A Toll-Like Receptor 2/6 Agonist Reduces Allergic Airway Inflammation in Chronic Respiratory Sensitisation to Timothy Grass Pollen Antigens

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Key Words

Toll-like receptor 2/6 • Hygiene hypothesis • Chronic allergic airway inflammation • Respiratory sensitisation • Rebalancing of T helper responses • Timothy grass pollen allergens

Abstract

Background: The hygiene hypothesis negatively correlates the microbial burden of the environment with the prevalence of T helper type 2 (Th2)-related disorders, e.g. allergy and asthma. This is explained by Th1 triggering through pathogen-associated molecular patterns via Toll-like receptors (TLRs). In this study, the biological effects of a TLR2/6 agonist as a potential treatment of allergic inflammation are explored. **Methods:** In a model of chronic allergic airway inflammation induced by intranasal administration of Timothy grass pollen allergen extract, early TLR agonism and/or interferon (IFN)- γ administration was compared to the therapeutic and immune-modulating effects of dexamethasone

with regard to the cellular inflammation and cytokine profiles. **Results:** Eosinophilic inflammation was clearly reduced by TLR2/6 agonism. This effect was also seen without simultaneous administration of IFN- γ . However, lymphocyte counts were not affected among the different treatment groups. More precise determination of the lymphocyte-mediated immune reaction showed that TLR2/6 agonism induced neither 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, in addition, CD11c+ antigen-presenting cells in lymph nodes. Our data clearly point to the potential to rebalance Th2-skewed allergic immune responses by therapeutic TLR2/6 agonist administration. **Conclusion:** The use of the TLR2/6 agonist is a promising therapeutic approach in diseases with an imbalance in T cell responses, such as allergy and asthma.

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Introduction

Allergic disorders of the respiratory tract, such as asthma and rhinitis, are believed to be caused by sensitisation to aeroallergens and the subsequent development of severe allergic inflammation [1, 2]. This inflammation is characterised by a preponderance of T helper type 2 (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 yet fully understood, genetic and environmental factors have been proposed. In this context, the hygiene hypothesis was formulated, which points to an association between protection against allergy and the microbial load of the environment [4–6]. Pathogen-associated molecular patterns 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. With regard to the observed protection of farmers’ children against allergy and asthma, genetic variations in TLR2, but not in TLR4 [13], are believed to be responsible [14]. TLR2 forms heterodimers displaying a certain ligand specificity; diacylated lipopeptides, e.g. macrophage-activating lipopeptide of 2 kDa (MALP-2) [15], are sensed via TLR2/6 dimers, whereas triacylated lipopeptides, such as Pam₃cysSK₄, require TLR2/1 [8]. Clinical and preclinical data imply that *Mycoplasma* infections, recognised via TLR2, seem to prevent the establishment of allergic asthma [16, 17]. With regard to the therapeutic potential of these findings, the small *Mycoplasma*-related synthetic TLR agonist MALP-2 [18] has proven to be a potent pharmaceutical compound in several in vitro [19], preclinical and clinical studies in the fields 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 observed allergic responses and are therefore frequently used. In recent times, mouse models of chronic allergic airway inflammation have been established which offer greater similarity to human disease [27, 28] than models of acute inflammation. Additionally, the first models have been described in which allergen extracts of house dust mite, an allergen relevant to human sensitisation, were able to 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 in both 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 administration of adjuvant-free standardised extract preparations approved for application in humans. It was demonstrated previously that preventive treatment of intraperitoneally ovalbumin-sensitised animals with MALP-2 or interferon (IFN)- γ promoted the development of allergic inflammation [20]. However, the combination of the two treatments showed a beneficial effect.

In this model of respiratory sensitisation to grass pollen allergens, we wanted to investigate the capacity of early administration of the synthetic TLR2/6 agonist bisacyloxypropylcysteine polyethylene glycol conjugate, a pegylated derivative of MALP-2, to rebalance allergic immune responses in sensitised animals. Furthermore, we wanted to investigate the impact on general immunological reactions. We also addressed the question whether TLR agonism per se is sufficient to modulate allergic responses or whether simultaneous administration of a Th1 inducer, such as IFN- γ , is required to attenuate allergic inflammation.

