

available at www.sciencedirect.comwww.elsevier.com/locate/yclim

Augmented *Phl p* 5-specific Th2 response after exposure of dendritic cells to allergen in complex with specific IgE compared to IgG1 and IgG4[☆]

Kristina Lundberg^a, Malin Lindstedt^{a,*}, Kristina Larsson^a, Linda Dexlin^a, Christer Wingren^a, Mats Ohlin^a, Lennart Greiff^b, Carl A.K. Borrebaeck^a

^a Department of Immunotechnology, Lund University, Lund, Sweden

^b Department of Otorhinolaryngology, Head and Neck Surgery, Lund University Hospital, Lund, Sweden

Received 16 November 2007; accepted 28 April 2008

KEYWORDS

Antibody-mediated responses;
Th2;
IgE;
Phl p 5;
Allergic Rhinitis;
Dendritic cells;
T-cells;
Antibody arrays

Abstract In this study we have elucidated the effects of allergen-specific antibodies on human DCs and T-cells. Monocyte-derived DCs from allergic patients were exposed to *Phl p* 5 alone or in complex with *Phl p* 5-specific human IgG1, IgG4 or IgE and further co-cultured with autologous memory CD4⁺ T-cells. We demonstrate that DCs treated with *Phl p* 5/IgE-complexes secrete higher levels of IL-1 α , IL-6, VEGF and MCP-3 compared to *Phl p* 5 alone. Furthermore, we show that the ability of DCs to present allergen to memory CD4⁺ T-cells and induce a Th2 cytokine profile is significantly augmented when the uptake is mediated by specific IgE antibodies, whereas IgG1 and IgG4 have no such effect. The differences in cytokine profiles depending on the antibody subtype could partly explain the ability of allergic individuals to amplify allergen-specific immune response and could thus be involved in the etiology of allergic responses.

© 2008 Elsevier Inc. All rights reserved.

Introduction

Dendritic cells (DCs) are key regulators of the Th1/Th2 balance as they upon uptake of foreign antigen mature and migrate to lymphoid tissue in order to present processed antigen to T-cells and stimulate an appropriate response [1]. In allergic diseases, such as allergic rhinitis and asthma,

allergen-challenged DCs induce a Th2 response and thereby elevated levels of IgE antibodies [2].

IgE-allergen complexes have been shown to be internalized by DCs from allergic donors and augment antigen presentation to T-cells [3–6]. Thus, allergic patients are subjected to IgE-mediated enhancement of the T-cell response and this route of internalization and presentation may lower the threshold for the induction of an allergic reaction in atopic subjects. IL-4 and IFN- γ production has been studied in naïve T cells after coculture with Langerhans cell-like DCs and inflammatory dendritic epidermal cell-like cells [7]. The *in vitro*-derived DC types were shown to induce differential polarization of naïve T cells after stimulation with *Bet v* 1 in combination with chimeric *Bet v* 1-specific

[☆] Supported by grants from the Swedish Research Council (VR-M) and Lund University.

* Corresponding author. Department of Immunotechnology, BMC D13, Lund University, SE-221 84 Lund, Sweden. Fax: +46 46 222 42 00.

E-mail address: Malin.Lindstedt@immun.lth.se (M. Lindstedt).

IgE, as compared to unstimulated cells. However, the cytokine profile of allergen-stimulated DCs and T cells after addition of human allergen-specific IgE, compared to allergen alone has not previously been studied.

Furthermore, T-cell proliferation has been reported in response to Fc γ -receptor crosslinking, as well as upon addition of IgG [8–11]. Also, addition of IgG to antigen-challenged bone marrow-derived DCs in mice have been shown to induce the production of IL-4 [9,11]. However, the Th1/Th2 cytokine profile, induced by DCs when presenting allergen in complex with different IgG subclasses has thus far not been established in humans. In addition, the efficiency of IgG-mediated allergen presentation as compared to IgE-mediated presentation has not yet been elucidated.

Specific immunotherapy (SIT) is established as an effective treatment of allergic rhinitis, functioning partly via protection caused by elevated levels of IgG antibodies, in particular IgG4 [12,13]. Although several studies have shown in vivo effects of SIT, such as altered levels of antibodies and the number of Th2 cells at the site of allergen challenge [14,15], further insight into the mechanism is required to improve the effectiveness and safety of immunotherapy. One important aspect is the implication of the elevated levels of IgG isotypes on the resulting T-cell response in allergic subjects. Accordingly, a comparison of IgG1, IgG4 and IgE in terms of DC-induced Th1/Th2 cytokine production may give important information regarding the mechanisms behind SIT.

In this study, we elucidate the effects of different antibody isotypes on human DCs and T-cells in grass pollen allergy. We developed fully human IgG1, IgG4 and IgE with identical paratopes specific for the major grass pollen allergen *Phl p 5*, thereby excluding effects due to different paratopes, as shown by Manca et al [16]. Thus, the setup allows evaluation of the different isotypes in conditions where binding activity and binding specificity are perfectly normalized. Supernatants from DCs treated with IgE-*Phl p 5* and *Phl p 5* alone were analyzed using protein microarrays, simultaneously measuring a large set of cytokines. Furthermore, we studied the ability of IgE, IgG1 and IgG4 to influence the presentation of allergen by DCs to memory CD4⁺ T-cells.

Methods

Medium and cytokines

Cell culture medium RPMI 1640 was supplemented with 5% (v/v) fetal bovine sera (Invitrogen Gibco, Carlsbad, CA), 50 μ L mL⁻¹ gentamicin (Invitrogen), and 2 mM L-glutamine (Sigma-Aldrich, St Louis, MO), and is referred to as R5 medium. Recombinant human (rh) GM-CSF was obtained from Immunex (Seattle, WA) and rhIL-4 was purchased from R&D Systems (Minneapolis, MN).

