

ORIGINAL ARTICLE

EXPERIMENTAL ALLERGY AND IMMUNOLOGY

Immune regulation by intralymphatic immunotherapy with modular allergen translocation MAT vaccine

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Keywords

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Abstract

Background: Allergen-specific immunotherapy (SIT) faces problems related to side effects and limited efficacy. Direct administration of allergen extracts into lymph nodes induces increased specific IgG production and T-cell responses using significantly lower allergen doses.

Methods: In this study, mechanisms of immune regulation by MAT vaccines *in vitro* and in allergen-SIT of cat-allergic rhinitis patients, who received 3 inguinal intra-lymph node injections of MAT-Fel d 1 vaccine, were investigated in PBMC and cell cultures for specific T-cell proliferation, Fel d 1-tetramer-specific responses, and multiple immune regulatory molecules.

Results: MAT-Fel d 1 vaccine was efficiently internalized by antigen-presenting cells. This was followed by procaspase 1 cleavage to caspase 1 and secretion of IL-1 β , indicating inflammasome activation. Mat-Fel d 1 induced specific T-cell proliferation and an IL-10- and IFN- γ -dominated T-cell responses with decreased Th2 cytokines at 100 times lower doses than Fel d 1. Induction of immune tolerance by MAT-Fel d 1-ILIT involved multiple mechanisms of immune suppression. Early Fel d 1-specific T-cell activation was followed by full T-cell unresponsiveness to allergen after 1 year in the MAT-Fel d 1 group, characterized by increased allergen-specific T regulatory cells, decreased circulating Fel d 1 tetramer-positive cells, increased IL-10 and FOXP3 expression, and change in the HR2/HR1 ratio toward HR2.

Conclusions: This study demonstrates the induction of allergen tolerance after 3 intra-lymph node injections of MAT-Fel d 1 vaccine, mediated by increased cellular internalization of the allergen, activation of inflammasome, and generation of allergen-specific peripheral T-cell tolerance.

Abbreviations

APC, antigen-presenting cell; HR, histamine receptor; ILIT, intralymphatic immunotherapy; MAT, modular antigen translocation; MHC, major histocompatibility complex; PBMC, peripheral blood mononuclear cells; SIT, allergen-specific immunotherapy; TAT, trans-activator of transcription; T_{reg}, T regulatory.

Currently, allergen-specific immunotherapy (SIT) is performed with vaccines based on allergen extracts that can cause severe, often life-threatening, anaphylactic reactions as well as new IgE sensitization to other allergens present in the extract. Low patient adherence and high costs due to long duration (3–5 years) of treatment have been commonly reported (1, 2). Several strategies have been developed to

tackle these issues, and it became possible to produce recombinant allergen-SIT vaccines with reduced allergenic activity (3, 4). Recently, the application of intralymphatic injections in mice and humans demonstrated that direct administration of allergen into lymph nodes led to upregulation of specific IgG and T-cell responses using 100 times lower doses of allergens compared with classical, subcutaneous treatment (5, 6).

In allergic individuals, low amounts of allergens favor Th2 responses and the switch of B cells toward IgE production. In contrast, higher doses of allergens given by allergen-SIT or natural exposure such as professional beekeepers and cat owners favor the development of a T regulatory (T_{reg}) response and allergy-protective immune responses (7, 8). Based on these observations, efficient immune regulation due to increased cellular uptake and augmented antigen presentation through the major histocompatibility complex (MHC) class-II antigen presentation pathway that mimics high-dose allergen exposure was investigated as a strategy for allergen-SIT.

We developed a novel vaccine for the treatment of cat allergy, a common cause of rhinoconjunctivitis and asthma (9, 10). The major cat allergen, Fel d 1, is an 18-kDa heterodimer protein produced in cat saliva and sebaceous glands and is responsible for symptoms in more than 90% of cat-allergic individuals. As illustrated in Fig. S1, the vaccine consisted of three functional elements of the modular allergen translocation (MAT) technique, which was fused to form a single recombinant protein. First, a cell membrane translocation module, the trans-activator of transcription (TAT) peptide of human immunodeficiency virus, was used, which enables the vaccine to pass through the cell membrane and to accumulate inside antigen-presenting cells (APCs) (11). Second, an MHC class-II targeting module truncated invariant chain domain, which directs the processing of the MAT molecule within the APC and improves the presentation of the allergen and its components to the immune system via the MHC class-II pathway. Finally, the allergen, Fel d 1 in this case to which the patient is allergic, determines the specificity of the induced immune response. In a randomized double-blind placebo-controlled trial, MAT-Fel d 1 was injected into inguinal lymph nodes (ILIT) of cat-allergic patients (ClinicalTrials.gov, NCT00718679) (12). In skin prick tests and intradermal tests, 16- and 23-fold higher concentrations of MAT-Fel d 1 were tolerated, respectively, in the absence of adverse effects. Nasal tolerance to allergen was observed in parallel to increased cat dander-specific IgG4 (12). In this study, we evaluated mechanisms of immune regulation by MAT vaccines *in vitro* and in allergen-SIT of cat-allergic rhinitis patients, who received 3 inguinal intra-lymph node injections of MAT-Fel d 1 vaccine. The data demonstrate that early T-cell activation was followed by allergen-specific peripheral T-cell tolerance one year later induced by multiple mechanisms that suggest the *in vivo* generation of allergen-specific T_{reg} cells.

Methods

Methods are described in the online Supporting Information (Data S1).

Results

MAT vaccines induce increased cellular internalization and inflammasome activation leading to T-cell stimulation in low doses

Because of the novel structure and constituents of the MAT vaccine, we first investigated the internalization and antigen presentation capacities *in vitro*. Significantly higher internalization of MAT-Fel d 1 into the cells and formation of intracellular particles up to 1 μ M diameter was observed (Fig. 1a–c). MAT-Fel d 1 was most efficiently internalized by monocytes. In addition, T cells, B cells, and NK cells internalized MAT-Fel d 1, whereas rFel d 1 was not internalized by nonphagocytic cells. rFel d 1 was internalized by monocytes with poor efficiency (Fig. 1c). After internalization, MAT-Fel d 1 showed visible cellular aggregates (Fig. 1d). MAT-Fel d 1 induced increased procaspase 1 cleavage to caspase 1 and very high amounts of rapid IL-1 β production compared with Fel d 1, suggesting inflammasome activation (Fig. 1e,f).

After the development of various different MAT vaccine prototypes with different allergens, we then investigated their effects on antigen-specific T-cell responses. PBMC of house dust mite or cat-allergic individuals and nonallergic controls were stimulated with MAT-Der p 1 and MAT-Fel d 1 vaccines. T-cell proliferation and cytokine production were investigated. MAT vaccines induced relatively strong T-cell proliferation particularly at low allergen doses (1 and 10 nM). MAT-Fel d 1 induced a high IFN- γ - and IL-10-dominant cytokine profile with 10 to 100 times lower allergen doses in allergic individuals. In contrast, IL-5- and IL-13-dominant Th2 profile was observed in response to allergens without MAT (Fig. 2).

