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Intranasal Immunotherapy Is More Effective Than Intradermal Immunotherapy for the Induction of Airway Allergen Tolerance in Th2-Sensitized Mice[1]

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Immunotherapy (IT) by injection more readily induces clinical tolerance to stinging insects than to respiratory allergens. However, while systemic immunization induces adaptive responses systemically, the induction of mucosal immunity generally requires local Ag exposure. Taken together, these observations suggest that the poor success rate of systemic IT for asthma could be a consequence of inadequate immune modulation in the airways. In support of this position, investigations presented in this report demonstrate that allergen IT more effectively induces airway allergen tolerance in Th2-sensitized mice, when delivered by the intranasal (i.n.) vs the intradermal (i.d.) route. Moreover, compared with native allergen, allergen immunostimulatory sequence oligodeoxynucleotide conjugate proved to be a more effective i.n. IT reagent for protecting allergic mice from airway hypersensitivity responses. Furthermore, for both native allergen and allergen immunostimulatory sequence oligodeoxynucleotide conjugate, i.n. and i.d. IT delivery were similarly effective in modulating systemic immune profiles in Th2-sensitized mice, while only i.n. IT had significant immunomodulatory activity on B and T cell responses in the airways. The present investigations may be the first to suggest that i.n. IT is more effective than i.d. IT for the treatment of asthma. Furthermore, our results suggest that modulating airway rather than systemic immunity may be the more important therapeutic target for the induction of clinical tolerance to respiratory allergens. ***The Journal of Immunology*****, 2003, 170: 3898–3905.**

Allergen immunotherapy (IT)[3] was initially reported to be effective for the desensitization of pollen allergic patients by Noon in 1911 (1), and for many years IT was a first-line therapy for respiratory allergic diseases (2). However, in the last decade, IT has gradually fallen out of favor, in large part due to its limited scope of efficacy and its risk of eliciting rare but life-threatening anaphylactic reactions (2–5). IT by injection induces clinical tolerance in >90% of patients with hypersensitivities to stinging insects but only 30–50% of patients with allergic rhinitis are able to discontinue medications (2, 6–8). Moreover, several studies suggest that systemic IT does not improve outcomes for asthma patients receiving appropriate medications and environmental interventions to reduce allergen exposure (3, 4).

In consideration of the higher success rate of systemic IT for inducing clinical tolerance to stinging insects vs aeroallergens, it should be noted that immunization (Th2 sensitization) by injection induces robust adaptive immune responses at systemic sites, but mucosal immune responses are generally very weak (9–11). In contrast, under appropriate conditions, Ag exposure via the airways can induce adaptive immune responses both at systemic and mucosal sites (9–14). Therefore, hypersensitivities to stinging insects are likely to be maintained primarily by systemic lymphocytes, while lymphocytes residing in the airway mucosa and associated lymphoid organs probably play a more important role in the maintenance of airway hypersensitivities and the clinical manifestations of asthma. If these assertions are correct, then the increased efficacy of systemic IT for stinging insect allergens compared with aeroallergens may be explained, at least in part, by targeting of the appropriate immune compartment in the former but not the later hypersensitivity state.

Given that immunological events in the lungs are likely to play a central role in the pathogenesis and maintenance of the asthmatic phenotype and the semiautonomous nature of the mucosal immune response, there has been long-standing interest in the use of intranasal (i.n.) IT for the treatment of allergic rhinitis and asthma. Several animal and human studies have already shown that i.n. IT can attenuate airway hypersensitivities (15–18). However, no controlled comparative studies of systemic and i.n. IT have been undertaken to date. Furthermore, clinical studies demonstrate that IT delivered by respiratory routes can readily induce an asthmatic response, raising concerns about the safety of i.n. IT, particularly for asthmatic patients (19, 20).

The present series of investigations were conducted to compare the effectiveness of i.n. and intradermal (i.d.) IT for the induction of tolerance to airway allergen exposure in Th2-sensitized mice. First, we compared i.n. and i.d. IT with a simple protein allergen. Immunostimulatory sequence oligodeoxynucleotide (ISS) has proven to be a powerful vaccine adjuvant for the prevention and reversal of allergic hypersensitivities (21), and allergen ISS conjugates (AICs; allergens physically linked to ISS) are particularly

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[3] Abbreviations used in this paper: IT, immunotherapy; i.n., intranasal; i.d., intradermal; ODN, oligodeoxynucleotide; ISS, immunostimulatory sequence ODN; AIC, allergen ISS conjugate; M-ODN, mutated control ODN; OVA:ISS, OVA conjugated to ISS; OVA:M-ODN, OVA conjugated to M-ODN; BALF, bronchoalveolar lavage fluid; DAB, diaminobenzidine; BLN, bronchial lymph node.

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promising IT reagents, because they have proven to be more immunogenic than allergen mixed with ISS and are less allergenic in models of IgE-dependent hypersensitivity (11, 22–25). Therefore, we also evaluated the potential of i.n. and i.d. AIC IT to protect against airway allergen challenge responses in Th2-sensitized mice. Finally, Th2-sensitized mice were i.n. challenged with allergen, a mixture of allergen and ISS, or AIC, to compare their potential to directly induce airway hypersensitivity responses.

Our results demonstrate that, for the IT reagents studied, i.n. delivery was consistently more effective than i.d. delivery for inducing airway allergen tolerance in Th2-sensitized mice. In addition, although i.n. and i.d. IT were similarly effective in modulating systemic immune parameters, only i.n. IT induced significant changes in the immunological status of the lungs of allergic mice. With respect to the efficacy of the reagents studied, allergen by itself was less effective than allergen mixed with ISS, and AIC was the most effective i.n. IT intervention for inducing airway allergen tolerance, as well as for inducing immunological changes in Th2-sensitized mice. Finally, when their direct asthmagenic potentials were compared, i.n. challenge with allergen delivered by itself induced the most robust airway hypersensitivity response in Th2-sensitized mice, followed by allergen mixed with ISS, and AIC induced the weakest airway hypersensitivity response. Taken together, these results highlight the importance of targeting airway immunity in the reversal of airway allergen hypersensitivities as well as the usefulness of AIC technology for the development of i.n. IT reagents.