Donor characterization

Eleven patients with allergic rhinitis participated in the study. The donors tested positive for extract of *Phleum pratense* (*Phl p*) in a skin prick test (Soluprick SQ purchased from ALK-Abelló, Hørsholm, Denmark) and had a history of intermittent allergic rhinitis. The atopic individuals were subjected to allergen (ALK-Abelló) challenges outside the

natural pollen season, i.e., an “artificial pollen season” [17]. Increasing doses of allergen were administered at 10 min intervals using a spray-device delivering 100 μ L per actuation. One actuation was administered into each nostril resulting in effective doses of 100, 300, 1000, and 3000 Standardized Quantity units per nasal cavity (where 100,000 SQ units correspond to the maintenance dose for the average patient in successful specific allergy vaccination according to the manufacturer). This scheme was followed until the subject responded with at least 5 sneezes or recorded a symptom score of 2 or more on a scale from 0 to 3 for either of the symptoms nasal secretion or nasal blockage, i.e., the threshold dose. In the present study, in contrast to our previous studies where this allergen dose was chosen for the allergen challenge series, we chose a dose one dose-step higher than the threshold dose in order to produce somewhat more marked symptoms. The allergen challenge was given once daily for seven days after which blood was collected. At that point, all donors had symptoms of ongoing allergic rhinitis (Table 1). The study was approved by the regional Ethics Committee and informed consent was obtained.

Allergen-specific IgE/Basophil degranulation assay

High levels of *Phl*-specific IgE was detected in serum from allergic participants (3–812 kU A L⁻¹) using the Pharmacia CAP System (Pharmacia Diagnostics, Uppsala, Sweden) according to the manufacturer's instructions (Table 1). The detection limit of the assay is 0.35 kU A L⁻¹. An ELISA using immobilized recombinant *Phl p 5a* (Biomay, Vienna, Austria) and peroxidase-labeled goat anti-human IgE (KPL, Guildford, UK) for detection, demonstrated high levels of *Phl p 5*-specific IgE in the donors' serum (Table 1). The BD FastImmune assay was performed on basophils from all donors to show that the *Phl p* extract induced basophil degranulation (Table 1). The experiment was performed according to the manufacturer's instructions (Becton Dickinson, San Diego, CA) using CD123-PE, HLA-DR-PerCP, and CD63-FITC antibodies.

Generation of *Phl p 5*-specific IgG1, IgG4 and IgE

Antibodies specific for *Phl p 5a* were created using the variable domain of the 4.3 antibody that had been selected by phage display technology from a library with an origin in human IgE encoding genes [18]. This antibody recognizes an epitope within a highly immunogenic fragment (residues 56–165, [19]) of *Phl p 5a*. The antibody cross-reacts with *Phl p 6* which has parts that show >95% sequence identity to the relevant region of *Phl p 5*. In contrast, it does not cross-react with any of the five unrelated tested *Phl p* allergens (*Phl p 1*, 2, 4, 7 and 11) [20]. IgG1 and IgG4 antibodies, with the specificity originating from the 4.3 antibody fragment, were produced using vectors originating from those established by Norderhaug et al. [21]. In addition, the same specificity was produced in IgE format by using a variant of the heavy chain-encoding vector designed for this purpose (a generous gift from Dr P. A. Würtzen, ALK-Abelló, Hørsholm, Denmark). Heavy and light chain-encoding vectors were transfected into CHO-K1 cells using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) and the cell supernatant of transiently transfected cells were

Table 1 Profiles of the study subjects, as determined by skin prick test, symptom scores, basophil degranulation and *Phl p*-specific, as well as *Phl p* 5-specific IgE

Donors	SPT to <i>Phl p</i> extract	Rhinorrhea score (0–3)*	Congestion score (0–3)*	Net basophil degranulation to <i>Phl p</i> extract stimulation (%)*	<i>Phl p</i> -specific IgE (kU A L ⁻¹)*	<i>Phl p</i> 5-specific IgE*
1	+	2	2	32	60	+
2	+	0	1	26	10	+
3	+	2	1	14	20	+
4	+	3	3	13	6	+
5	+	3	2	37	29	+
6	+	0	2	63	80	+
7	+	2	2	20	30	+
8	+	1	2	53	3	+
9	+	3	3	18	32	+
10	+	3	3	52	28	+
11	+	3	2	33	812	+

*Measured at the day of blood withdrawal, after 7 days of daily administration of allergen.

Abbreviations: SPT – skin prick test.

collected every 2–3 days until the concentration was below 0.25 µg/ml (for 6–8 days).

Concentration, specificity and purification of IgG1, IgG4 and IgE

Concentration of IgE was determined by a sandwich ELISA using immobilized goat anti-human IgE (Biosource, Camarillo, CA) and detection with peroxidase-labeled goat anti-human IgE (KPL). IgE concentration was determined relative a human IgE standard (Chemicon International Inc., Temecula, CA). Similarly, IgG concentrations were determined by using an immobilized rabbit anti-human lambda light chain antiserum and detection with peroxidase-labeled rabbit anti-human IgG (Dako Cytomation, Glostrup, Denmark). As standard an IgG preparation (Gammagard S/D) was used. *Phl p* 5 specificity of the antibodies was determined by an ELISA using immobilized recombinant *Phl p* 5a.

Phl p 5-specific IgG1 and IgG4 antibodies were purified using protein A columns and IgE was purified by goat anti-human IgE bound to an NHS-activated column (columns from GE Healthcare Bio-Sciences, Uppsala, Sweden), according to manufacturer's instructions. The eluted products were extensively dialyzed against PBS and filtered through 0.22 µm filters. Finally, concentration was determined by Micro BCA™ Protein Assay Reagent Kit (Pierce, Rockford, IL). All handling of the produced antibodies was carried out using endotoxin free lab materials.

