IFN-γ treatment are not mediated via the induction of tolerance.

Additionally, neither a reduction of IL-12p40, nor an increase in IL12-p70 or IL-6 was observed (fig. 3.26). Neutrophil counts in BALF were rather reduced in the IFN-γ treated

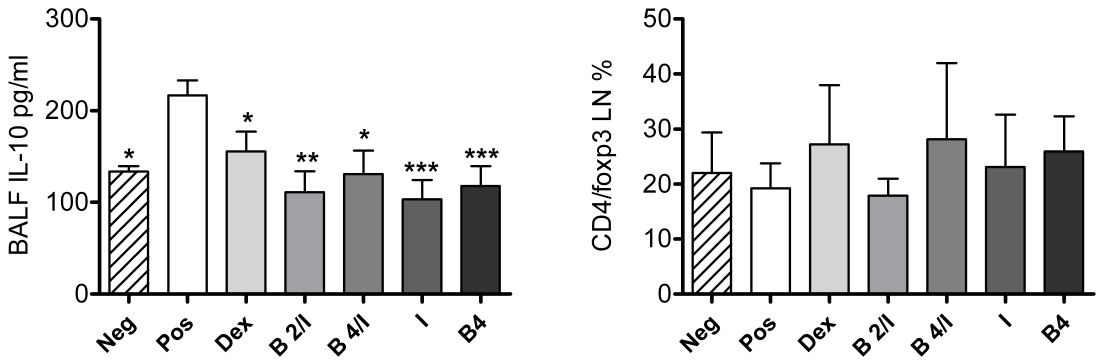

Figure 3.25: Regulation of the allergic response in preventively intervened respiratory sensitisation. Decreased IL-10 concentrations in BALF and unaffected occurrence of CD4+foxp3+ cells in mediastinal lymph nodes exclude an induction of tolerance. IL-10 concentrations in BALF as well as CD4+foxp3+ cells were assessed after 30 intranasal extract provocations.

group than increased, and their contribution to cellularity of BALF was not different to positive control (fig. 3.19). IFN-γ could only be detected in BALF in those groups, which received an intranasal IFN-γ instillation 12 h before animals were sacrificed and lavage was collected. No induction of IFN-γ was observed when the TLR-agonist was administered alone (fig. 3.26). These results exclude a Th_2/Th_1-shift of the immune response following TLR-agonist and IFN-γ administration.

