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Abstract

BACKGROUND: IgE-mediated allergy can be treated by subcutaneous allergen-specific immunotherapy (SIT). However, the percentage of allergic patients undergoing SIT is low, mainly due to the long duration of the therapy and the risk of severe systemic allergic reactions associated with the allergen administration. To improve the safety and attractiveness of SIT for patients, alternative routes of allergen administration are being explored, such as sub-lingual or oral administration. METHODS: The present study evaluated direct intralymphatic allergen administration as a means to enhance SIT with bee venom and cat fur allergens in mice. Allergen-specific antibody and T-cell responses were analysed by ELISA and flow cytometry. The therapeutic potential of intralymphatic immunisation in sensitised mice was analysed using an anaphylaxis model. RESULTS: Direct injection of the major bee venom allergen phospholipase A(2) or the major cat fur allergen Fel d 1 into inguinal lymph nodes enhanced allergen-specific IgG and T-cell responses when compared with subcutaneous injections. Moreover, only intralymphatic immunisation stimulated the production of the Th1-dependent subclass IgG2a, which is associated with improved protection against allergen-induced anaphylaxis. Biodistribution studies showed that injection into the lymph node delivered antigen more efficiently to subcutaneous lymph nodes than subcutaneous injection. CONCLUSIONS: As intralymphatic immunisation induced more than 10-fold higher IgG2a responses with 100-fold lower allergen doses than subcutaneous immunisation, this approach should allow to reduce both the number of allergen injections as well as the allergen dose, improving both efficacy and safety of SIT.

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Intralymphatic Injections as a New Administration Route for Allergen-Specific Immunotherapy

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Key Words
Cat allergen · Allergen-specific immunotherapy · Th1/Th2 balance · Vaccination

Abstract
Background: IgE-mediated allergy can be treated by subcutaneous allergen-specific immunotherapy (SIT). However, the percentage of allergic patients undergoing SIT is low, mainly due to the long duration of the therapy and the risk of severe systemic allergic reactions associated with the allergen administration. To improve the safety and attractiveness of SIT for patients, alternative routes of allergen administration are being explored, such as sub-lingual or oral administration. ***Methods:*** The present study evaluated direct intralymphatic allergen administration as a means to enhance SIT with bee venom and cat fur allergens in mice. Allergen-specific antibody and T-cell responses were analysed by ELISA and flow cytometry. The therapeutic potential of intralymphatic immunisation in sensitised mice was analysed using an anaphylaxis model. ***Results:*** Direct injection of the major bee venom allergen phospholipase A_2 or the major cat fur allergen Fel d 1 into inguinal lymph nodes enhanced allergen-specific IgG and T-cell responses when compared with subcutaneous injections. Moreover, only intralymphatic immunisation stimulated the production of the Th1-dependent subclass IgG2a, which is associated with improved protection against allergen-induced anaphylaxis. Biodistribution studies showed that injection into the lymph node delivered antigen more efficiently to subcutaneous lymph nodes than subcutaneous injection. ***Conclusions:*** As intralymphatic immunisation induced more than 10-fold higher IgG2a responses with 100-fold lower allergen doses than subcutaneous immunisation, this approach should allow to reduce both the number of allergen injections as well as the allergen dose, improving both efficacy and safety of SIT.

Introduction

Allergy treatment with corticosteroids and antihistamines can efficiently ameliorate IgE-mediated symptoms but cannot cure the disease and cannot stop its progression. The only treatment with a long-lasting effect is allergen-specific immunotherapy (SIT), classically performed by repeated subcutaneous allergen injections. Despite the high efficacy of SIT and despite its recommendation as a first-line therapy [1], less than 5% of allergic patients choose to undergo SIT, mainly due to the long duration of the treatment (3–5 years), the high number of injections required (30–70) and the risk of adverse allergic reactions [2]. Therefore, there is a high interest in improving current methods of SIT. Numerous studies have investigated new strategies to optimise the allergen molecules [3–5], the ad-

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juvants used [6–8], the dosage form [9–11], as well as the route of allergen administration [12–14].

