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Poly-γ-Glutamate microneedles as transdermal immunomodulators for ameliorating atopic dermatitis-like skin lesions in Nc/Nga mice

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ABSTRACT

Atopic dermatitis (AD), a common, relapsing, inflammatory disorder of the skin, is associated with T helper type 2 (Th2)-biased immune responses. Despite the efficacy of existing drugs for AD treatment, their safety and side effects cause concern. The present study describes the use of dissolvable poly-γ-glutamate (γ-PGA) microneedles (MNs) with immunomodulatory effects for effectively relieving AD-like symptoms in Nc/Nga mice. γ-PGA MNs can easily penetrate the epidermis and release γ-PGA into the dendritic cell–rich dermis to interact with dendritic cells for modulating immune responses. Transdermal administration of high-molecular-weight (HMW, 1100 kDa) γ-PGA MNs significantly reduced clinical dermatitis scores, epidermal thickness, and mast cell infiltration in mice by downregulating immunoglobulin (Ig)E and IgG1 levels (Th2-associated antibodies) compared with the AD control group. However, low-molecular-weight (200–400 kDa) γ-PGA MNs ameliorated AD-like skin lesions less effectively than HMW γ-PGA MNs, thus indicating that the MW of γ-PGA may affect its immunomodulatory properties. Notably, the mouse skin quickly recovered its barrier function within 4 h after MN application. No weight loss or abnormality was observed in the MN-treated mice during the 8-week treatment period. These results suggest that the γ-PGA MNs represent an innovative, safe, and reliable therapeutic strategy for AD management.

Statement of Significance

This study is the first to explore the feasibility of using poly-γ-glutamate (γ-PGA) microneedles (MNs) as transdermal immunomodulators for improving atopic dermatitis (AD) symptoms and to evaluate their immunomodulatory effect in mice with spontaneously developed AD. Transdermal administration of γ-PGA MNs enables the γ-PGA to localize in the skin for activation of dermal dendritic cells, thus modulating immune responses. We demonstrate that high-molecular-weight γ-PGA MNs can be retained in the skin for at least 6 days and effectively suppress AD-like skin lesions in mice by reducing infiltration of mast cells and downregulating Th2-associated antibody production (IgE and IgG1). The developed MN device has the potential to replace conventional therapy and to become an innovative treatment strategy for AD.

1. Introduction

Atopic dermatitis (AD) is a common, chronic skin inflammatory condition that affects people of all ages, particularly infants and children. The clinical symptoms of AD include redness, dryness, itching, eczematous lesions, and abnormal immune responses [1,2]. Patients with severe AD experience intense pruritus, recurrent eczematous skin lesions, and a fluctuating course that considerably impairs their quality of life [3]. Furthermore, patients with AD have a high risk of asthma, allergic rhinitis, hay fever, or other allergic diseases [4,5].

Genetic and immunological factors may cause AD, but environmental factors can trigger or aggravate it [6,7]. Although the mechanisms underlying AD pathogenesis are not sufficiently understood, numerous studies have reported that AD is closely related to an overactive immune system that responds aggressively to environmental irritants and allergens, which causes skin inflammation [8–11]. In particular, T helper type 2 (Th2)-polarized im-

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mune responses involve interleukin (IL)-4, IL-5, and IL-13 secretion, which contributes to the onset and development of AD [12–14]. These cytokines drive immunoglobulin (Ig)E and IgG1 production by B cells and induce mast cell degranulation that releases histamine, which causes allergic responses [15,16].

Topical corticosteroids (TCSs) and topical calcineurin inhibitors (TCIs) are most commonly used for treating AD. These two treatments have been reported to effectively reduce skin inflammation and relieve AD symptoms; however, the long-term use of TCSs and TCIs has been reported to cause multiple adverse events. TCSs may cause cutaneous atrophy, skin infections, and hypothalamic–pituitary–adrenal axis suppression [17,18]; TCIs can cause a risk of skin malignancy or lymphoma [19,20]. Therefore, the development of a safe and innovative therapeutic strategy for AD is urgently required.

Poly-γ-glutamate (γ-PGA) is a naturally occurring polypeptide that consists of glutamate units connected by γ-amide linkages [21]. It can be produced by some strains of Bacillus subtilis isolated from fermented soybean foods [22]. Its nontoxicity, biocompatibility, biodegradability, edibility, and hygroscopicity make it a promising biomaterial for use as a health food, thickening agent in the food industry, moisturizer in cosmetics, and drug carrier in pharmaceutical and biomedical industries [21,23,24]. Recent studies have reported that γ-PGA can be used as an immunomodulatory agent because it can activate dendritic cells (DCs) to produce Th1-type cytokines; furthermore, it was reported to exhibit antitumor activity [11,21,25,26]. Daily oral administration of γ-PGA (400 μg/day) for 7 weeks downregulated serum levels of IgE and Th2-type cytokines, including IL-4 and IL-5, in inflamed skin, thus ameliorating AD symptoms in mice [11]. Moreover, repeated intraperitoneal injection of γ-PGA (2 mg/injection) in mice promoted polarization of invariant natural killer T cells toward a Th1 phenotype by inducing basophil apoptosis, which resulted in the suppression of Th2 immune responses [27]. Although these results showed that administration of γ-PGA is a potential therapy for treating Th2-biased allergic diseases, daily ingestion of γ-PGA for a few weeks or multiple injections of γ-PGA is inconvenient for patients, thus leading to a lack of compliance. In addition, after oral administration, most of the ingested γ-PGA was degraded or destroyed in the gastrointestinal tract, which considerably limited its efficacy.

In this study, we developed dissolvable γ-PGA microneedles (MNs) and investigated the effect of transdermal delivery of γ-PGA on amelioration of AD-like symptoms in Nc/Nga mice, a widely used animal model for AD. The γ-PGA MNs were combined with a polycaprolactone (PCL) supporting substrate (Fig. 1). The substrate provided a greater length to enhance MN penetration during insertion, and it could be quickly removed from the skin after the γ-PGA MNs were dissolved by the interstitial fluid in the skin. We hypothesized that the dissolved γ-PGA directly stimulated DCs located in the dermis, thus regulating immune responses and ameliorating AD pathology. Transdermal delivery using MNs avoids problems such as harsh acidic and enzymatic gastric environments and drug absorption problems that orally administered drugs are liable to. Most importantly, MNs facilitate targeted delivery of active ingredients into DC-rich skin layers for efficient modulation of immune responses.

Herein, we fabricated MNs composed of low- and high-molecular-weight (LMW and HMW, respectively) γ-PGA and assessed whether the MW of γ-PGA affected its ability to modulate immune responses and its efficacy for AD treatment. The effects of MN treatment on skin lesions, transepidermal water loss (TEWL), serum antibody levels, mass cell infiltration, and epidermal thickness were investigated in Nc/Nga mice with spontaneously developed AD.

