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**IgG4 but no IgG1 antibody production after intralymphatic immunotherapy
with recombinant MAT-Feld1 in human**

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Short title: IgG4-stimulating allergy vaccine

Abbreviations:

AIT = allergy immunotherapy

ILIT = intralymphatic immunotherapy

MAT = modular antigen transporter

Abstract

Allergy immunotherapy (AIT) mediates protection against allergen exposure in part due to allergen-specific antibodies. While immunization typically stimulated IgG1 and IgG2, AIT is often associated with production of IgG4. Here, twenty cat-dander-sensitized patients were randomized to receive three injections of intralymphatic immunotherapy (ILIT) with MAT-Feld1 adsorbed to aluminum hydroxide or just aluminum hydroxide (placebo) in a double-blind setting (ClinicalTrials.gov NCT00718679). Whereas the clinical data, showing benefit of Mat-Feld1 ILIT was published in 2012 (Senti et al., J Allergy Clin Immunol, vol 129(5):1290-1296), the current study investigated the cat-allergen-specific antibody responses. Blood was drawn prior to ILIT, as well as 1, 3 and 12 months after first ILIT. The sera were analyzed to characterize the all IgG subclasses and IgE antibody responses. ILIT with MAT-Feld1 elicited high levels of total IgG that were maintained for at least 12 months. Interestingly, a strong increase in IgG4 and some increase in IgG2 were observed throughout the study, while production of cat-specific IgG1 and IgG3 was not stimulated by MAT-Feld1 ILIT. The IgE levels remained constant.

Keywords: Cat allergy; Intralymphatic immunotherapy; Recombinant allergen; Antibody subclasses

Introduction

Allergen-specific immunotherapy (AIT) in patients with IgE-mediated allergies can produce immune tolerance to later allergen exposure. AIT stimulates a complex of B- and T-cell responses to cope with allergic symptoms: regulatory CD4-positive T cells (Tregs) with anti-inflammatory functions are induced; lymphocytes, monocytes, and dendritic cells secrete anti-inflammatory IL-10 and TGF- β cytokines; the balance of protective T-helper type 1 (Th1) to pathological Th2 cells shifts towards Th1; AIT decrease the production of pro-inflammatory mediators with reduced migration of mast cells within target organs.; antibodies in mucosa (IgA) or blood (IgG) can lead to symptom improvement by inhibiting binding of allergen to IgE on mast cells and basophils (1).

Classical subcutaneous AIT is typically followed by an increase in allergen-specific IgG and little change in IgE, which rather increases in the early phase of AIT and returns to baseline. IgG1 is the most abundant IgG subclass and typically ascribed an important protective and therapeutic role after vaccination and AIT. Consequently, IgG1 is the major subclass utilized in engineered therapeutic antibodies (2). IgG1 is predominantly responsible for immune response against proteins, activation of complement cascade, and binds to the Fc receptor of phagocytic cells. IgG2 is the dominant IgG response to bacterial polysaccharides (3, 4). IgG3 is primarily observed in response to viruses (5-8), and IgG4 is the response to parasitic helminths (9). In AIT, high serum concentrations of IgG4 are considered beneficial (2) and although its function has been subject for discussion there is growing evidence that IgG4 has a blocking function (1, 3, 4).

We have presented pre-clinical (10-12) and clinical data (13, 14) describing intralymphatic immunotherapy (ILIT) with a recombinant allergen Feld1 (15) fused to the 12mer HIV-derived translocation sequence (GYGRKKRRQRRR) and to a part of the human invariant chain (16). This

modular antigen transporter Feld1 (MAT-Feld1) is efficiently taken up by antigen-presenting cells (17). The clinical data demonstrated that three MAT-Feld1 ILITs increased tolerance to conjunctival, nasal, and skin provocations with cat dander allergen extracts. ILIT was also associated with increased specific IL-10 and IFN γ production in PBMCs restimulated *in vitro* with allergens. In the current study, allergen-specific IgG subclasses were analyzed in sera from different stages of ILIT with MAT-Feld1.

Methods

Study design

Patients with confirmed cat dander allergy were randomized to receive either MAT-Feld1 (n=12) or placebo (n=8) ILIT (13). MAT-Feld1 (1, 3 and 10 μ g) adsorbed to aluminum hydroxide was injected thrice with four weeks intervals into inguinal lymph nodes. The placebo was aluminum hydroxide and saline. All patients consented to the study (NCT00718679). Blood was collected before and 1, 3, and 12 months after ILIT for analysis of cat-specific antibodies.

