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Characteristics of T-cell and B-cell immune responses to pollen allergens in Bulgarian patients with pollinosis

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ABSTRACT

Characterization of the pollen allergens and identification of the main T-cell and B-cell epitopes is of crucial importance, since the clarification of the cellular and molecular mechanisms will allow long-term control of this type allergic diseases and development of safe immunotherapeutic medication. The objective of this study was to identify which pollen allergens are responsible for the T-cell activation in atopic patients with pollinosis and to determine whether the same allergens are responsible for the IgE-mediated reactions. Thirty-seven patients with pollinosis and thirteen non-allergic subjects were recruited. Peripheral blood samples were collected out of the pollen season. T-cell responses (IFN- γ production) towards different pollen allergens and levels of pollen specific IgE and IgG in the sera were measured by ELISA. The T-cell reactivity in most patients was directed towards the grass pollen B1, tree pollen I and autumn pollen B5. 70% of the allergic individuals responded to allergens from *Lolium perenne*, 84% to *Dactylis glomerata*, 11% to *Phleum pratense*, 65% to *Betula pendula* and 70% to *Taxus baccata*. Elevated serum levels of specific IgE in the allergic patients ($p < 0.01$) were measured against the tree pollen I and autumn pollen, which include widespread deciduous trees (birch, willow, poplar and yew) and *Artemisia absinthium*, respectively. Our results show that the T-cell reactivity and antibody responses may be directed towards different or the same allergens. In addition, we propose that short synthetic peptides, which contain overlapping T- and B-cell epitopes can be used for specific immunotherapy treatment if they lack antibody recognition domains.

Key words: pollen allergy, T-cell responses, B-cell responses, allergens

Introduction

Allergic diseases are defined as multifactorial, heterogeneous disorders caused by the interaction of environmental and genetic factors (Takai & Ikeda, 2011). Their pathogenesis is determined by the immune system – overproduction of IgE, abnormalities in the receptor for IgE or impaired Th2 cell differentiation. Sources of allergens (such as pollen) can produce or contain proteases, which are allergens. It is assumed that the proteolytic activities of these proteases are involved in the pathogenesis of the allergy by facilitating the passage of allergens through tissue barriers, breaking down different molecules, which affects the

functions of cells and the immune responses (Takai & Ikeda, 2011).

During the last years, the allergic diseases have become a serious health problem for most European countries, which reported a drastic increase of their distribution only for several decades, and now affected at least 20% of the population (Evans III, 1993; Burney et al., 1997; D'Amato et al., 1998; Brožek et al., 2010).

There are comparatively few investigations concerning the frequency of pollen allergies in Bulgaria and specificity of the immune response of the allergic patients. Studying the range and quantity of pollen grains outdoors and indoors during the pollen seasons in 1989 and 1990, Yankova

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reported for changes in the health status of some individuals affected by pollinosis (Yankova, 1991). The species belonging to Poaceae have been determined as most frequent cause of pollinosis in Bulgaria (Petrunov & Yankova, 1991). Several years later, Yankova et al. observed a tendency for an increase of the ragweed pollinosis (Yankova et al., 1996; 2000). Experiments with pollen extracts have shown a specific IgE response to grass pollen in 92% of the studied allergic patients (Michova et al., 2006).

In 2010, a new allergen prepared from Bulgarian ragweed plants was characterized and it was found that 17.3% of all 81 tested patients with pollen allergy have positive reaction to the Bulgarian allergen from *Ambrosia artemisiifolia* (Nikolov et al., 2010).

In this study, we investigated the immune responses in Bulgarian patients with pollinosis aiming to identify the main sensitizing pollen allergens which activate T-cells and to determine whether the same allergens are responsible for the IgE-mediated reactions.

Materials and Methods

Allergens

Several pollen extracts have been used in this study: grass pollen (B1) – a mixed grass pollen allergen (*Dactylis glomerata*, *Festuca sp.*, *Lolium perenne*, *Secale cereale*, *Phleum pratense*, *Poa sp.*, *Holcus lanatus*, *Agrostis alba*, *Bromus sp.*, *Alopecurus sp.*, *Agropyron repens*, *Arrhenatherum elatius*, *Zea mays*, *Deschampsia caespitosa*); tree pollen I (B2-I) – a mixed tree pollen allergen (*Cornus mas*, *Ulmus sp.*, *Corylus avellana*, *Betula pendula*, *Alnus sp.*, *Carpinus betulus*, *Salix alba*, *Salix caprea*, *Populus sp.*; *Populus tremula*; *Fraxinus sp.*, *Taxus baccata*); tree pollen II (B2-II) – a mixed tree pollen allergen (*Quercus sp.*, *Fagus sylvatica*, *Acer campestre*, *Acer negundo*, *Acer pseudoplatanus*, *Juglans regia*, *Morus sp.*); tree pollen III (B2-III) – a mixed tree pollen allergen (*Pinus nigra*, *Pinus sylvestris*, *Picea abies*, *Larix europaeae*, *Platanus orientalis*, *Aesculus hippocastanum*, *Sambucus nigra*, *Tilia sp.*, *Ailanthus glandulosa*; *Gleditschia sp.*); spring pollen (B3) – a mixed pollen allergen (*Ranunculus sp.*, *Taraxacum officinale*, *Lilium sp.*, *Narcissus sp.*, *Tulipa sp.*, *Carex sp.*, *Brassica napus*); summer pollen (B4) – a mixed pollen allergen (*Paeonia sp.*, *Papaver sp.*, *Philadelphus coronarius*, *Rosa canina*, *Cannabis sativa*, *Ligustrum vulgare*, *Typha sp.*, *Matricaria sp.*); autumn pollen (B5) – a mixed pollen

allergen (*Amaranthus sp.*, *Artemisia absinthium*, *Plantago major.*, *Rumex acetosa*, *Rumex acetosella*, *Chenopodium sp.*, *Urtica dioica*, *Rumex patientia*); individual pollen extracts from *Lolium perenne*, *Dactylis glomerata*, *Phleum pratense*, *Plantago major*, *Artemisia absinthium*, *Urtica dioica*, *Taxus baccata*, *Corylus avellana* and *Betula pendula*. All pollen extracts we purchased from BulBio-NCIPD Ltd., Sofia, Bulgaria).

Based on our previous study related to the similarity between pollen T- and B-cell epitopes and the binding affinity to MHC II molecules (Moten et al., 2011) we have designed three peptide sequences that are analogs of the pollen allergens: PA-1 (GELQVIDKIDAAFKVAATAA), PA-2 (KEMGETLLRAVESYLLAHS) and PA-3 (GTKSEVEDVIPEGWKADTSY). These peptides were synthesized from Schafer-N (Copenhagen, Denmark).

Participants

Thirty-seven patients with pollinosis (15 male and 22 female, 26-52 years old, mean age 37) and 13 healthy non-allergic age-matched subjects (four male and nine female, 23-50 years old, mean age 35) were included in this study. All allergic patients had pollen induced seasonal allergic rhinitis for at least two years, positive skin prick test to some of the pollen allergens and serum specific IgE to pollen extracts (measured by ImmunoCAP, ImmunoDiagnostics Thermo Fisher Scientific, Uppsala, Sweden).

