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Immunotherapy with a Recombinant Hybrid Molecule Alleviates Allergic Responses More Efficiently than an Allergenic Cocktail or Pollen Extract in a Model of *Chenopodium album* Allergy

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Key Words
Recombinant hybrid molecule · Allergenic cocktail · Allergen-specific immunotherapy

Abstract
Background: The aim of this study is to assess the therapeutic potential of a recombinant hybrid molecule (rHM) alongside an allergenic cocktail from recombinant wild-type allergens as well as pollen extract on *Chenopodium album* allergy, using a BALB/c mouse model. ***Methods:*** The BALB/c mice had already been sensitized to *C. album* via intraperitoneal injections of alum-adsorbed allergenic cocktail and immunotherapy procedure was followed by subcutaneous injections of the rHM, allergenic cocktail and pollen extract at weekly intervals. Humoral immune responses were determined via measurement of specific antibodies in serum. Splenocytes of immunized mice were stimulated in vitro and then proliferation responses, cytokine secretion and mRNA expression of genes involved in immunotherapy were examined by ELISA and real-time PCR. ***Results:*** Sensitized mice were identified with high specific IgE against allergenic cocktail when compared with healthy mice. Immunotherapy with the rHM induced the highest ratio of the IgG2a/IgG1 levels compared to allergenic cocktail or *C. album* pollen extract. The rHM was able to induce proliferative responses as well as the allergenic cocktail in cultured splenocytes. Immunotherapy with the rHM significantly improved secretion of IFN-γ and IL-10, while secretion of IL-13 rapidly diminished. Interestingly, mRNA expression of GATA3 was strongly decreased in rHM-treated mice whereas mRNA expression of T-bet and Foxp3 was significantly increased. ***Conclusion:*** Our results prove that immunotherapy with the rHM effectively controlled allergic responses by shifting from a Th2-like immune response to a Th1-dominated immune response.
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Introduction

Chenopodium album pollen is one of the major causes of respiratory allergy diseases in desert and semidesert areas of the world [1]. Allergen-specific immunotherapy (SIT) is the only curative approach to treat *C. album* allergy diseases. SIT of *C. album* allergy is currently performed with allergen extracts and has poor efficacy and many side effects. There are key problems with the use of

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extracts for immunotherapy, including batch to batch variations, reduced immunogenicity of certain allergens, poor representation of allergens and the presence of non-allergenic materials [2–4]. In spite of a lot of scientific effort to improve the quality of allergen extracts, it has failed to overcome these fundamental problems. Therefore, novel approaches to perform SIT have focused on recombinant allergens. Moreover, concerning application of wild-type recombinant allergens in immunotherapy, there remain some side effects and immune responses are unpredictable [5, 6]. The next approaches were focused on synthesis of hypoallergenic molecules with reduced IgE binding. Several methods have been introduced for hypoallergenic molecule production that include oligomerization, fragmentation and site-specific mutagenesis in allergens [7, 8].

It seems that hybrid technology is one of the greatest options in synthesis of hypoallergenic molecules [9]. One of the advantages of hybrid technology is that amino acid sequences of Che a 1, Che a 2 and Che a 3 allergens are preserved. In terms of recombinant hybrid molecule (rHM) production from *C. album* allergens, all of the T cell epitopes remain intact, whereas conformational changes in the hybrid molecule may prevent specific binding of IgE to the rHM. Interestingly, a high level of immunogenicity is obtained when low molecular weight allergens are produced as chimeric proteins [10].These traits may introduce the rHM as an appropriate candidate for allergen immunotherapy.

Nowadays, subcutaneous immunotherapy is a well-documented treatment for allergic rhinitis and asthma. It seems that several immunological pathways are involved in clinical improvement followed by SIT. Briefly, modulation of allergen responses occurs through deviation of Th2-like response to Th1-like response that is associated with IL-4 and IL-13 reduction, and also to IFN-γ increase [11]. In mice, Th2 responses are accompanied by IgE and IgG1 production, while the Th1 responses lead to IgG2a synthesis. Moreover, increase of allergen-specific IgG antibodies, in particular IgG4 in human and IgG2a in mouse was observed after immunotherapy [12].