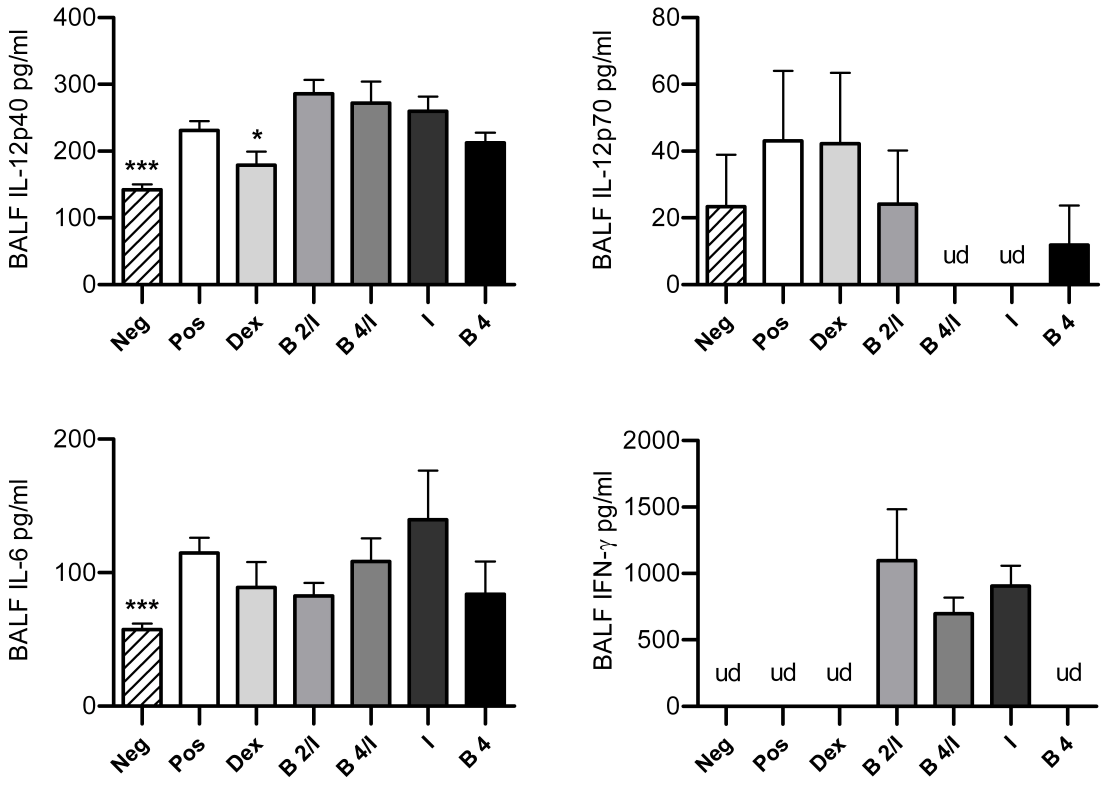

Figure 3.26: Cytokines in BALF in preventively intervened chronic respiratory sensitisation. Th-1 related cytokine IFN-γ could only be measured in animals that received IFN-γ applications the day before sampling. Neither a Th1-promoting ratio of IL-12p70 to IL-12p40, nor an exaggeration of proinflammatory IL-6 concentrations were detected in the different treatment groups (after 30 Timothy grass pollen extract provocations, ud = under detection limit).

4 Discussion

The main results of this thesis are:

- Mouse models of airway hyper responsiveness and allergic airway inflammation which use grass pollen allergen were developed and validated. These models reflect human allergic asthma by mimicking the acute, sub-chronic and chronic phase of the disease.
- Through the use of both, human relevant grass pollen allergen and confrontation routes, resemblance of sensitisation and effector phase was clearly improved.
- The proven beneficial pharmacological intervention of airway hyper responsiveness and allergic airway inflammation by a toll-like receptor agonists in combination with IFN-γ is not achieved by a Th2/Th1-shift or induction of tolerance, but rather due to a reduction of T-cell function.

4.1 Pollen allergens

Allergic diseases show high prevalence and incidence, especially in the Western world. This might be connected to the Western life style and the impact the so called modern way of life has on environmental factors. Pollen grains are a main source for seasonal allergens, causing allergic disorders as rhinitis, conjunctivitis, and asthma. If the plant is stressed, for example by air pollution, allergenicity of the pollen grains raises importantly [124]. Moreover, the allergenicity is further amplified by aggravated occurrence of particles, e.g. diesel exhaust particles, in urban areas [125]. For allergic rhinitis, pollen is believed to cause symptoms after contact to the nasal mucosa. However, for causing allergic asthma, pollen grains are in fact too big in size to be inhaled to the lung and penetrate the deeper

airways. Therefore, pollen starch granules (PSGs) are believed to be respirable allergen particles responsible for the onset of asthma [126]. These PSGs are released under certain climate conditions. Important factors for the generation, release and distribution of PSGs are temperature, humidity and wind shaking [127]. The ability of PSGs to provoke a humoral immune response has been shown in whole-pollen sensitised rats [128].

Also in the studies related to this thesis, PSGs were able to cause allergic inflammation in mice sensitised towards grass pollen allergen. However, there still remain variances in allergenicity of pollen grains, either due to environmental conditions, to natural biologic variation, or to the isolation technique applied. Moreover, due to their natural origin, a contamination of the PSG-preparation, e.g. with endotoxins, can not be excluded. Endotoxins on the one hand can cause sepsis; on the other hand they are ligands to toll-like receptors and therefore strong immunomodulators [129]. This makes PSGs to an allergen source hardly standardiseable and therefore not recommended in experimental models for pharmaceutical testings.

