

Original article

Targeting the allergen to oral dendritic cells with mucoadhesive chitosan particles enhances tolerance induction

Background: Sublingual immunotherapy (SLIT) efficacy could be improved by formulations facilitating allergen contact with the oral mucosa and uptake by antigen-presenting cells (APCs).

Methods: Two types of chitosan microparticles, differing in size and surface charge, were tested *in vitro* for their capacity to improve antigen uptake and presentation by murine bone marrow-derived dendritic cells (BMDCs) or purified oral APCs. T-cell priming in cervical lymph nodes (LNs) was assessed by intravenous transfer of carboxyfluorescein diacetate succinimidyl ester-labelled ovalbumin (OVA)-specific CD4⁺ T cells and flow cytometry analysis. Ovalbumin-sensitized BALB/c mice were treated sublingually with soluble or chitosan-formulated OVA twice a week for 2 months. Airway hyperresponsiveness (AHR), lung inflammation and T-cell responses in cervical and mediastinal LNs were assessed by whole-body plethysmography, lung histology and Cytometric Bead Array technology, respectively.

Results: Only a mucoadhesive (i.e. highly positively charged) and microparticulate form of chitosan enhances OVA uptake, processing and presentation by murine BMDCs and oral APCs. Targeting OVA to dendritic cells with this formulation increases specific T-cell proliferation and IFN- γ /IL-10 secretion *in vitro*, as well as T-cell priming in cervical LNs *in vivo*. Sublingual administration of such chitosan-formulated OVA particles enhances tolerance induction in mice with established asthma, with a dramatic reduction of both AHR, lung inflammation, eosinophil numbers in bronchoalveolar lavages, as well as antigen-specific Th2 responses in mediastinal LNs.

Conclusions: Mucoadhesive chitosan microparticles represent a valid formulation for sublingual allergy vaccines.

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Sublingual immunotherapy (SLIT) is a noninvasive and efficacious treatment of type I respiratory allergies (1–4). Nonetheless, it can still be further optimized, for example with the aim to decrease treatment duration and simplify administration schemes. In addition, a current trend in allergen-specific immunotherapy is to replace biological extracts with highly purified allergens produced by recombinant DNA technology (5, 6). In this context, appropriate delivery systems are needed to improve allergen targeting to the oral immune system. Specifically, mucoadhesive formulations could increase SLIT efficacy by enhancing contact duration of the allergen with the oral mucosa (7). Moreover, the pertinence of particulate formulations to facilitate allergen uptake by oral antigen-presenting cells (APCs) prone to induce tolerance (8, 9) should be investigated, given that such a strategy has been successful in other settings to target APCs with phagocytic capacity (10–13).

In this study, we focused on chitosan, a polysaccharide derived from chitin by deacetylation, as a candidate formulation for sublingual vaccines. Chitosan is a natural polymer of glucosamine found in crustaceans, insects or mushrooms (14). As a biodegradable, well-tolerated molecule, it lacks any irritating or sensitizing properties and chitosan-based products have been approved by regulatory authorities for human utilization in cosmetic, health and environmental applications (15).

Herein, we tested two types of chitosan particles with distinct size and charge characteristics for their ability to facilitate allergen uptake by various murine APC populations, and further to enhance allergen-specific tolerance when administered sublingually to ovalbumin (OVA)-sensitized mice with asthma. Among chitosan-based polymers, highly mucoadhesive microparticles were the most efficient in targeting the allergen to APCs as well as reducing airway hyperresponsiveness (AHR), lung

inflammation and T helper 2 (Th2) responses in the murine SLIT asthma model.

Methods

Animals, culture medium and reagents

Six-week-old female BALB/c mice were purchased from Charles River (L'Arbresle, France) and maintained on an OVA-free diet. DO11.10 OVA-specific T-cell receptor (TCR) transgenic female mice with approximately 50% of their CD4⁺ T cells expressing a TCR specific for the peptide 323–339 fragment of OVA (16) were bred in the Centre d'Exploration et de Recherche Fonctionnelle Expérimentale (Evry, France). International levels of ethical standards were applied for animal handling.

Complete culture medium consisted of RPMI 1640 supplemented with 10% foetal calf serum, 1% L-glutamine, 200 U/ml penicillin and 200 µg/ml streptomycin (all from Invitrogen, Carlsbad, CA, USA). Recombinant murine GM-CSF and interleukin-4 (IL-4) were obtained from Gentaur (Brussels, Belgium). Phosphate-buffered saline (PBS) and alum were purchased from Lonza (Basel, Switzerland) and Pierce (Rockford, IL, USA), respectively. For desensitization purposes, OVA grade V with low endotoxin content was purchased from Sigma (St Louis, MO, USA) and further purified on an endotoxin removing gel (Pierce) as described elsewhere (17). Residual endotoxin concentrations determined by Endochrom-K assay (R1708K, Charles River, Wilmington, MA, USA) were always less than 0.1 EU/µg protein.

Preparation and characterization of chitosan-OVA particles

Ovalbumin-loaded particles were prepared from either high or medium molecular weight (MW) chitosan, ranging from 310 to 375 kDa and 190 to 310 kDa, respectively (references 419419 and 448877; Sigma). Briefly, 0.05 g chitosan was dissolved in 25 ml aqueous acetic acid solution (1%), the suspension was homogenized with a Ultra Turrax T18 homogenizer (Ika, Staufen, Germany) at 9500 rpm for 2 min and the pH adjusted to 5 with 1N NaOH. Subsequently, 30 ml of a 20 mg/ml OVA solution was added to chitosan at 0.75 ml/min under magnetic stirring at room temperature and cross-linking was performed after adding 10 ml of a 0.1% tripolyphosphate aqueous solution (Sigma). Particle size and charge (zeta potential) were analysed with a Zetasizer Nano ZS (Malvern, Worcestershire, UK).

