

Evidence for a Role of TGF- β -Activated Kinase 1 and MAP3K7 Binding Protein 3 in Peanut-Specific T-Cell Responses

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Keywords

Allergen-specific T cells · Ara h 2 · Food allergy · Peanut allergy · T cells · TAB3

Abstract

Peanut allergy is considered to be the most common cause for food-induced anaphylaxis. Currently, no approved treatment is available. Avoidance is the only measure to prevent anaphylactic reactions to peanuts. T-helper cells are of special importance for the sensitization process and the maintenance of allergic inflammation. Identifying markers of allergen-specific T-cell responses may help to develop novel treatment approaches. Therefore, we aimed to define new T-cell target genes in Ara h 2-specific T cells and to investigate the possibility of using them as biomarkers of peanut allergy in peripheral blood mononuclear cells (PBMCs). We performed whole mRNA array analysis (whole human genome oligo microarray) of in vitro expanded Ara h 2-specific

T cells (CFSE^{low}CD3⁺CD4⁺) from 5 peanut-allergic (PA) and 5 non-peanut-sensitized individuals. Expression of selected genes as a result of a two-step bioinformatic approach was confirmed in a second cohort by quantitative PCR. TGF- β -activated kinase 1 and MAP3K7 binding protein 3 (TAB3), calcium/calmodulin-dependent protein kinase type IV (CAMK4) and HemK methyltransferase family member 1 (HEMK1) were significantly upregulated in Ara h 2-specific T cells of PA patients. In addition, the expression of these genes was also assessed in unstimulated PBMCs from a cohort ($n = 43$) of PA, atopic non-PA, and nonatopic controls. Interestingly, in unstimulated PBMCs, TAB3 expression was significantly downregulated in PA patients compared to atopic non-PA individuals. Thus, TAB3 may play a significant role at the level of T-cell activation and may also be a candidate biomarker for PA.

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Introduction

Food allergy is a major public health issue in childhood in westernized societies [1]. Food-allergic reactions may result in life-threatening anaphylaxis [2, 3], and peanut allergy (PA) in particular is the most frequent cause of such reactions. At present, no effective treatment of peanut allergy is approved by the regulatory authorities. Thus, vigilant avoidance represents the most important action. Especially children are in danger of anaphylaxis to low amounts of allergen. They contribute significantly to the frequent attendance at the emergency rooms due to unintentional ingestion of foods contaminated by hidden allergens [4–6]. The pathomechanisms that contribute to the development and maintenance of PA involve antigen-presenting cells, mast cells, T cells, and B cells [7, 8]. Beside data on successful oral immunotherapy [9], investigating novel markers in whole blood that reflect PA would be helpful and may be of benefit to monitor the response to immunotherapy.

Until now, few data are present regarding the signaling pathways in PA [10]. Genes involved in inflammation, cell survival, cell death, and proliferation are mainly regulated by the transcription factor nuclear factor “ κ light-chain enhancer” of activated B cells (NF κ B). Allergic inflammation is also impacted by the NF κ B pathway in the context of lung inflammation [11]. However, data in the context of PA are limited.

We explored novel genes which are upregulated in Ara h 2-specific, in vitro expanded T-helper cells of PA individuals and assessed their potential role as biomarkers under unstimulated conditions.

Materials and Methods

Study Population

The study was conducted at the Medical University of Vienna. PA was confirmed either by an oral food challenge or according to convincing patient's history defined by house staff allergists (multiple episodes of severe systemic allergic reactions/anaphylaxis after peanut consumption, and peanut-specific IgE and Ara h 2-specific IgE >0.35 kUA/L). Healthy controls were defined as having a negative atopic history and no evidence of allergic sensitizations (see list of allergens in Table 1).

Peripheral blood mononuclear cells (PBMCs) taken for allergen-specific T-cell generation were obtained from two independent cohorts of adults and adolescents (one for the gene arrays and one for the confirmation of the array results) of PA and non-peanut-sensitized individuals including atopic individuals with no PA.