Cell preparation and culture

In order to generate immature MoDCs, monocytes were purified from peripheral blood and cultured for seven days in R5 culture medium supplemented with rhIL-4 (50 ng mL⁻¹) and rhGM-CSF (150 ng mL⁻¹) as described elsewhere [22]. Immature MoDCs were exposed to one of the following: 25 µg mL⁻¹ *Phl p* extract (a gift from Dr P. A. Würtzen, ALK Abelló; endotoxin level: 0.025 EU/ml culture), 150 ng mL⁻¹ recombinant *Phl p* 5a (endotoxin level: 0.002 EU/ml culture), 1 µg mL⁻¹ *Phl p* 5-specific IgG1, IgG4 or IgE, or a

combination of recombinant *Phl p* 5 and specific antibodies (previously incubated for 60 min at 37 °C at a molar ratio of 1:1.3 to allow complex formation) or left unstimulated. After 48 h cell supernatants were collected and stored in –20 °C. The MoDCs were washed twice before co-culture with memory T-cells.

Isolation of memory CD4⁺ T-cells

CD4⁺ T-cells from autologous donors were purified by negative selection using the CD4⁺ T-cell isolation kit II (Miltenyi Biotec, Bergisch Gladbach, Germany). Memory CD27⁺/CD4⁺ T-cells were further isolated by negative selection using anti-CD45RA, anti-CD8, anti-glycophorin A antibodies (Dako Cytomation) and microbead-conjugated anti-CD27 antibodies (Miltenyi Biotec) for 15 min at 4 °C. Subsequently, the cell suspension was labeled with microbead-conjugated goat-anti-mouse antibodies (Miltenyi Biotec) for 15 min at 4 °C. Depletion of contaminating cells was performed with LS columns (Miltenyi Biotec), which resulted in 80–90% memory CD4⁺ T-cells enriched for CD27⁺ cells.

Functional T-cell assays

Differentially stimulated MoDCs were co-cultured with autologous, enriched CD45RO⁺/CD4⁺/CD27⁺ T-cells in R5 medium supplemented with 2 × 10⁻⁵ M 2-mercaptoethanol (Merck, Whitehouse, NJ) at a DC:T cell ratio of 1:20 for 7 days. During the last 16 h, 2.5 µCi mL⁻¹ thymidine (Amersham Biosciences, Uppsala, Sweden) was added to each well. Thymidine incorporation was measured using a Matrix 96 Direct beta scintillation counter (Packard, Meriden, CT). Samples were run in triplicates.

In addition, CD45RO⁺/CD4⁺/CD27⁺ T cells were co-cultured with differently stimulated MoDCs for 7 days for intracellular detection of cytokines. Staining was performed using CD4-PeCy5, IL-4-PE, IFN-γ-FITC, IL13-PE and IL5-PE (Becton Dickinson) as previously described [23]. Unspecific binding was reduced by blocking the cells with Chrome Pure

Table 2 Complete list of analytes assessed in the antibody microarray analysis of supernatants from MoDC treated with *Phl p 5* alone or in complex with specific IgE

Upregulated	IL-1 α , MCP-3, VEGF, IL-6
Unaltered	Eotaxin, IL-10, IL-11, IL-12, IL-13, IL-18, IL-1 β , IL-1R α , IL-2, IL-3, IL-5, IL-7, IL-8, IL-9, INF- γ , MCP-1, MCP-4, RANTES, TGF- β 1, TNF- α , TNF- β , IL-16
A total of 81 clones were used, measuring 26 unique analytes.	

Mouse IgG (Jackson ImmunoResearch Laboratories, West Grove, PA).

Production and purification of single chain fragments (scFv)

Eighty-one human recombinant antibody fragments against 26 unique protein analytes (A complete list of the chemokines, cytokines and growth factors assessed is presented in Table 2), selected from the n-CoDeR library [24], were kindly provided by BioInvent International AB (Lund, Sweden). All scFv's were produced in 100 mL *E. coli* cultures and purified from the expression supernatant using affinity chromatography on Ni-NTA agarose (Qiagen, Hilden, Germany).

Labelling of cell culture supernatants

Cell culture supernatants from MoDCs stimulated either with *Phl p 5* alone or in complex with specific IgE from the five atopic donors with the most distinct Th2 cytokine profile were biotinylated at a molar ratio of biotin:protein of 45:1 using the EZ-Link Sulfo-NHS-LC-Biotin (Pierce, Rockford, IL, USA). A labelling protocol, recently optimized for labelling of complex samples in an efficient and reproducible manner, was adopted [25,26]. The biotinylated samples were extensively dialyzed against PBS at 4 °C to remove unreacted biotin, and stored at -20 °C until further use.

Fabrication and processing of antibody microarrays

For production and processing of the antibody microarrays, we used a set-up previously optimized in our laboratory [25–27]. Briefly, 2 to 25 fmol (5 fmol average) of each scFv were dispensed in eight replicates onto black polymer MaxiSorp microarray slides (NUNC, Roskilde, Denmark), using a non-contact Biochip Arrayer1 (Perkin Elmer Life & Analytical Sciences, Wellesley, MA). Specifically bound biotinylated antigens were visualized using Alexa-647 conjugated Streptavidin (Molecular Probes, Eugene, OR, USA). Next, the slides were scanned with a confocal microarray scanner (ScanArray Express, Perkin Elmer) at a resolution of 5 μ m. The ScanArray Express software V2.0 (Perkin Elmer) was used to quantify the intensity of each spot using the fixed circle method. The local background was subtracted and, to compensate for possible local defects, the two highest and two lowest

replicates were excluded in order for each data point to represent the mean value of four replicated.

Data analysis and normalization of antibody microarray data

Chip-to-chip normalization of the data-sets was performed using a semi-global normalization approach. The 14% of the analytes that displayed the lowest coefficient of variation values over all samples and that did not exceed an intensity of 1200 were identified, corresponding to 12 analytes, and used to calculate a chip-to-chip normalization factor. The normalization factor N_i was calculated by the formula $N_i = \mu / \mu_i$, where μ is the average signal intensity of the twelve analytes for all samples, and μ_i is the average signal intensity for the twelve analytes for each sample. Each data-set generated from one sample was multiplied by the normalization factor. Mean intensity values for negative PBS controls from each sample were subtracted from the data-set from each sample before extraction of altered analytes. Significantly up- or down-regulated analytes were identified based on three criteria. Analytes with a p-value lower than 0.05 for each donor were selected. Subsequently, up- or down-regulated analytes detected in supernatants from MoDCs stimulated with *Phl p 5* as compared to those stimulated with *Phl p 5* in combination with specific IgE were based on significance and fold change ± 1.3 . Finally, significant analytes with fold change ± 1.3 in at least four out of five donors generated a list of up- or down-regulated analytes.

