A Toll-like receptor 2/6-agonist reduces allergic airway inflammation in chronic respiratory sensitisation to Timothy grass pollen antigens

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\textbf{Running heads:} TLR 2/6-agonist reduces allergic airway inflammation

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Abstract

**Background:** The hygiene hypothesis negatively correlates the microbial burden of the environment to the prevalence of Th2-related disorders, e.g. allergy and asthma. This is explained by Th1-triggering through pathogen-associated molecular patterns via toll-like receptors. In this study, biological effects of a potential use of toll-like receptor (TLR) 2/6 agonists for treatment of allergic inflammation are explored. **Methods:** In a model of chronic allergic airway inflammation induced by intranasal Timothy grass pollen allergen extract administrations, early-onset TLR agonisation and/or interferon-γ administration was compared to the therapeutic and immune-modulating effects of dexamethasone in respect to the cellular inflammation and cytokine profiles. **Results:** By TLR 2/6-agonisation, eosinophilic inflammation was clearly reduced. This effects were not limited to a simultaneous administration of IFN-γ. However, lymphocyte counts were not affected among the different treatment groups. Closer determination of lymphocyte-mediated immune reaction showed that TLR2/6 agonisation neither induced CD4+foxp3+ regulatory T-cells in draining lymph nodes, nor a pronounced Th1-immune response. In contrast, dexamethasone reduced both, sensitisation as well as allergic inflammation, and, moreover, CD11c+ antigen-presenting cells in lymph nodes. Our data clearly points to the potential to rebalance Th2 skewed allergic immune responses by therapeutic TLR2/6 agonist administration. **Conclusion:** The use of the toll-like receptor 2/6 agonist is a promising therapeutic approach in diseases with misbalanced T-cell responses, such as allergy and asthma.
**Key words:** Toll-like receptor 2/6, hygiene hypothesis, chronic allergic airway inflammation, respiratory sensitisation, rebalancing of Th-responses, Timothy grass pollen allergens

**Introduction**

Allergic disorders of the respiratory tract, such as asthma and rhinitis, are believed to be caused by a sensitisation to aeroallergens and the subsequent development of severe allergic inflammation [1, 2]. This inflammation is characterised by a preponderance of Th2-lymphocytes, Th2-related cytokines, e.g. interleukin (IL)-4 and IL-5, and eosinophils [3]. Although the underlying mechanisms causing allergic diseases are not fully understood yet, genetic and environmental factors are discussed. In this context, the hygiene hypothesis was formulated, which points to an association of protection against allergy, and the microbial load of the environment [4-6]. Pathogen-associated molecular patterns (PAMPs) on microbes are sensed by the innate immune system through a variety of specific receptors. Among them, toll-like receptors (TLRs), which are expressed in a variety of both structural [7] and immune cells [8-12], play an important role. For the observed protection of farmers’ children against allergy and asthma, genetic variations in TLR2, but not in TLR4 [13], is believed to be responsible [14]. TLR2 forms heterodimers displaying a certain ligand-specificity: diacylated lipopeptides, e.g. MALP-2 [15], are sensed via TLR2/6 dimers, whereas triacylated lipopeptides, as Pam3cysSkk4, require TLR2/1 [8]. Clinical and preclinical data imply that Mycoplasma-infections, recognised via TLR2, seem to prevent establishment of allergic asthma [16, 17]. As a therapeutic approach of these findings, the small Mycoplasma-related synthetic TLR-agonists MALP-2 (Macrophage activating lipopeptide of 2kD) [18] has proven to be a potent pharmaceutical compound in several in vitro [19], preclinical and clinical studies, in the field of allergy [20], adjuvant development [21-24], wound healing [25] and cancer therapy [26]. Recently, pegylated derivatives of MALP-2 were developed, which show improved stability, solubility and efficacy (patent PZT/DE 0307892 (WO 2004/00925A3 PEG Adjuvants)).