Capture of chitosan-OVA particles by bone marrow-derived dendritic cells and purified oral APCs

Bone marrow-derived dendritic cell (BMDCs) were generated as previously described (18). For antigen uptake assays, 5×10^4 cells/ml were suspended in complete medium. Fluorescein isothiocyanate-labelled OVA (FITC-OVA) or chitosan-formulated FITC-OVA was added (25 µg/ml final concentration) and cells were incubated for 15, 60 or 240 min at 4 or 37°C. For antigen processing assays, DQ-OVA (Invitrogen), which fluoresces following proteolytic cleavage, was used. 5×10^4 /ml cells were suspended in complete medium and incubated for 3 h with 10 µg/ml DQ-OVA or chitosan-formulated DQ-OVA at 4 or 37°C. Cells were then washed twice with cold HBSS and analysed using a FC500 Flow Cytometer (Beckman Coulter, Villepinte, France).

To isolate oral APCs, buccal floor and lingual tissues were removed from naïve BALB/c mice, and treated for 45 min at 37°C with 400 U/ml collagenase type IV (Roche diagnostic, Mannheim,

Germany), 50 µg/ml DNase I (Roche diagnostic) and 2 U/ml dispase (Invitrogen) in RPMI. After blocking residual enzymatic activity with 5 mM EDTA in PBS, oral tissues were dissociated in PBS. Single-cell suspensions were labelled with phycoerythrin (PE)-labelled anti-CD11b and allophycocyanin (APC)-labelled anti-CD11c antibodies (BD Biosciences, San Jose, CA, USA). CD11b⁺ CD11c⁺ cells, representing the main subset of oral APCs (19), were isolated using a MoFlo (Dako, Glostrup, Denmark) cell sorter. More than 99% pure oral APCs (as assessed by flow cytometry) were incubated at 10^4 cells/well in 100 µl complete medium with DQ-OVA or chitosan-formulated DQ-OVA (10 µg/ml final concentration). After 1 h at 4 or 37°C, cells were washed and analysed by flow cytometry.

In vitro proliferation assays and cytokine measurements

CD4⁺ T cells were purified from spleens of DO11.10 mice and labelled with carboxyfluorescein diacetate succinimidyl ester (CFSE) as previously described (17). Carboxyfluorescein diacetate succinimidyl ester-labelled DO11.10 T cells were then incubated with BMDCs and either OVA (1.5 or 15 µg/ml), chitosan-formulated OVA (1.5 or 15 µg/ml), or chitosan alone for 3 days. Ovalbumin-specific T cells were stained with the anti-clonotypic PE-KJ1.26 mAb (BD Biosciences) and proliferating T cells were evaluated by flow cytometry as cells with a decreased CFSE-associated fluorescence. Interferon-γ (IFN-γ), IL-10 and IL-5 secretion was measured in culture supernatants using a Cytometric Bead Array (CBA) Flex kit (BD Biosciences) according to the manufacturer's instructions (with detection limits of 0.5, 9.6 and 0.9 pg/ml, respectively).

Adoptive T-cell transfer and *in vivo* proliferation assay

To measure OVA-specific T-cell proliferative responses *in vivo* after sublingual administration, DO11.10 CD4⁺ T cells were purified and CFSE-labelled as described above. 5×10^6 of such T cells were adoptively transferred by retroorbital intravenous injection into BALB/c mice at day 0. Twenty-four hours later, mice were treated by the sublingual route with soluble OVA or chitosan-formulated OVA (500 µg OVA/dose). Control animals were treated with PBS or chitosan formulations alone. Cervical lymph nodes (LNs) were recovered at day 10. Ovalbumin-specific T cells were stained with the PE-KJ1.26 mAb and proliferating cells evaluated by flow cytometry.

Sublingual immunotherapy of OVA-sensitized mice

BALB/c mice sensitization was performed as described elsewhere (7). Mice were then treated sublingually twice a week during 2 months with OVA or chitosan-formulated OVA solutions (500 µg/dose). As controls, mice were treated with PBS or chitosan formulations alone. Mice were then challenged with OVA aerosols (1% w/v) twice on consecutive days (Fig. 4A).

Measurements of AHR were performed 24 h after the last challenge by whole body plethysmography (Buxco Europe Ltd, Winchester, UK) as described elsewhere (20) and airway resistance expressed as enhanced pause (Penh). A Penh index was obtained as a ratio between Penh values measured after either exposure to 100 mg/ml inhaled methacholine or nebulized PBS.

For tissue histology, lungs were recovered and fixed in phosphate-buffered formalin-zinc, and embedded in paraffin wax. Tissue sections were stained with haematoxylin, eosin and safran (HES) for the determination of cellular infiltrates. A semi-quantitative

assessment of perivascular, peribronchial and alveolar inflammation was performed on coded samples by a trained histologist.

For analysis of inflammatory cells in bronchoalveolar lavages (BAL), mice were anaesthetized by intraperitoneal injection of pentobarbital (50 mg/kg body weight), and BAL performed with 600 µl PBS. Bronchoalveolar lavage fluid was centrifuged at 800 *g* for 10 min at 4°C. Cell pellets were resuspended in PBS, spun onto glass slides by cytocentrifugation, fixed and stained with May-Grünwald Giemsa (Réactifs RAL, Martillac, France). Eosinophils were counted under light microscopy using a 200-fold magnification.

To assess T-cell responses, cervical and mediastinal LNs were removed. Cells were plated at 3×10^5 cells/well and stimulated with OVA (100 µg/ml) or medium alone. After 72 h at 37°C in 5% CO₂/95% air, IL-13, IL-5, IL-10 and IFN-γ were measured in culture supernatants using a CBA Flex kit.

Statistical analysis

Mean comparisons were performed using Student's *t*-tests. A *P*-value < 0.05 was considered as statistically significant.