MAT-Fel d 1-ILIT induces Fel d 1-specific T-cell tolerance in humans

Immunological effects of MAT-Fel d 1 were investigated in a single-center, double-blind, placebo-controlled, randomized, two-arm phase I/IIa clinical trial (12). A total of 12 patients received three injections of alum-adsorbed MAT-Fel d 1 directly into a superficial subcutaneous lymph node in the groin area. The injections were given at 4-week intervals. The initial MAT-Fel d 1 dose was 1 μ g, increasing to 3 μ g for the second, and 10 μ g for the third injection. Eight patients received three intra-lymph node injections of an alum suspension as placebo. We first analyzed allergen-specific T-cell proliferation in response to rFel d 1. Both the MAT-Fel d 1 and the placebo group showed a significant dose response with approximately six- to eightfold increased proliferation compared with unstimulated cells in 1.2 μ M doses. Nine weeks after the initiation of therapy, which is one week after the last injection, the MAT-Fel d 1 group showed increased proliferation in response to 0.3 and 0.6 μ M Fel d 1, suggesting an allergen-specific T-cell activation (Fig. 3). Interestingly, after one year, the MAT-Fel d 1 group showed a full peripheral T-cell unresponsiveness in allergen-stimulated T-cell proliferation, a typical finding for

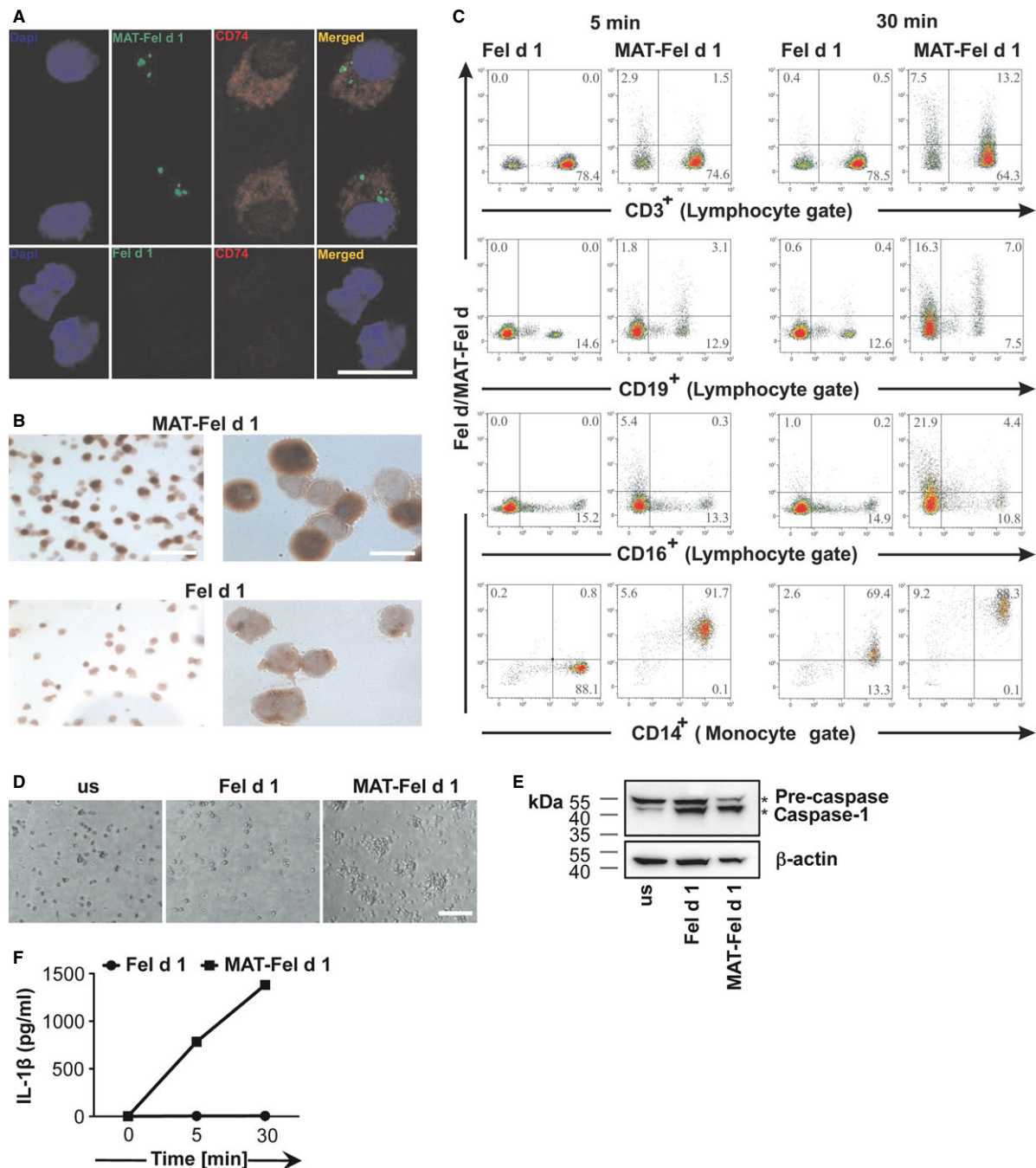


Figure 1 Increased rapid internalization of MAT-Fel d 1; (A) MAT vaccines aggregate inside the cell after internalization. PBMC were incubated with Alexa 488-labeled rFel d 1 and Mat-Fel d 1 for 30 min and counterstained with anti-CD74 mAb and analyzed by confocal microscope. Scale bar = 10 μ m; (B) PBMC were incubated with rFel d 1 and Mat-Fel d 1 for 30 minutes, and cells were cytopspinned and stained with anti-6xHis mAb by immunohistochemistry. Scale bars: left = 60 μ m, right = 10 μ m. (C) PBMC were incubated with Alexa 488-labeled rFel d 1 and MAT-Fel d 1 for 5 and 30 min at 37°C and counterstained with anti-CD3, anti-CD14,

anti-CD16, and anti-CD19 mAbs and analyzed by flow cytometry. One representative of three independent experiments is shown. (D) MAT-Fel d 1-stimulated T cells show significant cellular aggregates within 30 minutes. Scale bar = 100 μ m; (E) precaspase and caspase-1 protein expression was assessed in THP-1 cells after stimulation for 120 min with Fel d 1 or MAT-Fel d 1. (F) PBMC were incubated with Fel d 1 and MAT-Fel d 1, and secreted IL-1 β was measured in supernatants after 8 h. The dose of Fel d 1 and MAT-Fel d 1 was 0.6 μ M for all experiments.

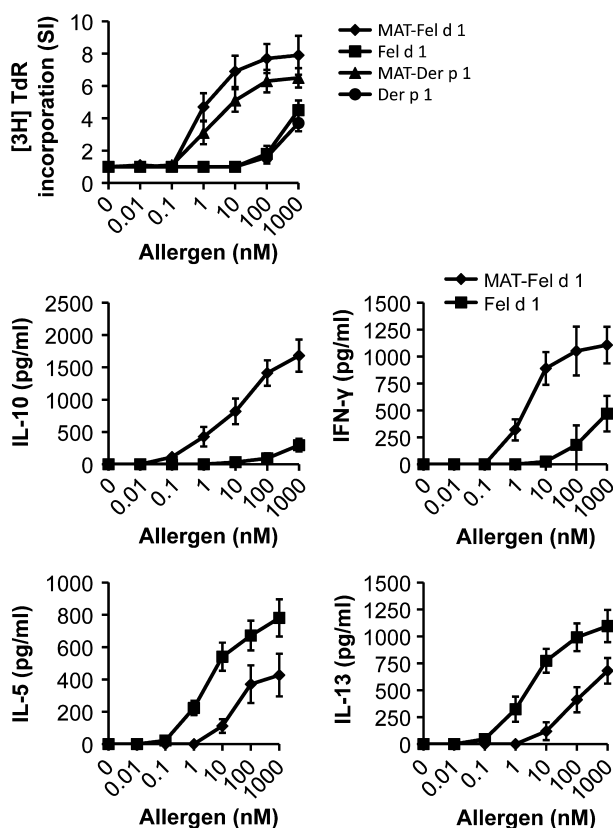


Figure 2 MAT vaccines induce increased specific T-cell proliferation, IFN- γ , and IL-10 production by PBMC. Dose-response comparison of rFel d 1 and MAT-Fel d 1 or MAT-Der p 1 on PBMC proliferation, and production of IFN- γ , IL-5, IL-10, IL-13 in house dust mite or cat-allergic individuals. Cumulative data from 6 allergic patients are shown.

