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Microneedles coated with peanut allergen enable desensitization of peanut sensitized mice**Running title:** Microneedle mediated peanut allergen immunotherapy

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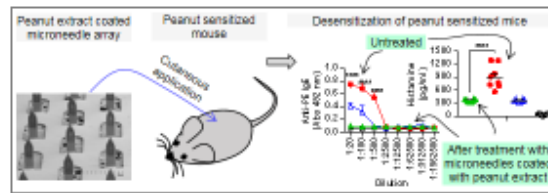
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Graphical abstract



Abstract

The prevalence of peanut allergies has escalated over the last 20 years, yet there are no FDA approved treatments for peanut allergies. In this study we evaluated the potential of microneedles to deliver peanut protein extract (PE) into skin and assessed if the ensuing immune responses could desensitize mice that were sensitized to peanuts. Peanut sensitized mice were either treated through cutaneous immunotherapy using PE-coated microneedles or not treated, and then orally challenged with PE. After oral challenge, the clinical symptoms of peanut-induced anaphylaxis were significantly lower in the microneedle treated mice as compared to untreated mice, and this was accompanied by down-regulation of systemic anaphylaxis mediators such as histamine and mast cell protease-1 (MCPT-1) in the microneedles treated group. Overall, there was an up-regulation of Th1 cytokines (IL-2 and IFN- γ) as compared to Th2 cytokines (IL-4 and IL-5) in splenocyte culture supernatants of the microneedle treated group as compared to untreated group,

suggesting that microneedles promoted immune modulation towards the Th1 pathway. Furthermore, mice treated with PE-coated microneedles were observed to retain integrity of their small intestine villi and had reduced eosinophilic infiltration as compared to the untreated but peanut sensitized mice, which further confirmed the desensitization capability of peanut cutaneous immunotherapy using coated microneedles. Thus, our current study represents a novel minimally invasive microneedle based cutaneous immunotherapy, which may provide a novel route of desensitization for the treatment of peanut allergies.

Keywords: Allergen; allergen immunotherapy; cutaneous immunotherapy; coated microneedles; food allergy; microneedles; peanut allergy; skin vaccination

1. Introduction

Currently, there are no approved FDA-approved treatments for peanut allergies. About 1% of the US population (~3 million people) [1, 2] has peanut allergies, and this number has tripled since 1990s [3, 4]. Peanut allergy presents early in life and is often lifelong and studies have shown that only 20% of children outgrow their peanut allergy [5-7]. Strict avoidance and a peanut-free diet are the only options when managing peanut allergies [8]. However, adherence to a peanut-free diet imposes severe limitations on the lifestyle of the patient and their families [9], and reduces their quality of life [10]. Importantly, restricted food diets in children can lead to nutritional deficiencies [11, 12]. Although food allergies are often considered to affect children, their occurrence in adulthood is also high and often underestimated [13, 14]. To mitigate emergency situations arising from accidental peanut exposure, patients are advised to carry an epinephrine autoinjector.

Recently, the cutaneous route has been the target for peanut desensitization through the application of a transdermal patch containing peanut proteins [15-17]. Findings from a phase IIb study using the transdermal patch technology (Viaskin®) formulated with 250 µg of peanut protein demonstrated a 50% response rate in children between 6-11 years of age when the patch was worn daily for 1 year (fresh patch reapplied every 24 h), however adults showed no significant response [18]. In a follow up phase III study in children 4-11 years of age, the Viaskin® transdermal patch when applied daily for 12 months demonstrated a statistically significant response rate of 35.3% in the treated group as compared to 13.6% in the placebo group. However, the pre-specified criteria for success for the study, that is, a greater than or equal to 15% value for the lower bound of the 95% confidence interval of the difference between treated and placebo response rate was not met [19].

The top layer of the skin called the stratum corneum is responsible for majority of the skin's barrier property, and it inherently provides a substantial barrier to the diffusion of protein molecules into the skin [20, 21]. As a result, passive diffusion of peanut proteins from a transdermal patch into the skin is expected to be quite low, which could potentially explain the low response rate observed from the Viaskin® transdermal patch. Furthermore, it has been shown that the skin permeability not only differs between children and adults, but it also differs across different body surfaces such as the dorsal forearm and upper inner arm [22-24]. This inter- and intra-person variability in skin permeability could explain the disparity in response rates that was observed between adults and children. In line with this reasoning, recently, when the Viaskin® patch alone was used in a phase I clinical study to evaluate safety and immunogenicity of a genetically-inactivated pertussis toxin protein in healthy adults (clinical trial NCT 03035370), a need was felt for a reproducible and a reliable method to enhance vaccine

effectiveness; which then prompted an investigation of a laser-based system to increase permeability of the skin by creating micropores in the top skin layer, prior to application of the Viaskin® transdermal patch [25].

To overcome the above mentioned limitations of the transdermal patch and to improve cutaneous immunotherapy, we recently proposed that, micron-sized needles widely known as microneedles (MNs) could be used to deliver the allergens into the skin [26-28]. Since MNs penetrate the stratum corneum layer and directly deliver the allergen into the skin, unlike a transdermal patch the MN delivery performance is not affected by the inherent differences in skin barrier property that exist between adults and children. Thus, MNs can offer better reliability, robustness, and dosage accuracy as compared to the conventional skin patch approach. We have recently demonstrated the potential of MNs-coated with ovalbumin [26-28] and Der p1 (house dust mite allergen) [29] for cutaneous immunotherapy in the context of respiratory allergens. In this study, we examined for the first time the ability to coat MNs with peanut protein extract (PE), and examined their specific immune responses and therapeutic efficacy to desensitize peanut allergy in a mouse model.

2. Materials and Methods

2.1 MNs fabrication and coating

MNs were made from 50- μ m thick stainless steel (304 grade) sheets via a wet etch process [30]. Briefly, first the MN array was designed in AutoCad® software. The design file was then given to Tech Etch Inc. (MA, USA) who fabricated the MN arrays using standard wet etching techniques. Upon receiving the fabricated MNs arrays, individual MNs in an array were

manually pushed to position them perpendicular to the base. The MN array measured 1 cm x 1 cm and contained 57 MNs each 700 μm long. The coating formulation contained PE (Greer, Laboratories, Inc., NC, USA) (20 %, w/v), carboxymethyl cellulose (low viscosity, USP grade, CarboMer, San Diego, CA, USA) (2%, w/v) as a viscosity enhancer, and Lutrol F-68 NF (BASF, Mt. Olive, NJ, USA) (0.5%, w/v) as a surfactant. MNs were coated with PE by dipping them in to the coating formulation by use of a computer-controlled machine developed in house as described earlier [30, 31]. To visualize coatings on MNs, PE was conjugated with *N*-hydroxysuccinamide (NHS)-activated fluorescein dye (Thermo Scientific, Rockford, IL, USA) and coated on the MN array. A stereomicroscope (Olympus SZX16, Olympus America Inc.) was used to observe the coatings on the MN array. To measure length and thickness of PE coating on MNs, coated MN arrays were visualized under scanning electron microscopy (Hitachi, S-4300SE/N, Japan) and high-magnification images were taken for analysis.