Non-allergic healthy controls were identified as having negative skin prick tests to the main groups of pollen allergens and no clinical history of allergy. None of the subjects had received allergen-specific immunotherapy or used antihistamines or corticosteroids during the past 2 months before the blood sampling. Only non-smokers were recruited in the study.

All tests and analyses were performed in adherence to the Declaration of Helsinki ethical guidelines. Allergic patients and control subjects were enrolled in the study after written informed consent, and the study was approved by the Ethics Committee at the Plovdiv University.

T cell response and pollen specific antibody levels

Peripheral blood samples were collected out of the pollen season (February) by a certified nurse into heparinized tubes (Vacutainer®, BD) and centrifuged at 700×g at room temperature for 15 minutes. The serum was transferred into 2 ml microtubes and stored at -70°C until analyses. From the

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cell fraction, peripheral blood mononuclear cells (PBMCs) were isolated by Lymphoprep™ (AXIS-SHIELD PoC AS, Oslo, Norway), washed and resuspended at 2×10^6 cells/ml in RPMI 1640 medium containing GlutaMAX™ Supplement and HEPES (Gibco®, Thermo Fisher Scientific), 5% autologous serum, penicillin (100 U/ml) and streptomycin (100 µg/ml). Cells were plated in 96-well round-bottom plate (Nunc, Roskilde, Denmark), stimulated in triplicates with the allergens, synthetic peptides (5 µg/ml), medium alone (negative control) or in the presence of 1 µg/ml phytohemagglutinin (PHA, Sigma-Aldrich) as a positive control, and cultured for 7 days at 37°C and 5% CO₂. After 7 days, 50 µl supernatant were harvested and pooled from the replicates and IFN-γ production was measured by IFN gamma Human ELISA Kit (Abcam).

The levels of pollen specific IgE and IgG in the sera were measured by ELISA. Microtiter plates (MaxiSorp Immuno Plate, Nalge Nunc International, Roskilde, Denmark) were coated with 50 µl/well pollen extract (1000 PNA/ml) or synthetic peptides (5 µg/ml) overnight at 4°C. After blocking for 1 h with phosphate-buffered saline (PBS), 0.05% (v/v) Tween 20 and 1% (w/v) nonfat milk, the plates were washed with PBS/Tween 20 and 1:10 diluted sera from the allergic and non-allergic (healthy) subjects were added in triplicates onto the coated plates (100 µl/well) and incubated at 4°C overnight. After washing, 50 µl of biotinylated monoclonal anti-human IgE antibody (1:1000 dilution, clone GE-1, Sigma-Aldrich) or biotinylated anti-human IgG (γ-chain specific) antibody (1:2000 dilution, Sigma-Aldrich) was added for 1 h at 37°C for detection of pollen specific IgE and IgG, respectively. Antibodies were detected using peroxidase-conjugated goat anti-biotin pAb (Calbiochem®, Merck Millipore) and 2,2-azino-di-[2-ethylbenzthiazoline sulfonate] diammonium salt (ABTS tablets, Roche Diagnostics) as substrate. The absorbance values were determined by a microplate reader (BioTek, USA) at 405 nm.

Statistical analysis

The statistical evaluation was performed by using StatView software (SAS Institute, USA). The non-parametric Mann-Whitney U test was used for comparing the T-cell response and pollen specific Ig levels between the patients with pollinosis and control subjects. Differences were considered as significant when $p < 0.05$.

Results

To investigate the mechanisms of sensitization of individuals and development of pollinosis, it is of crucial importance to know the primary T-cell epitopes. To determine which pollen allergens are able to activate the T-cells out of the pollen season, we have measured the production of IFN-γ from PBMCs in response to stimulation *ex vivo* with different pollen extracts and synthetic peptides. First we have analyzed the response against the grass pollen. Samples from all allergic individuals showed elevated levels of IFN-γ production after stimulation with grass pollen extract (Figure 1A). No production of IFN-γ was detected in the samples from the control subjects (Figure 1B). Next we assessed the T-cell response against individual pollen extracts from *Lolium perenne*, *Dactylis glomerata* and *Phleum pratense*, which are included in the grass pollen extract. Results demonstrated that 70% of the patients with pollinosis (but not control subjects) respond to allergens from *Lolium perenne* (Figure 1C,D), 84% (31 patients) respond to allergens from *Dactylis glomerata* (Figure 1E,F) and only 11% (4 patients) respond to allergens from *Phleum pratense* (Figure 1G,H). When we compared IFN-γ production after stimulation with grass allergens between the allergic patients and healthy controls, we found significant differences for the grass pollen (B1), *Lolium perenne* extract, *Dactylis glomerata* extract, but not for the *Phleum pratense* extract (Figure 2).

Further, we characterised IFN-γ production under the same conditions following PBMC stimulation with tree allergens (Figure 3). Significantly higher levels of IFN-γ were detected in the PBMC supernatants from the allergic patients (68%, 25 patients) only after stimulation with tree pollen I (Figure 3A). No T-cell responses were seen when the cells were stimulated with tree pollen II (Figure 3C,D) or tree pollen III (Figure 3E,F). Twenty-four of the investigated 37 allergic patients (65%) responded to *Betula pendula* and 26 patients (70%) responded to *Taxus baccata* (Figure 4). Pollen from these trees was included in the tree pollen I. No response was detected against *Corylus avellana* that also was included in the tree pollen I (Figure 4C,D). IFN-γ production of stimulated cultures with tree allergens was significantly higher in the pollinosis group compared to the non-allergic individuals. (Figure 5).