Mouse models are useful tools to investigate the contribution of different cells and mediators to the observed allergic responses and are therefore frequently used. In recent times, mouse models of chronic allergic airway inflammation were established, which provide a higher similarity to human disease [27, 28] than acute models. Additionally, the first models were described in which allergen extracts of house dust mite, an allergen relevant to human sensitisation, could induce allergy symptoms by intranasal administration [29]. Besides
moulds and faeces enzymes of house dust mite, a main seasonal allergen source is grass pollen, e.g. of Timothy grass *Phleum pratense*. Timothy grass pollen shows high immunogenicity both in humans and mice [30]. We established a model in which mice are sensitised to timothy grass pollen allergen via the respiratory tract by chronic intranasal administrations of adjuvant-free standardised extract preparations approved for the application in humans. In this model, we proved the capacity of early on-set administration of the synthetic TLR2/6-agonist bisacyloxypropylcysteine polyethylene glycol conjugate (B), a pegylated derivative of MALP-2, to rebalance allergic immune responses, and investigated the impact on general immunological reactions. Furthermore, we wanted to elucidate whether TLR agonisation is *per se* sufficient to modulate allergic responses, or whether it shows adjuvantic activity when administered in combination with a Th1 inducer, such as IFN-γ, as was shown for the closely related compound MALP-2 before [20].

**Methods**

**Chronic intranasal sensitisation to Timothy grass pollen extract**

Female BALB/c aged 6-8 weeks were obtained from Charles River (Sulzfeld, Germany) and housed under constant light/dark cycle and humidity in specified pathogen free conditions. All animal experiments were approved by the local governmental institutions and followed the German animal welfare law. For intranasal sensitisation, 50 µl standardised Timothy grass pollen extract (kindly provided by ALK Abello, Horsholm, Danmark) equalling a dose of 9 µg major allergen was administered under inhalative 2-bromo-3-chloro-trifluoromethane (Sigma Aldrich, Taufkirchen, Germany) narcosis in a vapour system (Draeger, Hamburg, Germany) twice a week on consecutive days (Fig. 1). Negative controls received phosphate buffered saline (PBS). Groups of n=10 animals per treatment group were assessed after 30 intranasal provocations (15 weeks). Satellite groups of n=10 were sacrificed after 10 and 22 provocations to monitor the course of sensitisation.

**Treatment**

A therapeutic treatment protocol was employed to investigate the effects on late-phase allergic responses (Fig. 1). After the tenth intranasal extract instillation, when they showed raised IgG1 levels in serum, mice were treated the first time, 8 h delayed to the second extract provocation. This procedure was repeated weekly until the end of the study. Sham-treatment was performed with PBS intranasally in negative and positive controls (Neg or Pos, respectively), while other groups received 4 ng of the TLR2/6-agonist...
bisacyloxypropylcysteine polyethylene glycol conjugate /5,000 I.U. IFN-γ (Strathmann Biotech, Hamburg, Germany) (B/I), 5,000 I.U. IFN-γ (I), or 4 ng bisacyloxypropylcysteine polyethylene glycol conjugate (B) alone in 50 µl PBS, respectively. In prestudies, these doses did not result in variances of body temperature or weight, changes of the outer appearance of naive mice, nor in a detectable cellular inflammation of the lung measured in bronchoalveolar lavage fluid (BALF) (data not shown). Positive treatment control received 0.5 mg/kg bodyweight dexamethasone (Ratiopharm, Ulm, Germany) (Dex) in 200 µl sterile saline intraperitoneally 18 and 2 h before each provocation.

Obtaining bronchoalveolar lavage fluid (BALF) and serum
Twenty-four hours after the last allergen challenge, mice were injected with an overdose of pentobarbital-Na (Merial, Halbergmoos, Germany). Mice were bled via the Vena cava caudalis and serum was conserved at -70°C. Bronchoalveolar lavage (BAL) was performed with 0.8 ml ice-cold sterile PBS twice. After determination of absolute cell counts in BALF using ACT8 cytometer (Beckmann Coulter, Munich, Germany), cytospots of BALF cells were prepared on a cyto-centrifuge (Shandon, Frankfurt/Main, Germany), stained according to Pappenheim, and differential cell counts were performed to quantify and qualify allergic inflammation.

Quantification of serum IgG1 -levels
To monitor sensitisation processes towards Timothy grass extract by determination of total IgG1, serum dilutions were incubated with capture antibody bound to a 96 well Maxisorp flat bottom plate (NUNC, Wiesbaden, Germany) and detected with a 1/75,000 dilution of the secondary antibody. Enzyme reaction was stopped after 15 min by adding 2 N H₂SO₄. IgG1-standard and antibodies were taken from the IgG1 quantification kit (Bethyl Diagnostics Natutec, Frankfurt/Main, Germany). Comparison of total serum IgG1 to unsensitised controls provide a good hint for the sensitisation processes to the different allergens included in the extract used in our model.