For the investigation of the markers of PA in unstimulated PBMCs, a third independent cohort was recruited (patient charac-

teristics are listed in Table 1; $n = 13$ PA, $n = 21$ atopic, non-peanut sensitized, $n = 9$ nonatopic). Atopic status was defined by specific-IgE antibodies (>0.35 kUA/L) against food sources (peanut, soybean, egg white, milk protein, codfish, wheat flour, and nuts, namely hazelnut, almond, coconut, and Brazil nut) and airborne allergens (timothy grass, rye, mugwort, birch, cat and dog dander, house dust mites, and *Cladosporium herbarum*) by screening via ImmunoCAP (Phadia 250; Thermo Fisher, Uppsala, Sweden) (for more information, please see also online suppl. material at www.karger.com/doi/10.1159/000496438).

Cell Culture and Isolation of Allergen-Specific T-Helper Cells

PBMCs were isolated from heparinized blood samples by density gradient centrifugation as described previously [12–14]. PBMCs were cultured in RPMI medium (50 μ g/mL gentamicin sulfate, 1% L-glutamine [Sigma-Aldrich, MO, USA]), 2% nonessential amino acids, 5% fetal calf serum (Thermo Fisher Scientific, MA, USA) alone or in the presence of Ara h 2 (10 μ g/mL). Native Ara h 2 with a minimal LPS content [15] of 0.14 EU per 10 μ g/condition (amebocyte lysate assay; Lonza, Walkersville, MD, USA) was purified as described previously [16]. For the gene array experiment, CFSE-labeled PBMCs ($n = 5$ PA and $n = 5$ nonatopic) were cultured, and on day 7 cells were stained with anti-CD3 Pacific orange, anti-CD4 Pacific blue (BD Biosciences, Franklin Lakes, NJ, USA), and the viability dye 7-AAD (BD Biosciences). Cells were separated with a MoFlo Astrios cell sorter (Beckman Coulter, CA, USA). A total of 1.5×10^3 to 11×10^3 allergen-specific T cells (7AAD⁻CFSE^{low}CD4⁺CD3⁺) were sorted after allergen-specific expansion starting with 1×10^7 fresh PBMCs on day 7. These Ara h 2-specific CD4⁺ T cells were used for the whole mRNA array experiments.

For the confirmatory experiments, PBMCs from a second set of PA and nonallergic individuals were obtained. Isolated PBMCs (1×10^7 per well) were cultured in vitro in a 6-well plate in RPMI and stimulated with Ara h 2 for 7 days. After 7 days of incubation, a supernatant was stored from the cultured cells for further analysis, and we proceeded with MACS separation according to the manuals of Miltenyi Biotec (Bergisch Gladbach, Germany; www.miltenyibiotec.com/130-096-533). Thereafter, CD4⁺ T cells were isolated via negative selection using the CD4⁺ T-cell isolation kit (Miltenyi Biotec) by autoMACS[®] Pro Separator (Miltenyi Biotec). In order to have a higher purity, dead cells were removed via magnetic sorting using a dead cell removal kit (Miltenyi Biotec) according to the datasheet protocol (order No. 130-090-101). Purity of the sorted cells was analyzed with a FACS Canto II (Becton Dickinson, Franklin Lakes, NJ, USA), and $>95\%$ were achieved.

Whole mRNA Gene Array

Isolated cells were lysed with SuperAmp[™] lysis buffer (Miltenyi Biotec) according to the manufacturer's instructions, and gene array expression analysis was performed by Miltenyi Biotec using Agilent whole genome oligo microarrays applying the SuperAmp[™] protocol. RNA was amplified according to an undisclosed procedure based on a global RT-PCR protocol. Amplified mRNA-derived cDNA samples were labeled with Cy3 and hybridized to a whole human genome oligo microarray 8x60K. After 2 final washing steps, the fluorescence signals of the hybridized microarrays were analyzed using Agilent's microarray scanner system, and the results were further processed with the feature extraction software (see online suppl. Fig. E1). For more detailed