2.2 *In vivo* delivery efficiency

FITC-conjugated PE was coated on MNs and the delivery efficiency of the coated MN array was determined by using a fluorescence spectrofluorometer (Cary Eclipse, Agilent Technologies, CA, USA) at excitation 480 nm and emission of 530 nm. The quantification was done as discussed previously [27, 29, 32]. In brief, three MN arrays were coated with FITC-conjugated PE and each array was placed into 1 ml of PBS solution to obtain the amount of PE coated on arrays (M1). Another set of coated MN arrays (n=3) were pressed into the hair-free mouse skin using the thumb of a hand and held in place by continuously applying thumb pressure for 3 min. After application, arrays were removed and placed in 1 ml PBS solution to obtain the amount of PE left on the array (M2). The target zone of the skin was wiped with a cotton swap pre-soaked

in PBS solution to obtain the amount left on the skin surface (M3). The delivery efficiency was calculated as $(M1-M2-M3)/M1 * 100$.

2.3 Mice and immunotherapy

Balb/c female mice aged 8-10 weeks (Charles River Laboratories, Inc., USA) were used in the experiments. Mice were housed under climate-controlled environment in air ventilated polystyrene cages enriched with plastic pipe pieces and wood beddings materials. All animal experiments were performed under approved protocols and ethical guidelines of Institutional Animal Care and Use Committee (IACUC) Texas Tech University, Texas, USA. Coated MNs were inserted in mouse skin as discussed previously [27, 29, 32]. In brief, mice were anesthetized with isoflurane. Hair from the back region of the mouse was trimmed using an electric hair trimmer, and subsequently a hair removing lotion was applied for complete hair removal. PE coated MN array was inserted into the mouse skin and manually pressed using thumb pressure to hold in position for 3 min to allow for the delivery of PE coated on microneedles. After application, the array was removed, and mice were returned to their cage until they gained consciousness. Blood was collected to assess the anti-PE antibody response using ELISA.

2.4 Splenocyte and bone marrow culture

Spleen and bone marrow were harvested. Splenocytes were cultured in triplicate at a concentration of one million cells per well for 72 h with (i) PE (200 µg/ml), (ii) culture medium negative control, (iii) positive control of 5µg/ml of concanavalin A (Sigma Aldrich, MO, USA). RPMI (Gibco, Life Technologies, USA) supplemented with fetal bovine serum (FBS) and penicillin-streptomycin antibiotics were used for cell culture. Supernatant of cultured splenocytes

were collected after 14 h for measurement of IL-2, and after 72 h for measurement of IFN- γ , IL-4 and IL-5 cytokines. Similarly, the bone marrow single cell suspension was cultured but without restimulation with PE for 96 hours. The supernatant was collected to assess anti-PE antibody response through ELISA.

2.5 Serological analysis

ELISA was used to measure PE specific IgG, its subtypes, and IgE in serum as discussed previously [26, 27]. Briefly, ELISA plates were coated overnight with PE at 20 $\mu\text{g/ml}$. Plates were then washed with ELISA buffer (1xPBST: 1xPBS with Tween 0.05%, v/v), incubated with blocking solution (5% skimmed milk, w/v in 1xPBST) for 1.5 h, and washed again with ELISA buffer. Fifty microliter serum diluted in PBST was added into each well. After 1.5 h, plates were washed with ELISA buffer, and goat anti-mouse detection antibodies labeled with horseradish peroxidase (goat anti-mouse IgG, IgE, IgG1, IgG2a; Southern Biotech, Birmingham, AL, USA) were added for 1.5 h. Plates were washed and peroxidase substrate solution (OPD substrate tablet in phosphate citrate buffer pH 5.0+ H_2O_2) was added. Reaction was stopped after 15 min by adding 3M H_3PO_4 and plates were read at 492 nm.

2.6 Development of a peanut sensitized mouse model

Mice were sensitized to peanut as described previously [33]. In brief, mice were orally administered six weekly doses of 1 mg PE mixed with 10 μg cholera toxin (CT) (Sigma Aldrich, MO, USA). To validate the model, four weeks later, mice were orally challenged with 20 mg PE dissolved in 300 μl saline solution. Post challenge, drop in body temperature was recorded in 15 min intervals up to 135 min by using a mouse rectal temperature probe (Thermalert TH-5, Physitemp, Clifton, NJ, USA) lubricated with petroleum jelly (Vaseline, Unilever,

Netherlands). The clinical scores of severity were recorded in mice as discussed previously [34]. Briefly, mice were scored using the following system; 0- no symptoms (healthy mice without any symptom), 1- hypersensitive to touch/impatience/aggression; 2- puffiness around the eyes, increase in respiratory rate with low activity; 3- difficult breathing, lying flat, swelling around the mouth and eyes; 4- complete loss of activity, no reaction upon pinching, loss of consciousness; 5- death. Five minutes post challenge, plasma was also collected to analyze the change in anti-PE antibody response through ELISA, and histamine concentration by using mouse histamine kit (Abbexa Ltd., Cambridge, UK).

2.7 MNs mediated cutaneous desensitization and analysis

To assess the therapeutic potential of MNs coated with PE, mice were first sensitized against peanut. Four weeks later, sensitized mice were treated with 5 µg of PE coated on MNs (three doses once a week). Three weeks later, treated mice were orally challenged with a high dose of PE (20 mg of PE). Post challenge, mice were clinically scored for 150 min as discussed above. Post challenge, blood was obtained from the mice to quantify IgE, histamine and mast cell protease-1 (MCPT-1) in serum. For MCPT-1 analysis, mouse MCPT-1 ELISA Ready-SET Go! (eBioscience, Inc. San Diego, CA, USA) kit was used. At the end of immunotherapy, mice were euthanized. Post euthanasia, spleens were collected and the splenocytes were cultured as discussed above, for measurement of secreted cytokines. Proximal, middle and distal parts of the small intestine were also harvested for histological evaluation. Tissues were fixed overnight in formalin solution (4%, v/v), followed by 24 h incubation in ethanol (70%, v/v), and were then embedded in paraffin wax. Tissues were sectioned and stained with hematoxylin & eosin (H&E) by IDEXX BioResearch (MO, USA). The stained sections were observed for morphological

changes and eosinophilic infiltration. Brightfield images of stained tissues were taken with a Nikon Eclipse Ti Microscope (Tokyo, Japan).

