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Intranasal delivery of allergen in a nanoemulsion adjuvant inhibits allergen-specific reactions in mouse models of allergic airway disease

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Abstract

Background: Atopic diseases are an increasing problem that involve both immediate hypersensitivity reactions mediated by IgE and unique cellular inflammation. Many forms of specific immunotherapy involve the administration of allergen to suppress allergic immune responses but are focused on IgE mediated reactions. In contrast, the effect of allergen-specific immunotherapy on allergic inflammation is complex, not entirely consistent and not well understood. We have previously demonstrated the ability of allergen administered in a nanoemulsion (NE) mucosal adjuvant to suppress IgE mediated allergic responses and protect from allergen challenge in murine food allergy models. This activity was associated with decreases in allergen specific IL-10 and reductions in allergic cytokines and increases in regulatory T cells.

Objective: Here we extend these studies to using 2 distinct models, the ovalbumin (OVA) and cockroach (CRA) models of allergic airway disease which are based predominantly on allergic inflammation.

Methods: Acute or chronic allergic airway disease was induced in mice using ovalbumin and cockroach allergen models. Mice received three therapeutic immunizations with allergen in NE, and reactivity to airway challenge was determined.

Results: Therapeutic immunization with cockroach or OVA allergen in NE markedly reduced pathology after airway challenge. The 2 models demonstrated protection from allergen challenge-induced pathology that was associated with suppression of Th2-polarized immune responses in the lung. In addition, the reduction of ILC2 numbers in the lungs of allergic mice along with reduction in epithelial cell alarmins, IL-25 and IL-33, suggest an overall change in the lung immune environment induced by the NE immunization protocol.

Conclusions & Clinical Relevance: These results demonstrate that suppression of allergic airway inflammation and bronchial hyperreactivity can be achieved using allergen-specific immunotherapy without significant reductions in allergen-specific IgE and suggest that ILC2 cells may be critical targets for this activity.

Key Messages

- Therapeutic immunization with allergen and NE suppresses allergic airway inflammation in two mouse models.
- Intranasal NE vaccines modulate allergen-specific cytokine milieu to suppress Th2 cytokine production in the lungs.
- NE-vaccine induced suppression of allergic airway disease is associated with reduced ILC2s and alarmins.

Introduction

Atopic disease has increased dramatically, both in incidence and severity, creating an expanding, unmet medical need.[1,2] An important consideration is that while the public focus has been on IgE mediated immediate hypersensitivity reactions, there are actually two components to atopic diseases. Along with immediate IgE reactions cellular immune responses of the Th2 phenotype cause significant and chronic inflammatory problems.[3,4] Therefore, an important goal

is to understand both the immediate IgE hypersensitivity reactions and the cellular inflammation that cause atopic diseases.

Approaches to treat allergic disease fall into two major categories; allergen specific and non-specific approaches. The various forms of allergen-specific immunotherapy involve chronically administered an allergen to a patient, either by injection or on the skin or mucosal surfaces, at progressively increasing doses. The focus of immunotherapy is predominantly to suppress allergen specific immediate IgE reactions, which has been accomplished with varying degrees of success.[5-10] The mechanism by which this therapy blocks IgE reactions is not entirely defined, but likely involves multiple immune alterations and unique changes in different individuals. Among these alterations, immunotherapy has been associated with the development of "blocking antibodies" that bind antigen preventing IgE binding and allergic reactions.[11] In addition, some approaches to immunotherapy can reduce IgE concentrations and/or allergen-specific Th2 cells after long term administration.[12,13] In most cases, however, it is felt that immunotherapy does not induce tolerance to the allergen as discontinuation of chronic administration of the allergen result in rapid recurrence of allergic sensitivity.[7,8,14] In addition, allergen immunotherapy has not been thought to be useful for treating atopic diseases related to cellular allergic inflammation. In particular, immunotherapy has not been effective for diseases like eosinophilic esophagitis, food protein-induced enterocolitis syndrome or uniformly useful for asthma or atopic dermatitis, where cellular inflammation with eosinophils and other allergic inflammatory cells predominate.[15-20]

In contrast to allergen-specific immunotherapy, recent advances in biological drugs have yielded therapeutics that block cytokines or inactivate allergic inflammatory cells, and these approaches have provided new insights into the treatment of allergic disease. Anti-cytokine therapy is not antigen specific but has been shown to reduce inflammation in a variety of diseases associated with allergic inflammation, particularly atopic dermatitis, asthma, allergic rhinitis and polyps and eosinophilic esophagitis.[17,21,22] The mechanism of action of these therapies involves blocking the cytokines produced by Th2 phenotype lymphocytes that promote the infiltration, proliferation and activation of allergic inflammatory cells, predominantly eosinophils, in local tissue. In contrast to biologicals that target IgE directly, some of the anti-

cytokine therapies can also cause long-term reductions in IgE.[23] Of interest, however, the reductions in IgE with these biologicals occur much slower than the improvement in inflammatory symptoms, and it is unclear whether anti-cytokine therapy acutely reduces immediate hypersensitivity reactions. Given these observations, there appears to be a dichotomy in the approach to treat the two mechanisms of atopic diseases and a fundamental lack of clarity on the relationship between immediate hypersensitivity reactions and chronic allergic inflammation.

We have recently examined a new approach to treating allergic disease.[24] We have been able to protect animals from anaphylaxis in murine models of peanut and milk allergy.[25,26] We accomplished this using specific allergen immunizations formulated in an adjuvant that redirects pre-existing Th2 immunity to a more balanced, Th1/Th17 phenotype. This process altered the allergen-specific T cell response by enhancing the production of Th1 cytokines, while also producing IL-10 and T regulatory cells.[25] This immunization is able to block immediate hypersensitivity reactions to allergen despite allergen specific IgE levels that would otherwise support anaphylaxis. Importantly, unlike traditional immunotherapy the inhibition of immediate hypersensitivity responses was maintained long after allergen/adjuvant administration was stopped.[26] This was associated with reductions in gut inflammation that suggested a direct effect on allergic inflammation not seen with other types of immunotherapy.

Given these observations we sought to evaluate the effect of this allergen-specific immunization in a model of atopic disease based predominantly on Th2 cellular inflammation rather than immediate hypersensitivity reactions (such as food anaphylaxis). Therefore, we examined the effect of allergen/adjuvant immunizations on allergic inflammation and clinical outcomes in two, well-defined allergic airway disease models.