2. Materials and Methods

2.1. Mice, Materials and Reagents

Specific pathogen-free male Nc/Nga mice were obtained from the Riken BioResource Center (Tsukuba, Japan) and housed in individually ventilated cages of an animal room under controlled environmental conditions (illumination: 13/11 h light/dark cycle, temperature: 22°C ± 1°C, and relative humidity: 50 ± 10%) before use in experiments. γ-PGA (Na^+ form, MW = 200–400 or 1100 kDa), PCL (MW = 70–90 kDa), and fluoresceinamine isomer I (FA) were purchased from Vedan Enterprise Corp. (Taichung, Taiwan), Sigma-Aldrich (St. Louis, MO, USA), and Acros Organics (New Jersey, USA), respectively. Metallic master structures of MNs and supporting substrates (Fig. S1, Supporting Information) were provided by Hong-Da Precision Industry Co., Ltd. (New Taipei City, Taiwan).

2.2. Ethics Statement

All animal studies were reviewed and approved by the Institutional Animal Care and Use Committee of National Cheng Kung University (NCKU). All animal-related experiments were conducted in compliance with the guidelines of the Laboratory Animal Center of NCKU.

2.3. Fabrication of γ-PGA MNs with PCL Supporting Substrates

A γ-PGA gel (60 wt%) was prepared by dissolving 3 g of the γ-PGA powder in 2 mL of deionized (DI) water, and the solution was stirred overnight. The obtained gel (approximately 1 g) was placed on a polydimethylsiloxane (PDMS) MN mold and a polytetrafluoroethylene (PTFE) plate was used to cover the gel (Fig. 2). A compression force (300 N) was applied to the PTFE plate for 30 s to push the gel into the mold cavities. The excess gel on the mold surface was collected for subsequent use. After drying in an oven at 37°C for 10 min to leave the filled γ-PGA gel half dry, a PCL supporting substrate was aligned with the mold cavities manually under a stereomicroscope to combine with the γ-PGA gel in the mold. Because the resulting γ-PGA gel was highly viscous, it can act as an adhesive to integrate the PCL substrate with the γ-PGA MN. The assembled system was dried in an oven at 37°C overnight to create a γ-PGA–PCL MN patch. The resulted patch contained 81 (9 × 9) MNs with a 1000 μm center-to-center distance between two needles. The height and base width of the γ-PGA MNs and PCL supporting substrates were 600 and 300 μm, respectively. Methods for preparing the PDMS mold and the PCL supporting substrate are provided in the Supporting Information.

2.4. Conjugation of γ-PGA with FA

First, FA powder was dissolved in ethanol (0.01 g/mL) and mixed thoroughly with an aqueous solution (20 wt%) of γ-PGA. Next, 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC·HCl, 0.08 g) was added to the mixture and stirred at 4°C for 8 h in the dark. Finally, the solution was transferred to a dialysis membrane (MWCO: 14 kDa, BioDes Inc., New York, USA) and dialyzed against DI water for 2 days in the dark to remove unreacted FA. The dialyzed solution was lyophilized and stored in the dark at 4°C until further use.

2.5. Skin Puncture Capability and Skin Retention Time

MNs were applied to samples of pig cadaver skin and mouse dorsal skin using a custom-made applicator, which provided an

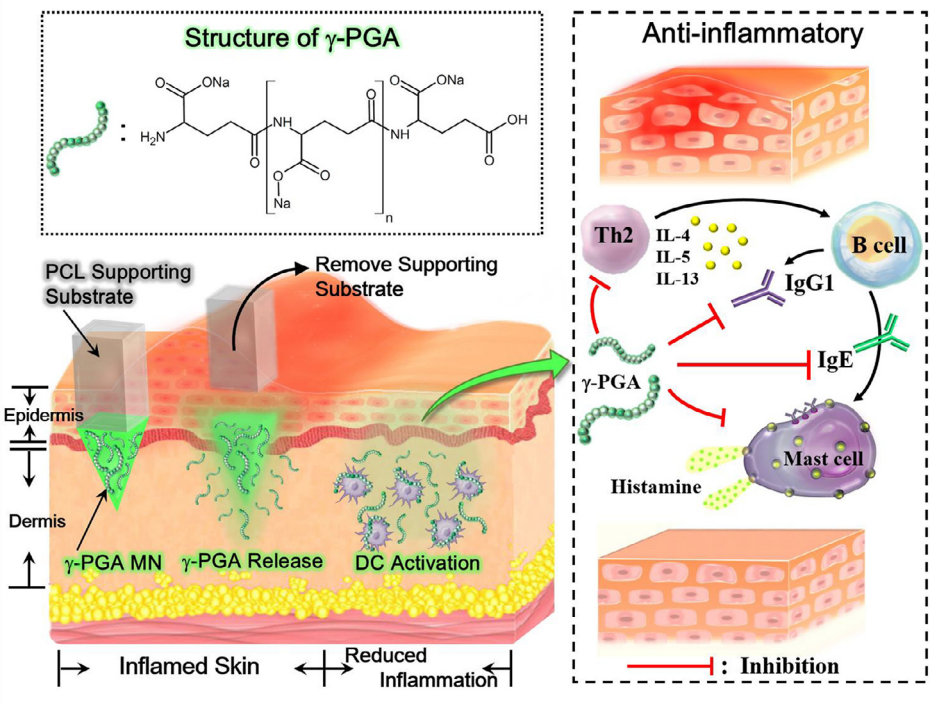

Fig. 1. Schematic illustration of transdermal delivery of poly-γ-glutamate (γ-PGA) microneedles (MNs) for alleviating skin inflammation by down-regulating the IgE and IgG1 production (Th2-associated antibodies) and reducing mast cell infiltration. Upon insertion, the polycaprolactone (PCL) supporting substrate can provide a greater length to enhance MN penetration and then be quickly removed from the skin when the MN is dissolved within the skin. The dissolved γ-PGA directly activates dendritic cells (DCs) in the dermis, thus regulating immune responses to ameliorate atopic dermatitis pathology.

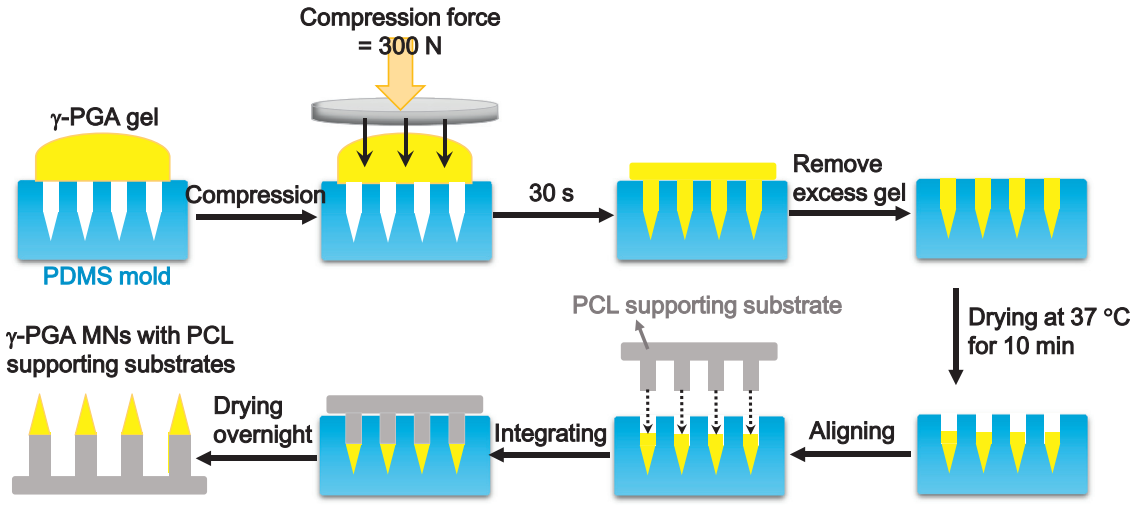

Fig. 2. Schematic illustration of the fabrication procedure for γ-PGA MNs with PCL supporting substrates.

insertion force of 10 N/patch for the pig skin and 8 N/patch for mouse skin. Before MN insertion, the mice were anesthetized, and their back hair was shaved. After insertion for 2 min, the PCL supporting substrate was removed from the skin. The MN-treated skin was excised and processed for histological staining or confocal microscopic observation at various time points. 3D reconstruction images were created using confocal scanning microscope images by using an XYZ stack for evaluation of the skin retention time of the FA–γ-PGA.