Materials and Methods

Reagents

Grade VI OVA was purchased from Sigma-Aldrich (St. Louis, MO), and phosphorothioate ISS (5′-TGACTGTGAACGTTCGAGATGA-3′) and mutated control oligodeoxynucleotide (M-ODN; 5′-TGACTGTGAAGGTTAGAGATGA-3′) were synthesized by Trilink Biotechnologies (San Diego, CA). The ISS and M-ODN selected for study have previously been shown to have high and low immunostimulatory activity, respectively (22). To produce OVA conjugated to ISS (OVA:ISS) and to M-ODN (OVA:M-ODN), maleimido groups were introduced onto exposed lysines of the OVA molecule by incubation with a 20 M excess of sulfosuccinimidyl 4-(*N*-maleimidomethyl)-cyclohexane-1-carboxylate (Pierce Chemicals, Rockford, IL) for 2 h, followed by purification on a NAP-25 column (Amersham Pharmacia, Uppsala, Sweden). A 5′ thiol group was added to the oligodeoxynucleotides (ODNs) by incubation with 20 μM tricarboxyethylphosphine (Pierce Chemicals), and activated ODNs were subsequently purified on a NAP-10 column. Maleimido-modified OVA and thiol-activated ODNs were incubated together overnight at room temperature. Crude OVA:ISS and OVA:M-ODN conjugate mixtures were then loaded on a Superdex 200 (10/30) gel filtration column attached to fast protein liquid chromatography to obtain fractions with an average OVA to ODN conjugation ratio of 1:3.5.

Sensitization and immunization protocols

Five- to 6-wk-old BALB/c female mice were Th2 sensitized by s.c. injection of OVA (25 μg) and alum (1 mg) in a volume of 50 μl of normal saline at weekly intervals for 3 wk. Two weeks after the last sensitization, mice received IT with OVA, OVA:ISS or OVA:M-ODN (10 μg based on OVA content), ISS alone (5.2 μg, equivalent to amount in 10 μg of OVA:ISS), or OVA mixed with ISS delivered i.d. (50 μl of PBS) or i.n. (30 μl of PBS divided equally between nares). Mice receiving i.n. IT were lightly anesthetized before each dose (isoflurane; Abbott Laboratories, North Chicago, IL). A total of four weekly doses were given to each mouse receiving IT.

Asthma challenge protocol

In studies of airway allergen tolerance induction (Figs. 1–4), allergen challenges were initiated 8 wk after the completion of IT. For allergenicity studies (Fig. 5), mice were airway allergen challenged 2 wk after the last OVA/alum sensitization. The airway allergen challenge consisted of two i.n. OVA challenges (5 μg in 30 μl PBS divided equally between nares) spaced 5 days apart. Mice were anesthetized before i.n. allergen delivery. Twenty-four hours after the last airway allergen challenge, mice were exposed to increasing concentrations of nebulized methacholine and bronchial resistance was measured (Penh) by whole-body plethysmography, as previously described (26). After methacholine challenge, mice were sacrificed and bronchoalveolar lavage was conducted with 0.8 ml of PBS delivered by tracheal catheter. Aliquots of bronchoalveolar lavage fluid (BALF) were analyzed with a hemocytometer and light microscope to determine total cell counts, and the rest of each BALF sample was centrifuged (700 $\times$ *g* for 5 min). BALF supernatants were saved for analysis of OVA-specific IgA and IL-5 content, while BALF cell pellets were resuspended in 1 ml of PBS, cytospun onto slides, and Wright-Giemsa stained. BALF cell differential percentages for eosinophils, lymphocytes, macrophages, and neutrophils were determined by a blinded observer counting a minimum of 200 cells in random high-power fields using a light microscope (×400 magnification). Absolute eosinophil, lymphocyte, macrophage, and neutrophil counts were calculated by multiplying total cell counts by the percentage of each cell type found in BALF exudates. The left lower lung segment of each mouse was embedded in OCT in 10 $\times$ 50 $\times$ 50-mm tissue wells, cryosectioned, and acetone-fixed onto poly(L-lysine)-coated slides. Tissue sections were then stained with eosinophil peroxidase substrate (diaminobenzidine (DAB) stain), counterstained with hematoxylin, and mononuclear cell and eosinophil infiltration of lung tissue was scored on a four-point scale by a blinded observer.

Ab and cytokine analysis

Blood samples were obtained 1 day before beginning airway allergen challenges for ELISA analysis of OVA-specific IgG2a, IgG1, and IgE in sera, and BALF samples were obtained for IgA determination 24 h after challenge, as previously described (11, 27). All samples were compared with high titer anti-OVA IgG1, IgG2a, IgE, and IgA standards (end-point titration $\geq$ 50,000 for IgG1, IgG2a, and IgA, and equal to 1,024 in the case of IgE). These standards were given arbitrary concentration units of 40,000 for IgA, 20,000 for IgG1 and IgG2a, and 10,000 for IgE. For IgG1, IgG2a, and IgA analysis, 96-well plates were coated with 5 μg/ml OVA (Sigma-Aldrich) in 50 μl of borate-buffered saline (BBS; pH 9.2) overnight at 4°C. Plates were then blocked with 1% BSA in BBS at 37°C for 2 h, washed with BBS/0.5% Tween 20 (Sigma-Aldrich), and incubated with standards and serial dilutions of samples overnight at 4°C. Plates were then incubated with alkaline phosphatase-linked anti-IgG1, -IgG2a, or -IgA (Southern Biotechnologies, Birmingham, AL) at a 1/2000 dilution, washed, and then incubated with *p*-nitrophenyl phosphate (2.63 mg/ml; Boehringer Mannheim, Indianapolis, IN). Absorbance at 405–650 nm was read at 1 h and compared with a standard curve on each plate using the DeltaSOFT II version 3.66 program (Biometallics, Princeton, NJ). Ag-specific IgE was determined in a manner analogous to that described for the other Ig isotypes. However, to remove IgG and thereby improve the sensitivity of the IgE assay, serum samples were incubated overnight with protein G-Sepharose beads according to the manufacturer's recommendations (Pharmacia, Piscataway, NJ) before IgE ELISA. Protein G-absorbed 1/10 and 1/40 dilutions of sera were added to OVA-coated and BSA-blocked ELISA plates, which in turn were incubated overnight (4°C) followed by incubation with goat biotinylated anti-mouse IgE at 8 μg/ml (BD PharMingen, San Diego, CA) overnight (4°C). ELISA plates were then incubated with HRP-linked streptavidin at a 1/2000 dilution (Zymed, San Francisco, CA) and then 3,3′,5,5′-tetramethylbenzidine substrate (Kirkegaard & Perry Laboratories, Gaithersburg, MD). The color reaction was stopped with an equal volume of 1 M phosphoric acid. Absorbance at 450–650 nm was then read and compared with the standard curve on each plate using the DeltaSOFT II program.