Materials and Methods

Antigen and adjuvants. Endotoxin-free ovalbumin (OVA) was purchased from Lionex (Branschweig, Germany). The cockroach allergen (CRA) was clinical grade, skin test CRA (Hollister Stier, Toronto, Canada) that was purified by centrifugation using Amicon Ultra-15 Centrifugal Filter Unit with Ultracel-3 membrane, 3000 MWCO to obtain endotoxin free CRA.

Nanoemulsion adjuvant (NE) was produced by a high speed emulsification of ultra-pure soybean oil with cetyl pyridinium chloride, Tween 80 and ethanol in water, with resultant NE droplets with average 350-400 nm diameter.[27,28] Aluminum hydroxide (alum, alhydrogel) was purchased from InvivoGen (San Diego, CA). Incomplete Freund's Adjuvant (IFA) was purchased from Sigma-Aldrich (St. Louis, MO). The absence of endotoxin in all reagents was confirmed using limulus assay.

Ovalbumin allergic airway disease model. Specific pathogen-free BALB/c mice (females 4-5 weeks old) were purchased from Jackson Laboratory and immunized as per the schedule shown in Figure 1. For all immunizations, mice were anesthetized under isoflurane anesthesia using the IMPAC6 precision vaporizer. Allergic sensitization was induced with intraperitoneal immunizations (i.p.) of 20 µg OVA adsorbed on 2 mg alum.[29] Intranasal (i.n.) immunizations were administered as 12 µl (6 µl /nare) of a formulation containing 20 µg of OVA mixed with 20% NE.[25,26] OVA mixed with PBS, PBS alone, and 20% NE only (no allergen) served as controls for the study. Mice were challenged intratracheally with 100 µg of OVA on three alternating days during week 16. Mice were sacrificed 2 days after the third challenge. All animal procedures were approved by the University of Michigan Institutional Animal Care and Use Committee (protocol numbers PRO00005743 and PRO00007671, approved 7/1/2014 and 6/19/2017, respectively).

Lung histology. At the time of sacrifice, lungs were perfused with 4% formaldehyde for fixation. After fixation, lungs were embedded in paraffin, sliced transversally into 5-µm thick sections, and stained with hematoxylin and eosin or periodic acid-Schiff (PAS) to detect cellular infiltration and mucus production. The lung sections were scored for inflammation using the following scoring system: 0, absent; 1, minimal; 2, slight; 3, moderate; and 4, severe.[30] Total number of airways were counted and scored as mucus positive or mucus negative to determine the percentage of airways producing mucus. Eosinophils were identified by morphometric analysis at 1000x magnification. Individual eosinophils were counted from 100 high powered fields (HPF) per lung.

Analysis of cytokine expression. To assess allergen-specific recall responses, red blood cell-depleted splenocytes or lymphocytes isolated from cervical lymph nodes or lungs were cultured

ex vivo ± OVA (20 μg/ml). After 72 hours, cytokine secretion was measured in cell culture supernatants using Luminex Multiplex detection system (Millipore, Billerica, MA). To quantify cytokines in lung tissue, lungs were isolated one day after the final OVA challenge and homogenized in 350 μL of T-PER tissue extraction buffer (Thermo Scientific), and frozen at -80°C. Samples were subjected to an additional freeze/thaw cycle, and then centrifuged at 10,000xg for 5 min at 4°C to remove debris. Cytokines in lung supernatants were analyzed using a Luminex Multiplex kit. ELISpot assays were run using kits from Mabtech according to manufacturer's instructions. Briefly, sterile 96-well multiscreen filter plates with PVDF membrane (Millipore) were coated overnight with anti-IFN-γ, IL-5 or IL-17 capture antibodies, blocked with 5% fetal bovine serum, and cells were added at 500,000 to 1,000,000 cells per well. Cells were cultured ± OVA (20 μg/ml) for 40 hours and cytokine secreting cells were detected by incubation with biotinylated antibodies to the respective cytokines followed by streptavidin-alkaline phosphatase. Spots were developed by addition of BCIP/NBT substrate and counted using an AID ELISpot reader system.

Mouse chronic CRA allergic airway disease model. Mice were sensitized by i.p. and subcutaneous (s.c.) injection of 500 protein nitrogen units (pnu) of CRA mixed 1:1 in IFA (Sigma-Aldrich, St. Louis, MO). Next, mice were challenged intranasally with 150 pnu of CRA on days 14, 18 and 22 after initial CRA sensitization to localize the response to the lung.[31] Mice were immunized on days 28, 56 and 84 with a formulation containing 20 μg of CRA mixed with 20% NE (12 μl/mouse; 6 μl /nare). CRA mixed with PBS and 20% NE only with no allergen were used as controls. Mice were challenged by intratracheal injection with 500 pnu CRA on days 98 and 102. Mice were sacrificed, and samples were taken one day after the last allergen challenge.

Quantitative RT-PCR Lung tissue was homogenized and RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA). mRNA concentration was quantified by NanoDrop, followed by cDNA synthesis using a High Capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster City, CA). Real-time quantitative PCR was performed using Power SYBR green PCR master mix (Applied Biosystems, Foster City, CA). Gene expression was quantified by ΔΔCt analysis and normalized to GAPDH levels within individual samples.

Measurement of airway hyperreactivity (AHR). AHR was assessed using a mouse plethysmograph specifically designed for low tidal volumes (Buxco Research Systems), as described previously.[32] Briefly, mice were anesthetized with sodium pentobarbital, intubated and ventilated at a volume of 200 µl with a frequency of 120 breaths/min. The plethysmograph was sealed, so changes in lung volume are represented by changed box pressure. Airway resistance was measured in by assessing tracheal pressure and comparing to the corresponding box pressure changes. Baseline levels were determined, and mice were challenged via tail vein with 0.35 mg/kg of methacholine. The peak airway resistance recorded to quantify AHR.