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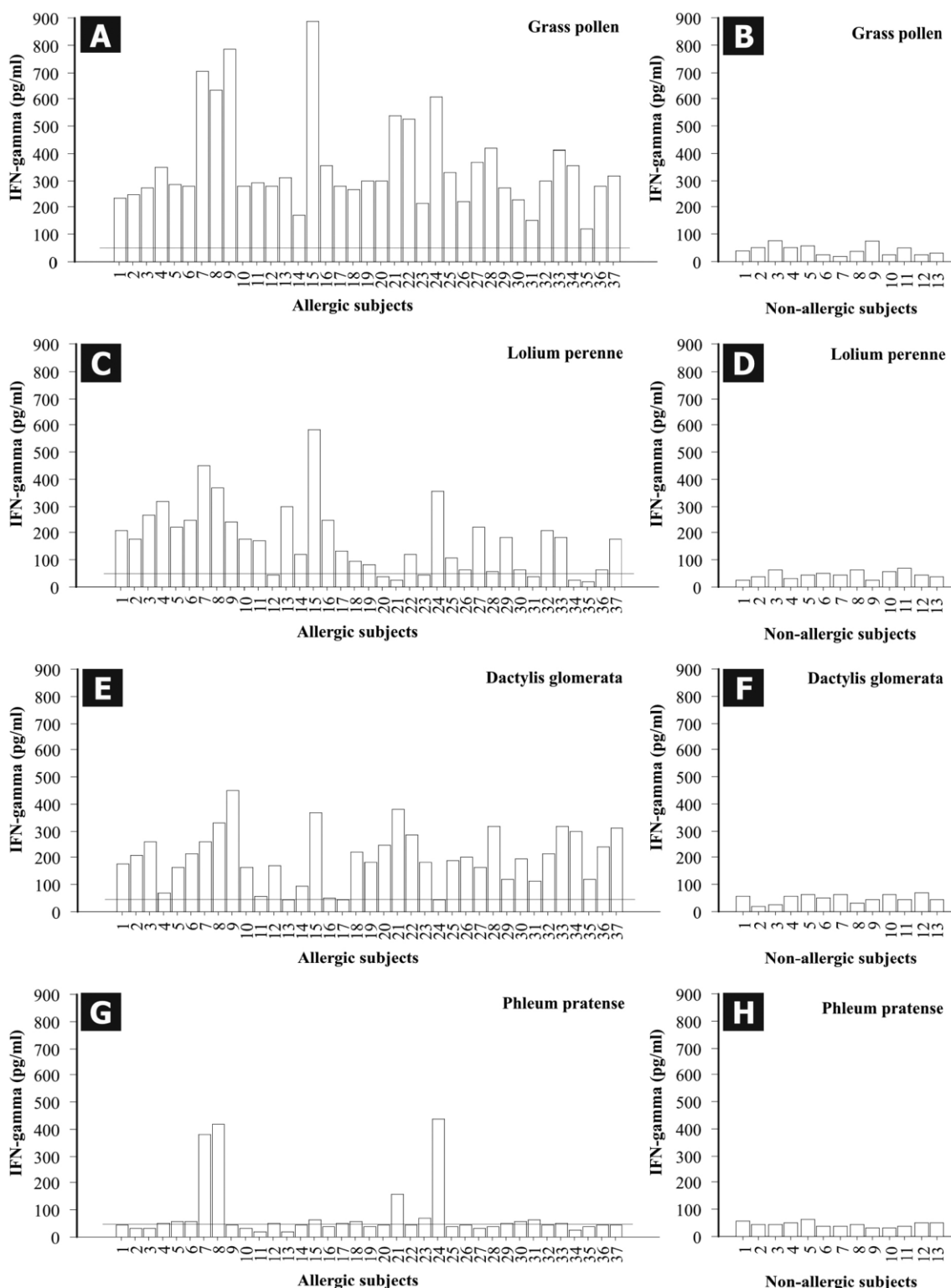


Figure 1. Biodiversity Individual IFN- γ responses upon stimulation of peripheral blood mononuclear cells (PBMCs) from allergic subjects and non-allergic controls with grass pollen extract (A, B), *Lolium perenne* extract (C, D), *Dactylis glomerata* extract (E, F) and *Phleum pratense* extract (G, H). The horizontal line represents the baseline level.

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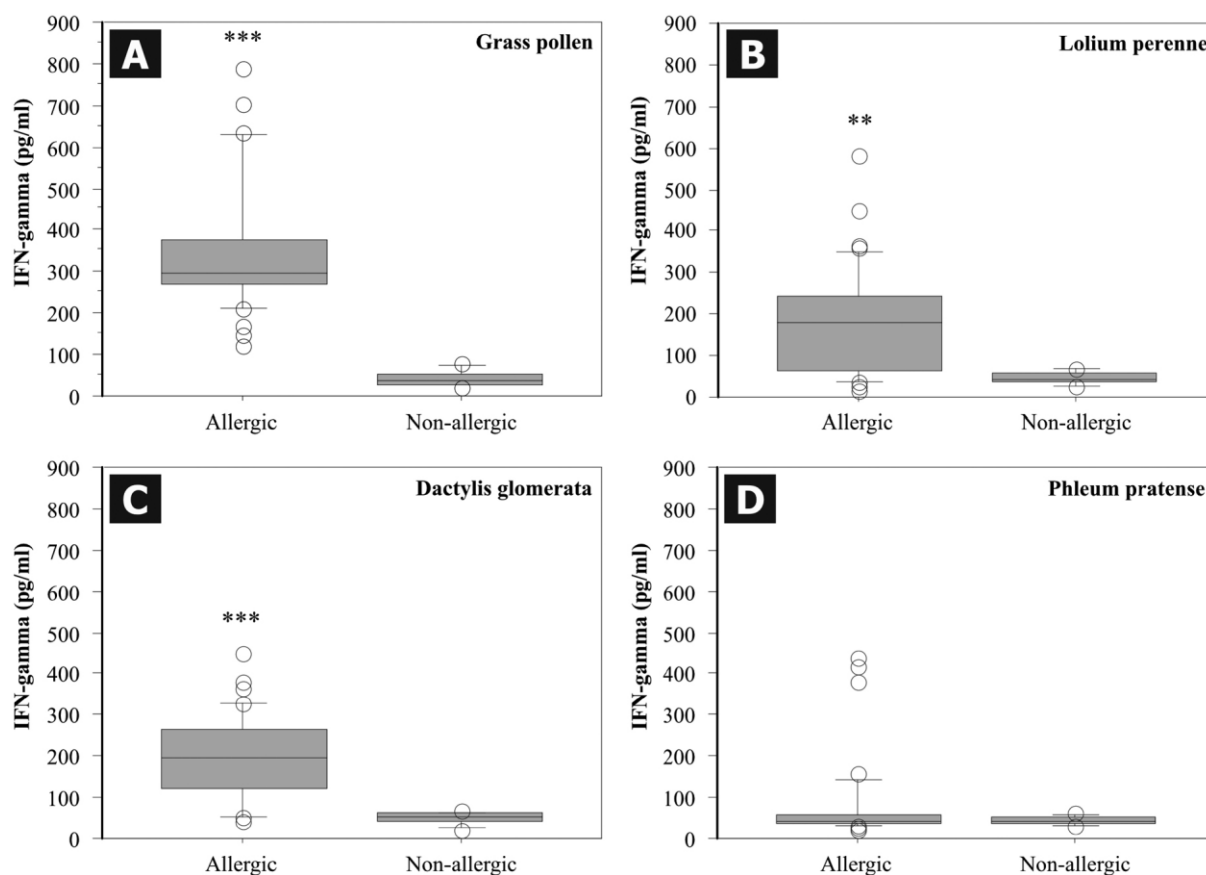


Figure 2. Comparison of IFN- γ production by PBMCs from allergic subjects ($n=37$) and non-allergic controls ($n=13$) stimulated *in vitro* with grass pollen extract (A), *Lolium perenne* extract (B), *Dactylis glomerata* extract (C) and *Phleum pratense* extract (D). The results are presented as mean \pm SE. Mann-Whitney U-test was used for statistical analysis. ** $p < 0.01$; *** $p < 0.001$.

We also investigated T-cell activation following *ex vivo* stimulation of PBMCs with seasonal pollen extracts: spring pollen, summer pollen and autumn pollen (see Allergens in section ‘Materials and Methods’). Only 7 patients with pollinosis (out of 37) responded to the autumn pollen and 2 of them responded also to the spring pollen. These responses were mainly against *Artemisia absinthium* (data not shown). IFN- γ production was not detected in any of the supernatants after stimulation with summer pollen (data not shown).