Cytokine responses were determined as previously described (11, 27). Splenocytes were prepared from airway allergen-challenged mice by gently teasing spleens to make single-cell suspensions. Bronchial lymph node (BLN) mononuclear cells were prepared by careful dissection of BLNs (pooled from four mice per group) followed by incubation in digestion medium (collagenase VIII (300 U/ml) and DNase 1 (1.5 μg/ml); Sigma-Aldrich) for 1 h. Single-cell suspensions of BLN cells were then obtained by pouring digests over fine nylon sieves. After washing, cell viability was always >95%. Splenocytes and BLN cells were cultured at 2.5×10^6 cells/ml in RPMI 1640 supplemented with 10% heat-inactivated FCS, 2 mM glutamine, 0.05 mM 2-ME, and 1% penicillin-streptomycin (complete medium) with or without OVA (50 μg/ml) for 72 h before harvesting supernatants. IL-5, IL-10, and IFN-γ levels in culture supernatants were determined by ELISA, using capture and biotinylated detecting Abs from BD PharMingen. Washing and blocking steps were analogous to those used in the Ig ELISA described previously. Detection of biotinylated

secondary Ab was achieved by adding 1/2000 diluted HRP-labeled streptavidin (Zymed) to ELISA plates, followed by 3,3′,5,5′-tetramethylbenzidine substrate (Kirkegaard & Perry Laboratories). The color reaction was stopped with an equal volume of 1 M phosphoric acid and absorbance was read at 450–650 nm. Standard curves were generated using known amounts of recombinant IL-5, IL-10, and IFN-γ (BD PharMingen). Each supernatant was compared with the standard curve on the plate to quantitate cytokine levels using the DeltaSOFT II program.

Statistics

Statistical analyses were conducted using Statview software (Abacus Concepts, Berkeley, CA). Two-tailed unpaired Student's *t* tests were conducted to analyze all data except for methacholine hypersensitivity, for which ANOVA was used. Results were considered statistically significant if *p* values were ≤ 0.05. For statistical analyses involving multiple comparisons, the Bonferroni correction was used to account for the increased probability of type I errors when multiple groups are statistically compared.

Results

Intranasal allergen IT is more effective than i.d. allergen IT for the prevention of airway hypersensitivity responses in allergic mice

In an initial series of investigations, we determined whether targeting airway immunity with i.n. allergen IT would be more effective than i.d. allergen IT in protecting Th2-sensitized mice from airway hypersensitivity responses. Mice were initially Th2 sensitized with OVA and alum and then received IT with OVA either by the i.n. or i.d. route (four weekly IT doses). Eight weeks after finishing IT, mice were i.n. OVA challenged and asthmatic responses were assessed. Mice receiving OVA IT via the i.n. route had a markedly attenuated airway hypersensitivity response compared with mice receiving i.d. OVA IT, as reflected in a significant decrease in methacholine sensitivity (Fig. 1*a*; $p < 0.05$), a >80% decrease in BALF IL-5 post-airway allergen challenge (Fig. 1*b*; $p < 0.05$), >60% decreases in both total cells and eosinophils recovered from BALF (Fig. 1*c*; $p < 0.05$ for both comparisons), and a 35% reduction in lung inflammation (Fig. 1*d*; $p < 0.05$).

Intranasal and i.d. allergen IT have similar effects on systemic immunity but divergent effects on airway immunity in allergic mice

Previous work has demonstrated the induction of immunological tolerance with i.n. allergen delivery, in both naive and Th2-sensitized mice (15, 28–31). Therefore, we determined whether i.n. and i.d. OVA IT had differential effects on the systemic and mucosal immune status of allergic mice. Serum was collected from mice 1 day before the first airway allergen challenge, while BALF and BLN and splenic mononuclear cells were obtained at sacrifice, to determine OVA-specific Ig levels and cytokine responses. Evaluation of the systemic immune compartment demonstrated no significant differences between mice receiving i.n. and i.d. OVA IT (Fig. 2*a*). However, allergic mice treated with i.n. OVA IT had a >10-fold increase in BALF IgA (Fig. 2*b*; $p < 0.05$) compared with mice receiving i.d. OVA IT. In addition, while IFN-γ and IL-10 responses from BLNs were relatively weak for both groups, i.n. OVA IT also inhibited BLN production of IL-5 by >85%, compared with i.d. OVA IT (Fig. 2*b*; $p < 0.05$).