Measurement of serum antibodies. Blood was collected at the end of the study, and sera were harvested by centrifugation. OVA- and CRA-specific IgG1, IgG2a and IgE antibodies were determined by ELISA in serially diluted serum, using OVA- and CRA-coated 96-well plates and alkaline phosphatase conjugated detection antibodies as described previously.[27]

Flow cytometry. The animals' lungs were removed and digested with 1 mg/ml collagenase A (Roche, Indianapolis, IN) and 20 U/ml DNaseI (Sigma, St. Louis, MO) in RPMI 1640 containing 10% FCS. Single cell suspensions were achieved by dispersion through an 18-gauge needle and filtration through 100-µm cell strainer. Cells were resuspended in PBS and stained by flow cytometry. All antibodies used for flow cytometry were purchased from BioLegend unless otherwise noted. Fc receptors were blocked with purified anti-CD16/ 32 and surface markers were identified using antibodies against the following antigens: B220, CD3, CD4, CD11b, CD25, CD45, CD90, Gr-1, ST2 and Ter119. Cells were fixed, permeablized and labeled for intracellular Foxp3 (eBioscience) and GATA3 (eBioscience). Cell types were defined as follows: *Treg:* CD4+CD25+Foxp3+. *Activated Th cells:* CD4+CD69+. *ILC2:*Lin-CD45+ CD90+ST2+GATA3+. For innate lymphoid cell staining, lineage markers were CD3, CD11b, B220, Gr-1, and TER119. Samples were acquired on a NovoCyte flow cytometer (Acea Biosciences). Data were analyzed using FlowJo (Treestar).

Statistics. Statistical comparisons were assessed by the Mann-Whitney test using GraphPad Prism version 8 (GraphPad Software). The p value < 0.05 was considered as significant.

Results

NE vaccines protect against airway inflammation in the OVA allergic airway disease model

As described previously, BALB/c mice were sensitized with OVA and alum to induce an allergic phenotype.[33,34] Animals were then immunized 3 times with either NE adjuvant-OVA vaccines or allergen in PBS as a control to demonstrate that the effects were specific to the vaccine and not due to intranasal allergen exposure (Figure 1A). Additional controls included intranasal instillation of PBS or NE adjuvant only without allergen. Following inhalation challenge with OVA, histopathological analyses of lung tissue were performed to characterize the effect of the NE allergy vaccine. As shown in Figure 1, mice sensitized with OVA-alum had significant infiltration of inflammatory cells in the lungs after allergen challenge ($p=0.0061$ vs nonsensitized group). This inflammation was greatly diminished in mice that received therapeutic the OVA-NE vaccine, as documented by significant decrease in cellular infiltrates ($p=0.0016$). The inflammation in the lungs of the OVA-NE immunized mice after antigen challenge was focal in nature and did not disrupt the pulmonary architecture. NE immunization also induced significant reductions in allergen-induced mucus production (Figure 1D; $p=0.0002$). Sensitized mice had mucus in approximately 28% of their airways after OVA challenge as compared with 8% of the airways in mice receiving the NE immunizations. In the NE-treated mice, the airways that contained mucus had significantly less mucus and fewer mucus-producing cells, suggesting an inhibition of the goblet cell hyperplasia observed in OVA-sensitized mice who were not immunized with NE. These effects were specific to the OVA-NE vaccine, as sensitized mice that received PBS, OVA alone or NE alone all had similar increases in inflammation and mucus.

Intranasal immunization with NE adjuvant suppresses acute allergic Th2 cytokine production and IgE in the OVA model.

To examine the effect of NE adjuvant alterations in the cellular immune response to OVA, cytokine production was evaluated by Luminex (Figure 2A, 2B) and ELISpot (Figure 2C) to quantify, respectively, both the amount of cytokine secreted and the number of cytokine producing cells. OVA-alum sensitization produced a Th2-polarized cellular response, with IL-5 and IL-13 production from lypmphocytes isolated from the cLN and spleen, and IL-9 production in the lungs (Figure 2A, 2B). Intranasal instillation of OVA alone or NE alone had no effect, as cytokine secretion from these mice was the same as sensitized mice that received i.n. PBS. Lymphocytes isolated from cervical lymph nodes of mice that received subsequent OVA-NE

immunizations produced significantly more Th1 cytokines, including IFN-γ and IL-2, and significantly less Th2 cytokines such as IL-5 and IL-13 (Figure 2B). Additionally, OVA-NE treatment significantly increased both IL-17 and IL-10 production (p=0.0001 and 0.0047, respectively). Similar patterns were observed in cultured splenocytes from these animals, with more dramatic reductions in IL-5 and IL-13 (Figure 2C). This indicated that the OVA-NE nasal immunizations altered both local and systemic immune responses.

ELISpot analysis of cytokine-producing cells revealed similar results. Upon *ex vivo* OVA stimulation with allergen, cells from OVA-alum sensitized mice produced predominantly IL-5 vs. IFN-γ (Figure 2C). This changed in animals immunized with OVA-NE, where IFN-γ and IL-17 cells predominated (Figure 2C). In addition, there were no significant differences in the cellular profile between sensitized mice that received OVA-NE immunization and nonsensitized mice that were immunized with OVA-NE alone (Figure 2C). This suggested the OVA-NE immunizations could redirect the Th2 phenotype of the OVA T cell response that were induced by sensitization towards a Th1/Th17 response.

The OVA-specific humoral immune responses were also characterized to determine if the suppression of Th2 immunity and induction of Th1/Th17 altered OVA-specific IgE and IgG subclasses. Allergen specific IgE was not detectable in the blood of non-sensitized animals. Titers of anti-OVA IgE increased dramatically after alum immunization, to 10^4, and subsequent immunization with the OVA-NE vaccine decreased IgE significantly (p=0.0139; 10-fold, Figure 3A). However, OVA-specific IgE remained significantly elevated compared to non-sensitized controls. In addition, while IgG2a was significantly increased by the OVA-NE vaccine, IgG1 titers were not changed (Figure 3B-C). Similar to the data described above, i.n. instillation of the vaccine containing both NE and OVA was required to modulate the immune response, as treatment with either component alone had no effect.

Nanoemulsion adjuvant vaccine reduces histopathology and disease parameters in chronic, CRA-induced allergic airway disease in mice.

To determine if the therapeutic immunization with NE was effective at preventing airway inflammation in a second chronic model of allergic airway disease, we used the well-defined, mouse model of chronic cockroach allergen (CRA)-induced airway where airway remodeling is