antigen-specific peripheral T-cell tolerance (Fig. 3). The placebo group showed a normal T-cell proliferation response to rFel d 1 in all three blood samples without any difference

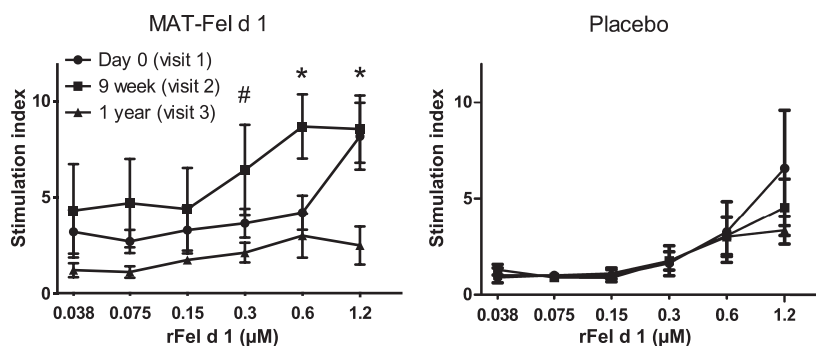


Figure 3 MAT-Fel d 1-ILIT induces full T-cell unresponsiveness after 1 year. PBMC proliferation in response to different doses of Fel d 1 was analyzed at 3 different time points: before immunotherapy, 9 weeks and 1 year, and after the beginning of immunotherapy in patients treated with MAT-Fel d 1 and placebo groups.

between the three time points (Fig. 3). There was no significant difference between the groups in response to purified protein derivative of *M. bovis* stimulation used as a control antigen (Fig. S2).

Next, we investigated antigen-induced cytokine profiles. We analyzed IL-4, IL-5, and IL-13 as Th2 cytokines, IFN- γ as a Th1 cytokine, IL-10 as a Tr1 cytokine, IL-17 as a Th17 cytokine, and TNF- α as an innate immune response and inflammation representing cytokine (Fig. S3). Within all cytokine comparisons between MAT-Fel d 1 versus placebo treatment, only IL-10 showed a significant increase 9 weeks after the beginning of the MAT-Fel d 1 group. As can be seen from very high standard errors, some patients showed a strong response for certain cytokines such as IL-5, IL-13, IFN- γ , and TNF- α in the MAT-Fel d 1 group after one year; however, the differences were not statistically significant.

Induction of immune tolerance by MAT-Fel d 1-ILIT involves multiple mechanisms of immune suppression

To further elucidate cellular and molecular mechanism of allergen-specific T-cell tolerance, changes in the frequency of circulating Fel d 1-specific T cells were investigated. Fel d 1-specific T cells were identified in peripheral blood mononuclear cells (PBMC) using MHC class-II peptide tetramers. The frequencies of Fel d 1-tetramer-specific T cells before SIT were in the range of 34–302 cells per 10^5 CD3⁺CD4⁺ T cells without any difference between the two groups. Their frequency showed a significant decrease after 9 weeks in the MAT-Fel d 1-treated group, but not in placebo-treated patients (Fig. 4). Unfortunately, only 7 of the patients had MHC class-II expression suitable for the available Fel d 1 tetramers.

After observing decreased numbers of circulating Fel d 1-tetramer-specific T cells in the MAT-Fel d 1-treated group, we investigated possible molecular mechanisms that may play a role. Active suppression/regulation and/or migration to central lymphoid organs were the two hypotheses that were investigated. The percentage of FOXP3-expressing CD4⁺

Proliferation was measured by ³H-thymidine incorporation after 5 days. Data are presented as means and standard errors (SE). #: $P < 0.05$ before immunotherapy compared with 1 year after immunotherapy. *: $P < 0.05$ 9 weeks compared with 1 year after immunotherapy.

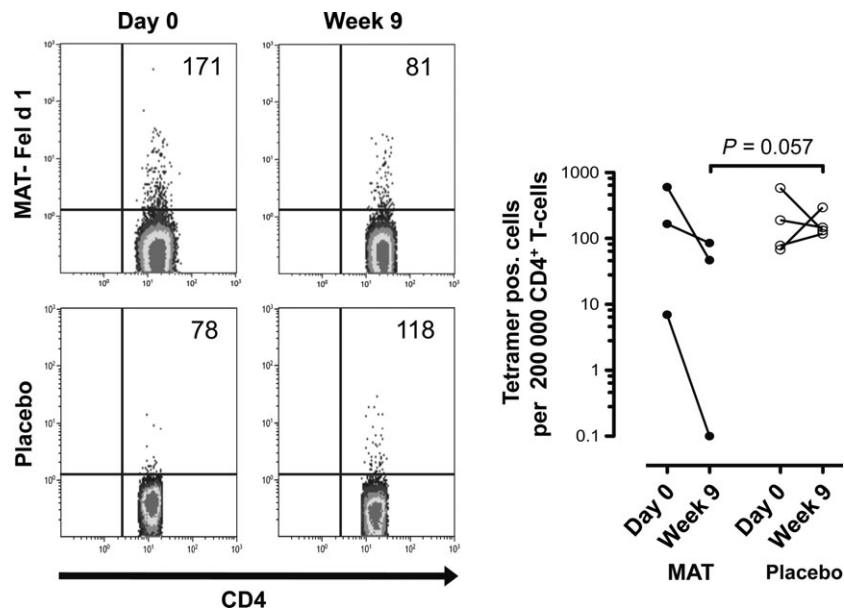


Figure 4 Fel d 1-specific CD3⁺CD4⁺ T cells determined using MHC class-II peptide tetramers. PBMC from MAT-Fel d 1-treated patients ($n = 3$) and placebo individuals ($n = 4$) expressing one of the following MHC class-II molecules: HLA-DRB1* 1104, DRB1*0701, and DRB1*1501 were incubated with PE-labeled MHC class-II, Fel d 1 peptide tetramer or corresponding CLIP and

stained with panel of antibodies according to staining protocol. Left: representative flow cytometric analysis showing the number of tetramer-positive cells per 200 000 CD3⁺CD4⁺ T cells. Right: frequency of tetramer-positive T cells per 200 000 CD3⁺CD4⁺ T cells. CLIP-stained cells were subtracted from tetramer-positive cells.

cells was in the range of 2.08–3.72%, and there was no difference in the MAT-Fel d 1 group compared with placebo before and after 9 weeks (Fig. 5a). CCR7 is expressed on a subpopulation of CD4⁺ cells, which are migrating to central lymphatic organs (13). Because there was a decrease in circulating Fel d 1-tetramer-specific T cells after 9 weeks, we analyzed whether CCR7 expression in these cells showed any change. Interestingly, in all analyzed individuals, the FOXP3-expressing CD3⁺CD4⁺CCR7⁺ T-cell population showed an increase after 9 weeks of MAT-Fel d 1-ILIT, while there were no changes in the placebo group (Fig. 5b). An increase in FOXP3 expression was observed in CCR7⁺ cells, as well as a tendency on Th2 cells toward a shift into T_{reg} cells by increasing FOXP3 expression after 9 weeks of MAT-Fel d 1 treatment (Fig. 5c). CRTh2, the prostaglandin D2 receptor, has been suggested to be particularly expressed on Th2 cells (14). There was no change in CRTh2 and CCR7 expression in the whole CD3⁺CD4⁺ population (Supplementary Fig. S4). Because of very low frequency of tetramer-positive Fel d 1-specific T cells, it was not possible to demonstrate the co-expression of FOXP3, CRTh2, and CCR7 on tetramer-positive cells. It was of great interest to investigate the changes in FOXP3 allergen-specific T cells after one year of MAT-Fel d 1-ILIT, because T-cell proliferation data showed the induction of full allergen-specific T-cell unresponsiveness in MAT-Fel d 1 group. As a possible molecular mechanism, FOXP3 mRNA showed a significant increase after one year in MAT-Fel d 1 group, without any change in the placebo group (Fig. 5d).