2.6. Determination of the Amount of γ-PGA Delivered into the Skin

The amount of γ-PGA delivered into the skin was determined by subtracting the amount of γ-PGA remaining on the PCL substrate after insertion and the residual γ-PGA on the skin surface from the amount originally on the PCL substrate before insertion. After skin insertion for 2 min, the PCL supporting substrate was removed from the skin. The substrates before and after skin insertion were separately immersed in DI water and stirred at room temperature to dissolve the FA–γ-PGA. The MN insertion site was tape-stripped 20 times consecutively with 3M Transpore tapes to collect the residual γ-PGA on the skin surface. The stripped tapes were cut into small pieces and then soaked in DI water to recover the FA–γ-PGA on the tapes. The amount of γ-PGA extracted from the skins or the tapes was calculated by measuring the fluorescence intensity of FA–γ-PGA of the obtained solution by using a multi-mode microplate reader (Infinite 200, Tecan Group Ltd, Männedorf, Switzerland).

2.7. TEWL Measurement

To measure the integrity of the skin barrier, TEWL was measured using a Delfin VapoMeter® (Delfin Technologies Ltd., Kuopio, Finland). TEWL values were recorded before and after MN insertion until they returned to baseline values, which indicated that the skin was sealed. The control mice underwent only shaving of back hair.

2.8. Circular Dichroism (CD) Spectroscopy

The structural analysis of γ-PGA was performed using CD spectropolarimeter (JASCO, J-810, Tokyo, Japan) before and after two weeks of storage at room temperature (RT) or 37 °C. The MN sample was dissolved in 2 mL of DI water with stirring and then diluted with DI water to a concentration of 40 μg/mL for spectral measurements. CD spectra were measured using a quartz cell with 1 cm path length and at a scanning speed of 200 nm/min.

2.9. Induction of AD Lesions and Treatment Using γ-PGA MNs

To induce AD-like skin lesions, the male Nc/Nga mice (age: 7 weeks old) were housed under air-uncontrolled conventional conditions where the room was not equipped with a high efficiency particulate air (HEPA) filter to protect from allergens. Therefore, environmental allergens, such as mold and dust mites, can trigger the development of AD. After 1 week, the mice were randomized into three groups (n = 4 mice per group) namely the control, LMW MN, and HMW MN groups. In the control group, the mice did not receive any treatment. In the LMW MN group, LMW (200–400 kDa) γ-PGA MNs were applied to the shaved dorsal skin every 2 weeks for a total of four doses. In the HMW MN group, HMW (1100 kDa) γ-PGA MNs were applied to the shaved dorsal skin every 2 weeks for a total of four doses. Blood samples were obtained from the facial vein to measure serum IgG1, IgG2a, and IgE levels.

2.10. Clinical Scoring of Skin Lesions

The total clinical severity score of AD was evaluated according to the following criteria: erythema, edema, crust formation, excoriations, and dryness. Each parameter was scored as 0 (none), 1 (mild), 2 (moderate), or 3 (severe) [28]. The sum of the individual scores was defined as the dermatitis score.

2.11. Enzyme-linked Immunosorbent Assay

Serum IgE, IgG1, and IgG2a levels were measured using commercial enzyme-linked immunosorbent assay (ELISA) kits (eBioscience, San Diego, CA, USA) according to the manufacturer's instructions.

2.12. Histological Analysis

Skin samples were embedded in Tissue-Tek® optimum cutting temperature solution, frozen using a cryostat, and sectioned. For each sample, a 10 μm-thick section was cut and stained with hematoxylin and eosin for morphological examination or with 0.01% toluidine blue for mast cell identification. The number of mast cells in a defined area of the skin was counted using ImageJ.

2.13. Statistical Analysis

Statistical analysis was conducted using the Student's t test. A value of $P < 0.05$ was considered to indicate a statistically significant difference. *: $P < 0.05$, **: $P < 0.01$, ***: $P < 0.005$.

3. Results and Discussion

3.1. MN Characterization

The proposed MN patch was prepared by separately molding the γ-PGA MNs and PCL supporting substrate and then integrating them to form a γ-PGA–PCL MN patch (Fig. 2). To observe and track γ-PGA distribution in the skin, γ-PGA was labeled with a fluorescent dye, FA. Fig. 3a and Fig. 3b show the optical microscopic images of HMW FA-γ-PGA MNs (fluorescent yellow) with PCL supporting substrates. When the FA−γ-PGA gel was filled into the mold cavities, not all of the FA−γ-PGA gel can be completely compressed into the bottom of the mold cavity. During the integration of the PCL substrate with the filled FA−γ-PGA gel, a small amount of gels that remained and adhered to the top wall of the mold cavity would form a thin yellow layer covered on the external surface of the PCL substrate. The thin FA−γ-PGA layer would make the PCL substrate appear slightly yellow (Fig. 3b).

The obtained MNs exhibited a pyramidal shape and were arranged in a 9 × 9 array on a 10 × 10 mm patch (Fig. 3a). Both the MN and supporting substrate have an average height of 600 μm and an average base width of 300 μm (inset of Fig. 3). The center-to-center spacing between adjacent structures was 1000 μm. The optical microscopic images of the LMW MNs are shown in Fig. S2a (Supporting Information). No notable differences were observed between the morphological features of the HMW and LMW MNs.

3.2. Skin Puncture Capability

To evaluate the skin insertion ability, we inserted the prepared MNs into a pig cadaver skin and a mouse skin by using a custom-made applicator. A clear array of micropores (9 × 9) was observed on the skin surface following MN insertion (Fig. 3c and Fig. 3d), which indicated that the MNs pierced pig or mouse skin with an insertion ratio of 100%. After applying the MN patch to the skin for various time periods, the patch was removed from the skin for observation the MN dissolution process. We found that MN dissolution occurred within 2 min of patch application. The short dissolution time was attributable to the hydrophilic and hygroscopic nature of γ-PGA, which is beneficial for a rapid transdermal drug delivery.

