

Original article

Targeting the MHC class II pathway of antigen presentation enhances immunogenicity and safety of allergen immunotherapy

Background: Current s.c. allergen-specific immunotherapy (SIT) leads to amelioration of IgE-mediated allergy, but it requires numerous allergen injections over several years and is frequently associated with severe side-effects. The aim of this study was to test whether modified recombinant allergens can improve therapeutic efficacy in SIT while reducing allergic side-effects.

Methods: The major cat allergen Fel d 1 was fused to a TAT-derived protein translocation domain and to a truncated invariant chain for targeting the MHC class II pathway (MAT-Fel d 1). The immunogenicity was evaluated in mice, while potential safety issues were assessed by cellular antigen stimulation test (CAST) using basophils from cat-dander-allergic patients.

Results: MAT-Fel d 1 enhanced induction of Fel d 1-specific IgG2a antibody responses as well as the secretion of IFN- γ and IL-2 from T cells. Subcutaneous allergen-specific immunotherapy of mice using the modified Fel d 1 provided stronger protection against anaphylaxis than SIT with unmodified Fel d 1, and MAT-Fel d 1 caused less degranulation of human basophils than native Fel d 1.

Conclusion: MAT-Fel d 1 allergen enhanced protective antibody and Th1 responses in mice, while reducing human basophil degranulation. Immunotherapy using MAT-Fel d 1 allergen therefore has the potential to enhance SIT efficacy and safety, thus, shortening SIT. This should increase patient compliance and lower treatment costs.

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Subcutaneous allergen-specific immunotherapy (SIT) is the only causal treatment of allergies, has a long-lasting effect and can stop progression of the allergy to multiple sensitizations or to asthma. Subcutaneous SIT typically requires 50–70 injections during 3–5 years (1) and bears a significant risk of allergic side-effects including anaphylaxis. Hence, the number of injections and also the risk of allergic side-effects should be reduced. Current efforts to enhance SIT focus on optimizing the allergen molecules (2–4), the adjuvants (5, 6), the route of administration (7–9), and also their dosage form, including use of particulate delivery systems, such as virus-like (10) or poly(lactide-co-glycolide) particles (11).

Approaches to increase the safety of SIT include pretreatment with anti-histamines or anti-IgE antibodies (12). Safety can also be improved by disrupting IgE-binding epitopes by heat denaturation (13), by chemical modifications to generate allergoids (14), using genetically engineered allergens with reduced IgE-binding capacity (2, 3), or using synthetic peptides that do not bind IgE but stimulate T-cell responses (4). However, reduced IgE-binding has often been followed by reduced immunogenicity of the allergens (15).

We have recently proposed a new concept for allergy vaccines based on targeting the MHC class II antigen presentation pathway (16). These so-called modular antigen transporter (MAT) recombinant allergens consist of an allergen fused to a TAT-derived translocation peptide and to the first 110 amino acids of the human invariant chain (Ii). The TAT peptide mediates cytoplasmic uptake of extracellular proteins (17, 18). Small molecules are believed to enter cells via electrostatic interactions in an energy-independent manner, whereas large molecules are taken up by energy-dependent macropinocytosis (19). Early in biosynthesis, MHC class II $\alpha\beta$ heterodimers assemble in the endoplasmic reticulum with an Ii trimer to form a nonameric complex (20). Ii binds to the MHC class II molecule and blocks the class II peptide-binding groove until the MHC II–Ii complexes are transported to the endosomes where Ii is removed by proteolysis, thus permitting loading of the groove with endosomal peptides (21). Fusing the allergen to Ii therefore directly links the allergen with the class II pathway of antigen presentation.

In this study, we used Fel d 1 as a model allergen and tested the influence of the above described modifications

on immune responses *in vivo*. To avoid potential differences in distribution and pharmacokinetics of the different proteins, the allergens were administered directly into the inguinal lymph nodes of mice (22).

Methods

Allergens

Cat-fur allergen extract was purchased from Stallergènes (Fresnes, France). rFel d 1 (19 kDa), TAT-Fel d 1 (21 kDa; with the HIV-TAT sequence GYGRKKRRQRRR) and MAT-Fel d 1 (34 kDa; with HIV-TAT plus amino acids 1–110 of the human invariant chain) were engineered on pQE30 expression vectors (Qiagen, Hilden, Germany) containing a N-terminal [His]₆-tag for protein purification and were produced in *Escherichia coli* as described (16).