Statistical analysis

The statistical significance of changes in Th2 cytokine production and proliferation was determined with Wilcoxon Matched-Pairs Signed-Rank Test. For the antibody microarray data, Student's *t*-test was utilized to determine significant difference between stimulation with IgE/*Phl p 5* complexes as compared to *Phl p 5* alone.

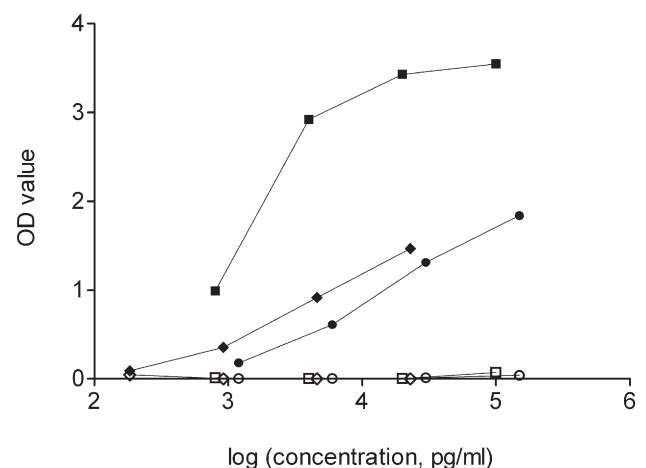


Figure 1 Binding of anti-*Phl p 5*-specific IgG1 (circles), IgG4 (diamonds) and IgE (squares) to immobilized recombinant *Phl p 5* (filled symbols) or BSA (open symbols) as assessed by ELISA.

Results

IgG1, IgG4 and IgE antibodies bind specifically to Phl p 5

In order to compare the effects of different isotypes of human antibodies on DC and T-cell function in grass pollen allergy, we used vectors encoding IgG1, IgG4 and IgE heavy chains and lambda light chain, all with variable regions coding for a Phl p 5 specificity, to produce fully human antibodies. Intact antibodies of IgG1, IgG4 and IgE isotypes were produced in CHO-K1 cells. Specificity analysis, using ELISA, showed binding of all three isotypes to Phl p 5, whereas none of the antibodies bound to the negative control antigen (Fig. 1). Thus, the generated human IgG1, IgG4 and IgE antibodies can be considered to be specific to their epitope.

Presentation of allergen extract as measured by T-cell proliferation

T-cell proliferation was measured after co-culture for seven days with MoDCs, which had been differentially stimulated, as previously described. To enable detection of altered T-cell responses due to the addition of specific antibodies at low allergen concentrations, the selected Phl p 5 concentration was considerably lower than the concentration of Phl p extract, 150 ng/ml and 25 µg/ml respectively. Phl p extract-stimulated MoDCs gave rise to a significantly higher T-cell proliferation as compared to unstimulated MoDCs ($p < 0.05$), whereas Phl p 5 stimulation of MoDCs did not alter the level of T-cell proliferation (Fig. 2), most likely due to the low sub-threshold concentration. Furthermore, T-cell proliferation

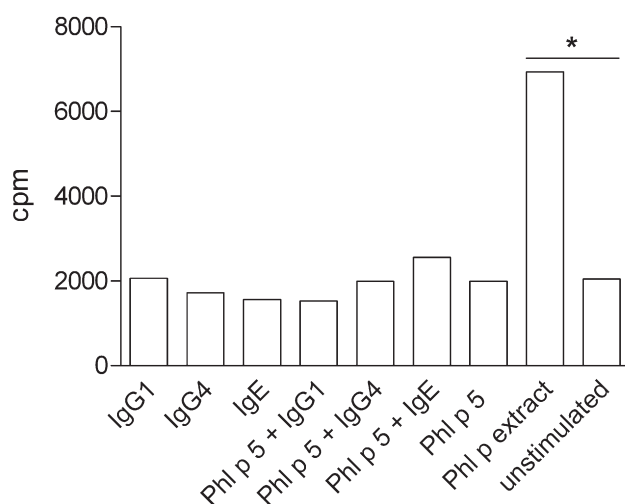


Figure 2 Measurement of T-cell proliferation after exposure of MoDCs to Phl p extract and Phl p 5 with and without antibodies. Phl p extract-stimulated ($25 \mu\text{g ml}^{-1}$) DCs induced proliferation of memory T-cell, whereas proliferation caused by a low level of recombinant Phl p 5 (150 ng ml^{-1}) with or without antibodies was undetectable. The average values of 11 donors are shown and the statistical significance was determined with Wilcoxon Matched-Pairs Signed-Rank Test (*= $p < 0.05$).

was also monitored after co-culture with MoDCs that had encountered the allergen in complex with a specific antibody and similarly, the T-cell proliferation was not altered by any of the complexes.

Th2 cytokine production is augmented after exposure of MoDCs to Phl p 5 in complex with specific IgE, compared to IgG1 and IgG4

In order to analyze alterations in the Th1/Th2 profile due to co-culture with MoDCs that had encountered Phl p 5-antibody complexes, the production of characteristic Th1 and Th2 cytokines produced by CD45RO⁺/CD4⁺/CD27⁻ T-cells was monitored. The exposure of MoDC to Phl p 5/IgE complexes significantly increased the subsequent percentage of T-cells producing IL-4, IL-13 and IL-5 after DC and T-cell co-culture (Fig. 3). Thus, addition of the specific IgE to Phl p 5 augmented Th2 responses in grass pollen allergic donor cells. In addition, the combination of IgE and Phl p 5 significantly increased the percentage of T-cells producing IL-4, as compared to IgG1 in combination with Phl p 5. Also, the percentage of T-cells producing IL-13 was significantly augmented when Phl p 5 was complexed with IgE, as compared to IgG1 and IgG4. In contrast, the proportion of T-cells producing the Th1 cytokine IFN- γ was unaltered by the addition of allergen, specific antibodies or their complexes (Fig. 3D). Altogether, these results demonstrate increased Th2 cytokine production after co-culture of effector memory CD4⁺ T-cells with MoDCs pulsed with allergen-IgE complexes, as compared to allergen in complex with IgG1 or IgG4.

