



Intranasal administration of nucleus-deliverable GATA3-TMD alleviates the symptoms of allergic asthma

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ABSTRACT

Although the T helper 2 (Th2) subset is a critical player in the humoral immune response to extracellular parasites and suppression of Th1-mediated inflammation, Th2 cells have been implicated in allergic inflammatory diseases such as asthma, allergic rhinitis, and atopic dermatitis. GATA binding protein 3 (GATA3) is a primary transcription factor that mediates Th2 differentiation and secretion of Th2 cytokines, including IL-4, IL-5, and IL-13. Here, a nucleus-deliverable form of GATA3-transcription modulation domain (TMD) (ndG3-TMD) was generated using Hph-1 human protein transduction domain (PTD) to modulate the transcriptional function of endogenous GATA3 without genetic manipulation. ndG3-TMD was shown to be efficiently delivered into the cell nucleus quickly without affecting cell viability or intracellular signaling events for T cell activation. ndG3-TMD exhibited a specific inhibitory function for the endogenous GATA3-mediated transcription, such as Th2 cell differentiation and Th2-type cytokine production. Intranasal administration of ndG3-TMD significantly alleviated airway hyperresponsiveness, infiltration of immune cells, and serum IgE level in an OVA-induced mouse model of asthma. Also, Th2 cytokine secretion by the splenocytes isolated from the ndG3-TMD-treated mice substantially decreased. Our results suggest that ndG3-TMD can be a new therapeutic reagent to suppress Th2-mediated allergic diseases through intranasal delivery.

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1. Introduction

Allergic asthma is a chronic bronchial inflammatory disease accompanied by airway hyperresponsiveness and obstruction [1]. Although there are existing asthma treatments, such as bronchodilators, antihistamines, and corticosteroids, their temporary effects or overall immunosuppression cannot resolve the underlying cause of asthma [2]. In addition, there are clinical limitations in their frequent use due to side effects such as oral infection, headaches, cataracts, and growth retardation in children [2–4]. Previous studies have revealed that T helper 2 (Th2) cell function is closely related to asthma progression, and most asthma patients have

abnormally enhanced Th2 function [5–7]. Excessive activation of Th2 cells triggers an intense inflammatory response in the respiratory system via their cytokines, such as IL-4, IL-5, and IL-13. IL-4 induces plasma cells to produce immunoglobulin E (IgE) that activates granulocytes, including mast cells and basophils, along with eosinophils recruited by IL-5, leading to airway hyperresponsiveness (AHR) [6,8,9]. In addition, bronchial stenosis caused by IL-13-mediated overproduction of mucus and persistent inflammation causes shortness of breath due to muscle spasms, called asthma attacks. Monoclonal antibodies (mAbs) such as mepolizumab (anti-IL-5) or dupilumab (anti-IL-4/IL-13R) are often used to block the action of these cytokines. But their therapeutic efficacy is limited due to the functional redundancy and pleiotropism of these inflammatory cytokines [2,10,11]. For this reason, the therapeutic strategy targeting GATA3, a transcription factor responsible for the Th2-mediated inflammatory microenvironment, is essential.

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GATA3 is an indispensable transcriptional factor for Th2 polarization and function [12]. In the nucleus, it induces chromatin remodeling of the Th2 cytokine promoters and activates transcription by binding to various regulatory regions to induce Th2 cell-related gene expression [13,14]. It has been shown that GATA3-deficient mice do not adequately produce Th2 cytokines both *in vitro* and *in vivo* and that GATA3 knockdown using siRNA reduces IL-13 production by preventing NFAT from binding to the IL-13 promoter upon T cell activation [15,16]. Furthermore, indirect inhibition of GATA3 expression with small molecules decreased the secretion of Th2 cytokines in patients with type 2 high asthma [17]. Although these studies have demonstrated the importance of GATA3 expression in Th2-mediated inflammation, the therapeutic strategy to modulate the functions of GATA3 involved in the transcriptional activity or protein-protein interaction has yet to be attempted. Therefore, we hypothesized that intranuclear delivery of GATA3-TMD would alter endogenous GATA3-mediated immunological processes. To address this possibility, ndG3-TMD, an intranuclear transducible form of GATA3-TMD that functions as a competitive protein inhibitor for endogenous GATA3, was generated. Subsequently, its transduction efficiency, dynamics, and ability to regulate the differentiation of naïve CD4⁺ T cells into various helper subsets were examined *in vitro*. The inhibitory effect of ndG3-TMD on the immunopathological function of Th2 *in vivo* was also assessed in the OVA-induced mouse model of asthma.

