



Contents lists available at ScienceDirect

## Immunobiology

journal homepage: [www.elsevier.de/imbio](http://www.elsevier.de/imbio)

## Differential T-cell responses and allergen uptake after exposure of dendritic cells to the birch pollen allergens Bet v 1.0101, Bet v 1.0401 and Bet v 1.1001

Ursula Smole<sup>a</sup>, Nina Balazs<sup>a</sup>, Karin Hoffmann-Sommergruber<sup>a</sup>, Christian Radauer<sup>a</sup>, Christine Hafner<sup>a,b</sup>, Michael Wallner<sup>c</sup>, Fatima Ferreira<sup>c</sup>, Roland Grössinger<sup>d</sup>, Esther C. de Jong<sup>e</sup>, Stefan Wagner<sup>a,\*</sup>, Heimo Breiteneder<sup>a</sup>

<sup>a</sup> Department of Pathophysiology, Centre for Physiology, Pathophysiology and Immunology Medical University of Vienna, Waehringer Guertel 18–20, 1090 Vienna, Austria

<sup>b</sup> Karl Landsteiner Institute for Dermatological Research, St. Poelten, Austria

<sup>c</sup> Department of Molecular Biology, Christian Doppler Laboratory for Allergy Diagnosis and Therapy, University of Salzburg, Salzburg, Austria

<sup>d</sup> Institute of Solid State Physics, Technical University of Vienna, Vienna, Austria

<sup>e</sup> Department of Cell Biology and Histology, Academic Medical Centre of the University of Amsterdam, Amsterdam, The Netherlands

## ARTICLE INFO

## Article history:

Received 7 September 2009

Received in revised form

6 November 2009

Accepted 6 November 2009

## Keywords:

Allergy

Allergen uptake

Bet v 1.0101

Bet v 1.0401

Bet v 1.1001

Dendritic cells

T cells

## ABSTRACT

The major birch pollen allergen Bet v 1 is present in pollen as a mixture of at least 14 isoforms that share high sequence and structural identities. These isoforms possess either a high or a low IgE-binding capacity which defines them as allergenic or hypoallergenic. Recently, we could demonstrate that only the allergenic isoform Bet v 1.0101 was able to induce an IgE response in birch pollen allergic individuals. The hypoallergenic isoforms Bet v 1.0401 and Bet v 1.1001 were unable to induce IgE synthesis. T-helper cell responses against allergens are characterised by increased levels of Th2 cytokines. Therefore, we examined extent and polarisation of the Th cell response and the kinetics of the allergen uptake after exposure of dendritic cells (DCs) to these isoforms. Monocyte-derived DCs (MDDCs) from birch pollen allergic and non-atopic individuals stimulated with Bet v 1.0101, Bet v 1.0401 or Bet v 1.1001 in combination with the maturation factors TNF- $\alpha$  and IL-1 $\beta$  resulted in a mature DC phenotype as measured by costimulatory molecule up-regulation. Only Bet v 1.0101-stimulated MDDCs from allergic subjects enhanced proliferation of autologous Th cells and the expression of the Th2 cytokines IL-5 and IL-13. Immature MDDCs of allergic individuals internalised equivalent amounts of the allergenic Bet v 1.0101 and the hypoallergenic Bet v 1.0401. In contrast, the uptake of the hypoallergenic Bet v 1.0401 by immature MDDCs of non-atopic individuals was significantly higher. These results provide evidence that DCs discriminate between allergens and highly related hypoallergens. This process may have an impact on the early phase of sensitisation.

© 2009 Elsevier GmbH. All rights reserved.

## Introduction

From spring to early summer, allergy in Europe as well as North America is mainly caused by pollen from white birch (*Betula verrucosa*) trees (D'Amato et al. 2007). Certain proteins from birch pollen have been initially defined as allergens by binding to IgE antibodies and thereby inducing degranulation of effector cells. Bet v 1, a member of the PR-10 protein family, is the most investigated birch pollen allergen. More than 98% of Austrian, Finnish, and Swedish birch pollen allergic patients display IgE antibodies to this allergen and up to 60% of these patients are mono-sensitized to Bet v 1 (Moverare et al. 2002). Bet v 1 consists of at least 14 isoforms that share > 96.5% sequence

identities and almost identical T-cell epitopes (Schenk et al. 2006). These isoforms possess either high or low IgE reactivity (Ferreira et al. 1996). Each birch pollen grain has the genetic background to produce a mixture of isoforms with varying IgE reactivity (Schenk et al. 2006). Recently, we have demonstrated that the isoform Bet v 1.0101 with high IgE reactivity and the two hypoallergenic isoforms Bet v 1.0401 and Bet v 1.1001 possessed highly variant immunogenic and allergenic properties (Wagner et al. 2008). Only Bet v 1.0101 had sensitising ability and the induced IgE antibodies partly cross-reacted with the two other isoforms.

The distinction between allergens and related hypoallergens is made by their capacity to bind and cross-link mast cell-bound IgE and to initiate the release of inflammatory mediators. Although the mechanisms of the allergic response are well described, the initial stages of the recognition of an allergen by the immune system that lead to the induction of a Th2 response and the synthesis of IgE are less clear. For aeroallergens, the main route of sensitisation is via the respiratory tract. However, only a rather limited number of inhaled antigens actually become allergens.

Abbreviations: DC, Dendritic cell; FITC, Fluorescein isothiocyanate; MDDC, Monocyte-derived dendritic cell; MF, Maturation factors

\* Corresponding author. Tel.: +43 1 40400 5103; fax: +43 1 40400 5130.

E-mail address: stefan.wagner@meduniwien.ac.at (S. Wagner).

0171-2985/\$ - see front matter © 2009 Elsevier GmbH. All rights reserved.  
doi:10.1016/j.imbio.2009.11.003

Please cite this article as: Smole, U., et al., Differential T-cell responses and allergen uptake after exposure of dendritic cells to the birch.... Immunobiology (2009), doi:10.1016/j.imbio.2009.11.003

This is an indication that, in addition to the genetic predisposition to atopy, particular proteins possess intrinsic characteristics responsible for inducing the de novo IgE synthesis. In a limited number of studies, it was demonstrated that such specific characteristics, for example the proteolytic activity of the house dust mite allergen Der p 1 or the glycan structure of the peanut allergen Ara h 1, were essential to direct the adaptive immune response toward a Th2-dominated response (Hammad et al. 2001; Shreffler et al. 2006). Additional molecular characteristics were indicated by the observation that the Brazil nut allergen Ber e 1 and the structurally similar but weak allergen SFA8 from sunflower seeds also induced different polarisations of the immune response (Kean et al. 2006).

