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Local treatment with BPPcysMPEG reduces allergic airway inflammation in sensitized mice

BPPcysMPEG reduces airway inflammation

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Key words

allergic airway inflammation, BPPcysMPEG, immunomodulation, Th1, TLR2/6 agonist

Abbreviations

Alum: aluminium hydroxide, ANOVA: analysis of variance, BAL: bronchoalveolar lavage, BPPcysMPEG: pegylated bisacyloxypropylcysteine, ConA: concanavalin A, DC: dendritic cells, ELISA: enzyme linked immunosorbent assay, iDC: immature dendritic cells, IL: interleukin, IFN: interferon, i.n.: intranasal, i.p.: intraperitoneal, MALP-2: macrophage-activating lipopeptide of 2 kDa, PBS: phosphate buffered saline, *P. pratense*: *Phleum pratense* L., Timothy grass, rPhl p 5: recombinant *P. pratense* major allergen 5, TCR: T cell receptor, Th: T helper, Treg: T regulatory

Abstract

According to the hygiene hypothesis, triggering the immune system with microbial components during childhood balances the inherent Th2 bias. In contrast, specific immunotherapy involves exposure of the patient to the allergen in order to achieve desensitization to subsequent contact. In a human *in vitro* allergy model the potential of the TLR2/6 agonist BPPcysMPEG to modulate antigen presenting cells and allergen-specific immune responses was evaluated. Specific immunomodulation via co-administration of the allergen and BPPcysMPEG enhanced expression of co-stimulatory molecules on DC and increased secretion of the proinflammatory cytokine TNF- α . Acting as an adjuvant, BPPcysMPEG elevated allergen-specific immune responses in co-culture with autologous lymphocytes. Although administration of BPPcysMPEG alone enhanced expression of co-stimulatory molecules on DC, proliferation of autologous lymphocytes was not induced.

Based on this finding, the potential of BPPcysMPEG to reduce allergic airway inflammation by preventive modulation of the innate immune system via TLR2/6 agonization was investigated in mice. Local administration of BPPcysMPEG altered cellular influx and cell composition in BAL fluid. Furthermore, the Th2-associated cytokines IL-4 and IL-5 were diminished. Allergen-specific restimulation of cells from mediastinal lymph nodes and splenocytes suggested an alteration of immune responses. The treatment with BPPcysMPEG induced a Th1-dominated cytokine milieu in mediastinal lymph nodes, while allergen-specific immune responses in splenocytes were diminished. The co-administration of allergen and BPPcysMPEG reduced cytokine secretion upon restimulation in mediastinal lymph nodes and splenocytes.

From these data we conclude that BPPcysMPEG was able to influence the immune system with regard to subsequent allergen contact by TLR2/6 agonization.

Introduction

Allergic immune responses to harmless antigens are characterized by an imbalance between T regulatory, T helper 1 (Th1), and T helper 2 (Th2) cells. In addition, Th17 cells have recently been described to contribute to the pathogenesis of allergic asthma (Zhao et al., 2009). The development of allergic airway diseases includes an initial sensitization phase to an aeroallergen. During this sensitization phase, priming of allergen-specific CD4-positive Th2 lymphocytes results in secretion of Th2-associated cytokines, which induce immunoglobulin class switching and thus IgE production of B cells, mucus secretion, and activation of endothelial cells. This facilitates migration of Th2 cells and eosinophils into the tissue. The binding of IgE to the high-affinity receptor of IgE (FcεRI) sensitizes mast cells and basophils for subsequent cross-linking by the allergen. This cross-linking of the IgE-FcεRI-complex leads to degranulation of preformed mediators including histamine, prostaglandines, leukotrienes, as well as cytokines and chemokines characterizing the immediate phase of an allergic immune response (Akdis, 2006; Romagnani, 2004). During the late phase of an allergic reaction, persistence of an allergic airway inflammation depends on the presence of CD4-positive T lymphocytes (Brusselle et al., 1994; Cohn et al., 1998; Gavett et al., 1994). In the milieu of a polarizing Th2 cytokine pattern, T lymphocytes differentiate into Th2 cells in the peripheral lymphoid organs upon antigen recognition. They are characterized by a distinct cytokine pattern and associated effector functions, including induction of hyper-IgE, eosinophil survival, mucus hyperproduction, and interaction with resident tissue cells (Abbas et al., 1996; Akdis et al., 2004; O'Garra, 1998).

Although the underlying mechanisms of allergic diseases are not fully understood, their increasing prevalence seems to be associated with environmental factors. The hygiene hypothesis postulates that the decreased incidence of infectious diseases and the urbanized lifestyle during childhood are related to an increased incidence of immunological diseases (Bach, 2002). During infection, highly conserved microbial structures, the pathogen-associated molecular patterns (PAMP), are recognized by pattern-recognition receptors (PRR) expressed on a variety of cells of the innate immune system. Signaling through PRR induces proinflammatory cytokine release, thereby providing a constant Th1 triggering of the immune system. Based on this, several approaches for the treatment of allergic diseases focus on targeting the innate immune system to induce a counterbalancing inflammatory immune response (Akdis et al., 2007; Lombardi et al., 2008; Sel et al., 2007). Toll-like receptors (TLR) are part of the PRR-mediated innate immunity and are widely expressed on a variety of cell types, including immune cells as well as non hematopoietic epithelial and endothelial cells (West et al., 2006). Ten TLR in humans and 13 in mice have been identified so far (Beutler, 2004). TLR2 recognizes a wide range of microbial products and generally functions as a heterodimer