Immune responses are induced in secondary lymphoid organs, where professional antigen-presenting cells present antigenic epitopes to lymphocytes, and where T- and B-cell interactions take place. If antigens stay outside organised lymphoid tissues, then even large amounts of antigens can be ignored by the immune system [15, 16]. Therefore, antigen localisation is an important regulator of the immune response. Accordingly, direct administration of antigens into lymph nodes should be a more efficient route of immunisation. We have already demonstrated this for the administration of naked DNA vaccines [17], major histocompatibility complex class I binding peptide vaccines [18] and tumour cells [19]. In the present study, we compared subcutaneous with direct intralymphatic administration of allergens in mice as a means for improving the efficacy of SIT.

Methods

Immunisation Protocols

Female CBA mice (6–8 weeks old; Harlan, Horst, The Netherlands) were immunised 3 times with 2-week intervals by injection with the major bee venom allergen phospholipase A_2 (PLA_2; n = 5; Sigma-Aldrich, Buchs, Switzerland) or recombinant Fel d 1 (n = 4), the major cat allergen. Fel d 1 was cloned into the pQE30 expression vector (Qiagen, Hilden, Germany) as an N-terminal $[His]_6$-tag fusion protein, produced in *Escherichia coli* and purified as described [20]. The lipopolysaccharide content of Fel d 1 was 1.27 ng/mg of protein, as determined by the Limulus amoebocyte lysate assay. Different doses of the allergens were mixed with aluminium hydroxide (Alhydrogel 3%; Brenntag Biosector, Fredrikssund, Denmark) and saline 1 h before intralymphatic injection into the inguinal lymph node [18] or subcutaneously at the base of the tail. The doses of aluminium hydroxide were 90 and 450 μg, for intralymphatic and subcutaneous injections, respectively, and the injection volumes were 10 and 50 μl. Serum was prepared from tail vein blood drawn at different time points and frozen at –20°C until analysed by ELISA.

Alternatively, the 2 routes of administration were tested in a therapeutic model in CBA mice that were first sensitised with cat fur allergen extract (Stallergènes, Fresnes, France) by 6-weekly intraperitoneal injections. The allergen extract (1 μg/dose) was adsorbed to aluminium hydroxide (900 μg/dose) and injected in a volume of 100 μl. Two weeks after the last sensitisation injection, groups of 4 mice each were desensitised with 1 μg Fel d 1 by intralymphatic or subcutaneous injections as described above. As positive control, a group of sensitised but not treated mice was used, while naïve mice were used as negative controls. For the induction of anaphylaxis, all 4 groups of mice were challenged, 3 weeks after the last desensitisation injection, with 30 μg of cat fur allergen extract in saline (50 μl i.p.), and the body temperature was measured with a calibrated digital thermometer before and 30 min after the challenge. All animal experiments were approved by and performed according to guidelines from the Swiss veterinary authorities.

Antibody Measurements

For antibody detection, 96-well microtitre plates (Nunc Maxisorb, Basel, Switzerland) were coated with 1 μg/ml cat fur allergen extract (Stallergènes) or 5 μg/ml of PLA_2 (Sigma) in carbonate buffer and incubated overnight at 4°C. After blocking the plates with 2.5% non-fat dry milk in PBS-0.05% Tween-20 (PBSTM), serial dilutions of individual sera in PBSTM were added to the plates and incubated for 2h. Then plates were incubated with 1 μg/ml biotinylated goat anti-mouse IgG1 or IgG2a (BD Pharmingen, San Diego, Calif., USA) in PBSTM, followed by incubation with a 1:1,000 dilution of streptavidin-conjugated horseradish peroxidase (BD Pharmingen). In the last step, plates were incubated with the enzyme substrate 2,2′-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) di-ammonium salt (Sigma-Aldrich) in 1 M sodium dihydrogen phosphate. The absorption was read at 405 nm on a Model 550 Microplate reader (Bio-Rad, Hercules, Calif., USA). Unless otherwise specified, all incubations were done at room temperature and were intercepted with PBST washes.

For detection of PLA_2-specific IgE antibodies, plates were coated with 2 μg/ml of anti-mouse IgE capture antibody (BD Pharmingen). As secondary reagent for binding to mouse serum, an in-house biotinylated PLA_2 was used at 3 μg/ml.