accompanied by intense peribronchial leukocyte recruitment, mucus hypersecretion, development of airway hyperreactivity (AHR) and significant peribronchial and airway thickening.[31,35-37] The inflammation in this chronic model is driven purely by Th2-polarized immune response, as the allergen preparation contains no endotoxin and the immunization uses no innate cell adjuvant for sensitization. The experimental design used in this model is outlined in Figure 3A. Following the standard 22-day sensitization period, the animals were treated intranasally with a CRA-NE vaccine every 4 weeks for a total of 3 administrations. For the CRA studies, control groups were administered CRA in PBS and NE only to demonstrate the observed effects were due to the CRA-NE vaccine and not either of the individual components. Our data in the OVA model (Figure 1) and our previous work with peanut allergy models demonstrated no significant effect of administering small doses of allergen intranasally compared with instillation of PBS.[25] Fourteen days after the final intranasal administration, the animals were CRA challenged twice by intratracheal administration. Examination of the histopathology of the lungs demonstrated a reduction in overall inflammation and mucus production in the airways of animals given NE-CRA vs animals who received CRA alone (Figure 4B). Eonsinophilic infiltration was increased in sensitized mice following CRA challenge, while this was significantly suppressed in mice that were treated with the CRA-NE vaccine (Figure 4C). In addition, mRNA levels of *muc5ac* and *gob5/clca3* were significantly reduced in the lungs of mice treated with the CRA-NE vaccine, indicating reduced mucus expression that correlated with less histopathology (Figure 4D). In addition, animals were also tested for changes in airway hyperreactivity (AHR) using a methacholine challenge. Animals that received the NE-CRA had a significant decrease in AHR compared to those receiving CRA only (Figure 4E). Together these data showed that inflammation, mucus hypersecretion and physiologic changes in the lung of mice markedly improved in NE-CRA immunized animals.

Nanoemulsion adjuvant vaccine induced reductions in pathology in CRA allergic airway disease model occurs without significant modulation of the humoral immune response .

Humoral immunity to the eliciting allergen was also characterized in the CRA model of allergic airway disease to determine if similar effects were induced by the NE vaccine in this chronic model. Surprisingly, unlike in the OVA model, NE vaccination did not significantly alter the

CRA-specific IgE, IgG1 or IgG2a in the chronic allergic airway disease model, where sensitized mice have high titers of all three antibody classes (Figure 5). Therefore, the reduction of inflammation and airway hyperreactivity induced by can occur with minimal modulation of the humoral immune response.

Nanoemulsion adjuvant-induced reduction in IL-13 is associated with decreases in ILC2s in the lung.

Because the protection conferred by the NE vaccines in the acute OVA model was associated with changes in Th2 cytokines, we hypothesized that NE vaccines protected predominantly by altering the allergen-associated cellular inflammation. In the CRA model, allergen-specific production of Th2 cytokines was very high in the LN, with no significant differences in CRA-NE immunized mice (Supplemental Figure 1). Given that these mice had significantly reduced inflammation following CRA challenge, cellular responses were next assessed in the lungs to determine if local changes were associated with protection from challenge. IL-13 is a key Th2 cytokine linked to the severity of disease in allergic airway disease, and we found that it was significantly decreased in the lungs of mice that received the CRA-NE vaccine (Figure 6A). We also examined lymphocyte populations in the lung that have been associated with allergic responses to assess whether the CRA-NE vaccine alters their numbers. There was no difference in the total CD4 or total CD8 cell populations in the lungs of vaccine treated vs. CRA treated mice (data not shown), and there was no difference in Treg cells ($CD4^+$, $CD25^+$, $Foxp3^+$) or activated Th1 cells ($CD4^+$, $CD69^+$) in the lungs (Figure 6B and 6C). In contrast, there were very significant decreases in ILC2 cells (Lin^-, $CD45^+$, $CD90^+$, $ST2^+$, $GATA3^+$) in the lungs of the mice treated with the NE adjuvanted CRA as compared with control sensitized animals (Figure 6D). This suggests that in this chronic allergic airway disease model the lung ILC2 cells are a prominent source of IL-13 and were significantly decreased by intranasal administration of NE-adjuvanted allergen.

Nanoemulsion adjuvant-immunized mice have reduced alarmins in the lungs following allergen challenge

The activation and proliferation of ILC2 cells depends upon alarmin cytokines, including IL-25 and IL-33.[38-41] We hypothesized that reduced lung ILC2s in NE-immunized mice may be due to

changes in the production of these cytokines. Cytokine levels were quantified in lungs isolated following CRA challenge. Expression of both IL-25 and IL-33 were significantly reduced in the lungs of mice that received the CRA-NE vaccine prior to allergen challenge (Figure 6E). We next wanted to determine if alarmins were similarly suppressed in the OVA model to identify this as a common target of the NE vaccines in the two distinct models. Similar decreases in these alarmins was confirmed in the lung isolates from OVA-NE immunized mice (Supplementary Figure 2). These data suggest that intranasal administration of NE-adjuvanted allergen reduces lung ILC2 cells through suppression of the alarmins IL-25 and IL-33.

Discussion

Allergic disease pathogenesis involves both allergen-specific IgE, which is responsible for immediate hypersensitivity reactions, and Th2-polarized cellular inflammation. Most therapeutic approaches to treat atopic diseases have been directed at either IgE or cellular immunity; however the interactions between these components as well as their relative contributions to atopic diseases are not entirely clear. Here we sought to evaluate the effects of allergen-specific immunotherapy using a NE adjuvant that biases immune responses towards a Th1 and Th17 phenotype while suppressing Th2 polarized immune responses and cellular inflammation in the tissue. We have previously reported that nasal immunization with allergen in NE results in markedly decreased reactivity in murine models of food allergy.[24-26] Of interest, this occurred without an absolute elimination of allergen specific IgE titers but was associated with reductions of allergen-specific production of IL-4, IL-5 and IL-13 and increased production of IL-2, IFN-γ, IL-10 and IL-17 in lymphocytes of treated animals.[24-26] There was increased production of allergen-specific IgG, and therefore blocking antibody that could also have played a role in the reduced immediate reactivity. Therefore, we decided to examine the activity of this therapeutic immunization strategy in two models of allergic airway disease that are based predominantly on cell-mediated Th2 inflammation rather than IgE reactivity.

Our results in the current studies demonstrate that allergen-specific immunotherapy could markedly reduce allergic inflammation in both the OVA ("acute") and CRA ("chronic") allergic

airway disease models. The OVA model was used to develop the therapeutic NE immunization protocol and demonstrate reduction in inflammation. These findings were confirmed in the more biologically relevant CRA model which employs cockroach allergen which is a common trigger of allergic airway disease in humans. The CRA model also induces a very robust Th2 cellular immune response and severe airway hyper-responsiveness. Therapeutic immunization with allergen formulated in NE resulted in suppression of inflammation associated with allergen challenge. Consistent with our previous work in food allergy models, protection from reactivity to allergen challenge was associated with reduction of IL-5 and IL-13, and increases in IFN-γ, IL-2 and IL-10 production by lymphocytes from regional lymph nodes and the spleen in the OVA model and lungs of the CRA model. This appeared to be independent of IgE reactivity, as antibody titers were either minimally changed (OVA model) or totally unchanged (CRA model) after treatment. Of interest, ILC2 cells, thought to be a major source of allergic cytokines during chronic asthma responses in humans and animals, were markedly reduced after the immunizations. This coincided with a reduction in epithelial-derived innate alarmin cytokines IL-25 and IL-33, which induce activation of ILC2s and maturation of Th2 cells. Together, these results indicate that NE adjuvant/allergen-specific immunotherapy is altering allergic inflammation, specifically alarmin expression and ILC2 accumulation, as the major effect on the allergic phenotype in these animals, and not simply altering the dynamics of IgE/allergen interactions.