Another mechanism for the release of allergens located on PSGs or in the pollen cytosol is the osmotic burst of the pollen grain after contact to the respiratory mucosa. With this burst, not only granules and proteins are ejected, but also lipids. These lipids are actually of biological relevance for the directional growth of pollen-tubes [130], however, pollen grains containing these pollen-associated lipid mediators (PALMs) are *per se* able to activate the mucosal membrane [131]. On DCs, prostaglandin-like compounds called E1 phytoprostanes (PPE1) inhibit the production of IL-12 and thereby promote Th2-responses [124]. Additionally, PALMs are chemoattractants for eosinophils [132]. The use of pollen extract preparations containing both protein-allergens and lipids, either for induction of allergic inflammation in sensitised mice or in particular for respiratory sensitisation, is therefore a method highly reflecting the onset of human disease and could be developed in this work for the acute, sub-chronic and chronic phase. In these novel and optimised animal models, very distinct questions can be investigated, for example as tested in this work from the field of immunomodulation, but also of desensitisation or specific immunotherapy.

For immunotherapy, different treatment regimes are investigated, using recombinant major allergens, e.g. of birch (rBet v1, [133]), olive tree (rOle e1, [134]) and Timothy grass (rPhl

p5 [119]), antibodies or combination vaccines against epitopes of recombinant allergens [135, 136]. The major allergen group 5 of Timothy grass is a RNAse involved in the plant´s defence against infections [137]. It is believed to be locally associated with PSGs, but also in the cytosol of the pollen grain [138, 118] and forms different isoforms and isoallergens [139].

In models in which mice are sensitised i.p. and treated by administration of recombinant allergens, allergic symptoms induced by extract provocations were alleviated. This was due to an induction of immunosuppression, mediated via Th3 or regulatory T-cells [133, 140]. In this thesis, continued extract challenges in the chronic model of i.p.-sensitised mice also led to an attenuation of allergic inflammation, in contrast to animals that were only sensitised by the respiratory route, i.e. by continued extract administrations. Therefore, induction of tolerance might partly be a mechanism induced by systemic sensitisation, and possibly not exclusively related to desensitisation with recombinant major allergens. The question concerning the effect that could be observed by administration of recombinant protein in the model of chronic respiratory sensitisation remains.

4.2 Mouse models of asthma and allergic airway inflammation

4.2.1 Identification of susceptible mouse inbred lines

The susceptibility to induction of asthma-like phenotypes is clearly related to the genetic background of the mouse inbred strains used [141]. BALB/c and A/J mice are particular susceptible for induction of long lasting allergic inflammation in the lung, while airway hyper responsiveness was not detected [122]. The susceptibility has been described in the following ranking: BALB/c > C57/BL6 > CBA. Possibly, this is due to differences in eosinophil survival [142].

As a prerequisite to develop improved mouse models, the susceptibility of different inbred mouse strains to sensitisation towards Timothy grass pollen allergens was tested. Estimation of the ability to reflect allergic inflammation, i.e. raised eosinophil counts, but

only moderate increase in neutrophils, revealed the following order: A/J > BALB/c > C57/BL6 > CBA/J. However, as shown before [122], even in this study airway hyper responsiveness in A/J mice could not be measured, and the acute, sub-chronic and chronic model was established in BALB/c.

4.2.2 Development of a protocol to induce symptoms of acute and chronic asthma

Acute models of allergic asthma are useful tools in basic and pharmaceutical research [143]. These models provided important insight into mechanisms, cells and mediators contributing to establishment and maintenance of allergic asthma. Especially pharmaceutical intervention processes during the effector-phase of the allergic reaction can be investigated in acute-phase models, e.g. prevention of eosinophil invasion of eosinophils into the lung by agonisation and antagonisation of the chemokine receptor 3 (CCR3) [144, 145]. In the acute model of allergic asthma developed in this work, all important requirements to an acute asthma model could be fulfilled, as there are an impressive induction of allergic inflammation depicted as raised eosinophil counts and Th2-cytokine levels in BALF, and an airway hyper responsiveness to unspecific stimuli. In this model, dexamethasone as a standard drug in asthma therapy shows comparable effects as in patients. This underlines the possibility to influence asthma parameters in this model and stresses the high similarity to human acute allergic asthma. Moreover, through the use of an allergen relevant to humans, very distinct questions can be answered in this new model, e.g. from the field of specific immune therapy to Timothy grass pollen allergens.