Results

Characterization of chitosan-OVA particles

Particles were prepared from either high or medium MW chitosan differing in length of the polymer as described in methods. Size and charge (i.e. zeta potential) of chitosan particles were analysed using a Zetasizer. Diameters of high and medium MW chitosan-OVA particles were 1.0–3.0 µm and 300–800 nm, respectively. High and medium MW chitosan-OVA particles exhibited a zeta potential of 7.3 ± 0.9 and 1.8 ± 0.6 mV, respectively. Enzyme-linked immunosorbent assay inhibition assays using sera from OVA-immunized mice demonstrated that OVA formulated in both types of chitosan particles remained well recognized by specific antibodies (data not shown).

Chitosan particles improve OVA uptake and processing by APCs

To investigate the effect of chitosan microparticles on allergen uptake by dendritic cells (DCs), *in vitro* capture studies were performed using FITC-OVA and chitosan-formulated FITC-OVA. As shown in Fig. 1, both high (A) and medium (B) MW chitosan formulations dramatically enhanced OVA uptake by murine BMDCs when compared with soluble OVA. The uptake of high MW chitosan-OVA occurred faster than medium MW chitosan-OVA, with binding saturation reached after 15 and 60 min, respectively. Noteworthy, a better uptake with chitosan-formulated FITC-OVA was observed at both 4 and 37°C, suggesting a binding between cationic particles and the surface of DCs. *In vitro* processing assays were undertaken in BMDCs using DQ-OVA and chitosan-formulated DQ-OVA. As presented in Fig. 1C,D, OVA formulation with both types of chitosan microparticles dramatically improved processing by BMDCs (detectable

at 37°C but not 4°C, as expected), when compared with soluble OVA. We further investigated the uptake of chitosan-formulated OVA by myeloid CD11b+ CD11c– murine APCs purified from oral tissues as described in methods. In contrast to previous results obtained with BMDCs, only high MW chitosan particles enhanced OVA targeting and processing in oral APCs (Fig. 1E).

High MW chitosan-OVA enhances T-cell proliferation and IFN-γ/IL-10 secretion *in vitro*

To determine whether the uptake of chitosan-formulated OVA by DCs improved subsequent T-cell proliferation and cytokine secretion, OVA-specific CD4+ naïve T lymphocytes isolated from DO11.10 mice were labelled with CFSE and co-cultured with BMDCs in the presence of medium, OVA, chitosan or chitosan-formulated OVA. After 3 days, supernatants were harvested for cytokine measurements and T-cell proliferative responses to OVA assessed by flow cytometry. As shown in Fig. 2A, targeting DCs with high MW chitosan-OVA enhanced T-cell proliferation, when compared with OVA alone. This was associated with a dramatic increase in IFN-γ and IL-10 secretion by T cells (Fig. 2B). In contrast, incubation with medium MW chitosan-OVA induced a lower T-cell proliferation (Fig. 2A), as well as little IFN-γ and IL-10 secretion (Fig. 2B). No significant change in IL-5 secretion was observed in those experiments (Fig. 2B). Also, no cytokines were detected in supernatants from control experiments performed in the absence of DO11.10 T cells (data not shown). Taken together, these experiments established that only high MW chitosan particles allowed an efficient antigen presentation by DCs to T lymphocytes.

T-cell priming occurs in cervical LNs after sublingual administration of high MW chitosan-OVA

To assess whether chitosan-OVA particles could enhance T-cell priming in draining LNs following sublingual administration, CFSE-labelled OVA-specific DO11.10 CD4+ T cells were adoptively transferred into BALB/c mice before sublingual treatment. After 10 days, cervical LNs were harvested and OVA-specific T-cell proliferative responses analysed by flow cytometry. As shown in a representative experiment in Fig. 3, T-cell proliferation was hardly detectable in cervical LNs recovered from mice sublingually treated with either PBS, high or medium MW chitosan alone, with 6.8%, 6.3% and 5.7% proliferating T cells, respectively. Sublingual administration of OVA induced a readily detectable (34.5%) T-cell proliferation in cervical LNs. Interestingly, the use of high MW chitosan-OVA further enhanced T-cell proliferation (46.7%), whereas medium MW chitosan-OVA was rather inhibitory (15.8%) when compared with OVA alone.

