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Intranasal nanoemulsion vaccine confers long-lasting immunomodulation and sustained unresponsiveness in a murine model of milk allergy

Short title: Nanoemulsion allergy vaccine confers long-term protection

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Abstract:

Background: Immunotherapy for food allergy requires prolonged treatment protocols and, in most cases, does not lead to durable modulation of the allergic immune response. We have demonstrated an intranasal (IN), nanoemulsion adjuvant that redirects allergen-specific Th2 responses towards Th1 and Th17 immunity, and protects from allergen challenge after only 2-4 monthly administrations. Here, we investigate the ability of this technology to provide long-term modulation of allergy in a murine model of cow's milk allergy.

Methods: Six weeks after sensitization to bovine casein, mice received four, monthly IN immunizations with nanoemulsion formulated with casein. Protection from casein challenge was assessed at 4 and 16 weeks after the final vaccine administration.

Results: The NE vaccine significantly blunted the physiological responses to allergen challenge, and this effect persisted for at least 16 weeks. The protection from challenge was associated with suppression of casein-specific Th2 immunity and induced Th1 and Th17 cytokines as well as induction of IL-10. Of interest, while immunized animals showed significantly decreased Th2 cytokine responses, cow's milk-specific IgE remained elevated in the serum at levels associated with reactivity in control sensitized animals. Protection was associated with suppressed mast cell activation and markedly reduced mast cell infiltration into the small intestine.

Conclusion: The sustained unresponsiveness of at least 16 weeks after vaccination suggests that the nanoemulsion vaccine alters the allergic phenotype in a persistent manner different from traditional desensitization, and this leads to long-term suppressive effects on allergic disease without eliminating serum IgE.

Keywords: allergy treatment, food allergy, immunotherapy vaccines and mechanisms, immunotherapy and tolerance induction, vaccines

Introduction

Food allergies have become common in industrialized nations and affect an estimated 5% of adults and 8% of children in the United States.[1,2] Milk is one of the eight major allergens responsible for the majority of serious food allergy reactions in the United States, and allergy to cow's milk is the most common food allergy in infants and young children.[3] While cow's milk allergy is often outgrown, high milk-specific IgE in infants is associated with persistent milk allergy as well as increased incidence of atopic dermatitis, asthma, rhinoconjunctivitis, and other food allergies. [4,5]

Strict avoidance of an offending food is the primary clinical approach to prevent allergic reactions and anaphylaxis, often coupled with the use of epinephrine for accidental exposure. Recently, however, progress has been made towards the development of immunotherapy for food allergy. This includes oral (OIT), sublingual (SLIT), and epicutaneous (EPIT) immunotherapies (reviewed in [6,7]), and single-allergen OIT for peanut allergy has been submitted to the FDA for approval.[8-10] OIT has also been studied for milk allergy in numerous clinical trials.[11] However, most evidence suggests that OIT is not effective to induce tolerance or durable sustained unresponsiveness (SU) to food allergens. SU, as defined by the ability to pass a double-blind, placebo-controlled food challenge (DBPCFC) 2-8 wks after cessation of the immunotherapy, has been reported to occur in 25-58% of trial participants,[12-14] and this included patients that may have naturally outgrown their allergy. Thus, while these immunotherapies have demonstrated efficacy, this lack of sustained protection suggests that OIT in most cases does not fundamentally change the allergen-specific allergic response.

An optimal goal for treating food allergies would be to generate immune tolerance in allergic individuals and suppress the underlying Th2-polarized cellular immune responses, including IL-4 and IL-13, and allergen-specific IgE antibodies.[1,15-17] Recent work has demonstrated that the induction of tolerance in allergies is associated with both the suppression of mast cell and basophil reactivity and changes in allergen-specific cells, including the upregulation of regulatory markers and increases in the number and/or function of regulatory T

cells (Tregs). Thus, the goal of approaches for long term SU or tolerance to prevent allergic reactions could include both the durable suppression of allergen-specific Th2 immunity and reductions in number and reactivity of effector cells.

Our group has developed an intranasal nanoemulsion (NE) adjuvant that modulates pre-existing allergen-specific Th2 immune responses towards a Th1 and Th17 phenotype.[18] We previously reported that in murine models of peanut allergy, this modulation resulted in protection when mice were challenged with peanut 2-3 weeks after final immunization.[19] As adjuvants are effective in inducing long-term changes in the immune response, we hypothesized that these NE-based vaccines might stably modulate the allergen-specific immune response resulting in durable SU. In this study, we test the ability of an intranasal NE-based vaccine to induce long-term modulation of established Th2 immunity and SU from allergic reactions in a mouse model of cow's milk allergy.

Materials and Methods

Antigen and adjuvants. 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.[20,21] Aluminum hydroxide (alum, alhydrogel) was purchased from InvivoGen. Casein purified from bovine milk, containing the four main types of casein found in cow's milk: α-s1 Casein, α-s2 Casein, β-Casein, and κ-Casein was purchased from Sigma and was solubilized in phosphate buffered saline (PBS) and sterilized by sequential filtration through 0.4 μm and 0.2 μm syringe filters. Endotoxin content of all vaccine components was determined by a limulus amebocyte lysate (LAL) assay (Pierce). There was no detectable endotoxin in PBS, NE or alum (Fig. S1). The stock solution of casein was approximately 5 endotoxin units (EU) per ml; however after dilution into the final vaccine formulation, the total amount of endotoxin given intranasally to each mouse is 0.004 EU per dose. As 0.004 EU is equivalent to approximately 0.4 pg of LPS, this amount of endotoxin is not anticipated to have major effects.