Immunization protocols

CBA female mice (6–8 weeks; Harlan, Horst, The Netherlands) were immunized thrice with 2-week intervals by intralymphatic (i.l.) injection (22) with 30 pmol of allergen, 90 µg of Al(OH)₃ (Alhydrogel 3%; Brenntag Biosector, Fredrikssund, Denmark) and saline (10 µl) into the inguinal lymph node or by s.c. injection with 300 pmol of allergen, 450 µg Al(OH)₃ and saline (50 µl). Moreover, the MAT-Fel d 1 immunization regime was optimized by testing different doses and numbers of i.l. injections. Serum was prepared from tail vein blood and frozen at –20°C until analyzed.

The therapeutic potential of the allergens was tested in mice sensitized by 6 weekly intraperitoneal (i.p.) injections of 1 µg cat-fur allergen extract with 900 µg Al(OH)₃ in 100 µl. Subsequently, mice were desensitized with the different allergen molecules administered by i.l. injections as described above. For induction of anaphylaxis, sensitized mice were challenged with 20 µg of cat-fur allergen extract in saline (50 µl i.p.). Body temperature was measured with a calibrated digital thermometer before and 30 min after the challenge.

Safety was assessed by challenging cat allergen-sensitized mice with 5 µM of the different Fel d 1 allergens molecules in saline as described above. All animal experiments were approved by and performed according to the guidelines from the Swiss veterinary service authorities.

Antibody measurements

For antibody detection, 96-well microtiter plates (Nunc Maxisorb, Basel, Switzerland) were coated with 1 µg/ml cat-fur allergen extract in carbonate buffer and incubated overnight at 4°C. After blocking the plates with 2.5% nonfat dry milk in PBS-0.05% Tween-20 (PBSTM), serial dilutions of individual sera in PBSTM were added to the plates for 2 h. The plates were then incubated with biotinylated goat anti-mouse IgG1 or IgG2a (BD Pharmingen, San Diego, CA, USA) in PBSTM, followed by incubation with streptavidin-conjugated horseradish peroxidase (BD Pharmingen). Finally, the plates were added with the enzyme substrate 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) di-ammonium salt (Sigma-Aldrich, Buchs, Switzerland) in 1 M sodium dihydrogen phosphate and absorption read at 405 nm on a Model 550 Microplate reader (BioRad, Hercules, CA, USA). Unless otherwise specified, all incubations were performed at room temperature and intercepted with PBST washes.

For detection of total IgE antibodies, plates were coated with 2 µg/ml of anti-mouse IgE capture antibody (BD Pharmingen) at 4°C overnight. As secondary reagent to bound mouse serum, biotinylated anti-mouse IgE (BD Pharmingen) was used at 2 µg/ml.

Cytokine-secretion assay

Mice were immunized thrice by i.l. injection with 30 pmol of MAT-Fel d 1, TAT-Fel d 1 or rFel d 1, as described above. One week later, spleens were isolated and single-cell suspensions prepared. Erythrocytes were removed by lysis, and triplicates of 8×10^5 cells/well were cultured in round-bottom 96-well plates with 10 µg/ml LoTox Fel d 1 (Indoors Biotechnology, Charlottesville, VA, USA) or left unstimulated in 200 µl of supplemented IMDM. Supernatants were collected after 20 h for determination of interleukin-2 (IL-2) and after 72 h for determination of IL-4, IL-10, and interferon-γ (IFN-γ) using the DuoSet ELISA (R&D Systems, Abingdon, UK).

Allergenicity in human leukocytes

Freshly withdrawn blood from ten patients with allergy to cat dander was prepared for analysis by the cellular allergen stimulation test (CAST-2000 ELISA; Bühlmann Laboratories AG, Schönenbuch, Switzerland). Leukocytes were isolated by dextran sedimentation and incubated with IL-3 together with cat-fur allergen extract, rFel d 1 or MAT-Fel d 1 at different concentrations for 40 min. Samples were then frozen at –80°C until analyzed for quantification of the leukotrienes released in the supernatant.