Methods

Chronic Intranasal Sensitisation to Timothy Grass Pollen Extract

Female BALB/c mice aged 6–8 weeks were obtained from Charles River (Sulzfeld, Germany) and housed under a constant light/dark cycle and constant humidity in specified pathogen-free conditions. All animal experiments were approved by local governmental institutions and complied with German animal welfare law. For intranasal sensitisation, 50 μ l of standardised Timothy grass pollen extract (kindly provided by ALK Abello, Horsholm, Denmark) equalling a dose of 9 μ g of major allergen was administered under inhalative 2-bromo-3-chloro-trifluoran

Abbreviations used in figures 1–5

Neg	Sham-sensitised, sham-treated negative controls
Pos	Sham-treated positive controls
Dex	Dexamethasone-treated positive treatment controls
B/I	TLR2/6 agonist/IFN- γ combination treatment
I	IFN- γ treatment
B	TLR2/6 agonist treatment

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

(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 10 animals per treatment were assessed after 30 intranasal provocations (15 weeks). Satellite groups of 10 animals were sacrificed after 10 and 22 provocations to monitor the course of sensitisation.

Treatment

A therapeutic treatment protocol was employed to investigate the effects of treatment on late-phase allergic responses (fig. 1). After the tenth intranasal extract instillation, when they showed raised IgG1 levels in serum, mice were treated for the first time, 8 h after the second extract provocation. This procedure was repeated weekly until the end of the study. Sham treatment was performed with intranasal PBS in negative and positive controls, while other groups received 4 ng of the TLR2/6 agonist bisacyloxypropylcysteine polyethylene glycol conjugate plus 5,000 IU of IFN-γ (Strathmann Biotech, Hamburg, Germany), 5,000 IU of IFN-γ alone or 4 ng of bisacyloxypropylcysteine polyethylene glycol conjugate alone in 50 µl of PBS. In preliminary studies, these doses did not result in variances in body temperature or weight, changes in the outer appearance of naive mice or detectable cellular inflammation of the lung measured in bronchoalveolar lavage fluid (BALF) (data not shown). Positive treatment control mice received 0.5 mg/kg body weight dexamethasone (Ratio-pharm, Ulm, Germany) in 200 µl of sterile saline intraperitoneally 18 and 2 h before each provocation.

Obtaining BALF and Serum

Twenty-four hours after the last allergen challenge, mice were injected with an overdose of pentobarbital-Na (Merck, Halbergmoos, Germany). Mice were bled via the vena cava caudalis, and serum was conserved at -70°C. Bronchoalveolar lavage was performed twice with 0.8 ml of ice-cold sterile PBS. After determination of absolute cell counts in BALF using an ACT8 cytometer (Beckmann Coulter, Munich, Germany), cytopots 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-bottomed plate (Nunc, Wiesbaden, Germany) and detected with a 1/75,000 dilution of the secondary antibody. The 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). The clear increase in total serum IgG1 after allergen exposure in comparison to unsensitised controls provides a strong indication of an ongoing sensitisation process. Since grass pollen extract consist of different allergens, it was beyond the scope of this study to establish an allergen-specific Ig assay.

In vitro Restimulation of Splenocytes

Spleens were removed and rinsed with medium (RPMI-1640 + 2 mM HEPES; Gibco Invitrogen, Karlsruhe, Germany). Erythrocytes were lysed from the obtained cell suspension by adding lysis buffer, then cells were washed and cultured in culture plates with 1×10^5 cells per well in RPMI-1640 (Gibco Invitrogen) plus 10% FCS and penicillin/streptomycin (Sigma Aldrich).

Stimulation of splenocytes was performed in a total volume of 200 µl/well with either medium or allergen extract (0.7 µg/well of major allergen; ALK Abello) for 96 h at 37°C. Supernatants in triplicate from 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 the detection of eotaxin-2 by ELISA (Duo Sets, R&D Bioscience, Wiesbaden-Nordenstadt, Germany) according to the manufacturer's instructions. The lower limit of quantification was 30.7 pg/ml. Additionally, BALF was analysed via bead array analysis using a Bioplex assay (Biorad, Munich, Germany) for the following targets (followed by lower limits of quantification 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). The Bioplex assay was also used to investigate supernatants of in vitro restimulation for IL-5 (0.3).

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 cryotek (Sakura Finetek, Zoeterwoude, The Netherlands) in PBS instilled through the trachea. The lungs were removed and embedded in OCT to be immediately frozen in liquid nitrogen. Five-micrometer-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 histomorphological alterations was performed by a pathologist blinded to the treatment group. 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). The amount of inflammatory infiltrates was graded from 0 to 3 using the following criteria: 0 = very low amounts of inflammatory infiltrates near the primary bronchus (considered background lesions, which 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, 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 for the different features of remodeling were combined and expressed as a total score.

Flow Cytometric Analysis of Vital Lung-Draining Lymph Node and Spleen Cells

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

Statistical Analysis

To detect significant differences between groups, one-way ANOVA followed by Dunnett's multi-comparison test and Student's *t* test were applied using the Graph Pad Prism 4 software. Results of $p < 0.05$ were regarded as statistically significant.