For detection of Fel d 1-specific IgE antibodies, plates were coated with anti-mouse IgE (BD Pharmingen) at 2 μg/ml and then incubated for 2 h with serum samples from immunised mice. Subsequently, rFel d 1 was added at 0.6 μM and the plates incubated for another 90 min. For detection, anti-Fel d 1-biotin antibody (Indoor Biotechnology, Warminster, UK) at 1:1,000 dilution (90 min) was used.

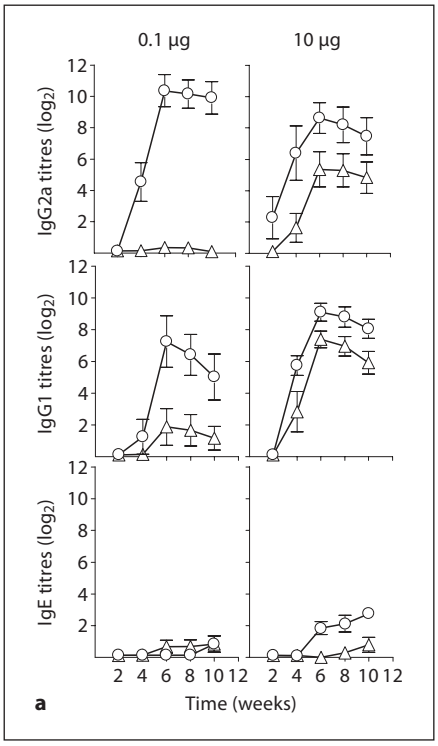
Flow Cytometry and Cytokine Secretion Assay

One week after the last injection of allergen, spleens were isolated and single-cell suspensions were prepared. For intracellular staining of interferon (IFN)-γ, red blood cell-free splenocytes 1×10^6 were stimulated overnight with 0.5 μg/ml ionomycin and 5 μg/ml heat-denatured PLA_2, the last 4 h in the presence of brefeldin A. After blocking of Fc receptors (anti-CD16/CD32), the splenocytes were fixed in PBS/PFA 1% for 10 min and permeabilised in PBS/NP40 0.1% for 3 min. Thereafter, the cells were stained with FITC-labelled antibodies specific for CD8 or CD4, as well as PE-labelled anti-CD44 and antigen-presenting cell-labelled anti-IFN-γ for 30 min at 4°C. All antibodies were purchased from BD Pharmingen.

For analysis of cytokine secretion by ELISA, triplicates of 8×10^5 cells were cultured with 10 μg/ml LoTox Fel d 1 from Indoor Biotechnology or left unstimulated in 200 μl of IMDM supplemented with 5% FCS, sodium pyruvate, L-glutamine, penicillin and streptomycin. The cytokines secreted in the supernatant were measured using the DuoSet ELISA from R&D Systems (Abingdon, UK) after 20 h (IL-2) or after 72 h (IL-4, IL-10 and IFN-γ). The detection limit for IFN-γ and IL-10 was 30 pg/ml and for IL-2 and IL-4 15 pg/ml.

Biodistribution

To compare the relative biodistribution of intralymphatically and subcutaneously administered proteins, CBA mice were injected with ^{99m}Tc pertechnetate-labelled human immunoglobu-



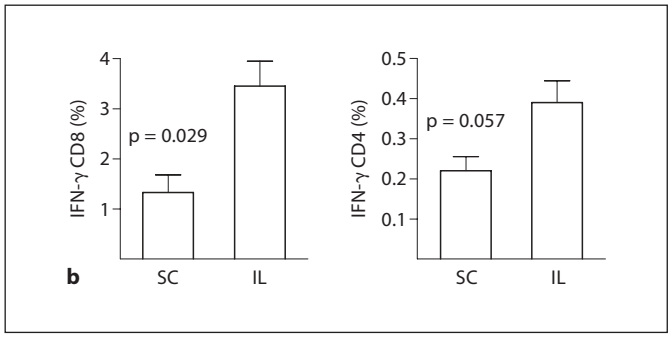


Fig. 1. Bee venom PLA_2-specific antibody and IFN-γ secretion. Mice were immunised thrice with 0.1 or 10 µg bee venom PLA_2 adsorbed on aluminium hydroxide by intralymphatic (IL) or subcutaneous (SC) injections. **a** Mice were bled at indicated time points, and the titres of sera analysed for PLA_2-specific IgG2a, IgG1 and IgE are illustrated. **b** Seven days after the last of 3 injections with 1 µg PLA_2, the splenocytes were re-stimulated in vitro and the IFN-γ secretion analysed by flow cytometry. The results illustrate the percentage of IFN-γ-producing CD4 or CD8 T cells that are also positive for CD44. p values indicated are obtained by Mann-Whitney analysis. All data are illustrated as means + SEM (n = 5).

