

ORIGINAL ARTICLE Experimental Models of Allergic Disease

Multivalent paediatric allergy vaccines protect against allergic anaphylaxis in mice

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Clinical
&
Experimental
Allergy

Summary

Background Almost a quarter of the world population suffers from IgE-mediated allergies. T cells and IgG-producing B cells can produce protection, but treatment for disease is laborious with unsatisfactory patient compliance.

Objective We sought to identify whether paediatric allergy vaccines affected later allergen sensitization and onset of disease when used prophylactically.

Methods A murine model of anaphylaxis was applied. Mice were first immunized with monovalent or multivalent allergy vaccines that also contained aluminium hydroxide and CpG oligodeoxynucleotide as adjuvants. Later, the mice were sensitized by multiple low-dose injections of aluminium-adsorbed allergen. After a dormant period, the mice were challenged systemically with high-dose allergen, and the clinical signs of anaphylaxis were recorded. Throughout the immunization and sensitization periods, blood was collected for serological testing.

Results Immunization with allergy vaccines produced antigen-specific protection against sensitization as measured by systemic anaphylaxis in mice. The long-term effect was observed both after juvenile (5–6 weeks) and neonatal (7 days) vaccination. Monovalent and pentavalent vaccines were protective to a similar level. Protection was associated with increased secretion of IgG2a and production of IFN- γ . Protection could also be transferred to sensitized mice via serum or via CD25-positive CD4 T cells.

Conclusion and clinical relevance Prophylactic and multivalent allergy vaccines in juvenile and neonatal mice protected against later sensitization and anaphylaxis. Such treatment may provide a rational measure for future management of allergen-related diseases and their strong socio-economic impact on daily life.

Keywords anaphylaxis, antibodies, childhood vaccine, murine model of allergy, T cells

Submitted 18 August 2013; revised 5 November 2013; accepted 20 November 2013

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Cite this as: Y. Waeckerle-Men, Y. Liang, S. von Moos, T. M. Kündig and P. Johansen, *Clinical & Experimental Allergy*, 2014 (44) 429–437.

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Introduction

The prevalence of allergic rhinitis in Europe has been suggested to be 23%, which accounts for approximately 170 million people [1]. Comparable frequencies are registered in other developed or industrialized regions of the world [2]. IgE-mediated allergies are almost exclusively treated by means of symptomatic medication, although allergen immunotherapy (AIT), the only disease-modifying treatment, has been shown to provide protection with long-term efficacy [3–5]. Allergen immunotherapy can also prevent additional sensitizations and progression to asthma [6]. However, less than

5% of the patients chose to receive AIT, mainly due to time constraints and therapy-induced side-effects. By comparison, vaccination coverage of against infectious diseases in children is more than 90%.

The goal of AIT is to produce immune tolerance or to stimulate immune reactions that can capture or neutralize innocuous allergens before these exert unwanted allergic effects. Protection is mainly mediated by B cells that secrete IgG antibodies and T cells that regulate overall type and size of immune responses to allergens upon exposure [7]. Hence, the mechanism of AIT is similar to that of childhood vaccines. Considering the fact that the prevalence of childhood infections in the western world is typically 0–0.01% [8], while that of life-threatening anaphylaxis is as much as 0.5–4%

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[9–13], the socio-economic benefit of prophylactic allergy vaccination should be obvious. However, prophylactic vaccination against allergy is not offered, and the major argument against vaccination is typically that allergy is not considered such a critical disease and that the load of recommended childhood vaccines is already too high in many people's opinion. For children with a hereditary risk of atopy, based on family history, occurrence of food allergies early in life and certain genetic polymorphisms [14–16], vaccination should nevertheless be considered.

To investigate the potential benefit of childhood allergy vaccination, we applied a murine model of anaphylaxis and found that vaccination can protect against sensitization and anaphylaxis later in life. Protection was mediated through a combination of IgG and CD4 T cells of regulatory (Treg) and T helper 1 (Th1) phenotypes.

Materials and methods

Animals

Female BALB/c mice were purchased from Harlan (Horst, the Netherlands) and were typically used at the juvenile age of 5–6 weeks. Alternatively, pregnant BALB/c were purchased and delivered at E12. The offspring was immunized on day 7 and 21 after birth. All animals were kept under specific pathogen-free conditions, and all procedures were approved by the veterinary office of the Canton of Zurich (licences 103/2009 and 68/2012).

Immunization and sensitization protocols

Groups of five juvenile female BALB/c mice were typically immunized by subcutaneous injection into the scruff of the neck with 20 µg ovalbumin (OVA; Grade V from Sigma-Aldrich, Buchs, Switzerland) in phosphate-buffered saline (PBS) and in a total volume of 200 µL. Alternatively, the mice were immunized with 20 µg of a recombinant major cat fur allergen, MAT-Fel d 1 [17], a divalent mixture of OVA and MAT-Fel d 1, or a pentavalent mixture of OVA, MAT-Fel d 1, 20 µg bee venom phospholipase A2 (Sigma-Aldrich), 5 IR house dust mite allergen extract (Stallergens, Antony cedex, France) and 5 IR birch pollen allergen extract (Stallergens). All vaccine preparations also contained 0.5 mg aluminium hydroxide (Alhydrogel 2% from Brenntag Biosector, Frederikssund, Denmark) and 20 µg phosphorothioate-stabilized oligonucleotides CpG 1826 (5'-TCC ATG ACG TTC CTG ACG TT-3') from Microsynth (Balgach, Switzerland). The immunization was repeated 14 and 28 days later. Two to three weeks after three immunizations, mice received an intraperitoneal