Intranasal AIC IT is more effective than i.d. AIC IT for the prevention of airway hypersensitivity responses in allergic mice

Previous investigations have demonstrated that AIC is significantly more immunogenic than allergen alone or allergen mixed with ISS (11, 22–25). Therefore, in parallel with studies of i.n. and i.d. OVA IT, we compared the anti-asthmatic potential of i.n. and i.d. OVA:ISS IT. In these experiments, Th2-sensitized mice treated with i.n. OVA:ISS IT had a significant reduction in methacholine sensitivity (Fig. 3*a*; $p < 0.05$), a >90% decrease in BALF IL-5 post-airway allergen challenge (Fig. 3*b*; $p < 0.05$), a >60% decrease in total cells and a >80% decrease in eosinophils recovered from BALF (Fig. 3*c*; $p < 0.05$ for both comparisons), and a >50% reduction in lung inflammation (Fig. 3*d*; $p < 0.05$), compared with mice receiving i.d. OVA:ISS IT. In fact, when compared with allergic mice receiving no IT, mice receiving i.d. OVA:ISS IT had only modestly attenuated airway hypersensitivity responses with significant differences in outcome measures noted only for methacholine sensitivity and BALF total cell and eosinophil counts ($p < 0.05$ for these comparisons). Moreover, mice receiving i.n. IT with OVA or OVA:M-ODN were generally better protected from asthma than mice receiving i.d. OVA:ISS IT (Fig. 3 vs Fig. 1). However, in comparison to i.n. OVA, and i.n. OVA:M-ODN IT, i.n. OVA:ISS IT was significantly more effective in protecting allergic mice from airway allergen challenge ($p < 0.05$ for comparisons of methacholine sensitivity, BALF IL-5, total cells and eosinophils in BALF, and lung inflammation).

As allergen-independent delivery of ISS has also been shown to protect Th2-sensitized mice from asthma for up to 4–6 wk, a control group of mice was treated with i.n. ISS alone (26). However,

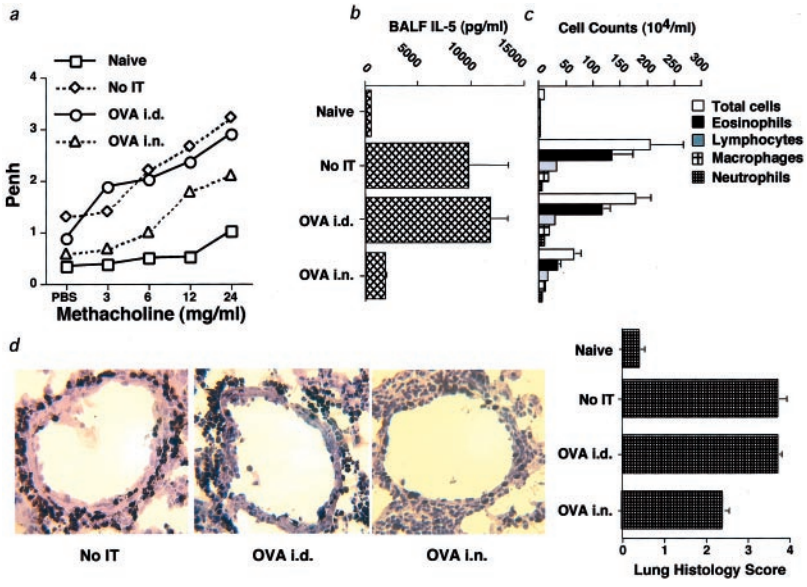

FIGURE 1. Airway allergen challenge outcomes in allergic mice receiving i.n. or i.d. OVA IT. Mice (n = 4 per group) were sensitized with OVA and alum before receiving four weekly doses of OVA IT via the i.n. or i.d. route. Eight weeks after the last IT dose, mice received two i.n. challenges with OVA spaced 5 days apart. The data presented in this composite figure are representative of three replicate experiments and bar graph data are presented as means ± SE. *a*, Twenty-four hours after the last airway allergen challenge, mice were exposed to increasing concentrations of nebulized methacholine and bronchial responses were measured. *b*, IL-5 content of post-allergen challenge BALF. *c*, Total cell counts, and absolute eosinophil, neutrophil, lymphocyte, and macrophage counts in post-allergen challenge BALF. *d*, Lung tissue was harvested after allergen challenge and stained with eosinophil peroxidase substrate (DAB stains eosinophils brown) and hematoxylin. Pulmonary inflammation was then scored on a four-point scale by a blinded observer.