Other recent findings reinforce that changes in Th2 cytokines and their signaling are important in suppressing allergic inflammation. Dupilumab, which blocks IL-4 and IL-13 signaling through the common alpha chain of these receptors and has been shown to be widely effective in blocking allergic inflammation in atopic dermatitis, asthma, and nasal polyps.[22,23,42,43] These are all diseases that are dependent on allergic inflammation rather than anaphylactic IgE reactions.[44] The speed of dupilumab action, with changes seen within days, suggests these cytokines are important in propagating allergic inflammation leading to pathogenic outcomes that alter lung function. Similar effects are observed with anti-IL-5 in diseases where eosinophils predominate and cause airway damage and remodeling of the airways. In contrast, anti-IgE has a more limited effect on atopic dermatitis and nasal polyps and is more effective with IgE mediated reactions, such as food anaphylaxis and urticaria.[45]

Therefore, the suppression of allergic cytokine production and effector cells such as eosinophils would appear to be central to preventing pathology in non-anaphylactic/IgE mediated allergic diseases. Thus, the ability of the nanoemulsion vaccine to primarily alter the type 2 immune responses, including T cells, eosinophils and ILC2, likely would provide a longer term and more significant impact on chronic allergic responses in the lungs.

The NE-based vaccines are administered intranasally at low volumes (6 µl/nare) that are retained in the nasal cavity and are not inhaled into the lungs or swallowed to enter the gastrointestinal tract.[27,46] However, antigen is taken up by dendritic cells in the nasal mucosa, leading to delivery to and retention in nasal associated lymphatic tissue and regional lymph nodes.[47] NE vaccines also upregulate mucosal homing markers on T cells, resulting in antigen-specific immune responses in mucosal immune sites, such as the lungs and intestine.[25,26,48] Mice that receive the NE-based vaccines have antigen-specific immune responses in both local (nasal mucosal, regional LN) and more distant (spleen, intestine) immune compartments, so the NE vaccines induce both local and more systemic effects. Specifically, in the chronic CRA model here, the effects of the NE vaccines were observed mostly in the lungs, suggesting that the local effects at the site of allergic inflammation are key drivers of modulation and suppression of disease.

The actual mechanism of how therapeutic NE immunizations alter the allergic phenotype in these animals is not entirely clear. This is not an induction of "tolerance" since despite increases in IL-10 production there is more allergen specific Th1, Th17 and IgG generated in response to allergen than before treatment. It also is not desensitization, as our prior work showed that the reductions in immediate reaction and immune modulation were maintained for long periods of time after treatment was stopped.[26] Therefore, the sustained unresponsiveness of allergic Th2 responses that was achieved with these immunizations appears to primarily divert the immune responses toward less pathogenic inflammation and cytokine profiles. There are several potential mechanisms that may be operative that alter the immune response. IL-10 is increased in these animals and has suppressive effects on allergic immune responses.[49,50] However, it is unlikely that IL-10 is solely responsible since the response preferentially suppresses the Th2 response while the Th1 response is increased. The protective effects are

likely due to an overall change in the cytokine milieu specifically in the lungs, which alters the recruitment of inflammatory cells, including ILC2s and eosinophils, thus suppressing allergic inflammation in the lung.

The NE formulation used here induces IL-17. IL-17 plays a potentially dual role in the allergic response in the lungs. While IL-17 can drive sensitization to allergen, once mice are already sensitized, exogenous IL-17 reduces both lung eosinophilic inflammation and AHR through inhibition of DC function, chemokines, and IL-4 and IL-5.[51] Other groups have demonstrated that the pathology associated with IL-17 in allergic responses occurs when IL-17 is produce along with high levels of Th2 cytokines.[52,53] Because NE vaccines suppress Th2 cytokines, particularly IL-13, the NE-induced IL-17 is likely not pathogenic and may in fact play a positive role in suppression of allergic disease. Th17 cell-mediated immunity may also suppress IgE responses, as has been recently indicated for Th17 immunity associated with human autoimmune disease. [54]

Another potential mechanism that contributes to the overall change in the immune environment in NE-immunized mice is that cellular migration into the lung is reduced due to alterations in chemotactic factors or adhesion molecules that may be altered due to the primary alteration of cytokine profiles. Eosinophil recruitment to the lungs was reduced in the NE-immunized mice. We have previously demonstrated that NE immunization reduces mast cell accumulation in the small intestine in food allergy models. Eosinophils and mast cells are effector cells responsible for reactivity to allergen in the lungs and intestine, respectively, and recruitment of both of these effector cell types can be regulated by ILC2s.[55,56] Since the driving force behind ILC2 cells are innate epithelial cytokines, TSLP, IL-25, and IL-33, changes in the production of these cytokines/alarmins may be important. One interesting possibility is that the mucosal Th1/Th17 immune response generated with the NE-adjuvant is directly suppressing the Th2 immunity through an effect on these cells through the reduction of alarmin production, consistent with the reduced IL-25 and IL-33 in the lungs of NE-immunized mice.

Th1 cytokines have been implicated in suppressing inflammation and reactivity in some models of allergic disease, and this could explain the reductions in ILC2 in the lungs of these animals. It also has been reported that Th2 cells are critical for activation of ILC2s in the lung in a house dust mite model of allergic airway disease.[57] ILC2 activation in allergic airway disease

may require two signals, an innate signal from alarmins and an adaptive signal from T cells producing IL-4/IL-13.[58] Th1 cells induced by NE may also directly impact ILC2s through the production of IFN-γ. IFN-γ restricts ILC2 accumulation through limiting the IL-33-dependent maintenance of ILC2s in the tissue.[59-61] Our data suggest that the increased IFN-γ from NE-induced Th1 cells suppresses IL-33-mediated ILC2 activation, resulting in reduced allergic disease. The NE adjuvant may be skewing the populations of innate cells, perhaps by downregulating ILC2s and increasing ILC1s or ILC3s or inducing a newly defined regulatory subset of ILCs.[62,63] Further investigation into this last possibility is warranted and is a focus of our ongoing work.