Mice and Immunizations. Specific pathogen-free BALB/c mice (females 3 weeks old) were purchased from Jackson Laboratory. Mice were 4 weeks of age at the onset of the experiment. The experimental design is shown in Figure 1. Allergic sensitization was induced with intraperitoneal immunizations (i.p.) of 125 μg bovine casein (Sigma) adsorbed on 1 mg alum at

weeks 0 and 2. Intranasal (i.n.) immunizations were administered as 12 µl (6 µl /nare) of a formulation containing 20 µg of casein mixed with 20% NE. Casein mixed with PBS alone served as a control. Systemic anaphylaxis was induced by i.p. injection of 100 µg casein and reactions were assessed as described below. For oral challenge experiments, mice were fasted for 5-6 hrs to ensure gastric emptying and then were challenged with 0.2 ml of 4% fat cow's milk by oral gavage. Mice were challenged orally every other day for a total of 7 gavages.[22] All animal procedures were performed according to the University of Michigan Institutional Animal Care and Use Committee and the National Institutes of Health guide for the care and use of laboratory animals.

Assessment of hypersensitivity reactions. Anaphylactic symptoms were evaluated for one hour following challenge using the following scoring system (modified from [23,24]): 0, no symptoms; 1, prolonged rubbing and scratching around the nose, eyes or head; 2, puffiness around the eyes or mouth, diarrhea, piloerection, and/or decreased activity with increased respiratory rate; 3, labored respiration, wheezing, stridor, and/or cyanosis around the mouth and tail; 4, tremor, convulsion, no activity after prodding and/or moribund; 5, death. Rectal temperature was monitored for 60 min following challenge. Mice were bled 60 minutes following challenge, and serum mouse mast cell protease-1 (MCPT-1) was determined by ELISA (eBioscience).

Measurement of serum antibodies. Sera were obtained by saphenous vein bleeding or by cardiac puncture post-euthanasia. Serum was separated from whole blood by centrifugation at 1500×g for 5 minutes after allowing coagulation for 30 to 60 minutes at room temperature. Serum samples were stored at −20°C until analyzed. Casein- and cow's milk- specific IgE antibodies were determined by ELISA as described previously[19]. Briefly, serially diluted serum samples were incubated on microtiter plates coated with 20 µg/ml bovine casein or cow's milk. IgE, IgG1 and IgG2a were detected with alkaline phosphatase conjugated anti-mouse IgE (Rockland) and IgG1 and IgG2a (Jackson ImmunoResearch) antibodies and Sigma Fast™ p-nitrophenyl phosphate substrate (Sigma) and quantified by measuring the optical density (OD) at 405 nm. The antibody concentrations are presented as endpoint titers defined as the reciprocal of the highest serum dilution producing an OD above background of naïve sera. The cutoff value is determined as the OD (mean+2 standard deviations) of the corresponding dilution of naive sera.[25,26]

Analysis of cytokine production. The cellular recall response was evaluated in lymphocytes isolated from mesenteric lymph nodes. Single cell lymphocyte suspensions were cultured *ex vivo* ± casein (25 µg/ml) at 37°C. After 72 hours, cytokine secretion was measured in cell culture supernatants using Luminex Multiplex detection system (Millipore).

Acute allergic skin response. The acute allergic skin response (ear swelling at 1h) was determined in anesthetized mice after intradermal (i.d.) injection of 10 µg casein in the ear pinnae. Ear thickness was measured in duplicate using a digital micrometer. The allergen specific change in ear swelling was compared with the non-specific ear swelling due to allergen injection in the PBS (sham)-sensitized group.

Mast cell quantification. 18 hours after challenge, the duodenum and jejunum were fixed in 10% formalin, embedded in paraffin and cut into 5-µm thick sections. Tissue sections were stained for chloroacetate esterase (CAE) activity as previously described.[22,27] Quantification of mast cells was performed by counting the number of CAE-positive cells from at least 25 fields of view at 40X magnification, and the data are reported as number of cells per field.

Serum transfer experiments. Mice were sensitized i.p. with casein and alum at wks 0 and 2 and immunized i.n. with casein-NE or casein-PBS at wks 6, 12, 18 and 20 as described above and in Figure 1. At week 24, mice were sacrificed and serum was harvested. Serum from mice receiving the same treatments was pooled and transferred into naïve recipient mice (200 µl/mouse) by injection into the tail vein. 24 hrs after serum transfer, recipient mice were challenged orally with cow's milk to assess reactivity.

Statistics. Statistical comparisons were assessed by the Mann-Whitney test using GraphPad Prism version 7 (GraphPad Software). The p value < 0.05 was considered as significant. Results presented here are the representatives of at least two independent experiments.

Results

Intranasal immunization with NE adjuvant suppresses allergic reactions and anaphylaxis

BALB/c mice were sensitized to the cow's milk protein casein at weeks 0 and 2 by i.p. injections of bovine casein adsorbed on alum.[19] Six weeks after sensitization, one group of mice received four, monthly i.n. immunizations with casein formulated in NE while the others received i.n. casein or PBS as controls. The mice were subsequently challenged intraperitoneally with casein to assess protection. At week 24, 4 weeks after the last i.n. immunization, sensitized control mice had profound reactions to challenge, indicated by core body temperature loss of greater than 5°C and severe symptoms of anaphylactic shock including pruritus, puffiness around the eyes and mouth, labored respiration or wheezing and lack of activity when prodded (Figure 1B). As compared to controls, the NE vaccine markedly suppressed physiological responses to allergen challenge. There was significant improvement in body temperature loss ($p=0.003$) as well as anaphylaxis symptom score ($p=0.002$), and mice that received the NE vaccine exhibited only mild symptoms of allergic reaction, as none experienced respiratory problems or shock. In order to quantify mast cell degranulation, MCPT-1 was measured in serum following challenge. Consistent with the clinical symptoms of allergic reaction, immunized mice had a significant reduction in MCPT-1, with average levels of 74 ng/ml compared with 528 and 840 ng/ml in control groups that received i.n. casein or PBS ($p=0.0079$). No significant differences in reactivity were observed in sensitized mice that received i.n. casein in PBS compared to sensitized mice that were not treated intranasally with allergen, demonstrating that the four i.n. exposures to 20 µg casein did not significantly modulate reactivity. Similarly, i.n. administration of NE without allergen had no effect on suppression of allergic reactivity (Fig. S2).