lin (TechneScan® HIG) from Mallinckrodt Medical B.V., Petten, The Netherlands; the half life of ^{99m}Tc is approximately 6 h. The radioactive protein was injected intralymphatically in the inguinal lymph node or subcutaneously in the inguinal region at 3 MBq per dose. Four animals each were euthanised 90 min and 17 h after injection. Lungs, spleen, liver and inguinal lymph nodes were dissected and analysed directly in a Cobra II gamma counter (Packard Bioscience, Dreieich, Germany). The absolute distribution to each organ was expressed as radioactive count per minutes.

Statistics

Differences between independent groups were tested by nonparametric statistical analysis. Data were presented as means ± standard error and compared using a 2-sided independent Mann-Whitney U test or Kruskal-Wallis one-way analysis of variance with Dunn's multiple comparison test. The significance level was set at 5%.

Results

Intralymphatic Immunisation with Bee Venom PLA_2 Enhanced Immune Responses

To asses the influence of the route of administration on the immunogenicity of allergens, mice were immunised thrice with 2-week intervals using either 0.1 or 10 µg of the bee venom major allergen PLA_2 either by subcutaneous or intralymphatic injections. As illustrated in figure 1a, a dose as small as 0.1 µg PLA_2 was sufficient to induce high PLA_2-specific IgG2a titers by intralymphatic injection. In order to induce comparable titers by subcuntaneous injections, 10 µg of PLA_2 were necessary. Similar differences were observed for IgG1. Neither intralymphatic nor subcutaneous injections induced significant IgE titres.

In a similar experiment, 7 days after the last immunisation with 1 µg PLA_2, splenocytes were re-stimulated in vitro for analysis of IFN-γ secretion by flow cytometry. As illustrated in figure 1b, intralymphatic injections elicited significantly higher CD8 T-cell responses than subcutaneous injections.

Intralymphatic Immunisation Also Enhanced Immune Responses to Cat Fur Allergen Fel d 1

The following experiments aimed at expanding the above observations to another allergen. The perennial major cat fur allergen Fel d 1 was intralymphatically injected into mice at doses of 0.1 and 1 µg and compared with the subcutaneous injection of 10 µg Fel d 1. Comparably high IgG1 levels were induced in all 3 groups of mice, independent of the route of administration or the

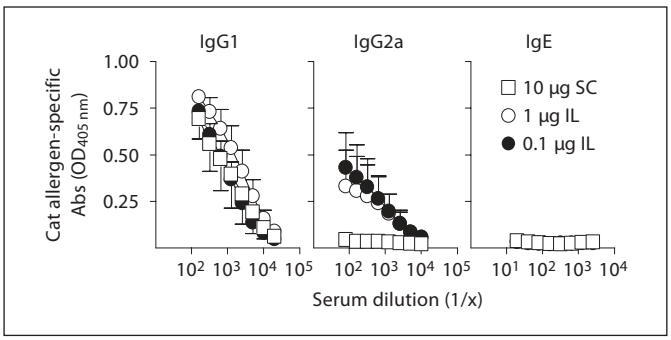


Fig. 2. Cat allergen-specific IgG1, IgG2a and IgE antibodies (Abs) measured by ELISA. Mice were immunised thrice, intralymphatically (IL) with 0.1 or 1 μg of Fel d 1 or subcutaneously (SC) with 10 μg Fel d 1. The sera were analysed after 2, 5 and 8 weeks of the last injection. The data from the last time point are illustrated and expressed as means ± SEM (n = 4). One representative out of 2 independent experiments is shown.