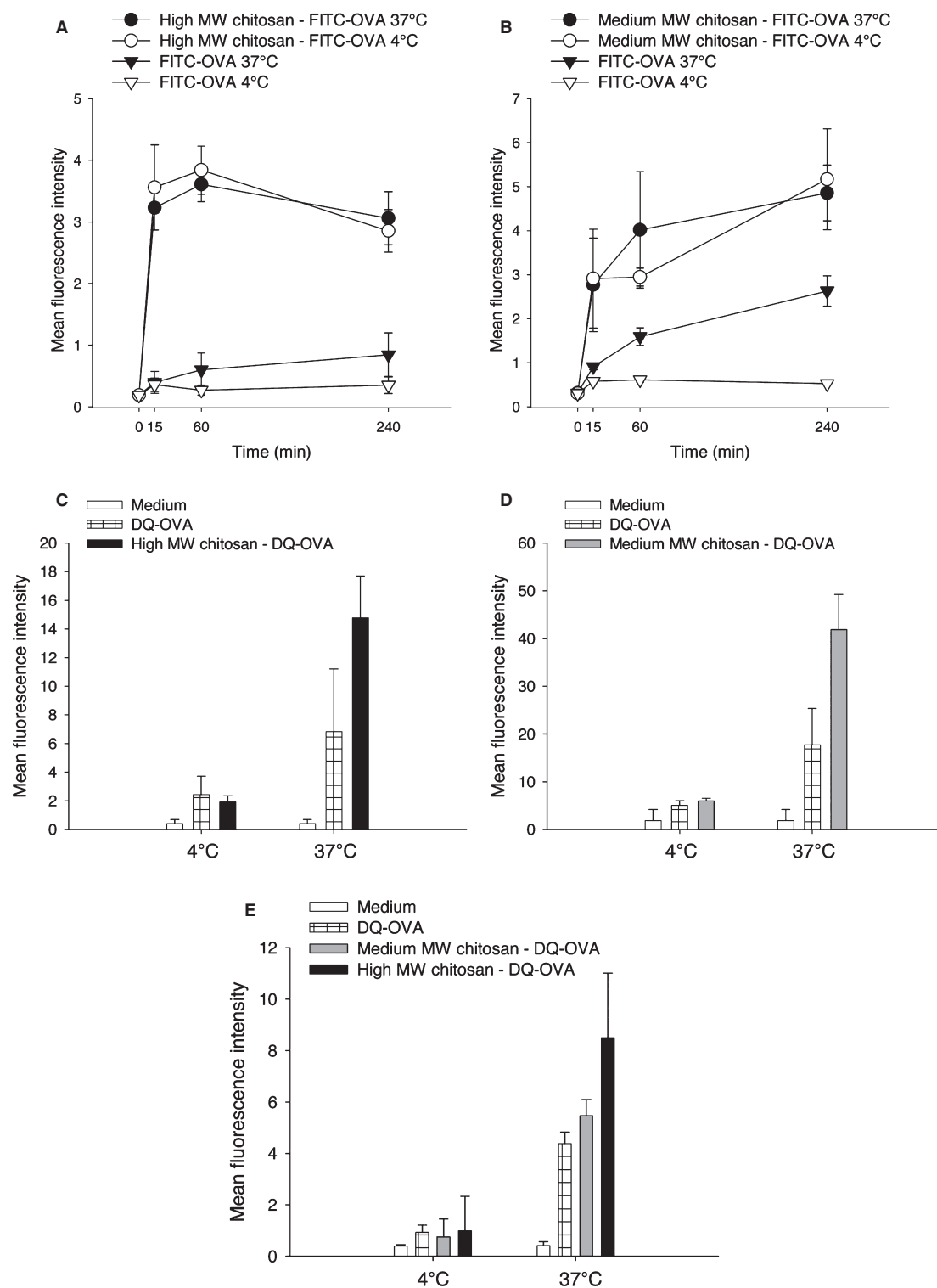


Figure 1. Chitosan particles improve OVA uptake and processing by APCs. For uptake studies, FITC-OVA as well as high (A) or medium (B) MW chitosan-formulated FITC-OVA were incubated with BMDCs for 15, 60 or 240 min, at 4 or 37°C. For processing assays, DQ-OVA as well as high or medium MW chitosan-formulated DQ-OVA (10 µg/ml) were incubated with either BMDCs (C and D) or purified oral APCs (E), for 3 h (BMDCs) or 1 h (oral APCs) at 4 or 37°C. Cells were washed and analysed using a FC500 flow cytometer. Mean ± SEM of 2 to 3 independent experiments.