In recent years, limitations of so called acute asthma models became more and more prominent and the need of chronic models emerged to deliver new opportunities to test efficacy of pharmacological intervention. Acute models are not always able to answer pharmacological questions: for example, chronic inflammation could be inhibited by cytosolic phospholipase A2 inhibitors, an effect which was not hinted at during the acute status [146]. Additionally, the beneficial effects of the cysteinyl leukotriene-receptor blocking compound montelukast were demonstrated in a chronic model. Montekulast-treatment led to a decrease in inflammation, reduction of IL-4 and IL-13 mRNA in lung

and BALF proteins. It also influenced fibrosis, mucus plugging, and smooth muscle hyperplasia [147], symptoms which can only be investigated in chronic models. This emphasises an important difference between the chronic and acute status: the ability to reflect remodelling processes. In chronic models, structural changes even persist after cessation of allergen challenges, uncoupled from inflammation [36, 34, 32].

However, although innovative these new chronic models described in the literature also have to face obstacles and limitations. Frequently, prolonged allergen challenges after sensitisation end in induction of tolerance [112], and resolution of eosinophilic inflammation [148], despite the fact that eosinophil viability factors are in fact increasing [110, 51, 52]. Also in the i.p.-model of sub-chronic allergic inflammation developed in this thesis, in which recombinant grass pollen allergen-sensitisations and extract challenges were set up, absolute eosinophilia in BALF decreased in sensitised mice after 22 provocations. Still there were significant enhancements compared to sham-sensitised mice at every time point, though. In this model, very rapidly a severe eosinophilic inflammation was induced, and severe histological alterations were observed. However, although dexamethasone controls showed a steady state level of inflammation, resolution of eosinophilia in positive controls resulted in the loss of a clear pharmacological treatment effect. These finding limits the model to a sub-chronic phase, i.e. read-out after ten provocations. Therefore, the model of respiratory sensitisation seems to be better applicable in pharmacological testing.

These limitations of chronic models do not evolve in protocols which use human relevant allergens, as for example timothy grass or house dust mite extract [115], and further on abandon the use of an adjuvant as in the chronic model developed in this thesis. However, an airway hyper responsiveness was not observed in this model. Although this limits the model for pharmacological testings, this finding might be an additional point closer to human pathology. From longitudinal studies it is implicated that the manifestation of all asthma related symptoms is a long lasting-process [149]. In a 15-week protocol of intranasal allergen extract administration, remodelling processes as for example epithelial hyperplasia and a constantly increasing eosinophilic inflammation could clearly be reflected. Supposably, airway hyper responsiveness might develop at a later time point. This assumption is supported from human studies, in which remodelling processes seem to occur long before clinical symptoms are recognised [150].

Importantly, this model also reflects allergy mechanisms in patients more thoroughly, concerning the human relevant allergen source, route of sensitisation, and comparable therapeutic effects of dexamethasone. Therefore, this innovative model was set into the focus of this work to investigate immuno-modulatory effects of TLR2-agonisations in chronic asthma.

4.3 Pharmacological intervention of allergic asthma

4.3.1 Dexamethasone in acute and chronic asthma

Dexamethasone is a standard therapeutic in patients with allergic asthma. For pharmacological studies in mice, it serves as a positive treatment control, to prove that the model is *per se* manipulable. The expected effects of dexamethasone was observed in an acute-phase, sub-chronic and chronic model in this thesis. However, even if in the acute model of Timothy grass pollen induced allergic asthma preventive actions could be shown, a preventive, i.e. long lasting, treatment with dexamethasone might bear more disadvantages than advantages for the patient because of the systemic immunosuppressive action of this drug.

In chronic respiratory sensitisation dexamethasone ameliorated inflammatory parameters, an observation that agrees with other studies [151, 33]. In allergic asthma, an exaggerated recruitment of $CD31^{hi}Ly\text{-}6C^{neg}$ bone marrow precursor cells is the prerequisite to a massive increase of DCs in mediastinal lymph nodes, as well as of eosinophils and DCs in the lung [152].

It is hypothesised that a reduction of CD11c+ cells due to dexamethasone treatment [153] in this model is one mechanism leading to less lymphocyte activation and inflammation, depicted in reduced chemokine and cytokine levels, but also to a decrease of inflammatory cells in BALF and lung tissue. Moreover, the reduction of T-cell cytokines, responsible for so called isotype-switching, and the diminished number of B220+ cells, results in low plasma IgG_1.