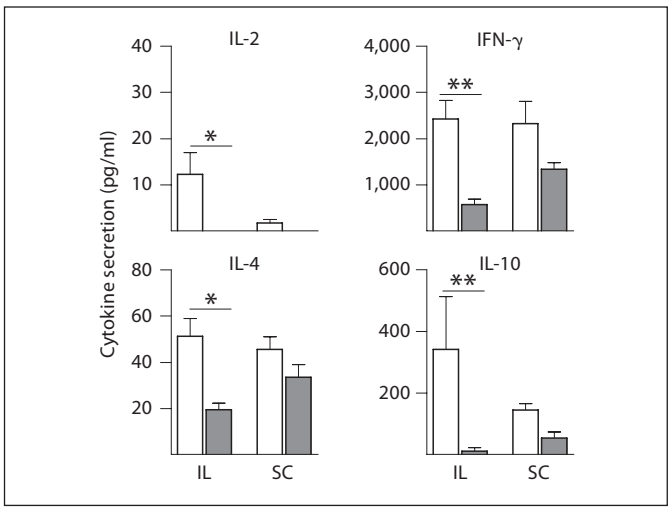


Fig. 3. Cytokine secretion assay. Groups of 4 mice were immunised thrice with 1 μg of Fel d 1 intralymphatically (IL) or subcutaneously (SC), and the splenocytes were analysed for secretion of different cytokines after in vitro re-stimulation with 10 μg/ml of Fel d 1. The cytokine concentrations in supernatants of stimulated (open bars) or unstimulated cultures (filled bars) are shown as measured by ELISA. Statistical differences were analysed by Kruskal-Wallis with a Dunn's post test of variance (* $p < 0.05$ and ** $p < 0.01$) (n = 4).

dose (fig. 2). However, only mice that received Fel d 1 by intralymphatic injection produced IgG2a, and 0.1 μg was sufficient to stimulate a significant response. Titres then remained constant for at least 8 weeks after the last injection.

Seven days after 3 immunisations with Fel d 1, splenocytes were re-stimulated in vitro with Fel d 1 for analysis of cytokine secretion by ELISA. The secretion of IL-2 was determined in the supernatants after 20 h, while IL-4, IL-10 and IFN-γ were measured after 72 h (fig. 3). The cytokine secretion was typically higher in cultured cells from mice that had been immunised by intralymphatic allergen administration, and the differences between stimulated and non-stimulated cells only reached statistical significance in the intralymphatically injected group.

Immunotherapy by Intralymphatic Fel d 1 Administration Enhanced Protection against Anaphylaxis

Mice were sensitised using repeated low-dose intraperitoneal injections of cat fur allergen extract, inducing significant levels of allergen-specific serum IgE and IgG1, but no detectable IgG2a (data not shown). These allergic mice were subsequently desensitised with Fel d 1, by 3 intralymphatic or subcutaneous injections with 2-week intervals. Again, a stronger IgG2a response was observed after intralymphatic allergen administration than after the subcutaneous injections (fig. 4a). Three weeks after completion of immunotherapy, mice were intraperitoneally challenged with a high dose of cat fur allergen extract and subsequently monitored for anaphylaxis, which in mice manifests as a drop in body temperature. As illustrated in figure 4b, immunotherapy by intralymphatic administration of Fel d 1 induced significant protection against anaphylactic temperature drop ($p = 0.048$). In contrast, subcutaneous immunotherapy was not sufficient to protect sensitised mice against the temperature drop.

Intralymphatic Injections Caused Accumulation of Allergen in Lymphoid Tissue

One possible explanation to the observed benefit of intralymphatic over subcutaneous immunotherapy could be an inefficient drainage to secondary lymphatic tissue after subcutaneous injections, i.e. intralymphatic injections deliver more antigen to the site of immune response induction. This difference in allergen dose available for stimulation of allergen-specific lymphocytes may also lead to an increase in the Th1 immune responses observed after intralymphatic allergen administration. Hence, to analyse the fate of an injected protein as a function of the administration route, mice received either intralymphatic or subcutaneous injections of a human immunoglobulin labelled with radioactive technetium. As illustrated in figure 5, analysis of the radioactivity in different organs 90 min after injection revealed that more