Histamine receptors (HRs) show distinct functions in T cells. Whereas HR1 is related to T-cell proliferation and IFN- γ production, HR2 plays a role in allergen tolerance in high-dose allergen-exposed individuals, such as beekeepers (7). We observed a tendency in the MAT-Fel d 1-ILIT toward decreased HR1 expression compared with placebo group (Fig. 6). There was also a tendency for increased HR2 in MAT-Fel d 1 group nine weeks and one year after therapy compared with the placebo group. The HR2/HR1 ratio showed a significant increase after 1 year compared with before treatment and placebo group in MAT-Fel d 1-treated group. There was no difference in HR4 expression between the two groups. In conclusion, after high-dose antigen exposure in the MAT-Fel d 1 group, T cells showed significantly higher FOXP3 expression and a switch toward HR2/HR1 dominance after one year when a complete Fel d 1-specific T-cell tolerance was established.

Discussion

The present study demonstrates immunological mechanisms of allergen-specific peripheral tolerance induction by MAT-Fel d 1 vaccine injected into lymph nodes of allergic patients at a 1000 times lower total allergen dose compared with conventional SIT. We analyzed the MAT-Fel d 1-induced immune response in a double-blind, placebo-controlled study in humans. Development of specific immune responses requires antigen presentation to CD4⁺ T cells through APC-displayed MHC-II peptide complexes and interaction with

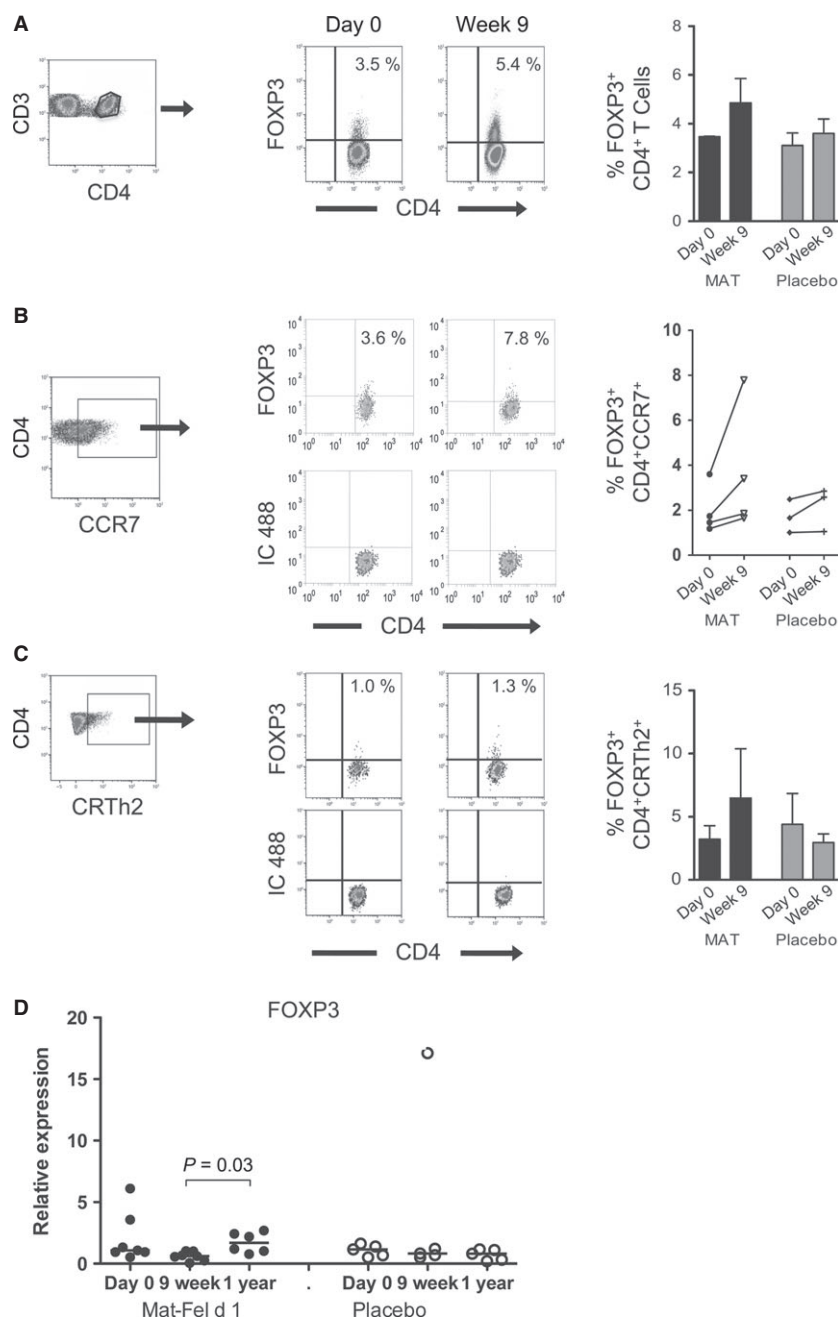


Figure 5 Frequency and mRNA expression of FOXP3⁺ T cells before and after ILIT. PBMC from MAT-Fel d 1-treated and placebo individuals (day 0 and week 9) were stained with a panel of antibodies and analyzed by flow cytometry. (A) Frequency of FOXP3⁺CD4⁺ T

cells, (B) a trend for increased frequency of FOXP3⁺CCR7⁺CD4⁺T cells, (C) frequency of FOXP3⁺ T cells among CRTh2⁺CD4⁺ T cells, (D) FOXP3 mRNA expression before, after 9 weeks, and 1 year from the start of ILIT. Data presented as mean $\pm$ SEM.

the T-cell receptor (15). The present study demonstrates that the soluble MAT vaccine forms particles inside the cells after internalization. The observed caspase 1 activation and rapid IL-1 β production following internalization of MAT-Fel d 1 suggest inflammasome activation. The maturation of IL-1 β is regulated by inflammasomes and particularly by NLRP3 (16, 17).

