Helios negative Regulatory T-cells as a key factor of immune tolerance in non-allergic beekeepers

**Running title:** Understanding bee venom immune tolerance

Ruiz-Leon B^{1,2,3,*}, Navas A^{1,2,*}, Serrano P^{1,2,3}, Espinazo M^{1,3}, Guler I^{1}, Alonso C^{1,2,3}, Jurado A^{1,2,3}, Moreno-Aguilar C^{1,2,3}

^{1}Maimonides Biomedical Research Institute of Cordoba (IMIBIC)/ Reina Sofia University Hospital/ University of Cordoba, Cordoba, Spain.
^{2}Department of Immunology and Allergy, Reina Sofia University Hospital, Cordoba, Spain
^{3}National Network ARADyAL. Health Institute Carlos III, Madrid, Spain.
*Both authors contributed equally

**Corresponding:**
Aurora Jurado Roger
Department of Immunology and Allergy, Reina Sofia University Hospital, Avd. Menéndez Pidal s/n, 14004. Córdoba. Spain.
E-mail: aurora.jurado.sspa@juntadeandalucia.es

This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the Version of Record. Please cite this article as doi: 10.18176/jiaci.0722
Abstract

Background: Although exposure to stings has been identified as the leading risk factor for anaphylaxis due to Hymenoptera venom allergy, professional beekeepers receive hundreds yearly without developing systemic reactions.

Objective: This study aims to analyse the mechanisms underlying bee venom tolerance in beekeepers.

Methods: A cross-sectional study was conducted. Participants were recruited and classified into three groups: allergic patients (AP) experiencing systemic reactions after bee stings, with a positive intradermal test and specific IgE (sIgE) to *Apis mellifera* venom (AmV); tolerant beekeepers (TBK) receiving ≥50 stings/year; and healthy non-exposed controls (HC). Serum levels of sIgE and specific IgG4 (sIgG4) to AmV, rApi m 1, rApi m 2, rApi m 3, Api m 4, rApi m 5 and rApi m10, as well as AmV-induced basophil degranulation, percentage of T-cell subsets, regulatory T-cells (Treg cells) and IL-10 production, were measured.

Results: APs had high levels of sIgE to AmV and all its allergic components (*p*<0.001) together with a high basophil activation rate (*p*<0.001) compared to TBKs. Conversely, compared to APs, TBKs showed higher levels of sIgG4 (*p*<0.001) and IL-10 (*p*<0.001) as well as an enhanced CTLA-4+ Treg population (*p*=0.001), expanded Helios+ Treg (*p*<0.003), and reduced T-helper 1 (*p*=0.008), T-helper 2 (*p*=0.004) and T-helper 17 (*p*=0.007) subsets.

Conclusions: A different profile, strongly marked by Treg activity, was found in TBKs. This natural tolerance would be led by the expansion of inducible Helios+ Treg cells at a peripheral level. Helios+ Treg population could be a novel candidate biomarker useful for monitoring tolerance.

Key words: Helios protein, Regulatory T-cells, Immune tolerance, Bee venom allergy, Beekeeping.
Resumen

Antecedentes: Aunque la exposición a las picaduras ha sido identificada como el principal factor de riesgo en la anafilaxia debido a himenópteros, los apicultores profesionales sufren cientos de ellas al año sin desarrollar reacciones sistémicas.

Objetivo: Analizar los mecanismos de tolerancia al veneno de abeja en apicultores.

Métodos: Se realizó un estudio transversal. Los participantes se clasificaron en tres grupos: pacientes alérgicos (AP) que habían sufrido reacciones sistémicas tras la picadura de abeja, con pruebas cutáneas positivas e IgE específica (sIgE) frente al veneno de *Apis mellifera* (AmV); apicultores tolerantes (TBK) que hubiesen recibido ≥50 picaduras/año; controles sanos no expuestos (HC). Se determinaron los niveles séricos de sIgE e IgG4 específica (sIgG4) frente a AmV, rApi m 1, rApi m 2, rApi m 3, Api m 4, rApi m 5 and rApi m10, así como la desgranulación de los basófilos inducida por AmV, el porcentaje de subpoblaciones de células T, células T reguladoras (Treg) y la producción de IL-10.