2. Materials and methods

2.1. Animals

7- to 8-week-old female BALB/c and C57BL/6 supplied from Orient Bio Inc. were used for *in vitro* Th2 differentiation or protein transduction analysis, and *in vitro* Th1, Th17 or iTreg differentiation, respectively. For *in vivo* experiment, 6-week-old male BALB/c mice purchased from Charles River Technology were maintained under pathogen-free conditions. Mouse care and experimental animal procedures were performed under approval from Yonsei University Animal Care Committee (IACUC-202204-1443-01).

2.2. Generation and purification of ndG3-TMD

GATA3-TMD (264–371 amino acid) combined with Hph-1 were cloned into pRSET(B) plasmid and transformed into *Escherichia coli* BL21 star (DE3)pLysS strain (Invitrogen, Carlsbad, CA, USA). The protein expression was induced by 1 mM isopropyl- β -D-thiogalactopyranoside (IPTG; Duchefa Biochemie, Haarlem, Netherlands) for 4 h at 37 °C. The cells were then harvested and resuspended in SP binding buffer (50 mM NaH₂PO₄, 300 mM NaCl, pH 7.0). Next, a soluble fraction of cell lysate obtained by sonication and centrifugation (13,000 rpm for 10 min at 4 °C) was bound by 400 μ l of SP sepharose fast flow (GE Healthcare, Pittsburgh, PA, USA) for 1 h at 4 °C. The mixture was loaded to chromatography columns (Bio-Rad, Hercules, CA, USA) and washed with SP wash buffer (50 mM NaH₂PO₄, 200 mM NaCl, pH 7.0) to remove the endotoxins. The target protein was then eluted with SP elution buffer (50 mM NaH₂PO₄, 2 M NaCl, pH 8.0). To remove the salt in the buffer, the protein solution was desalted with 10% glycerol PBS (phosphate-buffered saline) loaded PD-10 Sephadex G-25 column (GE Healthcare). Subsequently, Ni-NTA Agarose (Qiagen, Venlo, Netherlands) was added to the protein in Ni-NTA binding buffer (10 mM imidazole, 50 mM NaH₂PO₄, 200 mM NaCl, pH 8.0) and bound for 2 h at 4 °C. Then the protein solution was loaded on HisTrap chromatography columns (Bio-Rad). Non-specifically bound proteins were washed out with wash buffer (30 mM imidazole, 50 mM NaH₂PO₄, 200 mM NaCl, pH 8.0), and the protein of

interest was eluted with elution buffer (1 M imidazole, 50 mM NaH₂PO₄, 200 mM NaCl, pH 8.0). The protein was desalted again with a PD-10 column and stored at –80 °C.

2.3. Cell culture and protein transduction

All the cell cultures at 37 °C were conducted in a 5% CO₂ incubator. T cells or splenocytes were maintained in RPMI-1640 (Lonza, Basel, Switzerland) with 7.5% Fetal Bovine Serum (FBS; Lonza), 100 U/ml penicillin/streptomycin, 2 mM L-glutamine, 50 μ M β -mercaptoethanol. For HeLa and 293T cells, DMEM (Lonza) supplemented with 10% FBS, 100 U/ml penicillin/streptomycin, 2 mM L-glutamine, 1 mM sodium pyruvate and 100 μ M nonessential amino acid (NEAA) was used. Jurkat cells were cultured in RPMI-1640 with 10% FBS, 100 U/ml penicillin/streptomycin and 2 mM L-glutamine. Cells were treated with protein in serum-free media to examine protein cytotoxicity and delivery capacity. Transduction for T cell activation or differentiation and promoter binding assay was conducted in completed media. Transduced cells were washed with PBS before subsequent analysis.