Modular antigen translocation vaccines contain an invariant chain domain that may be responsible for intracellular aggregation. Intracellular MHC class-II molecules bind to the invariant chain, which ensures proper folding of the MHC-II complex and directs the invariant chain-protected complexes to endosomes/lysosomes, where peptide loading takes place (18). The invariant chain prevents binding of antigenic pep-

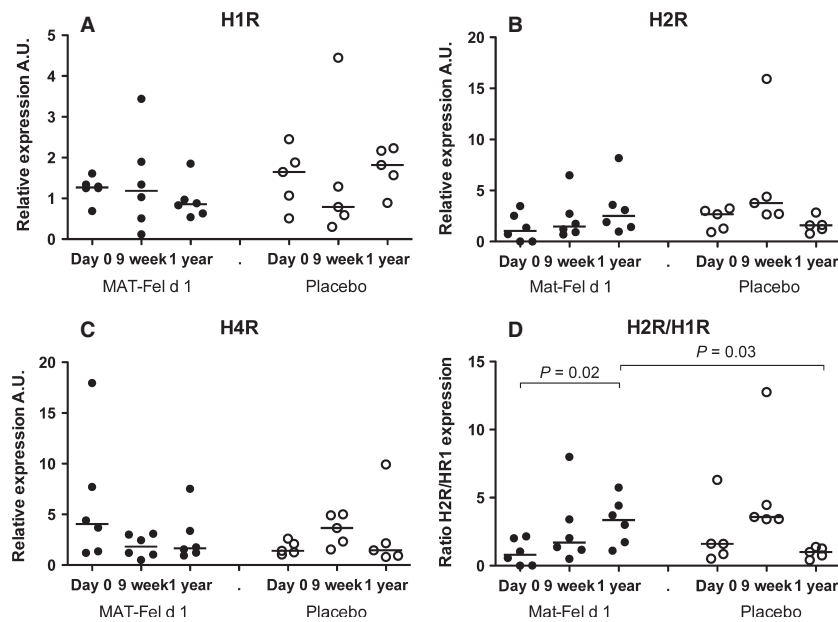


Figure 6 MAT-Fel d 1-ILIT regulates the expression of histamine receptors on T cells. PBMC isolated from MAT-Fel d 1- and placebo-treated individuals were stimulated with rFel d 1, expanded with IL-2 between day 5 and day 13, and re-stimulated with anti-

CD2/CD3/CD28 mAbs for mRNA expression and analyzed by qPCR. (A) H1R mRNA expression, (B) H2R mRNA expression, (C) H4R mRNA expression, (D) H2R/H1R ratio.

tides, which are present in the endoplasmic reticulum, to MHC-II molecules by blocking of the peptide-binding groove (19). A MAT-allergen construct is rapidly translocated into cytoplasm of APCs and induces allergen hyperpresentation as is demonstrated in the present study. These features enable to significantly decrease the allergen dose needed for an efficient SIT (20). The total allergen dose given in the present study was 14 μ g, whereas in a conventional subcutaneous SIT, it exceeds tens of mgs.

Due to above-mentioned mechanisms for efficient internalization and direct intra-lymph node injection, MAT-Fel d 1-ILIT can be considered as a high-dose immunotherapy. Two high-dose allergen exposure models leading to peripheral tolerance have been studied in humans: immune responses to bee venom allergens in beekeepers and immune responses to cat allergens in cat owners (21, 22). In both models, IL-10-secreting Tr1 cells specific for these allergens consistently represent the dominant subset. Tr1 cells use multiple suppressive mechanisms, particularly IL-10 and HR2. Both healthy and allergic individuals exhibit all three (i.e., Th1, Th2, and T_{reg}) allergen-specific T-cell subsets (23). The balance between these subsets is decisive for the outcome of the immune response, leading to the development of allergy or treatment effect.

Subsets of T_{reg} cells with distinct phenotypes and mechanisms of action include the CD4⁺CD25⁺FOXP3⁺ T_{reg} cells and the CD4⁺ IL-10-producing Tr1 cells. As observed in the present study, the numbers of circulating allergen-specific CD4⁺CD25^{high}FOXP3⁺ T_{reg} cells did not show a major difference between nonatopic and atopic individuals (24). How-

ever, numerous studies have reported that a functional defect in allergen-specific Treg cells is corrected by allergen-SIT (25). CD4⁺CD25⁺ T_{reg} cells from atopic donors have a reduced capability to suppress the proliferation of CD4⁺CD25⁻ T cells (26). The presence of local FOXP3⁺CD25⁺CD3⁺ cells in the nasal mucosa, their increased numbers after immunotherapy, and their association with clinical efficacy and suppression of seasonal allergic inflammation strengthen the concept of allergen tolerance based on T_{reg} cells in human subjects (27). FOXP3 expression shows a negative correlation with IgE level, eosinophilia, and the frequency of FOXP3⁺CD4⁺ T cells is significantly reduced in asthma and atopic dermatitis patients (28). CD4⁺CD25⁺ T_{reg} cells have been associated with spontaneous remission of cow's milk allergy (29).

In the present study, the T-cell proliferation response to Fel d 1 showed discrete early and late changes one week and 10 months after the completion of MAT-Fel d 1-ILIT. An early increase in proliferation was followed by full T-cell unresponsiveness to the allergen after 1 year, suggesting the establishment of long-lasting T-cell tolerance, because these individuals did not receive any vaccine during the 10 months after the 8th week (third injection) of the study. After 1 year, the full tolerance observed in MAT-Fel d 1 group looked similar to placebo. When we compare the data between the two groups after one year, MAT-Fel d 1 group shows more tolerance, but it was not significant. This can be also due to the placebo effect or avoidance of cat exposure during the 1-year period. Peripheral T-cell tolerance after 1 year was associated with increased FOXP3 expression and decreased

HR1 and increased the HR2/HR1 ratio in Fel d 1-specific T cells (30). HR2 plays an important role in allergen tolerance by suppression of T cells and dendritic cells (3, 7, 31). The decrease of HR1 expression in allergen-specific T cells may also play a role in allergen tolerance, because it was demonstrated that blocking of this receptor with H1-antihistamines induces a significantly decreased cell proliferation and increases IL-10 (32).

In the MAT-Fel d 1-ILIT, the expression of the lymph node homing receptor CCR7 was increased in $CD3^+CD4^+FOXP3^+$ T_{reg} cells, indicating recirculation of MAT-Fel d 1-activated T_{reg} cells in secondary lymphoid organs. Although tetramer stainings could be performed only in a limited number of individuals, the increase in $CCR7^+FOXP3^+$ T_{reg} cells correlated with decreased tetramer-positive cells in the circulation. Induction of peripheral antigen-specific T_{reg} cells critically depends on their capacity to enter secondary lymphoid tissues, where T_{reg} cells can exert their suppressive function *in vivo* (33). Similar to our findings, enhanced CCR7 expression was observed in $CD3^+CD4^+FOXP3^+$ T cells during the early course of wasp venom IT (34). It can be hypothesized that $CCR7^+$ central memory T cells, which have already encountered antigen, mainly reside in T-cell zones of lymph nodes to proliferate on re-encountering antigen, whereas the MAT-Fel d 1-mediated induction of $CCR7^+FOXP3^+$ T_{reg} cells might indicate homing of T_{reg} cells in secondary lymphoid organs to suppress allergen-specific T-cell effector responses and clonal expansion of Fel d 1-specific T cells.

As a general marker of circulating Th2 cells, CRTh2, the prostaglandin D2 receptor, is increased on T cells and eosinophils in patients with allergic asthma, and there is accumulation of CRTh2⁺ lymphocytes in human allergic nasal mucosa (14). The expression of CRTh2 did not change in the whole $CD3^+CD4^+$ T cells as well as the $FOXP3^+$ T cells, which is probably due to a very small fraction of the Th2 cells are specific for Fel d 1.

