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Original Article

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Microarray Patch Based Transdermal Allergen Immunotherapy Prevents Gliadin-induced Anaphylaxis in a Murine Model

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ABSTRACT

Purpose: Gliadins are the primary triggers in wheat-dependent exercise-induced anaphylaxis. Currently, there are no officially approved immune-modulating treatments for gliadin allergy. Recent *in vivo* studies have shown that hyaluronic acid based dissolving microarray patch (dMAP) could deliver house dust mite allergens through transdermal pathway and protect allergic asthma and atopic dermatitis *in vivo.* In this study, we explored the potential of dMAP for the transdermal delivery of gliadin proteins as a strategy to mitigate gliadin allergy.
Methods: C3H/HeJ mice were sensitized to gliadin with cholera toxin via oral administration, followed by oral or intraperitoneal gliadin challenge. To evaluate the protective effects of transdermal immunotherapy (TDIT), gliadin-loaded dMAPs were applied twice a week to gliadin-sensitized mice for 4 weeks. Afterward, the mice were challenged with gliadin.
Results: The manufacturing process of dMAP did not alter the allergenicity of gliadin. TDIT significantly improved the anaphylaxis clinical score and stabilized core body temperature in the gliadin anaphylaxis model. It reduced mast cell protease-1 and gliadin-specific immunoglobulin E (IgE), and increased specific IgG_1, IgG_{2a} and IgG_{2b} levels. *Ex vivo* splenocyte study revealed that TDIT enhanced T helper type 1 (Th_1) cell population, interferon-γ expression, regulatory T cell population, and interleukin (IL)-10 expression, as well as suppressed Th_2 cell population and associated cytokines (IL-4, IL-5, and IL-13). Furthermore, this TDIT preserved the structural integrity of small intestinal villi and reduced eosinophil and mast cell infiltration.
Conclusions: Gliadin TDIT using dMAP mitigates gliadin-induced anaphylaxis in a murine model, offering a promising novel immune modulating treatment for gliadin-induced anaphylaxis.

Keywords: Wheat allergy; gliadin; anaphylaxis; food allergy; microarray patch; immunotherapy

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Disclosure
The authors have no potential conflicts of interest to disclose.

INTRODUCTION

Wheat, a globally consumed staple cereal, is associated with severe allergic reactions, and the prevalence of wheat allergy has significantly increased over the past decade.[1,2] Physicians typically recommend a wheat-free diet for individuals diagnosed with wheat allergy.[3] However, patients frequently consume wheat-containing foods, either unintentionally or intentionally. Moreover, wheat-dependent exercise-induced anaphylaxis (WDEIA), a more common and severe manifestation of wheat allergy in adults, poses a significant challenge to clinical management.[4]

Wheat proteins can induce immunoglobulin E (IgE)-mediated allergic reactions. Gluten, the main component of wheat proteins, consists of gliadins and glutenins,[5,6] which are the major allergens in wheat allergy. Among these, gliadin demonstrates higher allergenicity than glutenin, and cross-reactivity between the two has been documented.[7] Gluten possesses unique properties: they are insoluble in water and salt, and primarily composed of glutamine and proline.[7,8] Notably, it is poorly digested by gastric, pancreatic enzymes, and intestinal brush border membrane proteases.[8] Consequently, gluten peptides are rarely absorbed under normal circumstances. However, during conditions such as intestinal infections or increased intestinal permeability, including exercise or aspirin exposure, these peptides may traverse the intestinal epithelial barrier.[9,10]

Oral allergen-specific immunotherapy (OIT) has emerged as a promising approach for desensitization and tolerance induction in food allergies.[11] However, there are no officially approved immune modulating therapy for wheat allergy. Due to gluten's indigestible nature, ensuring a consistent and predictable gluten absorption through oral administration presents a significant challenge. These unpredictable absorption kinetics often necessitate higher dose of wheat protein for OIT,[12,13] increasing the risk of adverse effects, such as exercise-induced anaphylaxis.[14-16] Furthermore, WDEIA patients exhibit lower success rate of desensitization or sustained immune tolerance with high-dose wheat OIT compared to other forms of wheat allergy.[13,15]

Alternative immunotherapies offering predictable absorption kinetics are essential for managing patients with gluten allergies. Epicutaneous immunotherapy (EPIT) has been extensively studied for treating food allergies, involving the application of a transdermal patch loaded with allergens to induce desensitization.[17] In a phase III study, the transdermal patch (Viaskin®) containing 250 μg of peanut protein successfully desensitized children to peanuts and improved their tolerance to peanut doses.[18] However, a phase IIb study showed limited efficacy of EPIT in adult,[19] likely due to variability in the skin barrier properties among individuals and across different skin regions.[20]

To overcome the limitations of EPIT, dissolving microarray patch (dMAP) technology has recently been introduced for allergen-specific immunotherapy.[21] Composed of dissolvable polymer needles embedded with target allergens, dMAP uses hyaluronic acid, a natural component of human skin, minimizing adverse reactions and robust dosing. These minimally invasive patches are painlessly and well-tolerated by patients.[22]

This study aimed to evaluate the feasibility of incorporating gliadin extract into dMAP for the first time. Furthermore, we investigated the efficacy and underlying mechanisms of gliadin-loaded dMAP transdermal immunotherapy (TDIT) in a murine model of gliadin anaphylaxis.

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MATERIALS AND METHODS

Animals and diets

Female C3H/HeJ mice (3–4 weeks old) were obtained from Central Lab Animal Inc. (Seoul, Korea). The mice were maintained on a gluten-free formulated according to AIN-93 standard and housed in pathogen-free conditions (room temperature of 21°C–24°C, relative humidity of 45% to 70%, and a 12-hour light/dark cycle). All animal procedures were performed according to the Institutional Animal Care and Use Committee (IACUC) regulations of Yonsei University College of Medicine (Seoul, Korea), which has been fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International (IACUC approval number: 2021-0051).

Development of a gliadin sensitized mouse model

Gliadins used in this study were extracted from bread flour (*Triticum aestivum* ssp. *aestivum* L; CJ CheilJedang, Seoul, Korea) purchased from local market. The wheat flour was treated with 50% propanol at 65°C for 20 minutes, followed by centrifugation at 10,000 × g for 5 minutes to isolate gliadins. The supernatant containing gliadins was collected and subsequently freeze-dried.[7]

Mice were immunized weekly for 6 weeks by oral gavage with 5 mg gliadin and 10 μg cholera toxin (CT) (List Biologicals, Campbell, CA, USA) dissolved in 200 μL of 0.4% carboxymethyl cellulose (CMC) solution (Sigma-Aldrich, St. Louis, MO, USA) per mouse. The 0.4% CMC was utilized as a solvent to facilitate gliadin disintegration. At the sixth week, mice were challenged with gliadin extract protein (**Fig. 1A**). Before oral challenge, the mice were fasted for 4 hours and then administered 500 μL (50 mg protein) of gliadin extract or 1 mg of gliadin extract intraperitoneally.