with either TLR1 or TLR6 (Ozinsky et al., 2000). Recent studies showed that triacylated lipoproteins are preferentially sensed by the TLR2/1 heterodimer, whereas diacylated lipoproteins are sensed by the TLR2/6 heterodimer (Takeuchi et al., 2002). Genetic variations in TLR2 have been found to affect susceptibility to allergies and asthma (Eder et al., 2004), whereas TLR4 did not (Raby et al., 2002). In addition, a recent study demonstrated that a TLR2 agonist in German cockroach frass mediates protection in allergic airway inflammation in mice (Page et al., 2009). The pharmaceutical efficacy of the *Mycoplasma fermentans*-derived macrophage-activating lipopeptide of 2 kDa (MALP-2), which signals through TLR2 and TLR6 (Takeuchi et al., 2001), was shown in several approaches concerning wound-healing (Niebuhr et al., 2008), vaccination (Borsutzky et al., 2006), tumor therapy (Schneider et al., 2004), infection (Reppe et al., 2009), and treatment of airway inflammation (Weigt et al., 2005). The synthetic MALP-2 derivate pegylated bisacyloxypropylcysteine (BPPcysMPEG) shows improved properties regarding solubility. A recent study by our group demonstrated the therapeutic potential of BPPcysMPEG in combination with IFN- γ to reduce allergic airway inflammation in chronic respiratory sensitization to Timothy grass pollen antigens (Fuchs et al., 2009). In the present study, we aimed to evaluate the efficacy of BPPcysMPEG alone to modulate antigen presenting cells and the subsequent immune response in a human *in vitro* allergy model. Based on these findings, the potential of preventive administration of BPPcysMPEG to reduce allergic airway inflammation by TLR2/6 agonization in a mouse model was investigated.

Material and methods

Patients

The study included male and female subjects (aged 18-55 years) with a history of allergy to grass pollen and a positive skin prick test for *Phleum pratense* pollen (ALK Scherax, Hamburg, Germany) at or within 12 months prior to their first visit. During a first visit, demographic data including medical and allergy (atopic) history, concomitant medication review, physical examination, vital signs, laboratory tests (hematology, biochemistry, and urinalysis), skin prick test (if not performed within the last 12 month), and a pregnancy test for female subjects were obtained. During a second visit, 200 ml of blood were drawn. The study was approved by the ethics committee of the Hannover Medical School (Hannover, Germany) and was performed according to standard operating procedures.

Human in vitro allergy model

Generation of immature dendritic cells (iDC) and co-culture of stimulated dendritic cells (DC) were performed as recently described (Weigt et al., 2004). In brief, iDC were generated from CD14-positive monocytes obtained from whole blood of allergic donors. On day 5, cells were stimulated with 20 µg/ml *P. pratense* extract (kindly provided by ALK Abello, Hørsholm, Denmark) with or without 25 ng/ml BPPcysMPEG (patent number WO 2004/009125 A2 Mühlradt/Morr) for two additional days. On day 7, cells were harvested and co-cultured with autologous lymphocytes for five days (Fig. 1 A).

Animal model

Female BALB/c mice (Charles River, Sulzfeld, Germany) aged 6-8 weeks were sensitized intraperitoneally (i.p.) on days 0, 14, and 21 with 5 µg recombinant *P. pratense* major allergen 5 (rPhl p5) (Biomay, Vienna, Austria) or saline adsorbed to 1 mg aluminium hydroxide (alum) (Pierce, Rockford, USA) in a total volume of 200 µl sterile saline. From day 28 to day 35, animals were treated daily with 1 µg BPPcysMPEG with or without the allergen, administered intranasally (i.n.). Controls received allergen or saline. I.n. challenges with *P. pratense* extract containing 9 µg of the major allergen Phl p5 in 50 µl sterile saline were performed on days 42 and 43. On day 44, animals were sacrificed (Fig. 1 B). The different study groups are depicted in Table 1. Animal experiments were performed in compliance with the German animal protection law under a protocol approved by the competent governmental authority.

Animal dissection

Animals were sacrificed by i.p. injection of an overdose of pentobarbital-Na (Merial, Hallbergmoos, Germany). Bronchoalveolar lavage (BAL) was performed twice with 0.8 ml ice-cold saline. The fluid was then centrifuged and aliquots of supernatant were stored at -80 °C until analysis of proteins. The cell pellet was resuspended in 0.5 ml PBS and a differential cell count was performed. Mediastinal lymph nodes and the spleen were removed and properties were determined.

Allergen-specific restimulation of cells from mediastinal lymph nodes and splenocytes

Using 96-well round bottom plates, 1×10^5 cells from mediastinal lymph nodes or splenocytes were cultured in RPMI 1640 (Lonza, Verviers, Belgium) containing 5% FCS (Sigma, Taufkirchen, Germany) with or without 20 µg/ml *P. pratense* extract in a total volume of 200 µl under normal cell culture conditions. Cells stimulated with concanavalin A (ConA) served as positive control. Culture supernatants were taken after five days and aliquots were stored at -20 °C until analysis of cytokines.

Differential cell count of lung cells

One $\times 10^5$ cells were spun onto glass slides using a cytocentrifuge, air-dried, and stained according to Pappenheim. Three hundred cells per slide were counted and differentiated by light microscopy according to standard morphologic criteria. Cell numbers were determined for macrophages, eosinophils, neutrophils, and lymphocytes.

Flow cytometric analysis

One $\times 10^5$ DC were washed with phosphate buffered saline (PBS), blocked with goat serum/PBS and stained with FITC-labeled murine-anti-human CD14 in combination with either PE-labeled murine-anti-human CD40, CD80, CD86, CD83, or HLA-DR, or with the corresponding isotype controls (all BD Biosciences, Heidelberg, Germany) for 30 min at 4°C. Cells were washed and analyzed using the Cytomics FC 500 Flow Cytometry System (Beckman Coulter, Krefeld, Germany).

Determination of proliferation

Human cells were pulsed with 5 µCi/ml ^3H -thymidine (Amersham Buchler, Braunschweig, Germany) for 18 hours and then harvested on filter mats (Canberra-Packard, Dreieich, Germany). After drying, 20 µl of liquid scintillator (Canberra-Packard, Dreieich, Germany) were

added and the plates were sealed. Counts per minute were determined on a Topcount Microplate Scintillation Counter (Canberra-Packard, Dreieich, Germany).