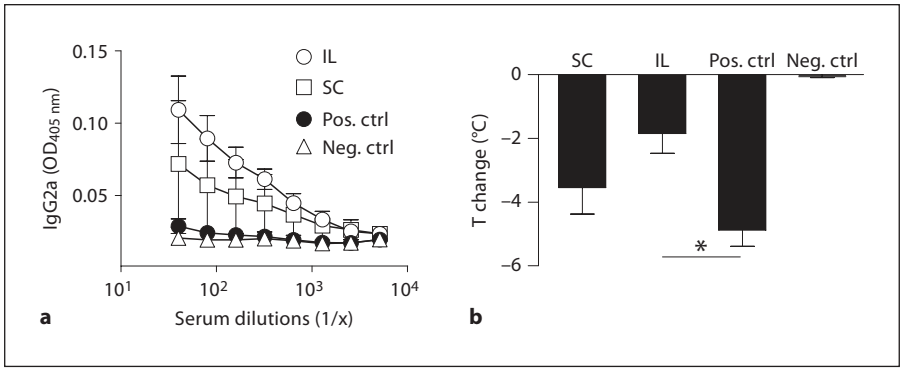


Fig. 4. SIT and test of anaphylaxis. Groups of 4 mice were sensitised against Fel d 1 by intraperitoneal injections of cat fur allergen extract and then treated by intralymphatic (IL) or subcutaneous (SC) injections of recombinant Fel d 1. Three weeks after the last SIT injection, mice were challenged with 30 μg of cat fur allergen extract. The negative control (ctrl) group contained naive mice, while the positive control was a group of sensitised mice that did not receive immunotherapy. Two weeks after the last SIT injection, mice were bled and IgG2a levels were determined (**a**). The body temperature was measured before and 30 min after the challenge (**b**). The groups were compared by Kruskal-Wallis with a Dunn's post test of variance (* $p < 0.05$).

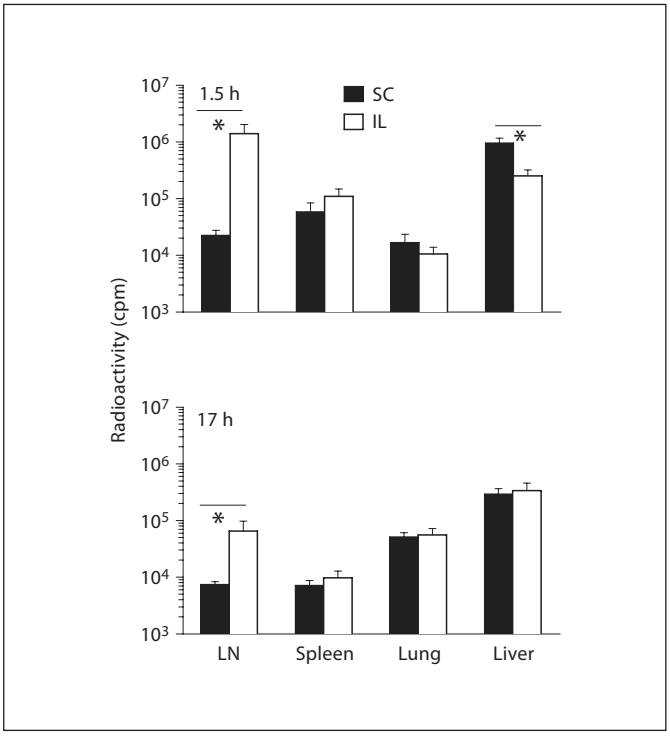


Fig. 5. Biodistribution of intralymphatically (IL) and subcutaneously (SC) injected protein. Groups of 4 mice were injected with ^{99m}Tc-labelled human immunoglobulin directly into an inguinal lymph node (open bars) or subcutaneously in the inguinal region (filled bars). After 90 min and after 17 h, the mice were euthanised, and the inguinal lymph nodes (LN), the spleen, the lungs as well as all liver lobes were isolated and immediately assayed for radioactive gamma decoy (cpm). Statistical differences are indicated when * $p < 0.05$, as analysed by the Mann-Whitney U test.

radioactivity was measured in inguinal lymph nodes after intralymphatic administration than after subcutaneous injection ($p < 0.05$). In contrast, subcutaneously administered protein drained more rapidly to the liver ($p < 0.05$). Seventeen hours after injection, radioactivity was generally reduced in all organs, but again, approximately 10 times more radioactivity could be detected in the inguinal lymph nodes of mice injected by the intralymphatic route than by the subcutaneous route ($p < 0.05$).