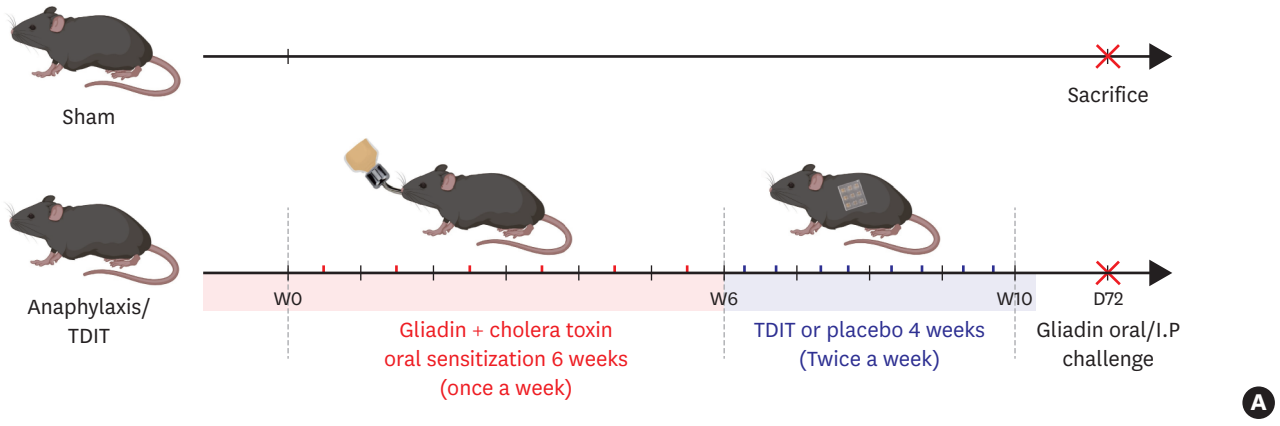

Fig. 1. TDIT suppresses allergic responses in the gliadin-anaphylaxis model. (A) Immunotherapy schedule, (B) diagram of the MNP on the skin, (C) enzyme-linked immunosorbent assay inhibition assay to evaluate the allergenicity of original gliadin and gliadin mixed with hyaluronic acid in terms of gliadin-specific IgE reactivity, (D, E) drop in body temperature after oral or IP challenge with gliadin, (F, G) clinical score after oral or IP challenge with gliadin. Results are expressed as mean ± standard error of the mean (n = 5 per group). Statistical analysis for temperature change was done with repeated ANOVA and others with one-way ANOVA with Bonferroni correction.
TDIT, transdermal immunotherapy; MNP, microneedle patch; IgE, immunoglobulin E; IP, Intraperitoneal; ANOVA, analysis of variance; dMAP, dissolving microarray patch.
$^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

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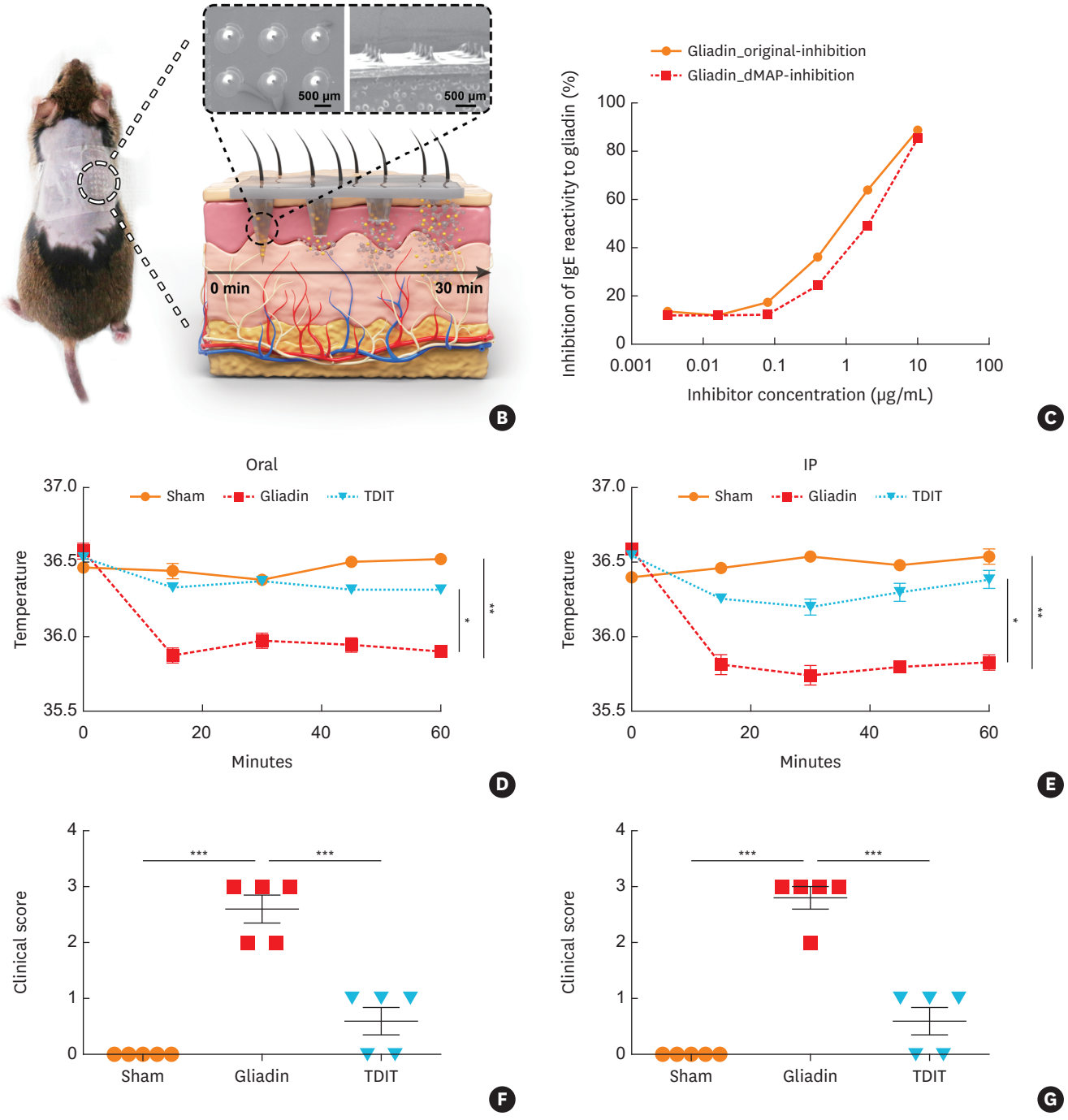

Fig. 1. (Continued) TDIT suppresses allergic responses in the gliadin-anaphylaxis model. (A) Immunotherapy schedule, (B) diagram of the MNP on the skin, (C) enzyme-linked immunosorbent assay inhibition assay to evaluate the allergenicity of original gliadin and gliadin mixed with hyaluronic acid in terms of gliadin-specific IgE reactivity, (D, E) drop in body temperature after oral or IP challenge with gliadin, (F, G) clinical score after oral or IP challenge with gliadin. Results are expressed as mean ± standard error of the mean (n = 5 per group). Statistical analysis for temperature change was done with repeated ANOVA and others with one-way ANOVA with Bonferroni correction.
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Preparation of dMAP and its' application to mice

Gliadin-loaded dMAP were prepared by mixing gliadin with sodium hyaluronate powder (intrinsic viscosity: 0.14 m^3/kg; Bloomage Freda Biopharm Co., Ltd.) in phosphate-buffered saline to achieve a loading of 25 μg/patch. Microneedles (MNs) were formed from a solution containing 18.5% (w/w) hyaluronate and the desired gliadin concentration. Controlled solution droplets were dispensed and shaped into MNs using an air blowing technique.[23] Each dMAP comprised a 76-MN array (1.4 mm needle pitch, 500 μm needle length) specifically designed for dermal penetration.

For dMAP application, mice were anesthetized with isoflurane. The hair on their back was clipped with an electric hair trimmer, and a hair removing lotion was applied to ensure complete hair removal. The gliadin-loaded dMAP was placed on the prepared skin and secured with thumb pressure for 3 minutes to facilitate gliadin delivery into dermis. The dMAP was applied twice a week for a duration of 4 weeks, and it was covered with self-adherent bandages for 2 hours before removal (**Fig. 1B**).

Challenge and monitoring of anaphylaxis

Mice were challenged by administering 1 mg of gliadin extract intraperitoneally or 50 mg of gliadin orally. Anaphylactic reactions were assessed based on a decrease in body temperature appearance of clinical symptoms.[24] The body temperature of the mice was measured prior to the challenge and at 15-minute intervals over 60-minute period. Clinical severity scores were recorded for each mouse. The score system consist of 0 - no symptoms, 1 - repetitive nose scratching and ruffled fur, 2 - mouth and eyes swelling, pilar erectus and reduced activity, 3 - difficulty breathing, mouth cyanosis and diarrhea, 4 - no reaction following stimulation of whiskers, tremor and/or seizure, 5 - death.[25] After the challenge, blood samples were collected from rom inferior vena cava.