*C. alb*um allergens have been well characterized recently and our laboratory has produced rChe a 1, rChe a 2 and rChe a 3 in an allergenic cocktail form and introduced it as a diagnostic tool for *C. album* allergy [13]. However, there has been no study to examine the immunotherapeutic potential of *C. album* recombinant allergens so far. Only a few hybrid allergens from special allergenic sources such as grass pollens [14, 15] have been produced and their allergenicity and immunogenicity has been checked in animal models. Therefore, for the first time, immunotherapeutic effects of the rHM along with allergenic cocktail and *C. album* pollen extract were monitored in a model of *C. album* allergy.

Materials and Methods

Antigens, Patients and Mice

The rHM is a heterotrimer of Che a 1, Che a 2 and Che a 3 expressed in *Escherichia coli* BL21 Codon Plus (DE3) RIL (Stratagene, La Jolla, Calif., USA) and purified by Ni-NTA chromatography. Immunologic experiments confirmed that the rHM had low capacity of IgE binding while its immunogenicity was preserved [16]. The recombinant Che a 1, Che a 2 and Che a 3 were previously expressed and purified from *E. coli* [13] and we had built an allergenic cocktail with equimolar concentrations of them. The allergenic cocktail concentration was adjusted to 5 μg. Eight *C. album*-allergic patients that simultaneously showed high levels of specific IgE against rChe, rChe a 2 and rChe a 3 were included in this study and a pooled serum was prepared for competitive ELISA [13]. Female BALB/c mice (6 weeks old) were purchased from Razi Vaccine and Serum Research Institute (Mashhad, Iran). Animal keeping procedures were conformed according to standard guidelines of animal care and were approved by the Animal Ethics Committee (No. 87542) of Mashhad University of Medical Sciences.

Skin Testing

To evaluate cutaneous reaction induction by the rHM, skin prick test was performed on sensitized mice after last aerosol challenge. Evans blue (100 μl of 0.5 mg/ml) was injected into the tail vein of sensitized mice. Subsequently, 10 μl of allergenic cocktail, the rHM (100 μg/ml), *C. album* pollen extract (1 mg/ml) and PBS were administered intradermally into the shaved abdominal skin. After 20 min, the appearance and distribution of blue color at the scratched point on the abdominal skin was measured. The contamination of the purified recombinant allergens and pollen extract to bacterial endotoxin was determined using the Limulus Amoebocyte Lysate test (GenScript, Piscataway, N.J., USA) according to the manufacturer's instructions. Endotoxin content in recombinant allergens and pollen extract were estimated to be lower than 5 EU/ml, which is suitable for in vivo experiments.

Induction of Allergic State in BALB/c Mice and Immunotherapy Protocol

Individual mice (n = 24) were injected intraperitoneally 4 times at weekly intervals with 5 μg of allergenic cocktail already adsorbed in 2 mg alum gel suspension (13 mg/ml, Sigma-Aldrich, Seelze, Germany) suspended in 0.2 ml of 15 mM PBS. The sensitization procedure was followed by 20-min exposure to an aerosol challenge of 1% allergenic cocktail on days 26 and 28 using Omron CX3 nebulizer. In parallel, control mice (n = 5) received four injections of alum plus PBS, and were aerosolized with PBS instead of the allergenic cocktail, using a similar schedule and routes as shown in online suppl. figure 1 (for all online suppl. material see www.karger.com/doi/10.1159/000347136). Sensitized mice were randomly divided into four groups (n = 5) and immunotherapy was carried out by subcutaneous injection of 5 μg of the rHM, 5 μg of allergenic cocktail and 50 μg of *C. album* pollen extract, 1 week

after the last aerosol challenge on two occasions (day 35 and 42). The control group received PBS and the treatment regimen was the same as above. One week after the last subcutaneous injection, mice were challenged twice with 1% allergenic cocktail in PBS. Forty-eight hours after the last aerosol challenge, mice were humanly scarified and serum samples were individually collected via cardiac puncture. Also, there was a weekly blood sampling via retro-orbital plexus before sensitization and immunotherapy.