In all tests, T-cells from healthy subjects did not show a distinct pattern of IFN- γ production upon *ex vivo* stimulation with pollen allergens.

Figure 6 presents the results for measured levels of specific IgG and IgE antibodies to the grass pollen extract in the serum samples of the allergic and non-allergic individuals. It can be seen that the levels of specific IgG

antibodies in the allergic patients against the grass pollen (Figure 6A) are significantly higher compared to the control non-allergic individuals ($p < 0.001$). There was no substantial difference in the levels of IgE specific antibodies against the grass allergens (Figure 6B). This can be explained on the one hand, that the blood samples were taken out of the pollen season (when there is not any allergic reaction) and, secondly, that the half-life of IgE is shorter compared with IgG.

In addition, all serum samples were tested for presence of specific IgG and IgE to the three different groups of tree pollen extracts (Figure 7). Elevated levels of specific IgG antibodies with statistical significance $p < 0.001$ were measured for all groups of tree pollens (Figure 7A,C,E).

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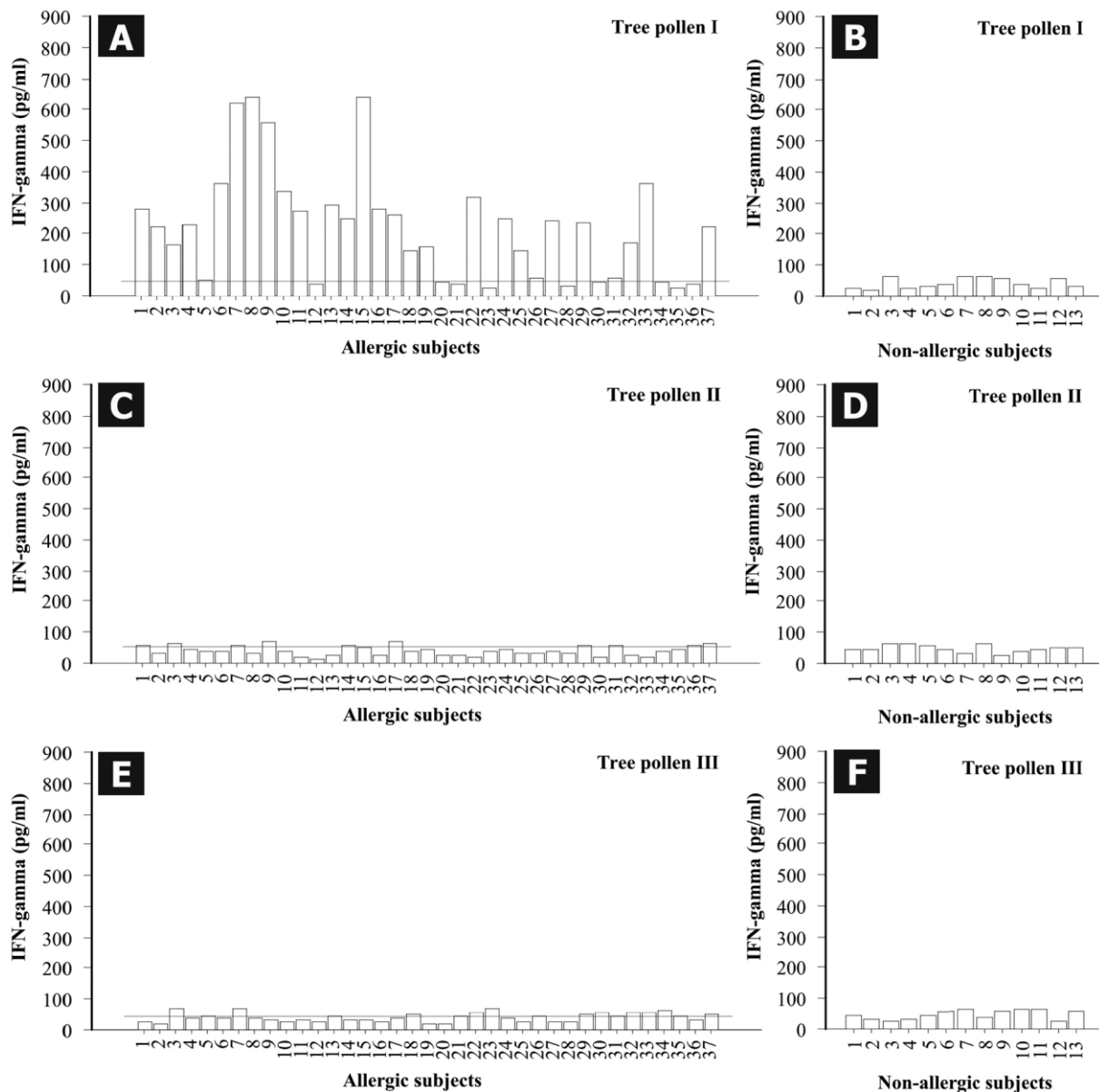


Figure 3. Individual IFN- γ responses upon stimulation of PBMCs from allergic subjects and non-allergic controls with tree pollen I extract (A, B), tree pollen II extract (C, D), and tree pollen III extract (E, F). The horizontal line represents the baseline level.

Higher levels of specific IgE in the sera of the allergic patients ($p < 0.01$) were measured against the tree pollen I (Figure 7B), while the other two did not show differences between allergic and non-allergic individuals (Figure 7D,F). Tree pollen I includes widespread deciduous trees (birch, willow and poplar). In this extract is included also pollen from yew (*Taxus baccata*), which is used for decorative purposes.

Collected serum samples were also tested for presence of specific immunoglobulins against pollen extracts, grouped according to the seasons: spring, summer and autumn (Figure 8). For the three groups of seasonal pollen we found statistically significant increase of pollen specific IgG in the allergic patients compared to the non-allergic control subjects (Figure 8A,C,E). Such an increase was also detected for the specific IgE against autumn pollen (Figure 8F).