Taken together, these data identify a novel, allergen-specific approach to suppress allergic inflammation in the lung. This approach fundamentally alters the lung immune environment and alters the response towards an allergen, as opposed to inducing classic immune tolerance or desensitizing against IgE-mediated hypersensitivity reactions. It will provide an important tool for examining the control of allergic inflammation and ILC2 cells that could provide long-lasting suppression of allergic disease.

Conflicts of interest

J.R.B. and J.J.O. are inventors of the adjuvant technology involved in this research, and patent applications has been submitted for this technology (PCT/US2015/054943 and PCT/US2020/060362). J.R.B. holds stock in Blue Willow Biologics, a company that has licensed this adjuvant technology from the University of Michigan. The other authors declare no conflicts of interest.

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Figure legends

Figure 1. NE immunization reduces lung histopathologic changes after allergen challenge. (A) Schedule of sensitization, immunotherapy and allergen challenge. Mice were sacrificed two days after the last challenge to assess lung histopathology. (B) Representative images of PAS staining of lungs. Scoring of severity of (C) inflammation,(D-E) mucus and (F) eosinophil accumulation in lungs. Data are expressed as mean ± standard deviation (n=8). Statistically significant differences (p<0.05) are indicated by *.

Figure 2. NE immunization suppresses Th2 immunity and induces Th1/Th17. Cellular recall immune responses to OVA protein were measured in lymphocytes harvested from the cLN, spleen and lungs following the experimental design shown in Figure 1. (A) Cytokine secretion in cLN, splenocyte and (B) lung lymphocyte cultures was determined by a Luminex multiplex assay. Cytokine production has been normalized to matched control unstimulated lymphocyte cultures from each individual animal and tissue. (C) Numbers of cytokine producing cells in the cLN were determined by ELISpot. Data are expressed as mean ± standard deviation (n=8). Statistically significant differences (p<0.05) are indicated by *.

Figure 3. Modulation of OVA-specific humoral immune response in NE-treated mice. Serum was obtained from mice at the end of the study in the OVA model. OVA-specific (A) IgE,

(B) IgG1 and (C) IgG2a antibodies were measured by ELISA. Statistically significant differences ($p<0.05$) are indicated by *. n.d. indicates not detected.

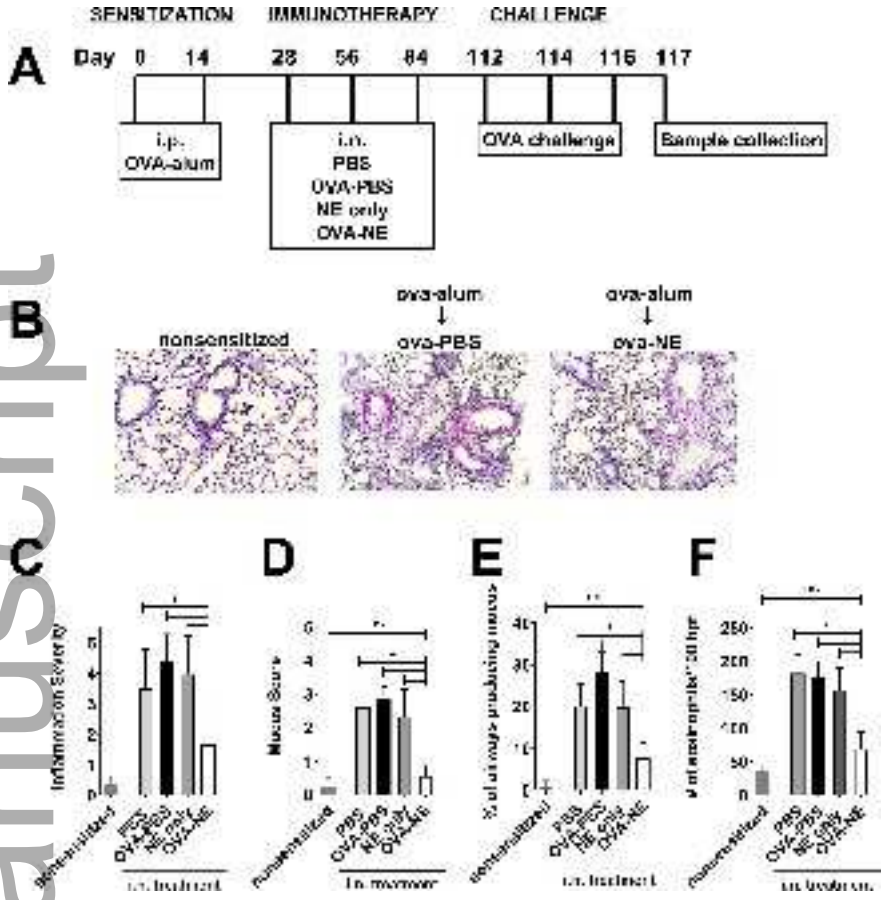
Figure 4. Nanoemulsion adjuvant vaccine reduces histopathology and disease associated parameters in severe allergic airway disease in mice. (A) Schedule of sensitization, immunotherapy and allergen challenge. (B) Histopathology was examined in harvested lung tissue 24 hrs after the final allergen challenge. Representative images of PAS staining of lungs are shown. (C) Eosinophils were identified in the lungs by morphometric analysis. (D) Lung mRNA was isolated from individual mice and subjected to quantitative PCR to analyze the mucus associated mRNA compared to age matched naïve, non-allergic mice. (E) Animals were subjected to airway resistance measurements 24 hour after the final allergen challenge using plethysmography. An IV Tail vein injection of methacholine (250 µg/kg) was used to induce AHR. Data are expressed as mean ± standard deviation (n=8). Statistically significant differences ($p<0.05$) are indicated by *.

Figure 5. Persistence of allergen-specific IgE in serum of treated mice in CRA model. Serum was obtained from mice at the end of the study in the CRA model. CRA-specific (A) IgE, (B) IgG1 and (C) IgG2a antibodies were measured by ELISA. n.d. indicates not detected.

Figure 6. NE immunization reduces ILC2 accumulation in the lungs in severe allergic airway disease model. (A) Lung mRNA was isolated from individual mice and subjected to quantitative PCR to analyze IL-13 mRNA compared to age matched naïve, non-allergic mice. Lungs from allergen challenged mice were dispersed into a single cell suspension using collagenase digestion. (B) Treg cells ($CD4^+$, $CD25^+$, $Foxp3^+$), (C) activated Th cells ($CD4^+$, $CD69^+$), and (D) ILC2 cells (Lin^-, $CD45^+$, $CD90^+$, $ST2^+$, $GATA3^+$) were identified by flow cytometry. (E) Following CRA challenge, lung mRNA was isolated from individual mice and subjected to quantitative PCR to analyze the IL-25 and IL-33 mRNA compared to age matched naïve, non-allergic mice. Data are expressed as mean ± standard deviation (n=8). Statistically significant differences ($p<0.05$) are indicated by *.