Assessment of Allergenic Cocktail-Specific Immunoglobulin Responses

The presence of specific antibodies were measured by a standard ELISA. Briefly, the wells of the ELISA microplate (Nunc, Roskilde, Denmark) were coated overnight at 4°C with 100 µl of recombinant allergenic cocktail (20 µg/ml in 15 mM Na_2CO_3 and 35 mM $NaHCO_3$, pH 9.6). Free sites were blocked with 2% bovine serum albumin (BSA) in PBS for 1 h at 37°C followed by washing five times with PBST (0.05% Tween-20 in PBS), and incubated overnight at 4°C with diluted serum. Mouse sera were diluted 1:5, 1:2,500 and 1:200 for IgE, IgG1 and IgG2a determination, respectively. For IgE determination, 100 µl of 1:2,000 diluted biotinylated rat anti-mouse IgE antibody (AbD Serotec Inc., Raleigh, N.C., USA) was added to each well and incubated for 2.5 h at room temperature. Then, 1:20,000 diluted horseradish peroxidase-conjugated streptavidin (1 mg/ml) was added to each well and reincubated for 1 h at room temperature. Subsequently, for determination of IgG1 and IgG2a levels, horseradish peroxidase-conjugated rat anti-mouse IgG1 and IgG2a antibodies were added to plates according to the manufacturer's instructions (Zymed Laboratories Inc., San Francisco, Calif., USA). Finally, signals were developed by addition of chromogenic substrate. Optical density at 450 nm was read using an ELISA microplate reader (Stat Fax® 2100 Microplate Reader, Awareness Technology, Palm City, Fla., USA) 20 min after the substrate was added.

Detection of Blocking Antibodies by Competitive ELISA

For competitive ELISA, sera from the last bleeding were used. Briefly, microplates were coated with 2 µg/well of allergenic cocktail and saturated with PBS-2% BSA as described earlier. Each well was overlaid with 1:2,500 diluted serum of nonsensitized mice or treated mice (PBS/rHM/allergenic cocktail/pollen extract) and incubated overnight at 4°C. Then, 100 µl of a pooled serum from *C. album*-allergic patients (1:5 diluted in PBS-1% BSA) was added to the same wells. IgE immune complexes were detected as previously described [13]. The inhibition percentage of human IgE binding to the same allergens was determined according to the following formula: inhibition (%) = $[1 - OD_A/OD_B] \times 100$, where OD_A and OD_B represent the optical densities corresponding to treated and nonsensitized mice sera, respectively.

Measurement of IL-4 and IFN-γ Levels in Supernatants of Cultured Splenocytes

Single-cell suspensions were obtained by homogenization of the mice spleens. Washed splenocytes were resuspended in a complete medium (RPMI 1640 containing 10% heat-inactivated fetal calf serum, 100 U/ml of penicillin and 100 µg/ml of streptomycin) and seeded in duplicate at 1×10^6 cells/ml in 96-well plates (Nunc). Splenocytes were cultured with rHM, allergenic cocktail (20 µg/ml), *C. album* pollen extract (50 µg/ml), phytohemagglutinin (PHA; 1 µg/ml) and medium alone at 37°C in a humidified atmosphere of 5% CO_2. Supernatants were harvested after 72 h. Consequently, IL-4 and IFN-γ levels were tested using ELISA method according to the manufacturer's instructions (Bender MedSystems GmbH, Vienna, Austria).

Comparing Proliferative Capacity of rHM and Allergenic Cocktail in Mice Splenocytes

Mice spleen cells (2.5×10^5 cells per well) were cocultured with rHM, allergenic cocktail and pollen extract as descried earlier. The proliferation response of splenocytes was determined using colorimetric immunoassay method, based on the BrdU incorporation to DNA during the cell cycle, according to the manufacturer's instructions (Roche Diagnostics, Germany). Finally, Cell proliferation was calculated as the stimulation index (SI) obtained from the following formula: $SI = [OD_A/OD_B]$, where OD_A and OD_B represent the optical density at 450 nm of stimulated cells with antigens and unstimulated cells, respectively.