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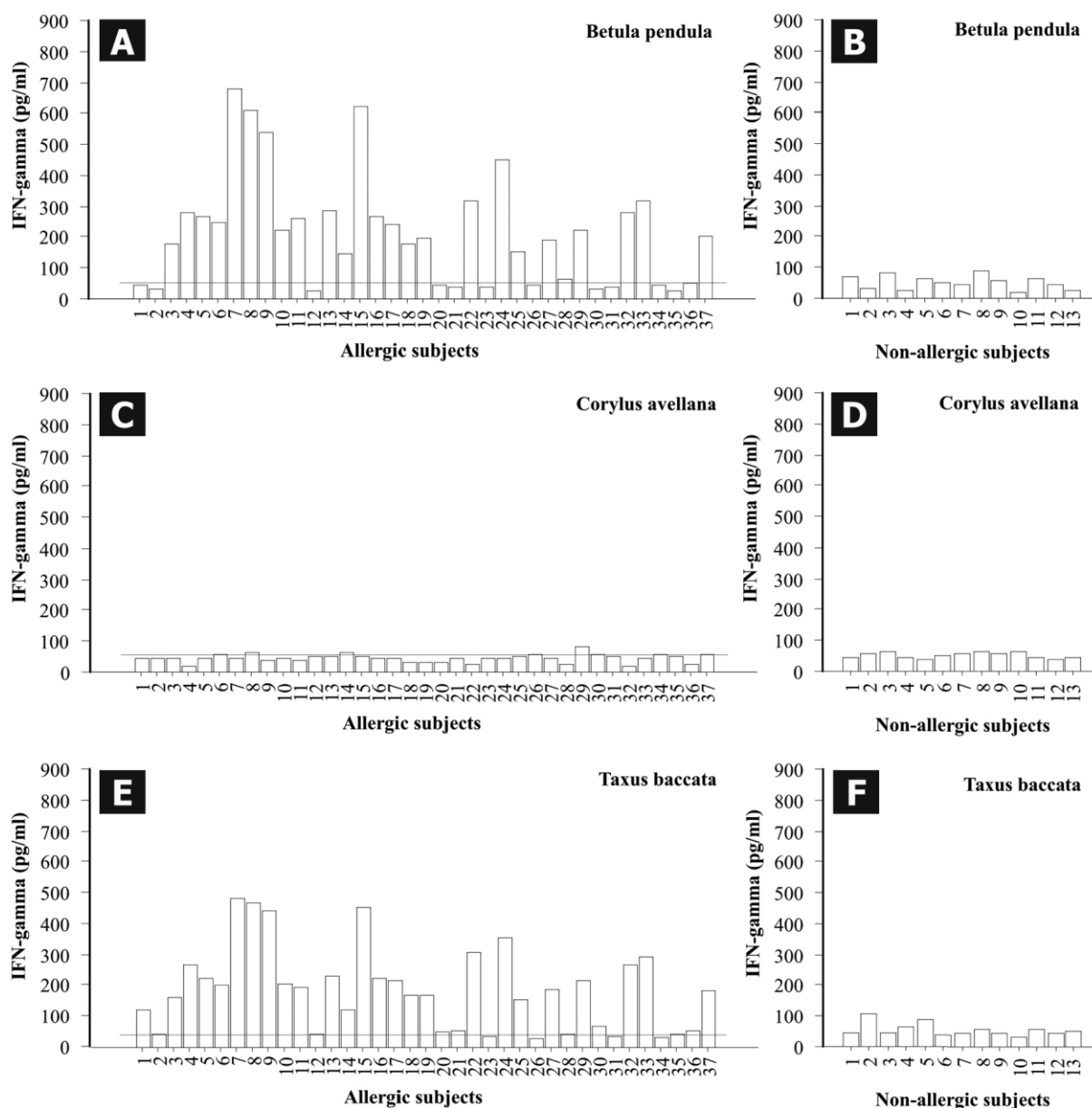


Figure 4. Individual IFN- γ responses upon stimulation of PBMCs from allergic subjects and non-allergic controls with *Betula pendula* extract (A, B), *Corylus avellana* extract (C, D), and *Taxus baccata* extract (E, F). The horizontal line represents the baseline level.

Detected specific IgE antibodies against tree pollen I (Figure 7B) and autumn pollen (Figure 8F) are probably against pollen of species that occur later in the fall and these antibodies still circulate in the blood (3-4 months after the pollen season).

When we screened the serum samples against individual pollen extracts, in the group of allergic patients were observed increased levels of both IgG and IgE antibodies specific for pollen extracts from *Lolium perenne* and *Betula pendula* (Figure 9).

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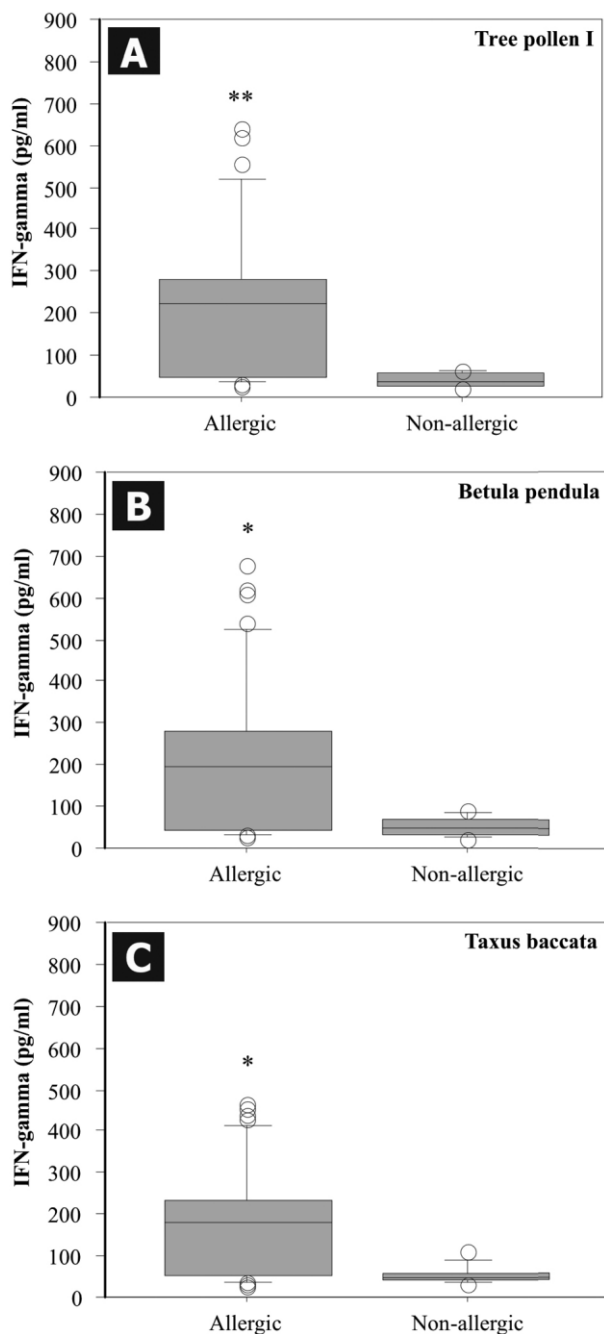


Figure 5. Comparison of IFN- γ production by PBMCs from allergic subjects ($n=37$) and non-allergic controls ($n=13$) stimulated in vitro with tree pollen I extract (A), *Betula pendula* extract (B) and *Taxus baccata* extract (C). The results are presented as mean \pm SE. Mann-Whitney U-test was used for statistical analysis. * $p<0.05$; ** $p<0.01$.