Antibody analysis

Cat-dander specific IgE in sera was measured using ImmunoCAP (Phadia, Upsala, Sweden) Cat-specific IgG and IgG subclass antibody concentrations were determined by ELISA. Microtiter plates were coated with 1 IR/mL cat dander extract (Stallergènes, Fresnes, France), and serial dilutions of the sera were analyzed using biotinylated antibodies to human IgG and its subclasses (BD BioSciences, Basel, Switzerland). The plates were developed with streptavidin-conjugated horse radish peroxidase and TMB substrate (eBioScience, Vienna, Austria). The optical density at a given serum dilution was measured at 450 nm. To test if antibodies were stimulated to the MAT part of the antigen, comparable ELISAs were performed using plates coated with the irrelevant MAT-Betv1 or Betv1, birch allergen.

As a non-AIT control for serum antibodies, a similar protocol was used to measure tetanus-specific antibodies in the same sera. Therefore, plates were coated with 1 Lf/ml tetanus toxoid (Institute Merieux, Lyon, France). Detection and development was performed as mentioned before.

Statistical analysis

One-tailed, non-parametric, statistical Mann-Whitney or Kruskal-Wallis tests were applied to measure differences between allergy and placebo ILIT at different time points during the therapy. Kruskal-Wallis statistics were corrected using Dunn's multiple comparison tests. Changes in antibody levels over time within the allergen ILIT group were analyzed using two-way ANOVA with Bonferroni corrections for multiple comparisons. Correlations between IgG subclasses were measured by calculating the non-parametric two-tailed Spearman's rank correlation coefficient ρ . The significance level was set to 0.05.

Results

ILIT with MAT-Feld1 did not significantly boost specific IgE although 9 out of 12 patients (83%) showed a slight increase after 3 months as compared to baseline (**Fig. 1A**). The corresponding numbers after placebo ILIT were 3 out of 8 patients (37.5%). Cat-specific total IgG increased after MAT-Feld1 ILIT, but not after placebo ILIT (**Fig. 1B**, significance indicated by *). The increase was observed after a single ILIT (1 month; $p < 0.05$), and rose after further treatment (3 months; $p < 0.001$). For the follow-up measurement after one year, the average IgG levels in the MAT-Feld1 ILIT group were higher than baseline, but as only 13 of 20 patients return for follow-up analysis, the statistical power was reduced. When analyzing only data from patients included in the follow-up analysis, statistical differences between allergy ILIT and placebo ILIT were observed at all post-ILIT time points (**Fig. 1B**, significance indicated by #). No IgG changes over baseline were observed in sera from patients treated with placebo ILIT (**Fig. 1B**).

Contrasting total IgG responses, production of cat-specific IgG1 antibodies was not stimulated after MAT-Feld1 ILIT (**Fig 2A**). The data further revealed increased allergen-specific IgG2 but not IgG3 production after ILIT. Cat-specific IgG4 in sera strongly and rapidly increased after allergen ILIT (**Fig. 2A**; $p < 0.01$ compared placebo); no further IgG4 production was observed after one year. When analyzing only IgG4 data from patients included in the follow-up analysis, differences between allergen and placebo ILIT were observed for all time points post ILIT (data not shown). Finally, cat-specific total IgG and IgG2 or IgG and IgG4 (**Fig. 2B**) were positively correlated as calculated using two-tailed Spearman r correlation ($P < 0.01$).

To control for general immunological changes during the time period of the trial, IgG responses to tetanus toxoid were measured, assuming that the study participants had been immunized against tetanus without receiving tetanus boosters during the trial. No changes were observed in the levels of tetanus-specific IgG when comparing pre- and post-ILIT levels. Furthermore, there were no differences between the allergen-ILIT group and the placebo-ILIT group at any time point during the trial. Similarly, no differences were measured between placebo and allergen ILIT groups with regards to tetanus-specific IgG1 and IgG4 antibodies (**Fig. 2C**).