Remodelling processes were not affected by dexamethasone-treatment in this model, a phenomenon frequently described in literature [154]. Moreover, the increased deposition

of collagen during remodelling processes is assumed to enforce dexamethasone tolerance [155]. However, effects of dexamethasone on remodelling seem to dependent strongly on the dose administered: high doses are able to reduce at least some features of remodelling, while low doses can act on infiltration of the lung [156].

On the other hand, dexamethasone also prevents the development of endogenous balancing mechanisms, e.g. the induction of tolerance, and therefore plays a dual role in therapy of allergy [70]. This prevention of tolerance was also shown in this work, as decreased concentrations of immuno-modulating IL-10 in BALF, and unaltered occurrence of regulatory T-cells in mediastinal lymph nodes.

4.3.2 Immuno-modulation of allergic asthma by TLR2-agonisation

The rationale for immuno-modulation by toll-like receptor agonist is delivered by the so called hygiene hypothesis [3, 4, 5]. This hypothesis relates the increasing prevalence and incidence of allergic diseases in the Western world with a cease in Th1-immune responses inducing triggers especially during early childhood. For example, bacterial infections are reduced due to a less frequent direct confrontation to bacteria, but also due to a decrease in the number of siblings [6, 7]. In current opinion allergic asthma can be caused by an imbalance of the Th1/Th2 axis. From this observations, a variety of experimental intervention strategies were developed, some of them already starting during pregnancy [157] or in newborns [158]. These strategies set the TLR4-ligand endotoxin into the scope of investigations to induce Th1-responses, ignoring the risk of inducing sepsis. Moreover, protection against allergy is more likely mediated via TLR2 than via TLR4 [80], which make TLR2-agonists a promising approach in immuno-modulation.

Because dendritic cells express toll-like receptors, immuno-modulation with TLR-agonists can causatively influence allergic diseases [159]. Dendritic cells are believed to be key players in immune responses, in sensitisation [160] but also in maintenance of allergy [12]. Dendritic cells resting in lung tissue favour a Th2-type response and need to be triggered with Th1-inducing stimuli [11]. These Th1-triggers can be delivered by synthetic toll-like receptor agonists.

TLR2-agonists in acute allergic asthma

Different studies using Mycoplasma-derived compounds underlined the immunomodulatory capacity in treatment of the acute-phase of allergic disorders.
In naïve mice, administration of the TLR2-agonist MALP-2 into the airways attracts neutrophils to the bronchoalveolar space within 24 h, underlining its ability to trigger Th1-responses. Two to three days after instillation, macrophages become more prominent. On macrophages, TLR2 agonists show clear activating effects [161]. After 72 h, lymphocytes reach their maximum contribution to cellularity of BALF. These effects decayed after 10 d [162]. Furthermore, changes in lung histology occur after MALP-2 aerosol administration, where the area of bronchus-associated lymphoid tissue is increased. The functional relevance of this finding remains to be investigated [163, 164].

In sensitised animals, different effects of TLR2-agonisation can be observed:
A therapeutic intervention of acute-phase allergic asthma could be achieved by intratracheal treatment with the TLR2/6 agonist MALP-2 in combination with the Th1 cytokine IFN-γ, which clearly reduced airway hyper responsiveness, eosinophilia and Th2-cytokines in BALF. At the same time, neutrophils and IL-12p70 were increased [98]. Similarly, treatment with a synthetic TLR2/1 ligand reduced cellular eosinophilic inflammation in the BALF, Th2-cytokine levels, i.e. IL-4 and IL-5, as well as airway hyper responsiveness, independent from IL-10 and TGF-β [99].
Additionally, TLR2/4-agonisation during allergen challenge in sensitised mice prevented allergic asthma. When incubated with DCs, the TLR2/4-agonist induced synthesis of IL-12 and TNF-α, which by itself induces IFN-γ production of T-lymphocytes. As a result, eosinophils, IL-4 and IL-13 were reduced, while neutrophil counts and IFN-γ were elevated. No increased activation of Th1-lymphocytes could be detected, though [96]. These studies point to a Th2/Th1 shift by TLR2-agonisation.
Investigations in an *in vitro* model of allergy demonstrated an induction of TNF-α and IL-10 synthesis, but not IL-12, when blood derived DCs were stimulated with MALP-2 [101], highlighting the possibility to induce tolerance.
However, also the contrary effect, the induction of Th2, could be observed: TLR2/1 agonisation aggravated allergic asthma when administered during the initial phase of the immune reaction. The type of TLR stimulation during this early phase seems to be a

determinant for the polarisation of the adaptive immune response [100].