Enzyme-linked immunosorbent assay (ELISA) inhibition assays in term of gliadin-specific IgE (sIgE)

For the inhibition analysis, 96 well ELISA plates were coated with gliadin at a concentration of 10 μg/mL and incubated overnight at 4°C. Simultaneously, patients' sera diluted 1:4 were pre-incubated with various concentrations of samples overnight at 4°C. Bound IgE was detected using biotinylated goat anti-human IgE (diluted 1:1,000; Vector, Burlingame, CA, USA) and streptavidin-peroxidase conjugated antibody (diluted 1:1,000; Sigma-Aldrich). Color development was carried out using 3,3′,5,5′-tetramethylbenzidine (TMB) substrate (Kirkegaard & Perry Laboratories, Gaithersburg, MD, USA). The reaction was stopped by adding 0.5 M H_2SO_4, and absorbance was measured at 450 nm. The percentage of inhibition was calculated using the formula:

$$1 - (\text{Absorbance With Inhibitor}/\text{Absorbance Without Inhibitor}) \times 100.$$

Gliadin-sIgE and MCPT-1 measurements

To quantify gliadin sIgE, $sIgG_1$, $sIgG_{2a}$, and $sIgG_{2b}$ levels in the serum, ELISA plates were coated overnight with gliadin at a concentration of 20 μg/mL. After washing with phosphate-buffered saline with Tween 20 (PBST), the microplates were blocked in 2% bovine serum albumin/PBST for 1 hour at room temperature. Diluted serum samples were added to the wells and incubated for 2 hours at room temperature. Subsequently, 100 μL of anti-mouse Ig antibodies (BioLegend, San Diego, CA, USA) diluted 1:1,000 were added to the wells and incubated for 2 hours. Following this, 100 μL of horseradish peroxidase-conjugated streptavidin (BioLegend) were added and incubated for 1 hour. After washing

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with PBST, 100 μL TMB (BD Biosciences, Franklin Lakes, NJ, USA) were added and allowed to react for 20 minutes at room temperature. The reaction was terminated by adding 50 μL of 1 M H_2SO_4 and the optical density was measured at 450 nm.

To analyze mast cell protease-1(MCPT-1), serum samples were analyzed using mouse MCPT-1 ELISA kit (Thermo Fisher Scientific Inc., Rockford, IL, USA) was used.

Flow cytometry analysis of splenocyte

Mice spleens were aseptically homogenized using a mesh in RPMI 1640 medium to prepare single-cell suspension for further analysis. Splenocytes were washed and stained for 30 minutes at 4°C with the following antibodies in FACS buffer. The antibodies used were anti-CD3-FITC (a marker for T-cells), anti-CD4-APC-R700 (a marker for T helper (Th)-cells), anti-CXCR3-Cyanine7 (a marker for Th_1-cells), anti-CCR4-APC (a marker for Th_2-cells), and anti-CCR6-PE (a marker for Th_{17}-cells) from BioLegend.

For intracellular staining of interferon (IFN)-γ, interleukin (IL)-4, and IL-13, single-cell suspensions were permeabilized using Perm/Wash buffer (BD Bioscience) according to the manufacturer's protocol. Cells were then stained with anti-CD4-APC, anti-IL-4-PE, anti-IL-13-PE-Cyanine7, and anti-IL-17-APC. To evaluate regulatory T (Treg) cells, staining was performed with anti-CD25-PerCP and anti-Foxp3-V450 (BioLegend). Fluorescence intensity was measured using a BD FACSVerse flow cytometer, and the data were analyzed using BD FACSuite software, version 1.0.6 (BD Biosciences).

Splenocyte stimulation with gliadin extract and cytokine production analysis

Mice spleens were aseptically ground in RPMI 1640 medium. Red blood cells were lysed using RBC lysis buffer (BioLegend), and the supernatant was removed by centrifugation at 300 × g for 5 minutes. The remaining cells were resuspended and seeded at a density of 5×10^6 cells/mL in 24-well culture plates. Splenocytes were stimulated with gliadin at a concentration of 50 μg/mL in RPMI medium supplemented with fetal bovine serum and penicillin-streptomycin. After 72 hours of incubation at 37°C with 5% CO_2, the cell culture supernatants were collected and stored at −80°C. Cytokine levels, including INF-γ, IL-4, IL-5, IL-10 and IL-17a, were quantified using ELISA kits (R&D Systems Inc., Minneapolis, MN, USA).

Histopathology of jejunum

Jejunum tissues were fixed in a 4% paraformaldehyde solution, embedded in paraffin, and stained with hematoxylin and eosin or toluidine blue to evaluate eosinophil and mast cell infiltration. Stained section was imaged using an upright microscope (BX53F; Olympus, Tokyo, Japan) equipped with a digital camera (U-TV0.63XC; Olympus). Mast cells and eosinophils were counted in 5 high-power fields per mouse.

Statistical analysis

Statistical analysis was performed using Prism software (GraphPad Inc., San Diego, CA, USA). Changes in temperature were evaluated by repeated analysis of variance (ANOVA), whereas differences between the other variables were compared by one-way ANOVA and Bonferroni *post hoc* test. $P < 0.05$ was considered statistically significant.

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RESULTS

TDIT mitigates drop in body temperature and reduces anaphylaxis clinical score in the gliadin-induced anaphylaxis model

We established a mouse model for gliadin-induced anaphylaxis to evaluate the efficacy of gliadin-loaded dMAP in desensitizing previously sensitized mice (**Fig. 1A and B**). Body temperature was monitored rectally at 15 minutes intervals over a 60-minute period.

We conducted an ELISA inhibition test to evaluate whether the manufacturing process of dMAP affects the allergenicity of gliadin. Our results showed no difference in maximal inhibition potency or the 50% inhibition concentration of gliadin extract and gliadin mixed with hyaluronic acid in terms of gliadin sIgE (**Fig. 1C**).

In mice with gliadin-induced anaphylaxis, body temperature decreased significantly within 20 minutes after the gliadin challenge compared to the sham group. However, in the TDIT group, body temperature remained stable without significant changes throughout the 60-minute observation following oral and intraperitoneal (IP) gliadin challenges (**Fig. 1D and E**).

TDIT-treated mice showed a substantial reduction in the severity of clinical symptoms upon oral or IP challenge, with a mean ± standard error (SE) clinical score of 0.6 ± 0.55 and a maximum clinical score of 1 (repetitive nose scratching and ruffled fur). In contrast, mock-treated gliadin anaphylaxis mice displayed more severe symptoms, with mean ± SE clinical scores of 2.6 ± 0.55 for oral challenge and 2.80 ± 0.45 for IP challenge, reaching a maximum score of 3 (marked by difficulty in breathing, lying flat, swelling around the mouth and eyes) (**Fig. 1F and G**).

TDIT protects against sIgE expression, IgE-mediated mast cell activation, and allergic inflammation in the jejunum

The gliadin anaphylaxis model exhibited significant elevation of gliadin sIgE ($P = 0.017$), $sIgG_1$ ($P = 0.010$), and tendency of increase in $sIgG_{2a}$ ($P = 0.490$) and $sIgG_{2b}$ ($P = 0.919$). Mice treated with TDIT showed a noteworthy reduction in sIgE ($P = 0.029$) (**Fig. 2A**). Additionally, TDIT treatment led to a marked increase in serum levels of $sIgG_1$ ($P < 0.001$), $sIgG_{2a}$ ($P < 0.001$), and $sIgG_{2b}$ ($P = 0.011$) (**Fig. 2B-D**). The gliadin challenge also induced elevated mucosal mast cell protease-1 (mMCP-1) levels in serum, indicating mast cell activation. TDIT effectively suppressed this increase ($P < 0.001$) (**Fig. 2E**).

Histopathological analysis revealed structural degradation of the jejunum in the gliadin anaphylaxis model, while TDIT treated mice, similar to the sham group, exhibited preserved jejunal structure. Furthermore, TDIT significantly reduced the infiltration of mast cells and eosinophils within the jejunum (**Fig. 3**).