In this study, we investigated the effects of MDDCs pulsed with allergenic or hypoallergenic Bet v 1 isoforms on T cells. We describe that only MDDCs of birch pollen allergic individuals pulsed with the allergenic isoform Bet v 1.0101 were able to induce increased Th2-like responses in naïve T cells. Both allergenic and hypoallergenic isoforms were internalised by immature MDDCs. Only MDDCs of non-atopic but not of birch pollen allergic individuals internalised significantly higher amounts of the hypoallergenic isoform Bet v 1.0401.

## Material and methods

### *Patients, allergens, and reagents*

Heparinised blood and sera were obtained from 9 individuals allergic to birch pollen who had not undergone specific immunotherapy and from 5 healthy controls with no history of reactions to birch pollen or any other allergen source. All allergic patients presented clinical reactivity to birch pollen and specific sensitisation to birch pollen was documented by allergen-specific IgE in the sera (CAP class  $\geq 2$ , measured with the ImmunoCAP<sup>®</sup>-specific IgE blood test, Phadia, Uppsala, Sweden). The healthy donors were matched with the allergic group with regards to gender and age. Blood sampling was authorised by the Ethical Committee of the Medical University of Vienna.

Recombinant Bet v 1.1001 (rBet v 1.1001) was purchased from Biomay (Vienna, Austria). For expression of Bet v 1.0101 and Bet v 1.0401, the cDNAs coding for Bet v 1.0101 (X15877) and Bet v 1.0401 (X77266) were cloned into the vector pET28b (Novagen, Merck Chemicals Limited, Nottingham, UK) and proteins were expressed in the *E. coli* strain BL21 (DE3) (Stratagene, La Jolla, CA). Proteins were purified by hydrophobic interaction chromatography using a phenyl sepharose column followed by anion exchange chromatography using a DEAE sepharose column. The endotoxin content of the recombinant proteins was  $< 3$  EU/mg protein as determined by the Limulus amoebocyte lysate (LAL) assay (Associates of Cape Cod, Inc., East Falmouth, MA).

### *Measurement of specific IgE by ELISA*

Serum IgE against Bet v 1.0101, Bet v 1.0401 and Bet v 1001 was measured by ELISA as previously described (Wagner et al. 2008)

### *Generation and induction of maturation of monocyte-derived immature dendritic cells*

Blood diluted in an equivalent volume of  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  free Hank's Balanced Salt Solution with Phenol Red (HBSS; BioWhittaker, Walkersville, MD) was centrifuged on Ficoll-Paque (GE Healthcare, Uppsala, Sweden) density gradients (1000 g, 30 min).

Interphase cells were washed in HBSS supplemented with 2% fetal calf serum (FCS; GIBCO invitrogen cell cultures, Carlsbad, CA). Monocytes and peripheral blood lymphocytes were isolated from purified PBMCs over a cushion of a Percoll (GE Healthcare) gradient (2100 g, 45 min).  $4 \times 10^5$  monocytes were cultured in 1 ml Iscove's modified Dulbecco's medium (IMDM; BioWhittaker, Walkersville, MD) containing gentamycin (86  $\mu\text{g/L}$ , Lactan, Graz, Austria) and 10% FCS, supplemented with GM-CSF (500 U/ml) and IL-4 (250 U/ml; both R&D Systems, Minneapolis, MN) in 24 well-culture plates (Greiner Bio-One, Kremsmuenster, Austria). The cells were re-fed on day 3 and on day 7, maturation was induced by culturing the cells for 48 h with 10  $\mu\text{g/ml}$  rBet v 1.0101, rBet v 1.0401 or rBet v 1.1001 in combination with 25 ng/ml TNF- $\alpha$  (Strathmann GmbH & Co. KG, Hamburg, Germany) and 10 ng/ml IL-1 $\beta$  (PeproTech, Inc., Rocky Hill, NJ) as MF (maturation factors), or with 1  $\mu\text{g/ml}$  LPS (Sigma). All subsequent tests were performed after harvesting and extensive washing of the cells to remove the allergens, the MF, or LPS.

### *Flow cytometry of dendritic cells*

Surface expression of the DC markers was analysed using multicolor flow cytometry. In brief,  $1 \times 10^5$  MDDCs either untreated or stimulated for 48 h were harvested, washed and suspended in FACS buffer consisting of phosphate buffered saline (PBS), 1% BSA, 0.1% sodium azide, 2% FCS, and 1% human serum (GIBCO invitrogen cell cultures) and then incubated with saturating concentrations of fluorescein isothiocyanate (FITC) – conjugated anti-CD40 and anti-CD80; phycoerythrin (PE) – conjugated anti-CD83 and anti-CD86; and allophycocyanin (APC) – conjugated anti-HLA-DR antibodies (all from BD Biosciences). Matched isotype control mAbs were used in control samples. Stained cells were acquired with a FACSCanto unit (BD Biosciences – Immunocytometry Systems, San Jose, CA) and analysed using BD FACSDiva software (BD Biosciences – Immunocytometry Systems).

### *Purification of naïve CD4<sup>+</sup> T cells*

CD4<sup>+</sup> T cells were obtained from peripheral blood lymphocytes after the Percoll gradient step described above using a CD4<sup>+</sup> T-cell isolation kit (Miltenyi Biotech, Bergisch Gladbach, Germany), according to the protocol of the manufacturer. Naïve CD4<sup>+</sup>CD45RA<sup>+</sup> cells were purified by negative selection using a PE-labelled anti-human CD45RO antibody (Dako, Glostrup, Denmark) and anti-PE beads (Miltenyi Biotech). T cells were frozen in IMDM containing 50% (v/v) FCS and 10% (v/v) dimethyl sulfoxide (DMSO) until use.

### *Co-culture of T cells and autologous allergen-pulsed dendritic cells*

Proliferation assays were performed with naïve CD4<sup>+</sup> cells co-cultured with autologous MDDCs that were either unstimulated, stimulated with the maturation factors TNF- $\alpha$  and IL-1 $\beta$  or the allergens in combination with the maturation factors for 6 days. The assays were performed with a DC/T-cell ratio of 1/10. Proliferation was assessed by adding 0.5  $\mu\text{Ci/ml}$  [<sup>3</sup>H]-thymidine during the last 16 h of culture and measuring the incorporated radioactivity by scintillation counting. Samples were run in triplicates.