To test whether ILIT with MAT-Feld1 induced antibodies against the modular allergen transporter MAT, ELISA plates were coated with the irrelevant birch allergen Betv1 or with MAT-Betv1, both recombinant. The ratios of IgG antibodies against MAT-Betv1 to that of Betv1 were calculated, higher ratios after active but not after placebo ILIT suggesting an antibody response against the MAT carrier. The ratios increased neither in sera from patients with allergen nor placebo ILIT (**Fig. 2D**). Hence, ILIT with MAT-Feld1 did not stimulate antibodies against the carrier.

Discussion

IgG4 is typically observed in AIT. However, it is still debated if IgG4 is the most important subclass mediating the protection or tolerance or just an epiphenomenon of AIT (18-20). Indeed, high IgG4 levels were once associated with failure of immunotherapy (21). The result of the present study comprising ILIT with MAT-Feld1 is surprising, as the immunotherapy strongly stimulated production of IgG4 but not IgG1, the most abundant IgG subclass after protein immunization. This subclass polarization may be associated with the allergen modification or with the route of administration. With regard to the former, MAT-allergens have been tested in a few pre-clinical studies, but only in one clinical trial, for which reason it is difficult to speculate on its effect on the relative stimulation of IgG1 and IgG4. In mice, MAT-Feld1 was found to stimulate more IgG2a, but similarly high IgG1 titers as compared to non-modified Feld1 (22). With regards to the route, a few ILIT trials have been performed (14, 23) but more are underway (NCT 02269566, NCT02665754). In two trials with grass pollen extracts, IgG was not assessed or reported (14, 23). In three trials, only IgE and IgG4 were assessed. Hylander et al. reported an overall unchanged allergen-specific IgG4 levels besides a transient rise in allergen-specific serum IgE, but increased IgG4 levels in patients showing improved clinics (24). Senti and Witten reported increased IgG4 and unchanged IgE (25, 26). Unfortunately, in the only study that compared ILIT and SCIT (14), IgG4 or other IgG subclasses were not measured. It is therefore difficult to compare the magnitude of IgG subclass production induced after the two methods. However, based on data available it should be evident that both AIT and SCIT stimulate IgG4. With regard to IgG1 and other subclasses, further ILIT studies with serological analysis would be required as to detect any immune-stimulating differences in the different AIT methods. Nonetheless, it would be important in such studies also to demonstrate that sera from AIT-treated but not placebo-treated subjects inhibit mast cell and basophil degranulation in vivo and vitro, and that the sera inhibit CD23-mediated IgE-facilitated allergen binding to B cells. Likewise, it would be

important to test if any of the IgG subclasses preferentially mediate such inhibition, as this potentially could serve as a biomarker for successful AIT.

If IgG4 is mediating tolerance after ILIT, it may be acting as a blocking antibody preventing the allergen from causing degranulation of IgE-bearing mast cells and basophils. Since IgE-allergen ligation is also associated with activation of T-helper (Th)2 cells and IL-4 secretion, IgG4 may also have an indirect immune modulating effect (20). In addition, IgG4 has been reported to favor tolerance by stimulating IL-10 production in regulatory T cells (20) as well as in B cells (27). Indeed, increased allergen-specific IL-10 production was observed in both ILIT trials that measured IL-10 (25, 26).

However, if immunoglobulins are important mediators of allergen tolerance after AIT, and since the clinical data suggest that ILIT is effective, one may conclude that tolerance is mediated by IgG4, at least partially, due to the fact that it is the IgG subclass most prominently induced by ILIT, and since neither IgG1 nor IgG3, and only marginal levels of IgG2 were observed. Nonetheless, future ILIT studies with MAT-allergens or other recombinant allergens should look more carefully into antibody responses and if possible correlate these with the clinical outcome – as this may teach us more about the involved mechanisms of action in ILIT.

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Conflict of interest

T. M. Kündig is named as inventor on a patent on intralymphatic immunotherapy. The patent is owned by the University of Zurich and licensed to MannKind Corp., USA. The other authors declare no conflict of interest.