This single center clinical study was performed between September and December 2005 with patients recruited from the allergy unit of the Zurich University Hospital. Inclusion criteria comprised a clear history of cat-dander allergy documented by quantitative Fel d 1-specific IgE ImmunoCAP determinations (Phadia, Uppsala Sweden) and positive skin prick test (wheal diameter ≥ 3 mm²). The study was approved by the local ethical review committee, performed according to Good Clinical Practice (GCP) guidelines and the Declaration of Helsinki, and independently monitored. All patients gave informed consent before entering the trial (<http://www.clinicaltrials.gov> Identifier: NCT00620880).

Statistics

To assess statistical variances, data was analyzed by nonparametric tests, i.e. two-tailed Mann–Whitney *U*-test or Kruskal–Wallis one-way analysis of variance with Dunn's multiple comparison test using shared *P*-values. The significance level was set at 95%.

Results

MAT allergens enhanced IgG2a antibody responses in mice

All three recombinant Fel d 1 allergens induced high levels of cat allergen-specific IgG1 antibodies (Fig. 1A), irrespective of the route of administration. The IgG1 levels were lower for the mice injected with MAT-Fel d 1 i.l. as compared with the other i.l. injected proteins ($P < 0.05$). The IgG2a responses (Fig. 1B) were significantly different among the three groups ($P < 0.05$), with MAT-Fel d 1 or TAT-Fel d 1 stimulating higher IgG2a levels than did rFel d 1. Moreover, the titers were higher upon i.l. than upon s.c. immunization for all the three proteins.

The ratio of IgG2a to IgG1 is a parameter for the relative strength of Th1–Th2 immune responses. All ratios were higher for i.l. than for s.c. injections, indicating a preferential Th1 polarization by the i.l. route (Fig. 1C). Immunization of mice with MAT-Fel d 1 induced signif-

icantly higher IgG2a-to-IgG1 ratios as compared with rFel d 1 ($P < 0.01$), both after s.c. and i.l. administration. All antibody responses were long-lasting, and the IgG2a-to-IgG1 ratios remained unchanged.

Vaccination with MAT-Fel d 1 allergen increased Th1 cytokine production

Upon re-stimulation of splenocytes, only cells from mice immunized with MAT-Fel d 1 secreted significant levels of IL-2 ($P < 0.05$) (Fig. 2A). Immunization with MAT-Fel d

1 also increased secretion of IFN- γ (Fig. 2B) as compared with TAT-Fel d 1 ($P < 0.05$) and rFel d 1. In contrast, IL-4 (Fig. 2C), and IL-10 (Fig. 2D) secretion was found to be stronger upon immunization with rFel d 1.

Immunotherapy with modified Fel d 1 enhanced protection against anaphylaxis

Based on the results above, MAT-Fel d 1 was chosen for further development for i.l. immunotherapy. First, to establish an optimal protocol for immunotherapy, mice were immunized with different doses and different numbers of i.l. injections with MAT-Fel d 1. Three i.l. immunizations with 0.1 μ g MAT-Fel d 1 were sufficient to induce both IgG1 and IgG2a antibody responses (Fig. 3A). The immune responses significantly increased for both subclasses at 1 μ g ($P < 0.05$), which represented an optimum, as no further increase of the response was observed at 10 μ g. At a dose of 1 μ g, optimal immunization regimen was achieved with three i.l. injections (Fig. 3B). One injection was inferior ($P < 0.05$), while four injections did not further improve the response, for which reason three injections of 1 μ g was chosen for the subsequent immunotherapy.