In conclusion, the present study demonstrates the induction of at least one-year-lasting efficient allergen tolerance established with 3 intra-lymph node injections using multiple mechanisms that can be summarized as increased cellular internalization of the allergen, activation of inflammasome, and generation of allergen-specific peripheral T-cell tolerance. MAT vaccines may open a new future for the curative treatment of allergic diseases.

Author contributions

Mübecel Akdis made substantial contributions to the development of the concept and design, acquisition, analysis, and interpretation of data, as well as drafting of the article. Cezmi A. Akdis contributed to interpretation and discussion of the data and drafting article. Horst Rose, Gabriela Senti, Thomas M. Kündig made contributions to design the clinical trial, selection and care of the patients, preparation of patient material. Marek Jutel contributed to the analyses, interpretation, and discussion of the data. William W. Kwok devel-

oped and provided Fel d 1-specific tetramers. Claudio Rhyner, Reto Cramerli cloned and produced MAT-Fel d 1 molecule that is used in the clinical trial and *in vitro* experiments. Thomas Eiwegger, Özge Soyer, Michael B. Soyka, Stefan Söllner, Oscar Palomares, and Willem van de Veen contributed to cell preparation, FACS analyses, and tetramer staining and data analysis, statistical analysis, and preparation of the graphs and data to be presented. Michael Soyka performed MAT and Feld1 internalization experiments. Cemalettin Bekpen, Duygu Demiröz, Angela Treis performed experiments related to the inflammasome activation by MAT-Fel d 1. Anna Zaleska performed antigen-specific stimulation experiments, FACS stainings and HLA typing, tetramer staining, and data preparation. She also contributed to the writing of the manuscript. All authors contributed to revising the article critically and approved the publication of the article.

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Conflicts of interest

Dr. M. Akdis reports grants from Swiss National Science foundation, grants from EU projects, during the conduct of the study. Dr. Rhyner reports grants from Swiss Government, during the conduct of the study. Dr. Soyka reports grants from Müller-Gierok Foundation, during the conduct of the study; he is part of the medical Advisory Board of MEDA Pharma. In addition, Dr. Kündig has a patent for 'Intralymphatic immunotherapy licensed to MannKind Corp., USA'. Dr. C. A. Akdis is consultant for the Actellion, Aventis, Stallergenes, Allergopharma, Circacia, Anergis, and he reports research grants from Swiss National Science foundation, grants from EU projects, CK-Care, Swiss-Polish research cooperation, and Novartis. The other authors declare no conflict of interest.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Schematic figure of the structure of the MAT-Fel d 1 vaccine.

Figure S2. MAT-Fel d 1-ILIT does not affect proliferative response to PPD of *M. bovis*.

Figure S3. MAT-Fel d 1-ILIT and cytokine production.

Figure S4. No changes in $CD3^+CD4^+CCR7^+$ or CRTh2⁺ T cells by Mat-Fel d 1-SIT.

Figure S5. Flow cytometry gating strategy.

Data S1. Methods.

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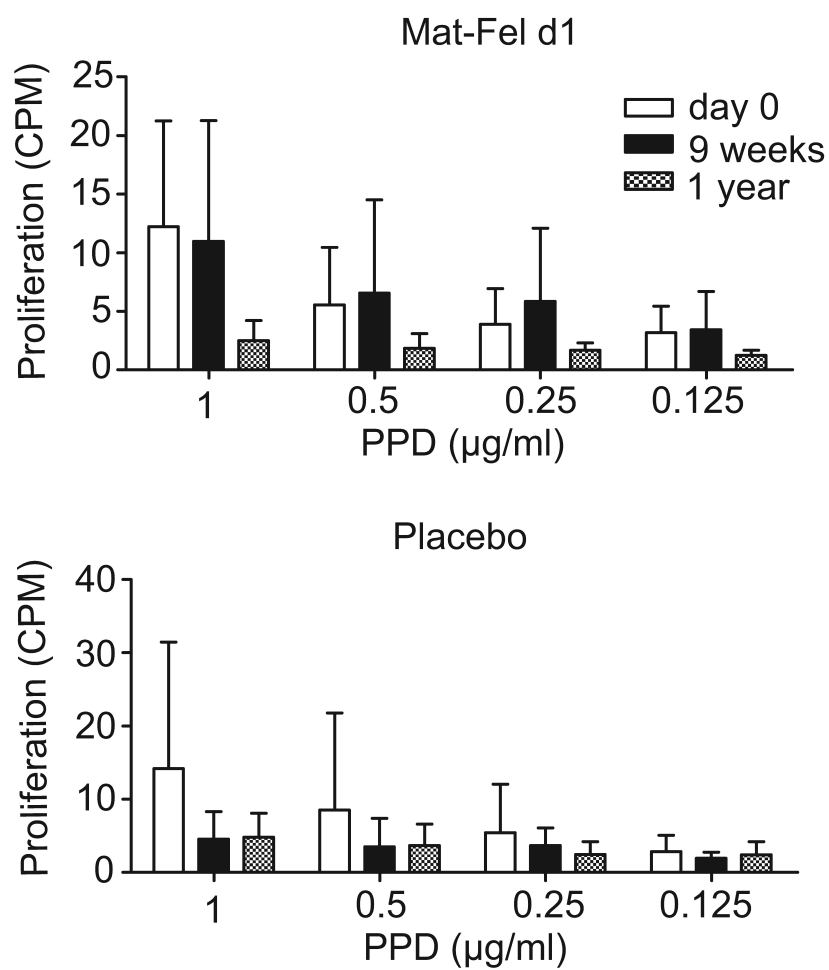


Figure S2
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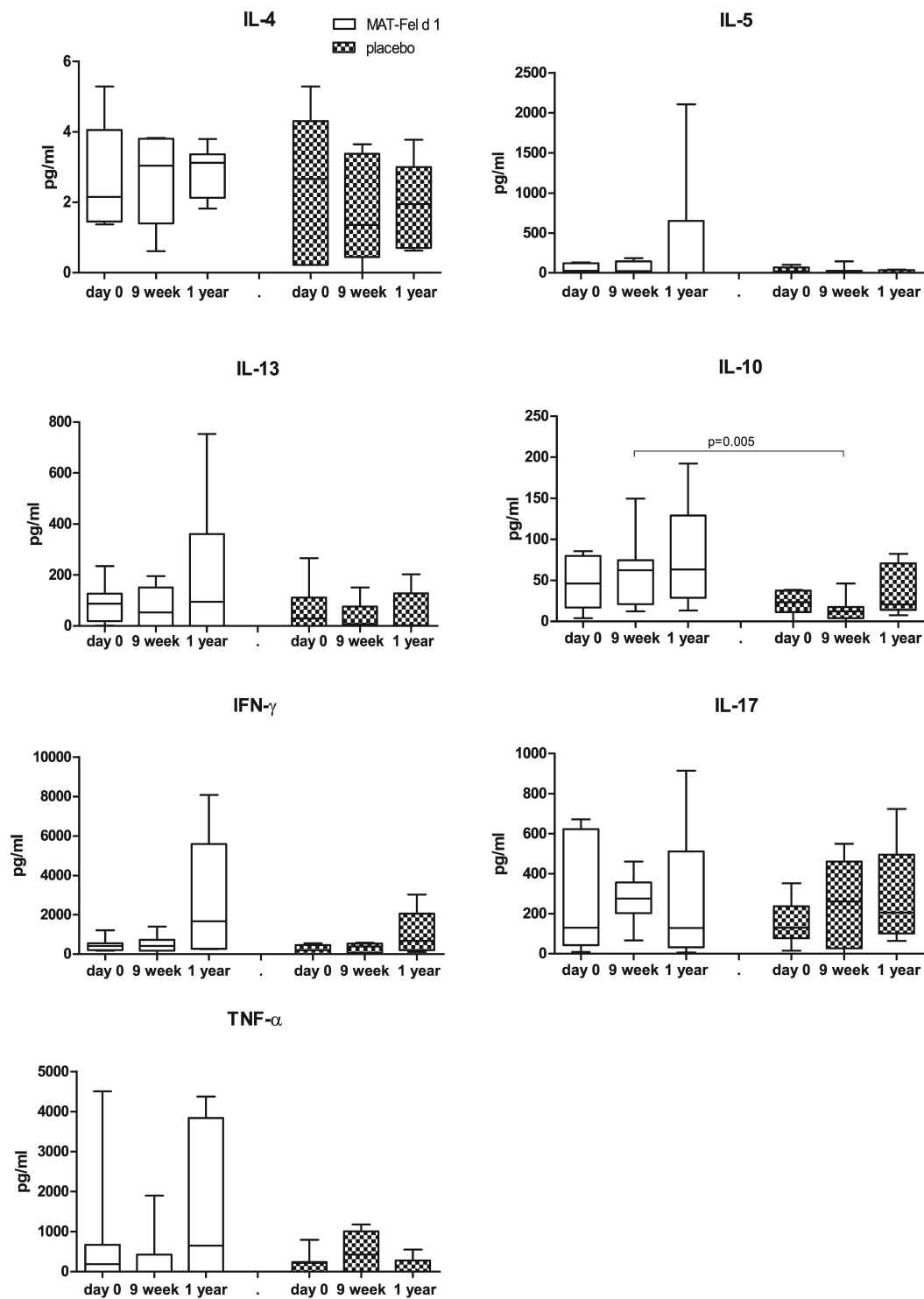