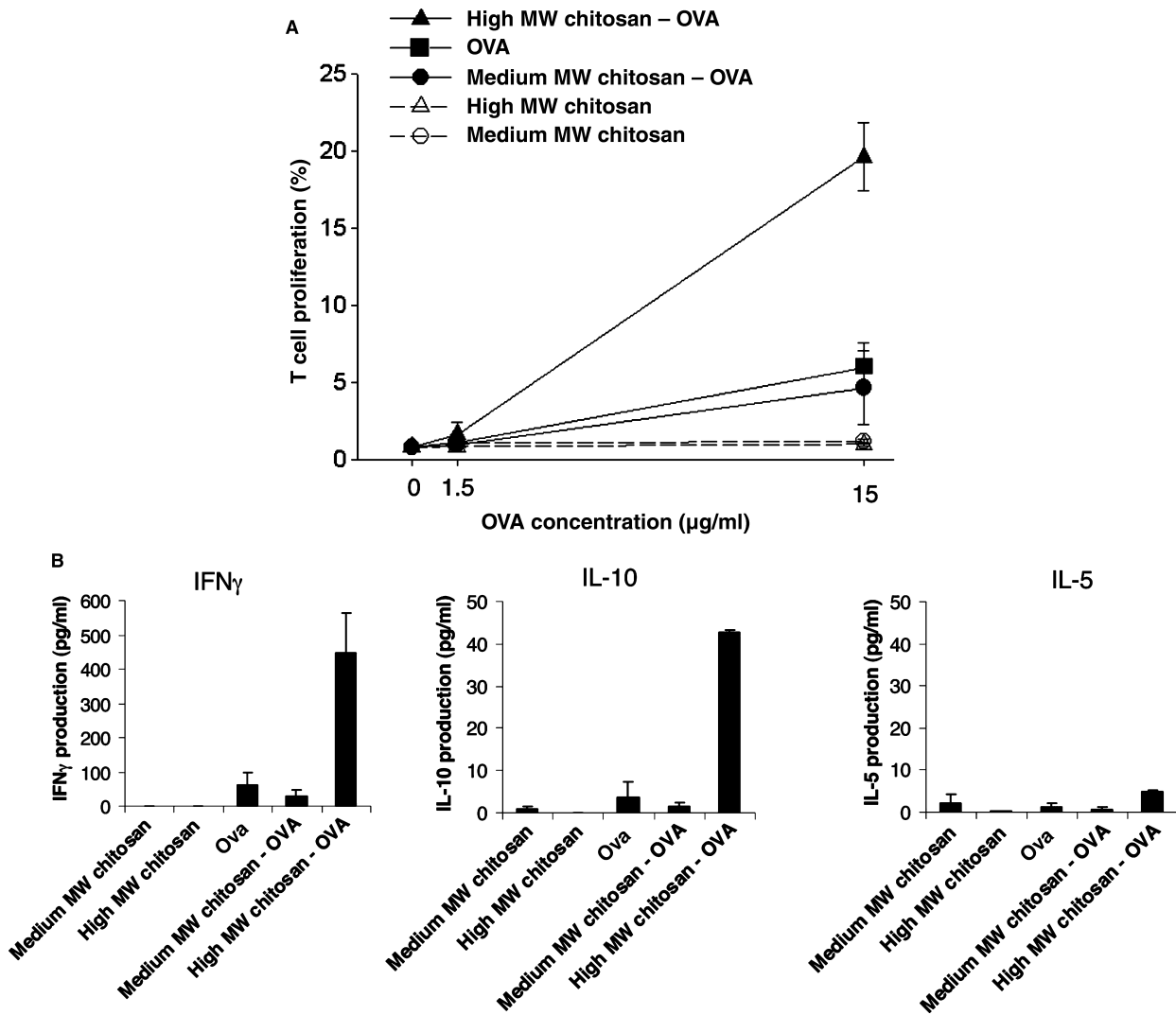


Figure 2. High MW chitosan particles increase CD4⁺ T-cell proliferation and IFN- γ /IL-10 secretion. Ovalbumin-specific naïve CD4⁺ T lymphocytes isolated from DO11.10 mice were labelled with CFSE and co-cultured with BMDCs in presence of OVA (1.5 or 15 μ g/ml), high or medium MW chitosan-OVA (1.5 or 15 μ g/ml), or chitosan formulations alone. After 3 days, cells were harvested, stained with the PE-KJ1.26 mAb, and CFSE-associated fluorescence was analysed in responder T cells using a FC500 flow cytometer (A). IFN- γ , IL-10 and IL-5 were measured in cell culture supernatants using a CBA assay (after stimulation with 15 μ g/ml OVA or chitosan-formulated OVA) (B). Mean $\pm$ SEM of three independent experiments.

Sublingual immunotherapy with high MW chitosan-OVA reduces established AHR and bronchial inflammation

Having established that chitosan microparticles can be used to target the antigen onto APCs, these formulations were tested in a SLIT model relying upon OVA-sensitized BALB/c mice (7). Such mice exhibit severe AHR, lung inflammation associated with cellular infiltrates and systemic OVA-specific Th2 immune responses. Ovalbumin-sensitized mice were sublingually treated for 2 months with either PBS, OVA, chitosan alone or chitosan-formulated OVA (Fig. 4A). Treatment with high MW chitosan-OVA dramatically reduced AHR in all animals tested, whereas treatment with soluble OVA

had only a moderate effect on AHR (Fig. 4B). In contrast, treatment with medium MW chitosan-OVA had no beneficial effect on AHR. Noteworthy, neither chitosan formulations alone nor chitin particles co-administered with OVA reduced AHR (data not shown).

The decrease of AHR observed after treatment with high MW chitosan-OVA was associated with a reduction in bronchial inflammation as shown by lung histology (Fig. 4C and Table 1). This reduction was also correlated with a significant decrease in eosinophil counts in BAL (Fig. 4D). Treatment with medium MW chitosan-OVA did not improve lung inflammation or eosinophil counts in BAL (Fig. 4C,D and Table 1).