Mice sensitized to cat-fur allergen extract have high levels of serum IgE and IgG1, but no detectable IgG2a (Fig. 4A; time point zero). Upon desensitization with

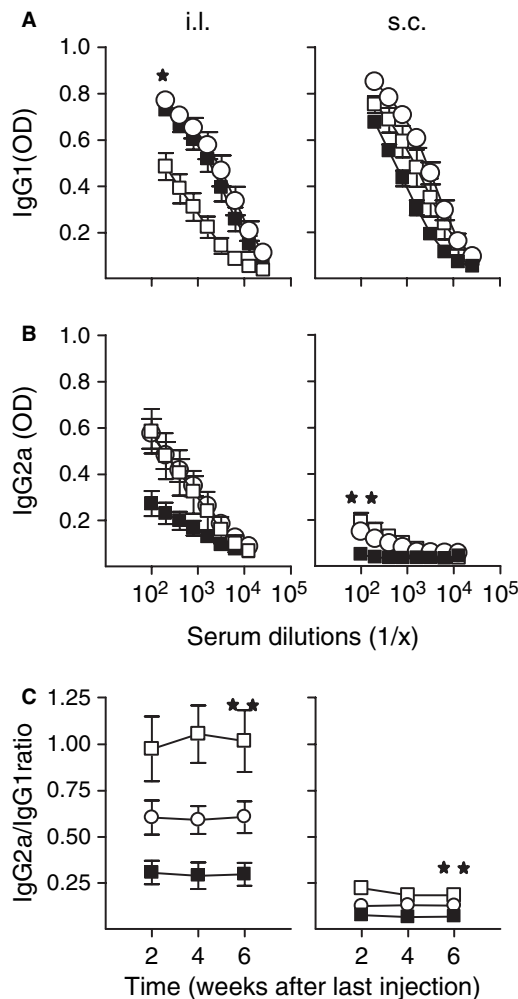


Figure 1. Cat allergen-specific serum antibodies of mice immunized thrice with 30 pmol intralymphatically (i.l.) or 300 pmol s.c. recombinant Fel d 1 (■) TAT-Fel d 1; (○) or MAT-Fel d 1; (□). Six weeks after the last injection sera were analyzed by ELISA for IgG1 (A) or IgG2a (B). Symbols represent means $\pm$ SEM ($n = 8$). The ratio of IgG2a to IgG1 (C) was calculated for the indicated time points at 1/400 serum dilution as a parameter for the Th1/Th2 balance. Statistical analyses were performed using the Kruskal–Wallis test with Dunn’s multiple comparison test (** $P < 0.01$; * $P < 0.05$), differences shown in the graphs are applied to rFel d 1 and MAT-Fel d 1.

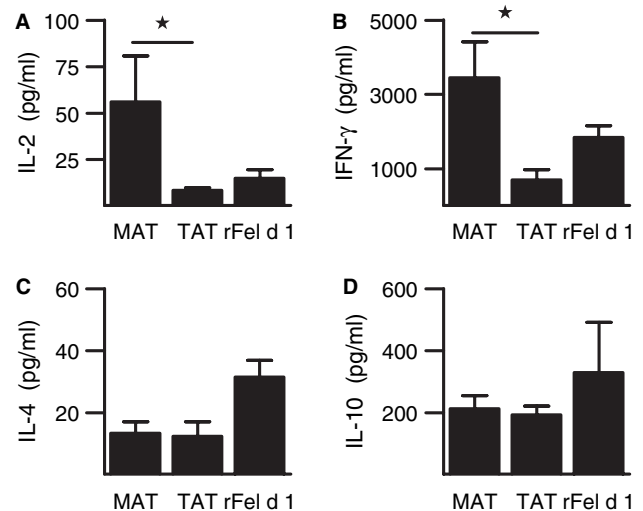


Figure 2. Cytokine secretion *in vitro*. Splenocytes from mice immunized with MAT-Fel d 1, TAT-Fel d 1 or rFel d 1 were analyzed for secretion of IL-2 (A), IFN- γ (B), IL-4 (C), and IL-10 (D) after *in vitro* re-stimulation with 10 μ g/ml LoTox Fel d 1. The results show the cytokine concentrations in supernatants after subtraction of spontaneous secretion from unstimulated splenocytes as determined by ELISA. Stimulation of cells from untreated mice did not induced detectable levels of the above-mentioned cytokines. Statistical differences are indicated (* $P < 0.05$ as analyzed by the Kruskal–Wallis test with Dunn’s multiple comparison test ($n = 4$)).