Altered levels of other immunoregulatory proteins are secreted by MoDCs treated with Phl p 5/IgE-complexes compared to Phl p 5 alone

Since Phl p 5/IgE-challenged MoDCs mediated an increased Th2 response, we further investigated whether Phl p 5-challenged MoDCs secrete altered levels of other immunoregulatory proteins in the presence of specific IgE. Supernatants from five donors with distinct Th2 cytokine production were selected and analysed using antibody microarrays. Subsequently, up- or down-regulated analytes detected in supernatants from MoDCs stimulated with Phl p 5, as compared to Phl p 5 in combination with specific IgE, were based on significance, determined by t -test, and a fold change of ± 1.3 in at least four out of five donors. The analysis demonstrated increased levels of IL-1 α , IL-6, VEGF and MCP-3 secreted by MoDC exposed to allergen in complex with IgE, as compared to allergen alone (Fig. 4). In summary, this data indicate that MoDCs secrete differential levels of a set of cytokines following addition of allergen/IgE complexes, as compared to allergen alone.

Discussion

In this study of antibody-mediated responses in allergy, we have compared the effect of fully human allergen-specific antibodies of IgE, IgG1 and IgG4 isotypes, on human DCs and

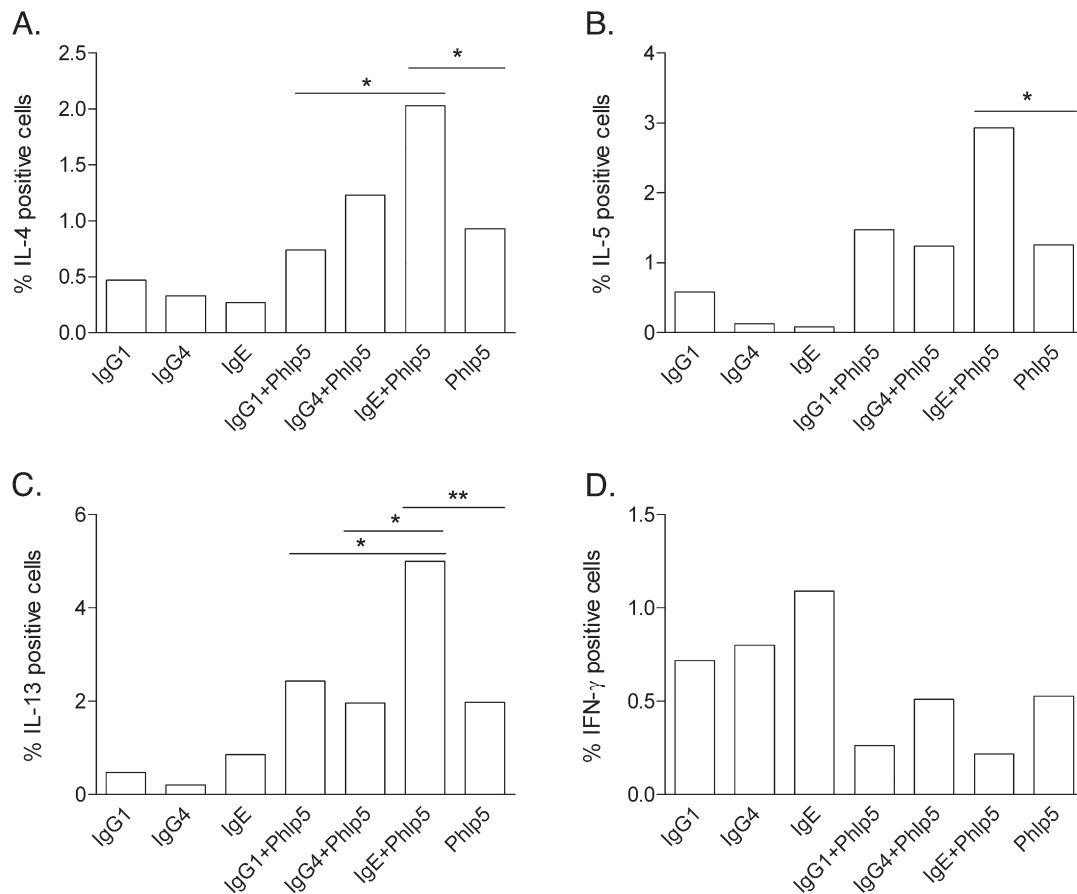


Figure 3 Th2 cytokine production after exposure of MoDCs to *Phl p 5* in complex with IgE, IgG1 and IgG4. The percentage of T-cells producing IL-4 (A), IL-13 (B), IL-5 (C) and IFN- γ (D) after DC/T-cell co-culture. DCs had previously been pulsed with *Phl p 5* and/or specific antibodies of the isotype indicated. The average values of 11 donors are shown and the statistical significance was determined with Wilcoxon Matched-Pairs Signed-Rank Test (*= $p < 0.05$, **= $p < 0.01$).

T-cells, under perfectly normalized conditions of antibody binding activity and specificity. We demonstrated an increased Th2 response after stimulation of DCs challenged with *Phl p 5* in combination with IgE, as compared to *Phl p 5* alone. In contrast, no such effect was seen when combining the allergen with either IgG1 or IgG4. We also showed differential expression levels of cytokines produced by MoDCs, following addition of allergen/IgE complexes.