2.8 Statistical analysis

Statistical analysis was conducted by use of Graphpad Prism 6 software. One-way ANOVA test was used to compare the data between the groups at different time points. Significance was considered for $p < 0.05$ for a 95% confidence interval.

3. Results

3.1 Successful coating of PE on MNs and delivery into mouse skin

We first assessed the ability to coat MNs with PE. Figure 1A demonstrates a MN array whose individual MNs have been coated with PE. Closer examination showed that all the MNs on the array were coated (Figure 1B), and that the coatings were uniform without patchy uncoated

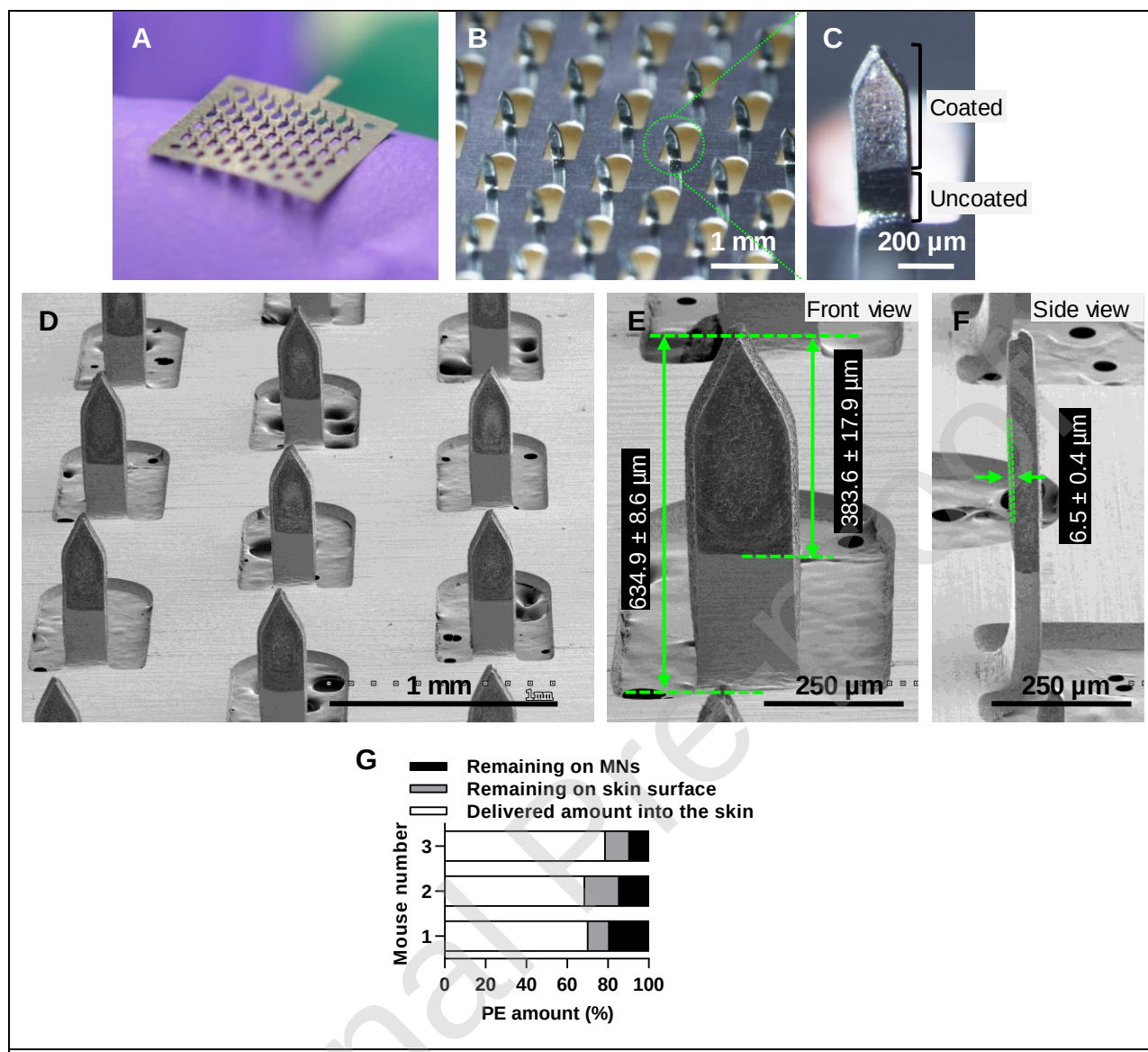


Figure 1. MN images and delivery efficiency. (A) Digital photograph of a MN array containing 57 total microneedles (MNs). Stereo microscopic image of (B) a coated MN array, and (C) a zoomed image of a single coated MN. Scanning electron micrograph of a PE coated MN array showing (D) a subset of coated MNs on the array, (E) zoom in of the front view of a coated MN, and (F) zoom in side view of a coated MN. The length and thickness of PE coating on the MN were analyzed through Image J software by using high resolution scanning electron microscopy images. The represented coating length and thickness are the average values obtained from four independent coated MNs. On each MN three randomly selected places were measured for coating thickness. (G) Delivery efficiency into mouse skin of fluorescein conjugated PE coated on a MN array. To assess the delivery efficiency of a MN array, PE was conjugated with N-hydroxysuccinamide (NHS) activated-fluorescein dye, purified and then coated on MNs array. After MN array insertion, delivery efficiency was determined with calibrated fluorescence spectroscopy, at an excitation wavelength of 480 nm and an emission wavelength of 530 nm. N=3 mice, each mouse received one coated MN array.

imperfections (Figure 1C, D). We next analyzed the length and thickness of PE coating on MNs through Image J software by using high resolution scanning electron microscopy images. The MNs after being bent perpendicular to the base substrate measured $634.9 \pm 8.6 \mu\text{m}$ in length, and were coated with a PE coating measuring $6.5 \pm 0.5 \mu\text{m}$ in thickness and $386.6 \pm 17.9 \mu\text{m}$ in length (Figure 1E, F). Next, we quantified the delivery efficiency using calibrated fluorescence spectroscopy. It was found that 72.3% (± 5.4) of the coated PE was delivered in to the skin, while 14.6% (± 4.9) PE was found on the skin, and 13.1% (± 3.4) PE remained on the MN surface (Figure 1G). The observed delivery efficiency of around 72% is consistent with previous studies with coated MNs where 73-78 % of the coated protein was delivered into mouse skin [27, 29, 32]. Post MN insertion, no symptoms of bleeding or inflammation were observed, which confirms the non-invasive nature of MNs as reported previously [35, 36].