Fig. 4 shows the histological images of the skin after 1 h of HMW FA-γ-PGA MN application. The green fluorescence indicates the distribution of the released FA-γ-PGA. As shown, the HMW MNs can successfully create microchannels in the pig cadaver skin and penetrate the epidermis to reach a depth of 525 ± 72 μm (n = 5). However, in the mouse skin samples, no MN-generated microchannel was observed, which may be attributed to that the disrupted skin resealed or healed. Previous studies also reported that the channels created in the living tissue would close within a few minutes to a few hours because of skin's elasticity and self-healing ability [29–31]. Therefore, it is not easily to show a clear skin rupture or directly measure the MN insertion depth from the living tissue. According to the fluorescence of FA-γ-PGA in the histological sections, the maximum permeation depth of HMW γ-PGA was 510 ± 23 μm. The LMW MNs also exhibited an insertion ratio of 100% (Fig. S2b, Supporting Information), and the maximum permeation depth of LMW γ-PGA was 475 ± 21 μm (Fig. S2c, Supporting Information) in the Nc/Nga mice.

Many studies have shown that skin deformation occurs around the MN piercing site due to the inherent elasticity of the skin, which results in incomplete MN insertion [30,32,33]. In this study, the total height of the microstructure (including the γ-PGA MN and the PCL supporting substrate) was 1.2 mm, but the penetration depth for the porcine cadaver skin was only approximately

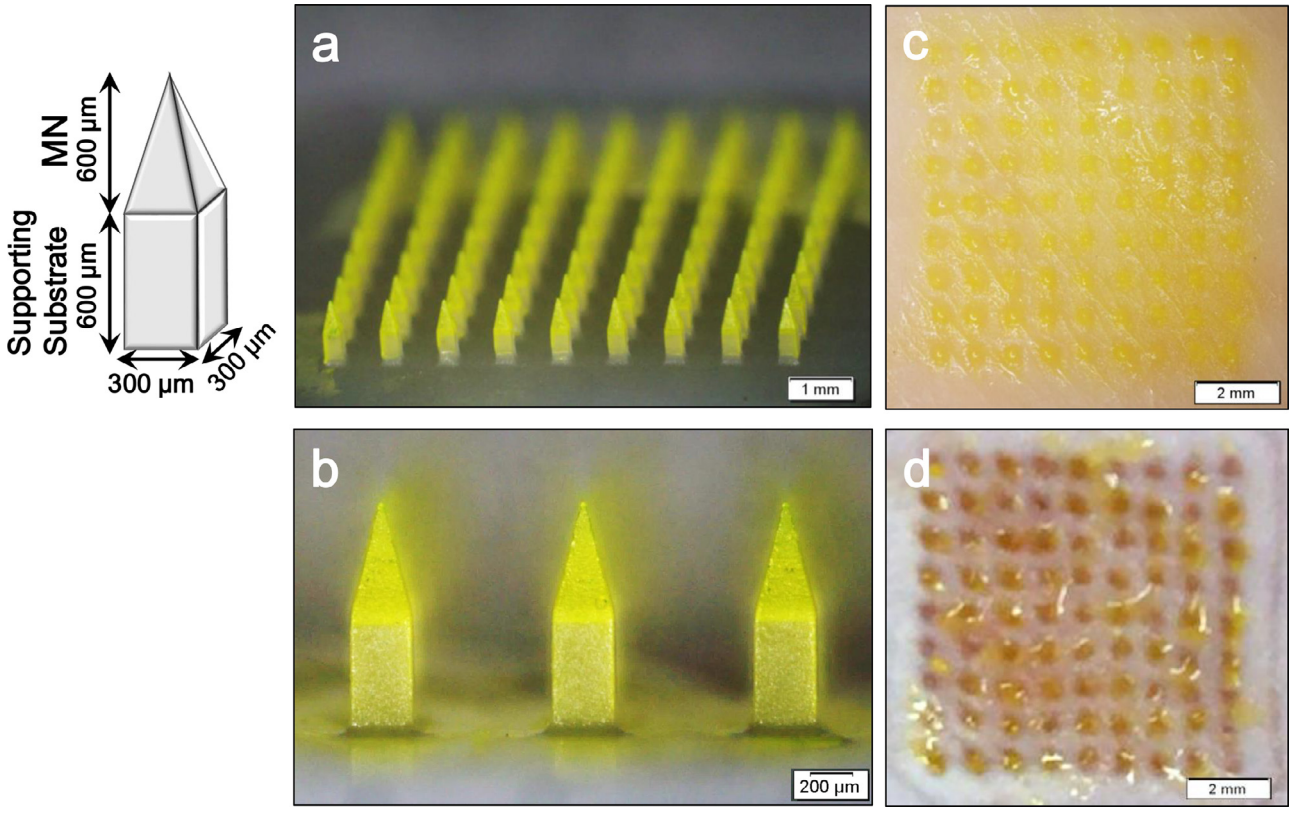

Fig. 3. Optical microscope images of the HMW FA–γ-PGA MNs with PCL supporting substrates (a and b) and the MN-treated pig cadaver (c) or mouse (d) skin. The inset shows the detailed dimensions of the MN device.

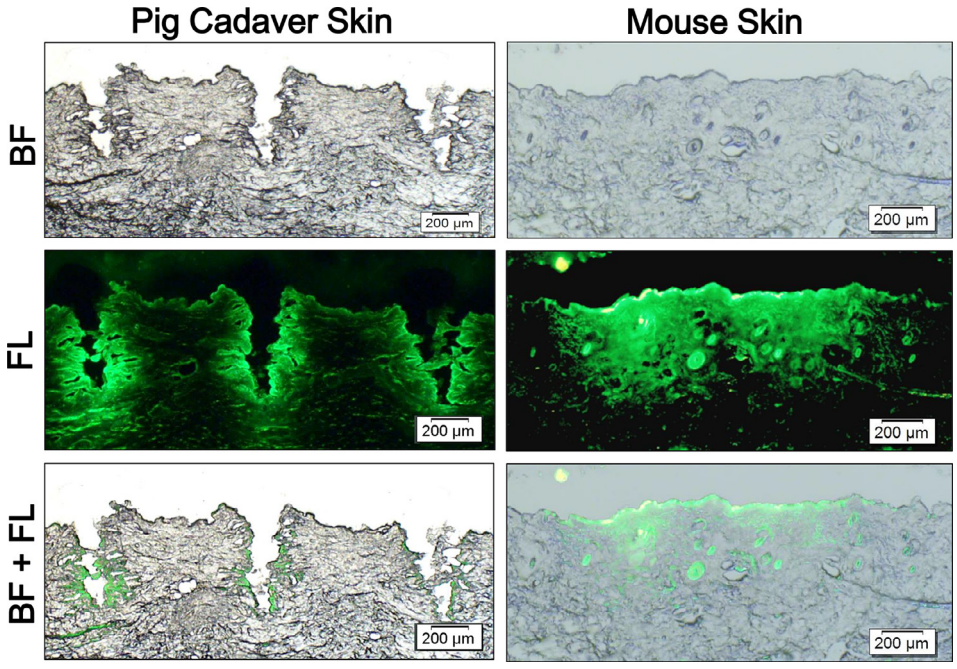

Fig. 4. Histological images of the skin after administration of HMW FA–γ-PGA MNs. The green fluorescence indicates the released FA–γ-PGA and its distribution. The camera parameters were set for fluorescence images with an exposure time of 12 ms and an ISO sensitivity (gain value) of 200. FL: fluorescence; BF: bright field.