Table 1. Characteristics of the study group

Demographics and baseline characteristics	Peanut allergic	Atopic	Nonatopic
Total	13	21	9
Males	8 (61.5)	13 (59.1)	5 (55.5)
Median age, years	7.7	7.2	13.9
Patient history			
Sensitization to food			
Milk	1 (7.7)	6 (28.6)	0
Egg	2 (15.4)	4 (19.0)	0
Wheat	3 (23.1)	5 (23.8)	0
Soybean	9 (69.2)	4 (19.0)	0
Fish	4 (30.7)	0	0
Oat	1 (7.7)	1 (4.8)	0
Rice	1 (7.7)	1 (4.8)	0
Fruits (apple, kiwi, orange)	3 (23.1)	0	0
Peanut	13 (100)	7 (33.3)	0
Tree nuts			
Hazelnut	5 (38.5)	9 (42.9)	0
Pistachio	0	1 (4.8)	0
Walnut	1 (7.7)	2 (9.5)	0
Almond	3 (23.1)	5 (23.8)	0
Coconut	1 (7.7)	2 (9.5)	0
Paranut	1 (7.7)	2 (9.5)	0
Airborne allergen sensitization			
Perennial allergens (HDM, cat, dog)	3 (23.1)	6 (28.6)	0
Fungi (<i>Alternaria</i> , <i>Aspergillus</i> , <i>Penicillium</i>)	1 (7.7)	0	0
Tree pollen (birch)	3 (23.1)	3 (14.3)	0
Timothy grass	5 (38.5)	5 (23.8)	0
Ryegrass	3 (23.1)	8 (38.1)	0
Mugwort	2 (15.4)	3 (14.3)	0
Barley	1 (7.7)	2 (9.5)	0
Allergies other than peanut			
Milk allergy	1 (7.7)	2 (9.5)	0
Egg allergy	2 (15.4)	2 (9.5)	0
Wheat allergy	1 (7.7)	0	0
Soybean allergy	2 (15.4)	0	0
Tree nuts			
Hazelnut	1 (7.7)	1 (4.8)	0
Pistachio	0	1 (4.8)	0
Walnut	1 (7.7)	1 (4.8)	0
Perennial allergens (HDM, cat, dog)	3 (23.1)	4 (19.0)	0
Pollen allergy			
Birch	4 (30.7)	3 (14.3)	0
Mugwort	1 (7.7)	1 (4.8)	0
Atopic disease			
Atopic dermatitis	4 (30.7)	10 (47.6)	0
Asthma	6 (46.2)	6 (28.6)	0
Allergic rhinitis and/or conjunctivitis	4 (30.7)	3 (14.3)	0
Anaphylaxis to peanut [29]			
I	1 (7.7)	0	0
IIa	2 (15.4)	0	0
IIb	2 (15.4)	0	0
IIIa	8 (61.5)	0	0

Table 1 (continued)

Demographics and baseline characteristics	Peanut allergic	Atopic	Nonatopic
Peanut-induced clinical symptoms			
Erythema of the skin	5 (38.5)	0	0
Pruritus	3 (23.1)	0	0
Dyspnea/asthma/wheezing	8 (61.5)	0	0
Cough	4 (30.7)	0	0
Tachycardia	3 (23.1)	0	0
Angioedema	7 (53.8)	0	0
Urticaria/hives	9 (69.2)	0	0
GI symptoms (diarrhea, vomiting, nausea)	4 (30.7)	0	0
Dizziness/hypotension/paleness	4 (30.7)	0	0

Numbers (%) of individuals are shown unless indicated otherwise. GI, gastrointestinal; HDM, house dust mites.

information, see the Materials and Methods section in the online supplementary material.

PCR Experiments

For the confirmatory experiments, total RNA was extracted from cells using the GenElute mammalian total RNA Miniprep kit (Sigma Aldrich, MO, USA) followed by a DNase treatment with a DNA-free™ DNA removal kit (Thermo Fisher Scientific, MA, USA). RNA was reverse transcribed using the iScript™ cDNA synthesis kit (Bio-Rad, Hercules, CA, USA). Relative expression of the genes was normalized against the housekeeping gene, elongation factor 1α (EF1a) as an internal control. Relative expression on PBMCs was calculated using the following formula: $2^{-(\Delta Ct \text{ GOI} - \Delta Ct \text{ lowest expressed sample})}$. Expression relative to the unstimulated control was determined using the formula: $2^{-(\Delta Ct \text{ stimulated cells} - \Delta Ct \text{ unstimulated cells})}$. Primer sequences are given in the online supplementary material (Table E1) (Invitrogen, Carlsbad, CA, USA).