3.2 PE-coated MNs induced PE specific immune response

To assess the ability of MNs to induce PE-specific immune response and to study the dose effect, naïve mice were treated once a week for three weeks with a MN array coated with either 1, 5 or 25 μg PE (the actual mass of PE coated being 1.1 ± 0.2 , 5.2 ± 0.6 , and 25.1 ± 0.5 , respectively) (Figure 2A). Immune responses were evaluated six weeks after the last PE dose. A dose-dependent effect was seen for anti-PE IgG and anti-PE IgG1 antibodies with 1 μg PE showing a considerably lower response than 5 μg and 25 μg PE doses (Figure 2B). There was no considerable difference between 5 and 25 μg PE doses (Figure 2B). Surprisingly no significant dose effect was seen for anti-PE IgG2a response (Figure 2B). The emergence of PE-specific IgG2a suggests activation of the Th1 pathway [37]. Anti-PE IgE response in serum was not significantly elevated as compared to naïve mice, especially at 1 and 5 μg PE doses (Figure 2B). Since high serum IgE antibody levels are associated with peanut allergy, and MN-based delivery

did not stimulate high serum IgE, this suggests that MN-based delivery of PE is not expected to exacerbate allergic responses in mice. To initiate immunotherapy in a patient, it is important to begin at a low dose so that adverse effects can be curtailed. Thus, since 5 μ g and 25 μ g PE dose coated on MNs both generated similar amount of IgG and IgG1 and this response was higher than that from 1 μ g PE, we selected 5 μ g PE for further evaluation.