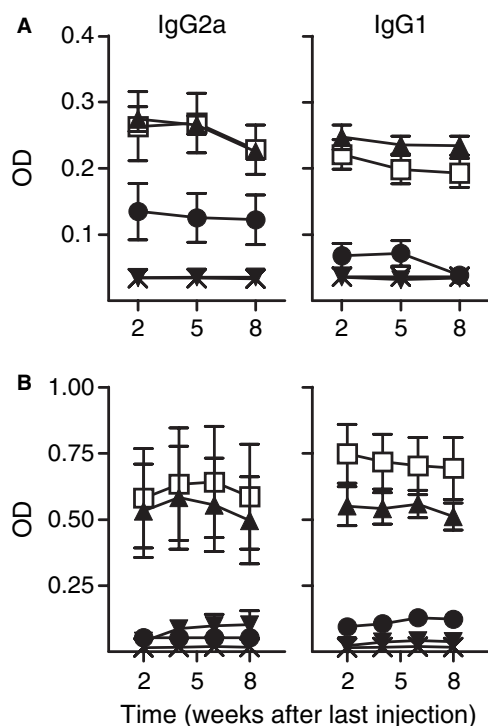


Figure 3. Cat allergen-specific IgG2a and IgG1 for the optimization of the immunization regime. (A) Mice were immunized thrice intralymphatically with 0 (x), 0.01 (▼), 0.1 (●), 1 (▲), or 10 µg (□) of MAT-Fel d 1. Sera were analyzed at 2, 5 and 8 weeks after the last injection. (B) Mice received from either no injections (x), or 1 (▼), 2 (●), 3 (▲), or 4 (□) intralymphatic injections of MAT-Fel d 1, respectively. Antibody levels were measured at 2, 4, 6 and 8 weeks after the last injection. For both experiments antibody levels are expressed as mean optical densities at 1/100 (A) or 1/400 (B) sera dilutions $\pm$ SEM ($n = 5$).

three i.l. injections of different allergens, MAT-Fel d 1 induced the highest IgG2a levels, followed by rFel d 1 and cat-fur extract. Four weeks after completed immunotherapy, mice were challenged with a high-dose of cat-fur allergen extract and subsequently monitored for anaphylaxis. Mice treated with MAT-Fel d 1 showed stronger protection against anaphylaxis than mice treated otherwise (Fig. 4B); however, it was only significantly different from the cat-fur allergen extract treated group ($P < 0.05$).

Modified allergens did not induce anaphylaxis in sensitized mice

To assess the safety of modified recombinant allergens in SIT, their unwanted potential to induce anaphylaxis in sensitized mice was measured. After the last of six sensitization injections with cat-fur allergen extract, all animals had comparable high levels of Fel d 1 specific IgG1 and IgE, whereas IgG2a was not detectable (not shown). Mice were then challenged with the different recombinant Fel d 1 allergens or with cat-fur allergen extract (Fig. 5). Three out of five mice challenged with