In this work, different time-points to intervene the manifestation of allergic asthma by TLR2-agonisation with the MALP-2-derivate BPPcysMPEG in combination with the Th1-related cytokine IFN-γ were investigated in acute asthma induced by Timothy grass pollen allergens. Here, this treatment showed impressive beneficial effects on inflammation and lung physiology. A preventive treatment started as early as before and during sensitisation, reduced allergic inflammation and showed a clear trend to improve lung function. But even in already sensitised mice, BPPcysMPEG/IFN-γ could influence airway hyper responsiveness when administered before allergen challenge, and allergic inflammation when treatment was performed after allergen confrontation. However, no hint for a Th2 towards Th1-shift was observed: neutrophil counts were not altered to positive controls, or were as shown in the therapeutic treatment significantly reduced to an extent comparable to the dexamethasone effect. The mechanism of action of BPPcysMPEG/IFN-γ-treatment was therefore investigated more thoroughly in the model of chronic respiratory sensitisation.

TLR2-agonists in chronic allergic airway inflammation

The examples of successful immuno-modulations from acute-phase models of allergic asthma demonstrate the various implementations of TLR2-agonisation in treatment of acute allergic inflammation. These effects can be achieved either by shifting Th2 towards Th1, or induction of tolerance, and rises the question of intervention effects in chronic models.

In our chronic and sub-chronic model, the impressive reduction of parameters of allergic inflammation were prominent, as well as the reduction of remodelling processes in the model of chronic respiratory sensitisation after preventive treatment with the MALP-2-derivate BPPcysMPEG. However, it was not hinted at a Th2/Th1-shift. In this study, the numbers of CD11c+ and B220+ cells, as well as of CD4+foxp3+ cells in lymph nodes and lymphocytes in BALF, were unaffected. Nevertheless, no induction of BALF neutrophilia or Th1-promoting cytokines as IL-12p70 was observed and TNF-α levels were below the lower limit of detection. Therefore, a T-cell mediated effect is hypothesised.

Effects of TLR2-ligands on T-cells have already demonstrated, for example the induction

of CD4+ cells apoptosis [165]. However, the unaffected number of lymphocytes and the absence of both Th1- as well as Th2-inducing factors in the study related to this thesis rises the question, if beneficial effects of BPPcysMPEG with or without IFN-γ on chronic allergic airway inflammation are mediated by induction of an overall T-cell anergy. This anergy seems to include Th-cells as well as regulatory T-cells. T-cell anergy is a phenomenon observed also in orally administered immunotherapy [166], which may once again underline the importance of TLR2-agonist and the use of human relevant allergen sources and sensitisation routes in the model of respiratory sensitisation for immunotherapy research.
Importantly, this probably is the first study in which TLR2-agonists were tested in a chronic model, and thereby the first study demonstrating a reduction of remodelling processes.

4.4 Conclusions

It was demonstrated, that symptoms resembling human bronchial asthma can be mimicked in mouse models using human relevant Timothy grass allergens. In these models, the acute, sub-chronic and chronic phase of allergic asthma can be reflected, as well as the respiratory sensitisation to the allergen. Additionally, as a prerequisite for pharmacological testing, similar treatment effects as in humans were observed by dexamethasone. In the acute-phase model of asthma, airway hyper responsiveness could be established as a read-out parameter. As a symptom occurring after a long-lasting process in asthmatic patients, the absence of airway hyper responsiveness in the chronic models might underline the patho-mechanistical similarity; however, it could also be related to mechanisms of aging in the experimental animal.

The hygiene hypothesis relates the increasing prevalence and incidence of Th2-related allergies to decreasing confrontation to bacterial stimuli, which would normally modulate dendritic cell function and balance the immune system towards Th1. Synthetic Mycoplasma-derived toll-like receptor agonists are believed to be Th1-triggers; however, this work could show that their preventive and therapeutic effect on airway hyper reactivity and allergic airway inflammation is not mediated via a Th2/Th1-shift or induction

of tolerance, but reduction of T-cell function. This overall effect makes this approach promising in prevention and therapy of diseases related to exaggerated T-cell responses, as allergic asthma or inflammatory bowl disease.