injection of 1 µg OVA and 250 µg aluminium hydroxide contained in 50 µL PBS for OVA-specific IgE sensitization. Alternatively, the mice were sensitized with a combination of 1 µg OVA and 1 IR cat fur allergen extract (Stallergens) with aluminium hydroxide. The sensitization was repeated four times with weekly intervals. Finally, the mice were challenged for measurement of anaphylaxis as described below. The whole procedure is outlined in Fig. 1a.

For paediatric immunization, groups of male and female BALB/c mice ($n = 5–8$) were immunized subcutaneously on day 7 after birth. A mixture of OVA (10 µg), aluminium hydroxide (30 µg) and CpG 1826 (3 µg) was contained in 10 µL PBS. The immunization was repeated on day 21 using a fivefold dose of all components contained in 50 µL PBS. All mice were then sensitized as above by four intraperitoneal injections on days 35, 42, 48 and 56. On day 76, the mice were challenged for induction and measurement of anaphylaxis.

Analysis of systemic anaphylaxis

The mice were challenged by intraperitoneal injection of 100 µg OVA or 30 µg cat fur allergen extract in 100 µL PBS. Prior to and after the challenge, the rectal temperature was measured using a digital thermometer (Thermalert TH-5 with a RET-3 probe, Physitemp, Huron, NJ, USA). After the last temperature measurement, all mice received 50 µg of the antihistamine clemastine (Tavegil, Novartis, Switzerland) intraperitoneally for alleviation of allergic symptoms. In some experiments, the challenge was repeated after 1–3 months to analyse the longevity of sensitization and protection.

Analysis of antibody responses

At different time-points during the immunization and sensitization periods, the mice were tail bled, and sera were prepared for analysis of allergen-specific IgE, IgG1 and IgG2a antibody responses as previously described [18]. The plates were coated with 2 µg/mL OVA for IgG1 and IgG2a detection and with anti-mouse IgE (AbD Serotec, Düsseldorf, Germany) for detection of IgE. Ovalbumin-specific IgE was detected with an in-house biotin-conjugated OVA, while IgG1 and IgG2a were detected with biotin-conjugated rat anti-mouse IgG1 or IgG2a (BD Pharmingen, San Diego, CA), respectively. All plates were developed with streptavidin-conjugated HRP and with TMB substrate (eBioscience, San Diego, CA, USA). The titres are defined as the highest dilution with absorbance higher than the absorbance of sera from untreated mice plus two standard deviations. Alternatively, the optical density (OD) at a given serum dilution was measured.