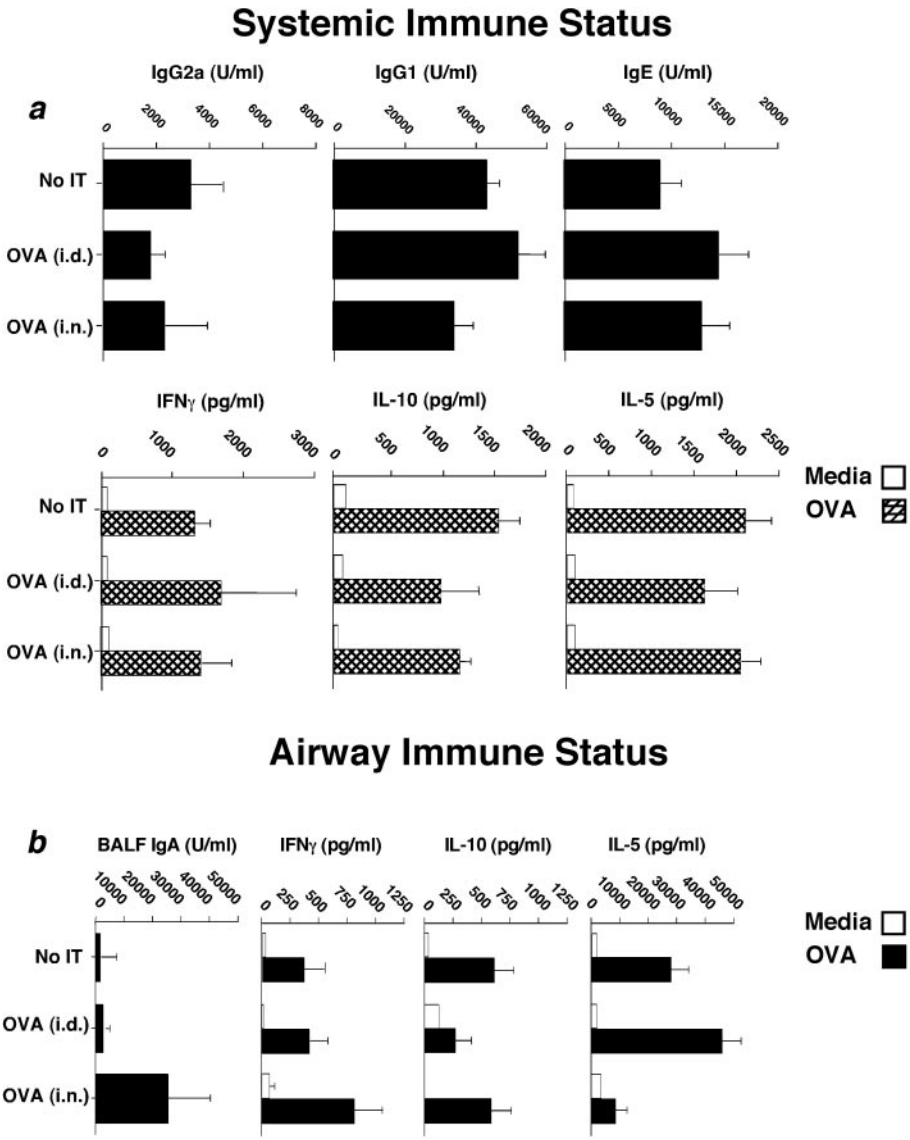

FIGURE 2. Immunomodulation in allergic mice receiving i.n. or i.d. OVA IT. One day before initiating the first of two airway allergen challenges, mice described in Fig. 1 were bled, and at sacrifice, BALF samples were obtained for Ig determinations. Splenocytes and BLN mononuclear cells were harvested and cultured with and without OVA, and day 3 culture supernatants were used to determine OVA-specific cytokine responses. Bar graph data are presented as means ± SE for four mice, except for cultures with BLN mononuclear cells, in which pooling from the four mice per group was required to have adequate numbers of cells for three replicate cultures. *a*, Systemic immune parameters of allergic mice receiving i.n. and i.d. OVA IT. *b*, Mucosal immune parameters of allergic mice receiving i.n. and i.d. OVA IT.

8 wk after the last dose, mice treated with ISS alone had asthma challenge outcomes that were comparable to those of Th2-sensitized mice that were not treated with IT (Fig. 3). To determine whether conjugation of OVA to ISS contributed to the efficacy of OVA:ISS as an i.n. IT reagent, a group of OVA (Th2)-sensitized mice received i.n. IT with OVA mixed with ISS (OVA plus ISS) at doses equivalent to those received with i.n. OVA:ISS IT. Our results demonstrated that i.n. IT with OVA plus ISS was generally more effective in protecting against airway hypersensitivity responses than other i.n. IT interventions. However, i.n. OVA:ISS IT was consistently more effective than i.n. OVA plus ISS IT in attenuating airway allergen challenge outcome measures in allergic mice with differences in BALF total cell and eosinophil counts and lung inflammation reaching statistical significance ($p < 0.05$).

Intranasal and i.d. OVA:ISS IT have similar effects on systemic immunity but divergent effects on airway immunity in allergic mice

As Th2-sensitized mice receiving i.n. OVA:ISS IT had significantly attenuated airway hypersensitivity responses compared with mice receiving i.d. OVA:ISS IT, we compared their underlying immunological status. However, mice receiving i.n. and i.d. OVA:ISS IT had no significant differences in their systemic immune profiles. Nonetheless, compared with mice receiving no IT, mice receiving i.n. and i.d. OVA:ISS IT had >40% reductions in OVA-specific serum IgG1 (not statistically significant) and >40% reductions in OVA-specific serum IgE ($p < 0.05$ only for i.n. OVA:ISS IT), and their splenocytes produced >85% less OVA-specific IL-5 ($p < 0.05$ for both groups) compared with mice receiving no IT (Fig. 4*a*). In addition, Th2-sensitized mice receiving OVA:ISS by both the i.d. and i.n. routes had 4- to 8-fold increases in OVA-specific serum IgG2a levels ($p < 0.05$ for both groups) and their splenocytes produced >3-fold more OVA-specific IFN-γ ($p < 0.05$ only for i.n. OVA:ISS IT) and >3-fold more OVA-specific IL-10 ($p < 0.05$ for both groups) compared with mice receiving no IT. It is particularly noteworthy that, unlike i.d. OVA:ISS IT, i.n. OVA:M-ODN IT did not modulate systemic immune parameters in Th2-sensitized mice (Fig. 4), as i.n. OVA:M-ODN IT was generally more effective than i.d. OVA:ISS IT in protecting against airway allergen challenge responses (Fig. 3). i.n. OVA plus ISS IT induced shifts in systemic immune parameters analogous to those seen with i.n. and i.d. OVA:ISS IT, but systemic immune modulation was generally less robust.

The divergent airway allergen challenge outcomes of allergic mice treated with i.n. and i.d. OVA:ISS IT (Fig. 3) could not be explained by divergent effects on systemic immunity (Fig. 4*a*).