Evaluation of Genes Expression by Real-Time PCR

Splenocytes were seeded at 2×10^6 cells/ml in 24-well plates in the presence of the rHM. Total RNA was extracted from splenocytes through total RNA isolation kit according to the manufacturer's instructions, and then cDNA was synthesized using the Easy™ cDNA Reverse Transcription kit (Pars Tous Biotechnology, Iran). Primers for T cell linage commitment including T-bet, GATA3 and Foxp3, along with IL-10 and IL-13 were designed as shown in online suppl. table 1. Real-time PCR was performed using 10 µl of SYBR® Real-Time PCR Master Mix (Pars Tous Biotechnology), 1 µl of each primer (10 pmol/µl), 4 µl of cDNA and 4 µl of DEPC water. Reactions were run using Stratagene Mx-3000P (Stratagene). After doing an RT-PCR for each of the target genes, cDNAs with high quality were selected and serially diluted to obtain six standard solutions in order to be able to draw a standard curve. The relative mRNA expression ratios were determined using the method introduced by Pffafl [17].

Statistical Analysis

All data were analyzed in GraphPad Prisms® 5.0 software (GraphPad Software Inc., San Diego, Calif., USA) and displayed as means ± SD. Differences between various groups were judged by one-way ANOVA with a post-test comparison using the Tukey test. All p values <0.05 were considered significant.

Results

IgE Responses to Allergenic Cocktail during Sensitization and Immunotherapy

Induction of allergy was serologically confirmed via measurement of high specific IgE against allergenic cocktail in all sensitized mice compared to nonsensitized mice (control group). Four weeks after the first intraperitoneal administration of allergenic cocktail specific IgE production reached the highest level in the sensitized mice while it was not even detectable in the control mice (fig. 1a). After subcutaneous immunotherapy, IgE levels in the rHM- (0.71 ± 0.09) and allergenic cocktail-treated mice

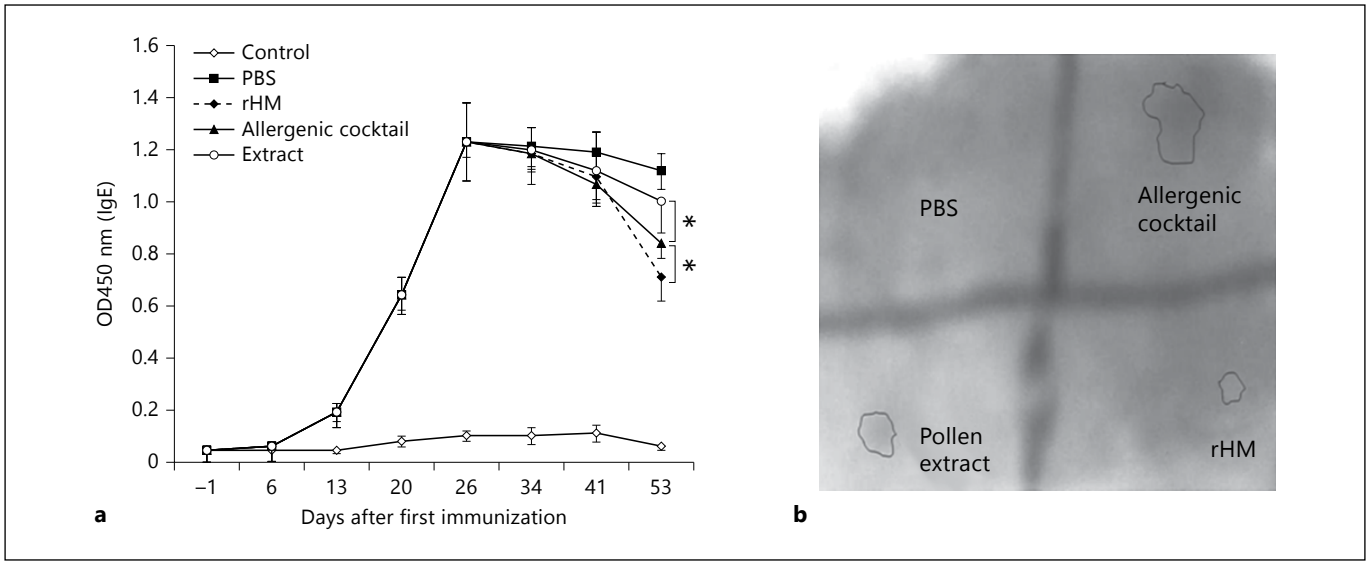


Fig. 1. Induction of specific IgE and cutaneous reaction after sensitization with allergenic cocktail in BALB/c mice. **a** Time course of specific IgE levels throughout intraperitoneal sensitization and subcutaneous immunotherapy. Specific IgE reached the highest levels after 4 weeks of first immunization and significantly decreased in either the rHM- or allergenic cocktail-treated mice. **b** Positive skin prick test was significantly observed in sensitized mice in response to allergenic cocktail and *C. album* pollen extract when compared to rHM. * $p < 0.05$.