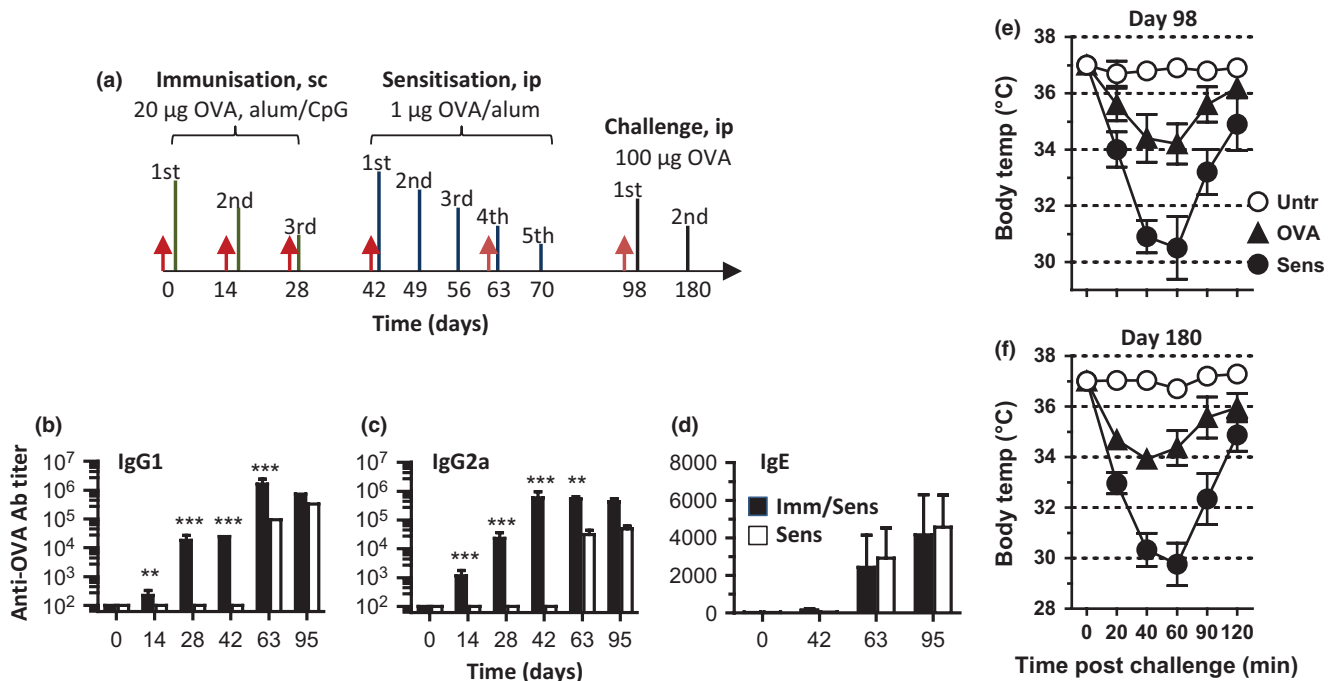


Fig. 1. Immunogenicity and protective effect of a prophylactic allergy vaccine in a juvenile murine model of active systemic anaphylaxis. (a) Scheme illustrating the immunization, sensitization and challenge schedules. BALB/c mice ($n = 5$) were immunized by subcutaneous injections of ovalbumin (OVA), aluminium hydroxide (alum) and CpG, sensitized for IgE with intraperitoneal injection of low-dose OVA and alum, and finally challenged intraperitoneally with a high dose of OVA in saline. (b–d) At different time-points before challenge, blood (arrows in A) was harvested from immunized + sensitized mice (filled bars) and sensitized mice (open bars) and analysed for OVA-specific IgG1 (b), IgG2a (c) and IgE (d). On days 98 (e) and 180 (f), mice were challenged with OVA for induction of anaphylaxis and the rectal body temperature was monitored during 120 min following the challenge. **: $P < 0.01$; ***: $P < 0.001$.

Analysis of lymphocyte responses

For analysis of lymphocyte responses, mice were killed and spleens harvested. Cultures with 2×10^5 erythrocyte-free splenocytes were incubated with 10 µg/mL OVA in RPMI 1640 medium supplemented with FCS, glutamine and antibiotics. Cytokine secretion was measured in the supernatants after four days by ELISA (eBioscience).

Adoptive transfer of immunity from immunized mice to sensitized mice

One group of female BALB/c mice was immunized three times and fortnightly with 20 µg OVA, aluminium hydroxide and CpG 1826 subcutaneously. Another group of female BALB/c mice was sensitized intraperitoneally with 1 µg OVA and 250 µg aluminium hydroxide five times with weekly intervals. Three weeks after last injection, the mice from the immunized group were killed. Blood was collected by heart puncture, and serum was prepared and pooled from all mice. Spleen and mesenteric lymph nodes were also harvested and combined. Erythrocytes were lysed to make single-cell suspensions, and the remaining cells were separated into CD4⁺, CD25[−] CD4⁺ or CD25⁺ CD4⁺ T cells using magnetic beads (Milte-

nyi Biotec, Bergisch Gladbach, Germany). The purified cells (10^7 cells in a total of 200 µL PBS) were then adoptively transferred by intravenous injection into the intraperitoneally sensitized mice. One group of sensitized mice received 100 µL of the pooled antiserum by intravenous injection. One day after the transfer, all mice were challenged with OVA and the temperature change was measured as described above. In some experiments, the challenge was repeated after several weeks to analyse the longevity of the transferred protection.

Statistical analysis

ELISA data were analysed by a nonparametric two-way Kruskal–Wallis test, using the Dunn's test to correct for multiple testing. Differences in the change of temperature over time after the anaphylaxis test were tested by a two-way ANOVA using time (min after challenge) and treatment (immunization protocol) as variable parameters with Bonferroni post hoc test to correct for multiple testing. The correlation between protection against anaphylaxis (change in body temperature) and antibodies was analysed using a two-way Pearson's product moment correlation for normally distributed data. All statistical analyses were carried out using the software GraphPad Prism.

Results

Antibody responses stimulated by immunization and sensitization

Immunization of female juvenile mice comprised three fortnightly subcutaneous injections and the sensitization five weekly intraperitoneal injections (Fig. 1a). Two weeks after the first immunization, anti-OVA IgG1 and IgG2a seroconversion was measured (Fig 1b and c). By day 42, the IgG1 and IgG2a titres further increased to approximately 2×10^4 and 1×10^6 , respectively. Prior to sensitization on day 42, IgE titres could not be detected (Fig. 1d). During sensitization, the anti-OVA IgG1 titres further increased approximately 100-fold (Fig. 1b). In contrast, OVA-specific IgG2a titres remained as established by immunization. However, the sensitization resulted in OVA-specific IgE sensitization (Fig. 1d).

Intraperitoneal sensitization of non-immunized naïve mice also stimulated OVA-specific IgG1 (Fig. 1b) and IgG2a (Fig. 1c) production, but the endpoint titres were approximately tenfold lower than those measured in sera from immunized mice. The OVA-specific IgE titres obtained in naïve mice and in immunized mice did not differ ($P > 0.05$).