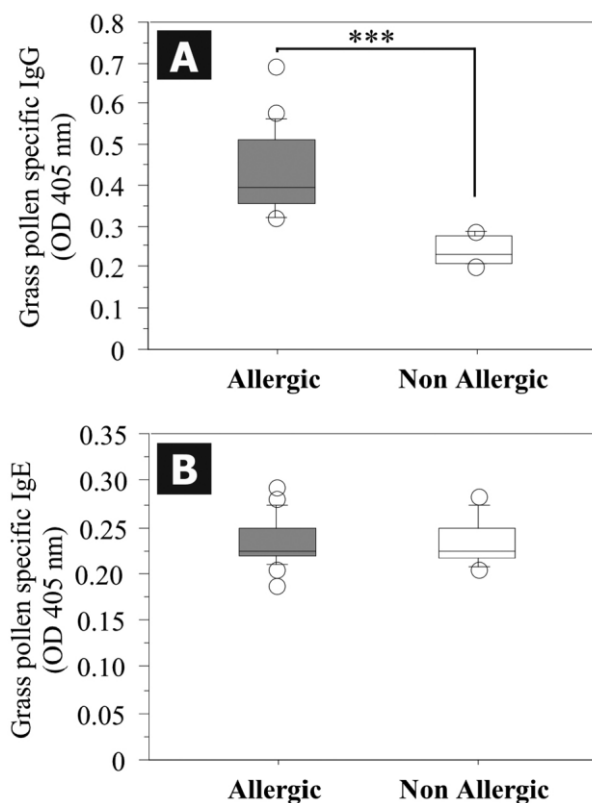


Figure 6. Serum levels of specific IgG (A) and IgE (B) antibodies to grass pollen extract in allergic subjects ($n=37$) and non-allergic controls ($n=13$) out of the pollen season. The results are presented as mean \pm SE. Mann-Whitney U-test was used for statistical analysis. *** $p<0.001$.

In order to investigate whether the cellular and humoral immune responses in the pollen allergic subjects are directed against the same epitopes, we have used specifically designed synthetic peptides that include frequently observed sequences of pollen T-cell and B-cell epitopes (Moten et al., 2011). We detected significant T-cell reactivity in the patient group with pollinosis after stimulation of PBMCs with the peptides PA-1 and PA-2, but not with PA-3 (Figure 10A). In contrast, in the sera of the allergic patients we found specific IgE that recognize PA-2 and PA-3, but not PA-1 (Figure 10B). Results showed that in most cases T- and B-cell epitopes are distinct, but sometimes could partially overlap.

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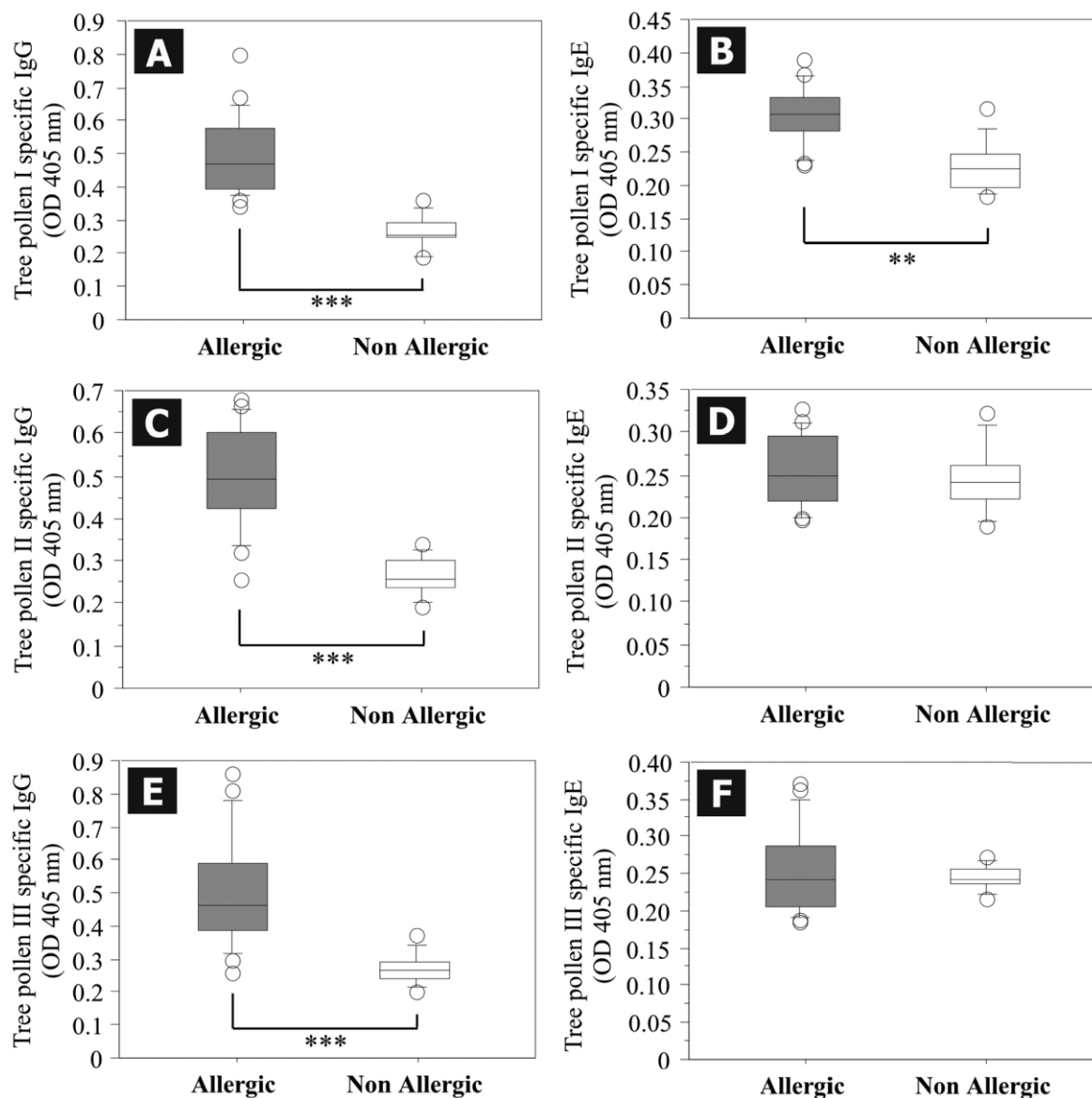


Figure 7. Serum levels of specific IgG and IgE antibodies to tree pollen I extract (A, B), tree pollen II extract (C, D) and tree pollen III extract (E, F) in allergic subjects (n=37) and non-allergic controls (n=13) out of the pollen season. The results are presented as mean \pm SE. Mann-Whitney U-test was used for statistical analysis. ** $p < 0.01$; *** $p < 0.001$.

Discussion

In the present study, we wanted to investigate which pollen allergens are responsible for the T-cell activation in atopic patients with pollinosis when they do not have any clinical symptoms and whether those patients have antibodies in the blood recognizing the same allergens. Our data showed that the T-cell reactivity in most patients was directed

towards the grass pollen B1 (mainly to *Lolium perenne* and *Dactylis glomerata*), tree pollen I (mainly to *Betula pendula* and *Taxus baccata*) and to autumn pollen B5 (mainly to *Artemisia absinthium*). In some of the patients these T-cell responses were against several pollen allergens, which is in agreement with previous studies that report high levels of T-cell cross-reactivity between allergens present in different species (Mohapatra et al., 1994).