2.4. Immunoblot

Cells treated with proteins in a dose- or time-dependent manner were lysed with RIPA buffer (Sigma Aldrich, St. Louis, MI, USA). Protein extracts were then blotted by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a polyvinylidene fluoride (PVDF) membrane (Millipore, Billerica, MA, USA). The membrane was blocked with 5% Bovine serum albumin (BSA; Amresco, Solon, OH, USA) in TBST (Tris-buffered saline with Tween 20) for 1 h, then incubated with anti-DYKDDDDK (FLAG) antibody (1:3,000; Cell Signaling, Danvers, MA, USA) in TBST for 3 h at room temperature (RT). After washing with TBST three times, the membrane was treated with anti-mouse IgG (1:10,000; Abcam, Cambridge, MA, USA) and incubated for 1 h at RT. ECL reagent (Bio-Rad) was applied to the membrane, and the chemiluminescence was detected by ChemiDoc (Bio-Rad).

2.5. Immunocytochemistry

HeLa cells seeded on Lab-Tek II chamber slides (Nunc, Portsmouth, NH, USA) were incubated with 2 μ M of ndG3-TMD for 2 h at 37 °C and washed with PBS twice. Cells were then fixed and permeabilized with 4% formaldehyde and 0.2% Triton X-100 (Sigma Aldrich) for 10 min each. After blocking the slide with 0.5% BSA added PBS for 1 h, cells were stained with anti-FLAG-FITC antibody (Sigma Aldrich) for 1 h and with 4',6-diamino-2-phenylindole (DAPI) for 10 min. The procedure was conducted at RT, and the cells were washed with PBS between steps. The samples were visualized by confocal microscope (LSM 980, Zeiss).

2.6. Cytotoxicity assay

5 $\times$ 10⁴ mouse splenocytes in a 96-well plate were incubated with protein (0.01–5 μ M) for 16 h at 37 °C. Cells were then treated with cell counting kit-8 (CCK-8) reagent (Dojindo Laboratories, Kumamoto, Japan) and incubated for another 4 h. The cell viability was determined by measuring the absorbance at 450 nm using a microplate reader (Bio-Rad).

2.7. Luciferase reporter assay

1 μ g of IL-4, IFN- γ or IL-17A promoter DNA and 1 μ g of pEGFP-N1 plasmid vector encoding wild type GATA3, T-box expressed in T cells (T-bet), or retinoid orphan receptor gamma t (ROR- γ t) were

co-transfected into 5×10^5 293T cells using Lipofectamine 3000 with Plus reagent (Invitrogen). After 6 h of incubation at 37 °C, 2 µM of proteins or PBS in the completed culture medium was added to the culture. Cells were harvested 24 h after the protein treatment and lysed with luciferase cell culture lysis reagent (Promega, Madison, WI, USA). The lysate was mixed with Luciferase Assay Reagent (Promega), and luciferase activity was measured by Glo-Max 96 Microplate Luminometer (Promega).

2.8. Mouse T cell isolation and in vitro differentiation

Naïve CD4 T cells were isolated from the mouse spleen using a CD4⁺CD62L⁺ T cell isolation kit (Miltenyi Biotec, Cologne, Germany). For T cell differentiation, the naïve T cells were activated with 1 µg/ml of plate-bound anti-CD3 ϵ and anti-CD28 (BD Bioscience, Franklin Lakes, NJ, USA) in a 96-well culture plate. T cell subset-skewing antibodies and cytokines - for Th2 differentiation, 50 ng/ml of IL-4 (PeproTech, Rocky Hill, NJ), 5 µg/ml of anti-IFN- γ (XMG1.2, eBioscience, San Diego, CA, USA) and 2.5 ng/ml of IL-2 (PeproTech); for Th1 differentiation, 25 ng/ml of IL-12 (PeproTech); for Th17 differentiation, 1 ng/ml of TGF- β (R&D Systems, MN, USA), 25 ng/ml of IL-6 (R&D Systems) and 2 µg/ml of anti-IFN- γ (eBioscience); for induced Treg differentiation, 5 ng/ml of TGF- β (R&D Systems) and 100 U/ml IL-2 (PeproTech) - were used with or without the proteins. Cells were incubated at 37 °C for 6 days for Th2 and 3 days for other T cell subsets.