Anaphylaxis prevented by prophylactic immunization

On day 98, mice were challenged with high-dose OVA to provoke anaphylaxis. Sensitized mice reacted with an average 6.2°C drop in body temperature (Fig. 1e). The mice showed clear clinical signs of allergic anaphylaxis, with piloerection, hunched posture, abdominal breathing and lack of fight-and-flight instincts. Mice that were immunized prior to the sensitization showed less anaphylaxis symptoms and a lower change in the body temperature ($P < 0.001$), the average temperature drop being 2.8°C; all symptoms were transient. To analyse the longevity of the protective effect of immunization, the mice were rechallenged three months later (day 180). Immunization still exerted a highly significant effect by preventing anaphylaxis in mice ($P < 0.001$), while non-immunized mice were still anaphylactic (Fig. 1f). Mice that were neither immunized nor sensitized did not show signs of anaphylaxis, and immunization with an irrelevant allergen (bee venom PLA2) or with the adjuvants aluminium hydroxide and CpG only was not protective (data not shown).

Combined allergy vaccines

While the above experiments show that prophylactic allergy vaccines may be feasible, prophylactic allergy vaccines for high-risk persons, for example children of

atopic parents, would necessarily include a mixture of allergens, equivalent to combined childhood vaccines, such as MMR or DTP-Hib. Therefore, combination vaccines were tested in the murine model of anaphylaxis equivalent to the protocol described above (Fig. 1a).

The immunogenicity of OVA with regard to IgG1 (Fig. 2a) and IgG2a (Fig. 2b) was not affected when OVA was combined in a divalent vaccine with the cat fur allergen MAT-Fel d 1 or in a pentavalent allergy vaccine that also contained bee venom PLA2, house dust mite and birch pollen allergen extracts. Weekly sensitizations with OVA and cat fur allergen extract further increased the anti-OVA IgG1 titres, independent on the primary vaccine used. In naïve mice, the sensitization with OVA and cat fur allergen extract induced anti-OVA-IgG1 and IgG2a (Fig 2a and b) similar to that of sensitization with OVA alone (cf. Fig 1b and c). The anti-OVA IgE responses differed slightly as a function of the primary immunization protocol ($P = 0.02$). There was a tendency of reduced IgE titres with more allergens in the vaccine. The pentavalent vaccine significantly reduced IgE as compared with the sensitized controls ($P = 0.046$).

The mice were challenged on day 98 with OVA and on day 102 with cat fur allergen extract. All OVA-containing vaccines provided partial protection against OVA-induced anaphylaxis, and no difference was observed between the monovalent, divalent and pentavalent vaccine groups (Fig. 2d). The drop in body temperature after challenge with cat fur allergen extract was only approximately 2.5°C (Fig. 2e), hence, much smaller than for OVA. Also, the mice recovered within approximately 60 minutes as compared to 90–120 min after OVA challenge. Immunization with the 'irrelevant' allergen OVA did not provide protection against cat allergy anaphylaxis. All allergy vaccines that contained the cat allergen MAT-Fel d 1 provided partial protection. A statistical significant effect was measured for the monovalent and the divalent MAT-Fel d 1 vaccines ($P = 0.009$).

Correlation between protection and IgG, but not IgE

Data from the experiments above were pooled to analyse correlation between OVA-specific anaphylaxis and the antibody production (Fig. 2f). No correlation was observed between IgE levels in blood and protection against anaphylaxis ($P > 0.05$ by Pearson). Strong correlation was measured between anaphylaxis and IgG1 ($P = 0.002$) or IgG2a ($P = 0.001$).

Paediatric vaccination against allergy

The above-described immunization experiments were all started when mice were juvenile (5–6 weeks old).