(0.84 ± 0.06) was significantly ($p < 0.05$) suppressed compared to the PBS- (1.12 ± 0.7) and pollen extract-treated mice (1.00 ± 0.12). Immunotherapy with the rHM reduced IgE levels more than with allergenic cocktail. There was no significant difference between the IgE level in PBS-treated mice and those which received *C. album* in the immunotherapy regimen (fig. 1a).

The rHM Acts as Hypoallergen for BALB/c-Sensitized Mice

Randomly performed skin prick tests initially confirmed that cutaneous reactions are clearly visible after intradermal application of *C. album* allergenic cocktail in sensitized mice. In addition, the rHM showed the lowest potential of cutaneous reaction when compared with *C. album* pollen extract or allergenic cocktail (fig. 1b). The obtained result revealed that the rHM can act as a hypoallergenic molecule in sensitized mice.

Development of Protective Antibodies after Immunotherapy with rHM

Specific IgG2a level was significantly higher in mice treated with 5 μg of the rHM (0.85 ± 0.15) compared to other groups, as shown in online suppl. fig. 2a ($p < 0.05$). Also, allergenic cocktail- (0.63 ± 0.06) and *C. album* pollen extract-treated mice (0.48 ± 0.08) had significantly higher levels of specific IgG2a in comparison with PBS-treated mice (0.29 ± 0.06), but there was no difference between them in the case of IgG2a. The level of specific IgG1 in treated mice with the rHM (1.07 ± 0.2) had a tendency to decrease, but, as the results depicted in online suppl. fig. 2b demonstrate, specific IgG1 levels stayed at its maximum state in mice treated with allergenic cocktail (1.19 ± 0.11) and pollen extract (1.23 ± 0.07). There was no significant change between them and those which received PBS (1.36 ± 0.12). Interestingly, IgG1 antibody was kept at the highest level in mice that had not received any treatment after sensitization (PBS group). As expected, we detected no specific antibodies in the serum of control mice.

Production of IgG Blocking Antibodies in rHM-Treated Mice

Preincubation of the ELISA plate with sera from mice treated with the rHM inhibited *C. album*-allergic patients IgE binding to allergenic cocktail from 44.3 to 65.8% (median 53.5%), whereas 33.6–57.7% (median 44.9%) and 35.8–52.4% (median 42.7%) inhibition was observed with sera from allergenic cocktail- and pollen extract-treated mice, respectively. It must be pointed out that the inhibition percentage obtained with the sera of the rHM-treated mice was somewhat higher than the allergenic cocktail

group, but no significant difference was observed between them, as shown in online suppl. fig. 3a. In addition, 36.3–60.4% (median 40.6%) inhibition was observed by sera from the PBS group.

Immunotherapy with rHM or Allergenic Cocktail Induces the Same in vitro Proliferative Responses