Resultados: En comparación con los TBKs, los APs presentaron niveles elevados de sIgE a AmV y todos sus componentes alergénicos (*p<0.001*) junto con una elevada tasa de activación de basófilos (*p<0.001*). Por el contrario, en comparación con los APs, los TBKs tenían niveles más elevados de sIgG4 (*p<0.001*) e IL-10 (*p<0.001*) así como aumento de las poblaciones Treg CTLA-4⁺ (*p=0.001*), Treg Helios⁺ (*p=0.003*), y una reducción de las subpoblaciones T-helper 1 (*p=0.008*), T-helper 2 (*p=0.004*) y T-helper 17 (*p=0.007*).

Conclusiones: En los TBKs se encontró un perfil diferente marcadamente caracterizado por la actividad T reguladora. Esta tolerancia natural podría conducir a la expansión de células Treg Helios⁺ inducibles a nivel periférico. La población Treg Helios⁺ podría constituir un novedoso biomarcador candidato, útil en la monitorización de la tolerancia.

Palabras clave: Proteína Helios, Células T reguladoras, Tolerancia inmunológica, Alergia al veneno de abeja, Apicultura.
Background

A single sting from a hymenopterans insect (bees and wasps) can induce an extremely severe and potentially life-threatening allergic reaction in sensitized individuals [1, 2]. Around 0.3% to 8.9% of the population can experience systemic adverse events secondary to the allergic mechanisms triggered by a Hymenopteran sting [3, 4]. Unlike other allergic diseases, atopy is not a requirement prior to the emergence of allergy to these insects, with the number of stings being one of the main risk factors identified [5]. The unavoidable exposure to stings in some professional groups therefore implies an element of susceptibility to systemic reactions, consequently reducing the work opportunities of allergic individuals [6]. Beekeeping — a form of small livestock farming where several individuals, habitually from the same family, work together to perform highly qualified and seasonal tasks [7] — is undoubtedly the socio-economic sector most affected by this health problem.

Many professional beekeepers receive hundreds of bee stings every year without developing systemic reactions [6]. As such, they represent a valuable human in vivo model to explore the mechanisms of tolerance induction to allergens, because of their natural exposure to high doses of bee venom. This particular type of exposure has been previously reported to naturally favour the appearance of high levels of specific IgG4 (sIgG4) to AmV [8], although the underlying causes of naturally-acquired immunity have not yet been completely clarified.

Allergen-specific immunotherapy has been shown to induce basophil and mast cell desensitization, regulation of IgE-IgG4 secretion and generation of T-regulatory (Treg) cells, resulting in suppression of effector T-helper 1 (Th1), Th2 and Th17 cells that, respectively, produce large amounts of INF-γ, IL-4 and IL-17 [9]. Additionally, the suppression of
inflammatory cytokines from dendritic cells is thought to play a role in the constellation of changes derived from the Treg enhancement [10].

The immunological status of tolerant beekeepers resembles that of bee sting allergic individuals who have achieved protection through Apis mellifera immunotherapy (AmIT). This similarity suggests that the immunological phenomena inherent to the T-cell regulation, probably induced by AmIT, could also be involved in the natural tolerance acquired by beekeepers. Since it has recently been found that there are different forms of AmV allergy according to the sensitization profile in terms of individual allergenic components present in the venom [11, 12], the sensitization profile could be of interest to identify different patterns of protective immunological response in the natural environment. In any case, no reliable biomarkers are identifying the risk of experiencing systemic reactions after a bee sting or assessing the real level of protection of exposed individuals, given that the sIgG4 level is considered an exposure biomarker only and not a protective one [9].

Considering this background, the aim of this study was to analyse tolerance and allergy based on classical parameters as well as unpublished ones, in a group of beekeepers who do not experience systemic reactions and in a group of patients who develop anaphylaxis secondary to bee sting venom, examining the possible immunological differences between them and the putative underlying immune tolerance mechanisms in healthy beekeepers exposed to bee stings looking for a reliable marker of protection, suitable for translating research findings into clinical practice.
Methods

See the methods section in this article’s supplementary material for full details about skin test procedure, total IgE, sIgE and sIgG4 levels, tryptase levels, basophil activation test, identification of Th1/Th2/Th17 cell subpopulations, identification of regulatory T cell subpopulations, cytokine IL-4 and IL-10 production and statistics.