2.9. Enzyme-linked immunosorbent assay (ELISA)

Cell culture media were harvested at the end of the activation or differentiation to measure cytokine levels. Whole blood collected from the mice was placed at RT for 30 min and centrifuged at 1,000 g for 10 min at 4 °C to obtain serum. Cytokine levels of culture media or serum were analyzed using mouse IL-4, IL-13, IL-5, IL-10, IL-17A, IFN- γ , and IL-2 ELISA kit (eBioscience) according to the manufacturer's directions. A mouse IgE ELISA kit (BioLegend, San Diego, CA, USA) was used to measure the serum IgE levels.

2.10. Flow cytometric analysis and antibodies

Cells were treated with a cell stimulation cocktail (plus protein transporter inhibitor; eBioscience) and incubated at 37 °C for 4 h for intracellular cytokine staining. For the surface molecules, Fixable Viability Dye eFluor 780, anti-CD4 (GK1.5) Alexa fluor 700, anti-CD4 (RM4-5) APC, and anti-CD69 (H1.2F3) APC and anti-CD25 (PC61) PE were purchased from eBioscience and BD biosciences. Cells were stained with antibodies diluted in PBS at 4 °C for 30 min. Cells were fixed and permeabilized with Fixation/permeabilization buffer (eBioscience) and treated with staining antibodies in permeabilization buffer for intracellular staining (eBioscience). Anti-IL-17A (eBio17B7) PE, anti-IL-10 (JES5-16E3) PE, anti-IFN- γ (XMG1.2) PE, anti-T-bet (4B10) PE, anti-FOXP3 (FJK-16s) PE, anti-IL-4 (11B11) PE, anti-ROR- γ t (B2D) PE and anti-GATA3 (TWAJ) PE from eBioscience, and anti-FLAG PE from BioLegend were used. Flow cytometric data were analyzed with FlowJo v.10.8 to generate dot plots and histograms.

2.11. OVA-induced asthma model

All groups consisted of four mice, and each experiment set was conducted twice. On days 0 and 14, mice were sensitized by intraperitoneal injection of 100 µg OVA (Ovalbumin; Sigma Aldrich) emulsified with 20 mg aluminum hydroxide (Sigma Aldrich). On days 21, 22, and 23, mice were anesthetized with a mixture of Zoletil (50 mg/ml) and Rompun (23.32 mg/ml) and challenged by

intranasal injection of 150 µg OVA. Some mice received 75 µg of ndG3-TMD or PBS 30 min before each OVA challenge.

2.12. Measurement of airway hyperresponsiveness

Each group of mice was inhaled with aerosolized methacholine (MCh) for 180 s by a nebulizer, followed by airway hyperresponsiveness measurements for an additional 180 s. Inhalation and the measurements were repeated with increasing MCh concentrations (0–100 mg/ml). Airway reactivity is indicated by increased enhanced pause (Penh) values.

2.13. Bronchoalveolar lavage (BAL) analysis

On day 26, BAL fluids were obtained through lung lavage four times with 1 ml of Hank's balanced salt solution (HBSS, Gibco, Rockville, MD, USA) using a tracheal tube. A hemacytometer determined total cell counts in the BAL fluid, and the samples were cytocentrifuged and stained with Diff-Quick for immune cell counts. Macrophage, neutrophil, eosinophil, and lymphocyte counts were determined by counting at least 200 cells in a blinded manner under an immersion oil field using Leukostat.

2.14. Histology

The right ventricles were cannulated, and the lungs were rinsed with 5 ml of PBS to remove intravascular red blood cells (RBC). Lungs and tracheal tubes were harvested from mice and stored in 4% paraformaldehyde for 24 h. After being embedded in paraffin, the tissues were made into thin sections with a thickness of 4 µm. The sections were stained with hematoxylin and eosin (H&E) for evaluation of lung morphology and periodic acid-Schiff (PAS) for observation of mucus-containing goblet cells.

2.15. Statistical analysis

Data in each graph shows the mean $\pm$ SEM and statistical significance of at least three replicates. Student's t-test for single-dose protein experiments and one-way ANOVA for multi-dose experiments were performed to find statistical significance by setting the PBS group as a control; ns (not significance); * ($P < 0.05$); ** ($P < 0.01$); *** ($P < 0.001$); **** ($P < 0.0001$).