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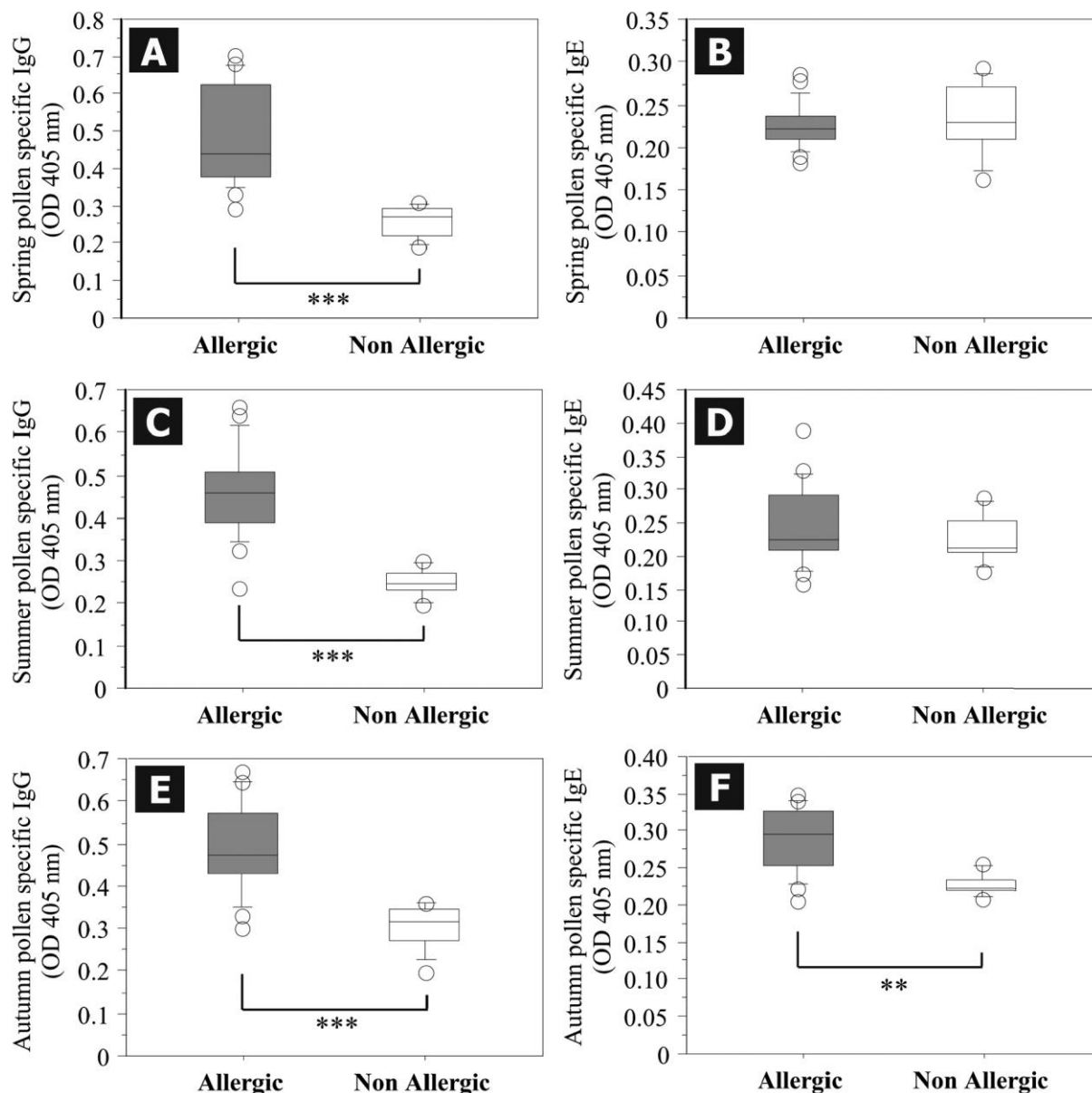


Figure 8. Serum levels of specific IgG and IgE antibodies to spring pollen extract (A, B), summer pollen extract (C, D) and autumn pollen extract (E, F) in allergic subjects (n=37) and non-allergic controls (n=13) out of the pollen season. The results are presented as mean \pm SE. Mann-Whitney U-test was used for statistical analysis. ** $p < 0.01$; *** $p < 0.001$.

In other patients, the T-cell response was directed only to one allergen. Little or no cross-reactivity at the level of T cell epitopes was also reported (Oseroff et al., 2010), although these authors tested only different Timothy grass Phl p allergens, but not allergens from different species. Taken together, these results show the complexity of the immune response and that it is controlled by different signaling

pathways. The most accepted explanation is that different allergens activate distinct Th cell subsets with distinct patterns of cytokine production (Bozza et al., 2009; Oseroff et al., 2010). It is well known that regulatory CD4⁺CD25⁺Foxp3-positive T-cells are able to inhibit Th1 and Th2 cytokine production of CD4⁺CD25⁻ T-cells (Bellinghausen et al., 2003; 2005).

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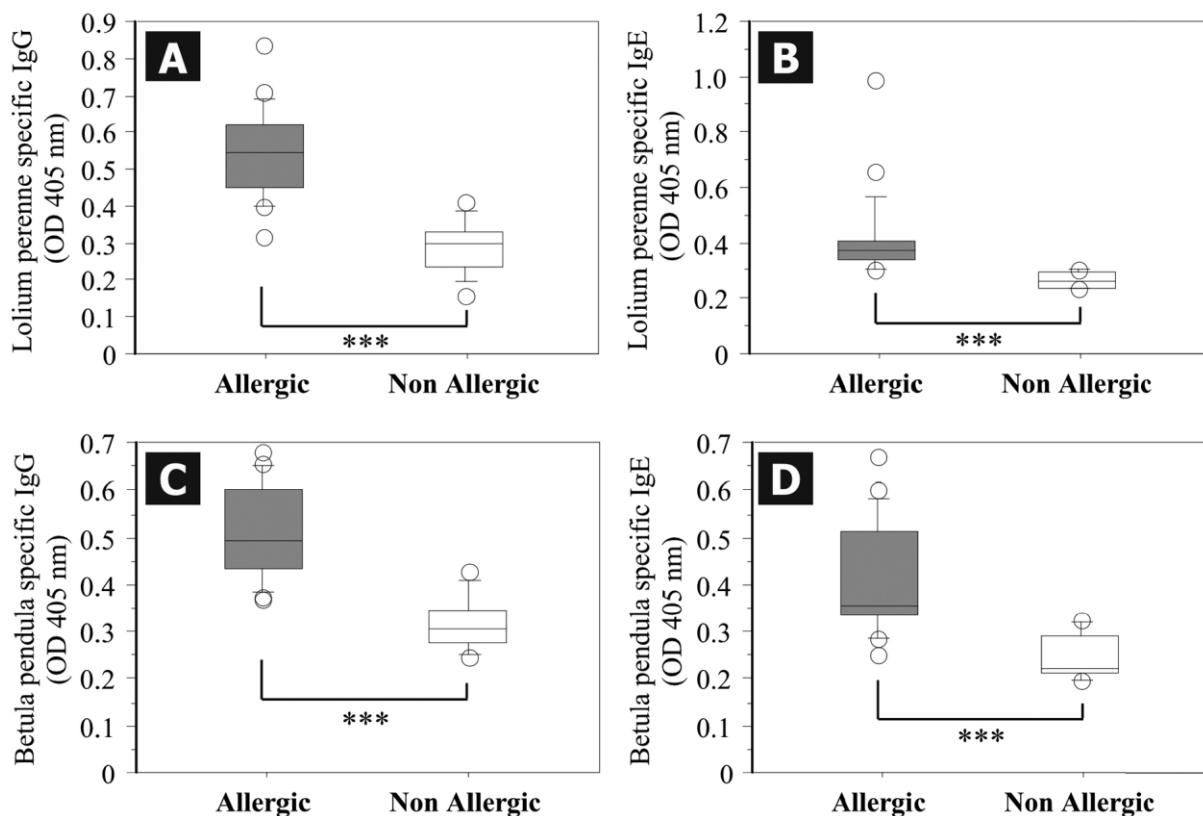