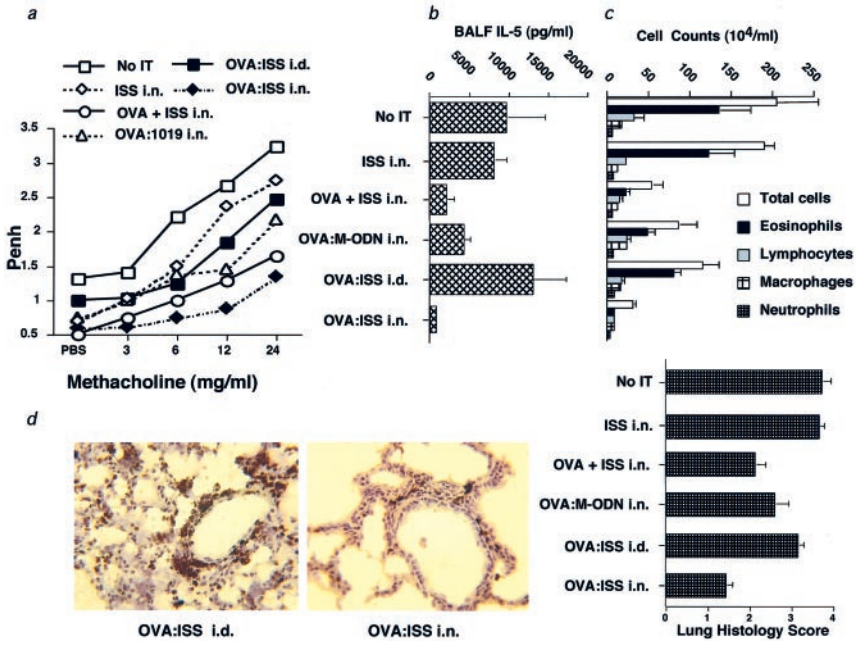

FIGURE 3. Airway allergen challenge outcomes in allergic mice receiving i.n. or i.d. OVA:ISS IT. Mice ($n = 4$ per group) were sensitized with OVA and alum before receiving four weekly doses of OVA:ISS IT via the i.n. or i.d. route. Control mice received i.n. OVA:M-ODN, ISS alone, or no IT. Eight weeks after the last IT dose, mice received two i.n. challenges with OVA spaced 5 days apart. The data presented in this composite figure are representative of three replicate experiments, and bar graph data are presented as means ± SE. *a*, Twenty-four hours after the last airway allergen challenge, mice were exposed to increasing concentrations of nebulized methacholine and bronchial responses were measured. *b*, IL-5 content of post-allergen challenge BALF. *c*, Total cell counts, and absolute eosinophil, neutrophil, lymphocyte, and macrophage counts in post-allergen challenge BALF. *d*, Lung tissue was harvested after allergen challenge and stained with eosinophil peroxidase substrate (DAB stains eosinophils brown) and hematoxylin. Pulmonary inflammation was then scored on a four-point scale by a blinded observer.

Therefore, we also compared the pulmonary immune profiles of these mice (Fig. 4*b*). Mice receiving i.n. OVA:ISS IT developed 20-fold or greater increases in OVA-specific BALF IgA compared with mice treated with i.d. OVA:ISS IT ($p < 0.05$). Moreover, i.n. OVA:ISS IT induced ~3-fold increases in OVA-specific IFN-γ and IL-10 production and a >90% decrease in IL-5 production from harvested BLN mononuclear cells compared with i.d. OVA:ISS IT ($p < 0.05$ for all cytokines). Like i.n. OVA:ISS IT, i.n. OVA plus ISS and i.n. OVA:M-ODN IT were significantly more effective than i.d. OVA:ISS IT in inducing IgA responses in the respiratory mucosa of Th2-sensitized mice ($p < 0.05$ for both groups) and in inhibiting IL-5 production from their BLN mononuclear cells ($p < 0.05$ for both groups). However, unlike i.n. OVA:ISS IT, neither i.n. OVA plus ISS IT nor i.n. OVA:M-ODN IT induced significant IFN-γ or IL-10 responses from BLN mononuclear cells.

OVA elicits a much stronger airway hypersensitivity response than OVA:ISS in airway allergen challenge studies

We have previously demonstrated that OVA:ISS has significantly less allergenic potential than native OVA in Ig-dependent hypersensitivity models, such as models of anaphylaxis, and the Arthus reaction, whereas challenge with OVA and OVA mixed with ISS led to very similar outcomes in these hypersensitivity models (32). Mechanistic studies demonstrated that the reduced allergenicity of OVA:ISS compared with OVA and OVA mixed with ISS was due to masking of allergen Ig binding epitopes by OVA-conjugated ODNs and not due to the immunostimulatory activity of the ODNs. In contrast to results in Ig-dependent hypersensitivity models, unconjugated ISS has been shown to attenuate the late-phase asthmatic hypersensitivity response of Th2-sensitized mice undergoing airway allergen challenge (33). Therefore, we next compared the airway hypersensitivity responses of allergic mice i.n. challenged with OVA, OVA plus ISS, or OVA:ISS (Fig. 5). As in previous investigations, Th2-sensitized mice i.n. challenged with OVA plus ISS developed an attenuated airway hypersensitivity compared with mice i.n. challenged with OVA alone, as reflected in a decrease in methacholine sensitivity ($p < 0.05$) and an almost 3-fold decrease in BALF eosinophilia ($p < 0.05$). However, compared with OVA plus ISS, OVA:ISS was even less asthmagenic, as reflected in a further decrease in methacholine sensitivity ($p < 0.05$) and an additional 3-fold reduction in BALF eosinophilia ($p < 0.05$).

Discussion

IT injections have not proven as effective for aeroallergen as for stinging insect desensitization (2, 6–8). However, systemic immunization, even with adjuvants, induces weak adaptive immune responses in the airways, while i.n. immunization can elicit both systemic and mucosal responses (9–11). Based on these considerations, we hypothesized that i.n. IT would more effectively target airway immunity than i.d. IT and therefore might more effectively protect against allergic hypersensitivity responses in the airways. The results of our investigations support these suppositions and, to our knowledge, are the first to suggest that i.n. IT is more effective than i.d. IT for the induction of airway allergen tolerance in Th2-sensitized mice.

In initial investigations, OVA-allergic mice received a course of i.d. or i.n. IT with native OVA. Eight weeks after completing IT, mice were i.n. challenged with OVA and pulmonary outcome measures were evaluated. Allergic mice receiving i.n. OVA IT had markedly attenuated airway allergen challenge responses compared with mice receiving i.d. OVA (Fig. 1). Previous investigations have demonstrated that mice exposed to allergen via the nose before or after systemic Th2 sensitization developed immunological tolerance (mucosal tolerance) (15, 28–31). However, in our studies, allergic mice receiving i.n. or i.d. OVA IT had little change in their systemic immune profiles compared with those of mice receiving no IT. In contrast, mice receiving i.n. OVA IT developed higher levels of IgA in their airways and an attenuated BLN IL-5 response, compared with those of i.d. OVA IT-treated mice (Fig. 2). Therefore, it appears that while i.n. OVA IT reduced the capacity of the airways to develop a hypersensitivity response to i.n. allergen challenge, i.n. OVA IT did not induce a global attenuation of allergen-specific immunity in Th2-sensitized mice.