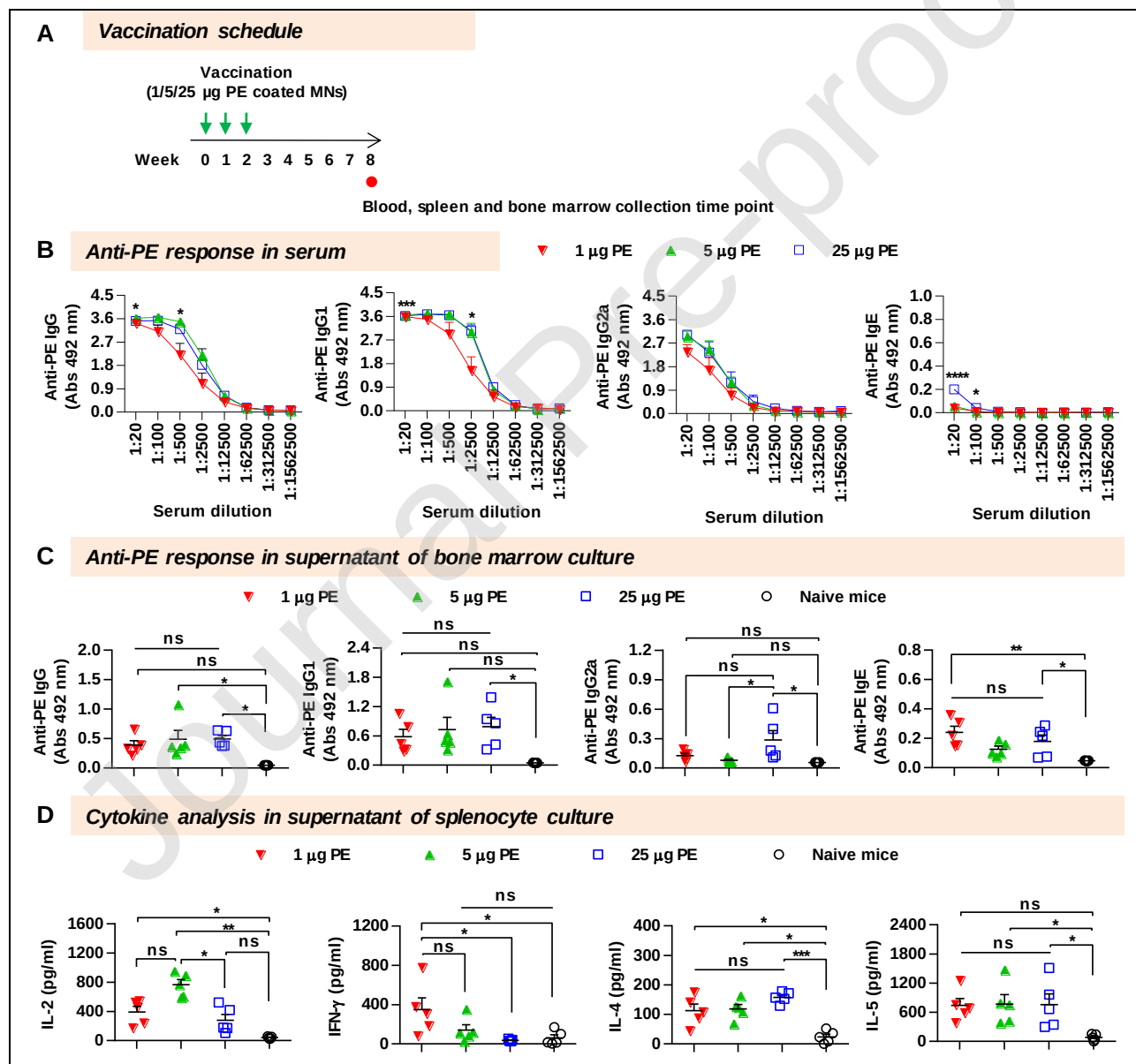


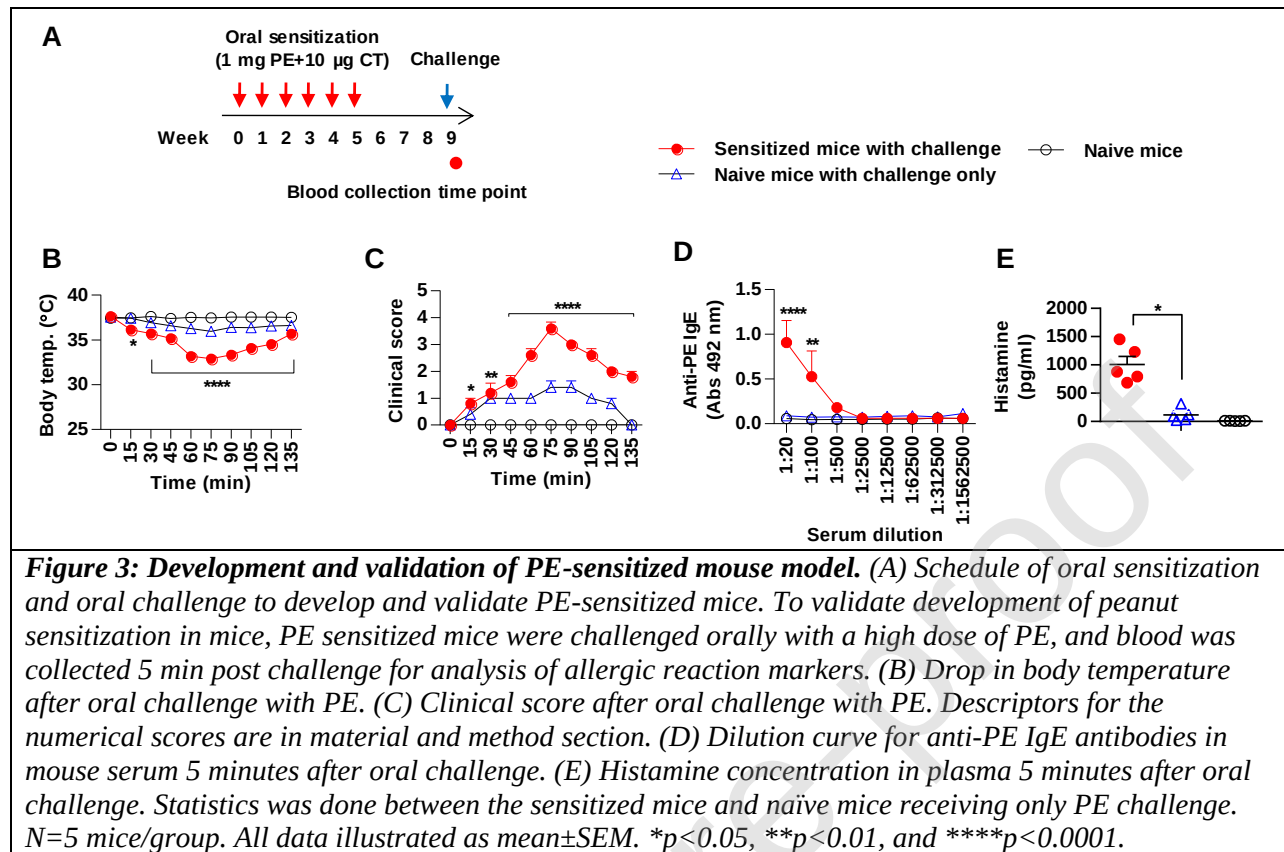
Figure 2. Immune response after delivery of PE from coated MNs. (A) PE delivery schedule: PE-coated MNs were used to administer different doses of PE to mice once per week for three weeks. Eight weeks later, mice were bled to assess the anti-PE antibody response, and the next day they were euthanized and tissues (spleen and bone marrow) were collected for analysis. (B) Serum dilution curves for anti-PE IgG, IgG1, IgG2a and IgE antibodies. Individual mouse serum was used in the analysis. (C) Anti-PE antibody response in culture supernatant of bone marrow cells that were harvested from the vaccinated mice and cultured in RPMI medium for 96 h. Anti-PE IgG, IgG1, IgG2a and IgE antibodies were analyzed in the supernatant of the bone marrow cultures after 96 h of culture. (D) Cytokine analysis in supernatant of splenocyte culture. Spleens were collected aseptically and splenocytes were cultured and restimulated with PE at a concentration of 200 $\mu\text{g/ml}$. Post 14 h, supernatant was collected to analyze IL-2 while post 72 h to analyze IFN- γ , IL-4 and IL-5 cytokines. The values are presented after subtraction of media alone cytokine levels. Data for concanavalin A is not plotted. N=5 mice/group. All data illustrated as mean $\pm$ SEM. * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$, and ns: not significant.

To generate a long-lasting immunotherapy effect, it is important that the anti-PE antibody response should be long-lived. Since most long-lived antibody secreting cells migrate to the bone marrow [38, 39], we cultured bone marrow cells ex-vivo without any antigen stimulation and tested the supernatant for anti-PE antibodies. As shown in Figure 2C, in the supernatant collected from bone marrow cultures a higher anti-PE IgG response was seen in the 5 and 25 μ g PE groups as compared to the naïve group; however, the anti-PE IgG1 and IgG2a responses were higher in only the 25 μ g PE group. Oddly, the anti-PE IgE response although overall low, was higher in 1 and 25 μ g PE groups as compared to naïve mice, but not in the 5 μ g PE group (Figure 2C).

Modulation of the immune system towards a Th1 response is considered beneficial in successful desensitization. Thus, we assessed the type of Th response induced by PE-coated MNs by culturing splenocytes in vitro under PE restimulation. All groups receiving PE (1, 5 or 25 μ g) secreted Th1 surrogate markers (IL-2 & IFN- γ) and Th2 surrogate markers (IL-4 & IL-5) (Figure 2D). However, no clear trend was evident with respect to Th1 or Th2 bias. Off note, this response was observed after just three weekly doses with PE-coated MNs and in just six weeks post treatment of mice.

3.3 Development of peanut sensitized mouse model

To assess the therapeutic efficacy of PE coated MNs, we first sensitized mice through oral administration of PE with CT as described previously [33], and validated peanut sensitization by orally challenging them with PE and recording different symptoms and markers indicative of an allergic reaction (Figure 3A). Drop in body temperature is a major symptom of food-induced anaphylaxis in mice [40], which we measured through the rectal route every 15 min for 135 min. Naïve mice that received the oral challenge showed slight drop in body temperature with respect



to naïve mice who received no challenge; however, peanut sensitized mice showed a significant temperature drop after oral challenge (Figure 3B). Physical symptoms of illness were also higher in peanut sensitized mice (Figure 3C). Anti-PE IgE is an important marker for sensitization, which was found to be elevated in sensitized mice 5 min post challenge (Figure 3D). During an allergic reaction, inflammatory mediators, such as histamine, are released from granulocytes [41], and this was quantified in plasma, and found to be significantly elevated in sensitized mice 5 min after the oral challenge (Figure 3E). Overall, these markers indicated successful development of peanut sensitization in mice [42].

3.4 MNs-based cutaneous immunotherapy (MNs-CIT)

After validating the peanut sensitized mouse model, we next proceeded to test the ability of PE-coated MNs to desensitize peanut sensitized mice. Following successful peanut sensitization, mice were treated weekly with 5 µg PE-coated MNs for three weeks. Three weeks later, mice were challenged orally with PE (20 mg/mouse) (Figure 4A) and different markers of allergic reaction were examined.

The therapeutic advantage of MNs-CIT was clearly visible in the clinical score (Figure 4B). The severity of clinical symptoms was significantly lower in mice that received MNs-CIT. In particular, in the MNs-CIT group the maximum clinical score rose to 2 (puffiness around the eyes, increase in respiratory rate with low activity) in 37.5 % of the mice, while 100 % of the mice in the untreated group became considerably ill and reached a maximum score of 3 (difficulty in breathing, lying flat, swelling around the mouth and eyes) (Figure 4B). The overall duration of symptoms was also significantly prolonged in untreated mice, which remained at a score of '2' for 135 mins and did not return to '0' score even 150 mins after the challenge. In contrast, MNs-CIT mice returned to '0' score within 90 min (Figure 4B).