Discussion

IgE-mediated allergies, such as allergic rhinoconjunctivitis and asthma, have become highly prevalent, affecting up to 35% of the population in industrialised countries [21–23]. The best long-lasting treatment available for these patients is SIT [24]. However, the major problems of current SIT regimes are the long-term commitment of the patient, the high treatment costs, and the significant risk of allergic adverse reactions associated with the allergen administration [2]. One approach for improving SIT focuses on the route of allergen administration. In this respect, sublingual immunotherapy, in which the allergen is given as soluble tablets or drops, and local nasal immunotherapy have proven promising and especially attractive for patients [1, 25–27]. However, the treatment duration of sublingual immunotherapy and nasal SIT remains similar to that of current subcutaneous SIT [28], and the required allergen doses are at least 50–100 times

higher than the dose needed for subcutaneous immunisation [29].

The results presented in the current study show that direct intralymphatic injection enhances immunogenicity of allergens as compared with the classical subcutaneous SIT route of administration. These results are in line with already published data showing enhanced immunogenicity of intralymphatic injection using plasmid DNA vaccines [17], major histocompatibility complex class I binding peptides [18] and tumour cells [28]. Other groups have also demonstrated enhanced efficacy by targeted lymph node immunisation with immunostimulatory complexes [30], bacteriophages [31], a recombinant simian immunodeficiency virus vaccine [32], and a canary pox virus-based cancer vaccine [33]. In addition, intralymphatic administration required less than 1% antigen [17] or immune stimulatory molecules [34] to induce potent cytotoxic T-cell immune responses than did subcutaneous antigen administration. In the present study, intralymphatic immunisation was shown to stimulate humoral immune responses comparable with that of subcutaneous SIT, but using only 1% of the allergen dose. Moreover, while subcutaneous SIT induced mostly IgG1 antibodies, intralymphatic allergen administration stimulated both Th1- and Th2-associated antibody subclasses. The enhanced humoral immune responses observed after intralymphatic immunisation were consistent with a general increase in the secretion of cytokines after restimulation of the lymphocytes in vitro.

The reason why the intralymphatic route of immunisation was more efficient than subcutaneous allergen administration in stimulating immune responses, especially of the Th1 type, is most likely a matter of dose and localisation, as proposed by the geographical concept of immune reactivity that emphasises the importance of antigen localisation [15]. As conventional T- and B-cell responses can only be induced in organised lymphoid tissues, the direct injection of antigens into subcutaneous lymph nodes would obviously facilitate the stimulation of stronger immune responses. As demonstrated in the biodistribution experiments using radioactively labelled proteins, only a few percentage of the subcutaneously injected material drained and remained in the lymphatic tissue (spleen and inguinal lymph nodes), while most of the material ended up in the liver. In contrast, after intralymphatic administration, the entire allergen dose is available for antigen presentation in the lymph node. This, and the fact that high antigen doses favour Th1-like immune responses [35, 36], could explain why efficient lymphatic targeting of the allergen is important for the stimulation of protective immune responses in SIT.

As lower therapeutic doses are required to induce comparably strong immune responses, intralymphatic immunotherapy should improve the safety of immunotherapy and reduce the risk of local and systemic allergic side effects. Although the presence of mast cells as well as basophils has been described in the lymph node [37], we found excellent local tolerance of allergen injection into inguinal lymph nodes in our clinical trial [38].

Recently, intralymphatic immunotherapy has been tested in humans for the treatment of both grass pollen and bee venom allergies, demonstrating the clinical feasibility and efficacy of this approach [38]. The injection into subcutaneous lymph nodes has also been applied in the treatment of other diseases, such as cancer [33].

In conclusion, the intralymphatic route of administration may represent a promising method to increase the efficacy of SIT, to reduce the required allergen dose and number of injections, and to reduce the risk of adverse events as compared with conventional subcutaneous immunotherapy.

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