Results

Intranasal Administration of Timothy Grass Pollen Allergens Causes IgG1-Mediated Sensitisation Which Can Be Prevented by Dexamethasone Treatment

In previous experiments, IgE serum levels could not be correlated with the allergic phenotype of the mice nor with the beneficial outcome of the different treatments (data not shown). However, the concentration of IgG1 in serum, which monitors chronic sensitisation to grass pollen allergens in mice [31], increased throughout continuous Timothy grass pollen extract challenges compared to negative control animals receiving PBS (fig. 2a). The treatment with dexamethasone significantly reduced serum IgG1 levels at the last time point.

In line with this finding, the number of B220+ cells in mediastinal lymph nodes was increased in positive controls and reduced by dexamethasone treatment, indicating 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- γ nor a combination of both could decrease IgG1 levels or the number of B220+ cells in mediastinal lymph nodes.

TLR2/6 Agonism Attenuates Allergic Inflammation of the Lung

Allergic inflammation in positive control animals was demonstrated 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, table 1). Dexamethasone treatment led to a significant reduction of both cellular inflammation and IL-5 levels. All groups that received treatment with the TLR2/6 agonist and/or IFN- γ showed attenuated eosinophil counts and a reduction of IL-5 and IL-4, although in the IFN- γ treatment group, the difference in IL-4 failed to reach statistical significance. The attenuation of allergic inflammation in groups which received TLR2/6 agonist and/or IFN- γ treatment was independent of the number of lymphocytes, which remained unaffected.

Additionally, differences between the experimental groups were measured by the levels of chemokines promoting allergic inflammation, i.e. eotaxin-2 (CCL24) and RANTES (CCL5) (table 1). Both eotaxin and RANTES levels in BALF were increased in positive control mice compared to mice which received saline instillations only (negative controls). Dexamethasone, but also the combination of the TLR2/6 agonist and IFN- γ and IFN- γ alone reduced the eotaxin-2 concentration in

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. **c–f** Cellular inflammation assessed in BALF by differential cell counts, i.e. total cell counts (**c**), lymphocytes (**d**), neutrophils (**e**) and eosinophils (**f**). All data were assessed after 15 weeks of intranasal allergen extract instillation. Results are shown as means $\pm$ SEM. * $p < 0.05$ versus sham-treated positive controls.

Table 1. BALF cytokine and chemokine levels

	Neg	Pos	Dex	B/I	I	B
<i>Th1</i>						
IL-12p40, pg/ml	142.1 $\pm$ 8.3*	231.3 $\pm$ 13.8	179 $\pm$ 20.7*	272.1 $\pm$ 32	259.7 $\pm$ 21.7	212.5 $\pm$ 15.4
IL-12p70, pg/ml	n.d.	43.1 $\pm$ 21.1	42.2 $\pm$ 21.3	n.d.	n.d.	n.d.
IFN- γ , pg/ml	n.d.	n.d.	n.d.	695.1 $\pm$ 123	906.4 $\pm$ 152.4	n.d.
IL-6, pg/ml	57.4 $\pm$ 4.5*	114.8 $\pm$ 11.4	88.9 $\pm$ 18.9	108.2 $\pm$ 17.3	139.7 $\pm$ 36.8	83.8 $\pm$ 24.6
<i>Th2</i>						
Eotaxin-2, pg/ml	88.8 $\pm$ 10.1*	602.3 $\pm$ 56.6	186 $\pm$ 39.4*	318 $\pm$ 79.4*	195.4 $\pm$ 30*	447.4 $\pm$ 76.4
RANTES, pg/ml	n.d.	100.1 $\pm$ 17.4	70.5 $\pm$ 28.8	108.1 $\pm$ 15.3	124.7 $\pm$ 8.9	56.4 $\pm$ 14.9*
IL-4, pg/ml	n.d.	249.6 $\pm$ 24.9	204.6 $\pm$ 76.6	139.0 $\pm$ 24.9*	159.2 $\pm$ 53.6	171.4 $\pm$ 32.7*
IL-5, pg/ml	33.6 $\pm$ 3.6*	218.6 $\pm$ 17.8	81.5 $\pm$ 17.6*	115.2 $\pm$ 14.1*	110.3 $\pm$ 15.5*	120.7 $\pm$ 14.4*
<i>Treg</i>						
IL-10, pg/ml	133.6 $\pm$ 5.9*	216.6 $\pm$ 16.7	166.5 $\pm$ 12.7*	150.6 $\pm$ 16.3*	125.4 $\pm$ 9.9*	137.7 $\pm$ 11.3*

Values are means $\pm$ 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 controls; Pos = sham-treated positive controls; Dex = dexamethasone-treated positive treat-

ment controls; B/I = TLR2/6 agonist/IFN- γ combination treatment; I = IFN- γ treatment; B = TLR2/6 agonist treatment; n.d. = not detected; Treg = regulatory T cells. Ten mice per group were analysed.