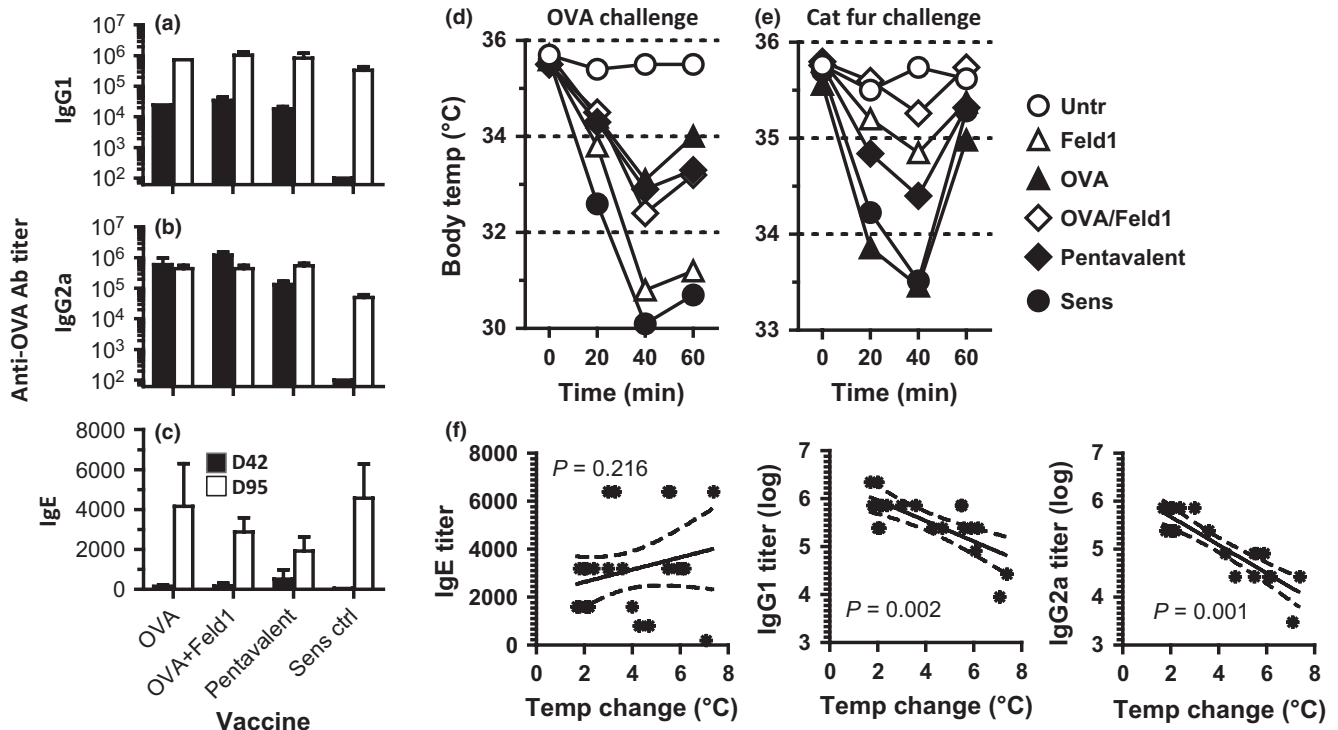


Fig. 2. Immunogenicity and protective effect of multivalent allergy vaccines. BALB/c mice ($n = 5$) were immunized, sensitized and challenged as described in Fig. 1a. Subcutaneous vaccines containing aluminium hydroxide and CpG as adjuvant were used: monovalent ovalbumin (OVA) or MAT-Fel d 1; divalent OVA and MAT-Fel d 1; pentavalent OVA, MAT-Fel d 1, bee venom PLA2, house dust mite allergen extract and birch pollen allergen extract. The mice were sensitized for IgE with five weekly intraperitoneal injections of OVA and cat allergen extract adsorbed on aluminium hydroxide. Blood was harvested and analysed of OVA-specific IgG1 (a), IgG2a (b) and IgE (c) on days 42 (filled bars) and 95 (open bars). (d-e) On day 98, the mice were challenged with OVA (d) and on day 102, the same mice were challenged with cat fur allergen extract (e) and the body temperature was monitored. (f) Pearson's correlation of antibodies measured on day 95 and temperature changes measured after the OVA challenge measured on day 98 ($n = 26$).

Paediatric vaccines were tested in infant mice on days 7 and 21 with a slightly modified and shortened protocol: only two immunizations instead of three; lower antigen and adjuvant doses for priming; only four instead of five intraperitoneal sensitization doses (Fig. 3a). Paediatric allergy vaccine stimulated immunity that inhibited susceptibility to later sensitization. The antibody analysis revealed more IgE and IgG2a in mice that were immunized than in mice that were sensitized only (Fig. 3b). Anaphylactic reactions were also strongly suppressed in immunized mice (Fig. 3c). Interestingly, the effect of immunization was stronger in infant males than in females. One group of immunized and sensitized mice and one group of mice that were only sensitized were killed 120 min after the challenge for analysis of the relative amount of inflammatory cells in peritoneum and in blood (data not shown). The results suggested only minor and not significant differences in mast cells and basophils ($CD34^+$), eosinophils ($CD11b^+$, $SiglecF^+$, $CD11c^-$), B cells ($B220^+$) and T cells ($CD4^+$ and $CD8^+$) between immunized and non-immunized mice. There was a slight reduction in mast

cells/basophils and a slight increase in lymphocytes of immunized mice compared with mice that were only sensitized. Finally, when splenocytes were restimulated in vitro with OVA for 4 days, cells from OVA-immunized mice produced more $IFN-\gamma$ than did cells from sensitized only and untreated mice (Fig. 3d).

Adoptive transfer of immunity from immunized donors to sensitized recipients

To analyse the relative contribution of serological factors and lymphocytes in mediating protection against systemic anaphylaxis, one set of female juvenile mice was immunized by subcutaneous injections of OVA admixed with aluminium hydroxide and CpG. Another set of age- and sex-matched animals was sensitized by intraperitoneal injections of OVA and aluminium hydroxide. Both immunization and sensitization were carried out as described above. The immunized mice were killed, and heart blood was collected for preparation of serum; all sera were pooled. Spleens and lymph nodes were also harvested and combined for $CD4$ T cell isolation using