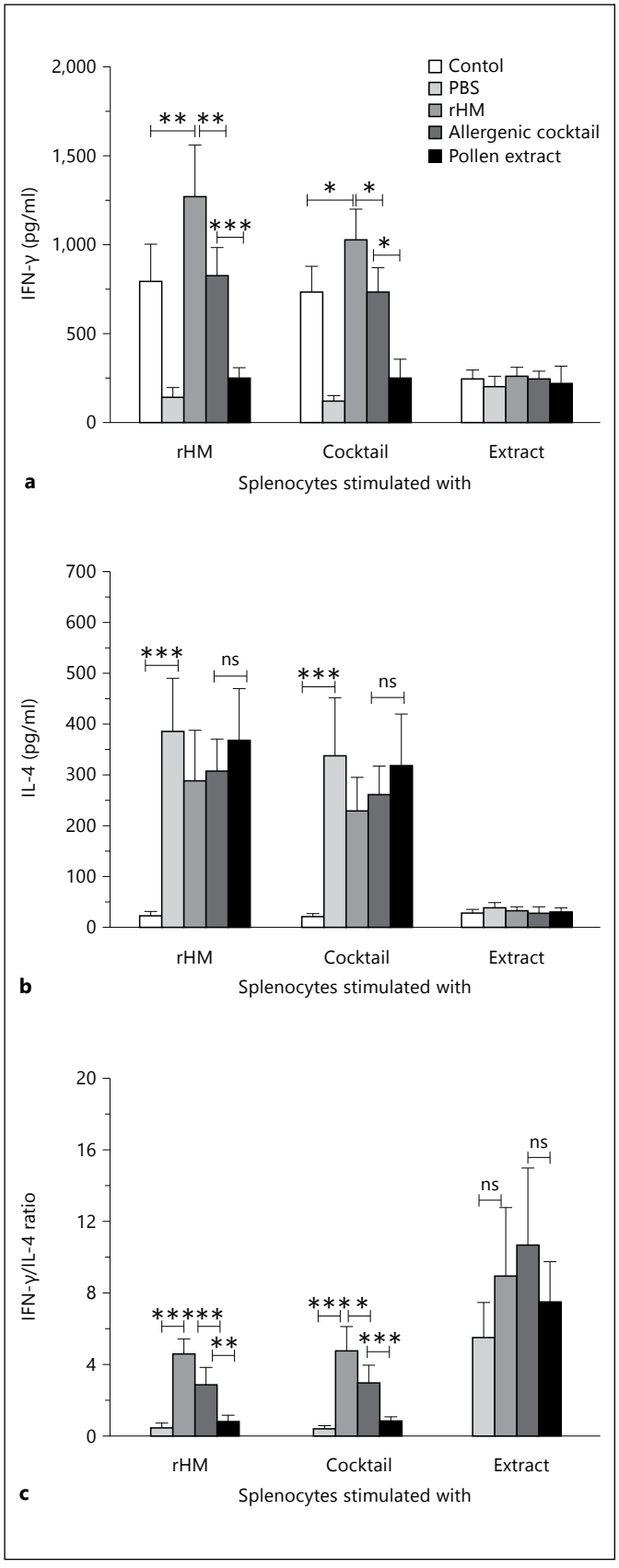
To evaluate the immunogenicity of the rHM along with allergenic cocktail and pollen extract, splenocytes proliferative response to the mentioned antigens were determined. High proliferative responses were determined to the PHA as a T cell mitogen (data not shown). Stimulated splenocytes of treated mice showed a heterogeneous profile of proliferative responses. As shown in online suppl. fig. 3b, the rHM and allergenic cocktail as antigens caused more proliferative responses than pollen extract. In all of the performed experiments, rHM was able to induce the same proliferative response as the allergenic cocktail. Proliferative responses by these antigens were not statistically different. Compared to other groups, the lowest SI was obtained in the pollen extract-treated group. In addition, no significant difference on SI was observed between the different groups when their splenocytes were stimulated in vitro using *C. album* pollen extract.

Alteration of Th1/Th2 Cytokine Ratios

IFN-γ and IL-4 measurement confirmed that IFN-γ and IL-4 concentrations were at the lowest levels (113 ± 27 pg/ml) and (9 ± 6 pg/ml), respectively, in nonstimulated splenocytes. In contrast, IFN-γ (5,448 ± 2,183 pg/ml) and IL-4 (717 ± 235 pg/ml) cytokines were detected at maximum levels in cells stimulated with PHA. The levels of IFN-γ in the rHM- (1,271 ± 290 pg/ml) or allergenic cocktail-treated mice (828 ± 157 pg/ml) were significantly ($p < 0.001$) higher than in the PBS- (140 ± 60 pg/ml) and pollen extract-treated mice (251 ± 57 pg/ml) when the rHM was used as recall antigen. More analysis of obtained data showed that the level of IFN-γ in the rHM-treated mice was significantly higher than in allergenic cocktail-treated mice (fig. 2a). IFN-γ levels in allergenic cocktail-stimulated splenocytes were slightly lower than in the rHM-stimulated ones, but there was no significant difference ($p > 0.05$) between them. The average of IFN-γ production in the supernatants of *C. album* pollen extract-stimulated splenocytes was significantly lower ($p < 0.01$) than the IFN-γ level in rHM- or allergenic cocktail-stimulated splenocytes (fig. 2a). The level of IL-4 was elevated in the rHM- (288 ± 99 pg/ml), allergenic cocktail- (307 ± 63 pg/ml), pollen extract- (368 ± 102 pg/ml) and PBS-treated mice (385 ± 106 pg/ml) and it was significantly higher ($p < 0.001$) than IL-4 levels in the control group (21 ± 9 pg/ml), when rHM was used as a recall antigen (fig. 2b). IL-4 production in allergenic cocktail-stimulated splenocytes was similar to the condition in which the rHM was used as stimuli. The lowest significant level ($p < 0.001$) of IL-4 was observed in the control group or in all treated mice when splenocytes were stimulated with *C. album* pollen extract (fig. 2b). As shown in figure 2c, the ratio of IFN-γ/IL-4 in mice treated with the rHM was significantly higher ($p < 0.001$) than those that received allergenic cocktail or pollen extract.