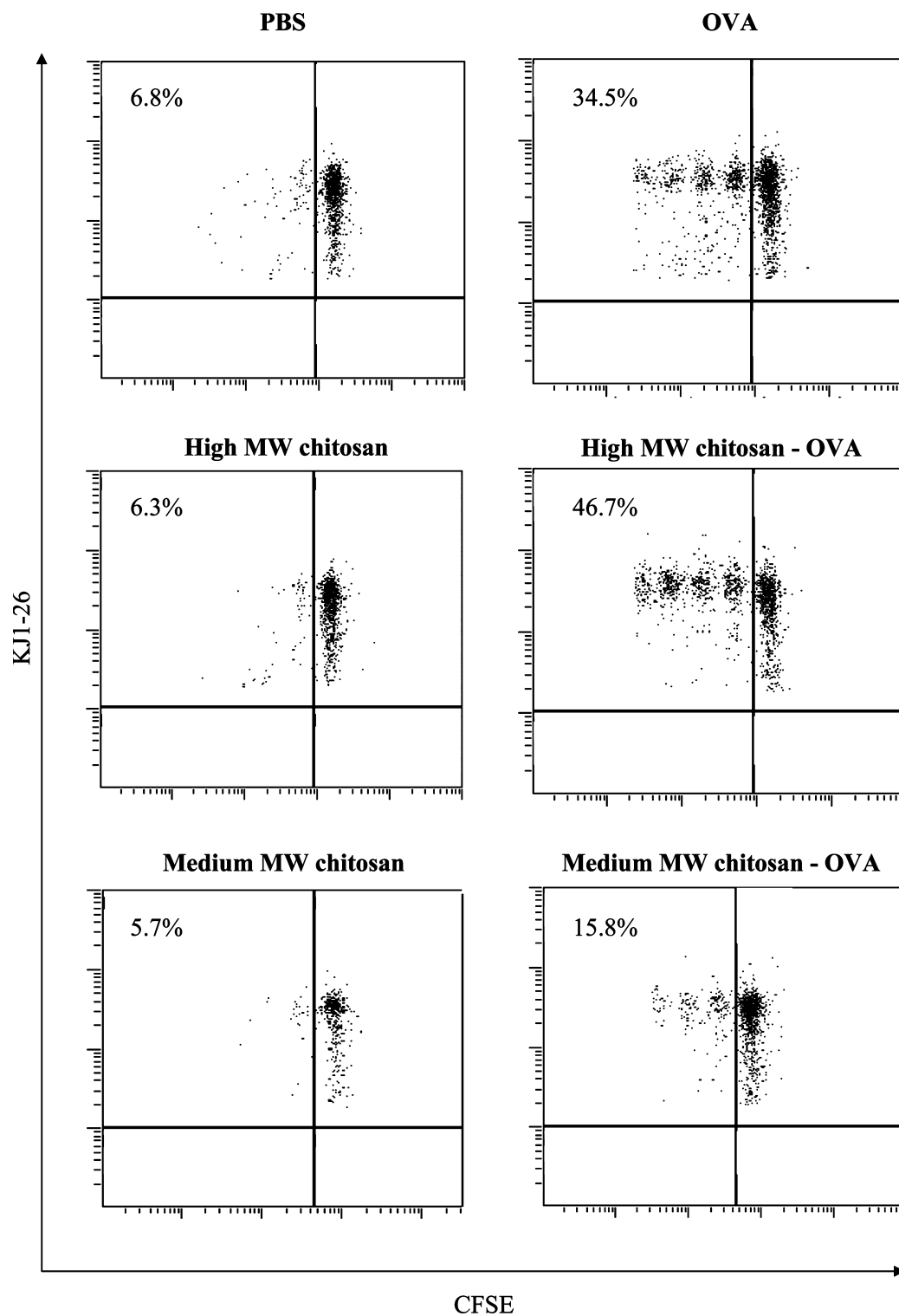


Figure 3. High MW chitosan particles enhance T-cell priming in cervical LNs. Purified CFSE-labelled DO11.10 CD4⁺ T cells were adoptively transferred into BALB/c mice at day 0. Twenty-four hours later, mice were treated by the sublingual route with soluble OVA or chitosan (high or medium MW)-formulated OVA. Control animals were treated with PBS or chitosan formulations alone. At day 10, cervical LNs were removed. Proliferating cells were detected by flow cytometry. Data are representative of two cumulative experiments (each conducted with 2 to 4 mice per group). Mean $\pm$ SEM of combined data are: PBS ($5.9 \pm 1.0\%$), high MW chitosan ($7.1 \pm 1.0\%$), medium MW chitosan ($4.7 \pm 1.1\%$), OVA ($32.7 \pm 5.7\%$), high MW chitosan-OVA ($44.5 \pm 0.9\%$) and medium MW chitosan-OVA ($19.1 \pm 6.7\%$).