### *Quantification of cytokine production by ELISA*

Human IFN- $\gamma$ , IL-5, IL-10, and IL-13 levels were measured in culture supernatants by ELISA according to the instructions of the

distributor of the used pairs of antibodies (Thermo Fisher Scientific, Waltham, MA). The detection limit was 6.2 pg/ml.

#### FITC labelling of Bet v 1.0101 and Bet v 1.0401

Two hundred micrograms of recombinant protein were labelled with FITC (Sigma-Aldrich, Sigma, St. Louis, MO, USA) using a standard labelling protocol (Hidaka et al. 1999). Briefly, the labelling solution containing FITC dissolved in dry DMSO at a concentration of 1 mg/ml was added 1:20 (v/v) to the allergen solution (1 mg/ml, in 0.1 M sodium carbonate, pH 9) and incubated for 8 h at 4 °C in the dark. The labelling reaction was stopped by the addition of ammonium chloride to a final concentration of 50 mM, and the solution was dialysed against PBS overnight at 4 °C. Determination of the fluorescein/protein ratio of the conjugate was measured at 495 and 280 nm using a Hitachi U-1800 spectrophotometer (Tokio, Japan). The FITC-labelled allergens were stored at –20 °C until use.

#### Uptake of FITC-labelled Bet v 1.0101 and Bet v 1.0401

Samples of  $2 \times 10^4$  immature MDDCs per ml in IMDM supplemented with 10% FCS were incubated with 1, 2.5 or 5 µg of FITC-labelled proteins. Incubation was performed at 37 °C or for control purposes at 4 °C with various time points (0, 5, 10, 30, 60, and 120 min). After incubation, the cells were washed with ice-cold FACS buffer. A minimum of  $5 \times 10^3$  cells per sample was analysed by flow cytometry. Results are quantitatively expressed as mean percentage of FITC positive cells of 5 experiments and represent uptake under experimental conditions minus background autofluorescence (untreated cells).

#### Statistical analysis

Statistical significance of differences was determined using the Wilcoxon signed ranks test. Differences were considered statistically significant for  $P < 0.05$ .

## Results

#### IgE responses to rBet v 1.0101, rBet v 1.0401 and rBet v 1.1001

Sera of 9 patients were tested for IgE reactivity to the three Bet v 1 isoforms. The patient inclusion criteria were allergic symptoms following birch pollen exposure and birch pollen CAP test results  $\geq 2$ . Blood was taken from patients A1–A3 outside and from patients A4–A9 during the birch pollen season. Sera of all 9 allergic individuals displayed IgE to Bet v 1.0101 whereas 7 sera (A1–A4, A7–A9) showed distinct ( $> 0.2$  above cut-off base line) IgE reactivity to Bet v 1.0401 and 4 sera (A2, A4, A7, A9) to Bet v 1.1001 (Fig. 1). Sera of the five healthy control individuals did not display any IgE to the allergens (data not shown).

#### Phenotyping

Immature MDDCs from seven individuals allergic to birch pollen (A3–A9) and three healthy controls were left untreated or were incubated with the maturation factors TNF- $\alpha$  and IL-1 $\beta$ ; or with rBet v 1.0101, rBet v 1.0401 or rBet v 1.1001 for 48 h in combination with the maturation factors. As positive control, LPS, a well-characterised agent to mature MDDCs, was used.

Exposure of MDDCs to the maturation factors induced DC maturation similar to that of LPS (data not shown) with an induction of the DC-specific marker CD83 and up-regulation of

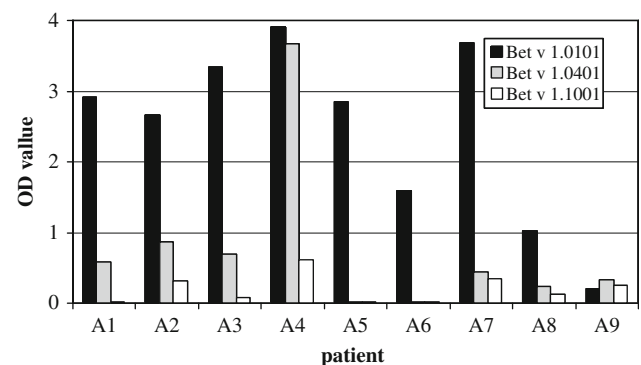


**Fig. 1.** IgE antibody levels in sera of 9 individuals with birch pollen allergy. IgE levels (OD<sub>405</sub> values) were determined for three Bet v 1 isoforms.

the costimulatory molecules CD80 and CD86. Compared to untreated cells of allergic individuals, the costimulatory molecules CD80 and CD86 were significantly up-regulated by stimulation with the combination of the Bet v 1 isoforms plus the MF (Fig. 2, CD80 and CD86). There were no differences observable upon stimulation with the isoforms plus the MF and the MF alone. For MDDCs of the non-atopic individuals, an increase in the expression of CD80 and CD86 was also measured, but significance for these changes was not calculable for the three samples (Fig. 2, CD80 and CD86). HLA-DR expression was in general higher for MDDCs from allergic individuals compared to non-atopic individuals but unchanged upon stimulation within the two groups (Fig. 2, HLA-DR). Expression of CD40 showed no differences (Fig. 2, CD40). In summary, the cells showed a mature phenotype that was induced by the addition of the maturation markers without any additional effect of the Bet v 1 isoforms.

#### Proliferation of naïve T-cells of birch pollen allergic individuals is enhanced by Bet v 1.0101-activated MDDCs

Naïve CD4<sup>+</sup> T-cells isolated from nine individuals allergic to birch pollen (patients A1–A9) and from 5 non-atopic individuals were stimulated with autologous MDDCs pulsed for 48 h with LPS, with the maturation factors TNF- $\alpha$  and IL-1 $\beta$ , or simultaneously with the maturation factors and rBet v 1.0101, rBet v 1.0401 or rBet v 1.1001 to analyse the immunogenic capacity of the proteins. Proliferation was measured after 6 days of culture by incorporation of [<sup>3</sup>H]-thymidine.

Proliferation of T cells of allergic individuals was significantly increased upon stimulation with MDDCs pulsed with LPS (Fig. 3, left graph). All three Bet v 1 isoforms induced in combination with the MF a significant increase in T-cell proliferation compared to the unstimulated medium control (Fig. 3, right graph). Compared to the background level of T-cell stimulation induced by the MF, only Bet v 1.0101 showed a significant increase in proliferation. This increase was also significant compared to the isoform Bet v 1.1001.