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List of Figures

List of Abbreviations

APC	Allophycocyanin, fluorochrome
AHR	Airway hyper responsiveness
BAL/BALF	Broncheoalveolar lavage (fluid)
BPPcysMPEG	Bisacyloxypropylcystein-conjugate, TLR-agonist
BSA	Bovine serum albumin
BW	Body weight
DC	Dendritic cell
ECP	Eosinophilic cationic protein
EDTA	Ethylene-di-nitrilo-tetra acetic acid
EPX	Eosinophilic peroxidase
FACS	Fluorescent-activated cell sorting
FCS	Fetal calf serum
FELASA	Federation of European Laboratory Animal Science Associations
FOV	Field of view
FITC	Fluorescein Isothiocyanat, fluorochrome
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, buffering agent
IFN	Interferon
IL	Interleukin
i.n.	Intranasal
i.p.	Intraperitoneal
i.t.	Intratracheal
MALP-2	Macrophage-activating lipopeptide, 2kD, TLR2/6-agonist
MBP	Major basic protein
MCh	Acetyl-β-methylcholine chloride
MHC	Major histocompatibility complex
MMP	Matrix metalloproteinase
PALMs	Pollen-asociated lipid mediators
PAMP	Pathogen-associated molecular pattern
PBS	Phosphate-buffered saline

PE	Phycoerythrin, fluorochrome
P. pratense	*Phleum pratense* L., Timothy grass
PSG	Pollen starch granules
RANTES	Regulated upon activation, normally T-cell expressed, and secreted, chemokine
rPhl p5	(recombinant) major allergen group 5 of *P.pratense*
7AAD	7-Amino-Actinomycin D, dye for dead-cell exclusion
TGF	Transforming growth factor
Th	T helper
TIMP	Tissue inhibitors of matrix metalloproteinase
TLR	Toll-like receptor
TNF	Tumor necrosis factor

Appendix

Publications in scientific journals

1. Forssmann U, Hartung I, Baelder R, **Fuchs B**, Escher SE, Spodsberg N, Dulkys Y, Walden M, Heitland A, Braun A, Forssmann WG, Elsner J: **n-Nonanoyl-CC chemokine ligand 14, a potent CC chemokine ligand 14 analogue that prevents the recruitment of eosinophils in allergic airway inflammation.** *J Immunol* 2004, **173(5)**:3456-66.

2. Baelder R, **Fuchs B**, Bautsch W, Zwirner J, Kohl J, Hoymann HG, Glaab T, Erpenbeck V, Krug N, Braun A: **Pharmacological targeting of anaphylatoxin receptors during the effector phase of allergic asthma suppresses airway hyperresponsiveness and airway inflammation.** *J Immunol* 2005, **174(2)**:783-9.

3. Nassenstein C, Kammertoens Th, Veres T, Uckert W, Spies E, **Fuchs B**, Krug N, Braun A: **Neuro-immune crosstalk in asthma: Dual role of the neurotrophin receptor p75NTR.** *submitted to J Allergy Clin Immunol*

4. Gupta S, **Fuchs B**, Schulz-Maronde S, Heitland A, Escher SE, Münch J, Kirchhoff F, Mack M, Braun A, Forssmann W-G, Elsner J, Forssmann U. **Intravascular inactivation of CCR5 by n-Nonanoyl-CC chemokine ligand 14 and the prevention of allergic airway inflammation.** *submitted to J Allergy Clin Immunol*

5. Veres T, Rochlitzer S, Shevchenko M, **Fuchs B**, Prenzler F, Nassenstein C, Fischer A, Welker L, Holz O, Müller M, Krug N, Braun A: **Spatial interactions between dendritic cells and sensory nerves in allergic airway inflammation.** *submitted*

6. **Fuchs B**, Knothe S, Rochlitzer S, Müller M, Greweling M, Lauenstein HD, Nassenstein C, Müller M, Ebensen T, Krug N, Guzman CA, Braun A: **Prevention of allergic airway inflammation by dexamethasone and TLR2-ligand treatment in chronic respiratory sensitisation to grass pollen allergens.**, *to be submitted in May 2007*

7. **Fuchs B**, Braun A: **Modulation of asthma and allergy by addressing toll-like receptor 2.** *Journal of Occupational Medicine and Toxicology, invited review, to be submitted in May 2007*

8. **Fuchs B**, Braun A: **Mouse models of chronic asthma - chances and obstacles.** *Current Drug Targets, invited review for publication in September 2007*

Contribution to scientific congresses

Oral presentations

1. **Testing of toll-like receptor agonists in improved asthma models.** *6th Workshop "Animal models of asthma"* , Hannover, 2007/20.01.