TDIT suppresses Th_2 and Th_{17} while enhancing Treg cell responses in the gliadin anaphylaxis model

To explore the impact of dMAP TDIT on Th_1 and Th_2 cell responses in the gliadin-anaphylaxis model, we assessed the expression of Th_1 and Th_2 cells in the spleen post-sacrifice. The model, demonstrated elevated level of $CCR4^+$ Th_2 cell ($P = 0.013$) and a disrupted Th_1/Th_2 cell ratio ($CXCR3^+/CCR4^+$) ($P = 0.027$). TDIT significantly reduced $CCR4^+$ Th_2 cell expression and restored the Th_1/Th_2 balance (**Fig. 4**). Intracellular staining further revealed that IFN-γ expression in Th cells, which was reduced in the anaphylaxis model, was restored by TDIT

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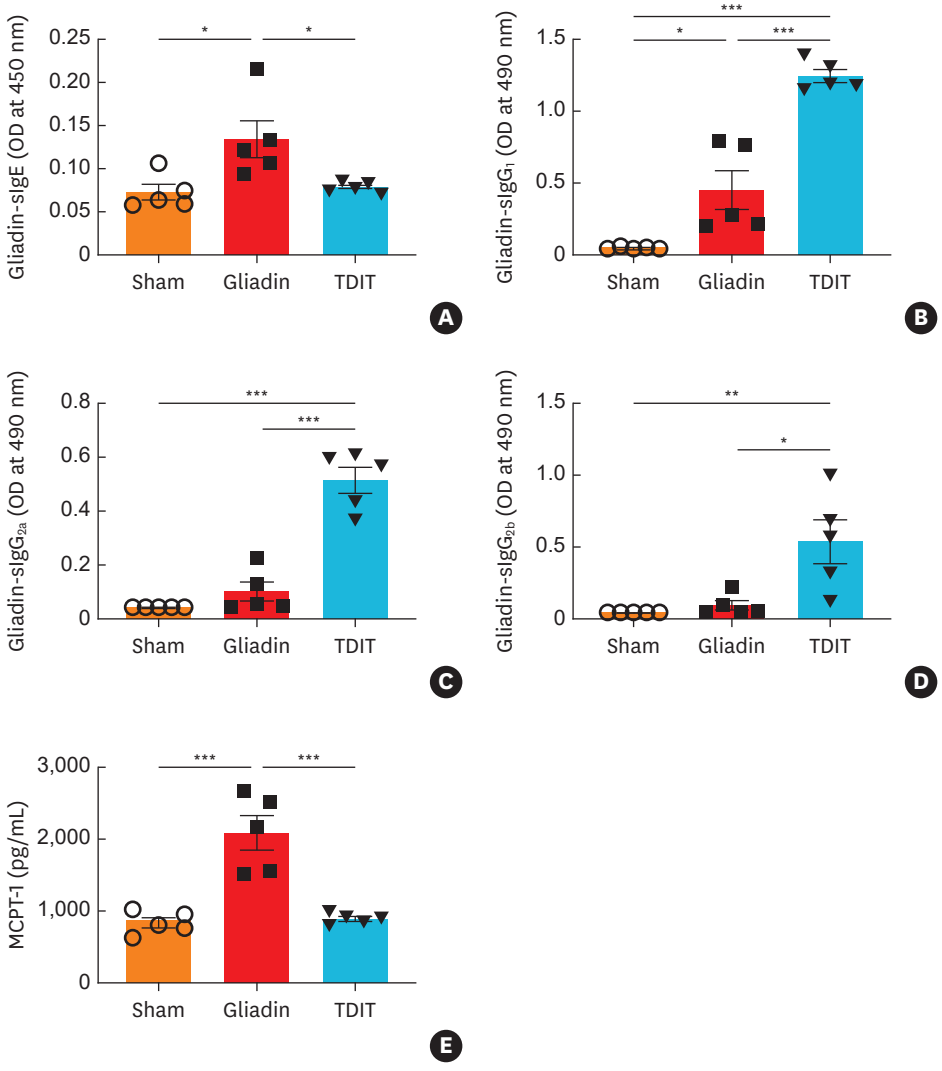

Fig. 2. TDIT protects gliadin sensitized mice against IgE-mediated anaphylaxis. Levels of gliadin sIgE (A), IgG_1 (B), IgG_{2a} (C), and IgG_{2b} (D). (E) MCPT-1 was assessed after gliadin challenge. Results are expressed as mean ± standard error of the mean (n = 5 per group). Statistical analysis was done with one-way analysis of variance with Bonferroni correction.
TDIT, transdermal immunotherapy; Ig, immunoglobulin; sIg, specific immunoglobulin; OD, optical density.
$^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

(**Fig. 5A, D, and G**). Conversely, the elevated expression of intracellular IL-4 ($P = 0.008$) and IL-13 ($P = 0.048$) in Th cells was significantly attenuated by TDIT (**Fig. 5B, C, E, and F**). Consistent with these findings, ELISA analysis demonstrated the increased production of IL-4 ($P = 0.008$), IL-5 ($P = 0.048$), and IL-13 ($P = 0.017$) from gliadin stimulated splenocytes was mitigated by dMAP TDIT, while decreased IFN-γ production was restored (**Fig. 5H-J**).

The gliadin anaphylaxis model also showed a reduced proportion of Treg cells in the spleen ($P = 0.030$), while was significantly increased by dMAP TDIT ($P < 0.001$) (**Fig. 6A and D**). This was accompanied by an upregulation in IL-10 expression from gliadin stimulated splenocytes ($P = 0.005$) (**Fig. 6H**). Furthermore, the model exhibited an elevated Th_{17} cell population ($P = 0.013$) and intracellular IL-17 expression in Th cells ($P = 0.007$). TDIT effectively suppressed these Th_{17}-associated responses (**Fig. 6B, C, and E-G**).

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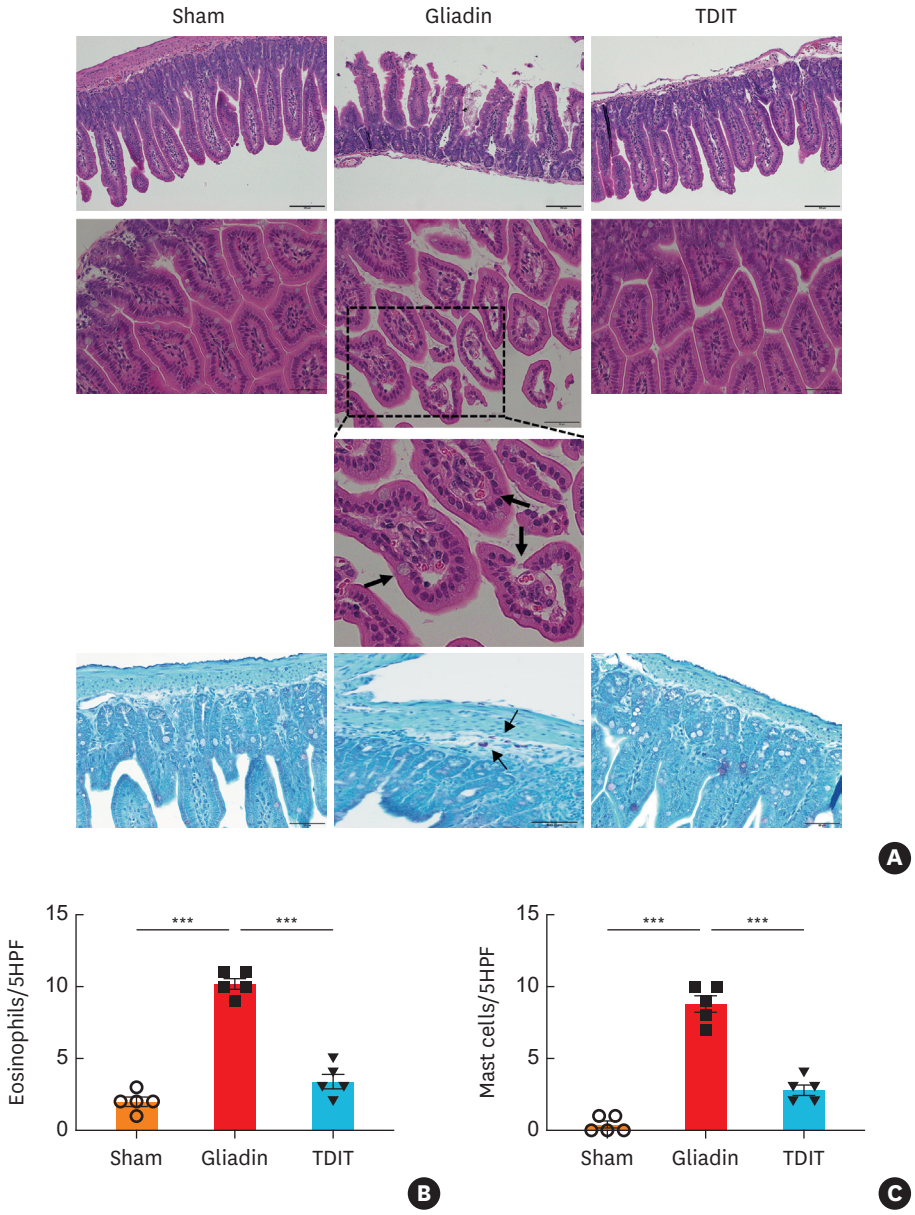