In vitro restimulation of splenocytes
Spleens were removed and rinsed with medium (RPMI 1640+2mM HEPES, Gibco Invitrogen, Karlsruhe, Germany). Erythrocytes were lysed from the obtained cells suspension by adding lysis buffer, cells were washed and cultured in culture plates with 1*10⁵ cells per
well in RPMI 1640 (Gibco Invitrogen, Karlsruhe, Germany) +10% FCS and penicillin/streptomycin (Sigma Aldrich, Taufkirchen, Germany).

Stimulation of splenocytes was performed in a total volume of 200 µl/well with either medium or allergen extract (0.7 µg/well major allergen) (ALK-Abello, Horsholm, Denmark) for 96 h at 37°C. Supernatants of triplets of every individual were pooled and stored at -20°C for measurement of IL-5.

Cytokine quantification in BALF and supernatant of in vitro restimulation

Cell-free BALF was preserved for detection of eotaxin-2 by ELISA technique (R&D bioscience, Duo Sets, Wiesbaden-Nordenstadt, Germany) according to the manufactures instructions. Lower limit of quantification was 30.7 pg/ml. Additionally, BALF was analysed via bead-array analysis using Bioplex assay (Biorad, Munich, Germany) for the following targets (followed by lower limits of quantifications in pg/ml): IL-4 (38), IL-5 (22), RANTES (41), IL-6 (19), IL-10 (99), IFN-γ (135), IL-12p40 (27) and IL-12p70 (36). Supernatants of in vitro restimulation were investigated for IL-5 (0.3) via Bioplex assay.

Histology of cryosections and quantification of inflammatory and remodeling processes in lung tissue

Right lung lobes were filled with a 1:4 dilution of OCT kryotek (Sakura Finetek, Zoeterwoude, The Netherlands) in PBS instilled through the trachea. The lungs were removed and bedded in OCT to be immediately frozen in liquid nitrogen. Five µm thick sagittal sections displaying the main bronchus were Giemsa stained. Photographs were taken at the distal part of the main bronchus with a digital camera connected to a Zeiss AxioVision microscope (Jena, Germany).

Evaluation of histo-morphologic alterations was performed by a blinded pathologist. Lesions seen in the Giemsa-stained sections were scored for their 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 which are most common in older mice and graded as 0. 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.

Scoring results of the different features of remodeling were combined and expressed as a total
score.

Flowcytometric analysis of vital lung-draining lymph node and spleen cells

Spleens and mediastinal lymph node single cell suspensions were prepared for FACS
staining. Both types of cell suspensions were incubated with directly fluorochrome-labelled
antibodies: For determination of vital leukocytes, 7AAD (Beckman Coulter, Krefeld,
Germany)/CD45 was stained. CD11c-APC (eBioscience Natutec, Frankfurt/Main, Germany)
positive cells, also positive for CD80-FITC (AbD Serotec, Dusseldorf, Germany) and CD86-
FITC (BD Bioscience, Heidelberg, Germany) (data not shown) of spleen- and lymph-node
suspensions, as well as B220-FITC+ (BD Bioscience, Heidelberg, Germany) lymph-node
cells and appropriate isotype controls were analysed. For determination of APC-foxp3+
lymphocytes, CD4-PE (BD Pharmingen, Heidelberg, Germany) and intracellular staining was
performed according to the manufacturer´s instruction (eBioscience Natutec, Frankfurt/Main,
Germany). Flow cytometric measurement and analysis was done on a FC500 (Beckman
Coulter, Krefeld, Germany).

Statistical analysis

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(*) were observed as statistically different.

Results

Intranasal administration of Timothy grass pollen allergens causes IgG\textsubscript{1} -mediated
sensitisation, which can be prevented by dexamethasone treatment
In previous experiments, IgE serum levels could not be correlated to the allergic phenotype of the mice, nor to the beneficial outcome of the different treatments (data not shown). However, the concentration of IgG\textsubscript{1} in serum, which monitors chronic sensitisation to grass pollen allergens in mice [31], increased throughout continuous Timothy grass pollen extract challenges compared to Neg control animals receiving PBS (Fig. 2A). The treatment with dexamethasone significantly reduced serum IgG\textsubscript{1} levels at the latest time point.