Anaphylactic reaction in mice is also associated with histamine release and mast cell degranulation [42]. Both mast cell protease-1 (MCPT-1) and histamine were found to be elevated in the untreated group as compared to the MNs-CIT group (Figure 4B), indicating the ability of MNs-CIT to diminish the allergic response.

Another key mediator of allergic response is peanut-specific IgE, which mediates type 1 hypersensitivity reactions [42] by binding to high affinity FcεRI receptors on the surface of the mast cells and basophils thus triggering the allergic response [43]. Peanut-specific IgE in serum

was observed to be significantly lower in MNs-CIT mice than the control untreated mice (Figure 4C), indicating the effectiveness of MNs-CIT immunotherapy.

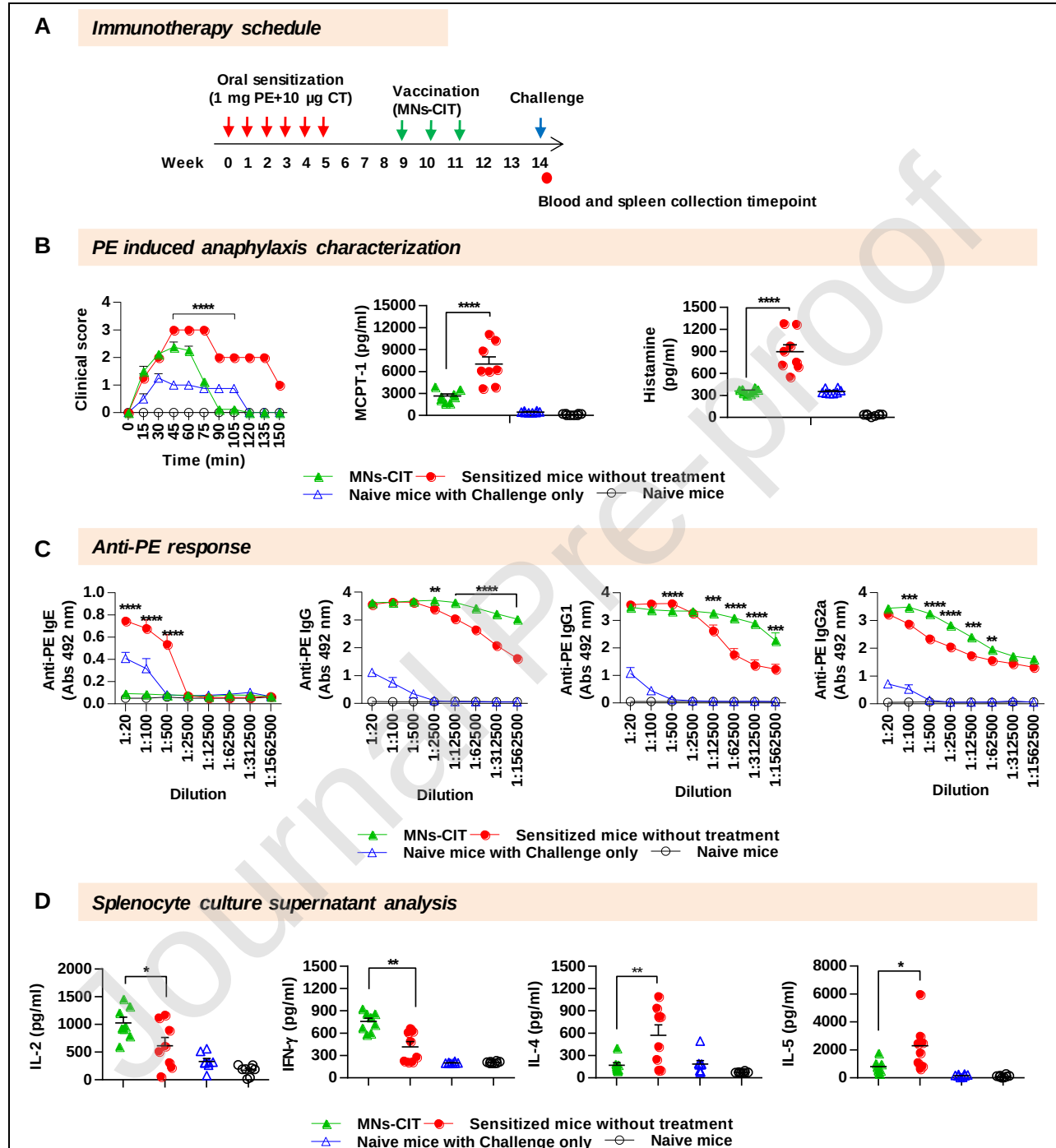


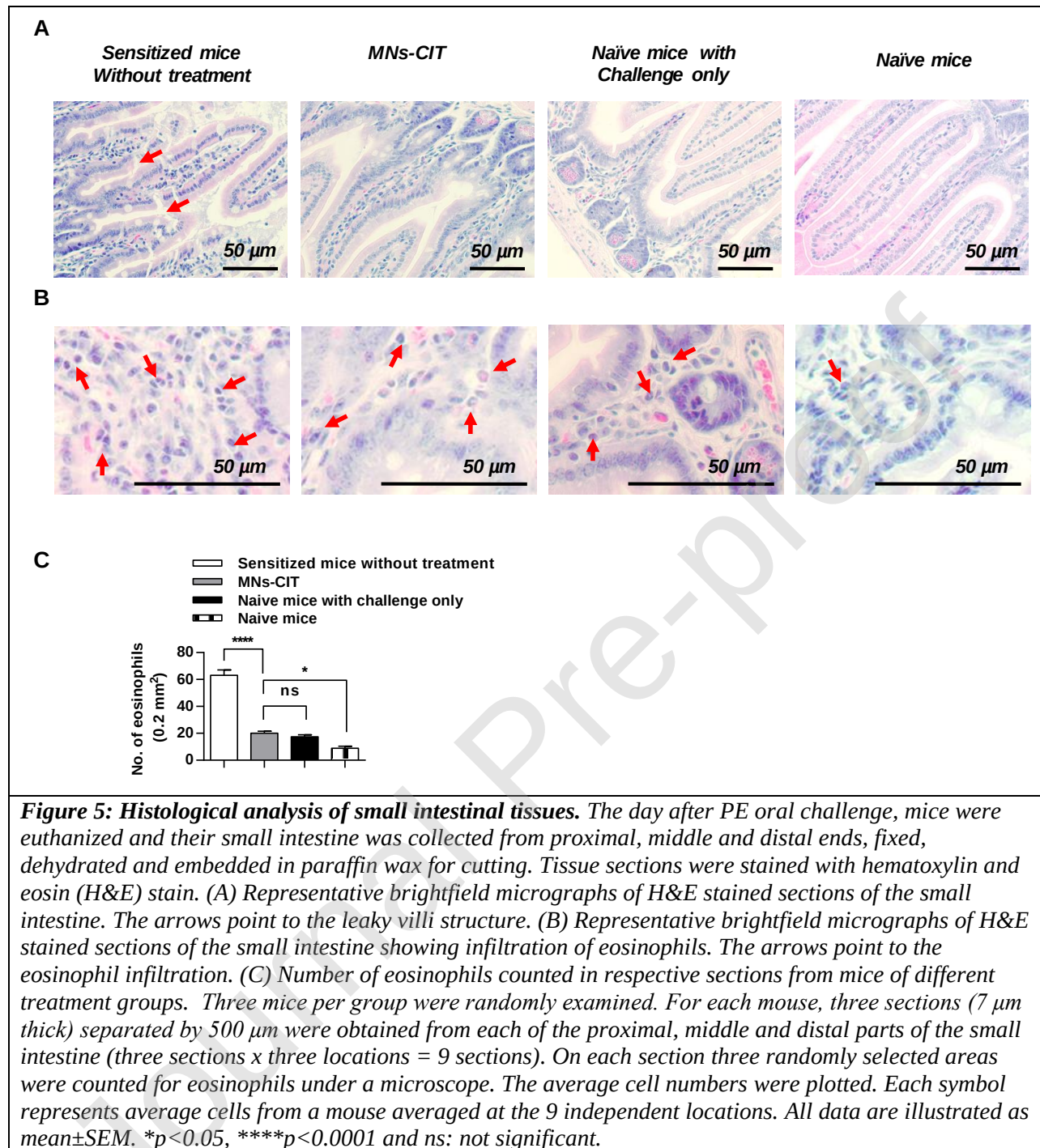
Figure 4: MNs mediated cutaneous immunotherapy. (A) Immunotherapy schedule: Mice were sensitized orally every week up to six weeks. Four weeks later, sensitized mice were treated with MNs-CIT followed by oral challenge with a high dose of PE. Five minutes post challenge, mice were bled to collect plasma and serum for the measurement of markers of anaphylactic reaction. The next day, mice were euthanized

*and tissues (spleen and bone marrow) were collected. (B) Characterization of anaphylaxis: After oral challenge with PE, clinical score, plasma MCPT-1 level, and plasma histamine level was analyzed. Individual mouse plasma was used for analysis of MCPT-1 and histamine. (C) Anti-PE antibody response in serum collected at 5 minutes post challenge. (D) Splenocyte culture supernatant analysis: Spleens were collected aseptically post euthanasia and splenocytes were cultured and re-stimulated with PE. Th1 and Th2 cytokines were analyzed in culture supernatants. The values are presented after subtraction of media alone cytokine levels. Data for concanavalin A (control) is not plotted since the levels are high and this results in data compression along the y-axis. Data representative of two independent studies (1st study N=8 mice/group presented here, while the repeat study included N=5 mice/group and data not presented here). All data illustrated as mean±SEM. * $p<0.05$, ** $p<0.01$, *** $p<0.001$, and **** $p<0.0001$.*

While the exact correlate of desensitization to peanut allergy is not completely understood, a therapeutic regime of allergy immunomodulation is thought to involve the activation of the Th1 pathway [44]. In mice, IgG2a and IgG1 antibody subtypes are a surrogate marker for the Th1 and Th2 pathways, respectively. We thus measured PE specific IgG, IgG1 and IgG2a antibodies in serum after oral challenge. All three antibodies were