Fig. 3. Histopathology of jejunum tissues. (A) Histological analysis of small intestine (original magnification: 100×), and the arrows point to the eosinophils (original magnification: 50×) and mast cells in tissue (original magnification: 50×). (B) Counts of eosinophils in tissue. (C) Counts of mast cells in tissue. The data in (B) and (C) were counted from 5 HPF. Results are expressed as mean ± standard error of the mean (n = 5 per group). Statistical analysis was done with one-way analysis of variance with Bonferroni correction.
HPF, high-power fields; TDIT, transdermal immunotherapy.
***$P < 0.001$.

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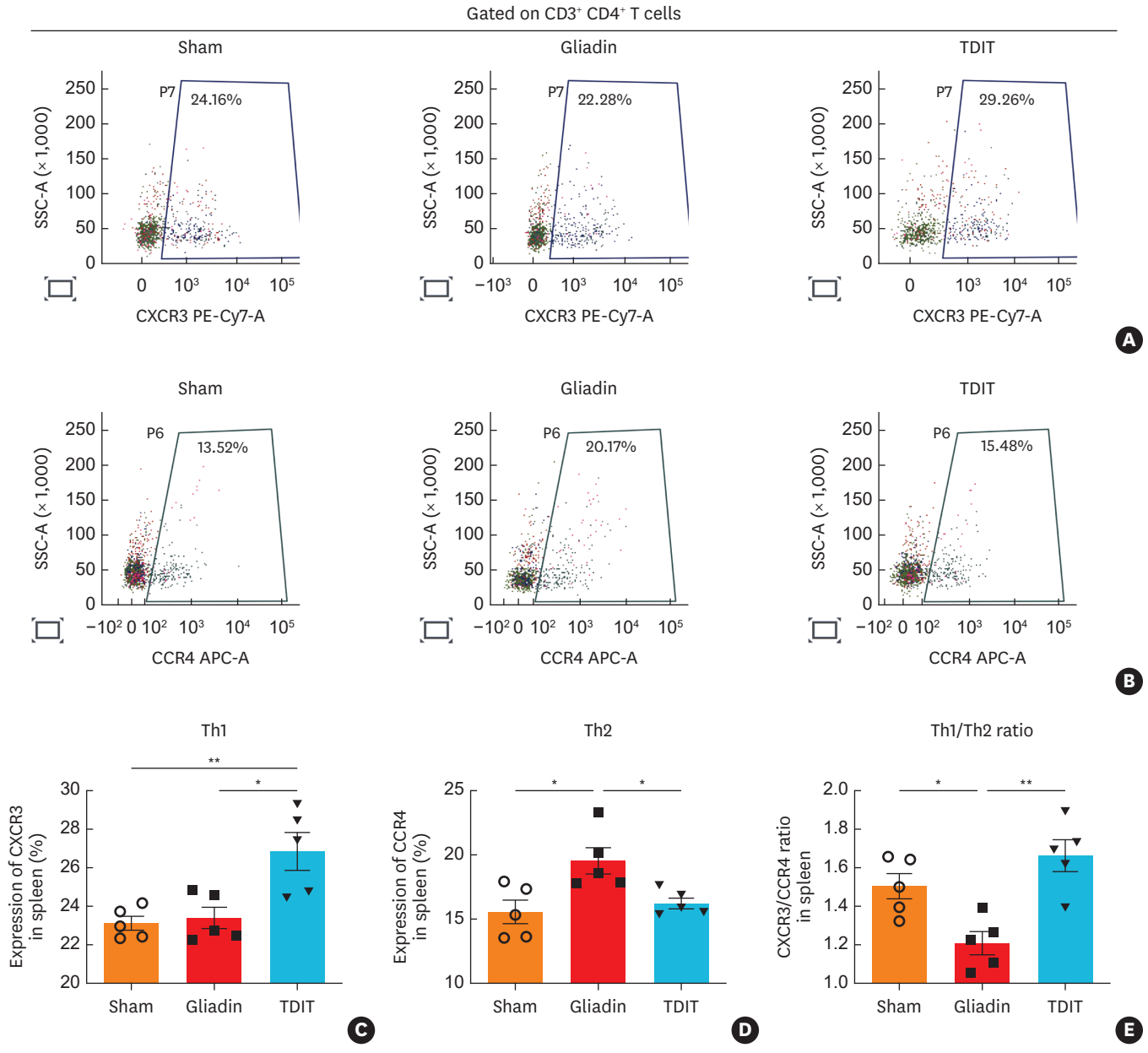

Fig. 4. Effects of TDIT on the Th_1 and Th_2 cells responses in gliadin anaphylaxis model. Proportion of Th_1 (A, C), Th_2 cells (B, D) and the ratios of Th_1/Th_2 cells (E). Results are expressed as mean ± standard error of the mean (n = 5 per group). Statistical analysis was done with one-way analysis of variance with Bonferroni correction.
TDIT, transdermal immunotherapy; Th, T helper.
*P < 0.05, **P < 0.01.