AIC has been found to be a more potent immunogen than native allergen, both for i.n. and i.d. vaccination, and Santeliz et al. and others (11, 22–25) have reported that i.d. AIC immunization was more protective than i.d. allergen immunization in a murine asthma model. Therefore, in parallel with studies of i.n. and i.d. OVA IT, we compared the antiasthmatic activities of OVA:ISS IT delivered via the

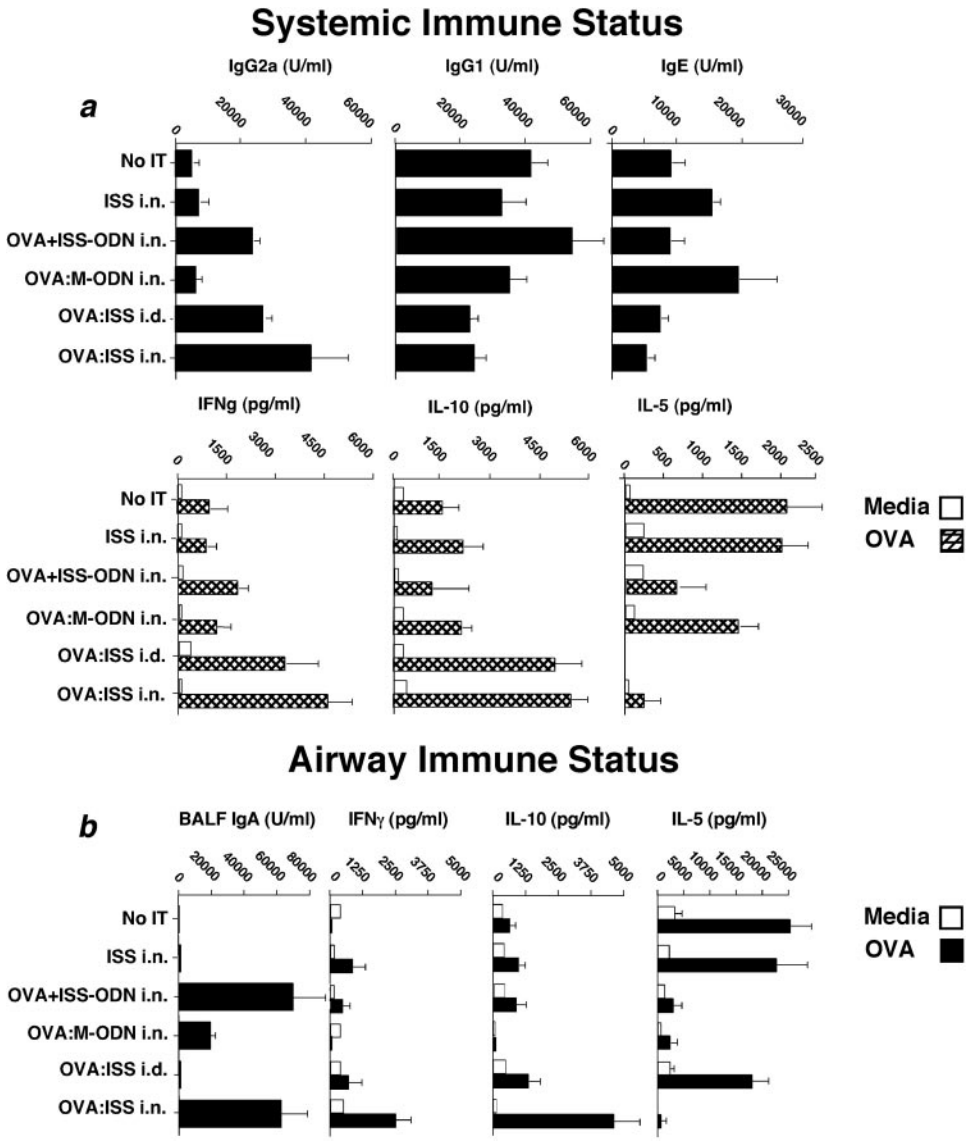

FIGURE 4. Immunomodulation in allergic mice receiving i.n. or i.d. OVA:ISS IT. One day before initiating the first of two airway allergen challenges, mice described in Fig. 3 were bled, and at sacrifice, BALF samples were obtained for Ig determinations. Splenocytes and BLN mononuclear cells were harvested and cultured with and without OVA, and day 3 culture supernatants were used to determine OVA-specific cytokine responses. Bar graph data are presented as means ± SE for four mice, except for cultures with BLN mononuclear cells, in which pooling from the four mice per group was required to have adequate numbers of cells for three replicate cultures. *a*, Systemic immune parameters of allergic mice receiving i.n. and i.d. OVA:ISS IT. *b*, Mucosal immune parameters of allergic mice receiving i.n. and i.d. OVA:ISS IT.

i.n. and i.d. routes. Intradermal OVA:ISS IT provided Th2-sensitized mice with limited protection against airway allergen challenge, but it was generally less effective than either i.n. OVA or i.n. OVA:M-ODN IT (Fig. 3 vs Fig. 1). In contrast, i.n. OVA:ISS IT was substantially more effective in inducing airway allergen tolerance than all other IT interventions tested, including i.d. OVA:ISS IT and i.n. IT with OVA mixed with ISS. Previous studies have shown that the innate immune response to ISS delivered alone provided allergic mice with allergen-independent protection from asthma for up to 4–6 wk (26). However, in the present studies, treatment with ISS alone did not protect Th2-sensitized mice from airway allergen challenge 8 wk after the last ISS dose (Fig. 3).