Study design

A total of 54 individuals over 18 years were included in this cross-sectional observational study at the Department of Immunology and Allergy at Reina Sofia University Hospital (Córdoba, Spain). Participants were stratified into three groups according the following criteria:

I. Allergic patients (AP, n = 20): subjects with a) at least one episode of anaphylaxis after a bee sting; b) positive intradermal cutaneous response to AmV at a concentration of 0.1 µg/mL or less; c) serum specific IgE (sIgE) levels to AmV higher than 0.35 kU/L; d) no previous AmIT; and e) consecutively seen in our clinic between January and December 2016.

II. Tolerant beekeepers (TBK, n = 17): active beekeepers reporting more than 50 stings per year for more than 10 years without experiencing extensive local or systemic reactions. They were recruited outside the beekeeping season.

III. Healthy controls (HC, n = 17): non-allergic individuals non-exposed to bee stings.
All study participants underwent a physical examination and were given a structured questionnaire to identify their geographic area, and number and severity of systemic episodes after stings [13].

**Results**

*Characteristics of the study population*

Of 54 subjects, 35 (64.8%) were males and 19 (35.1%) females. Patient ages ranged from 18 to 68, median 43. None of them had any immune disease. Distribution of the variables age, sex, geographic location and total serum IgE level was non-Gaussian in all groups (AP, TBK and HC). Among the recruited subjects, we found a significantly higher proportion of males in the AP (80.0%) and TBK groups (82.4%) than in the HC group (29.4%; \( p = 0.0010 \)). Therefore, a comparison of the analysed variables in AP and TBK stratified by sex was carried out without finding any intragroup differences. Both APs and TBKs usually lived in rural locations, as compared to HCs (\( p = 0.00279 \)). In the AP group, seven patients (35.0%) were diagnosed with severity grade 2, and 13 (65.0%) were diagnosed with severity grade 3 for systemic allergic reactions. The mean baseline serum tryptase of APs was 4.65 ± 2.52 µg/l.

After using the individual REMA score, two patients were found to have a score ≥2 and were diagnosed with indolent systemic mastocytosis. No statistically significant differences among groups were found in terms of the other study characteristics shown in Table 1. The raw data for all the variables analysed for each study subject (healthy controls, allergic patients and tolerant beekeepers) are shown in Supplementary Table 1.
Specific IgE and IgG4

slgE and slgG4 values to AmV, rApi m 1, rApi m 2, rApi m 3, Api m 4, rApi m 5 and rApi m 10 measured in the three groups are shown in Figure 1. Considering a cut-off of 0.35 KU/L, the 70.6% of TBK and the 6% of HC were sensitized to AmV. We found a significantly higher level of slgE to AmV and its components in the AP group compared to the HC group, except for rApi m 2 ($p = 0.064$; Figure 1C). Even though slgE to AmV, rApi m 1 and rApi m 2 values were significantly higher in APs compared to TBKs (Figure 1A-C), no significant differences were found in the slgE level to rApi m 3, Api m 4, rApi m 5 or rApi m 10 between them (Figure 1D-G). Sensitization to rApi m 1 was most prevalent among APs (75%), followed by sensitization to rApi m 10 (55%), rApi m 5 (50%), Api m 4 (30%), rApi m 2 (15%) and rApi m 3 (15%). We also found 13 different sensitization profiles in our allergic population, the most frequent being monosensitization to rApi m 1, followed by sensitization to rApi m 1 + rApi m 5 + rApi m 10 (Figure 2).

Considering slgG4 to whole AmV and its components, we found that the TBK group exhibited significantly higher levels compared to HCs and APs (Figure 1A-G). Comparison of APs and HCs revealed no differences except for slgG4 to AmV and rApi m 1 (Figure 1A and B).