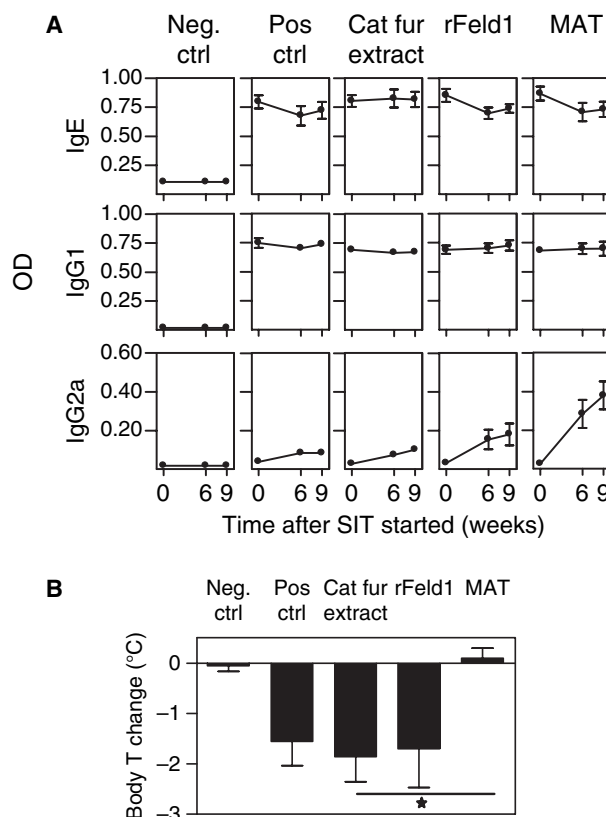


Figure 4. Murine anaphylaxis model. (A) Cat allergen-specific IgG1 and IgG2a antibodies and total IgE analyzed in sera from sensitized mice before SIT (time point 0), as well as 6 and 9 weeks after the first SIT injection. The negative control group was naive mice, and the positive control was a group of sensitized mice that did not receive SIT. (B) Four weeks after the last SIT injection, mice were challenged with 20 µg of cat-fur allergen extract i.p.. The body temperature drop was measured before and 30 min after the challenge. Data were analyzed by the Kruskal–Wallis test with Dunn’s multiple comparison test (* $P < 0.05$; $n = 5$).

rFel d 1 and five out of five challenged with cat-fur allergen extract experienced anaphylaxis while none of the mice challenged with MAT-Fel d 1 showed a notable drop in body temperature.

Reduced degranulation of human basophils after incubation with MAT-Fel d 1

Further evaluation of the potential safety of MAT-Fel d 1 in human immunotherapy was performed by testing the allergen-induced degranulation of human basophils from cat-fur allergic patients in CAST-ELISA. While cat-fur allergen extract or rFel d 1 caused strong basophil degranulation and leukotriene release at 0.05 nM, a 100-fold higher concentration of MAT-Fel d 1 allergen was required to induce comparable basophil degranulation (Fig. 6).

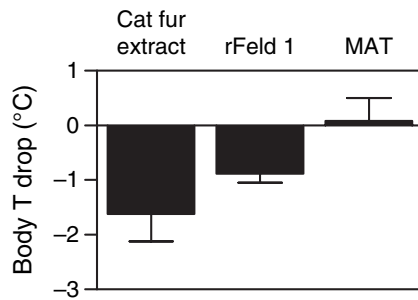


Figure 5. *In vivo* safety. Mice were sensitized with cat-fur allergen extract and 3 weeks after the last injections challenged with the pure extract or the different Fel d 1 proteins. Body temperature was measured before and 30 min after the challenge. Results are illustrated as means $\pm$ SEM ($n = 5$). MAT-Fel d 1 and rFel d 1 were compared by the Mann-Whitney *U*-test ($*P < 0.05$).

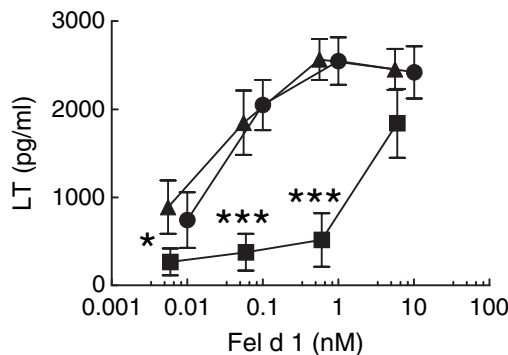


Figure 6. CAST-ELISA. Blood from 10 cat allergic patients was used for measuring the leukotrienes (LT) released after incubation with MAT-Fel d 1 (■), rFel d 1 (▲), or cat-fur allergen extract (○) at four different concentrations. Results are illustrated as means $\pm$ SEM and statistical comparison between MAT-Fel d 1 and rFel d 1 was performed using the Mann-Whitney *U*-tests ($*P < 0.05$, $***P < 0.001$).

Discussion

Although SIT offers medical advantages over symptomatic treatments of IgE-mediated allergies, merely 3–4% of allergic patients choose to undergo SIT, mainly because of the long treatment duration and the frequently associated allergic side-effects. For use in SIT it would therefore be highly desirable to have allergens that enhance efficacy and at the same time reduce allergic side-effects, i.e. to render SIT faster and safer. In the present study, different modified recombinant Fel d 1 allergens were tested for their therapeutic potential in SIT. The allergens were in part modified by fusing them to a protein translocation sequence to improve cellular uptake and a truncated invariant chain for targeting to the MHC class II pathway of antigen presentation (16).