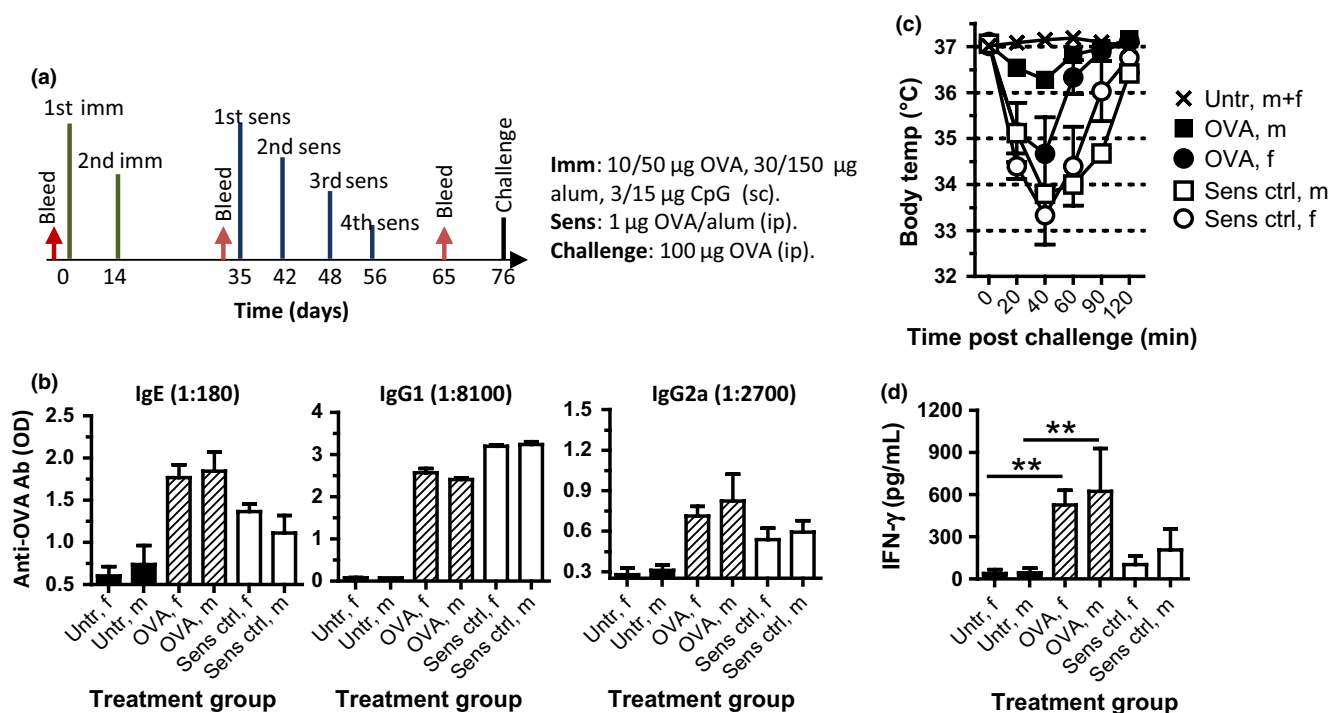


Fig. 3. Immunogenicity and protective efficacy of paediatric allergy vaccination. (a) Scheme illustrating the immunization, sensitization and challenge schedules. Female (f) and male (m) BALB/c mice were immunized by subcutaneous injections of ovalbumin (OVA), aluminium hydroxide (alum) and CpG at the age of 7 and 21 days ($n = 5-8$). Sensitization and challenge were carried out equivalent to earlier. Blood (arrows) was harvested on days 0, 35 and 65. (b) OVA-specific IgG1, IgG2a and IgE as measured in blood on day 65 from mice that were untreated (filled bars), immunized + sensitized (hatched bars) or sensitized (open bars). The antibody response is given as optical density (OD) in the ELISA at the indicated serum dilution. (c) Analysis of anaphylaxis after the OVA challenge on day 76. (d) Analysis of IFN- γ secretion from splenocytes restimulated *in vitro* with OVA ($n = 5$). **: $P < 0.01$.

magnetic beads. Then, the sensitized mice were randomly grouped to receive either OVA-immune serum or OVA-immune CD4 T cells by intravenous injection. A third group of sensitized mice was left untreated. Twenty-four hours later, the mice were challenged with OVA. The scheme of events is illustrated in Fig. 4a.

CD4 T cells ($P = 0.046$) and especially serum ($P < 0.003$) provided partial protection against anaphylaxis (Fig. 4b, left panel). The treated mice showed less clinical symptoms of anaphylaxis, and the drop in temperature reached an average of 7.2°C in the sensitized controls, 2.8°C in serum-treated mice and 3.5°C in CD4 T cell-treated mice. Four weeks later, both serum-treated (a 3.6°C drop) and CD4 T cell-treated (3.0°C) mice had lost some protection relative to the sensitized control (5.8°C) group when all mice were rechallenged (Fig. 4b, right panel). No statistical significant difference was observed between the serum-treated and CD4 T cell-treated mice, neither at 24 h nor at 28 days after the transfer. Transfer of serum or CD4 T cells from mice immunized with an irrelevant allergen (bee venom PLA2) did not protect against OVA-induced anaphylaxis (data not shown).

As CD25-positive regulatory CD4 T cells are ascribed an important role in AIT [19], we repeated

the transfer experiment, but instead of transferring the whole CD4 T cell population, the cells were separated into CD25-positive and CD25-negative CD4 T cells by magnetic sorting. Again, partial protection was observed as a function of treatment ($P < 0.001$). The body temperature of the unprotected sensitized controls dropped from approximately 37.0°C to an average of 30.7°C, while the temperature in serum-treated animals dropped to 34.0°C ($P < 0.05$; Fig. 4c, left panel). The temperature in mice that received CD25-negative or CD25-positive CD4 T cells dropped to 32.1°C and 32.9°C, respectively, within 60 min of the challenge ($P = 0.027$ compared to sensitized controls). The mice were then left to rest for another 40 days and challenged again with OVA (Fig. 4c, right panel). Mice that had received anti-OVA serum and those that had received CD25-positive CD4 cells showed a statistically significant ($P < 0.007$) tolerance against OVA-specific anaphylaxis. In contrast, CD25-negative CD4 T cells had lost their effect. Similar transfer experiments with C57BL/6 mice showed comparable results, with a transient effect of CD25-negative CD4 T cells, and a more sustained effect transferred serum and CD25-positive CD4 T cells (data not shown).