The proliferation levels of T-cells derived from the non-atopic control individuals were generally lower after stimulation with pulsed MDDCs compared with the proliferation of T-cells derived from birch pollen allergic individuals (Fig. 3). No significant difference was observed between the various stimuli.

#### Cytokine production of T-cells stimulated with allergen-pulsed MDDCs

In order to investigate the cytokine production of the T-cells after stimulation with autologous pulsed MDDCs, supernatants

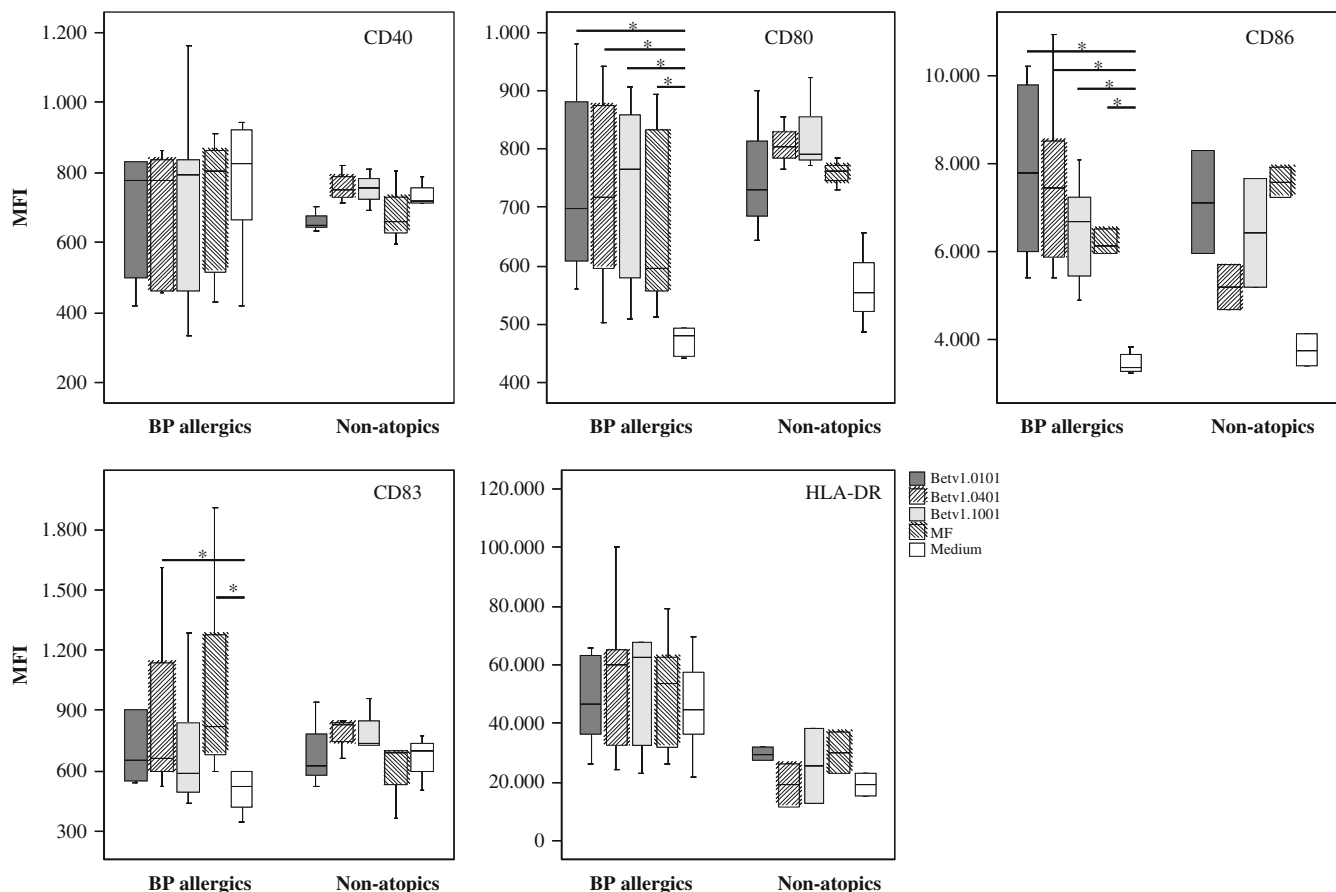


**Fig. 2.** Bet v 1 isoforms induce DC maturation in combination with the maturation factors TNF- $\alpha$  and IL-1 $\beta$ . MDDCs from subjects with birch pollen (BP) allergy ( $n=7$ ) and non-atopic individuals ( $n=3$ ) were pulsed with antigens (48 h) and analysed for expression of surface antigens. Mean fluorescence intensities (MFI) are summarised in box plots. Each box represents the interquartile range containing 50% of the data. The line across the box indicates the median. Significances were calculated using the Wilcoxon signed rank test (\*,  $p < 0.05$ ).