Supplementary Figure 1. CRA allergic airway disease model is purely Th2 driven without induction of Th1 or Th17. Cellular recall immune responses to CRA protein were measured in lymphocytes harvested from the lung draining LNs following the experimental design shown in Figure 3. Cytokine secretion from lung-draining lymph node cultures was determined by a Luminex multiplex assay. Cytokine production has been normalized to matched control unstimulated lymphocyte cultures from each individual animal. Data are expressed as mean ± standard deviation (n=8).

Supplementary Figure 2. NE immunization reduces alarmins in the lungs in the OVA model. (A -B) Following OVA challenge, lungs were isolated from individual mice and homogenized for the analysis of (A) IL-25 and (B) IL-33 protein Data are expressed as mean ± standard deviation (n=8). Statistically significant differences ($p<0.05$) are indicated by *.



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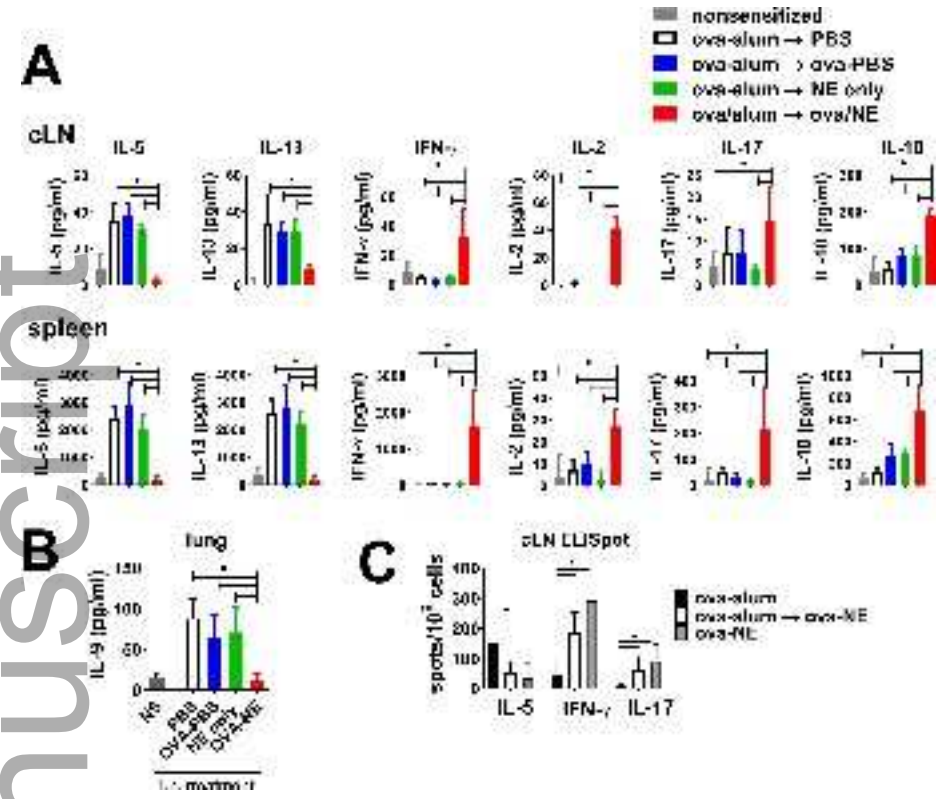

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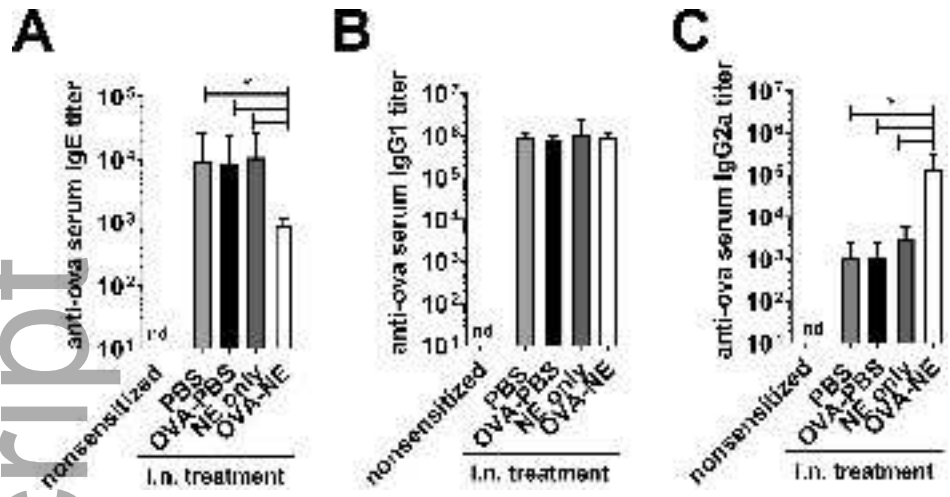