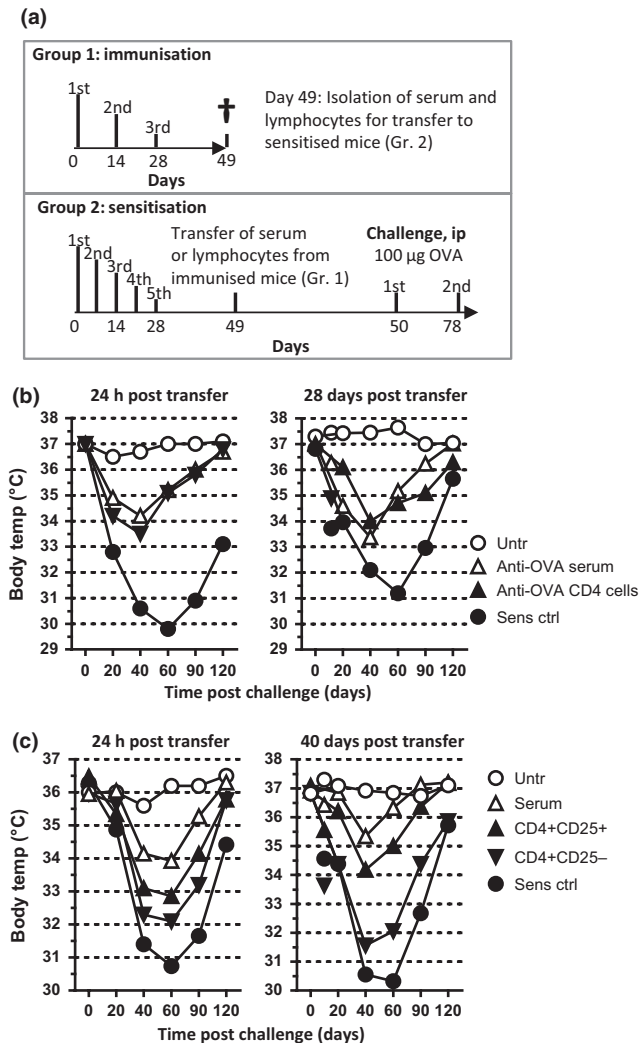


Fig. 4. Adoptive transfer of immunity from immunized mice to sensitized mice. (a) Scheme illustrating immunization (group 1) and sensitization (group 2) for testing the effect of serological and cellular factors on the protection against anaphylaxis in mice. One group of BALB/c mice (group 1) was subcutaneously immunized with ovalbumin (OVA) on alum and CpG. Another group of BALB/c mice (group 2) was intraperitoneally sensitized with OVA and alum. Three weeks after the last of three sensitizations, mice from group 1 were killed. Serum and CD4 lymphocytes were purified and transferred intravenously to the sensitized mice of group 2. Naïve mice were used as untreated controls (Untr) and sensitized mice that did not receive serum or lymphocytes served as sensitized controls (Sens ctrl). (b) Twenty-four hours and 28 days after the transfer, the mice were challenged with OVA and the body temperature monitored. (c) The experiment was repeated with transfer of CD4 lymphocytes separated into CD25-positive and CD25-negative CD4 T cells. Twenty-four hours and 40 days after the transfer, the mice were challenged with OVA and the body temperature monitored.

[†]Symbolising time point of death.

Discussion

The world experienced an epidemic rise of allergic rhinitis and asthma during the last quarter of the 20th

century [2], and these IgE-mediated diseases have been associated with societal and lifestyle changes. As a consequence, there are growing needs to reduce the escalating personal, social and economic costs of allergy. While risk factors have been identified [20, 21], no action is taken until manifestation of allergic symptoms become evident. Then, pharmacological treatment is typically given. Alternatively, a few patients chose to receive AIT, which can provide protection against later exposure to the allergen, but which is extremely the time-consuming.

Despite the knowledge of risk factors, prophylactic allergen vaccines are not offered and are barely considered [4] or studied although the similarities with childhood vaccines should make such an approach evident [22]. If prophylactic vaccines would be administered before the children are exposed to allergens or to any of the many risk factors that should facilitate sensitization, this would allow the development of protective immunity or tolerance during a time period when the immune system still has plasticity enough to produce protective immunity; such immunity is typically characterized by IgG production and stimulation of T helper type 1 (Th1) CD4 T cell responses. In sensitized children, the immune system is already committed to IgE production and a pool of Th2 memory cells that would control immune responses to the allergen throughout life. For that reason, AIT must be maintained for several years to allow reprogramming of the immune system. In contrast, childhood vaccination could immediately generate a pool of IgG-producing and memory B cells as well as Th1 memory cells. Subsequent and natural allergen exposure would then not easily be able to reprogramme the immune-response phenotype towards Th2 and IgE production.