Figure S3
Zaleska et al.

Supporting Information

METHODS

MAT-vaccines and other antigens

Deglycosylated recombinant Fel d 1 (Felis domesticus allergen 1, rFel d 1) purified from *Pischia pastoris* culture (Indoor biotechnologies, United Kingdom) and MAT-Fel d 1, a recombinant non glycosylated fusion protein consisting of 291 amino acids was supplied by ImVisioN GmbH, manufactured according to cGMP regulations by Richter-Helm BioLogics GmbH & Co KG (Hamburg, Germany) and kept at -80 °C before use (1). MAT-Der p 1 was produced inhouse. Cloning and expression of the recombinant allergens used in the present study have been previously reported (2). rFel d 1 was 97% pure and the content of lipopolysaccharide was undetectable. The purity of MAT-Fel d 1 (IVN201) was 99% and lipopolysaccharide was undetectable.

Study population

Blood samples from 20 adult, cat allergic individuals qualified for specific ILIT with MAT-Fel d 1 were studied. All participants met the inclusion criteria that involved: a history and objective symptoms of cat-related rhinitis determined by nasal symptoms as a result of nasal provocation with cat dander extract, positive skin prick test (wheal diameter $\geq 3 \text{ mm}^2$ with flare) and intradermal test (wheal diameter $\geq 7 \text{ mm}^2$ with flare) with cat dander extract and presence of cat dander specific IgE in serum. The research protocol was approved by the ethics committee of Canton of Zürich and written consent was obtained from all participants before starting. The ILIT study was performed by the Clinical Trial Center of the University Hospital in Zurich from 2008 to 2009. Participants were divided into 2 groups, 12 of them had undergone intralymphatic immunotherapy with MAT-Fel d 1 vaccine adsorbed to aluminium hydroxide and 8 individuals were assigned to placebo group receiving only aluminum hydroxide. The treatment took 8 weeks and in total, and all patients received 3 intralymphatic injections into superficial inguinal lymph nodes with 4 weeks intervals in increasing dose: 1 μg (day 0), 3 μg (4

weeks), 10 µg (8 weeks). Blood samples were taken 3 times: before immunotherapy on day 0 and one week after the last injection (week 9) and after 1 year. Isolated PBMC and plasma were investigated at the Swiss Institute of Allergy and Asthma Research (SIAF), Davos. All patients finished the therapy of 8 weeks and blood samples were obtained in the 9th week. Blood samples of five of the patients treated with MAT-Fel d 1 and 2 patients from the placebo group could not be obtained in the one year visit.

T cell cultures and cytokine detection

PBMC were isolated by Ficoll (Biochrom, Germany) density gradient centrifugation of peripheral venous blood. Cells were washed three times and resuspended in RPMI 1640 medium supplemented with 1 mM sodium pyruvate, 1 % MEM nonessential amino acids and vitamins, 2 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, 1% kanamycin, 50 µM beta-mercaptoethanol (all from Gibco, Invitrogen) and 10% heat-inactivated FCS (Sigma Aldrich). 6×10^5 cells were stimulated with titrated rFel d 1 (Indoor biotechnologies) and with MAT-Fel d 1 starting with 20 µg/ml in 600 µl of medium described above in 48-well flat-bottom plates in duplicates. Allergen specific T-cell proliferative response was measured by ³H-thymidine incorporation after 5 days. As a control, thymidine incorporation by unstimulated cells was measured and all results were shown as a stimulation index. Non-allergic control individuals did not show any T cell proliferation and cytokine release in response to MAT-allergens or recombinant allergens. On day 7 antigen-specific responding T cells were supplemented with IL-2 (100 U/ml, Proleukin Proreo Pharma) and expanded. On day 13 the cells were collected, washed 3 times and re-stimulated with a combination of mAbs to T cell surface markers: anti-CD2 (clone 4B2 and 6G4 each 0.5 µg/ml) anti-CD3 (clone OKT3; 0.5 µg/ml) and anti-CD28 (clone B/G5; 0.5 µg/ml all from Sanquin) for 8 hours prior to RNA isolation. The secretion of cytokines into culture supernatants was determined on day 5 by cytometric bead array (Bio-Rad).

Isolation of RNA, cDNA synthesis and quantitative real-time PCR

T cells were lysed with RNeasy lysis buffer and total RNA was isolated using the RNeasy mini kit (Qiagen). Reverse transcription was performed with TaqMan reverse transcription reagents (Fermentas) using random hexamer primers according to the manufacturer's protocol. RT-PCR reaction was performed using iTaq SYBR green supermix with ROX (Bio-Rad) to amplify specific gene products. The PCR primers were designed based on sequences reported in GenBank with the Primer Express Software version 1.2 (Applied Biosystem) as follows: EF-1 α forward 5' CTG AAC CAT CCA GGC CAA AT 3', EF-1 α reverse 5' GCC GTG TGG CAA TCC AAT 3', FOXP3 forward 5' CCC GGC CTT CCA CAG AA 3', FOXP3 reverse 5' CAC CCG CAC AAA GCA CTT G 3'(Microsynth). cDNAs for H1, H2 and H4 receptor expression were amplified with TaqMan PCR master mix (Applied Biosystems). Primers and probes were as follows: H1R forward 5'-TCT CGA ACG GAC TCA GAT ACC A-3', H1R reverse 5'-CCT GTG TTA GAC CCA CTC CTC AA-3', H1R probe FAM-ACA GAG ACA GCA CCA GGC AAA GGC AA-TAMRA, H2R forward 5'-GCT GGG CTA TGC CAA CTC A-3', H2R reverse 5'-GGT GCG GAA GTC TCT GTT CAG-3', H2R probe FAM-CCC TGA ACC CCA TCC TGT ATG CTG C-TAMRA, H4R forward 5' GGG TCT TGA AGA TTG TTA CTC TGA TG 3', H4R reverse 5' CTA GAA TCA TTG GCC CAT TCA CT 3' H4R probe FAM-GCC AGC ACC CAA ACG GCC A-TAMRA (Microsynth). Relative quantification and calculation of range of confidence was performed on a 7900HT Fast Real-Time PCR instrument (Applied Biosystems) using the comparative $\Delta\Delta$ CT method (Applied Biosystems). EF- α was used as an endogenous control. All amplifications were conducted in triplicates.