500 μm (Fig. 4). This insertion depth indicates that γ-PGA MN can penetrate through the epidermis and deliver dissolved γ-PGA into the upper dermis. Although administration of the proposed MNs may cause a slight pain, dermal puncture may be a useful strategy to directly stimulate or activate DCs located in the dermis.

3.3. Amount of γ-PGA Delivered

To quantify the amount of γ-PGA delivered into the skin of the Nc/Nga mice, we analyzed the fluorescence intensity of FA-γ-PGA on the PCL supporting substrate before and after insertion into mouse skin and that left on the skin surface. The total amounts

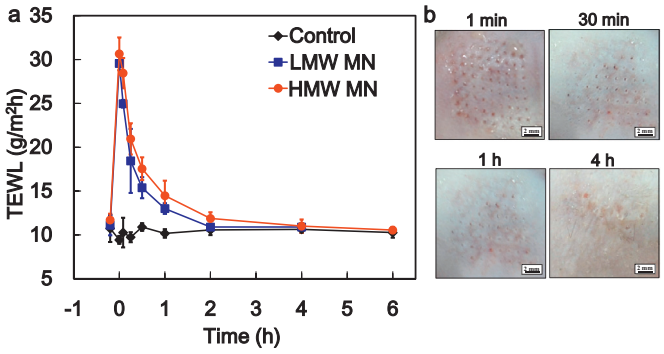

Fig. 5. Skin barrier recovery. Transepidermal water loss (TEWL) from the mouse skin after MN treatment (n = 4–5 mice/group) (a): the intact skin without MN insertion (control); the skin inserted with LMW MNs or HMW MNs. Optical images of the MN-inserted mouse skins at different time points (b). Data in (a) are presented as mean ± SD.

of γ-PGA on the LMW and HMW MN patch were 1943 ± 317 and 1930 ± 60 μg (n = 5), respectively. We found that the percentages of γ-PGA delivered by the LMW and HMW MNs were 39.6 ± 9.8% and 46.7 ± 8.6% (n = 5), respectively. The percentages of γ-PGA left on the skin surface was approximately 10.2% and 8.9% for the LMW and HMW MNs, respectively. There was no statistical difference in the delivery efficiency between these two groups (P> 0.05). The calibration curve of fluorescence intensity and its corresponding FA-γ-PGA concentration was shown in Fig. S3 (Supporting Information).

3.4. Skin Barrier Recovery

The stratum corneum (SC) is a vital barrier of the skin that prevents irritant penetration and minimizes water loss. The microchannels created by MNs disrupted the integrity of the SC. The measurement of TEWL is a reliable indicator of the integrity of the skin's barrier function, which essentially refers to the skin's capability to retain moisture. Upon MN treatment, the values of TEWL increased immediately, but they returned to baseline values within 4 h (Fig. 5a), which indicated that the skin recovered its barrier function in 4 h after application. Fig. 5b also shows that the MN-generated micropores gradually resealed and finally disappeared at 4 h after application. The skin recovery rates did not differ significantly between the LMW and HMW groups.

Rapid restoration of skin integrity is highly desirable because it reduces the risk of exposure to exogenous irritants and allergens. Many studies have reported that moisturizers can help restore skin barrier function and improve AD symptoms [34–36]. Because of the hygroscopic and moisturizing effects of the γ-PGA, it has been considered a natural moisturizer for skin care and exhibits a water-holding capacity comparable with that of hyaluronic acid [21,37]. Additionally, γ-PGA, a polypeptide, can be easily degraded into glutamate, an amino acid that can provide nutrition to the skin [21,23,38]. Therefore, γ-PGA is particularly promising as a MN material for AD treatment.

3.5. Skin Retention

The skin not only acts as the first line of defense against various infections but also as an active organ for immunomodulation, which provides an appropriate habitat for immune cells and immunomodulators [39]. MN delivery enables direct and efficient delivery of its payload into the skin. We hypothesized that prolonging the retention time of immunomodulators in the skin enhanced the immunomodulatory effect by increasing the chances of interaction between DCs and immunomodulators. To study the skin retention time of γ-PGA, FA-γ-PGA MNs were applied to the dorsal skin of mice, and the MN-treated skin samples were investigated through confocal laser scanning microscopy. Fig. 6a and 6b show the Three-dimensional (3D) reconstruction images of the skin after insertion of the LMW and HMW MNs and a representative image of the γ-PGA MNs. Green fluorescence indicated that the γ-PGA had penetrated into the skin. In both groups, these fluorescent signals were strong and easily detected on Day 0, but they gradually became weaker with time, possibly because of γ-PGA diffusion into adjoining tissues. HMW γ-PGA remained visible in the punctured skin until Day 6; however, LMW γ-PGA was not observed after 2 days. These results demonstrated that HMW γ-PGA can be retained in the skin for at least 6 days and showed superior skin retention than LMW γ-PGA.

3.6. Storage Stability of γ-PGA MNs

We used CD spectroscopy to investigate the stability of the γ-PGA MN formulation. Fig. 7 shows the CD spectra of γ-PGA solution, freshly prepared HMW MNs and the MNs after two weeks of

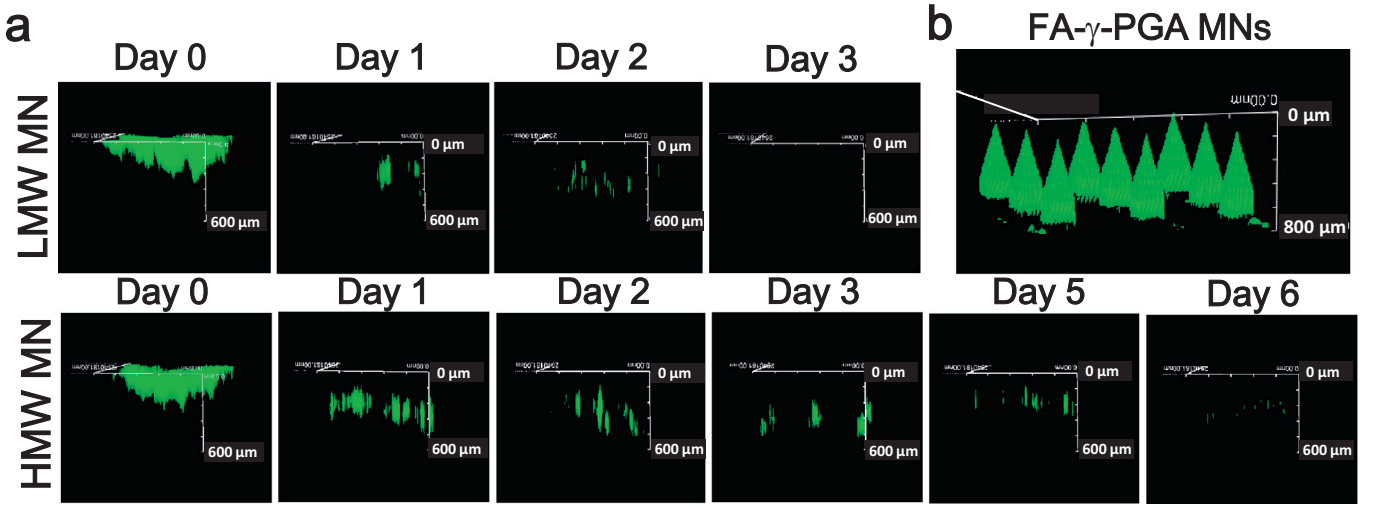

Fig. 6. Skin retention of γ-PGA. 3D reconstruction images of the skin after insertion of the LMW and HMW FA–γ-PGA MNs (a) and the 3D reconstruction image of the FA–γ-PGA MNs (b). The green fluorescence in (a) indicates the FA–γ-PGA in the skin.