3. Results

3.1. Intracellular delivery kinetics of ndG3-TMD into the nucleus of the cells

GATA3 consists of two transactivation domains (TAD) s at the N-terminus and two following zinc finger (ZFN) domains mediating DNA binding, with basic regions on each C-terminal side of the ZFN. To generate ndG3-TMD, GATA3-TMD consisting of ZFNs and linker domain was fused with a human-origin Hph-1-PTD whose intracellular transduction potential was demonstrated previously [18–21]. ndG3-TMD contains two consecutive Hph-1 for enhanced transduction efficiency and a FLAG-tag for *in vitro* protein detection. ndG3-TMD without Hph-1 sequences (G3-TMD) or ndG3-TMD with the reduced DNA binding ability(ndG3-TMD-R276E) by Arg-276 to Glu-276 mutation was also generated as negative controls [22] (Fig. 1A and B). Expression and purification of these proteins were conducted in an *Escherichia coli* expression system.

First, the intracellular delivery potential of the purified proteins was evaluated in mouse splenocytes, and the transduced proteins inside the cells were detected by flow cytometry or Western blot analysis using anti-FLAG antibodies. As shown in Fig. 1C, ndG3-TMD

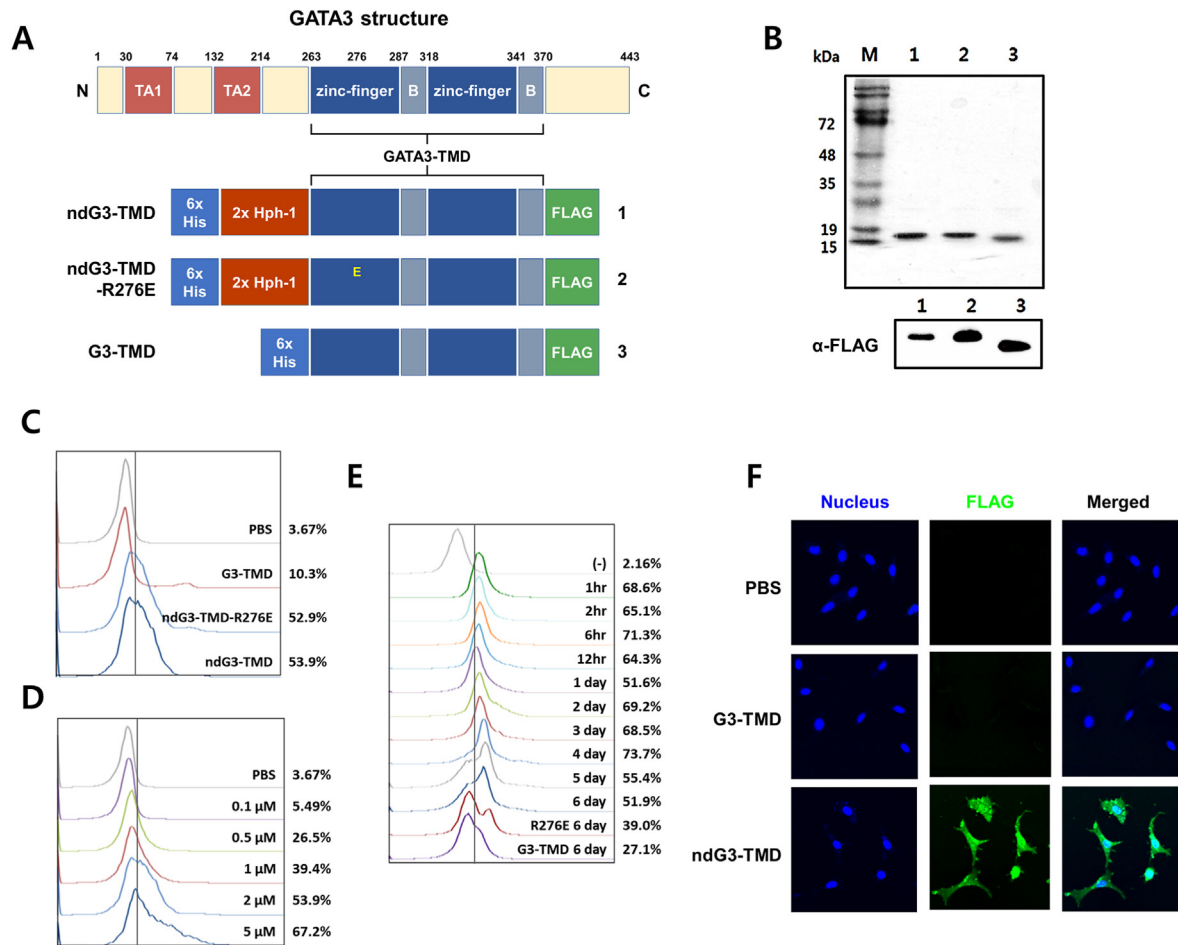


Fig. 1. Generation of a nucleus-deliverable form of GATA3-TMD

(A) Schematic of mouse GATA3, ndG3-TMD, and its non-binding (ndG3-TMD-R276E) and non-deliverable (G3-TMD) controls. (B) The purity and size of the produced proteins were confirmed by SDS-PAGE and Western blot. (C) The delivery efficacy of each protein in mouse splenocytes under the same conditions (2-h, 2 μM) was investigated using flow cytometry. (D–E) The ndG3-TMD delivery efficiency in splenocytes according to concentration and time was evaluated at 2 h and 2 μM transduction, respectively. (F) The intracellular localization of proteins delivered to HeLa cells was detected by FITC-conjugated anti-FLAG and observed by confocal microscopy.