In order to investigate durability of protection, mice were challenged 16 weeks after the final i.n. immunization. The increased time between sensitization and challenge did not influence the severity of allergic reactions for control mice (Figure 1C). In contrast, immunized mice were protected from challenge as body temperature loss, anaphylaxis symptom score and MCPT-1 were significantly improved ($p=0.0001$, 0.0002, and 0.002, respectively). The protection from challenge appeared complete, as there were no differences in reactions in NE immunized mice compared with non-sensitized mice. This indicated that therapeutic

immunization with NE results in complete protection 16 weeks after the final intranasal administration, with the protection appearing more pronounced than at earlier time points.

Intranasal immunization with NE adjuvant suppresses allergy-associated Th2 responses

We have previously reported that allergen-NE immunization modulates allergen-specific Th2-polarized immunity while inducing IL-10, Th1 and Th17 immune responses.[18,19] To confirm this in the milk allergy model, mesenteric lymph node cells were stimulated *ex vivo* with casein to characterize the recall response to allergen after the 4 wk SU challenge at week 24 (Figure 2A). Upon stimulation with casein, cells from casein-alum sensitized mice produced predominantly Th2-type cytokines (IL-4, IL-5, IL-13) with lower levels of IFN-γ. IL-10, IL-17 and IL-22 (Figure 2A). Lymphocytes from mice that received subsequent casein-NE immunizations produced significantly more Th1 cytokines and significantly less Th2 cytokines. Casein-NE significantly increased IL-10 ($p=0.01$) and the Th17 cytokines IL-17 and IL-22 ($p=0.0079$) and decreased IL-4 and IL-13 ($p=0.016$ and 0.032, respectively). Consistent with reactivity data described above, i.n. administration of casein did not have any significant effects on cytokine production, compared with sensitized mice that were not treated i.n. with casein. Lymphocytes from PBS control mice (non-sensitized but challenged) did not produce measurable cytokine upon restimulation with casein.

Cytokine production was also characterized after the 16 wk SU challenge (week 36) to determine if modulation of the allergen-specific cytokine response persisted for 16 weeks after the final i.n. immunization. Consistent with the strong protection from challenge at this time point, IL-4 and IL-13 production remained significantly suppressed ($p=0.001$ and 0.007) (Figure 2B). Interestingly, from weeks 24 to 30 there was an approximately 3.5-fold reduction in IL-4 and IL-13 in the casein-NE-immunized animals while the production of these cytokines was increased in the non-immunized mice possibly as a result of reactivity upon challenge.

NE immunization-induced enhancement of IFN-γ, IL-17, and IL-22 was maintained at week 36 ($p=0.042$, 0.01 and 0.0007). However, at this later time point, there was no significant difference in IL-10 production between mice that received i.n. instillations of casein-NE or casein-PBS. While IL-10 production remained constant at approximately 4000 ng/ml in mice that received casein-NE, there was a five-fold increase in IL-10 production in mice receiving casein-PBS. For all cytokines tested, there was largely no detectable antigen-specific cytokine

production in the non-sensitized control group at week 24, and all cytokines increased at week 36 to low, but detectable, levels. This suggests minimal effect from the prior casein challenge on casein-specific cytokine production at week 36 in non-sensitized animals. Overall, these data demonstrate that modulation of Th2 cytokine production not only remains modulated for at least 16 weeks after the final immunization but becomes stronger in the months after immunization.

NE reduces mast cell infiltration into tissue and protects despite presence of allergen-specific IgE in the serum

Allergen-specific antibodies were quantified from the serum throughout the course of the study (Figure 3). Sensitization and immunization with casein induced similar levels of antibodies specific for casein and cow's milk, demonstrating that these antibodies bind to casein epitopes that are present in cow's milk. Immunization with casein-NE largely prevented further increases in allergen-specific IgE that occurred in non-immunized mice. Surprisingly, at week 24 despite being protected from challenge, serum casein- and cow's milk-specific IgE titers were not significantly different in casein-NE- and casein-PBS-immunized mice (Figure 3A). At week 36, allergen-specific IgE was significantly decreased in the serum of NE-immunized mice ($p=0.007$); however, IgE antibody titers remained significantly elevated at titers where non-immunized mice would be expected to react to challenge (Figure 3A). While allergen-specific IgG1 titers were not significantly affected by the casein-NE treatment (Figure 3B), IgG2a titers were increased more than 20-fold in the serum of mice that received casein-NE ($p<0.0001$), demonstrating a shift in the profile of cow's milk-specific antibodies (Figure 3C).