Thus, the percentages of T-cells producing the Th2 cytokines IL-4, IL-13 and IL-5 were increased after interaction with MoDCs that had previously been pulsed with *Phl p 5*/IgE

complexes, as compared to *Phl p 5* alone. No such effect was evident for the Th1 cytokine IFN- γ . Accordingly, IgE in complex with antigen promotes a T-cell response that facilitates further IgE production. In agreement with our results, previous studies have shown that IgE facilitates presentation of allergen by DCs to T-cells in allergic diseases [4,7]. However, polyvalent ligation of IgE has been shown to be required for efficient internalization and subsequent presentation [3]. Thus, the use of antibodies specific for a single epitope and the use of a theoretically monovalent *rPhl p 5* could have restricted the uptake and subsequent T-cell

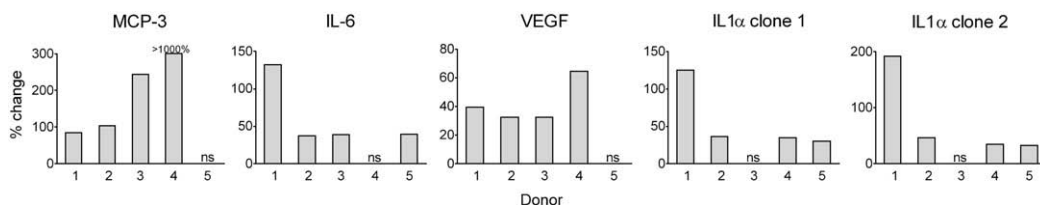


Figure 4 Altered secretion of immunoregulatory proteins in cell supernatants of MoDCs stimulated with *Phl p 5* in complex with specific IgE as compared to *Phl p 5* alone as analyzed using recombinant antibody microarrays. Up- or downregulated analytes were based on significance ($p < 0.05$), determined by paired Student's *t*-test, and a fold change of ± 1.3 in at least four out of five donors. In the case of IL-1 α , two different clones were shown to be upregulated in the analysis. ns; not significant.

presentation in our study. Nevertheless, the significantly increased percentage of T-cells producing Th2 cytokines after co-culture with DCs exposed to *Phl p 5*/IgE-complexes indicates functional IgE-allergen uptake and presentation. Possibly, the presence of a dimer component in *rPhl p 5*, as has been shown to exist for *rBet v 1* [28], may explain the effect seen in this study. Although a clear increase in Th2 cytokines was evident due to the presence of IgE, no such effect was seen in the T-cell proliferation assay. This was most likely due to the low concentration of *Phl p 5*, chosen in order to be sub-stimulatory. It would be of interest to test higher concentrations of the *Phl p 5* with and without additional antibodies to evaluate the effect on proliferation. However, due to cell limitation this could not be included in the present study. Nevertheless, the proliferation assay shows T-cell proliferation in response to *Phl p* extract in agreement with previous studies [23]. Overall, the increased Th2 cytokine response after co-culture with MoDCs previously exposed to *Phl p 5*/IgE-complexes supports a role for IgE in amplifying the allergic reaction in grass pollen allergic individuals, by further stimulating Th2 responses and thereby maintaining a harmful cycle that promotes allergic disease. In addition, this implies that a lower dose of allergen is required to induce an allergic response in sensitized individuals.

This study investigates not only the role of IgE, but also that of IgG1 and IgG4, in inducing a Th1/Th2 response in grass pollen allergy. We demonstrate that IgG1 or IgG4 in complex with *Phl p 5* do not affect the ability of MoDCs to influence the Th1/Th2 cytokine response, as compared to *Phl p 5* alone. In contrast, Anderson et al have shown IL-4 production by T-cells in mice, when antigen-challenged bone marrow derived DCs had been stimulated with antigen-specific IgG prior to T-cell co-culture [11]. This discrepancy may be due to significant differences between the mouse and human IgG subclasses and Fc γ receptors [29]. To ensure that the discrepancy in the cell assay was not due to a lack of complete IgGs and/or interfering monovalent antibodies or single chains, an SDS-PAGE was performed in non-reducing conditions. Indeed, over 90% of the IgG1 and IgG4 proteins were shown to be complete antibodies (data not shown). Overall, the results in the T-cell cytokine assay demonstrate that the human IgG1 and IgG4 were substantially less potent than IgE to cause an increased Th2 cytokine production at low allergen concentrations.

SIT has been shown to induce a multiplicity of immunological effects, such as increased levels of specific IgG4 in serum [12,13] and skewing towards a regulatory/suppressor T cell response [15]. However, it is not clear at present how these effects are exerted on the immune system. Our data show that, in contrast to IgE, IgG1 and IgG4 neither induced T-cell production of Th2 cytokines (IL-4, IL-13 and IL-5) nor the Th1 cytokine IFN- γ . Our data implies that IgG may inhibit a Th2 response not only by blocking the activation of effector cells, but also by hindering the ability of IgE-allergen complexes to stimulate a Th2 response induced by DCs. However, to further validate this hypothesis it would be desirable to undertake additional experiments where the IgG isotypes are combined with the IgE. Nevertheless, the results indicate that the effect of IgG on allergen presentation may play an important role in the success of specific immunotherapy, where increased levels of IgG are obtained.

The augmented Th2 response after T-cell co-culture with MoDC pulsed with *Phl p 5*/IgE-complexes, led us to investigate the secretion of 26 immunoregulatory proteins by MoDCs. This approach enabled us to elucidate a differential expression pattern of immunoregulatory proteins, which is in contrast to most other studies, concentrating on one or a few proteins. The analysis demonstrated an upregulation of IL-6, VEGF, MCP-3 and IL-1 α as a result of stimulation of MoDCs with *Phl p 5*/IgE-complexes in at least four out of five donors. In agreement with our result, these mediators have previously been shown to be upregulated in Th2 related diseases [30–34]. MCP-3 is an important eosinophil chemoattractant known to play a key role in the allergic response [30]. This cytokine has also been shown to be upregulated on gene level in Th2 cells stimulated with allergen-challenged MoDCs from allergic subjects [23]. Furthermore, Lee et al. have shown that VEGF is a mediator of vascular and extravascular remodelling and inflammation which enhances antigen sensitization and is crucial in adaptive Th2 inflammation [31]. The observed upregulation of IL-6 is in agreement with several other studies, suggesting that this cytokine has a role in mediating Th2 responses (reviewed by Diehl and Rincon [32]). Moreover, Gosset et al. have shown increased levels of IL-1 α in nasal lavage fluids after allergen provocation [33]. In addition, IL-1 α has been shown to be increased in allergic contact dermatitis [34]. The set of immunoregulatory proteins, which were differentially secreted, may influence the ability of DCs to augment a Th2 response.