Determination of cytokines

Cytokines in cell culture supernatants of stimulated DC were determined with enzyme linked immunosorbent assay (ELISA) technique using DuoSet® ELISA Development Systems (R&D Systems, Wiesbaden, Germany) according to the manufacturer's instructions. Cytokines in cell culture supernatants of the allergy model *in vitro* were determined with electrochemiluminescence detection technology using a human Th1/Th2 (10-Plex) Ultra-Sensitive Kit (Meso Scale Discovery, Gaithersburg, USA) according to the manufacturer's instructions. The detection limit of this assay was 0.6 pg/ml. Similarly, cytokines in cell culture supernatants and BAL fluid of the mouse model of allergic airway inflammation were determined using a mouse Th1/Th2 (9-Plex) Ultra-Sensitive Kit (Meso Scale Discovery, Gaithersburg, USA) according to the manufacturer's instructions. The detection limit of this assay was 2.4 pg/ml.

Statistical analysis

Values obtained from the *in vitro* as well as from the *in vivo* model are given either as mean \pm standard error of the mean (SEM) of a minimum of four independent experiments or as a pool sample from five individual experiments. Statistical analysis was performed using the software GraphPad Prism®, version 4.03. To identify statistically significant differences, results were analyzed by analysis of variance (ANOVA) and Bonferroni's Multiple Comparison Test. Probability values of * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ were considered significant. To identify tendencies, results were analyzed with the unpaired t-test. Probability values of $p < 0.1$ indicated a trend.

Results

Immunomodulatory potential of BPPcysMPEG in a human in vitro allergy model

In a human *in vitro* allergy model the potential of BPPcysMPEG to mediate immunomodulation was evaluated. First, the effects of the TLR2/6 agonist on maturation of DC were determined. Second, its modulatory potential on co-cultures of DC with autologous lymphocytes was analyzed. The maturation of iDC was characterized by an altered pattern of co-stimulatory molecules on their cell surface and by secretion of proinflammatory cytokines. BPPcysMPEG enhanced expression of CD40, CD80, CD83, and HLA-DR as compared to medium control (Fig. 2 A). In addition, the presence of BPPcysMPEG stimulated secretion of the proinflammatory cytokine TNF- α by DC either alone as compared to medium control, or in combination with the allergen as compared to the allergen control (Fig. 2 B). Neither IL-10 nor IL-12 was induced (data not shown). Furthermore, stimulated cells were co-cultured with autologous lymphocytes and analyzed for proliferation and cytokine secretion to determine whether BPPcysMPEG modulates the outcome of an immune response. Fig. 3 A shows that *P. pratense* extract-induced allergen-specific proliferation was enhanced when BPPcysMPEG was co-administered with the allergen. However, BPPcysMPEG did not induce proliferation on its own (Fig. 3 A). In accordance with the proliferation data, co-administration of BPPcysMPEG enhanced the allergen-induced secretion of the Th2-associated cytokine IL-5 (Fig. 3 B) as well as the Th1-associated cytokine IFN- γ (Fig. 3 C) and the proinflammatory cytokine TNF- α (Fig. 3 D) as compared to the allergen control.

Thus, the human *in vitro* allergy model using monocyte-derived DC and autologous lymphocytes clearly displayed the potential of BPPcysMPEG to induce maturation of DC, as well as to enhance a specific immune response to a co-administered allergen via TLR2/6 agonization.

Local treatment with BPPcysMPEG reduced allergic airway inflammation in mice

The immunomodulatory properties exhibited by BPPcysMPEG in the *in vitro* studies provided the rationale to evaluate its potential to reduce parameters of allergic airway inflammation *in vivo*. To this end, preventive immunotherapy with BPPcysMPEG was performed in sensitized mice. Administration of BPPcysMPEG alone did not alter total cellular influx as compared to the positive control (data not shown). Cell differentiation showed increased numbers of alveolar macrophages (Fig. 4 A), whereas the number of eosinophils tended to be decreased as compared to the positive control (Fig. 4 B). The levels of neutrophils (Fig. 4 C) and lymphocytes (Fig. 4 D) were not altered by the treatment with BPPcysMPEG alone as compared to the positive control. Similarly, co-administration of allergen and BPPcysMPEG did not alter total cellular influx as compared to the allergen control (data not shown). While the influx of alveolar

macrophages (Fig. 4 A) was enhanced, the influx of eosinophils (Fig. 4 B) and lymphocytes (Fig. 4 D) was reduced as compared to the allergen control. Levels of neutrophils were not altered as compared to the allergen control (Fig 4 C).

For evaluation of the cytokine balance, the production of both Th1 and Th2 cytokines in BAL fluid was measured using electrochemiluminescence detection technology. The treatment with BPPcysMPEG tended to reduce levels of IL-2 (Fig. 5 A) as well as diminished those of the Th2 cytokines IL-4 (Fig. 5 B) and IL-5 (Fig. 5 C) as compared to the positive control. In contrast, IL-12 levels (Fig. 5 D) tended to be elevated as compared to the positive control, thereby indicating a shift towards a Th1 immune response. The level of IFN- γ , however, was below the detection limit (data not shown). Co-administration of allergen and BPPcysMPEG reduced both IL-2 (Fig. 5 A) and IL-12 levels (Fig. 5 D) as compared to the allergen control. In contrast, IL-4 (Fig. 5 B) and IL-5 levels (Fig. 5 C) were not altered as compared to the allergen control.

The question of whether the *in vivo* treatment with BPPcysMPEG affected the local or the systemic immune response was then further addressed by determining the properties of cells from mediastinal lymph nodes and splenocytes. Upon allergen-specific restimulation, treatment with BPPcysMPEG induced a Th1-dominated cytokine milieu in mediastinal lymph nodes as compared to the positive control (Fig. 6 A), when calculating the alteration of IL-4 and IFN- γ levels. In contrast, allergen-specific cytokine secretion in splenocytes was reduced as compared to the positive control (Fig. 6 B). Co-administration of allergen and BPPcysMPEG diminished the secretion of both IL-4 and IFN- γ upon allergen-specific restimulation in cells from mediastinal lymph nodes (Fig. 6 A) and splenocytes (Fig. 6 B), respectively as compared to the allergen control.