To confirm that the NE vaccine could protect from IgE-mediated allergic reactions, the acute allergic skin response to intradermal injection of casein was measured. NE treatment reduced the acute allergic skin responses compared to the casein-sensitized control mice (Figure 4B). As this further suggested that the NE-immunized mice were protected from IgE-mediated allergic reactions, we tested the ability of NE to protect from reactivity in an additional experimental food allergy model in which intestinal mast cells drive the clinical phenotype.[22,28,29] As illustrated in Figure 4A, two weeks after the final i.n. immunization, mice were challenged orally with cow's milk every other day for 2 weeks. NE-immunized mice were significantly protected from allergic reaction to oral cow's milk challenge, as indicated by a greater than 10-

fold reduction in MCPT-1 ($p=0.0002$) (Figure 4C). Mast cells were quantified in the small intestine to determine if NE immunization protected through blocking the accumulation of mast cells in the tissues. There was an almost 10-fold increase in small intestine mast cells in sensitized mice, however, mast cell infiltration was significantly lower in NE-immunized mice ($p<0.0001$) and was identical to mast cell numbers observed in non-sensitized mice (Figure 4D and S3). These data suggest that while allergen-specific IgE circulates in the blood, the NE-immunized mice are protected from challenge due to the lack of mast cell accumulation in the tissue.

In order to further assess the role of the humoral immune response in the protection conferred by NE immunization, a passive sensitization experiment was performed. Serum was harvested from mice following sensitization and immunization and transferred into naïve recipient mice. Recipient mice were challenged to determine if the serum from NE-immunized mice would confer reactivity. Mice that received serum from sensitized mice that were immunized with casein-PBS reacted to challenge, as demonstrated by a decrease in core body temperature and increase in MCPT-1 (Figure 5). Mice that received serum from casein-NE immunized mice had significantly reduced reactions. These data demonstrate that while serum in these mice contains allergen-specific IgE, it does not confer sensitively to the allergen.

Discussion

To date, no immune approach to address food allergies has demonstrated long-term protection from reactivity, identified as SU, which is an unmet need for the millions of patients with food allergies. We have investigated the use of adjuvants to induce a durable modulation of allergen-specific immune responses that is effective with few immunizations. Our previous results demonstrated that NE-based allergy vaccines could suppress peanut allergy in several mouse models a few weeks after administration of the last immunization. The present studies investigate whether the immunomodulation of the allergen-specific response by NE vaccine could induce durable immune alterations capable of conferring long-term SU and protection from allergen challenge. We demonstrated that NE-immunized mice were protected for 16 weeks following NE immunization and these mice were completely unresponsive to allergen challenge at that late time point. Protection from allergen challenge correlated with continued

suppression of allergen-specific Th2 immune responses, suggesting that the changes in the allergic immune phenotype were also durable.

While allergen-specific IgE is commonly used to diagnose food allergies, many patients with allergen-specific IgE in the blood do not react upon consumption of that food.[30,31] In this study, while cow's milk-specific IgE was significantly decreased in the serum of NE-immunized mice at the end of the study, IgE titers remained significantly elevated to similar levels where sensitized control (non-NE vaccinated) mice reacted to challenge. Since these NE-immunized mice were protected from IgE-mediated allergic reactions, this suggests that similar to food allergic humans, serum allergen-specific IgE is not completely predictive of clinical reactivity. In addition, while serum casein-specific IgE was not completely suppressed in NE-immunized mice, there was complete suppression of mast cell accumulation in the intestine after oral challenge, consistent with reports that food allergic reactions in murine models of food allergy are dependent upon tissue mast cell infiltration.[22,28]

Serum transfer from NE-immunized mice was used to dissect the humoral and cellular immune mechanisms responsible for the protection induced by the NE vaccines. While transfer of serum from sensitized and non-immunized mice passively sensitized mice, serum from sensitized and NE-immunized mice did not result in reactivity in recipient mice. The serum from NE-immunized mice contained allergen-specific IgE; however, the transfer of this IgE did not transfer reactivity. This suggests that modulation of the allergen-specific antibody profile may play a key role in the protection from challenge observed following NE immunization. Modulation of the humoral immune response can lead to protection through multiple mechanisms. NE immunization may induce blocking antibody that can neutralize the allergen before it binds to IgE on effector cells.[32,33] Mast cell degranulation can also be suppressed through activation of the inhibitory Fc receptor FcγRIIb.[33-35] Allergen-specific IgG2a is significantly increased in NE-immunized mice at both the 24 week and 36 week challenges and may be involved in the inhibition of mast cell activation either through activation of FcγRIIb or by blocking allergen from binding to IgE. Together, these data suggest that while allergen-specific IgE circulates in the blood, NE-immunized mice may be protected from clinical reactions due to reduced mast cell recruitment, accumulation and activation in the gut. These

results also suggest that tissue mast cell infiltration would be a strong predictor of food allergy reactivity compared to allergen-specific serum IgE.

Other studies have demonstrated efficacy of immunotherapies for food allergies in mice but have typically challenged mice within two weeks of the last therapeutic intervention.[36-45] As this is a timepoint where the effect of desensitization could not have worn off, we modified this approach by evaluating SU in excess of three months after discontinuation of therapy. While our studies were performed in mice, comparisons to studies of OIT performed in both mice and humans can provide insights into how this might translate into protection in humans. Studies of OIT in mice have demonstrated that protection is lost 2 weeks after stopping OIT. [46] In humans, however, OIT has led to SU intervals of 2-8 weeks in a fraction of patients. Despite this, most OIT human trials also have shown that as time post treatment increases the percentage of patients who pass the DBPCFC decreases. [47,48] Thus, it is significant that the NE vaccines induce protective immunity that persists in all mice for at least 16 weeks off treatment. It also suggests that the complete unresponsiveness at 16 weeks after the last immunization in mice could translate into a significant duration of protection in humans.