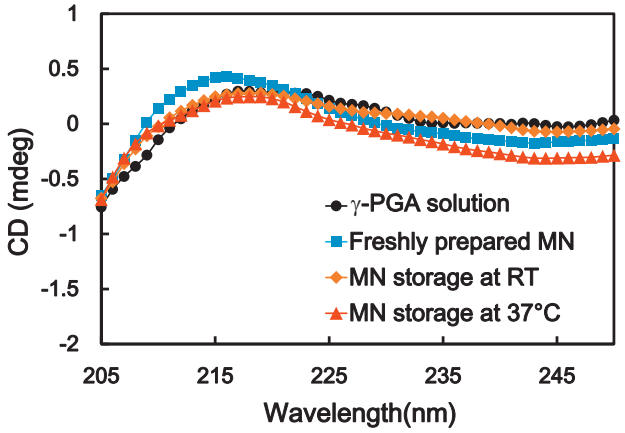

Fig. 7. Circular dichroism spectra of γ-PGA solution, freshly prepared HMW γ-PGA MNs and the MNs after two weeks of storage at room temperature (RT) or 37 °C.

storage at room temperature (RT) or 37 °C. We found all the samples have similar spectra, indicating that no considerable change in secondary structure occurred. These results showed that the proposed MN formulation was able to maintain the structural stability of γ-PGA at RT or 37 °C for at least two weeks.

3.7. Amelioration of AD Symptoms in the Nc/Nga Mice

It is important to note that rodent skin may not be the best model to evaluate skin permeation due to the differences in the thickness and structure between the rodent and the human. However, in this study, our purpose is to explore the feasibility of using γ-PGA MNs as transdermal immunomodulators for improving AD symptoms. Therefore, a suitable animal model of AD is very important to evaluate the efficacy for AD treatment. Many studies have reported that the Nc/Nga mice spontaneously develop AD-like skin lesions when exposed to nonsterile conditions and exhibit pathological and behavioral characteristics similar to those observed in AD patients [11,40–42]. Therefore, the Nc/Nga mouse model has been considered a suitable animal model of human AD [11–13,40–43].

To evaluate the efficacy of using γ-PGA MNs for alleviating AD-like skin lesions, γ-PGA MNs were applied to the dorsal skin of the Nc/Nga mice every 2 weeks until week 6 (Fig. 8a), and images of the skin lesions were recorded to determine dermatitis scores. These mice were exposed to environmental aeroallergens under conventional surroundings to generate AD symptoms 1 week before treatment. Compared with the unexposed mice (Fig. 8b), mild skin dryness, erythema, and excoriation were observed after being housed in nonsterile conditions for 1 week (Fig. 8c, at Week 0), and these symptoms become increasingly severe with disease progression at Week 8 (the AD control group, Fig. 8d).

After MN treatment for 8 weeks, we observed that transdermal delivery of the HMW γ-PGA ameliorated the AD-like skin lesions (Fig. 8f), whereas the LMW γ-PGA showed a relatively lower amelioration effect on skin lesions (Fig. 8e). The severity of AD was assessed by grading five signs, including erythema, edema, crust, excoriation, and dryness. The dermatitis score was defined as the sum of the individual scores for each of five items. Fig. 8g shows the mean dermatitis score of the HMW MN group was significantly lower than that of the AD control mice ($P < 0.05$) at Week 6 and Week 8. Although the mice treated with the LMW MNs for 8 weeks showed a reduction in the clinical score compared with the control mice, the score of the LMW MN group was not significantly different from that of the control group ($P > 0.05$). Table S1 (Supporting Information) shows the scores of each AD

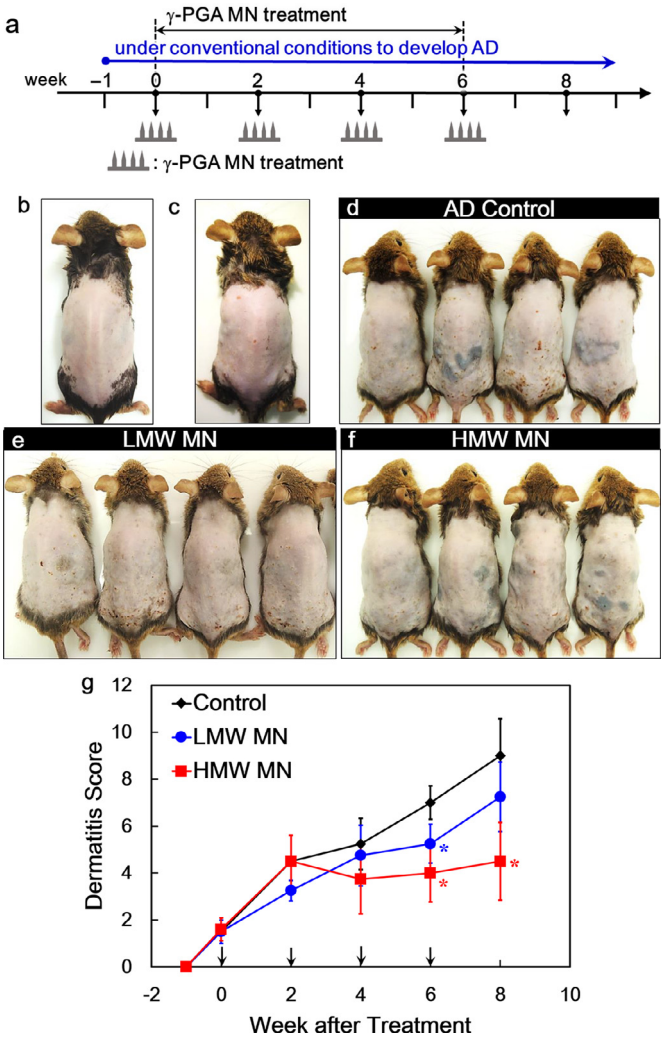

Fig. 8. Amelioration of AD-like skin lesions in the Nc/Nga mice. Experimental schedule for the induction of AD-like skin lesions and the MN treatment (a). After exposure to the conventional conditions for 1 week, the MNs were applied to the dorsal skin once every 2 weeks for a total of four doses. Representative photographs of the mouse skins before (b) and after being housed in conventional conditions for 1 week (c). Representative photographs of skin lesions taken at Week 8: the AD control group (d, without treatment), the LMW MN group (e), and the HMW MN group (f). Dermatitis scores of the mice (g). Data are presented as the mean ± SD ($n = 4$ mice/group). Arrows in (g) indicates the time point of the MN application. *: $P < 0.05$, compared with the control group.

symptom at Week 8. As shown, the HMW MN group had significantly lower scores on the erythema and dryness items than the Control group ($P < 0.05$). There was no significant difference in the individual symptom score between the HMW MN and LMW MN groups ($P > 0.05$). These results indicated that transdermal administration of the HMW γ-PGA MNs efficiently prevented the progression of spontaneous AD symptoms in the Nc/Nga mice.