In line with this finding, the number of B220+ cells in mediastinal lymph nodes was increased in Pos and reduced by dexamethasone treatment, implicating a reduced frequency of antigen presenting cells and activated lymphocytes, including antibody-secreting plasma cells (Fig. 2B).

Neither treatment with the TLR2/6 agonist, IFN-\(\gamma\) nor a combination of both could decrease IgG\textsubscript{1} levels or the number of B220+ cells in mediastinal lymph nodes.

**TLR2/6-agonisation attenuates allergic inflammation of the lung**

Allergic inflammation in Pos control animals was displayed by exaggerated total cell counts, granulocytes and lymphocytes, as well as increased concentrations of the Th2-cytokines IL-4 and IL-5 in BALF (Fig. 2C-F, Tab. 1). Dexamethasone treatment led to a significant reduction of both cellular inflammation and IL-5 levels. All groups that received a treatment with the TLR2/6-agonist and /or IFN-\(\gamma\) showed attenuated eosinophil counts and a reduction of IL-5 and IL-4, although in the later cytokine quantified the difference in the IFN-\(\gamma\)-treatment group failed to reach statistical significance. The attenuation of allergic inflammation in groups which received a TLR2/6-agonist and /or IFN-\(\gamma\)-treatment was independent from the number of lymphocytes, which remained unaffected.

Additionally, differences between the experimental groups were measured in chemokines promoting the allergic inflammation, i.e. eotaxin-2 (CCL24) and RANTES (CCL5) (Tab. 1). Both, eotaxin and RANTES levels in BALF were increased in Pos control mice compared to mice which received saline instillations only (Neg). Dexamethasone, but also the combination of the TLR2/6-agonist/IFN-\(\gamma\) and IFN-\(\gamma\) alone reduced the eotaxin-2 concentration in BALF. Moreover, the administration of the TLR2/6-agonist could diminish RANTES levels in BALF. The anti-allergic effects of dexamethasone, TLR2/6-agonist together with IFN-\(\gamma\) and IFN-\(\gamma\) alone were underlined by the histological assessment of hyperplasia, remodelling and signs of tissue inflammation (summed up to a total score of lung remodelling); these data revealed a significant improvement in TLR2/6-agonist treated animals (Fig. 3).
Although allergic inflammation was prominent in mice sensitised via the respiratory tract, no alterations in lung physiology could be detected when airway hyperreactivity towards methacholine aerosols was assessed in head-out body-plethysmography (data not shown).

Mechanisms of allergy reduction by dexamethasone, but not of the TLR2/6-agonist, are accompanied by a reduction of CD11c+ antigen presenting cells in lung draining lymph nodes

In Pos controls, measurement of CD11c+-positive antigen-presenting cells in lung draining lymph nodes showed elevated numbers compared to controls (Fig. 4). This increase was restricted to local lymph nodes and was not observed in the spleen. Dexamethasone reduced the frequency of CD11c+-cells in lymph nodes. Dexamethasone additionally reduced the baseline occurrence of CD11c+ cells in spleen, underlining its systemic suppressive effects. Treatment with either the TLR2/6-agonist and/or IFN-γ failed to reduce CD11c+-cells both in lymph nodes and spleens. Therefore, beneficial effects on allergy outcome in these treatment groups are independent from the number of CD11c+ or B220+ antigen-presenting cells.

Reduction of the allergic phenotype in the lung by TLR2/6-agonist and/or IFN-γ treatment is not caused by an increased frequency of regulatory T cells or Th1-responses

In groups treated with the TLR2/6-agonist alone or in combination with IFN-γ, but also when animals received IFN-γ, lymphocyte counts in BALF were not altered compared to sensitised and saline-treated animals. However, important mediators of T-cell responses, i.e. IL-4 and IL-5 (see above, Tab. 1), showed differences among the groups. To clarify whether these differences can be explained by changes in the polarisation of the lymphocyte population, additional experiments were performed.

Measurement of CD4+/foxp3+-regulatory T-cells in lymph nodes showed no differences among the groups (Fig. 3). When IL-10 levels were determined, positive control animals showed increased concentrations in the BALF. Dexamethasone, but also the TLR2/6-agonist, IFN-γ and the combinations of both substances caused a reduction in IL-10 levels. Therefore, we found no evidence for an increased occurrence of regulatory T-cells or raised IL-10 as a mechanism of action.