Basophil activation test (BAT)

When using AmV as a stimulus at a concentration of 0.1 µg/mL (Figure 3A), we found that the percentage of degranulated basophils was higher in the AP group than in the others, achieving statistical significance only regarding the TBK group ($p = 0.024$). No differences were found between TBKs and HCs ($p = 0.417$). However, when using AmV at a concentration of 1 µg/mL (Figure 3B), the AP group showed a significantly higher proportion of degranulated basophils
than both TBK ($p < 0.001$) and HC individuals ($p < 0.001$). The proportion of degranulated basophils in the TBK group was also significantly higher than that of the HC group ($p = 0.038$). The percentage of basophil degranulation in the TBK group showed a significantly negative correlation with sIgG4 levels to AmV ($p = 0.005$) as well as positively correlated with sIgE levels to AmV ($p = 0.020$) (Figure 4).

**CD4+ lymphocyte subsets and cytokine IL-10 and IL-4 production**

A similar proportion of peripheral Treg cells, defined as CD4$^+$CD25$^{high}$CD127$^{low}$, was found among groups (AP vs. TBK, $p = 0.93$; AP vs. HC, $p = 0.42$ and TBK vs. HC $p = 0.58$). However, the Helios$^+$ Treg population was significantly enhanced in the TBK group with respect to the AP group ($p = 0.003$) and to the HC group ($p = 0.010$; Figure 5A). CTLA-4$^+$ Treg cells (Figure 5B) were significantly increased in the TBK group with respect to AP ($p < 0.001$) and HC groups ($p = 0.007$). When considering effector T-cells, all Th1, Th2 and Th17 subsets (Figure 5C-E) appeared significantly reduced in the TBK group with respect to the AP (Th1, $p = 0.008$; Th2, $p = 0.004$ and Th17, $p = 0.007$) and HC groups (Th1, $p = 0.001$; Th2, $p = 0.004$ and Th17, $p < 0.001$). No differences were found with regard to the remaining cell biomarkers studied.

IL-10 levels (Figure 6) were significantly higher in TBK than in AP individuals ($p < 0.001$) and slightly higher than in the HC group as a trend ($p = 0.069$). Similarly, IL-10 was higher in the HC group than in the AP as a trend ($p = 0.079$). Although IL-4 levels were higher in the AP group than in TBK and HC, no significant differences were found between them (Figure 6).
Definition of immunological profiles

Variables identified as candidate biomarkers were separated into allergic (sIgE and BAT) and tolerance ones (sIgG4, IL-10, Helios⁻ Treg and CTLA-4⁺ Treg) and included in a heatmap (Figure 7), where different expression levels were adjusted according to a colour scale to display individual behaviours and analyse two profiles. In general, TBKs exhibited a high level of sIgG4 to AmV and its components, a significantly expanded CTLA-4⁺ Treg population, expanded Helios⁻ Treg cells and high levels of IL-10. Conversely, APs showed high levels of sIgE together with high rates of basophil activation. Individual number 37 must be highlighted as an outlier, exhibiting a high sIgE/IgE ratio, very low IL-10 levels and a strongly expanded (98.6%) Helios⁻ Treg subset.

Discussion

Biomarkers of both allergic and immune tolerance responses were evaluated in two subject populations highly exposed to bee stings: an AP group, who experiences anaphylaxis, and TBKs, receiving hundreds of stings each year with no reactions; additionally, a non-exposed group of HCs was compared.

The results showed a well-defined regulatory-suppressor profile for the TBKs group characterized by expanded proportions of CTLA-4⁺ Treg and Helios⁻ Treg cell populations and 

ex vivo high rates of basophil activation without clinical symptoms. Additionally, as it has been previously described, TBKs showed increased production of IL-10, increased amounts of sIgG4 to AmV and all its components (rApi m 1, rApi m 2, rApi m 3, Api m 4, rApi m 5 and rApi m 10), reduced numbers of effector T-cell populations Th1, Th2 and Th17, as well as lower levels of
sIgE to bee venom and its allergenic components, compared with APs. None of these findings was present in APs or HCs.