Figure 9. Serum levels of specific IgG and IgE antibodies to *Lolium perenne* extract (A, B) and *Betula pendula* extract (C, D) in allergic subjects (n=37) and non-allergic controls (n=13) out of the pollen season. The results are presented as mean \pm SE. Mann-Whitney U-test was used for statistical analysis. *** $p < 0.001$.

At high allergen concentrations such regulatory T-cells from grass-pollen allergic patients fail to inhibit proliferation but not cytokine production of the effector T-cells, while in non-atopic subjects they retain full regulatory capacity (Bellinghausen et al., 2005). In addition, an impaired IL-10 production in atopic subjects confirmed that the T-cell regulation in these individuals is reduced (Domdey et al., 2010). Consistent with these reports, Mittag et al. demonstrated significantly decreased frequency of ryegrass pollen induced Foxp3^{hi} cells outside the pollen season within the dividing CD4⁺ T-cells in allergic subjects compared to non-atopic individuals (Mittag et al., 2010).

The question about overlapping of the T- and B-cell epitopes, and whether T-cell reactivity and IgE responses are directed against the same epitopes has been a matter of debate long time. It has been shown that the dominant T-cell epitope p21-35 derived from Der p2 (a major allergen of the house dust mite *Dermatophagoides pteronyssinus*) shares

four amino acids (at positions 28, 29, 30 and 31) with the B-cell epitope (Wu et al., 2000). On the other hand, Oseroff et al. demonstrated that there is no correlation between the T cell responses and IgE levels against the common Timothy grass Phl p allergens (Oseroff et al., 2010). The authors were able to detect strong T-cell reactivity in the absence of specific IgE antibodies. Our results showed that in some patients with pollinosis both T- and B-cell responses were towards the same pollen allergens, but in other patients these responses are directed to different pollen allergens. It remains possible that these epitopes are distinct since we have tested pollen extracts, but not synthetic peptides representing the certain epitopes. Although in most cases the T-cell epitopes within an allergen are distinct from the IgE binding domains (Wallner & Geffer, 1996) it was shown that a short peptide containing partially overlapping T- and B-cell epitopes has the capacity to trigger both T-cell proliferation and antibody production (Wu et al., 2000).

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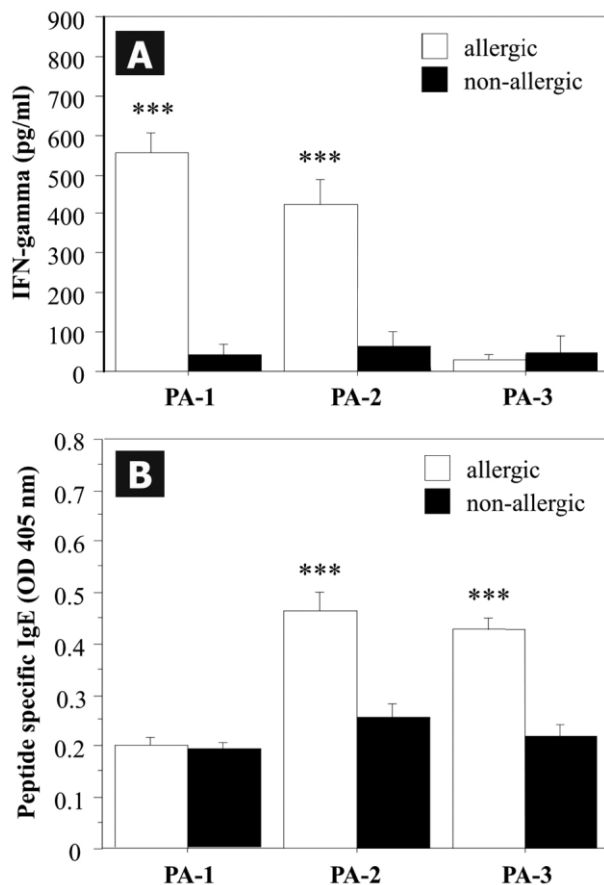


Figure 10. Immune responses of allergic subjects ($n=37$) and non-allergic controls ($n=13$) to synthetic peptides (PA-1, PA-2 and PA-3) that contain overlapping T-cell and B-cell pollen epitopes. **A** – IFN- γ responses upon stimulation of PBMCs with the peptides PA-1, PA-2 and PA-3. **B** – Serum levels of specific IgE antibodies binding to the peptides PA-1, PA-2 and PA-3. The results are presented as mean \pm SE. Mann-Whitney U-test was used for statistical analysis. *** $p < 0.001$.

This is of great importance since T-cell epitope-containing peptides have been proposed and used for treatment of IgE-mediated allergic diseases (Wraith, 1995; Wallner et al., 2013).

In the present study, we show that one of the three investigated short synthetic peptides (PA-1) that contain partially overlapping T- and B-cell pollen epitopes was able to stimulate T-cells from allergic patients without antibody recognition. Results suggest that this peptide has the capacity to be used for specific immunotherapy treatment (SIT) of

patients with pollinosis. On the other hand, it was proposed that due to lack of posttranslational modifications, some of the recombinant allergens may not have the same immunological reactivity as their native counterparts (Suck et al., 2002). Recently, Martínez-Cóccera et al. demonstrated that treatment of patients with a single species *Phleum pratense* allergen extract significantly diminished the immediate and delayed cutaneous responses not only to the *P. pratense*, but also to a grass mix allergen extract (Martínez-Cóccera et al., 2010). This suggests that there is a common mechanism for regulation of the immune response in patients with pollen allergy.

Conclusion

In conclusion, we show that in the majority of patients with pollen allergy there is cross-reactivity at the level of T cell activation and the T-cell responses are mainly against allergens from *Lolium perenne*, *Dactylis glomerata*, *Betula pendula*, *Taxus baccata* and *Artemisia absinthium*. Our data confirm the complexity of the immune response and that the T-cell reactivity and antibody responses may be directed towards different or the same allergens. We propose that short synthetic peptides that contain partially overlapping T- and B-cell pollen epitopes can be used for specific immunotherapy treatment after screening for antibody recognition domains.

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