3.8. Downregulation of Serum IgE and IgG1 Levels

Because AD development is closely related to increased IgE levels and Th2-biased immune responses, we investigated whether transdermal delivery of γ-PGA modulated the production of IgE and IgG1 or IgG2a in Nc/Nga mice. Consistent with previous studies [15,44,45], the serum total IgE, IgG1, and IgG2a levels increased significantly in the dermatitis-induced mice (the control group, Fig. 9). Upon HMW MN treatment, the increase in serum total IgE and IgG1 levels was considerably suppressed (Fig. 9a and Fig. 9b).

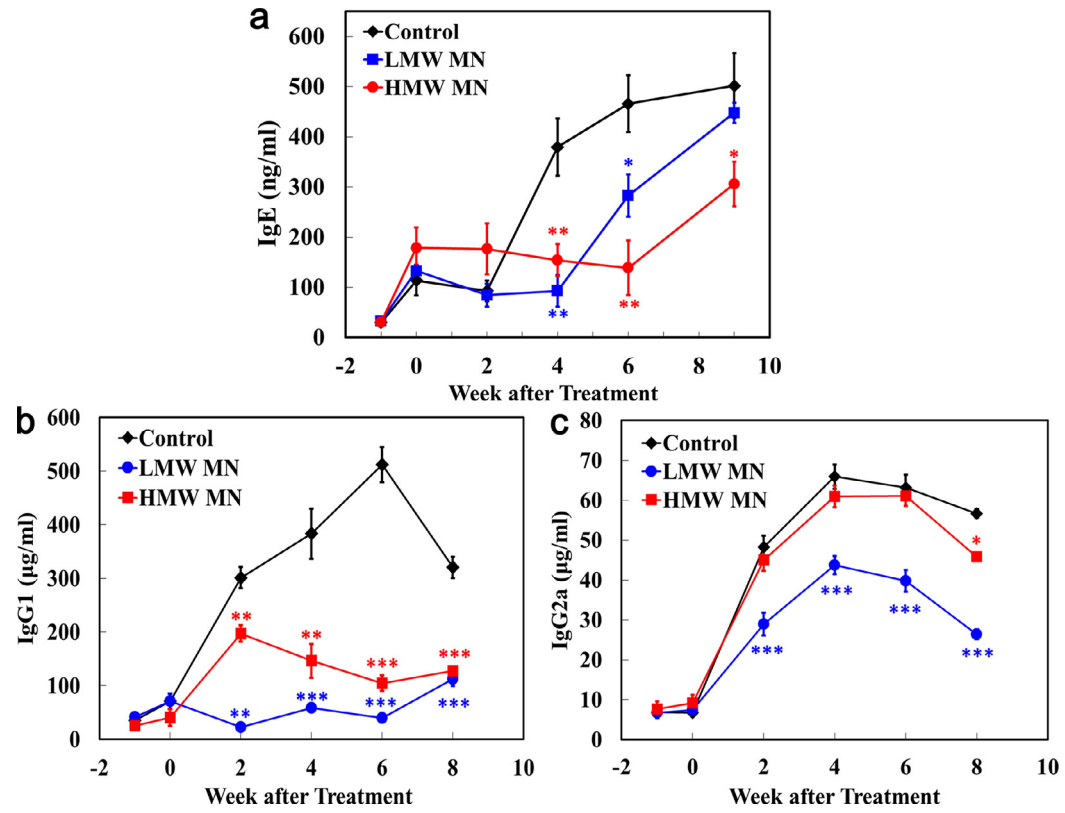

Fig. 9. Downregulation of serum IgE and IgG1 Levels. Nc/Nga mice were treated with γ-PGA MNs once every 2 weeks for a total of four doses. Serum IgE (a), IgG1 (b) and IgG2a (c) Levels were measured by ELISA. Data are presented as the mean ± SD (n = 4 mice/group). *: $P < 0.05$, **: $P < 0.01$, ***: $P < 0.005$ compared with the control group.

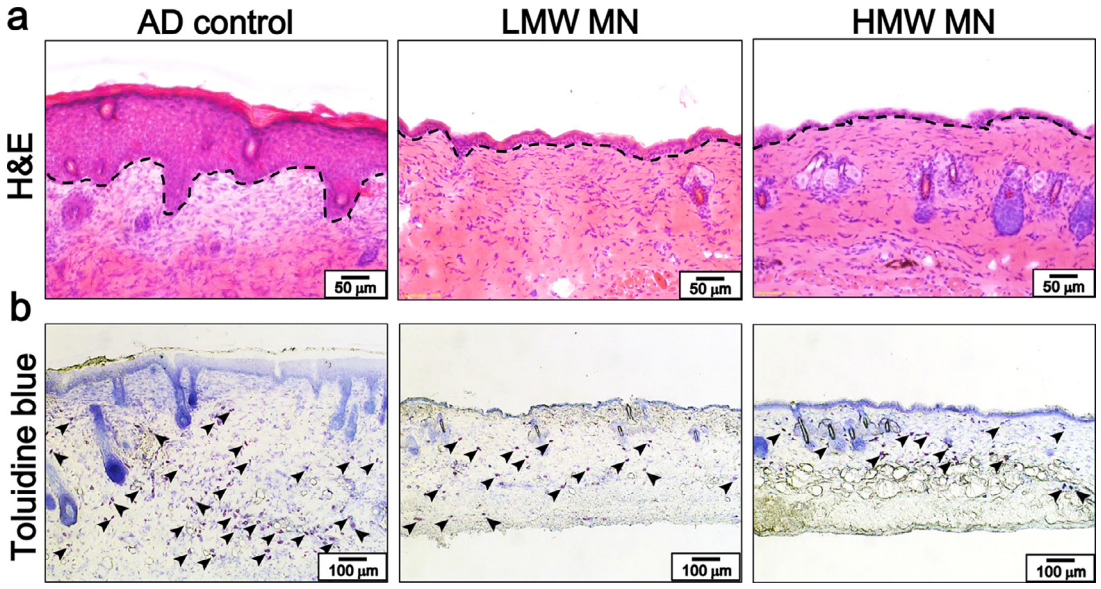

Fig. 10. Suppression of tissue inflammation and mast cell infiltration. Representative H&E- (a) or toluidine blue-stained (b) skin sections of Nc/Nga mice at Week 8. Dotted lines in (a) indicate epidermal hypertrophy. Arrowheads in (b) indicate the mast cells.