An increased number of Helios+ Treg cells was found in the TBK group compared to APs and HCs. Helios is a member of the Ikaros transcription factor family, preferentially expressed at the mRNA level by Treg cells, and it has been shown to identify thymic-derived Treg cells (tTreg) which mediate tolerance to self-antigens. Conversely, peripheral or induced Helios+ Treg cells (iTreg) are directed to external antigens [19, 20]. Therefore, Helios expression could allow the identification of Treg populations phenotypic and functionally different, with unique non-redundant TCR repertoires aimed at the detection of own versus non-self antigens [20-22], being proposed as a marker for the distinction between central and peripherally induced Treg cells [21]. Our findings are consistent with this hypothesis and suggest that TBK subjects develop an expanded extrathymic Helios+ Treg cell (iTreg) subset because of high antigen exposure. Moreover, the number of stings (hundreds per year in the TBK group) probably would play a crucial role in developing allergenic tolerance driven by changes in the Treg activity in and out of the apiarian season [23]. The Helios+ Treg population could represent the effector subset responsible for suppressing the initially observed allergic response to bee venom, as demonstrated in the case of aeroallergens [24] and food allergens [25]. However, it contrasts with the increase of Treg Helios+ cells due to the immunotherapy against Der p 1 recently described [26]. Furthermore, it has been reported that B cell regulatory activity is also enhanced due to the high dose of venom received by beekeepers during beekeeping season [27].

Almost half of the tolerant beekeepers exhibited high rates of basophil activation, which is consistent with the detection of sIgE levels in this group but raises a question about the
underlying mechanisms for their absence of symptoms; interestingly, the basophil activation index in TBKs correlated negatively with sIgG4 levels to AmV (Figure 4). Several of the characteristic protagonists in the TBK group regulatory-suppressor profile may help explain this exciting finding. Indeed, it has been reported that elevated sIgG4 levels in TBK could impair in vivo basophil activation through cell surface Fcγ receptors [28], or alternatively, a vast repertoire of IgE in the AP group based on different concentrations, affinities and clonalities could powerfully stimulate basophil cells [29]. A combination of both mechanisms could also be the reason for this finding. In any case, the utility of BAT has been widely evaluated using different types of allergens, including bee-venom, for monitoring the achievement of tolerance. Despite the decrease regarding baseline BAT reactivity throughout immunotherapy, some reports did not observe any difference once reached tolerance. Thus, Kucera et al. found that 56.3% of non-reactors to sting challenge allergic patients after VIT had positive results in BAT [30]. The identification of TBK who do not exhibit systemic reactions but have positive BAT results is in line with that report, supporting that the degranulation of basophils is not the best method to evaluate the tolerance acquired either by VIT or naturally through high amounts of bee venom exposure.

Subjects from the TBK group showed high levels of sIgG4 to bee venom and its allergenic components. Similar results have been reported among beekeepers, although details of the underlying mechanism of production and their function are not well known [31]. Nonetheless, this elevation is particularly significant in beekeepers tolerant to stings [8, 32]. IgG4 plays a role as a biomarker of exposure, but sIgG4 levels are not a reliable marker for individual tolerance, and simultaneous elevation of sIgE and sIgG4 to AmV may also occur [33]. However, its protective role in allergic diseases by inhibiting mast cell degranulation has been
demonstrated [34]. Basophils and mast cells, in addition to FcεRI, express the FcγRII receptor [35]. Of all the IgG subclasses, IgG4 has the highest affinity for the FcγRIIb inhibitor receptor [36]. Coaggregation of FcγRIIA induces basophil degranulation, but coaggregation of FcεRI and FcγRIIb by binding to IgE and IgG4 immune complexes can inhibit mast cell degranulation [37]. Additionally, IgG4 can inhibit the degranulation of mast cells and basophils by behaving as a blocking antibody, competing with IgE for allergen binding [35]. Grass pollen-specific IgG4 antibodies from a patient who had received immunotherapy inhibited the activation of basophils by blocking the interaction between the allergen and IgE [38]. Similarly, serum from peanut-allergic patients who had received immunotherapy containing specific IgG4 antibodies against peanut allergens inhibited the ability of sIgE to activate basophils and mast cells [34]. More recently, the ability of subcutaneous immunotherapy against Der p to inhibit BAT test has been evaluated. The authors postulate that the increase in specific IgG4, rather than the reduction of specific IgE, correlates with the basophil activation test inhibition in patients with immunotherapy. To this end, the authors propose a mechanism by which specific IgG4 would compete with the ability of specific IgE to bind the allergen [39].