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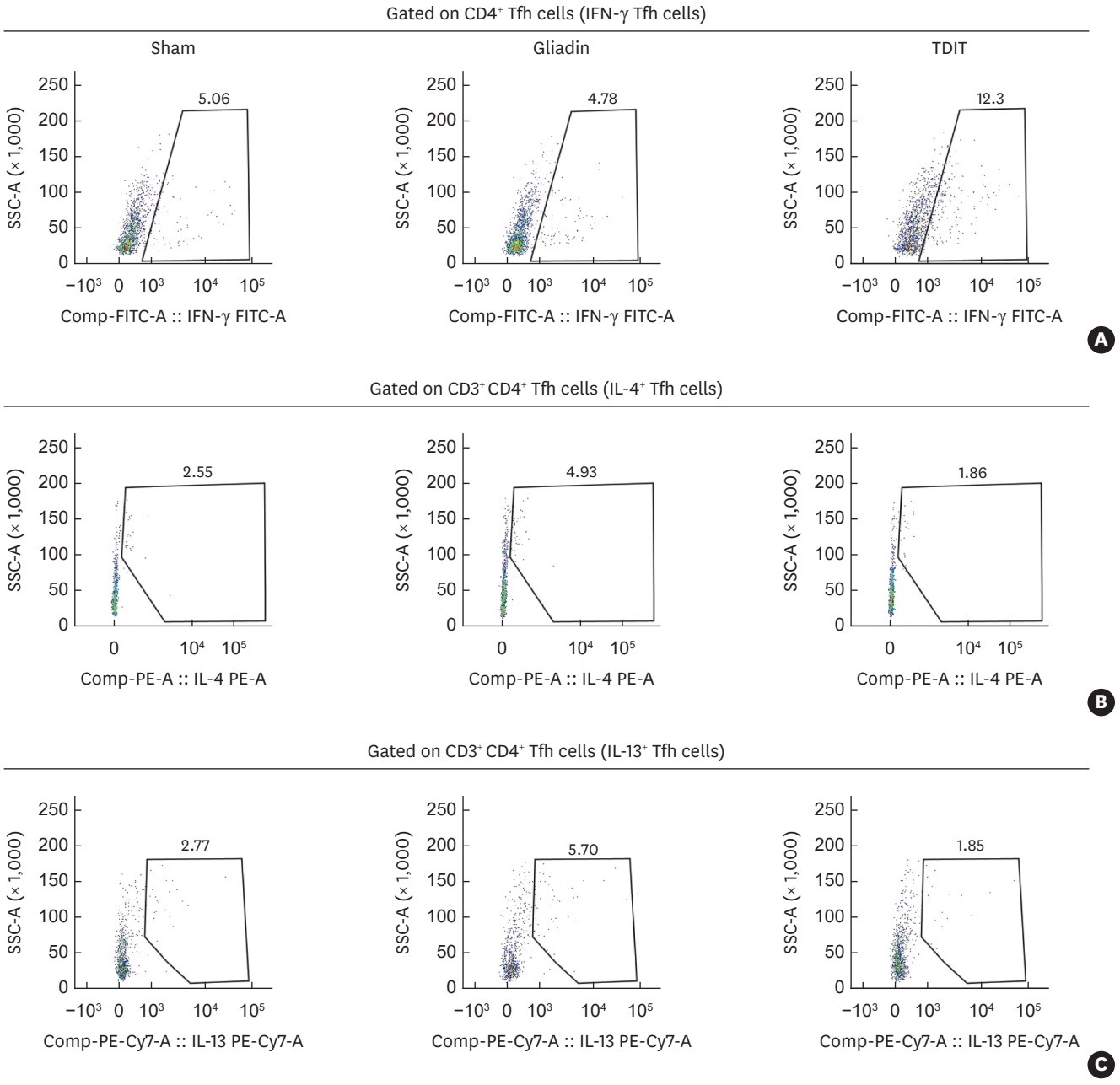

Fig. 5. Effects of TDIT on the production of Th_1 and Th_2 cytokines in the spleen cell cultures. Flow cytometry analysis of IFN-γ Tfh cells (A), IL-4+ Tfh cells (B), and the expression of IL-13+ Tfh cells among these groups (C). The expression percentage of IFN-γ Tfh (D), IL-4+ Tfh (E), and IL-13+ Tfh cells subpopulations (F). Cytokine levels of IFN-γ (G), IL-4 (H), IL-13 (I), and IL-5 (J) in supernatants after stimulation with 50 μg/mL gliadin for 72 hours to spleen cells were evaluated using enzyme-linked immunosorbent assay. Results are expressed as mean ± standard error of the mean (n = 5 per group). Statistical analysis was done with one-way analysis of variance with Bonferroni correction.
TDIT, transdermal immunotherapy; Th, T helper; IFN, interferon; Tfh, follicular helper T; IL, interleukin.
*P < 0.05, **P < 0.01, ***P < 0.001.

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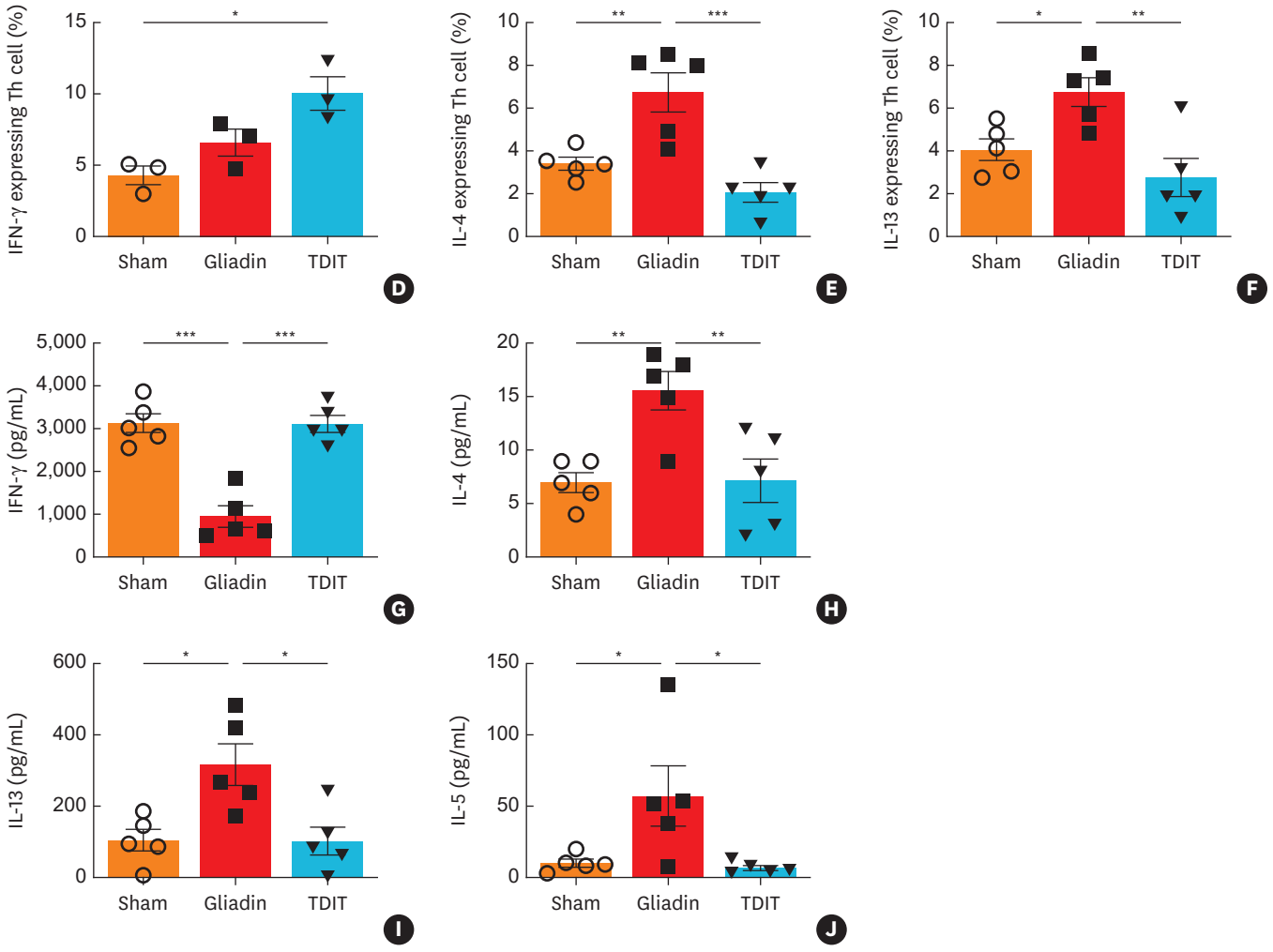