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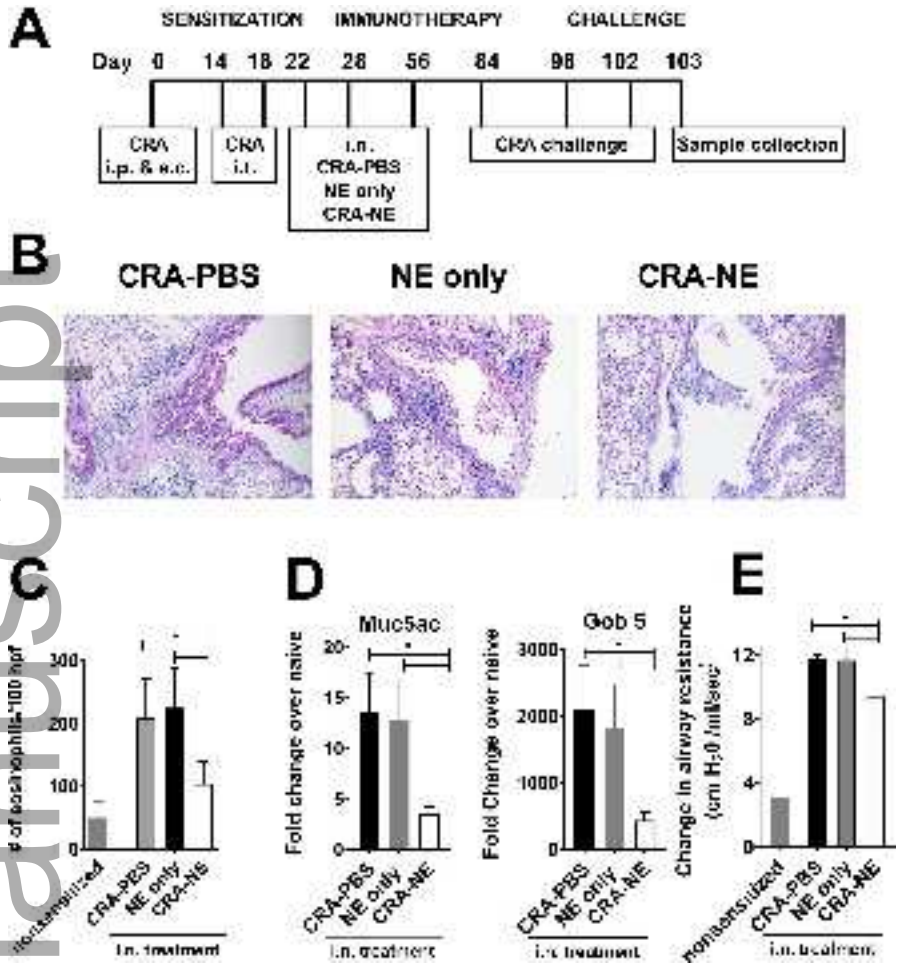

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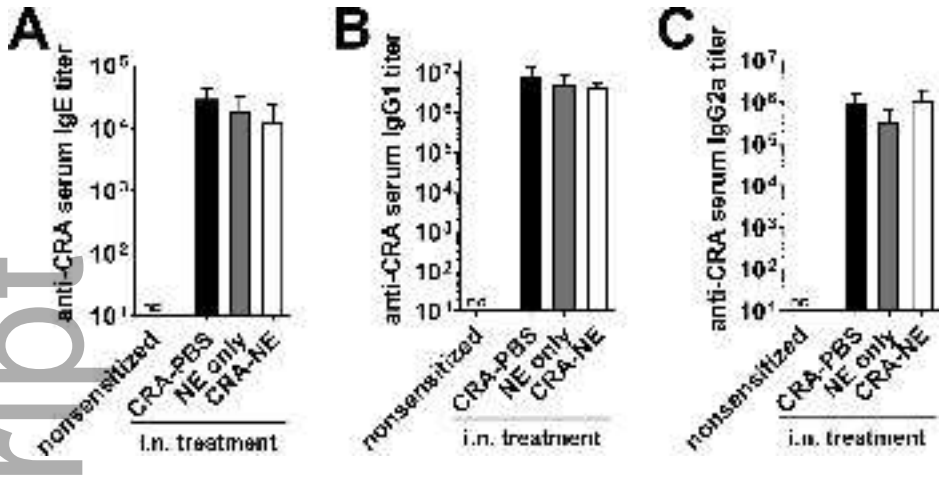

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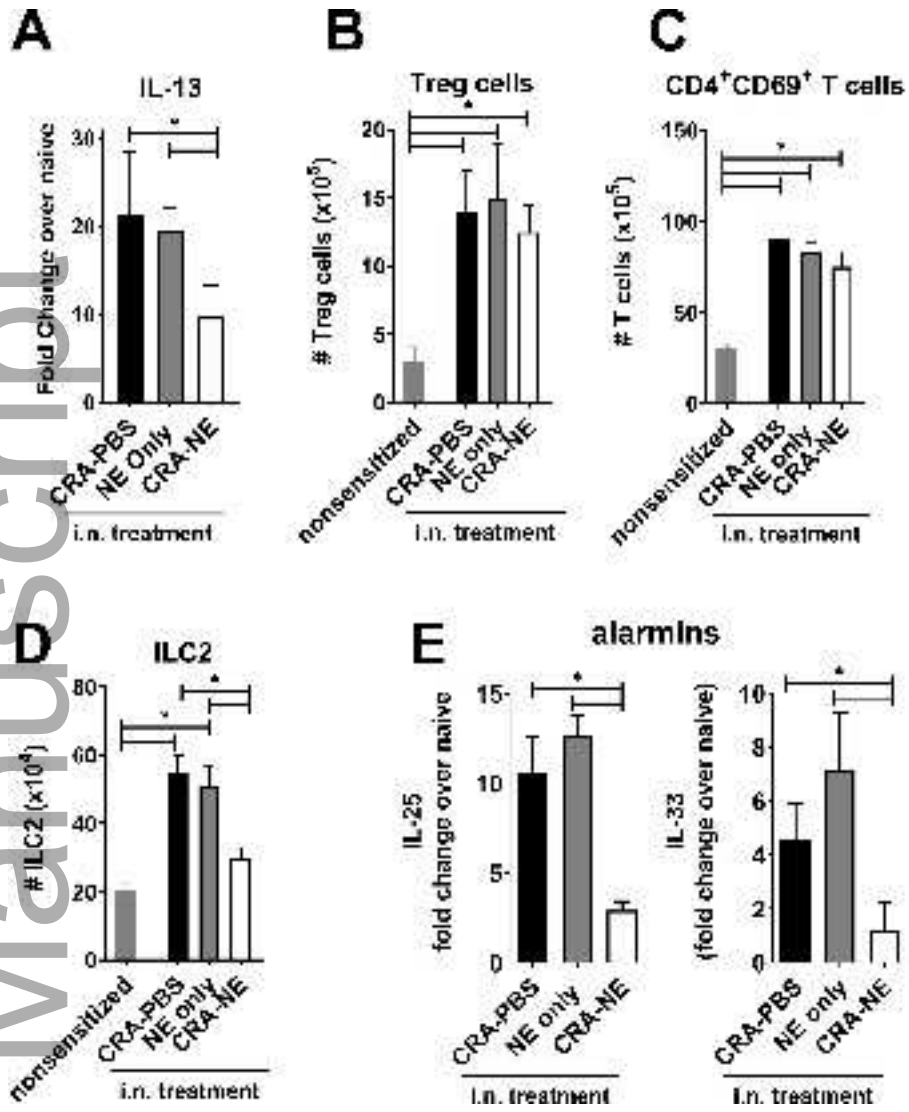

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