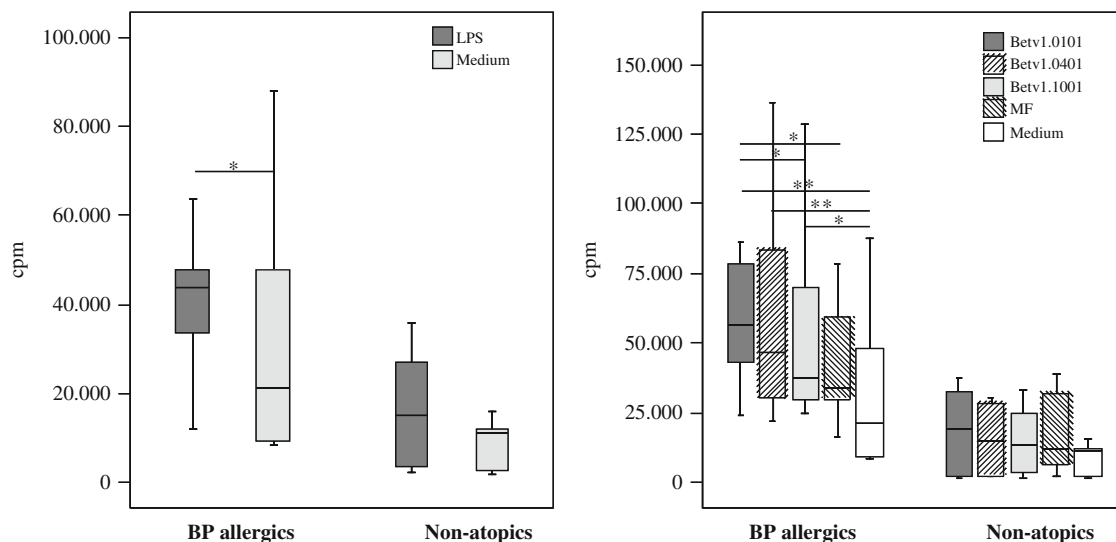


**Fig. 3.** Enhanced proliferation of naive CD4<sup>+</sup> T cells after stimulation with rBet v 1.0101 pulsed MDDCs.  $1 \times 10^5$  CD4<sup>+</sup> T cells were co-cultured with  $1 \times 10^4$  allergen-pulsed autologous MDDCs. After 6 days of cultures, proliferation was assessed by [<sup>3</sup>H]-thymidine incorporation. Summarised results from 9 birch pollen (BP) allergic and 5 non-atopic individuals are shown as counts per minute (cpm) in box plots. Significances were calculated using the Wilcoxon signed rank test (\*,  $p < 0.05$ , \*\*,  $0 < 0.01$ ).

were collected after 5 days of co-culture and cytokine levels determined.

Bet v 1.0101-pulsed DCs induced significant higher IL-5 expression of T-cells compared to the two other isoforms, to the MFs and to the unpulsed medium control (Fig. 4, IL-5). Bet v

1.1001 showed a significant increase in IL-5 production compared to the medium control, but neither Bet v 1.0401 nor Bet v 1.1001 pulsed MDDCs-induced elevated IL-5 levels compared to the background IL-5 level induced by the MFs alone. The highest IL-13 values were measured for T-cells stimulated with Bet v 1.0101

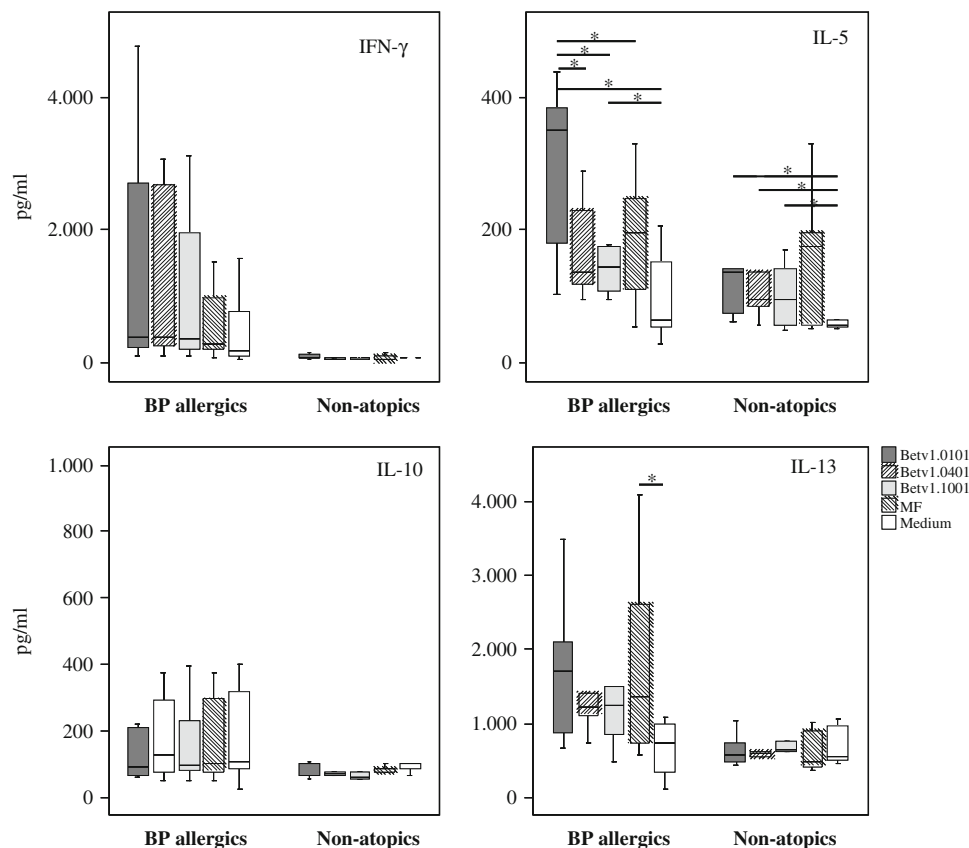


**Fig. 4.** Bet v 1.0101 but not the hypoallergenic isoforms induces an up-regulation of the Th2 cytokine IL-5. Allergen-pulsed MDDCs were co-cultured with autologous naïve T cells for 5 days and cytokine levels measured in the supernatants. Summarised results from 9 birch pollen (BP) allergic and 5 non-atopic individuals are shown as pg/ml in box plots. Significances were calculated using the Wilcoxon signed rank test (\*,  $p < 0.05$ ).

pulsed MDDCs but the differences to the control groups were not significant (Fig. 4, IL-13). The expression of IFN- $\gamma$  and IL-10 by T-cells were weakly but not significantly influenced by the differently pulsed MDDCs (Fig. 4, IFN- $\gamma$  and IL-10).

When the different allergens were presented by MDDCs prepared from the non-atopic control subjects, only low levels of cytokines were detected in supernatants of the T-cell cultures (Fig. 4). DCs of the non-atopic control subjects pulsed with the three isoforms plus the MF-induced elevated IL-5 levels in the T-cell cultures compared to the medium control but not to the T-cell cultures pulsed with DCs stimulated with the MF (Fig. 4, IL-5).

#### Uptake of FITC-labelled Bet v 1.0101 and Bet v 1.0401

Possible differences in the uptake of the allergenic isoform Bet v 1.0101 and the hypoallergenic isoform Bet v 1.0401 were investigated using MDDCs from birch pollen allergic patients ( $n=5$ ) and MDDCs from non-atopic donors ( $n=5$ ). Internalisation was studied using three different concentrations of FITC-labelled proteins (1, 2.5, or 5  $\mu\text{g}$ ) for incubation periods of 0–120 min at 37 °C. Trypan blue was added to the samples thereby quenching fluorescence from extracellular proteins. As control, cells not exposed to FITC-labelled proteins were used.