Statistical Analyses

Potential targets with a *p* value <0.025 after correction for multiple testing were assessed using the two-stage approach proposed by Heller et al. [17]. False discovery rate (FDR) was calculated by applying the Benjamini-Hochberg FDR correction [18] (see online suppl. material for information on detailed statistical analysis). Statistical analyses for the real-time PCR results and the graphs were done via GraphPad Prism 6 software (GraphPad Software, San Diego, CA, USA). *p* values ≤0.05 were considered statistically significant.

Results

Ara h 2-Specific CD4⁺ T Cells Show Upregulated Expression of TAB3, CAMK4, and HEMK1

Whole mRNA array of Ara h 2-specific T cells, defined as 7AAD-^{CFSE}^{low}CD3⁺CD4⁺ cells (online suppl. Table E1), revealed a number of potential targets after correc-

tion for multiple testing. Based on the statistical preselection and published studies (PubMed resource, a service of the National Center for Biotechnology Information [NCBI]) of the Ara h 2-specific T-cell gene array data, 10 potential novel target genes were identified. CAMK4, BMPRIa (bone morphogenetic protein receptor type 1A), COMMD1 (copper metabolism [Murr1] domain containing 1), TAB3, GIMAP8 (GTPase, IMA family member 8), HEMK1, IL-13 (all upregulated in PA individuals), PTPN11 (protein tyrosine phosphatase, nonreceptor type 11), CSNK1E (casein kinase 1ε), and PYCR1 (pyrroline-5-carboxylate reductase like) (all 3 were downregulated in PA individuals) showed a different regulation in PA individuals.

In an independent set of experiments, the relevance of the findings was confirmed using a similar approach by magnetic cell sorting of CD4⁺ T cells after generating short-term Ara h 2-specific T-cell lines (purity >95%), which allowed to obtain higher amounts of mRNA. Three genes were significantly upregulated in PA individuals: TAB3, CAMK4, and HEMK1 (Fig. 1). Seven other genes had similar expression patterns than those observed in the gene array, but did not reach statistical significance (online suppl. Fig. E2). As reported, TAB3 is involved in the NFκB pathway, even if it is not clear to which extend protein expression is linked to NFκB activation [19]. We examined the differences in the NFκB pathway by comparing TAB3 expression and NFκB phosphorylation (NFκB^{phos}). Although TAB3 expression was not significantly altered, NFκB p65 phosphorylation was reduced in T cells from PA individuals, and the relative increase in TAB3 expression matched mRNA data (online suppl. Fig. E4).

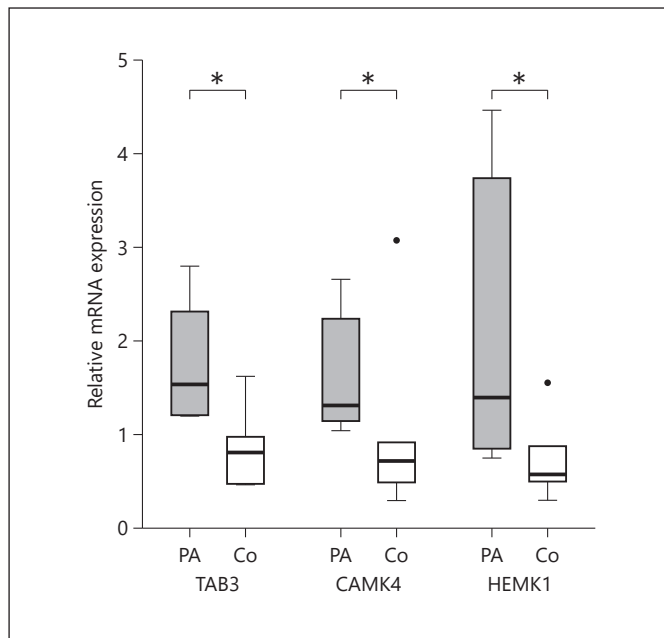


Fig. 1. TAB3, CAMK4, and HEMK1 expression in Ara h 2-stimulated allergen-specific CD4 T cells ($n = 5$ peanut allergic [PA] individuals, $n = 7$ nonatopic [Co] individuals). Mann-Whitney U test: * $p < 0.05$.