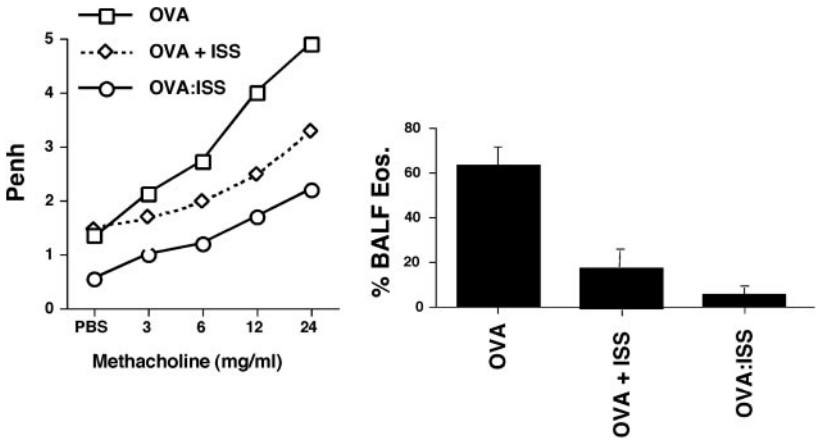

FIGURE 5. Asthmagenic potential of OVA, OVA plus ISS, and OVA:ISS in Th2-sensitized mice. Mice were Th2 sensitized with OVA and alum. Allergic mice then received two i.n. challenges with OVA, OVA plus ISS, or OVA:ISS spaced 5 days apart. Twenty-four hours after the last airway allergen challenge, mice were exposed to increasing concentrations of nebulized methacholine and bronchial responses were measured. Mice were then sacrificed and the percentage of eosinophils in BALF exudates was assessed.

Interestingly, despite their differential effectiveness in protecting OVA (Th2)-sensitized mice from asthma, i.n. and i.d. OVA:ISS IT were similarly effective in reducing allergen-specific serum IgE and splenic IL-5 responses and in inducing serum IgG2a, and splenic IFN-γ, and IL-10 responses (Fig. 4). However, consistent with results comparing i.n. and i.d. OVA IT, i.n. OVA:ISS IT induced a much stronger IgA response in the airways of allergic mice than did i.d. OVA:ISS IT. Furthermore, i.n. OVA:ISS IT was more effective than i.d. OVA:ISS IT in inhibiting allergen-specific IL-5 production and in inducing IFN-γ and IL-10 production from BLN mononuclear cells harvested from these mice.

The improved efficacy of i.d. OVA:ISS vs i.d. OVA IT against asthma (Fig. 3 vs Fig. 1) may well be explained by a marked attenuation of systemic Th2-biased immune profiles with the former but not the latter IT intervention (Fig. 4 vs Fig. 2). Moreover, effective allergen IT has been linked to the induction of allergen-specific IFN-γ and IL-10 responses in clinical trials (34, 35) and i.d. OVA:ISS IT induced OVA-specific production of these cytokines from cultured splenocytes, whereas i.d. OVA IT did not. However, i.n. OVA IT and i.n. OVA:M-ODN IT failed to modulate systemic immunity and yet these interventions were generally more effective than i.d. OVA:ISS IT in protecting allergic mice from asthma (Figs. 1 and 3). Taken together, these results suggest that reversal of Th2-biased immunity at systemic sites has only limited effectiveness in protecting allergic mice from airway allergen challenge and that the increased efficacy of i.n. vs i.d. IT in attenuating the airway hypersensitivity state cannot be explained by differential effects on systemic immunity.

In contrast to the relatively weak correlation between the antiasthmatic and systemic immunomodulatory effects of the IT interventions tested, their ability to modulate airway immunity correlated well with their ability to protect against airway allergen challenge. For example, i.n. OVA IT induced a significant increase in BALF IgA and decrease in BLN IL-5 production compared with i.d. OVA IT, and only i.n. OVA IT protected against allergic hypersensitivity responses in the airways. Moreover, i.n. OVA:ISS IT induced a 3-fold increase in airway IgA production compared with i.n. OVA and i.n. OVA:M-ODN IT and a 30-fold increase compared with i.d. OVA:ISS IT and was substantially more effective than these interventions in protecting allergic mice from airway hypersensitivity responses. Given that airway delivery of allergen-specific IgA mAb has previously been found to attenuate pulmonary hypersensitivity responses in Th2-sensitized mice (36), the local IgA response to i.n. IT may well contribute to its improved effectiveness over i.d. IT in protecting Th2-sensitized mice from i.n. allergen challenge. In further consideration of the improved efficacy of i.n. OVA:ISS IT compared with other IT interventions, i.n. OVA:ISS IT was found to induce the most robust IFN-γ and IL-10 responses from BLN mononuclear cells. Therefore, improved modulation of cellular immunity in the airways by i.n. OVA:ISS IT compared with other IT interventions may also contribute to its overall improved efficacy against the asthmatic phenotype.

One clinical concern that remains with i.n. IT is that, compared with systemic IT, it more readily induces asthmatic reactions in susceptible patients (19, 20). However, ISS conjugation has previously been shown to mask allergen Ig binding epitopes, and when compared with native OVA in anaphylactic challenge studies, OVA:ISS induced a much attenuated immediate hypersensitivity response (22). The present airway allergen challenge studies (Fig. 5) further demonstrate that OVA:ISS induces significantly weaker late-phase airway hypersensitivity responses than does native OVA in Th2-sensitized mice. In addition, these investigations suggest that both epitope masking by allergen-conjugated ODNs and the intrinsic antiasthmatic activities of ISS contribute to the low asthmagenic potential of OVA:ISS, because airway hypersensitivity responses to i.n. OVA plus ISS challenge were weaker than to i.n. OVA challenge, whereas i.n. OVA:ISS challenge responses were even more attenuated than with i.n. OVA plus ISS challenge. Consistent with the results presented in this report, recent clinical trial results demonstrate that AIC is less allergenic and more clinically effective than allergen extracts for systemic IT in allergic rhinitis patients (37, 38). Such results suggest that AIC technology may be particularly well suited for developing i.n. IT reagents for use in allergic patients.

It has yet to be determined why IT delivered by injection is more effective for inducing clinical tolerance to stinging insects than to aeroallergens. In demonstrating the improved efficacy of i.n. compared with i.d. IT, both for the modulation of airway immunity and the induction of airway allergen tolerance in Th2-sensitized mice, the present results further suggest that poor targeting of immunity in the lungs may provide at least a partial explanation. This series of investigations provide a strong rationale for the continued evaluation of i.n. IT for the treatment of asthma and allergic rhinitis. Furthermore, our findings suggest that modulation of airway rather than systemic immunity may be the more important therapeutic target for IT in the induction of clinical tolerance to respiratory allergens.

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