significantly elevated in MNs-CIT group as compared to untreated mice (Figure 4C). To further characterize the Th1/Th2 bias, expression of Th1 and Th2 cytokines were analyzed in the supernatant of splenocyte cultures. Splenocytes were cultured and re-stimulated with PE along with positive or negative controls, and different cytokines were analyzed. The Th1 cytokines IL-2 and IFN- γ were significantly higher in MNs-CIT group as compared to the sensitized but untreated group (Figure 4D). In contrast, Th2 cytokines IL-4 and IL-5 were significantly lower in the MNs-CIT group as compared to sensitized but untreated group. Th2 cytokines trigger the activation of allergen reactive Th2 cells and enhance the recruitment of IgE producing plasma B cells [45]. Together, these results suggest that MNs-CIT exert their beneficial immunotherapy effect via the Th1 pathway.

3.5 Histological analysis

To assess the ability of MNs-CIT to minimize anaphylactic shock, we also examined histological sections of the small intestine of mice. Reduced inflammation was seen in the small intestine of MNs-CIT group, however, sensitized but untreated mice showed irregularity in the intestinal villi structure (Figure 5A), which may also be associated with changes in intestinal permeability. Such functional and structural changes in small intestine have been reported previously as an outcome of sensitization against food allergens [46, 47]. Similarly, eosinophilic infiltration is characteristic of peanut allergy [33], which were found to be in low numbers in the MNs-CIT group, while significantly higher eosinophils were found in sensitized but untreated mice (Figure 5B, C). Negative controls, i.e., naïve mice and naïve mice that received PE challenge showed intact morphology of intestinal villi and low numbers of eosinophils (Figure 5A, B, C).



4. Discussion

In this study our objective was to assess the desensitization capability of MNs coated with PE for peanut cutaneous immunotherapy. The idea that the skin can be used for allergen desensitization is reinforced by a recent clinical study which used a transdermal patch for peanut allergen immunotherapy [19]. In that clinical study, a lower response rate was seen in patients. Considering the inherent limitation of poor passive diffusion of proteins into the skin, it is possible to postulate that if skin permeability can be increased, the positive outcome of the treatment involving allergen delivery through the skin could be improved. In this regard we developed MNs to help deposit the peanut allergen in to the skin in a more controlled and consistent manner, and investigated the ability of this delivery system to desensitize peanut sensitized mice.