In conclusion, we have shown that the ability of MoDCs from grass pollen allergic subjects to present allergen to memory CD4⁺ T-cells and induce a Th2 cytokine profile is significantly augmented when the uptake is mediated by antigen-specific IgE antibodies, whereas antigen-specific IgG1 and IgG4 have no such effects. Furthermore, MoDCs produced different levels of several immunoregulatory proteins when pulsed with *Phl p 5*/IgE complexes as compared to *Phl p 5* alone, and several of these proteins have previously been implicated in Th2 responses. Consequently, our data suggests a route whereby IgG antibodies, by blocking uptake of IgE-allergen complexes by DCs, can prevent a strong Th2 response which ultimately results in increased IgE production. This finding may contribute to clarify on the one side a specific amplifying process of established allergic responses, on the other the mechanism of action of specific immunotherapy.

References

- [1] M. Moser, K.M. Murphy, Dendritic cell regulation of TH1–TH2 development, *Nat. Immunol.* 1 (2000) 199–205.
- [2] D.B. Corry, F. Kheradmand, Induction and regulation of the IgE response, *Nature* 402 (1999) B18–23.
- [3] D. Maurer, E. Fiebiger, B. Reininger, C. Ebner, P. Petzelbauer, G.-P. Shi, H.A. Chapman, G. Stingl, Fc ϵ receptor I on dendritic cells delivers IgE-bound multivalent antigens into a cathepsin S-dependent pathway of MHC class II presentation, *J. Immunol.* 161 (1998) 2731–2739.
- [4] D. Maurer, S. Fiebiger, C. Ebner, B. Reininger, G.F. Fischer, S. Wichlas, M.H. Jouvin, M. Schmitt-Egenolf, D. Kraft, J.P. Kinet, G. Stingl, Peripheral blood dendritic cells express Fc ϵ RI as a complex composed of Fc ϵ RI α - and Fc ϵ RI γ -chains and