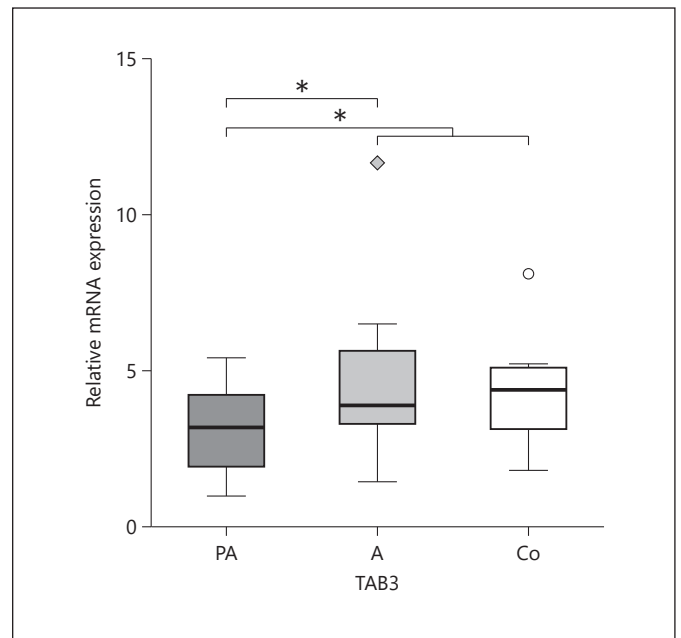


Fig. 2. TAB3 mRNA expression in unstimulated PBMC from peanut-allergic, non-peanut-sensitized atopic, and nonatopic individuals ($n = 12$ PA, $n = 18$ A, and $n = 9$ Co). PA, peanut allergic; A, atopic, non-peanut sensitized; Co, nonatopic. Mann-Whitney U test: * $p < 0.05$.

TAB3 Also Acts as a Marker of PA in PBMCs

In order to evaluate the potential of these 3 genes as probable biomarkers in unstimulated PBMCs, we enrolled 43 well-characterized individuals with either PA, atopy without PA, or nonatopic healthy controls (Table 1). The cohort showed a predominance of male participants. The PA and nonatopic group were matched for age (7.8 vs. 7.2 years) while the control group was older (13.9 years). Atopic individuals tend to suffer more likely from atopic dermatitis (48 vs. 31%), and PA patients had a higher prevalence of asthma (46 vs. 29%). Both groups had comparable rates of co-sensitizations to food allergens, whereas sensitization to environmental allergens, e.g., house dust mites, cat and dog dander (29%), and timothy grass (38%), was more prevalent among subjects in the atopic group. Sixty-two percent of the PA subjects had a history of severe anaphylaxis after peanut exposure (Table 1).

We identified 2 genes, PYCRL and TAB3, which were significantly different among the study groups. TAB3 was significantly downregulated in unstimulated PBMCs of PA individuals ($p < 0.05$). Atopic patients displayed remarkably high expression of TAB3 compared to PA pa-

tients ($p < 0.05$). PA PBMCs differed significantly in comparison to peanut nonallergic individuals (atopic and nonatopic controls; $p < 0.05$) (Fig. 2). PYCRL was significantly upregulated in PA patients compared to nonatopic individuals ($p < 0.05$). We did not observe significant differences in PBMCs in the expression of other genes (online suppl. Fig. E3).

Discussion

The induction, regulation, and maintenance of allergic immune responses require activation of CD4⁺ T cells [8]. In order to target T cells for the treatment of allergic diseases, it is vital to better understand their characteristics. Inherent changes in their effector cell characteristics may not only have the potential to be used for novel treatment approaches, they may also be used for the development of novel biomarkers for PA. This study adds to the growing body of evidence markers of allergen-specific T cells [10] by describing TAB3 as a new marker of expanded Ara h 2-specific T cells. The “marker” TAB3 is moreover remarkable since it displays distinct

expression in unstimulated PBMCs of PA individuals (Fig. 2).