Desensitization and tolerance are two distinct outcomes of immunotherapy, and it is important to distinguish between the two. Desensitization is an increase in the threshold amount of food that can be ingested without reaction while on the therapy. Desensitization is mediated by changes in effector cells, such as mast cells, but does not involve modulation of the underlying allergen-specific immune mechanisms. Thus, desensitization is temporary and the individual remains allergic to the allergen, reacting to the antigen soon after the therapy is stopped. Conversely, true tolerance is more permanent protection from reaction after discontinuation of the immunotherapy. Tolerance involves modulation of effector cells as well as allergen-specific immune cells, typically through the induction of regulatory mechanisms. [49,50] While the sustained unresponsiveness observed with OIT is likely mostly due to desensitization, the durable protective immunity and immunomodulation induced by the NE vaccines presented here are not consistent with desensitization and suggest an immune phenotype more consistent with tolerance.

We have previously reported that therapeutic immunization with NE induces Tregs.[18,19] Increases in both Tregs and IL-10 suggests that the suppression of Th2 immunity and subsequent protection from allergen challenge is due to the induction of regulatory mechanisms. A similar induction of Tregs and IL-10, as well as IFN- γ, has been reported in grass pollen allergic individuals with decreased Th2 immune responses after allergen immunotherapy.[61, 62] Induction of IL-10 is not sufficient for suppression of reactivity, as in this study casein-sensitized mice that received i.n. casein-PBS retain reactivity at week 36 despite having increased IL-10. This suggests that induction of IL-10 without the suppression of Th2 immune responses is not sufficient to suppress reactivity.

In summary, administering allergen with NE induces long-term immune changes that provide protection from allergen challenge for months after discontinuation of therapy. The cytokine profiles in allergic mice after immunization with the NE vaccines showed Th2 cytokines remained significantly suppressed and Th1/Th17 allergen-specific responses were augmented. This suggests allergen/NE immunization reprograms the immune system towards a balanced immune response that may have more lasting effects in suppressing allergic disease than traditional allergen desensitization immunotherapy.

Conflicts of interest

J.R.B. holds stock in Blue Willow Biologics and is an inventor of the nanoemulsion technology that the University has licensed to Blue Willow Biologics. One of these technologies is involved in this research. J.R.B. and J.J.O. are inventors on a patent application that has been submitted based on this technology (PCT/US2015/054943). The other authors declare no conflicts of interest.

Author contributions

J.J.O. and J.R.B. designed the study. J.J.O., J.J.L., K.W.L., H.K.L., A.M.M., and T.D.T. performed experiments and interpreted data. J.J.O. and J.R.B wrote the manuscript.

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Figure Legends

Figure 1. Protection conferred by NE immunization is sustained for at least 16 weeks. (A) Mice were sensitized with casein-alum (CS-alum) and immunized i.n. with 4 administrations of casein-NE (CS-NE) or casein-PBS (CS-PBS). Mice were challenged intraperitoneally with casein at (B) week 24, 4 weeks after the final i.n. immunization, or (C) week 36, 16 weeks after the final i.n. immunization. In order to assess sustained unresponsiveness (SU), the degree of allergic reactions was measured by change in core body temperature, clinical anaphylaxis score, and serum MCPT-1 levels as determined by ELISA. Statistically significant differences ($p<0.05$) are indicated by *.

Figure 2. NE immunization suppresses Th2 immunity and induces Th1/Th17 in mice sensitized to cow's milk protein. Cellular recall immune responses to bovine casein were measured in mLN lymphocytes harvested from mice at weeks (A) 24 and (B) 36. Cytokine secretion in culture supernatant was determined by a Luminex multiplex assay. Cytokine production has been normalized to control unstimulated lymphocyte cultures for each sample. Values are calculated as [stimulated] – [unstimulated] = Total (pg/ml) for each cytokine. Data are expressed as mean ± standard deviation. Statistically significant differences ($p<0.05$) are indicated by *.

Figure 3. NE immunization modulates allergen-specific IgE and IgG in serum. Serum casein- and cow's milk-specific (A) IgE , (B) IgG1 and (C) IgG2a were measured by ELISA

throughout the course of the therapeutic protocol. Statistically significant differences ($p<0.05$) are indicated by *.

Figure 4. NE immunization protects from mast cell-mediated allergic reactions. (A) Mice were sensitized with casein-alum (CS-alum) and immunized i.n. with 4 administrations of casein-NE (CS-NE) or casein-PBS (CS-PBS). At week 24, mice were challenged either (B) intradermally (i.d) with casein or (C) orally with cow's milk for a total of 7 challenges over 2 weeks. (B) The effect of NE immunization on the acute allergic skin response was measured before and 1 hr. after the i.d. challenge with casein. The effect of NE immunization on allergic reaction to oral challenged was measured by (C) serum MCPT-1 levels and (D) the number of mast cells in the jejunum after the 7th oral cow's milk challenge. Histology images were obtained at 40x magnification and cells counted per field of vision. Statistically significant differences ($p<0.05$) are indicated by *.

Figure 5. Serum from NE-immunized mice does not induce passive sensitization in recipient mice. (A) Serum from casein-sensitized mice that received i.n. PBS, casein-PBS or casein-NE was harvested at week 24 and transferred into naïve recipient mice. 24 hours later, recipient mice were challenged orally with cow's milk and reactivity was measured by (B) change in body temperature and (C) serum MCPT-1 levels. Statistically significant differences ($p<0.05$) are indicated by *.

Figure S1. Endotoxin content of vaccine components. The endotoxin content of vaccine components was determined by a limulus amebocyte lysate (LAL) assay. Values are reported as (A) EU/ml in reagent stock solutions and (B) total endotoxin in each dose. ND, not detectable.