Fig. 5. (Continued) Effects of TDIT on the production of Th_1 and Th_2 cytokines in the spleen cell cultures. Flow cytometry analysis of IFN-γ Tfh cells (A), IL-4$^+$ Tfh cells (B), and the expression of IL-13$^+$ Tfh cells among these groups (C). The expression percentage of IFN-γ Tfh (D), IL-4$^+$ Tfh (E), and IL-13$^+$ Tfh cells subpopulations (F). Cytokine levels of IFN-γ (G), IL-4 (H), IL-13 (I), and IL-5 (J) in supernatants after stimulation with 50 μg/mL gliadin for 72 hours to spleen cells were evaluated using enzyme-linked immunosorbent assay. Results are expressed as mean ± standard error of the mean (n = 5 per group). Statistical analysis was done with one-way analysis of variance with Bonferroni correction.
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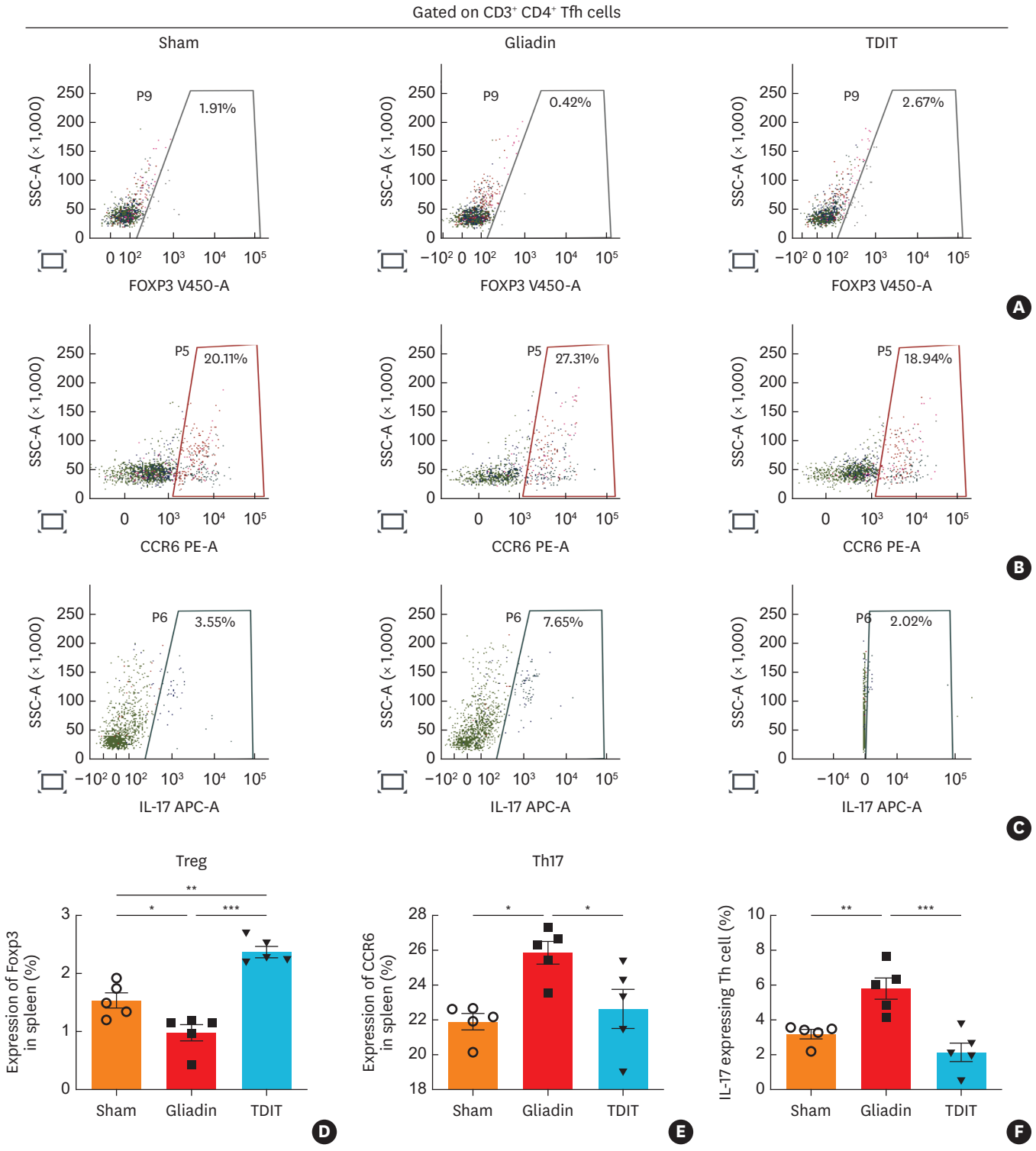

Fig. 6. Effects of TDIT on the production of Th_{17} and Treg cells in the spleen cell culture. Proportion of Treg (A), Th_{17} cells (B), and IL-17A$^+$ Tfh cells (C). The expression percentage of Treg (D), Th_{17} (E), and IL-17A$^+$ Tfh cells subpopulations (F). Cytokine levels of IL-17 (G) and IL-10 (H) in the supernatant of spleen cell culture measured by enzyme-linked immunosorbent assay. Results are expressed as mean ± standard error of the mean (n = 5 per group). Statistical analysis was done with one-way analysis of variance with Bonferroni correction.
TDIT, transdermal immunotherapy; Th, T helper; Treg, regulatory T; Tfh, follicular helper T; IL, interleukin.
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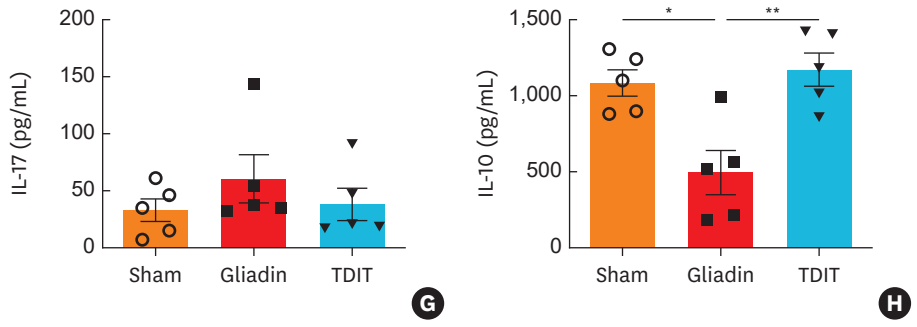

Fig. 6. (Continued) Effects of TDIT on the production of Th_{17} and Treg cells in the spleen cell culture. Proportion of Treg (A), Th_{17} cells (B), and IL-17A$^+$ Tfh cells (C). The expression percentage of Treg (D), Th_{17} (E), and IL-17A$^+$ Tfh cells subpopulations (F). Cytokine levels of IL-17 (G) and IL-10 (H) in the supernatant of spleen cell culture measured by enzyme-linked immunosorbent assay. Results are expressed as mean ± standard error of the mean (n = 5 per group). Statistical analysis was done with one-way analysis of variance with Bonferroni correction.
TDIT, transdermal immunotherapy; Th, T helper; Treg, regulatory T; Tfh, follicular helper T; IL, interleukin.
*P < 0.05, **P < 0.01, ***P < 0.001.

DISCUSSION

Wheat exhibited unique characteristics compared to other common food allergens. Its gluten component can trigger 2 distinct immune-mediated adverse reactions: IgE-mediated allergic or anaphylactic responses and non-IgE-mediated celiac disease. In contrast to IgE-mediated allergic diseases, celiac disease is strongly linked to the HLA DQ2 or DQ8 genes and involves the activity of intestinal tissue transglutaminase.[3,26] Investigations into celiac disease provide clinically significant insights into comprehending the distinctive aspects of IgE-mediated gluten allergy, particularly in relation to the challenges associated with poor digestibility and subsequent limited intestinal absorption of gluten peptides.[8,9] Gluten-induced anaphylaxis often necessitates the presence of cofactors such as aspirin or exercise for clinical manifestation. These cofactors are believed to enhance gluten absorption in the intestine. However, exact mechanism by which exercise enhances absorption remains unclear.[10] Aspirin or nonsteroidal anti-inflammatory drugs are known to disrupt the phospholipid monolayer of mucus and the phospholipid bilayer of the intestinal epithelium. Additionally, they compromise the tight junctions and interfere with oxidative phosphorylation in mitochondria. These mechanisms work together to increase intestinal permeability.[27] Finally, the characteristic poor intestinal absorption of gluten highlights the challenge of reliably delivering a consistent and predetermined dosage of gluten during OIT.