HLA-DR typing

For HLA-DR typing, genomic DNA was extracted from PBMCs using the QIAamp DNA Micro Kit (Qiagen). HLA-DRB1 low-resolution (2-digit) and high resolution (4-digit) typing was performed using the Histo Type DR mini kit (BAG Healthcare) and SSP Uni Tray kits (Invitrogen), respectively. Both typing steps were based on PCR sequence-specific primers that led to the identification of individuals expressing: HLA-DRB1* 1104, DRB1*0701, DRB1*1501 and DRB1*0401.

Flow cytometry, MHC class II Fel d 1 tetramer staining and microscopy

The following mAbs were used for flow cytometry: Pacific blue-labeled anti-CD3 and Alexa Fluor 488-labeled anti-FOXP3 (BioLegend), APC Cy7-labeled anti-CD4, PECy7-labeled anti-CCR7, Alexa Fluor 647-labeled anti-CRTh2, and Alexa Fluor 700-labeled anti-CD14 (all from BD Biosciences). In this study, specific HLA-DRB1* 0701, HLA-DRB1* 1104, HLA-DRB1 0401 and DRB5*0101 Fel d 1 peptide tetramers and the corresponding CLIP peptide tetramers were used to identify and quantify Fel d 1-specific CD4⁺ T cells among peripheral blood mononuclear cells, isolated from 7 different donors with specific alleles for DRB1* 0701 (1 donor), DRB1* 1104 (1 donor), DRB1* 0401(1 donor) and DRB1* 1501 (4 donors). Fel d 1 peptide sequences coupled to the tetramer molecules were as follows: Fel d 1 chain 2 9-28 (IFYDVFFAVANGNELLLDLS) for HLA-DRB1* 0701, Fel d 1 chain 1 49-68 (MTEEDKENALSLLDKIYTSP) for HLA-DRB1* 1104 and Fel d 1 chain 1 17-36 (VANGNELLLDLSLTKVNATE) for HLA-DRB1* 0401. For HLA-DRB1* 1501 individuals we provided HLA-DRB5*0101 peptide tetramer coupled to Fel d 1 chain 1 17-36 (TPDEYVEQVAQYKALPVVLE), as DRB5* 0101 is linked to DRB1* 1501 by linkage disequilibrium. In all cases, the corresponding tetramer molecules coupled to the CLIP (KMRMATPLLMQALPM) were used as negative controls.

For tetramer staining 1×10^6 cells were resuspended in 100 μ l of complete RPMI and incubated with 10 μ g/ml of PE-labeled HLA-DR Fel d 1 tetramer or corresponding CLIP (Benaroya Research Institute, Seattle, USA) for 2 h at 37⁰C in dark. After the incubation cells were stained for the surface markers: CD3, CD4, CCR7 and CRTh2 for 15 min at room temperature in dark. Following washing with 0.5% BSA, PBS ph 7.4 and 2 mM EDTA cells were fixed and permeabilized using Alexa Fluor 488 anti-human FOXP3 Flow Kit (BioLegend) and stained with anti-FOXP3 mAb for 30 min at room temperature. After washing cells were resuspended in PBS with 0.1% paraformaldehyde and 2mM EDTA and analyzed by flow cytometry. Corresponding isotype-matched Ab were used as controls in all stainings. The gating strategy is shown in (**Supplementary Fig. S5**).

For confocal microscopic analysis cyto-spinned PBMCs were fixed with 4 % paraformaldehyde for 10 min at room temperature. Cells were permeabilized with 0.1 % saponin in 1X PBS for 10 min after washing with 1X PBS for 10 min. Cells were blocked with blocking solution containing 3 % BSA, 0.1 % saponin in 1 X PBS before staining appropriate antibody 1:100 dilutions of Alexa 647 labeled anti-CD74 in blocking solution. After staining for 1 hour at RT, cells were subjected to several washing steps as follows; 1X PBS 10 min, 0.1 % saponin in 1X PBS for 10 min repeated 2 times. Finally, cells are mounted by using gold antifade mounting reagent (Invitrogen) containing dapi (blue) and analysed by confocal microscopy after over night at 4°C incubation.

Western Blot

Western blots were performed by using primary antibodies (polyclonal rabbit anti Caspase-1 p10 antibody (sc-515, Santa Cruz) or monoclonal mouse anti β -actin (8H10D10, Cell Signaling) in 2.5% BSA in TBS-T at 4°C. Membranes were incubated with horseradish peroxidase–conjugated secondary antibodies (anti-rabbit, anti-mouse, Cell Signaling) for 1 hour at room temperature. Proteins were visualized with a chemiluminescence reagent (ECL plus, GE Healthcare) with Image Reader LAS-1000 Pro version 2.5 software and analyzed with an Aida Image Analyzer.

Statistical analysis

Statistical analyses were performed with GraphPad prism 5 (GraphPad Software, Inc, La Jolla, CA). Unless otherwise stated in figure legends, data are expressed as medians and were analyzed using nonparametric tests: the Wilcoxon signed rank test and the Mann-Whitney *U* test and ANOVA. Where required, statistics were corrected by Dunn's multiple comparison test. P value <0.05 was considered as significant.

Supplementary Figure S1. Schematic figure of the structure of the MAT-Fel d 1 vaccine.

Supplementary Figure S2. MAT-Fel d 1-ILIT does not affect proliferative response to PPD of *M bovis*. PBMCs isolated from patients treated with MAT-Fel d 1 (visit 1,2 n=11; visit 3 n=6) and placebo group (visit 1,2 n=7; visit 3 n=5) were cultured with titrated doses of PPD. Proliferation was measured by ³H-thymidine incorporation. There was no difference between the groups during the time course and in the analysis between MAT-Fel d 1 and placebo treatments.

Supplementary Figure S3. MAT-Fel d 1-ILIT and cytokine production. Cytokine concentration in supernatants was determined by multiplex assays after 5 days of stimulation of PBMC with rFel d 1 before, 9 weeks after and 1 year after the beginning of immunotherapy.

Supplementary Figure S4. No changes in CD3⁺ CD4⁺ CCR7⁺ or CRTh2⁺ T cells by Mat-Fel d 1-SIT. Lymphocytes were gated first by forward and side scatter, monocytes defined as CD14⁺ cells were gated out and the alive, lymphocytic population CD3⁺ CD4⁺ was used to gated in and CCR7⁺ or CRTh2⁺ T cells were analyzed. Data presented as mean ± SEM.

Supplementary Figure S5. Flow cytometry gating strategy. Lymphocytes were gated first by forward and side scatter, monocytes defined as CD14⁺ cells were gated out. The alive, CD3⁺ CD4⁺ lymphocytic population was gated in: **(a)** Fel d 1-tetramer-specific T cells stained with PE-labeled tetramer and clip, **(b)** CCR7⁺ or CRTh2⁺ T cells. Corresponding isotype controls were used to set gates.

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