High Level Expression of Genes Involved in Suppression of Allergic Response following Immunotherapy

The expression level of T-bet in response to rHM was significantly increased ($p < 0.05$) in rHM-treated mice (3.1 ± 0.2) compared to the allergenic cocktail (1.7 ± 0.3), pollen extract (0.9 ± 0.2) and PBS groups (0.9 ± 0.1).There was no significant difference between T-bet expression in the rHM-treated mice and the control group (4.3 ± 0.4). Simultaneously, GATA3 mRNA level in restimulated splenocytes of mice which were already treated with rHM (1.8 ± 0.2) or allergenic cocktail (2.0 ± 0.3) significantly decreased in comparison with PBS- (7.9 ± 0.3) or pollen extract-treated mice (3.4 ± 0.1), as shown in online suppl. fig. 4b. However, GATA3 mRNA reached the normal level in the control group (1.0 ± 0.1) after immunotherapy with just rHM. The profile of the T-bet/GATA3 expression ratio was almost the same as results of T-bet expression for each group. The highest ratio of T-bet/GATA3 was observed in the control group (4.2 ± 0.2) and this ratio in rHM-treated mice (1.8 ± 0.3) was significantly higher than in pollen extract- (0.3 ± 0.1) or PBS-treated mice (0.1 ± 0.0), as shown in online suppl. fig. 4c. Foxp3 expression was significantly induced in mice that received the rHM (5.0 ± 1.1) and its expression level was significantly higher than other groups, as shown in online suppl. fig. 4d. Interestingly, the IL-10 expression pattern was the same as Foxp3 expression and significantly elevated in rHM-treated mice (3.8 ± 0.6) as compared to other groups (online suppl. fig. 4e). As shown in online suppl. fig. 4f, the rHM- (2.2 ± 0.1), allergenic cocktail- (4.1 ± 0.9) and pollen extract-treated mice (5.4 ± 0.4) had a significant tendency to decrease IL-13 expression levels compared to PBS-treated mice (9.7 ± 0.8). Furthermore, IL-13 expression levels in rHM-treated mice were significantly lower than pollen extract-treated mice, whereas no significant change was observed between allergenic cocktail- and pollen extract-treated mice.



Discussion

It seems that one of the strategies to improve SIT is to use single allergens with low capacity of IgE binding while T cell epitopes are preserved [18]. We have already constructed an rHM of three identified *C. album* pollen allergens, Che a 1, Che a 2 and Che a 3, via overlapping extension PCR. Using immunological experiments, we have confirmed that the constructed rHM has strongly lost its allergenicity and proposed that rHM may act as a suitable option in immunotherapy [16]. Since immunogenic properties of recombinant allergens and hypoallergenic derivatives must be evaluated in vivo before being used in immunotherapy of allergic patients [19], we studied immunotherapeutic potential of constructed rHM on type I allergy.