Figure S2. Intranasal administration of NE without allergen does not suppress the allergic response. Mice were sensitized with casein-alum (CS-alum) and immunized i.n. with 4 administrations of PBS, casein-PBS (CS-PBS), casein-NE (CS-NE) or NE only (no antigen). Mice were challenged with casein and serum MCPT-1 levels were determined by ELISA. Statistically significant differences ($p<0.05$) are indicated by *.

Figure S3. Representative images of mast cell staining of intestines of mice after challenge. Mice were sensitized and treated as described in Figure 4. The numbers of mast cells in the jejunum was determined after the 7th challenge by choloracetate esterase staining. Histology images were obtained at 40x magnification and cells counted per field of vision. Representative images are shown for each treatment group. Arrows identify representative positively stained mast cells.

Figure 1

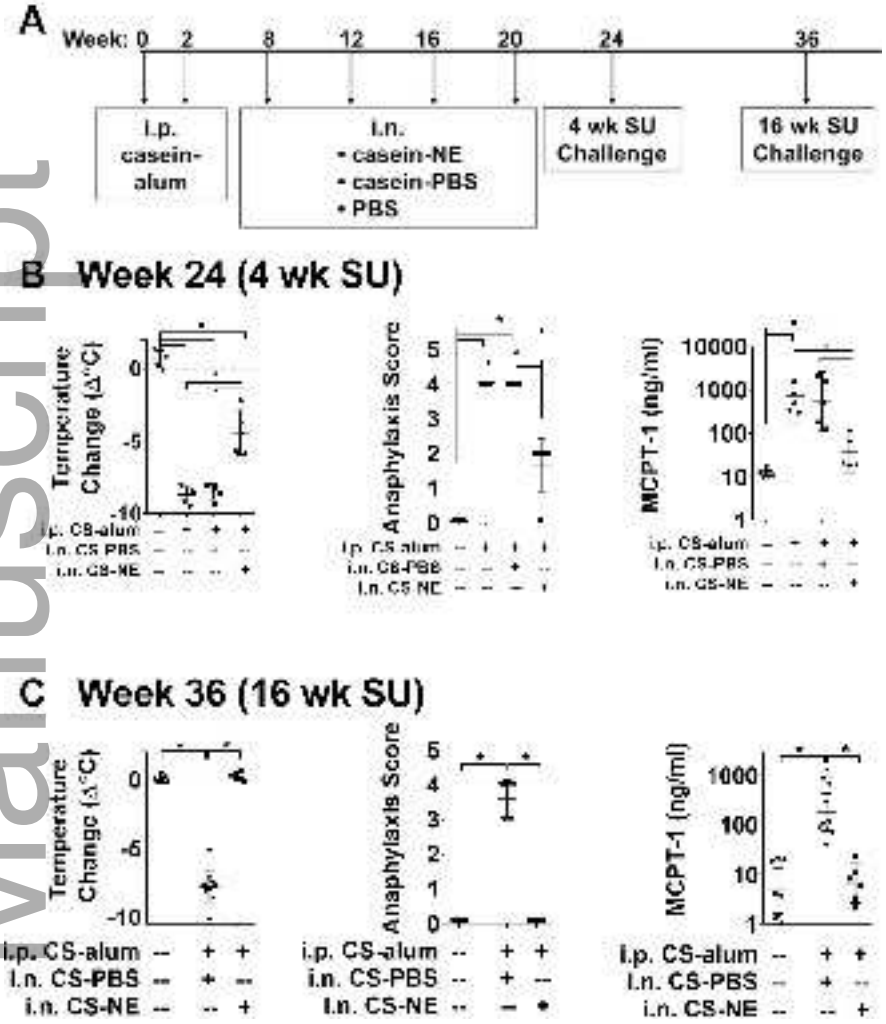

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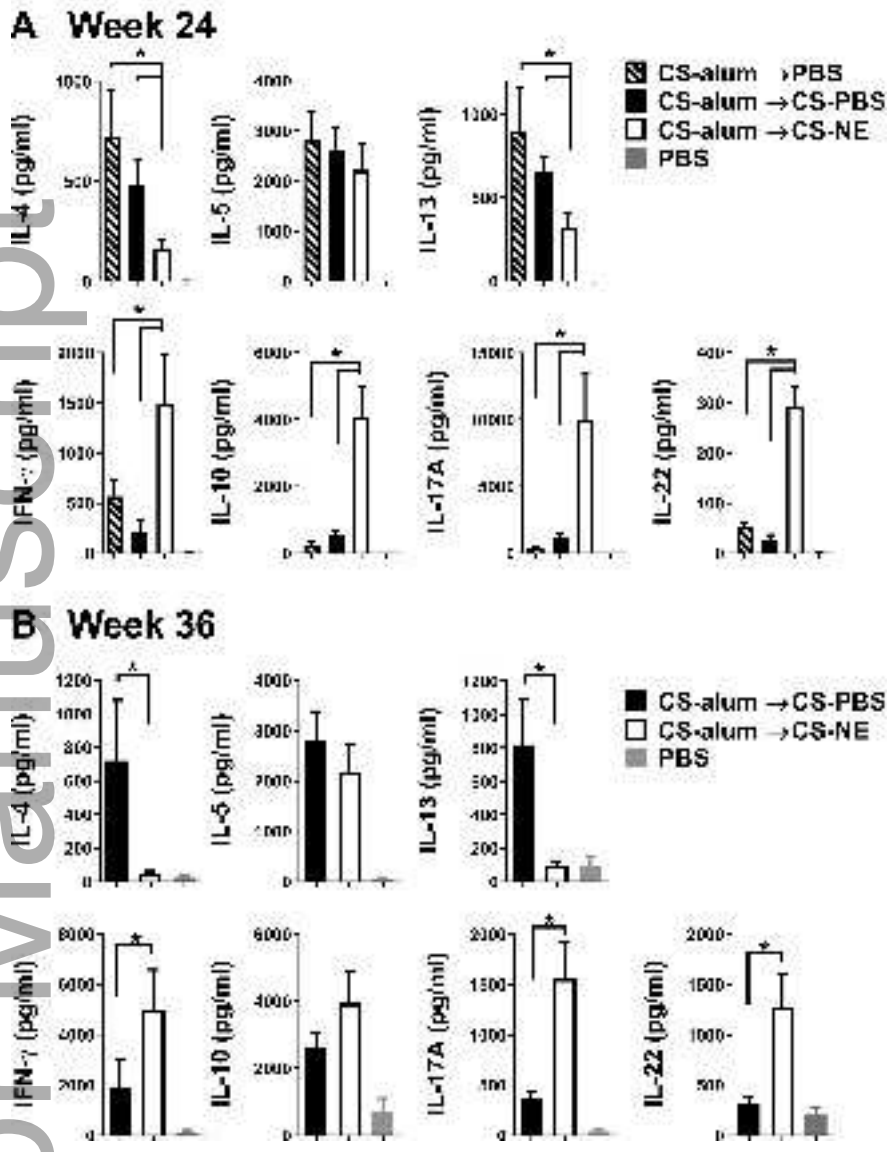
Figure 2