IgG4 exhibits another intriguing property, consisting of its ability to develop a process called Fab-arm exchange in vivo, which gives rise to bi-specific antibodies. This characteristic and the limited ability of IgG4 to form immune complexes may aid the blocking property [35,40].

The production of cytokines, such as IL-10, is associated with peripheral T-cell tolerance and the presence of Tregs [10]. Both IL-10 levels and the percentage of CTLA-4+ Treg cells were elevated in the TBK group. These findings could be associated with a tolerogenic phenotype of antigen-presenting cells. IL-10 inhibits the expression of molecules involved in antigen presentation (HLA and B7), thereby influencing Th1, Th2 and Th17 activation [41]. All three
effector subsets were consistently reduced in TBKs, which is further evidence of the robust regulatory activity in this group. Although a typically Th2 to Th1 shift has been described after hymenopteran-venom tolerance induction, with an increase in INF-γ levels and a decrease in IL-4 and IL-13 cytokine secretion [42, 43], an in vivo expansion of IL-10 producing allergen-specific cells at the expense of both Th1 and Th2 subsets has also been described [44]. Indeed, peripheral T cell tolerance is characterized by a decrease in Th2 and Th1 cells [45]. Moreover, in a ‘modified’ Th2 response, IL-10 production in the presence of IL-4 drives class switching to IgG4, without IgE production [35].

High sIgE levels play a critical role in the development of severe reactions to bee venom following insect stings. To our knowledge, this is the first report in which such a thorough analysis of molecular components of bee venom was performed. Thus, after testing the six allergenic components belonging to AmV in the three populations of this study, our results showed that in the TBK group, whose sIgE levels to AmV and its major allergens Api m 1-Api m 2 were significantly lower than those of APs, were consistent with another study comparing the same three groups [8]. However, they differed from the findings of another recent study that compared asymptotically sensitized subjects (without specifying whether they were beekeepers) [46], although both studies only analysed Api m 1 allergen. When comparing beekeepers with non-exposed HC, Matysiak et al. found significant differences regarding the sIgE to bee-venom ($p = 0.038$), but no to Api m 1 ($p = 0.055$) [33]. In contrast, we found significant differences in the sIgE to all molecular components analysed, excepting rApi m 3. The different results described between both studies could be due to the clinical characteristics of recruited individuals (Matysiak et al. examined 30 beekeepers, 2 of which still had systemic reactions), but both show that TBK usually have detectable sIgE levels [33].
As it has been previously described, among APs there was a predominance of allergic response markers defined by markedly elevated levels of sIgE to bee venom and its allergenic components (rApi m 1, rApi m 2, rApi m 3, Api m 4, rApi m 5 and rApi m 10), as well as a high rate of basophil activation to AmV.

Interestingly, considering paradoxical behaviours, among the 17 healthy beekeepers, basophils degranulated in nine (52.9%), but in none of the healthy individuals. Furthermore, one individual belonging to the TBK group was characterized by a marked basophil activation rate and a lower sIgG4 response. TBK number 37 shown in the heatmap graph must be highlighted because of the strong presence of an allergic profile and weak tolerance markers. Considering all these findings as a whole, it could represent a particular type of beekeeper risk profile, eventually susceptible to a higher risk of systemic reactions after further bee stings.

The main limitation of this study is that it was conducted in a single centre with limited sample size. The diversity of sensitization profiles demands a higher number of patients from different geographical origins, to reach sound information. Moreover, it would be desirable to reduce the technical complexity of the methods used to translate them into daily clinical practice. Despite these drawbacks, Helios Treg population seems to be a novel candidate biomarker, which together with the well-known CTLA4 and IL-10, will allow us to monitor the tolerance process from bench to bed. As the next proposal, it is suggested to thoroughly address the regulatory-suppressor activity, including its underlying mechanisms, along a venom immunotherapy course.
Conclusions

A well-defined regulatory-suppressor profile, which is strongly marked by Treg activity, was found for tolerant beekeepers. This natural tolerance would be led by the expansion of inducible Helios+ Treg cells at a peripheral level. Treg function remains the grounds on which tolerance is based, even in extreme exposure conditions.