The percentage of FITC positive cells increased from 1 to 5  $\mu\text{g}$ /ml of FITC-labelled proteins for non-atopic and allergic donors, demonstrating the uptake to be dose dependent (Fig. 5). The uptake was also time dependent with the cells internalising most proteins between 30 and 60 min. MDDCs incubated with either FITC-labelled proteins at 4 °C or with FITC alone displayed no notable uptake as fluorescence was close to the background autofluorescence of control cells.

Interestingly, non-atopic individuals showed a significant lower uptake of Bet v 1.0101 compared to Bet v 1.0401 with an up to 43% lower uptake for 1  $\mu\text{g}/\text{ml}$ , up to 31% for 2.5  $\mu\text{g}/\text{ml}$  and up to 30% for 5  $\mu\text{g}/\text{ml}$  FITC-labelled proteins (Fig. 5, Non-atopics). For allergic individuals at the two lower concentrations a slightly lower uptake of Bet v 1.0101 was observed but this was not significant. At the highest concentration of FITC-labelled proteins the uptake of the two isoforms was comparable (Fig. 5, BP allergic).

#### Discussion

Here, we investigated the ability of naturally occurring allergenic and hypoallergenic Bet v 1 isoforms to polarise T-cell responses via DC activation. Only MDDCs of birch pollen allergic individuals stimulated with the allergenic isoform Bet v 1.0101 resulted in a significant increase of the T-cell proliferation and of Th2 cytokine levels regardless of whether the cells had been isolated in- or outside the pollen season. This Th2 polarisation was not a consequence of differential internalisation as the extent and the kinetics of the uptake of allergenic and hypoallergenic isoforms by MDDCs of allergic individuals were comparable. In contrast, MDDCs of non-atopic individuals showed a significantly higher uptake of the hypoallergenic isoform.

In the periphery, immature DCs are well equipped with a series of receptors for pathogen-associated molecular patterns. Signalling through these receptors triggers DC migration to the secondary lymphoid organs where they develop into a mature state characterised by high expression levels of MHC and costimulatory molecules and the ability to present captured

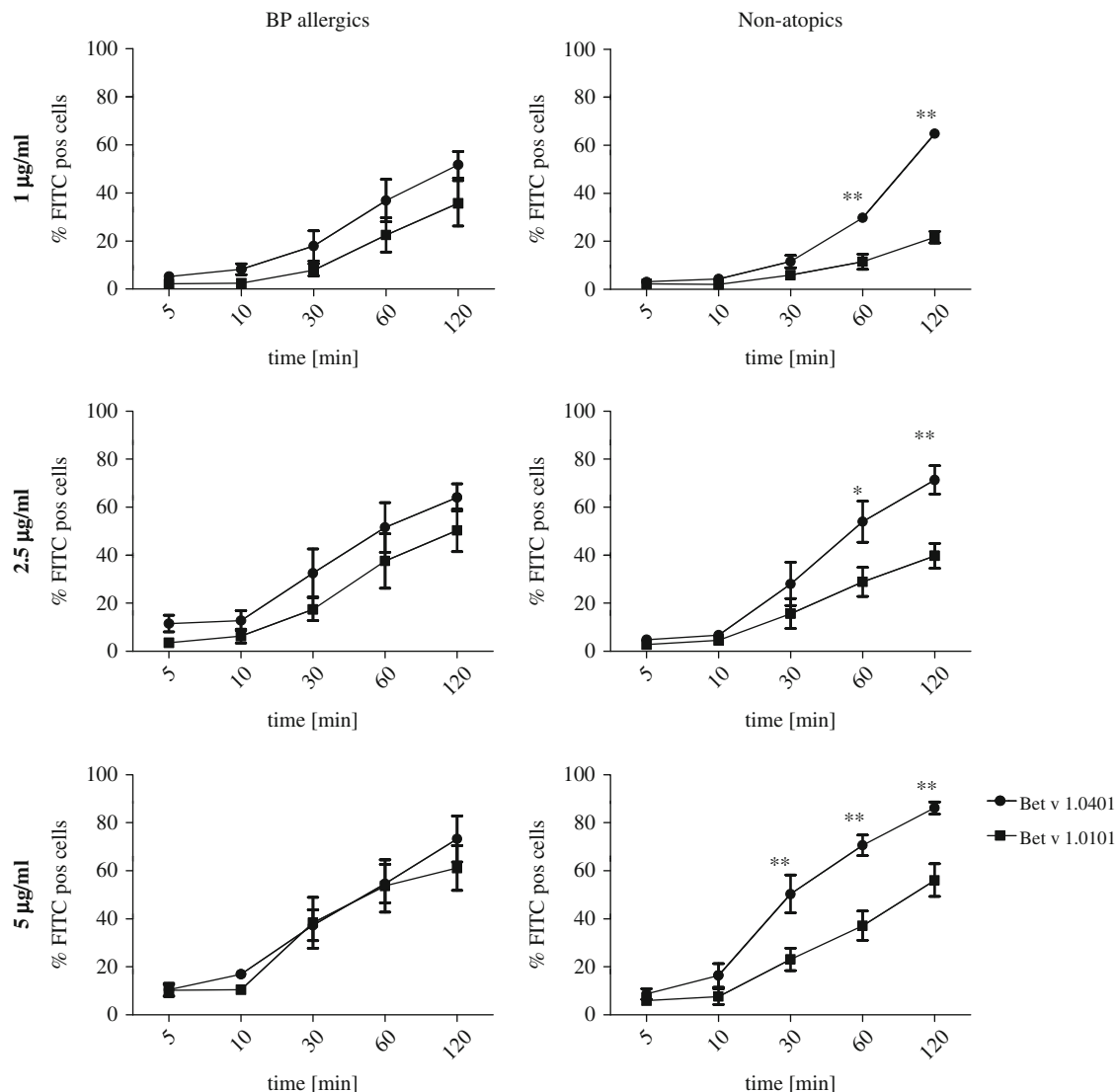


**Fig. 5.** Uptake of Bet v 1.0101 and Bet v 1.0401 by MDDCs of birch pollen (BP) allergic and non-atopic donors. Immature MDDCs were incubated with three different concentrations of FITC-labelled proteins for 120 min at 37 °C. Uptake was measured by means of flow cytometry and expressed as mean percent of FITC positive cells of data from each five allergic and healthy donors  $\pm$  standard deviation. Significances were calculated using the Wilcoxon signed rank test (\*,  $p < 0.05$ , \*\*,  $0 < 0.01$ ).