Discussion

Modulation of T cell responses in allergic diseases can be achieved in several ways, including the triggering of the innate immune system with microbial components and allergen-specific immunotherapy (Ebner et al., 1997; Secrist et al., 1993). By exploiting a human *in vitro* allergy model, we tried to predict the potential of BPPcysMPEG to modulate antigen presenting cells and a subsequent allergen-specific immune response. Antigen presenting cells play a decisive role in the induction of immune responses and modulation of this cell population might influence T cell polarization (Lombardi et al., 2008). The stimulation of iDC with BPPcysMPEG with or without the presence of the allergen caused maturation of these cells, as indicated by the upregulation of co-stimulatory molecules and the secretion of proinflammatory cytokines. As shown for the precursor molecule (Weigt et al., 2003), the stimulation with BPPcysMPEG did not lead to secretion of IL-12, but in contrast to MALP-2, BPPcysMPEG did not induce the secretion of IL-10. The exclusive induction of TNF- α suggested the induction of a general inflammatory immune response rather than a shift of an allergen-specific T cell response towards Treg or Th1. As expected, co-culture of allergen and BPPcysMPEG-treated DC with autologous lymphocytes induced enhanced allergen-specific proliferation. This was accompanied by increased secretion of both Th1- and Th2-associated cytokines. Thus, in the presence of the relevant allergen, BPPcysMPEG acted as an adjuvant on allergen-specific proliferation and cytokine secretion. In contrast, administration of BPPcysMPEG alone induced maturation of DC, but these DC did not induce proliferation of autologous lymphocytes. This clearly demonstrated the potential of BPPcysMPEG to modulate a pivotal cell subpopulation of the innate immune system via TLR2/6 agonization.

Recent studies also showed that amelioration of allergic airway disease can be achieved by using components which induce Th1 cell responses rather than regulatory responses (Drachenberg et al., 2001; Patel et al., 2005). The potential of MALP-2 co-administered with IFN- γ to induce a shift towards a Th1 counterbalancing immune response was shown both *in vitro* (Weigt et al., 2004) and *in vivo* (Weigt et al., 2005). In addition, according to the results obtained with the precursor molecule, BPPcysMPEG in combination with IFN- γ had the potential to reduce parameters of allergic airway inflammation (Fuchs et al., 2009). In these studies, however, the efficacy of the TLR2/6 agonist was shown to depend on the presence of IFN- γ . As the data obtained from the human *in vitro* allergy model indicated, that BPPcysMPEG was sufficient to affect the maturation of DC, the efficacy of preventive treatment with BPPcysMPEG either alone or with the allergen was investigated in sensitized mice. The treatment with BPPcysMPEG without allergen should mimic the situation of a constant triggering of the innate immune system with a microbial component to reverse a Th2 skew upon allergen exposure. The

model indicated that TLR agonization with BPPcysMPEG diminished allergic airway inflammation by shifting the allergen-specific immune response towards Th1. However, this was not accompanied by the induction of neutrophilia, indicating that the treatment did not induce an opposing Th1- or Th17-mediated inflammatory immune response. The administration of BPPcysMPEG exerted its immunomodulatory effects locally in the lung by abrogation of Th2 cytokines, which was associated with a trend towards enhanced Th1 cytokine levels. The shift towards Th1 was observed in mediastinal lymph nodes as well. In contrast to the results obtained from BAL fluids, however, this was achieved by induction of Th1-associated cytokines rather than by alteration of Th2-associated cytokines. The local bias came along with a reduction of the secretion of Th1- and Th2-associated cytokines by splenocytes.

In the present study, the effects of allergen-specific immunotherapy either in the presence or absence of BPPcysMPEG were controversial. Although the enhanced influx of eosinophils and lymphocytes after allergen treatment indicated an amplification of allergic airway inflammation, this effect was not accompanied by enhanced levels of Th2 cytokines. In addition, the IL-12 level was enhanced, suggesting a shift towards Th1. In the presence of BPPcysMPEG the measured parameters were diminished as compared to allergen-treated mice, but were still enhanced as compared to positive control. The failure of allergen-specific immunotherapy might be due to the animal model, as many studies concerning allergen-specific immunotherapy were performed in long-term models (Kildsgaard et al., 2007). Furthermore, the allergen dose as well as time and route of administration are crucial for specific immunotherapy (Akdis et al., 2009), and might account for the exacerbated inflammation in this model. The involvement of other cell types *in vivo*, as well as the kinetics of cytokine appearance in BAL fluid probably contributed to the controversial results.

Taken together, the obtained data showed that exclusive agonization of the TLR2/6 receptor with BPPcysMPEG was able to modulate the maturation of DC *in vitro* resulting in a proinflammatory immune response, thereby potentiating the immune response to a co-administered allergen *in vitro*. In a mouse model of allergic airway inflammation, triggering the immune system via TLR agonization alone seemed to be effective to balance the immune response to a subsequent administered allergen. The *in vitro* data indicated that the activation of innate immune cells resulted in a proinflammatory immune response, which might account for the abrogation of allergic airway inflammation in mice. In conclusion, modulation of innate immune mechanisms with BPPcysMPEG seems to be a valid option for the treatment of a broad range of allergies independently of the underlying allergen.

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Figures

Tab. 1: Study groups of the mouse model of allergic airway inflammation.