- can use this receptor for IgE-mediated allergen presentation, *J. Immunol.* 157 (1996) 607–616.
- [5] N. Novak, C. Tepel, S. Koch, K. Brix, T. Bieber, S. Kraft, Evidence for a differential expression of the FcεRIγ chain in dendritic cells of atopic and nonatopic donors, *J. Clin. Invest.* 111 (2003) 1047–1056.
 - [6] D. Maurer, C. Ebner, B. Reininger, E. Fiebiger, D. Kraft, J.P. Kinet, G. Stingl, The high affinity IgE receptor (FcεRI) mediates IgE-dependent allergen presentation, *J. Immunol.* 154 (1995) 6285–6290.
 - [7] N. Novak, R. Valenta, B. Bohle, S. Laffer, J. Haberstok, S. Kraft, T. Bieber, FcεRI engagement of Langerhans cell-like dendritic cells and inflammatory dendritic epidermal cell-like dendritic cells induces chemotactic signals and different T-cell phenotypes in vitro, *J. Allergy Clin. Immunol.* 113 (2004) 949–957.
 - [8] Z. Banki, L. Kacani, B. Mullauer, D. Wilflingseder, G. Obermoser, H. Niederegger, H. Schennach, G.M. Sprinzl, N. Sepp, A. Erdei, M.P. Dierich, H. Stoiber, Cross-linking of CD32 induces maturation of human monocyte-derived dendritic cells via NF-κB signaling pathway, *J. Immunol.* 170 (2003) 3963–3970.
 - [9] N.A. Fanger, D. Voigtlaender, C. Liu, S. Swink, K. Wardwell, J. Fisher, R.F. Graziano, L.C. Pfeifferkorn, P.M. Guyre, Characterization of expression, cytokine regulation, and effector function of the high affinity IgG receptor FcγRI (CD64) expressed on human blood dendritic cells, *J. Immunol.* 158 (1997) 3090–3098.
 - [10] A. Yada, S. Ebihara, K. Matsumura, S. Endo, T. Maeda, A. Nakamura, K. Akiyama, S. Aiba, T. Takai, Accelerated antigen presentation and elicitation of humoral response in vivo by FcγRIIB- and FcγRI/III-mediated immune complex uptake, *Cell. Immunol.* 225 (2003) 21–32.
 - [11] C.F. Anderson, M. Lucas, L. Gutierrez-Kobeh, A.E. Field, D.M. Mosser, T cell biasing by activated dendritic cells, *J. Immunol.* 173 (2004) 955–961.
 - [12] M. Larche, C.A. Akdis, R. Valenta, Immunological mechanisms of allergen-specific immunotherapy, *Nat. Rev., Immunol.* 6 (2006) 761–771.
 - [13] K.T. Nouri-Aria, P.A. Wachholz, J.N. Francis, M.R. Jacobson, S.M. Walker, L.K. Wilcock, S.Q. Staple, R.C. Aalberse, S.J. Till, S.R. Durham, Grass pollen immunotherapy induces mucosal and peripheral IL-10 responses and blocking IgG activity, *J. Immunol.* 172 (2004) 3252–3259.
 - [14] S.R. Durham, S. Ying, V.A. Varney, M.R. Jacobson, R.M. Sudderick, I.S. Mackay, A.B. Kay, Q.A. Hamid, Grass pollen immunotherapy inhibits allergen-induced infiltration of CD4+ T lymphocytes and eosinophils in the nasal mucosa and increases the number of cells expressing messenger RNA for interferon-γ, *J. Allergy Clin. Immunol.* 97 (1996) 1356–1365.
 - [15] M. Jutel, L. Jaeger, R. Suck, H. Meyer, H. Fiebig, O. Cromwell, Allergen-specific immunotherapy with recombinant grass pollen allergens, *J. Allergy Clin. Immunol.* 116 (2005) 608–613.
 - [16] F. Manca, D. Fenoglio, A. Kunkl, C. Cambiaggi, M. Sasso, F. Celada, Differential activation of T cell clones stimulated by macrophages exposed to antigen complexed with monoclonal antibodies. A possible influence of paratope specificity on the mode of antigen processing, *J. Immunol.* 140 (1988) 2893–2898.
 - [17] C. Ahlstrom-Emanuelsson, C.G. Persson, C. Svensson, M. Andersson, Z. Hosszu, A. Akerlund, L. Greiff, Establishing a model of seasonal allergic rhinitis and demonstrating dose-response to a topical glucocorticosteroid, *Ann. Allergy, Asthma, & Immun.* 89 (2002) 159–165.
 - [18] U. Andreasson, S. Flicker, M. Lindstedt, R. Valenta, L. Greiff, M. Korsgren, C.A.K. Borrebaeck, M. Ohlin, The human IgE-encoding transcriptome to assess antibody repertoires and repertoire evolution, *J. Mol. Biol.* 362 (2006) 212–227.
 - [19] S. Flicker, S. Vrtala, P. Steinberger, L. Vangelista, A. Bufer, A. Petersen, M. Ghannadan, W.R. Sperr, P. Valent, L. Norderhaug, B. Bohle, H. Stockinger, C. Suphioglu, E.K. Ong, D. Kraft, R. Valenta, A human monoclonal IgE antibody defines a highly allergenic fragment of the major timothy grass pollen allergen, *Phl p 5*: molecular, immunological, and structural characterization of the epitope-containing domain, *J. Immunol.* 165 (2000) 3849–3859.
 - [20] H. Persson, M.K. Sadegh, L. Greiff, M. Ohlin, Delineating the specificity of an IgE-encoding transcriptome, *J. Allergy Clin. Immunol.* 120 (2007) 1186–1192.
 - [21] L. Norderhaug, T. Olafsen, T.E. Michaelsen, I. Sandlie, Versatile vectors for transient and stable expression of recombinant antibody molecules in mammalian cells, *J. Immunol. Methods* 204 (1997) 77–87.
 - [22] M. Lindstedt, B. Johansson-Lindbom, C.A.K. Borrebaeck, Global reprogramming of dendritic cells in response to a concerted action of inflammatory mediators, *Int. Immunol.* 14 (2002) 1203–1213.
 - [23] M. Lindstedt, A. Schiott, A. Bengtsson, K. Larsson, M. Korsgren, L. Greiff, C.A. Borrebaeck, Genomic and functional delineation of dendritic cells and memory T cells derived from grass pollen-allergic patients and healthy individuals, *Int. Immunol.* 17 (2005) 401–409.
 - [24] E. Soderlind, L. Strandberg, P. Jirholt, N. Kobayashi, V. Alexeiva, A.M. Aberg, A. Nilsson, B. Jansson, M. Ohlin, C. Wingren, L. Danielsson, R. Carlsson, C.A. Borrebaeck, Recombining germline-derived CDR sequences for creating diverse single-framework antibody libraries, *Nat. Biotechnol.* 18 (2000) 852–856.
 - [25] C. Wingren, L. Dexlin, D. Szul, C.A.K. Borrebaeck, Design of recombinant antibody microarrays for complex proteome analysis: choice of sample labeling-tag and solid support. *Proteomics* (in press).
 - [26] J. Ingvarsson, A.G. Sjöholm, L. Truedsson, B. Jansson, C.A.K. Borrebaeck, C. Wingren, Design of recombinant antibody microarrays for serum protein profiling: targeting of complement proteins. *J. Proteome. Res.* (in press).
 - [27] C. Wingren, C.A.K. Borrebaeck, Antibody microarrays: current status and key technological advances, *Omics* 10 (2006) 411–427.
 - [28] I. Scholl, N. Kalkura, Y. Shedziankova, A. Bergmann, P. Verdino, R. Knittelfelder, T. Kopp, B. Hantusch, C. Betzel, K. Dierks, O. Scheiner, G. Boltz-Nitulescu, W. Keller, E. Jensen-Jarolim, Dimerization of the major birch pollen allergen Bet v 1 is important for its in vivo IgE-cross-linking potential in mice, *J. Immunol.* 175 (2005) 6645–6650.
 - [29] M.D. Hulett, P.M. Hogarth, Molecular basis of Fc receptor function, *Adv. Immunol.* 57 (1994) 1–127.
 - [30] M. Lampinen, M. Carlson, L.D. Hakansson, P. Venge, Cytokine-regulated accumulation of eosinophils in inflammatory disease, *Allergy* 59 (2004) 793–805.
 - [31] C.G. Lee, H. Link, P. Baluk, R.J. Homer, S. Chapoval, V. Bhandari, M.J. Kang, L. Cohn, Y.K. Kim, D.M. McDonald, J.A. Elias, Vascular endothelial growth factor (VEGF) induces remodeling and enhances TH2-mediated sensitization and inflammation in the lung, *Nat. Med.* 10 (2004) 1095–1103.
 - [32] S. Diehl, M. Rincon, The two faces of IL-6 on Th1/Th2 differentiation, *Mol. Immunol.* 39 (2002) 531–536.
 - [33] P. Gosset, F. Malaquin, Y. Delneste, B. Wallaert, A. Capron, M. Joseph, A.-B. Tonnel, Interleukin-6 and interleukin-1[α] production is associated with antigen-induced late nasal response, *J. Allergy Clin. Immunol.* 92 (1993) 878–890.
 - [34] C.Y. Levin, H.I. Maibach, Irritant contact dermatitis: is there an immunologic component? *Int. Immunopharmacol.* 2 (2002) 183–189.