Recent data sets on allergen-specific T cells mainly related to directly sorted allergen-specific CD4⁺ T cells from the peripheral blood [20, 21] derived from CD154 or tetramer-based approaches which result in the identification of largely unaltered allergen-specific T-helper cell populations. Our approach focused on sorted activated Ara h 2-specific T-helper cells. Peanut allergen-specific T cells were of the Th2 type, secreting IL-13 and IL-5 cytokines in response to peanut stimulation. Other subsets of CD4⁺ T cells, as regulatory T cells, Th1, and Th17, were also detectable in PA patients but not significant [22]. In line with these reports, allergen-specific cytokine production in the supernatant was dominated by Th2 cytokines in allergic individuals but not in nonallergic individuals (SN day 7) [unpubl. data]. TAB3 is an adaptor molecule that, in combination with protein kinase TAK1, phosphorylates the subunit of NFκB, IκBα/β and leads to NFκB activation [23, 24]. Data on the potential role of TAB3 in the context of allergy is limited. In a mouse model, ovalbumin-induced eosinophilic airway inflammation failed in the absence of one of the NFκB subunits. This effect was dependent on CD4 T-cell cytokine production [25]. Expression of neutrophils, eosinophils, and lymphocytes was less abundant in the bronchioalveolar lavage fluid of p50-deficient mice. Interestingly, while IL-5 production was dependent on p50 subunit expression, IL-2 was higher in the absence of the p50 subunit. However, IL-5 was completely absent in the supernatant of p50-impaired mice. IFN-γ and IL-4 secretion was almost similar in both mouse groups [25]. A possible explanation may be that patients with allergies have a lower constitutive TAB3 expression reflecting their atopic status. Studies described in cancer research showed TAB3 as one of the important molecules in a cascade of genes forming the NFκB activation pathway [24, 26].

In atopic diseases such as asthma, inflammatory molecules are regulated by transcription factors such as NFκB [27]. Assessment of sputum and bronchial biopsy specimens revealed the presence of the subunit of NFκB p65 in high levels in patients with asthma compared to non-asthmatic controls. This is in line with our data suggesting a role in the context of a typical type 2 disease. Importantly, this data set is not the result of a single approach. At an allergen-specific level, the findings for TAB3 derived from pathway analysis were replicated in an independent cohort of the same size. The mechanism of TAB3 at the molecular level in allergy or PA remains to be elucidated. In resting cells, NFκB is segregated in the

cytoplasm in an inactive form by inhibitory proteins of the NF of κ light polypeptide gene enhancer in the B-cell inhibitor (IκB) family. TAB3 is an adaptor upstream of NFκB phosphorylation [23]. TAB3 binds to the K63 polyubiquitin chain of TRAF6 (tumor necrosis factor receptor-associated factor) and activates TGF-β-activated kinase 1 (TAK1), which is a member of the MAPK kinase family and inhibitor of NFκB (IκB) kinase (IKK). Thereafter, TAB3 in complex with protein kinase TAK1 phosphorylates c-Jun N-terminal kinases (JNKs) and the subunit of NFκB IκBα/β [28]. Our experiments suggest a differential regulation of the NFκB pathway in the context of allergen-specific T cells.

In addition, we have conducted our experiments in different compartments (allergen-specific stimulated CD4⁺ T cells and unstimulated PBMCs) from well-characterized subjects. Based on the allergen-specific T-cell data, we decided to have a look whether there was a role for TAB3 as biomarker for PA. Given the low frequency of allergen-specific T cells in the peripheral blood (<200 CD4⁺ T cells per million CD4 T cells), this was indeed a high-risk approach. However, the fact that PA is the most frequent cause of anaphylaxis in childhood and adolescence, this did not exclude the possibility of an inherent mechanism to food allergy beyond general allergy-related ones. T cells are not the sole cell population where TAB3 plays a role, and we hypothesized that TAB3 may rather act as an indicator of a heightened propensity of PA in unstimulated PBMCs. In fact, TAB3 mRNA expression is significantly lower in PA individuals than atopic patients with multiple sensitizations and in some cases even with food allergies but without PA. From a biomarker perspective, these findings are important since PA-specific markers in the PBMC compartment are not available.

In conclusion, we provide evidence that TAB3 might play a role in allergen-specific T-cell responses and is a potential specific marker for PA.

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Statement of Ethics

Ethics approval was given by the Ethics Committee of the Medical University of Vienna (EK No. 428/2008). All participants and their parents or guardians were informed about the study, and their written informed consents were obtained.

Disclosure Statement

The authors have no conflicts of interest to declare.

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