Group	Sensitization	Treatment
Negative	Saline	Saline
Positive	rPhl p 5	Saline
BPPcysMPEG	rPhl p 5	BPPcysMPEG
Allergen	rPhl p 5	Allergen
Allergen + BPPcysMPEG	rPhl p 5	Allergen + BPPcysMPEG

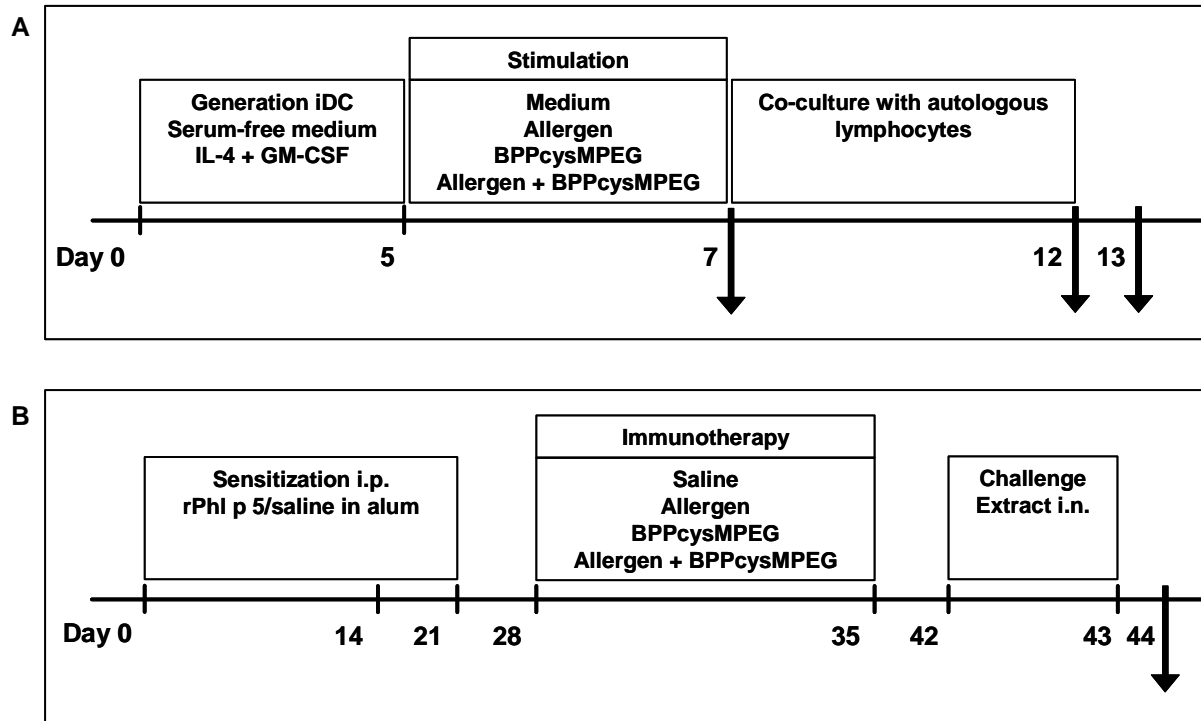


Fig. 1: Study design of the human *in vitro* allergy model and the mouse model of allergic airway inflammation.

(A) Monocytes were isolated from whole blood of allergic donors and cultured in the presence of IL-4 and GM-CSF to generate iDC. Cells were stimulated with allergen, BPPcysMPEG or a combination of both for two days. Medium served as control. On day 7, stimulated cells were harvested and co-cultured with autologous lymphocytes for five additional days. Black arrows indicate different days of analysis. On day 7, secreted cytokines were measured in cell culture supernatants of stimulated DC. Harvested cells were analyzed for the expression of cell surface marker. On day 12, secreted cytokines were measured in cell culture supernatants of the co-cultures. On day 13, proliferation of autologous lymphocytes was determined. (B) Mice were sensitized i.p. on days 0, 14, and 21 with rPhl p5 or saline adsorbed to alum and challenged i.n. with *P. pratense* extract on days 42 and 43. I.n. treatment with BPPcysMPEG alone or a combination of allergen and BPPcysMPEG was performed for seven days starting on day 28. Saline- or allergen-treated animals served as controls. A black arrow indicates the day of analysis.