Subcutaneous allergen-specific immunotherapy aims at regulating allergen-specific immune responses, i.e. it aims

to trigger Th1 T cells and neutralizing antibody responses. The presence of Th1 cytokines such as IFN- γ inhibits the production of IL-4 and thereby prevents the IL-4-mediated IgE-switch of B cells. IgE enhances the expression of high affinity IgE receptors (Fc ϵ RI) (23), hence, allergy is self-promoting. Our study revealed that mice immunized with MAT-Fel d 1 had higher levels of IFN- γ and lower levels of IL-4 than mice immunized with the unmodified Fel d 1 allergen. In line with this, immunotherapy of sensitized mice using MAT-Fel d 1 induced higher IgG2a levels and conferred better protection against a challenge with a high dose of the cat-fur allergen extract; enhanced production of Th1-assisted antibodies, IgG2a in mice and IgG1 and IgG4 antibodies in humans (24), have been reported after SIT (13).

In addition to being more efficient in stimulating Th1 immune responses, our study revealed that MAT-Fel d 1 also reduced basophil degranulation and leukotriene release by 100-fold when compared with unmodified Fel d 1 or the cat-fur allergen extract. This potentially increased safety profile of MAT-Fel d 1 was confirmed in sensitized mice that showed no anaphylaxis after a high-dose challenge with MAT-Fel d 1. This increased safety of the MAT-Fel d 1 can be explained by its reduced IgE-binding capacity (data not shown) and the more rapid cellular uptake of TAT-containing proteins (18, 25). These properties prevent the allergen from binding to Fc ϵ RI on mast cells and basophils. In conclusion, the results demonstrated that MAT-Fel d 1 not only increased SIT efficacy but also safety.

Over the last 10 years, several studies have reported enhanced presentation of antigens through the MHC class-II pathway by different strategies that involved the Ii (26–28). The so called Ii-Key peptides, consist of a short fragment of the human Ii (hIi aa 77–92 or aa 77–80) fused to an antigenic peptide (28). The Ii-Key binds to an allosteric site of the MHC-II molecules on the cell surface and the antigenic peptide binds to the epitope-binding groove, increasing the loading of MHC class II molecules and, therefore, enhancing the immunogenicity of the loaded antigen. The MAT allergens used in our study had a longer sequence of the hIi (aa 1–110). This targets the MHC class-II molecules to the endoplasmic reticulum (16) where it occupies the peptide binding groove, and thereby avoids interaction with other antigens. The complex formed by the MAT-allergen and the MHC class-II molecule is directed through the Golgi to the endo/lysosomal compartment where the Ii is degraded together with the fused allergen. Now, the allergenic peptides can be loaded to the MHC class-II molecules and be transported to the cell surface (29). As a result, MAT allergens increase the efficiency of allergen presentation, which furthermore result in an increase in the effective allergen dose available for stimulation of protective immune response. Indeed, high allergen doses suppress IgE production while low doses are supportive of its production (30), and human immunotherapy with

high allergen doses is more efficient than using lower allergen doses (31). While this in theory could be advantageously used for SIT, the risk of allergic adverse events limits the possibility of increasing the therapeutic allergen dose and explains the need for a controlled dose-escalation during the early phase of SIT. However, the safety profile as well as the cell-penetration and MHC-targeting properties of MAT allergens may allow SIT with high allergen doses, making such allergen highly attractive in human therapy.

This study, as well as other studies in mice (22) and humans (manuscript in preparation), also demonstrated that i.l. allergen administration may strongly improve SIT efficacy and allow lower allergen doses to be used, which again would improve safety as compared with s.c. SIT. Alternative routes of allergen administration, e.g. sublingual (SLIT), oral and intranasal applications (7–9) have been introduced as a means to improve SIT. However, as most proteins have short half-lives, most of such administered allergens are degraded before exerting their

immunotherapeutic effects. For this reason, i.l. injections, in which the allergen is administered directly into a s.c. lymph node, i.e. to the site of action on the immune system, represent an advantage over other administration routes.

In conclusion, our data demonstrated that the two main problems of current SIT, its relatively low efficiency requiring a high number of injections, as well as the risk of allergic reactions, could both be improved by using MAT allergens.

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