Declarations

Ethics approval and consent to participate

This study was approved by the local institutional “Ethics Committee of Reina Sofía Hospital” with the committee’s reference number FCO-VAC-2015-01. All the participants provided written informed consent.

Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

Funding

Ministerio de Sanidad. Instituto de Salud Carlos III. FIS PI15/021701

European Regional Development Fund. ARADyAL RD16/0006/0018

Agencia de Innovación y Desarrollo de Andalucía. Project S0169
Acknowledgments

We thank María José Barasona, Lourdes Fernández, Vanessa Sáiz, Estrella Cañones and Mercedes Guerra for being part of the collaborative clinical team and Jhonas Lidholm from Thermo Fisher for his help in making the Api m 4 prototype.

References


**Tables**

Table 1: Clinical and demographic characteristics of the study population.

<table>
<thead>
<tr>
<th></th>
<th>AP (n = 20)</th>
<th>TBK (n = 17)</th>
<th>HC (n = 17)</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male sex; n (%)</td>
<td>16 (80.0)</td>
<td>14 (82.4)</td>
<td>5 (29.4)</td>
<td>0.0010</td>
</tr>
<tr>
<td>Age (years); median (maximum-minimum values)</td>
<td>40.5 (68-18)</td>
<td>48 (64-27)</td>
<td>42 (59-21)</td>
<td>0.353</td>
</tr>
<tr>
<td>Rural location†; n (%)</td>
<td>8 (40.0)</td>
<td>7 (41.2)</td>
<td>0</td>
<td>0.00279</td>
</tr>
<tr>
<td>Systemic allergic reactions*</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Severity Grade 2; n (%)</td>
<td>7 (35.0)</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Severity Grade 3; n (%)</td>
<td>13 (65.0)</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Total serum IgE (kU/L); median (maximum-minimum values)</td>
<td>159.8 (9,897.5-1.5)</td>
<td>57.4 (1,269.7-4.9)</td>
<td>21.5 (1,149.2-1.5)</td>
<td>0.454</td>
</tr>
</tbody>
</table>

†Rural location was considered as one with <10,000 inhabitants. *EAACI 2018 grading [13].
Figure legends

**Figure 1.** Box-plots for the serum specific IgE level (sIgE) and specific IgG4 level (sIgG4) to whole honey bee venom (*Apis mellifera*) and its components (rApi m1, rApi m2, rApi m3, Api m4, rApi m5 and rApi m10; A-G panels) of allergic patients (AP), tolerant beekeepers (TBK) and healthy controls (HC).
**Figure 2.** Different sensitization profiles found in the allergic patient group according to the serum specific IgE level to each *Apis mellifera* venom component, considering 0.35 kU/L as the positive cut-off value. The proportion of allergic patients exhibiting the different identified profiles is displayed.
Figure 3. Percentage of degranulated basophils (%CD63⁺) of allergic patients (AP), tolerant beekeepers (TBK) and healthy controls (HC) when using 0.1 µg/mL (A) and 1 µg/mL (B) of whole honey bee venom as a stimulus. Mean and standard error of the mean are shown.
**Figure 4.** Correlation between the percentage of BAT positivity in tolerant beekeepers and the levels of sIgG4 and sIgE. $r =$ Pearson’s correlation coefficient.
Figure 5. Percentage of Helios<sup>-</sup> Treg cells (A), CTLA-4<sup>+</sup> Treg cells (B) and Th1 (C), Th2 (D) and Th17 (E) subsets of allergic patients (AP), tolerant beekeepers (TBK) and healthy controls (HC). Mean and standard error of the mean are displayed.
**Figure 6.** Box-plots for IL-10 and IL-4 levels (pg/mL) quantitated in culture supernatant of allergic patients (AP), tolerant beekeepers (TBK) and healthy controls (HC).
**Figure 7.** Heatmap including those study parameters identified as tolerance biomarkers. The colour scale is adjusted as red for higher expressions and yellow for lower expressions. Annotations at the top of the heatmap show the study groups. The dendrogram shows clustering of samples (rows) which is based on hierarchical clustering with Euclidean distance metric and average linkage.