2. **Asthma preventive effect of BPPcysPEG/IFN-γ treatment in a mouse model induced by grass pollen allergen.** *25th Congress of the European Academy of Allergology and Clinical Immunology (EAACI)* , Vienna, Austria, 2006/13.06.

3. **An agonist of the CC-chemokine receptor 3 as a novel approach in the treatment of allergic diseases.** *13th Annual Meeting of the Norddeutsche Immunologen* , Borstel, 2003/13.11.

Posters

1. **Toll-like receptor 2 agonist and IFN-γ modulate asthma parameters in mice chronically sensitised to grass pollen allergens via the respiratory tract.**, **Fuchs B**, Rochlitzer S, Knothe S, Müller M, Krug N, Guzman CA, Braun A, *26th Congress of the European Academy of Allergology and Clinical Immunology (EAACI)* , Gothenburg, Sweden, 2007/09.-13.06.

2. **Function of pituitary adenylate cyclase activating peptide receptor 1 in a murine model of allergic asthma.**, Lauenstein HD, Quarcoo D, **Fuchs B**, Nassenstein C, Plappert L, Hamelmann E, Fischer A, Krug N, Welte T, Braun A, Groneberg DA, *American Thoracic Society International Conference* , San Francisco, USA, 2007/18.-23.05.

3. **Activation of Toll-like receptor 2 shows preventive effects in experimental**

asthma induced by grass pollen allergen., **Fuchs B**, Lauenstein HD, Krug N, Guzman CA, Braun A, *16th European Congress of Immunology* , Paris, France, 2006/06.-09.09.

4. **Asthma preventive effect of BBPcysPEG/IFN-γ treatment in a mouse model induced by grass pollen allergen.**, **Fuchs B**, Lauenstein HD, Krug N, Guzman CA, Braun A, *EAACI /GA2LEN Summer school "Mouse models of Allergy and Asthma"* , Antalya, Turkey, 2006/26.-29.03.

5. **Grass pollen allergen induces asthma symptoms in a murine model of acute allergic asthma.**, **Fuchs B**, Erpenbeck VJ, Hohlfeld JM, Krug N, Braun A, *European Respiratory Society*, Copenhagen, Danmark, 2005/20.09.

6. **Grass-pollen induced acute asthma in mouse models.**, **Fuchs B**, Erpenbeck VJ, Krug N, Braun A, *World Allergy Congress* , Munich, 2005/26.06.-01.07.

7. **Gräserpollen-Mausmodell der allergischen Entzündung.**, **Fuchs B**, Erpenbeck VJ, Weigt H, Sommer S, Krug N, Hohlfeld JM, Braun A, *11. Mainzer Allergieworkshop*, Mainz, 2005/11.-12.03.05.

8. **CRIC3, a chemokine receptor inactivator as a novel tool in the treatment of allergic diseases. Fuchs B**, Forssmann U, Hartung I, Baelder R, Escher SE, Spodsberg N, Dulkys Y, Walden M, Heitland A, Braun A, Forssmann WG, Elsner J, *American Thoracic Society International Conference*, Orlando, USA, 2004/26.05.

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Erklärung

Hierdurch erkläre ich, dass die Dissertation

Development and applications of murine asthma models induced by grass pollen allergens: Modulation of the immune response by a toll-like receptor agonist

selbstständig verfasst und alle benutzten Hilfsmittel sowie evtl. zur Hilfeleistung herangezogene Institutionen vollständig angegeben wurden.

Die Dissertation wurde nicht schon als Diplom- oder ähnliche Prüfungsarbeit verwendet.

Hannover, den 03.05.2007

Barbara Fuchs