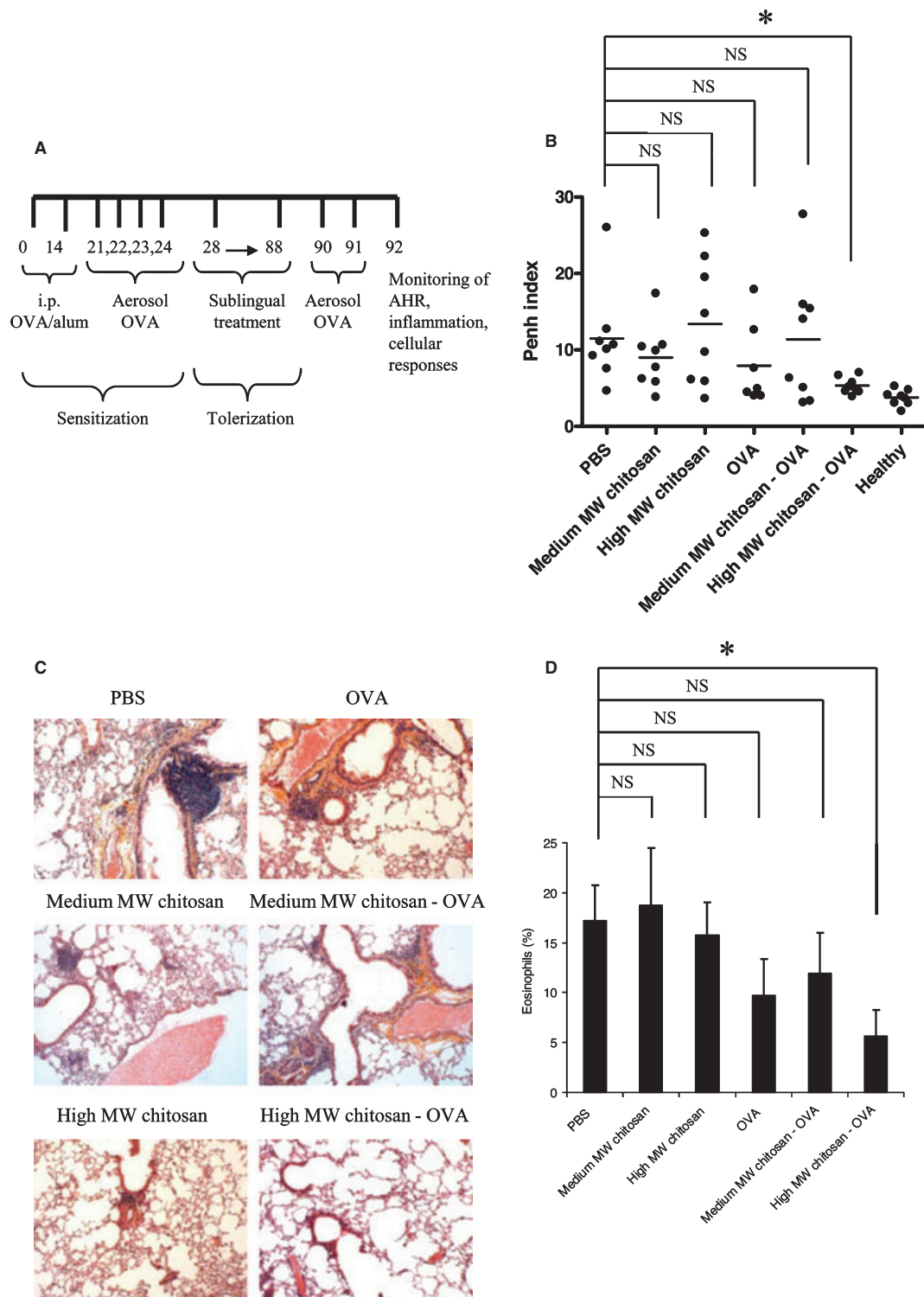


Figure 4. Therapeutic SLIT with high MW chitosan-OVA reduces established AHR and lung inflammation. Mice were sensitized by intraperitoneal injections with OVA/alum followed by aerosol challenges. Mice were then sublingually treated with PBS, OVA, chitosan (high or medium MW)-formulated OVA or chitosan alone. After OVA aerosol challenges, AHR, lung inflammation and cellular responses were investigated (A). AHR was determined by measuring the Penh index in all groups of mice (B) (— = mean). For tissue histology (C), paraffin lung sections were stained with HES. Representative views are shown (with a 100-fold magnification). Eosinophils were counted in BAL (D) (mean $\pm$ SEM). $n = 7-8$ mice per group. * $P < 0.05$ compared with PBS-desensitized mice. NS, not significant. Data are representative of two independent experiments.

Table 1. Therapeutic SLIT with high MW chitosan-OVA reduces perivascular, peribronchial and alveolar inflammation

Treatments	n	Perivascular inflammation			Peribronchial inflammation			Alveolar inflammation		
		–	+	++	–	+	++	–	+	++
PBS	7	1/7	5/7	1/7	1/7	5/7	1/7		4/7	3/7
Medium MW chitosan	4		4/4			4/4			4/4	
High MW chitosan	8	2/8	6/8		2/8	6/8		3/8	5/8	
OVA	7	4/7	3/7		4/7	3/7		1/7	6/7	
Medium MW chitosan-OVA	4		3/4	1/4		3/4	1/4		4/4	
High MW chitosan-OVA	8	7/8	1/8		7/8	1/8		6/8	2/8	

Lungs from mice treated with either PBS, OVA, chitosan (high or medium MW)-formulated OVA or chitosan alone were removed, fixed and stained with HES for the determination of cellular infiltrates. A semi-quantitative assessment of perivascular, peribronchial and alveolar inflammation was performed on coded samples. Results are expressed as numbers of animals exhibiting signs of inflammation with a given degree of severity (–, negative; +, intermediate; ++, high). Data are representative of two independent experiments.

Sublingual immunotherapy with high MW chitosan-OVA reduces OVA-specific Th2 responses

Serum titres of OVA-specific IgE or IgG antibodies were not modified by any of the sublingual treatments (data not shown). T-cell responses were assessed in mediastinal and cervical LNs recovered from mice after a 2-month SLIT regimen. As shown in Fig. 5, T cells in mediastinal LNs from animals treated with high MW chitosan-OVA exhibited lower IL-13 (A), IL-10 (B) and IL-5 (C) production in response to the allergen (although none of those differences were statistically significant), when compared with animals treated with PBS or chitosan alone, and to a lesser extent, with OVA or medium MW chitosan-OVA. In contrast, slightly higher IL-10 levels were detected in cervical LNs from mice sublingually administered with high MW chitosan-OVA (Fig. 5D).

Discussion

Sublingual immunotherapy is currently established as a safe and efficacious treatment for type I respiratory allergies in humans (1–4). Adjuvants or mucosal formulations could further improve sublingual vaccines, allowing to decrease the allergen dose and simplify immunization schemes. The present study aimed to identify a mucosal formulation to target the allergen to oral APCs in order to enhance SLIT efficacy. We focused herein on chitosan polymers, which represent attractive delivery systems, given both their mucoadhesiveness and their capacity to form particles (14). The nature of the chitosan molecule is known to impact both size and charge characteristics of particles formed (21). Specifically, two types of chitosan formulations were tested, including a medium MW polymer (190–310 kDa), which forms 300–800 nm particles, and a high MW type (310–375 kDa) assembling into larger (1–3 µm) and highly positively charged particles.

In vitro uptake assays revealed that both formulations enhance OVA uptake and processing by BMDCs when

compared with soluble OVA. However, the two chitosan formulations differ in their kinetics of uptake, with high MW chitosan being captured significantly faster by BMDCs. In addition, only the high MW chitosan improved OVA uptake and processing by purified oral APCs, thus resulting in a strong T-cell proliferation with IFN-γ and IL-10 secretion. Consistent with this observation, sublingual administration of high but not medium MW chitosan-OVA increased T-cell priming in cervical LNs, when compared with soluble OVA. Furthermore, when tested in BALB/c mice with established asthma to OVA (7), SLIT with high MW, but not medium MW chitosan-OVA reduced AHR, bronchial inflammation, eosinophil numbers in BAL, as well as specific Th2 responses in mediastinal LNs. Interestingly, an increase in antigen-specific production of IL-10 was observed in cervical LNs. This is consistent with our observation that targeting the antigen to DCs *in vitro* with high MW chitosan particles elicited IL-10 and IFN-γ production by naïve CD4⁺ T cells, i.e. a pattern associated with tolerance induction via the sublingual route (7, 17, 22).