The efficacy of wheat OIT largely depends on achieving and maintaining the appropriate maintenance dosage.[28] For optimal results, OIT often necessitates the administration of high dosages of wheat products.[12,13] This requirement is influenced not only by the poor intestinal absorption of gluten but also by low protein content of common wheat-based foods such as noodle and bread. These factors can make it challenging for children, to consistently adhere to the maintenance dosage. To address this issue, some researchers have employed a 70% purified wheat protein extract, containing gluten, to reduce the volume of wheat products required for maintenance.[28]

In contrast to the other gluten anaphylaxis models,[25,29] we utilized C3H/HeJ mice, a strain known for its increased intestinal permeability compared to other mouse strains.[30] Consequently, this strain is widely utilized as a model for studying oral food allergies and anaphylaxis.[31] Additionally, CT was used as an adjuvant for gliadin sensitization. CT enhances

antigen presentation by various antigen-presenting cells, promotes the production of IL-1β, and increases intestinal permeability.[32] With these modifications, we successfully induced IgE-mediated anaphylaxis through either oral or IP gliadin challenges.

Using this model, we demonstrated the significant protective effects of gliadin-loaded dMAP TDIT on body temperature and anaphylaxis clinical scores. Importantly, no adverse reactions like erythema, inflammation, or reactivity, were observed following dMAP TDIT. In this anaphylaxis model, elevated levels of gliadin sIgE and increased mMCP-1 were observed during the anaphylaxis phase. Treatment with dMAP TDIT led to enhanced production of gliadin sIgG subclasses, particularly IgG_1 (analogous to human IgG_4) and IgG_{2a} (analogous to human IgG_1). An increase in $sIgG_4$ response is generally considered a positive marker of successful allergen immunotherapy, as it competes with sIgE for allergen binding on mast cells and basophils.[33]

Gliadin-loaded dMAP TDIT also modulated cytokine expression in splenocytes. Shifting the Th_1/Th_2 cytokine balance. Specifically, dMAP TDIT increased production of IFN-γ while reducing IL-4, IL-5, and IL-13 in gliadin-stimulated splenocytes. Similar outcomes have been observed in EPIT studies. For example, a murine EPIT study using cashew allergens showed elevated cashew $sIgG_1$ and $sIgG_{2a}$ peaking at week 10, followed by a significant decline.[24] This EPIT approach also reduced Th_2 cytokines production (IL-4, IL-5, IL-13) in cashew-reactivated splenocytes. Our findings are consistent with these EPIT results, supporting the conclusion that innovative approaches like dMAP TDIT effectively mitigate allergic reactions by restoring the Th_1/Th_2 balance.

Treg cells are pivotal in maintaining immune tolerance during allergen immunotherapy and fostering intestinal immune tolerance.[34,35] Our findings revealed that dMAP TDIT increased the proportion of Fox $p3^+/CD25^+$ Treg cell in spleen, while concurrently reducing Th_{17} cell levels. Enhanced gliadin specific Treg activity may play a critical role in suppressing the development of gliadin specific Th_2 immune responses, thereby modulating the Th_1/Th_2 immune response and reducing allergic reactions.

After repeated administration of gliadin and CT via oral gavage in this model, eosinophilic allergic inflammation was observed in the jejunum, accompanied by microvilli distortion. Treatment with dMAP TDIT effectively mitigated this eosinophilic inflammation, suggesting that dMAP TDIT has the potential to restore the intestinal barrier function compromised by prolonged gliadin exposure.

Within the realm of TDIT, the Epicutaneous Viaskin Patch (EVP) represents the forefront of clinical advancement. In a phase III clinical trial for pediatric peanut allergy patients, the EVP successfully met its primary endpoints, including the responder endpoint after 12 months of treatment.[36] However, the efficacy of EPIT in treating peanut allergies has been modest,[18,36] necessitating further innovation in TDIT approach. The dMAP TDIT offers several advantages over EPIT with the EVP. Dissolving MN provides enhanced control over allergen delivery, introducing allergens directly into the epicutaneous layer and upper dermis, effectively bypassing the stratum corneum.[23,37,38] Stratum corneum limits penetration of macromolecular allergens, requiring prolonged application of EVP to the skin. In contrast, dMAP TDIT approach may require lower allergen doses to elicit a comparable immune response and reduce exposure time, potentially improving patient adherence. In this study, we utilized dMAP loaded with only 25 μg of gliadin, applying at twice a week interval.

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Direct delivery of allergens to the immune cells of skin through dMAP can minimize individual differences in skin barrier strength and passive diffusion.[39,40] This dMAP is made from hyaluronic acid, a natural constituent of human skin widely used in anti-aging and wrinkle reduction cosmetics. It has been applied for extended periods to targeted skin areas without notable adverse reactions.[41,42] However, several studies have reported that hyaluronic acid can induce goblet cell hyperplasia in the nasal epithelium through receptor-mediated signaling pathway.[43] This underscores the need for long-term clinical trials to thoroughly evaluate the safety and efficacy of dMAP TDIT.

However, the absence of substantial evidence demonstrating a definitive advantage of dMAP TDIT over EPIT in both animal models and adult human subjects underscores the need for further research. Future studies should prioritize comprehensive clinical trials to assess the efficacy of dMAP TDIT across various age groups and determine its potential to address the limitations associated with EPIT.

In conclusion, the current investigation demonstrated that MN TDIT effectively mitigated allergic reaction induced by gliadin. This was achieved by inhibiting gliadin sIgE mediated immune responses while restoring intestinal inflammation, and thereby barrier function. Furthermore, dMAP TDIT successfully rebalanced the Th_1/Th_2 immune responses and increased FOX $p3^+/CD25^+$ Treg cell population in the sensitized mice, contributing to improvement in gliadin allergy symptoms (**Fig. 7**). Based on these promising findings, further clinical trials are warranted to affirm the safety and efficacy of dMAP TDIT as a potential treatment for gliadin allergy.

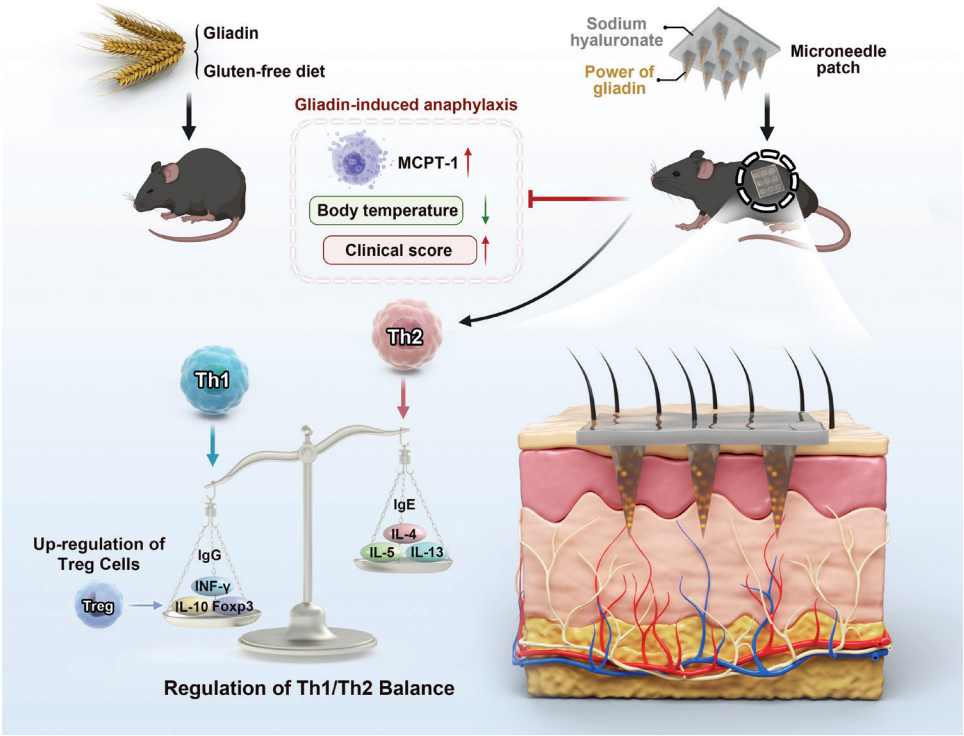

Fig. 7. Mechanisms of transdermal immunotherapy in a mouse model of gliadin-induced anaphylaxis.
Th, T helper; Treg, regulatory T; Ig, immunoglobulin; INF, interferon; IL, interleukin.

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