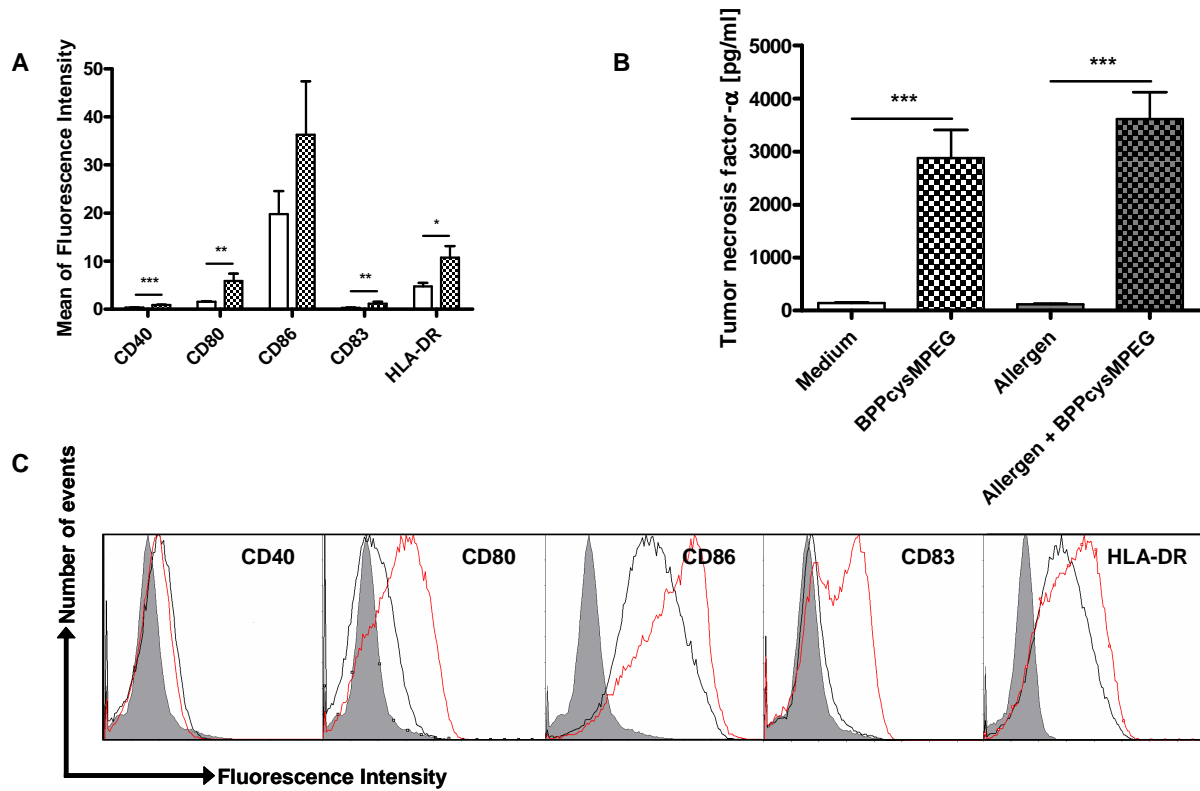


Fig. 2: BPPcysMPEG-stimulated maturation of DC *in vitro*. Monocytes were isolated from whole blood of allergic donors and cultured in the presence of IL-4 and GM-CSF to generate iDC. Cells were then stimulated with either medium (white bars) or BPPcysMPEG (checked bars) for 48 hours and analyzed for the expression of co-stimulatory molecules (A). In addition, secretion of TNF- α after stimulation with medium (white bar), BPPcysMPEG (checked bar), allergen (grey bar), or a combination of allergen and BPPcysMPEG (grey checked bar) was determined (B). The overlay plots depict the isotype control (filled grey histogram), the medium control (black histogram), and BPPcysMPEG (red histogram) for the indicated co-stimulatory molecules (C). Data are shown as mean \pm SEM of a minimum of six independent experiments (A+B) or as a representative experiment (C). Statistical significances are indicated by * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$.

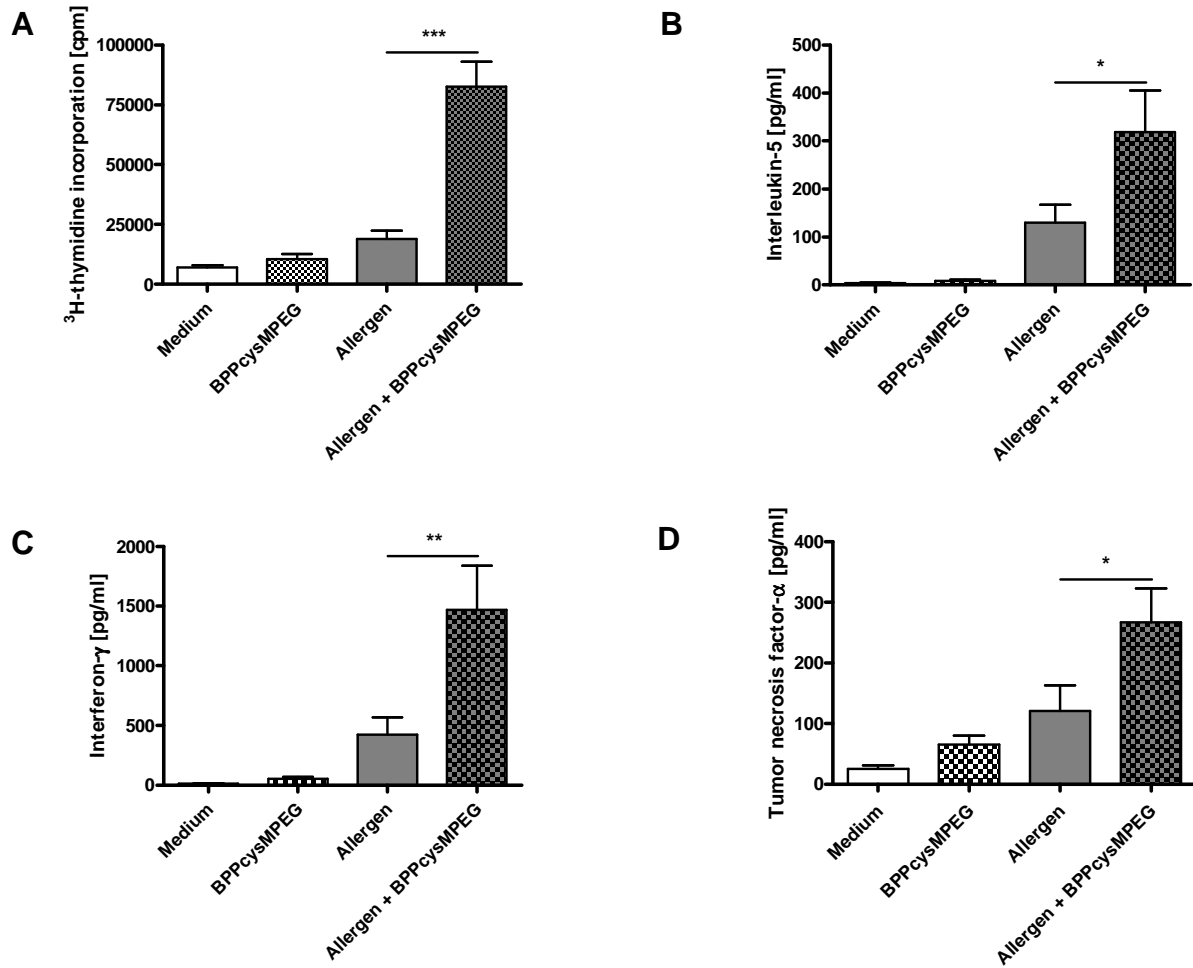


Fig. 3: BPPcysMPEG induced enhanced allergen-specific proliferation and cytokine secretion in a human *in vitro* allergy model. Autologous lymphocytes were cultured with allergen- and BPPcysMPEG-stimulated DC (grey checked bars) for five days. Medium- (white bars), allergen- (grey bars), or BPPcysMPEG-stimulated DC (checked bars) served as controls. Proliferation (A) was measured by ^3H -thymidine incorporation. Cytokines in cell supernatants (B-D) were determined using electrochemiluminescence detection technology. Data are shown as mean \pm SEM of eleven independent experiments. Statistical significances are indicated by * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$.