Furthermore, apart from dexamethasone, the treatment strategies showed no reduction of IL-12p40 levels in BALF nor were IL-12p70 levels elevated in any experimental group (Tab. 1). IFN-γ could only be detected in BALF when mice received intranasal IFN-γ during treatment. No induction of IFN-γ was observed when the TLR2/6-agonist was administered alone.
BALF IL-6 levels were raised in Pos controls. No tested treatment influenced IL-6 levels significantly. These results exclude an induction of a Th1-dominated immune response following TLR2/6-agonist and IFN-γ administration.

**TLR2/6-agonist alone or in combination with IFN-γ reduces splenocyte reactivity and thereby prevents manifestation of allergic airway inflammation**

In restimulation experiments when splenocytes were incubated with grass pollen allergen, Pos control released significantly higher levels of the allergy-related cytokine IL-5 compared to Neg control (Fig. 5).

A reduction of IL-5 was observed in groups treated with either dexamethasone or the TLR2/6-agonist in combination with IFN-γ. This further underlines the anti-allergic actions of this therapeutic strategy.

Together with our *in vivo* data, the results provide evidence that treatment with a TLR2/6-agonist in combination with IFN-γ or alone reduces T-cell responses, and thereby prevents the manifestation of allergic inflammation.

**Discussion**

In this study, we tested the outcome of dexamethasone, a standard drug for allergic symptoms, and a TLR2/6-agonist alone or in combination with IFN-γ in a model of chronic respiratory sensitisation to pollen allergens of Timothy grass. This model might reflect the processes during sensitisation in patients more thoroughly, considering a) it employs a more relevant allergen than ovalbumin, normally used in murine models, b) it uses a more physiological route of sensitisation, and c) the efficiency of dexamethasone comparable to what is seen in patients. In our model dexamethasone ameliorated inflammatory as well as remodelling parameters. For these positive observations, the reduction of CD11c+ cells seems to be the crucial step. It is suggested that the massive increase of mediastinal lymph node- and lung-dendritic cells (DCs) as well as eosinophils in allergic asthma is related to an exaggerated recruitment of CD31hi Ly-6Cneg bone marrow precursor cells [32]. We hypothesise that dexamethasone treatment leads to a reduction of CD11c+ cells [33] in our model, resulting in less lymphocyte activation and inflammation, as shown by the reduced chemokine and cytokine levels, and the decrease of inflammatory cells in BALF and lung tissue. Moreover, the reduction of T-cell cytokines responsible for isotype-switching, and the diminished number of B220+ cells, results in low serum IgG1. Additionally, remodelling processes were less developed in dexamethasone treated animals.
Different studies using *Mycoplasma*-derived compounds underlined their immunomodulatory capacity in the treatment of allergic disorders. Treatment with a synthetic TLR2/1 ligand reduced total cells and eosinophil counts in BALF, as well as IL-4 and IL-5 levels and airway hyperresponsiveness (AHR). These reductions were independent from IL-10 and TGF-β [34], rather implicating a shift to a Th1-reaction than an induction of tolerance to be responsible for these observations. 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 the TLR2/6 agonist MALP-2 [19]. Additionally, a therapeutic intervention could be achieved by the use of MALP-2 *in vivo*. Intratracheal treatment with this agonist in combination with the Th1-cytokine IFN-γ clearly reduced AHR, eosinophilia and Th2 cytokines in BALF, whereas neutrophils and IL-12p70 were induced [20]. These examples demonstrate the various implementations of TLR2- agonisation in treatment of allergic disorders in terms of modulation of the immune response to Th1 or tolerance. Recently, TLR4-induced IFN-γ was reported to enhance TLR2 expression and receptor sensitivity [35]. However, an enhanced reduction of inflammatory parameters by a combinatory treatment with IFN-γ and a TLR2/6-agonist was in our hands not significantly pronounced.