Contribution of authors

Performing experiments: M. Zehnder, G. Gafvelin

Writing paper: S. N. Freiburger, P. Johansen, H. Grönlund

Study design: P. Johansen, S. N. Freiburger, H. Grönlund, T. M. Kündig

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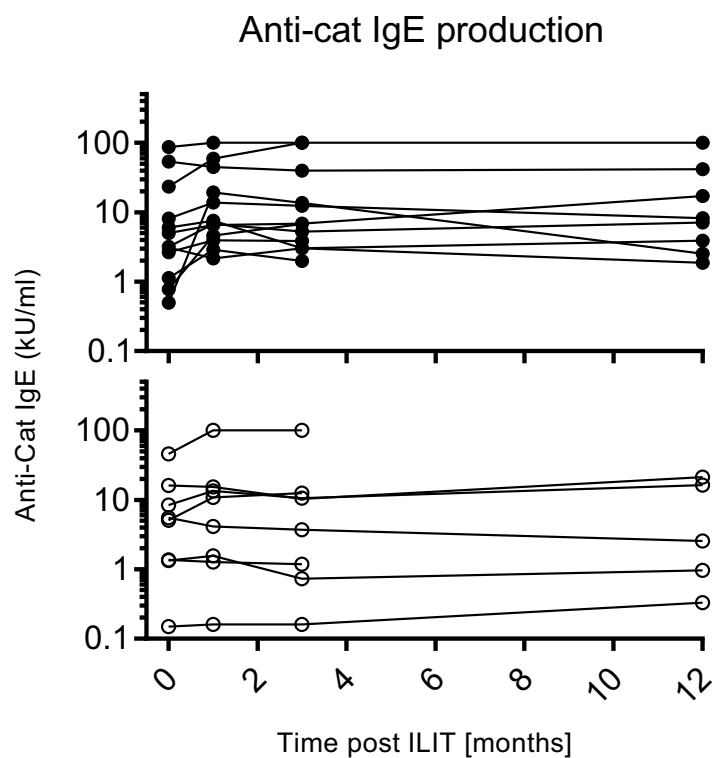
Figure legends

Figure 1. MAT-Feld1 ILIT induces cat-dander specific IgG, but not IgE. Study subjects were randomized to receive three ILITs with MAT-Feld1 (filled circles: n=12) or with placebo (open circles; n=8). Injections were given with four week intervals, i.e., on day 0 and on months 1 and 2. Blood sera were analyzed for cat-dander specific IgE (**A**) or IgG (**B**) before ILIT as well as on months 1, 3, and 12. Sera were diluted 1:800 for total IgG detection. Each line represents one patient. The dashed line indicates the specific antibody levels in non-sensitized control persons (mean of 4). Asterisks indicate statistical significances of differences in antibody levels between patients treated with MAT-Feld1 and placebo ILIT of all patients. *: p<0.05, **: p<0.01, ***: p<0.001; ****: p<0.0001. Hashes indicate statistical significances of differences in antibody levels between patients treated with MAT-Feld1

and placebo ILIT, only considering patients that came for follow-up after 12 months. #: $p < 0.05$, ##: $p < 0.01$, ###: $p < 0.001$; ####: $p < 0.0001$.

Figure 2. MAT-Feld1 ILIT induces cat-dander specific IgG4, but not IgG1. Study subjects were randomized to receive three ILITs with MAT-Feld1 (filled circles: $n=12$) or with placebo (open circles; $n=8$) as described in Figure 1. **A)** Blood sera were analyzed for cat-dander specific IgG1 (top left), IgG2 (top right), IgG3 (bottom left), IgG4 (bottom right). Sera were diluted 1:800 for IgG1 and IgG4 detection and 1:200 for IgG2 and IgG3 detection. The dashed line indicates the specific antibody levels in non-sensitized control persons (mean of 4). **B)** The correlation between cat-specific total IgG and IgG2 (left) and IgG4 (right) was calculated using Spearman's rank correlation coefficient (ρ). The p-value for the correlation is indicated. **C)** Blood sera were analyzed for anti-tetanus IgG (bottom left), IgG1 (top left), and IgG4 (top right). Sera were diluted 1:200 for IgG1 and IgG4 detection and 1:800 for detection of total IgG. **D)** Blood sera were analyzed for IgG antibodies against the irrelevant allergens Betv1 and MAT-Betv1. The ratio of IgG antibodies against Betv1 and MAT-Betv1 is shown. Each line represents one patient. Asterisks indicated statistical significances of differences in antibody levels between patients treated with MAT-Feld1 and placebo ILIT. *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$; ****: $p < 0.0001$.

A



B