* $p < 0.05$ versus sham-treated positive controls.

Fig. 3. Lung histology. Cryosections were Giemsa stained and scored for overall severity of alterations, severity of infiltration and severity of hyperplasia by a pathologist blinded to the treatment group (see Methods). Scores in the different categories were summarised and are depicted as the total score for remodeling in the different experimental groups. Results are means $\pm$ SEM. * $p < 0.05$ versus sham-treated positive controls.

BALF. Moreover, administration of the TLR2/6 agonist was able to diminish RANTES levels in BALF. The anti-allergic effects of dexamethasone, TLR2/6 agonist together with IFN- γ and IFN- γ alone were further investigated by the histological assessment of hyperplasia, remodelling and signs of tissue inflammation (summed for a total score for 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 in CD11c+ Antigen-Presenting Cells in Lung-Draining Lymph Nodes

Measurement of CD11c+ antigen-presenting cells in lung-draining lymph nodes showed elevated numbers in positive controls compared to negative 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 the TLR2/6 agonist and/or IFN- γ failed to reduce CD11c+ cells in both lymph nodes and spleens. Therefore, beneficial effects on allergy outcome

Fig. 4. **a** The frequency of regulatory T cells was assessed as the percentage of CD4+foxp3+ lymph node cells. **b, c** Frequency of local and systemic antigen-presenting cells. CD11c+ cells in lymph nodes (**b**) and spleen (**c**). The data were assessed after 15 weeks of intranasal allergen extract instillation. Results are means $\pm$ SEM. * $p < 0.05$ versus sham-treated positive controls.

Fig. 5. IL-5 in supernatants of in vitro allergen-restimulated splenocytes. Following respiratory sensitisation, splenocytes were incubated with 0.7 μ g of major allergens for 96 h, then supernatants were collected and IL-5 levels were determined. Results are means $\pm$ SEM. * $p < 0.05$ versus sham-treated positive controls.

in these treatment groups were independent of 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 in animals that only 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 (table 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. 4). 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 combination of both substances caused a reduction in IL-10 levels. Therefore, we found no evidence of 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 (table 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. IL-6 levels in BALF were raised in positive controls. No tested treatment influenced IL-6 levels significantly. These results exclude the possibility of induction of a Th1-dominated immune response following administration of TLR2/6 agonist and IFN- γ .

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, positive controls 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 effect 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 occurring during sensitisation in patients more accurately, considering that (1) it employs a more relevant allergen than ovalbumin, normally used in murine models, (2) it uses a more physiological route of sensitisation and (3) the efficiency of dexamethasone is comparable to what is seen in patients. In our model, dexamethasone ameliorated inflammatory as well as remodelling parameters. The reduction of CD11c+ cells seems to be the crucial step for these positive results. It is suggested that the massive increase in mediastinal lymph node and lung dendritic cells as well as eosinophils in allergic asthma is related to an exaggerated recruitment of CD31^{hi}/Ly-6C^{neg} 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 result 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. These reductions were independent of IL-10 and TGF- β [34], indicating that a shift to a Th1 reaction is responsible for these observations rather than an induction of tolerance. 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 dendritic cells were stimulated with the TLR2/6

agonist MALP-2 [19]. Additionally, a therapeutic effect 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 airway hyperresponsiveness, eosinophilia and Th2 cytokines in BALF, whereas neutrophils and IL-12p70 were induced [20]. These examples demonstrate the various possible implementations of TLR2 agonism in the 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, enhanced reduction of inflammatory parameters by a combinatory treatment with IFN- γ and a TLR2/6 agonist was not significantly pronounced in the present study.

The reduction of parameters of allergic inflammation after treatment with the synthetic MALP-2 derivative bisacyloxypropylcysteine used in this study of 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 Th1 response.

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 in 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 agonism was tested in a chronic model of respiratory sensitisation, demonstrating a reduction in chronic inflammation. Our data also showed that attenuation of allergic inflammation, i.e. eosinophil counts and cytokines, by early-onset administration of TLR2/6 agonist alone or in combination with IFN- γ does not interfere with systemic reactions, such as the occurrence of regulatory T cells or the induction of a Th1-type immune response as was described for the related TLR2/6 agonist MALP-2, but rather is associated with rebalancing of Th-type responses. Also, we did not observe inflammation-promoting processes when IFN- γ or the TLR2/6 agonist were administered alone as described previously [20]. This effect makes this treatment a promising approach in diseases caused by an imbalance in T cell responses, such as allergy and asthma.

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