To test paediatric allergy vaccines experimentally, we applied a murine model of anaphylaxis and found that immune responses induced by immunisation protected mice against later sensitization to the same allergens. While allergic mice reacted with anaphylaxis within few minutes of the allergy provocation, immunized mice were markedly protected. Hence, immunological priming of protective allergen-specific immune responses early in life is feasible. Similar studies in mice have shown a Th1-biased immune responses of DNA [23, 24] and mRNA [25, 26] vaccination with protective effects on allergic airway inflammation. Also prenatal exposure to aerosolized allergen can prevent allergen-induced sensitization and airway inflammation [27].

As potentially atopic children may be susceptible to many allergens, childhood vaccines should comprise several allergens. The allergens most frequently associated with allergic rhinitis are grass and tree pollens,

pet allergens, house dust mites, cockroaches and moulds [28, 29]. In Europe and USA, grass (*Phl p 1*), birch (*Bet v 1*), ragweed (*Amb a 1*), house dust mite (*Der p 1* & *Der p 2*) and cat (*Fel d 1*) are species that account for a majority of all IgE-mediated perennial and seasonal allergies. One important pharmaceutical issue of combination vaccines would therefore be if the mixing of allergens influences the performance of any of the individual allergens. Hence, various polyvalent combination vaccines, including a pentavalent vaccine, were prepared and tested. Mono-, di- and pentavalent vaccines stimulated comparable immune responses against the OVA or cat fur allergen, which were representatively analysed. Early vaccination with any of the cocktail vaccines stimulated long-lasting protection against OVA- or cat fur allergen-induced anaphylaxis. Hence, the mixing of several allergens in one vaccine does not seem to notably affect the immunogenicity of each individual allergen. This is important and should simplify the pharmaceutical development and standardization of childhood allergy vaccines.

Gender has long been considered a risk factor for allergies. Men are more susceptible to infections, while females are at greater risk of illnesses caused by an overactive immune system, such as autoimmunity and allergy. This has in part been ascribed to sex steroid hormones. Oestrogens can have potential effects on antigen presentation, Th2 polarization, IgE isotype switching and mast cell degranulation [30]. In line with this, we found that allergy vaccines produced a higher level of IgG2a and IFN- γ in male mice than in female mice, a result that may explain the slightly lower degree of anaphylaxis in males than in females.

The mechanism of AIT has been ascribed to the stimulation of allergen-neutralizing antibodies as well as regulatory and Th1-type T cells. The current experiments revealed that also in prophylactic vaccines, both serological and cellular factors can provide protection. When sensitized mice were pre-treated by systemic administration of antiserum, CD25-positive CD4 T cells or CD25-negative CD4 T cells from immunized mice, they were protected from allergic anaphylaxis. In line with this, that splenocytes from immunized mice produced larger amounts of the Th1 cytokine IFN- γ than did cells from sensitized mice, when the cells were restimulated with allergen in vitro. However, while serum and CD25-positive CD4 T cells produced lasting protection, the protection by CD25-negative CD4 T cells was only transient. This may suggest that the half-life of Treg cells is longer than that of Th1 cells. The protective role of antibodies was furthermore supported

by other results showing that the titres of IgG1 and IgG2a in immunized mice rose tenfold higher than the titres in sensitized mice. Indeed, a positive correlation between IgG titres and protection was found. In contrast, immunization had no crucial effect on the stimulation of IgE antibodies, the end titres being similar in sensitized mice and in immunized plus sensitized mice. Hence, while immunization had a strong impact on the clinical outcome of systemic anaphylaxis as measured after a high-dose challenge with the allergen, IgE was not a factor importantly regulated by immunization.

Whereas earlier recommendations suggested that allergen avoidance in early childhood reduced the risk of later sensitization, allergen avoidance is now even considered to increase the risk of sensitization [31]. Moreover, there is growing evidence that diet and gastrointestinal exposure in pregnancy and the early postnatal period can modify gene expression and disease susceptibility [32, 33]. In line with this, several clinical observations suggest that recurring exposure to allergens during early life can lead to the development of allergen-specific tolerance [22]. Most probably, gastrointestinal or intranasal uptake of aeroallergens may promote local IgA immune responses, which then further promote systemic tolerance.

In closing, it is a fact that the prevalence of IgE-mediated allergies has reached a level of socio-economic dimensions. A scientifically and economically rational way to combat this problem would be to offer prophylactic allergy vaccines, similar to prophylaxis against childhood infections. However, there will be ethical and personal constraints that will complicate a general recommendation of allergy vaccination, for example a justification that the benefits considerably outweigh any risk-related issues of vaccination, for which reason further research is required. Nonetheless, with the current knowledge of sensitization risks and mechanisms of immune-response stimulation and regulation, it is the reason to believe that allergy vaccination will once become one measure to fight the epidemic nature of allergen-related diseases.

Acknowledgements

Dr. Yuan Liang was supported with a grant from the Swiss Confederation Scholarship Exchange Programme (*Stipendium der Schweizerischen Eidgenossenschaft*). Mrs. Franziska Zabel provided experimental aid.

Conflict of interest

The authors disclose no conflict of interest.

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