antigens to T-cells (Wilson and Villadangos 2005). Upon stimulation with the Bet v 1 isoforms plus the maturation factors TNF- $\alpha$  and IL-1 $\beta$ , an up-regulation of the costimulatory molecules CD80 and CD86 of MDDCs from allergic and from non-atopic individuals was observed (Fig. 2). These up-regulations indicate a mature phenotype of the MDDCs that is only dependent on the maturation factors as no difference in the expression of costimulatory molecules was observed for MDDCs stimulated with the maturation factors alone or in combination with the allergens (Fig. 2). The addition of TNF- $\alpha$  and IL-1 $\beta$  in combination with allergens was used in previous studies for DC maturation (Bellinghausen et al. 2008; König et al. 2007; Shreffler et al. 2006). The requirement of such a stimulus to certain allergens to induce DC maturation was demonstrated as stimulation of DCs by Bet v 1 or Der p 1 alone did not result in DC maturation and T-cell proliferation (De Wit et al. 2000; Gerstmayr et al. 2007). Interestingly, HLA-DR expression was in general higher for allergic than non-atopic individuals (Fig. 2). This indicates a more activated status of the DCs as it was earlier described for birch pollen allergic versus healthy individuals (Casas et al. 2007).

Maturation of MDDCs from birch pollen allergic individuals induced by Bet v 1.0101 resulted in an enhanced activity as demonstrated by the increased proliferation of naïve autologous T-cells (Fig. 3).

We used naïve T-cells as a model system to reflect the first contact of the allergens with the immune system and to exclude effects based on cross-reactivity on the T-cell epitope level as it has been demonstrated for memory cells with Bet v 1-related food allergens (Jahn-Schmid et al. 2005).

The increased proliferation of T-cells resulting from the co-culture with DCs exposed to Bet v 1.0101 indicated a functional allergen uptake and presentation by DCs. Consistent with these proliferation results, Th2 cytokine levels were also elevated (Fig. 4). Interestingly, although MDDCs from allergic and non-atopic individuals were pulsed with the same maturation factors and had developed into mature cells, only stimulation with Bet v 1.0101 of MDDCs of the allergic subjects resulted in enhanced T-cell proliferation and elevation of Th2-type cytokines. This indicates that these DCs could discriminate between proteins despite high sequence identities over 96% and a very high degree

of structural identity. A similar observation was made by Kean and co-workers who measured Th2 cytokine induction by DCs pulsed with the Brazil nut allergen Ber e 1 but not with the homologous minor allergen SFA8 from sunflower seeds (Kean et al. 2006).

In this study, an *in vitro* culture system was used. Although such a system provides information on potential immunological mechanisms, it is artificial. Hence, the behaviour of cells under the conditions used *in vitro* might not be the same as *in vivo*. The observation that only Bet v 1.0101-pulsed MDDCs were able to induce enhanced Th2 polarisation is in accordance with our previous study using human sera (Wagner et al. 2008). Investigating the antibody responses of individuals allergic to birch pollen revealed that only Bet v 1.0101 was able to induce the synthesis of IgE that partly cross-reacted with the isoforms Bet v 1.0401 and Bet v 1.1001 (Wagner et al. 2008). Similar results were published for the yeast allergen Mala s 11 (Vilhelmsson et al. 2007). IgE antibodies induced by Mala s 11 were cross-reactive with the homologous human MnSOD but only Mala s 11 possessed the ability to induce DC maturation (Vilhelmsson et al. 2007). This indicates that the *in vitro* system using MDDCs reflected the behaviour of the cells *in vivo*.

In order to investigate the influence of protein uptake by MDDCs on the induction of T-cell proliferation we performed uptake studies using the allergenic isoform Bet v 1.0101 and the hypoallergenic isoform Bet v 1.0401. Interestingly, a significant difference was observable only for MDDCs of non-atopic individuals (Fig. 5). These cells internalised lower amounts of the allergenic isoform Bet v 1.0101. The observed higher uptake of Bet v 1.0101 by MDDCs of allergic individuals could possibly be explained by pre-existing specific IgE or IgG bound to Fc receptors on DCs. However, since the MDDCs used in this study were generated from monocytes cultured for 7 days in the absence of human serum, the presence of Bet v 1.0101-specific IgE or IgG in the cell cultures can be virtually excluded. This hypothesis was corroborated by the fact that the removal of possible bound IgE by lactic acid (Maurer et al. 1995) did not influence the uptake of the FITC-labelled allergens (data not shown).

A necessary and sufficient DC-derived signal for Th2 differentiation is not known. In a recent review the process of Th2 cell sensitization of allergens was associated with three fundamental processes – the sampling of the allergens across the epithelium barrier, epithelial-cell activation and DC activation (Hammad and Lambrecht 2008). For the highly investigated Th2-promoting *Schistosoma mansoni* eggs very recently the glycoprotein omega-1 was identified as a Th2-polarising protein (Everts et al. 2009; Steinfeldt et al. 2009). The molecular properties of this protein as well as of allergens necessary for Th2 polarisation remain unclear. In additional studies using the model of allergenic versus hypoallergenic isoforms of Bet v 1 the intrinsic characteristics of Bet v 1.0101, which induce Th2 polarisation and the molecular mechanisms by which this process is transduced by DCs will have to be investigated. Understanding the mechanisms of the induction of Th2 adaptive immunity will enable the generation of higher immunogenic and lower allergenic preparations for allergen-specific immunotherapy or intervention strategies at the earliest stages of allergic disease pathogenesis.

## Acknowledgements

This work was supported by the APART program of the Austrian Academy of Sciences (to S.W.) and grant SFB F01802 of the Austrian Science Fund (to H.B.).