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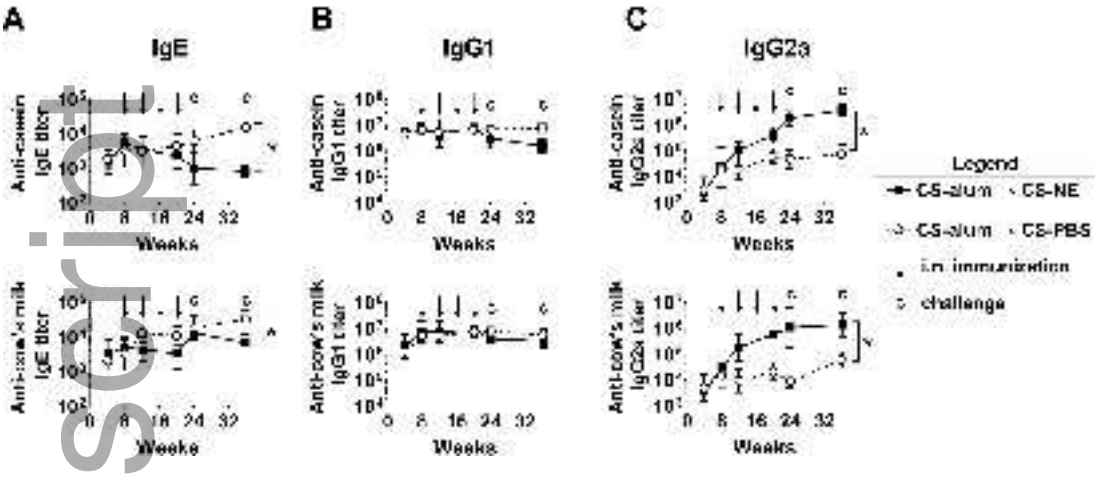

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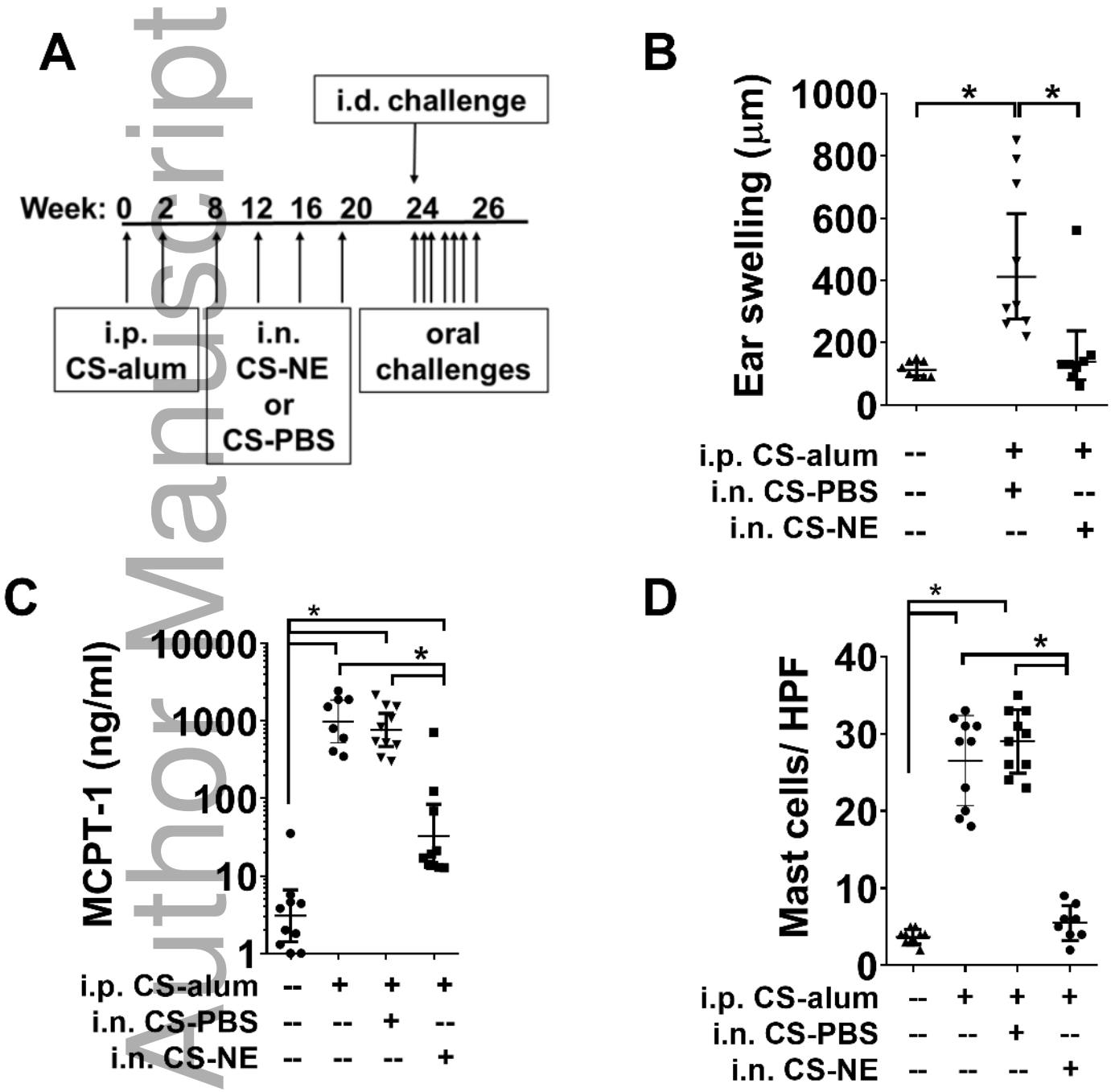