The reduction of parameters of allergic inflammation after treatment with the synthetic MALP-2-derivate bisacyloxypropyl cystein used in this study in chronic respiratory sensitisation presumably depends on a different mechanism than the induction of a shift in lymphocyte populations, or the shift of a Th2- response towards a full blown Th1. In our study, the numbers of CD11c+, B220+ and CD4+foxp3+ cells in lymph nodes and lymphocytes in BALF were unaffected. Nevertheless, no further increase of BALF neutrophilia in comparison to untreated sensitised animals or Th1-promoting cytokines (e.g., IL-12p70) was observed. To our knowledge this is the first study in which TLR2/6-agonisation was tested in a chronic model of respiratory sensitisation, demonstrating a reduction of chronic inflammation. Our data also showed that attenuation of allergic inflammation, i.e. eosinophil counts and cytokines, by early on-set administration of the TLR2/6-agonist alone or in combination with IFN-γ does not interfere with systemic reactions, such as occurrence of regulatory T-cells or the induction of a Th1-type immune response as it was described for the related TLR2/6 agonist MALP-2, but rather to rebalancing of Th-type responses. Also, we did not observe inflammation promoting
processes when IFN-\(\gamma\) or a TLR2/6-agonist were administered alone as described previously [20]. This effect makes this treatment a promising approach in diseases caused by misbalanced T-cell responses, such as allergy and asthma.

Acknowledgements

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### Table 1

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Tab. 1: BALF cytokine and chemokine levels (Mean ± (SEM)). BALF was obtained after 15 weeks of intranasal timothy grass pollen extract instillations. From the fifth week on, treatment was performed as follows: Neg sham-sensitised sham-treated negative control, Pos sham-treated positive control, Dex dexamethasone-treated positive treatment control, B/I TLR2/6-agonist/IFN-γ-combinatory treatment, I IFN-γ treatment, B TLR2/6 agonist treatment. n=10 mice per group were analysed. (nd= not detected, * = p<0.05 vs. Pos).

Fig. 1: Experimental protocol and treatment groups included in the study. n=10 mice per group received 15 weeks of intranasal timothy grass pollen extract instillations. From the fifth week on, treatment was performed as written in the figure. Sampling was performed 24h after the last extract provocations. BALF, serum, spleens and lung-draining lymph nodes were collected for subsequent analyses. Satellite groups of n=10 were analysed after 5 and 11 weeks to monitor the course of sensitisation.

Fig. 2: Allergic sensitisation and inflammation in respiratory sensitisation towards grass-pollen allergens. A IgG1 levels in serum measured by ELISA, B B220+ cells in lung-draining lymph nodes as measured after FACS-staining. Cellular inflammation assessed in BALF by differential cell counts: total cell counts, lymphocytes, neutrophils, and eosinophils (C-F). All data was assessed after 15 weeks of intranasal allergen extract instillation. Neg sham-sensitised sham-treated negative control, Pos sham-treated positive control, Dex dexamethasone-treated positive treatment control, B/I TLR2/6-agonist/IFN-γ-combinatory treatment, I IFN-γ treatment, B TLR2/6 agonist treatment (Mean ± (SEM), * = p<0.05 vs. Pos).

Fig. 3: Lung histology. Kryosections were Giemsa-stained and scored for overall severity of alterations, severity of infiltration, and the severity of hyperplasia by a blinded pathologist (see Methods). Scores in the different categories were summed up and are depicted as the total score of remodeling in the different experimental groups. Neg sham-sensitised sham-treated negative control, Pos sham-treated positive control, Dex dexamethasone-treated positive treatment control, B/I TLR2/6-agonist/IFN-γ-combinatory treatment, I IFN-γ treatment, B TLR2/6 agonist treatment (Mean ± (SEM), * = p<0.05 vs. Pos).
Fig. 4: **Frequency of regulatory T cells.** The number of regulatory T cells was assessed as CD4+ CD4+foxp3+ lymph node cells (B). **Frequency of local and systemic antigen-presenting cells.** CD11c+ cells in lymph nodes (C) and spleen (D). The data was assessed after 15 weeks of intranasal allergen extract instillation. Neg sham-sensitised sham-treated negative control, Pos sham-treated positive control, Dex dexamethasone-treated positive treatment control, B/I TLR2/6-agonist/IFN-γ-combinatory treatment, I IFN-γ treatment, B TLR2/6 agonist treatment (Mean ± (SEM), * = p<0.05 vs. Pos).

Fig. 5: **IL-5 in supernatants of in vitro allergen-restimulated splenocytes.** Following respiratory sensitisation, splenocytes were incubated with 0.7 µg major allergens for 96h, supernatant were collected and IL-5 levels were determined. Neg sham-sensitised sham-treated negative control, Pos sham-treated positive control, Dex dexamethasone-treated positive treatment control, B/I TLR2/6-agonist/IFN-γ-combinatory treatment, I IFN-γ treatment, B TLR2/6 agonist treatment (Mean ± (SEM), * = p<0.05 vs. Pos).