After sensitization, IgE reached the highest level in sensitized mice in accordance with other studies that showed intraperitoneal injection of allergens to cause Th2 immune responses [20, 21]. Suppression of specific IgE is one of the main mechanisms to interrupt the pathogenesis of allergy during allergen immunotherapy [22, 23]. Compared to the PBS- or pollen extract-treated mice, the rHM- and allergenic cocktail-treated mice showed a significant decrease in IgE production. Furthermore, the rHM induced high-level production of IgG2a-specific allergens that prevented IgE-specific antibody development. It may be explained through induction of stronger T cell responses by the rHM. Nevertheless, in the context of allergic alleviation through antibodies, the rHM was more efficient than the allergenic cocktail. Moreover, sera of treated mice with the rHM blocked human IgE binding to recombinant wild-type allergens. SIT with various hypoallergens in a mouse model has also resulted in the induction of blocking IgG antibodies [24]. These blocking IgG antibodies are able to modulate allergic responses through several mechanisms [25–28].

Fig. 2. Cytokine profile of stimulated splenocytes with either the rHM or allergenic cocktail or *C. album* pollen extract. **a** IFN-γ significantly increased in response to rHM or allergenic cocktail as the recalled antigen. There was significant difference between IFN-γ levels in the rHM-treated mice compared with allergenic cocktail-treated mice. **b** IL-4 levels were significantly increased in all treated mice compared to the control group and there was no significant change between all treated mice. **c** IFN-γ to IL-4 ratio was significantly higher than other groups when the rHM or allergenic cocktail was used as antigen. No significant change in this ratio was observed when *C. album* pollen extract was used as antigen. Values are mean ± SD. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$; ns = non-significant with One-way ANOVA test.

It has been proven that an alteration in the Th1/Th2 ratio could play a key role in regulation of antibody responses in vivo [22]. Analysis of cytokine and transcription factor data confirm that the rHM along with allergenic cocktail are able to stimulate splenocytes and force them to produce high levels of IFN-γ and T-bet, while *C. album* pollen extract unspecifically generated them at the lowest level (fig. 2a). It must be pointed out that rHM was able to inhibit expression of GATA3 and IL-13 while PBS and pollen extract were not. IL-13 is not a perfect Th2 cytokine but it is able to help IgE antibody production in allergic patients when it is produced in high levels [11]. In addition, upregulation of the IFN-γ/IL-4 ratio results in reduction of the Th2-mediated IgE, which was significantly low in the rHM-treated mice. IL-4 is able to induce antibody class switching on B cells and converts the IgM to IgE, and INF-γ is able to inhibit this process [22]. The suppressive effects of *C. album* pollen extract on splenocytes proliferation and cytokine production have been explained by the presence of unrelated allergenic components in pollen extracts that may affect immune responses.

Induction of allergen-specific suppressive activity through regulatory CD25+ CD4+ T cells and IL-10 is one of the key mechanisms in SIT [29, 30]. Notably, immunotherapy with the rHM can induce Foxp3 and IL-10 expression. Therefore, presence of IL-10 may cause suppression of IgE production and inhibition of IL-13 gene expression in treated mice with rHM. Induction of Foxp3 along with IL10 caused rHM to have more efficient suppressive effects on allergic immune responses compared to the allergenic cocktail. In addition, reduction of GATA3 and induction of Foxp3 expression in rHM-treated mice may be explainable using data which demonstrates overexpression of GATA3 in T cells correlated with deficiency in Foxp3 induction [31–32].

Our results are in accordance with the recently demonstrated data showing that molecules consisting of several copies of the same allergen of homologous allergens, or even of immunologically unrelated allergens, are of use in immunotherapy [10, 33]. There are several reasons to explain why increased immunogenicity of the rHM switches immune responses to Th1 following immunotherapy. In this designed rHM some of the conformational B cell epitopes were destroyed, whereas the T cell epitopes were preserved. Additionally, it is possible that processing of the rHM results in peptides which may bind better to the MHC or T cell receptors than those resulting from wild-type allergens. Alternatively, processing of the rHM which contains a few foreign amino acids linked to individual allergens may result in the formation of new T cell epitopes. Moreover, the rHM with reduced IgE reactivity does not use IgE-mediated antigen presentation while using phagocytic or pinocytic antigen uptake mechanisms. These mechanisms would induce Th1-like cytokine production by T cells and also higher IgG and lower IgE production by B cells [34–36].

Conclusion

In summary, the obtained results manifest that use of the rHM can downregulate allergic response in a BALB/c mice model of type I allergy. Taken together, our data confirm that the rHM can act as a new immunotherapeutic option with more efficiency compared to the recombinant wild-type allergens or pollen extract in the context of *C. album* allergy.

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