and ndG3-TMD-R276E were efficiently transduced into the cells within 2 h of transduction, but G3-TMD cannot be delivered inside the cells. The transduction of ndG3-TMD was dose-dependent, and the delivered ndG3-TMD remained stable for more than 4 days after transduction (Fig. 1D and E). When the intracellular localization of the delivered ndG3-TMD in the cells was examined by confocal microscopy, ndG3-TMD could be detected in the cytoplasm and the nucleus, and the proportion of ndG3-TMD in the nucleus gradually increased (Fig. 1F and Supplementary Fig. 1). Taken these results together, ndG3-TMD can be delivered into the nucleus of the cells effectively and stably in a dose- and time-dependent manner.

3.2. ndG3-TMD inhibits GATA3-mediated transcription without affecting T cell receptor (TCR) signaling

To exclude the possibility that ndG3-TMD affects the immune cell function by cellular cytotoxicity, mouse splenocytes were treated with various concentrations of ndG3-TMD for 16 h, and the cell viability was analyzed by CCK-8 assay. As shown in Fig. 2A, no cellular cytotoxicity was observed in the cells treated with the highest concentration (5 μM). As ndG3-TMD contains the GATA3 ZFNs with DNA-binding ability, we examined whether ndG3-TMD can modulate endogenous GATA3-mediated transcription by the

promoter binding assay. 293T cells were co-transfected with the expression plasmid containing wild-type GATA3 and the reporter construct in which the luciferase expression is driven by the IL-4 promoter. Then, the transfected cells were treated with either ndG3-TMD or ndG3-TMD-R276E for 24 h. As shown in Fig. 2B, ndG3-TMD significantly reduced GATA3-mediated IL-4 promoter activity, whereas ndG3-TMD-R276E, with the reduced DNA binding ability, showed a negligible effect on the luciferase activity (Fig. 2B). To evaluate the functional specificity of ndG3-TMD toward IL-4 promoter, 293T cells were co-transfected with the expression plasmid containing wild-type T-bet or ROR-γt gene and the reporter construct in which IFN-γ promoter or IL-17A promoter induces the expression of luciferase, respectively. When the transfected 293T cells were treated with ndG3-TMD, no significant change in the luciferase activity was observed (Fig. 2C). These results suggest that ndG3-TMD selectively inhibits the transcriptional functions of endogenous GATA3.