Notably, we found that only the LMW MN group exhibited a significant decrease in IgG2a levels ($P < 0.005$ from week 2 to week 8) compared with the control mice (Fig. 9c). However, administration of the HMW MNs did not substantially affect the IgG2a levels. These results showed that transdermal administration of HMW γ-PGA negatively regulated Th2-associated Ig production (IgE and IgG1) but not Th1-associated Ig (IgG2a) production. By contrast, the treatment with LMW MNs simultaneously inhibited both Th1- and Th2-associated antibody production.

A recent study reported that oral administration of γ-PGA with MW of 2000 kDa not only suppressed Th2 immune responses but also augmented the Th1 immunity [11]. Similarly, a previous study also reported that oral treatment with 2000 kDa γ-PGA enhanced Th1 responses, including induction of NK cell activation and IFN-γ secretion, to a greater degree than LMW γ-PGA (10 and 100 kDa) [25]. These findings suggest that the MW of γ-PGA is a critical factor affecting its immunomodulatory effects. In the present study, although transdermal administration of HMW MNs

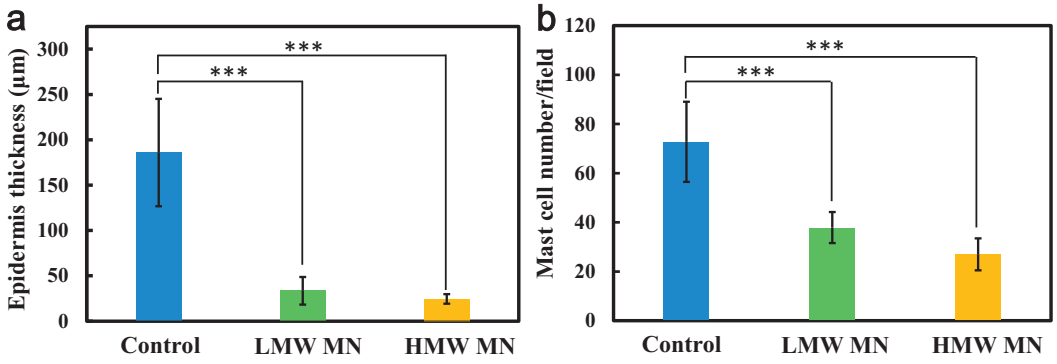

Fig. 11. Reduction of epidermal thickness and mast cell accumulation. The epidermal thickness (a) and mast cell number (b) were determined from the H&E- and toluidine blue-stained skin sections at Week 8. Data are presented as the mean ± SD (n = 4 mice/group). ***: $P < 0.005$ compared with the control group.

efficiently downregulated IgE and IgG1, it did not enhance IgG2a production. This may be because the MW of the HMW γ-PGA MNs (only 1100 kDa) was relatively low (< 2000 kDa) and may not have been able to drive Th1 immune development. Additionally, administration of γ-PGA through the oral and transdermal route are entirely different from each other and may exhibit dissimilar effects on immune modulation.

3.9. Reduction of Epidermal Thickness and Mast Cell Accumulation

To investigate whether skin inflammation was ameliorated by γ-PGA MN application, we observed the histopathological changes in and examined the extent of inflammatory cell infiltration in the skin lesions. Mast cells are the key effector cells of allergic disease and the primary source of histamine in AD. Therefore, skin sections were stained with toluidine blue to identify the mast cells. When exposed to a nonsterile environment for 9 weeks, the control mice exhibited typical AD features, including epidermal hyperplasia (dotted lines in Fig. 10a) and massive infiltration of mast cells (arrowheads in Fig. 10b), which coincided with the typical AD symptoms observed in Fig. 8d. Fig. 11a shows that the epidermal thickness was significantly lower in the mice treated with LMW (33.5 ± 15.2 μm, $P < 0.005$) or HMW MNs (24.5 ± 4.3 μm, $P < 0.005$) than in the control mice (186.0 ± 59.2 μm) at week 8. Similarly, the accumulation of mast cells in the dermal layer was significantly inhibited in the LMW (37.8 ± 6.3 cell/field, $P < 0.005$) and HMW MN-treated (27.0 ± 6.5 cell/field, $P < 0.005$) mice compared with the control group (72.7 ± 16.3 cell/field) (Fig. 11b). These results demonstrated that treatment with the γ-PGA MNs efficiently inhibited epidermal thickening and mast cell recruitment.

Currently available therapies for AD are limited and present various adverse effects; thus, a safe and effective treatment for AD is urgently needed. γ-PGA produced by microorganisms with generally regarded as safe status has been recognized as a safe and nontoxic food and skin care ingredient [11]. In the present study, we demonstrated that treatment with γ-PGA MNs significantly ameliorated AD symptoms (Fig. 8) by downregulating the production of Th2-dependent IgE and IgG1 antibodies (Fig. 9). Furthermore, no statistically significant differences in body weight were observed between the control and the MN-treated groups (LMW and HMW MNs) (Fig. S4, Supporting Information), and no apparent abnormality was observed in all the mice during the 8-week observation period, which showed that administration of γ-PGA MNs once every 2 weeks for four total treatments did not produce serious side effects or notable toxicity in mice.

Although a previous study has shown that oral γ-PGA effectively prevented AD development in mice, γ-PGA was not detected in the serum and was cleared up in the digestive system within 24 h after ingestion [11]. The ingested γ-PGA could be taken up by M cells in the intestine and presented to DCs for modulating the immune responses. Rapid excretion of γ-PGA provides relatively less time to γ-PGA for interaction with DCs; consequently, daily oral administration of γ-PGA is required to maintain its efficacy. In the present study, transdermal administration of γ-PGA MNs enabled the delivered γ-PGA to localize in the skin at least for 2–6 days (Fig. 6); this retention depended on the MW. The proposed MN formulation can be administered to mice just once every two weeks, instead of daily dosing, which provides more convenience to patients. We expected that prolonged skin retention of γ-PGA provided additional time for activation of epidermal and dermal DCs, thus enhancing its immunomodulatory effect and reducing the required dosing frequency. Therefore, transdermal delivery of γ-PGA by using an MN device is a more attractive therapeutic regimen than daily oral treatment because it may have higher bioavailability, higher patient acceptance, and longer duration of action. More studies in the future will be required to clarify the differences in γ-PGA's immune modulation between the oral and transdermal routes.

4. Conclusion

The present study is the first to use γ-PGA MN system as transdermal immunomodulators and evaluate its immunomodulatory effect in mice with spontaneously developed AD. Our results showed that transdermal delivery of HMW γ-PGA effectively suppressed the development of AD-like skin lesions in Nc/Nga mice, which was evidenced by reduction in inflammation, infiltration of mast cells, and serum IgE and IgG1 levels. After applying γ-PGA MNs to the skin, the skin can quickly recover its barrier function within 4 h without inducing notable skin irritation. Additionally, continuous treatment with γ-PGA MNs for 8 weeks did not result in a marked weight loss and abnormal clinical symptoms. Our results suggest that dissolvable γ-PGA MNs exert an immunomodulatory effect in Nc/Nga mice and have potential to be a mild, convenient, and effective treatment option for management of AD.

Declaration of Competing Interest

The authors declare that they have no competing interests.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.actbio.2020.07.029.

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