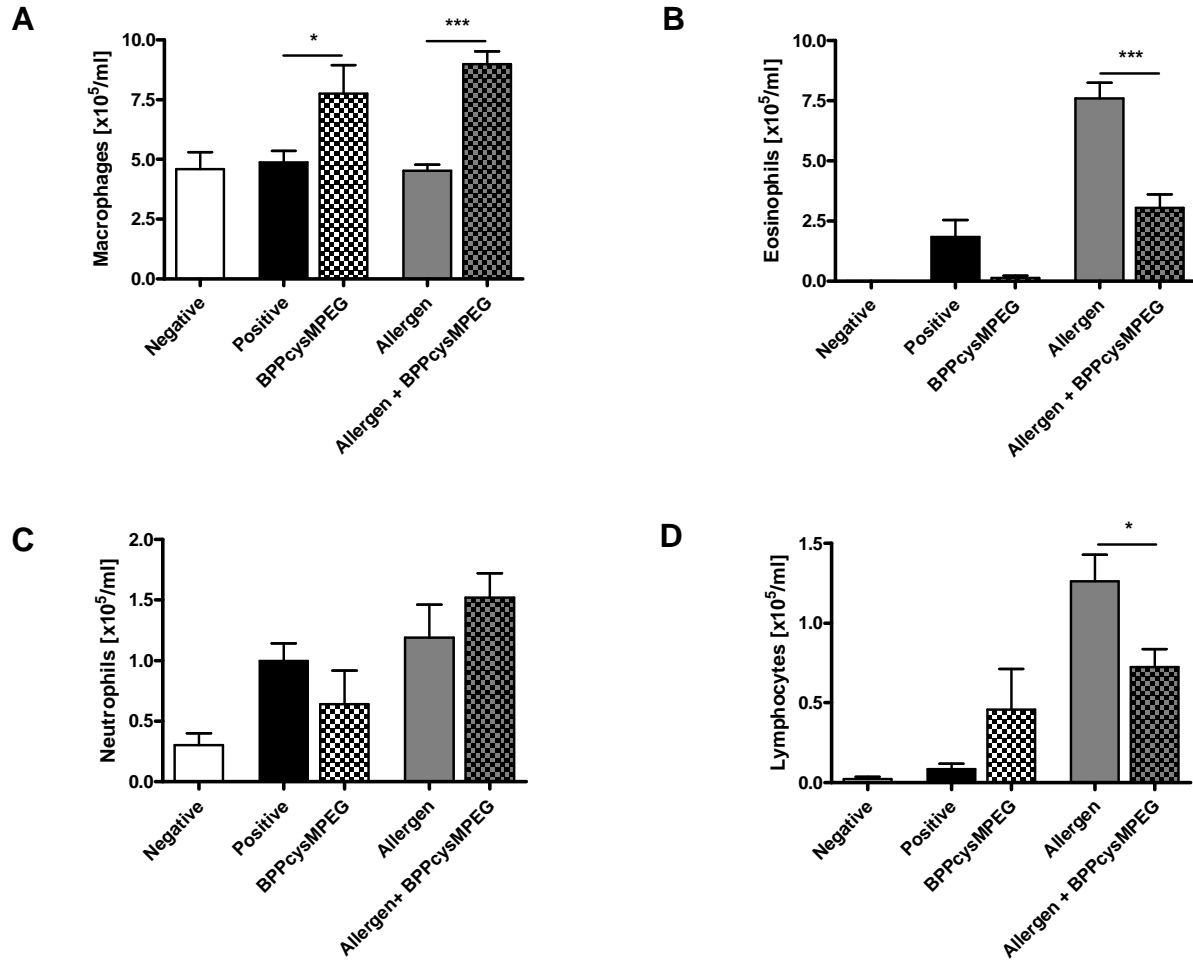


Fig. 4: Local administration of BPPcysMPEG altered cell composition in BAL fluid *in vivo*. Mice were sensitized i.p. on days 0, 14, and 21 with rPhl p5/alum or saline/alum and challenged i.n. with *P. pratense* extract on days 42 and 43. I.n. treatment with allergen (grey bars), BPPcysMPEG (checked bars), or a combination of both (grey checked bars) was performed for seven days starting on day 28. Positive controls (black bars) and negative controls (white bars) were treated with saline. BAL fluid was obtained 24 hours after the last challenge and cell differentiation was performed. Data are shown as mean \pm SEM of a minimum of four experiments. Statistical significances are indicated by * $p < 0.05$ and *** $p < 0.001$.