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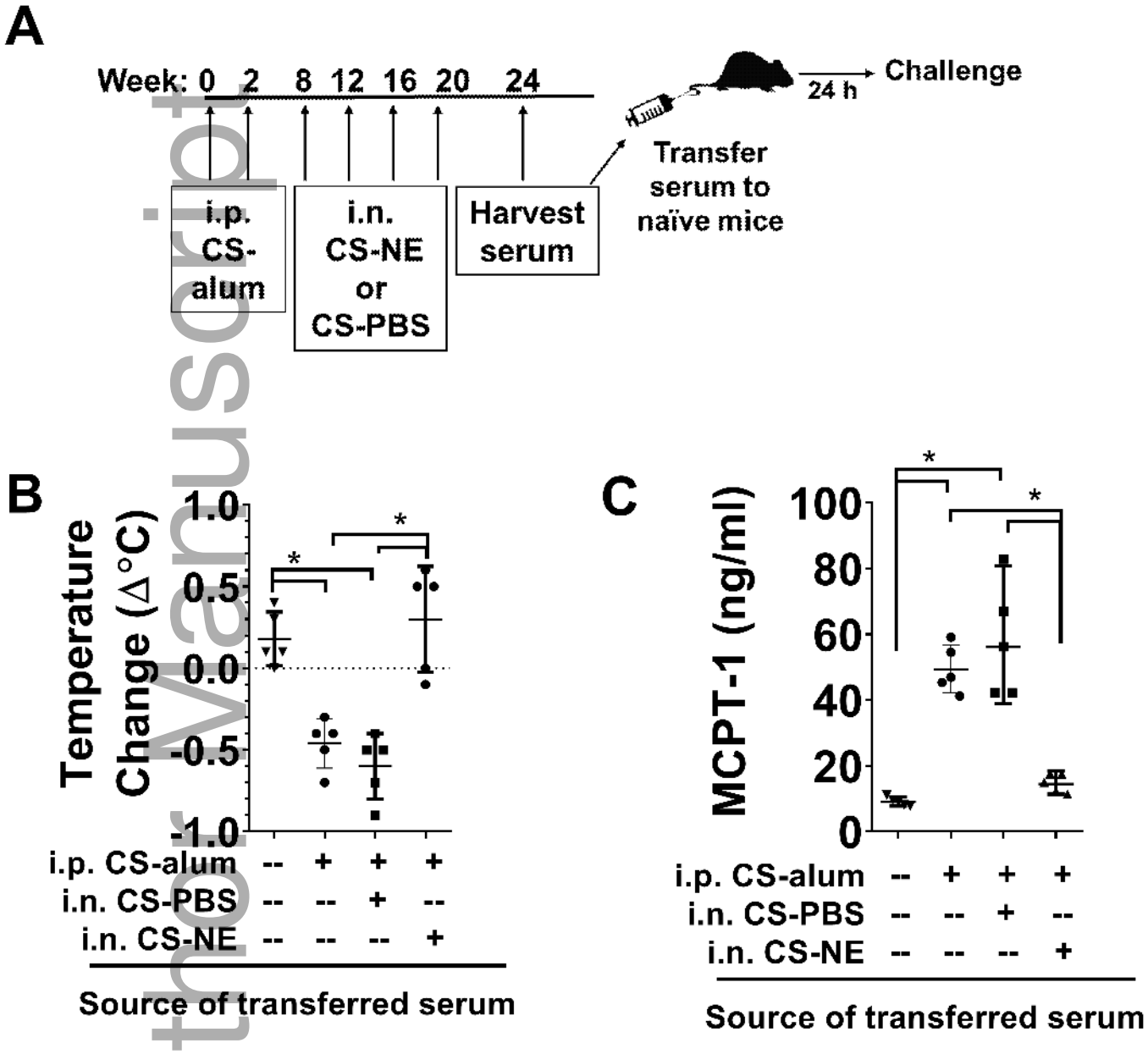
Figure 5



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