To implement the MN-based cutaneous immunotherapy, we found that insufficient data was available regarding the amount of peanut allergen that should be coated on MNs and delivered into the skin. Ideally this dose should be safe for delivery into a human subject. The clinical trial that used skin patches containing PE was not informative regarding a potential test dose since although in the clinical trials 250 μ g PE was applied on the skin of patients, the fraction of PE that actually penetrates the skin was not quantified [19]. We were however, able to glean some information on potential PE dose from the two clinical studies that had involved subcutaneous injections of PE to perform subcutaneous peanut immunotherapy [48, 49]. Those studies had shown promise and the patients were able to tolerate higher doses of peanut ingestion, however, due to unacceptably high severe reactions in patients during immunotherapy, the studies were not pursued further. In these two studies the PE dose was presented as a dilution factor and the actual amount of PE that was administered was not provided. However, based on

extraction studies it is known that on average about 10-15% by mass of the starting peanut flour gets extracted into the buffer as protein [50, 51]. Thus, assuming a 10% extraction of PE from the peanut flour, then for example, a 1:100 (w/v) extract, which refers to an extract obtained by adding 1 g peanut flour into 100 ml of a buffer will contain about 0.1 g PE per 100 ml of the buffer. Using this calculation approach, we estimated that the starting and maintenance doses of the subcutaneous immunotherapy injections were 0.5 μg (0.05 ml of 1:10,000 w/v $\equiv$ 10 $\mu\text{g}/\text{ml}$) and 500 μg (0.5 ml of 1:100 w/v $\equiv$ 1000 $\mu\text{g}/\text{ml}$), respectively. In these studies, before, during and after immunotherapy injections, patients were also tested for skin reactivity using prick skin test with different dilutions of PE to identify the dilution that generated the skin reaction of the same size (a wheal size of about 3 mm mean diameter). The range of PE dilutions that generated skin reactivity was from 1:100 to 1:1,000,000. From these prick tests, no systemic reactions were reported. Unfortunately, for prick tests, while the PE dilution is known, it is unclear how much PE actually gets delivered in to the skin as a result of pricking the skin. Nonetheless these tests offer confidence that as opposed to subcutaneous injections, if smaller doses are provided through the skin a safer treatment profile might be achieved. By this reasoning it is also suggestive that a MN based delivery of PE into the skin, which is akin to a prick test of skin, might offer a more suitable safety profile than subcutaneous injections. Overall, this analysis pointed to doses of PE in the range of few micrograms to tens of micrograms.

Guided by our dose estimation, we first evaluated three doses, 1, 5 and 25 μg of PE coated on MNs for their ability to generate PE-specific antibodies. The 5 μg and 25 μg PE coated on MNs generated comparable antibody responses and higher than 1 μg dose. In order to reduce the occurrence of adverse events a lower dose is preferred; thus we selected the lower of the two, i.e., 5 μg dose for further efficacy testing. Interestingly, bone marrow cells of mice receiving PE-

coated MNs were found to secrete anti-PE IgG, IgG1 and IgG2a responses. This could suggest stimulation of a long-lived antibody response, which may have importance in maintaining long-term tolerance. More studies are required to evaluate this possibility.

We developed a mouse model of peanut allergy to test the MN-based cutaneous immunotherapy. Sensitization of mice to peanut was confirmed through measurement of multiple markers and symptoms known to correlate with an anaphylactic response. Upon oral challenge with PE, sensitized mice exhibited a drop in body temperature, had elevated clinical symptoms of allergic response, elevated anti-PE IgE antibodies, and elevated serum histamine, all pointing towards successful sensitization of mice against peanut.

To test the efficacy of PE-coated MNs, peanut-sensitized mice were treated thrice (once per week) with MNs coated with 5 μ g PE. Upon oral challenge, mice that had received treatment with MNs coated with PE exhibited significantly reduced allergic reactions, including lower clinical score, reduced duration of reactivity, and lower amount of histamine as compared to sensitized mice that received no treatment. The anti-PE IgE antibody was also significantly lower in MN-treated mice, and lower numbers of eosinophils were observed in their small intestine. These different indicators demonstrated that PE-coated MNs have the potential to desensitize peanut sensitized mice.

Although correlates of successful allergen immunotherapy are not fully understood, multiple serological and cellular changes occur and these are thought to promote a successful immune-remodeling effect [52-54]. These changes include, activation of regulatory T cells [15], suppression of systemic allergen-specific IgE, and elevation of competing IgG antibodies (IgG2a in mice, and IgG4 in humans). For example, in patients who were treated with PE via the

subcutaneous immunotherapy route, higher serum IgG levels were seen to correlate with a reduced skin prick test response [48]. In this current work, MN-treated mice had significantly higher levels of anti-PE IgG antibodies, and higher amounts of IFN- γ and IL-2 cytokines indicative of involvement of a Th1 pathway.

From an allergen delivery standpoint, MNs rupture the stratum corneum, which is the topmost layer of the skin and one that constitutes majority of the barrier function against uptake of the allergen molecules. MNs bypass this layer and deliver the allergen directly into the underlying skin layers [55-58]. Antigen presenting cells such as Langerhans cells and dermal dendritic cells that reside in the skin can process the delivered allergen, and migrate to the nearest lymph nodes to prime naïve T cells to T helper cells for activation of B cells for antibody secretion [59]. MNs have not been previously used for allergen immunotherapy, however, they have been studied in the context of influenza vaccine delivery. Trafficking of dendritic cells and Langerhans cells to regional lymph nodes was seen upon MN-based vaccination in the skin, which probably contributes to effective induction of T and B cell responses [60-64]. Increased levels of cytokines important for recruitment of monocytes, neutrophils, and dendritic cells were observed at the site of immunization within 12 hours after MN-based vaccination [65].

MNs provide a more robust control for allergen delivery than a transdermal patch because PE-coated MNs deliver the allergen directly into the layers of the skin. As such coated MN arrays are unaffected by skin permeability, which can differ from a child to an adult and also within the same individual depending on anatomical location [22-24]. MNs address another drawback of topical allergen delivery by consistently delivering a known amount of the allergen, while in contrast, from a transdermal peanut patch the exact amount of allergen that permeates into the skin is unknown. MNs are also attractive because they are minimally invasive and

painless [28, 66, 67], and thus could offer a patient friendly approach of peanut allergy desensitization.

In this work we have provided a proof-of-concept validation that PE-coated MNs can desensitize peanut sensitized mice. We used a treatment regimen of just three weekly doses of PE-coated MNs and were still able to observe the desensitization effect in mice. A longer treatment regimen involving more doses might improve the desensitization effect, and this longer treatment might be required in humans when translating the approach into clinics. It is, however, noteworthy that unlike transdermal patches, which require a daily application, the PE-coated microneedles might be effective with weekly administrations, which could improve patient experience. Additional studies will be required to study these effects. In conclusion, the presented data successfully demonstrated the capability of PE-coated MNs to deliver the PE into mice skin and induce the PE specific immune response to desensitize the PE allergic immune response in a peanut allergy mouse model.

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pursuing this technology for developing microneedles for peanut and other food allergy immunotherapies. H.S. Gill, A.K. Shakya and C.M. Davis have equity in this startup company. This potential conflict of interest has been disclosed and is being managed by Texas Tech University and Baylor College of Medicine. No part of this study was paid for by the company, nor was the company involved in any data interpretation or analysis. The rest of the authors declare that they have no relevant conflicts of interest.

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