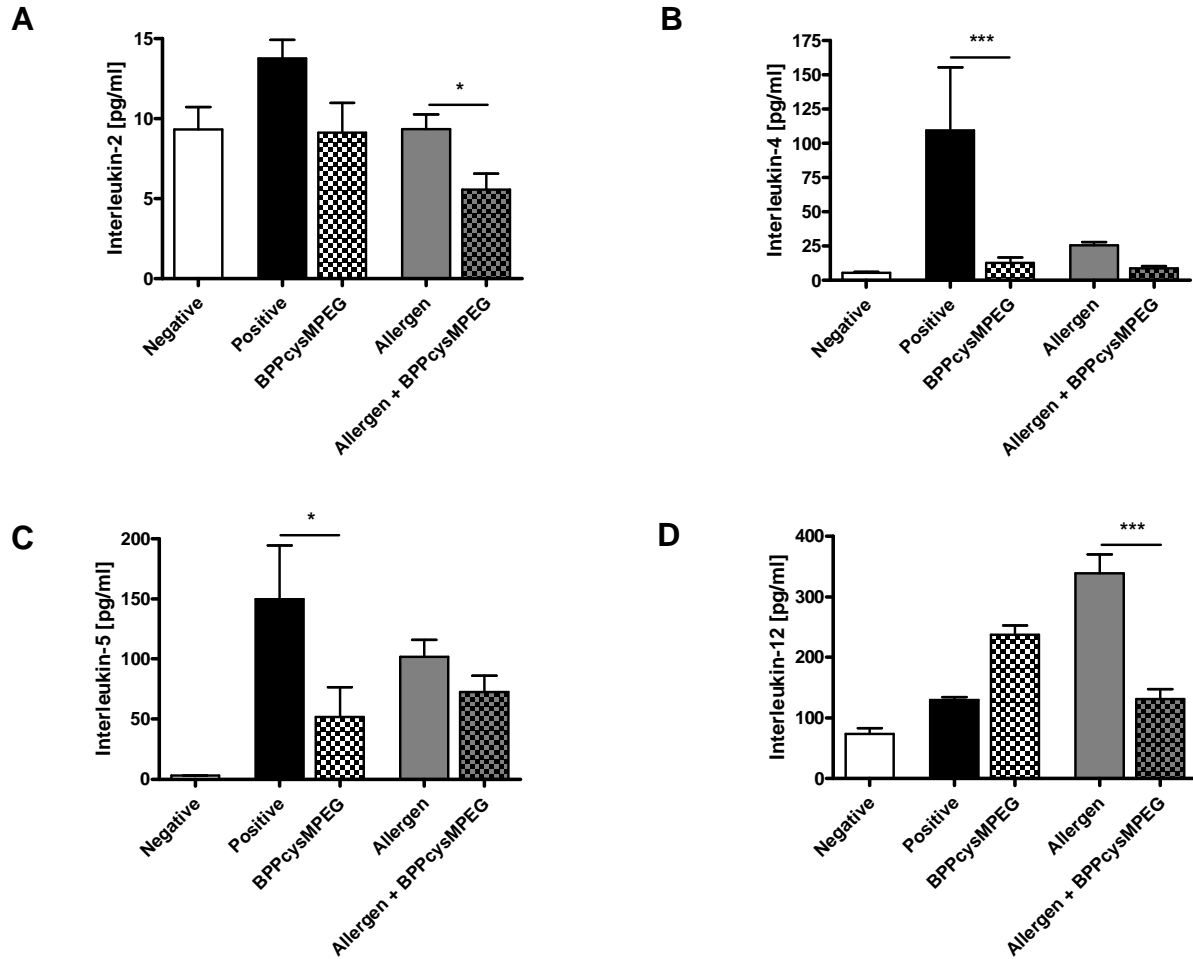


Fig. 5: Local administration of BPPcysMPEG altered cytokine pattern in BAL fluid *in vivo*. Mice were sensitized i.p. on days 0, 14, and 21 with rPhl p5/alum or saline/alum and challenged i.n. with *P. pratense* extract on days 42 and 43. I.n. treatment with allergen (grey bars), BPPcysMPEG (checked bars), or a combination of both (grey checked bars) was performed for seven days starting on day 28. Positive controls (black bars) and negative controls (white bars) were treated with saline. BAL fluid was obtained 24 hours after the last challenge. Cytokines in BAL fluid were determined using electrochemiluminescence detection technology. Data are shown as mean \pm SEM of a minimum of four experiments. Statistical significances are indicated by * $p < 0.05$ and *** $p < 0.001$.

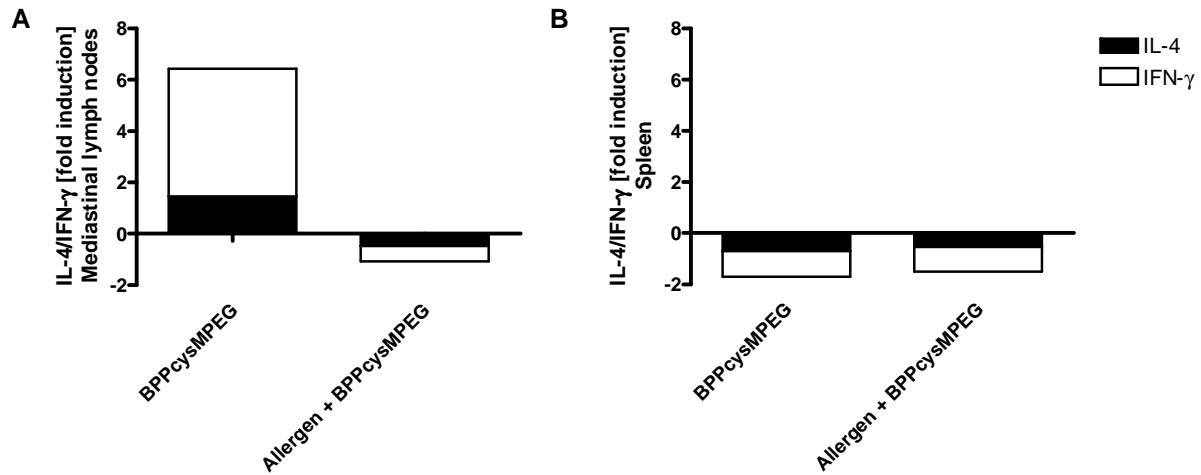


Fig. 6: Local administration of BPPcysMPEG with or without the allergen altered cytokine pattern in mediastinal lymph nodes and spleen. Cells from mediastinal lymph nodes (A) and splenocytes (B) were restimulated with *P. pratense* extract for five days. Cytokines in cell supernatants were determined using electrochemiluminescence detection technology. Data are shown as alterations in IL-4 and IFN- γ levels either after treatment with BPPcysMPEG as compared to treatment with saline, or after treatment with allergen and BPPcysMPEG as compared to treatment with allergen alone from a pool sample of five individual experiments.