## References

- Bellinghausen, I., Haringer, B., Lafargue, B., Strand, D., König, B., Decker, H., Saloga, J., 2008. Allergological implication of the quaternary hexameric structure of the cockroach allergen Per a 3. *Clin. Exp. Allergy* 38, 539–548.
- Casas, R., Skarsvik, S., Lindstrom, A., Zetterstrom, O., Duchon, K., 2007. Impaired maturation of monocyte-derived dendritic cells from birch allergic individuals in association with birch-specific immune responses. *Scand. J. Immunol.* 66, 591–598.
- D'Amato, G., Cecchi, L., Bonini, S., Nunes, C., Annesi-Maesano, I., Behrendt, H., Liccardi, G., Popov, T., van Cauwenberge, P., 2007. Allergenic pollen and pollen allergy in Europe. *Allergy* 62, 976–990.
- De Wit, D., Amraoui, Z., Vincart, B., Michel, O., Michils, A., Van Overvelt, L., Willems, F., Goldman, M., 2000. Helper T-cell responses elicited by Der p 1-pulsed dendritic cells and recombinant IL-12 in atopic and healthy subjects. *J. Allergy Clin. Immunol.* 105, 346–352.
- Everts, B., Perona-Wright, G., Smits, H.H., Hokke, C.H., van der Ham, A.J., Fitzsimmons, C.M., Doenhoff, M.J., van der Bosch, J., Mohrs, K., Haas, H., Mohrs, M., Yazdanbakhsh, M., Schramm, G., 2009. Omega-1, a glycoprotein secreted by *Schistosoma mansoni* eggs, drives Th2 responses. *J. Exp. Med.* 206, 1673–1680.
- Ferreira, F., Hirtenlehner, K., Jilek, A., Godnik-Cvar, J., Breiteneder, H., Grimm, R., Hoffmann-Sommergruber, K., Scheiner, O., Kraft, D., Breitenbach, M., Rheinberger, H.J., Ebner, C., 1996. Dissection of immunoglobulin E and T lymphocyte reactivity of isoforms of the major birch pollen allergen Bet v 1: potential use of hypoallergenic isoforms for immunotherapy. *J. Exp. Med.* 183, 599–609.
- Gerstmayr, M., Ilk, N., Schabussova, I., Jahn-Schmid, B., Egelseer, E.M., Sleytr, U.B., Ebner, C., Bohle, B., 2007. A novel approach to specific allergy treatment: the recombinant allergen-S-layer fusion protein rSbsC-Bet v 1 matures dendritic cells that prime Th0/Th1 and IL-10-producing regulatory T cells. *J. Immunol.* 179, 7270–7275.
- Hammad, H., Charbonnier, A.S., Duez, C., Jacquet, A., Stewart, G.A., Tonnel, A.B., Pestel, J., 2001. Th2 polarization by Der p 1 – pulsed monocyte-derived dendritic cells is due to the allergic status of the donors. *Blood* 98, 1135–1141.
- Hammad, H., Lambrecht, B.N., 2008. Dendritic cells and epithelial cells: linking innate and adaptive immunity in asthma. *Nat. Rev. Immunol.* 8, 193–204.
- Hidaka, H., Hidaka, E., Tozuka, M., Nakayama, J., Katsuyama, T., Fidge, N., 1999. The identification of specific high density lipoprotein3 binding sites on human blood monocytes using fluorescence-labeled ligand. *J. Lipid Res.* 40, 1131–1139.
- Jahn-Schmid, B., Radakovics, A., Luttkopf, D., Scheurer, S., Vieths, S., Ebner, C., Bohle, B., 2005. Bet v 1142–156 is the dominant T-cell epitope of the major birch pollen allergen and important for cross-reactivity with Bet v 1-related food allergens. *J. Allergy Clin. Immunol.* 116, 213–219.
- Kean, D.E., Goodridge, H.S., McGuinness, S., Harnett, M.M., Alcocer, M.J., Harnett, W., 2006. Differential polarization of immune responses by plant 2S seed albumins, Ber e 1, and SFA8. *J. Immunol.* 177, 1561–1566.
- König, B., Petersen, A., Bellinghausen, I., Bottcher, I., Becker, W.M., Knop, J., Saloga, J., 2007. Human dendritic cells transfected with allergen-DNA stimulate specific immunoglobulin G4 but not specific immunoglobulin E production of autologous B cells from atopic individuals *in vitro*. *Immunology* 122, 239–246.
- Maurer, D., Ebner, C., Reininger, B., Fiebiger, E., Kraft, D., Kinert, J.P., Stingl, G., 1995. The high affinity IgE receptor (Fc epsilon RI) mediates IgE-dependent allergen presentation. *J. Immunol.* 154, 6285–6290.
- Moverare, R., Westritschnig, K., Svensson, M., Hayek, B., Bende, M., Pauli, G., Sorva, R., Haahtela, T., Valenta, R., Elfman, L., 2002. Different IgE reactivity profiles in birch pollen-sensitive patients from six European populations revealed by recombinant allergens: an imprint of local sensitization. *Int. Arch. Allergy Immunol.* 128, 325–335.
- Schenk, M.F., Gilissen, L.J., Esselink, G.D., Smulders, M.J., 2006. Seven different genes encode a diverse mixture of isoforms of Bet v 1, the major birch pollen allergen. *BMC Genomics* 7, 168.
- Shreffler, W.G., Castro, R.R., Kucuk, Z.Y., Charlop-Powers, Z., Grishina, G., Yoo, S., Burks, A.W., Sampson, H.A., 2006. The major glycoprotein allergen from *Arachis hypogaea*, Ara h 1, is a ligand of dendritic cell-specific ICAM-grabbing nonintegrin and acts as a Th2 adjuvant *in vitro*. *J. Immunol.* 177, 3677–3685.
- Steinfeldt, S., Andersen, J.F., Cannons, J.L., Feng, C.G., Joshi, M., Dwyer, D., Caspar, P., Schwartzberg, P.L., Sher, A., Jankovic, D., 2009. The major component in schistosome eggs responsible for conditioning dendritic cells for Th2 polarization is a T2 ribonuclease (omega-1). *J. Exp. Med.* 206, 1681–1690.
- Vilhelmsson, M., Johansson, C., Jacobsson-Ekman, G., Cramer, R., Zargari, A., Scheynius, A., 2007. The *Malassezia sympodialis* allergen Mala s 11 induces human dendritic cell maturation, in contrast to its human homologue manganese superoxide dismutase. *Int. Arch. Allergy Immunol.* 143, 155–162.
- Wagner, S., Radauer, C., Bublin, M., Hoffmann-Sommergruber, K., Kopp, T., Greisenegger, E.K., Vogel, L., Vieths, S., Scheiner, O., Breiteneder, H., 2008. Naturally occurring hypoallergenic Bet v 1 isoforms fail to induce IgE responses in individuals with birch pollen allergy. *J. Allergy Clin. Immunol.* 121, 246–252.
- Wilson, N.S., Villadangos, J.A., 2005. Regulation of antigen presentation and cross-presentation in the dendritic cell network: facts, hypothesis, and immunological implications. *Adv. Immunol.* 86, 241–305.