Our results are in agreement with other studies investigating the effect of chitosan formulations in murine models of allergy (23–25). For example, oral or intranasal administration of chitosan particles containing plasmid DNA encoding either IFN-γ (23), the house dust mite Der p 1 allergen (24) or the dominant peanut allergen Ara h 2 (25), was shown to enhance tolerance induction in sensitized mice. However, in a murine SLIT model of papain-induced asthma, chitosan alone rather caused a nonspecific decrease in Th2 responses (26). In addition, particles made from chitin (i.e. an acetylated form of chitosan) were found to be tolerogenic in murine sensitization models when administered without the allergen, via the oral (27) or intra-nasal (28, 29) routes. Collectively, these studies reveal that both chitin or chitosan can mediate an adjuvant effect by themselves, but that such immunomodulatory properties vary considerably depending upon the chemical form as well as the route of administration considered. In the present study,

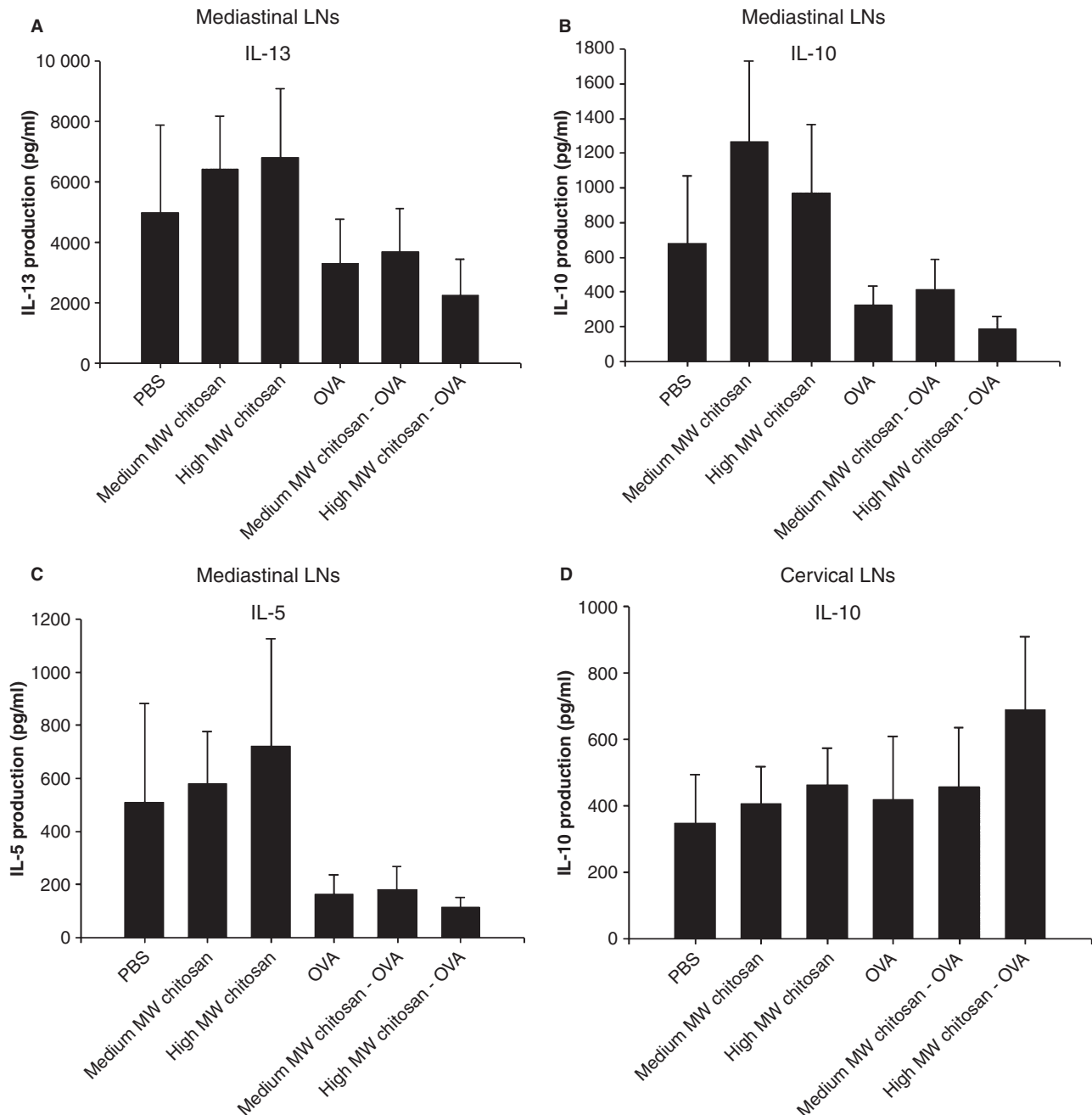


Figure 5. Therapeutic SLIT with high MW chitosan-OVA reduces OVA-specific Th2 responses in mediastinal LNs. IL-13 (A), IL-10 (B) and IL-5 (C) secretions in mediastinal LNs, and IL-10 production in cervical LNs (D) were measured by CBA assay (mean $\pm$ SEM). $n = 6$ mice per group.

neither chitosan formulations alone nor chitin particles mixed with OVA without any covalent association improved SLIT efficacy. Our results rather suggest that highly positively charged chitosan particles act by facilitating antigen contact with the mucosa, thereby enhancing uptake by oral APCs. These results are in line with our previous observation that allergen formulation in mucoadhesive nanoparticles made of polymerized mal-

to-dextrin dramatically enhances tolerance induction via the sublingual route (7).

The distinct results obtained with both high and medium MW chitosans imply that particle size and surface charge are critical parameters affecting immune responses. Internalization of chitosan particles can be a consequence of nonmutually exclusive mechanisms, such as (i) fluid-phase endocytosis (macropinocytosis),

previously described for particles with 0.5–5 µm diameter; (ii) phagocytosis, allowing particles larger than 0.5 µm to be internalized and (iii) capture through the interaction with specific receptors (e.g. lectins) on the cell surface (30, 31). Differences in the surface charge of DNA-loaded microparticles, poly (D,L-lactide-co-glycolide) microparticles, or airborne particulate matters, were previously reported to influence dramatically the magnitude and nature of immune responses generated (32–34). In addition, variations in particle size have been shown by others to significantly impact DC targeting properties (10, 30) as well as the balance of Th1/Th2 responses elicited (35).

In conclusion, allergen formulations combining both mucoadhesiveness and a particulate form appear well suitable for sublingual immunization. Specifically, highly positively charged chitosan microparticles represent an attractive delivery system to improve SLIT in humans.

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