To confirm the functional specificity of ndG3-TMD toward Th2 cells, mouse splenocytes were stimulated with anti-CD3/CD28 mAb in the presence of ndG3-TMD, and the level of Th2 cytokine secretion was examined by ELISA. The secretion of IL-4 and IL-13 from the splenocytes was significantly reduced by ndG3-TMD treatment, but G3-TMD- or ndG3-TMD-R276E treatment did not show the inhibitory effect (Fig. 2D). To rule out the possibility that

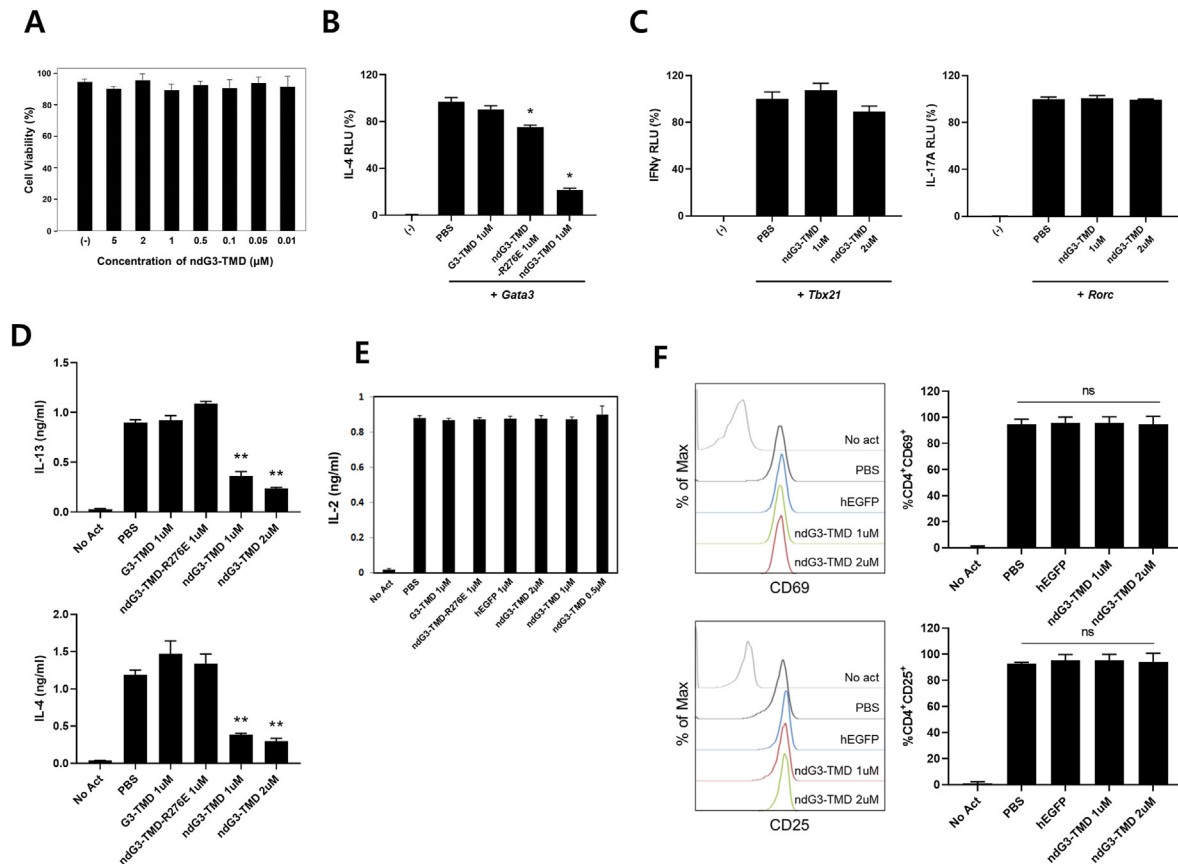


Fig. 2. Specific inhibition of GATA3-mediated transcription by ndG3-TMD

(A) The cytotoxicity of ndG3-TMD in mouse splenocytes was measured by CCK-8 assay. Cell proliferation inhibited by different concentrations of ndG3-TMD was determined by the relative value to the absorbance of the PBS control. (B–C) Function of ndG3-TMD specific to IL-4 promoter was examined by using IFN- γ and IL-17A promoter as the controls. 293T cells were co-transfected with a wild-type transcription factor (GATA3, T-bet, or ROR- γ t) and a promoter-luciferase gene (IL-4, IFN- γ , or IL-17A). The relative value of luciferase activity for the PBS-treated group was calculated. (D) Effect of ndG3-TMD on Th2 cytokine production upon TCR stimulation in mouse splenocytes were measured by ELISA. (E) The level of IL-2 secretion and (F) CD69 and CD25 expression upon T cell activation in the presence of ndG3-TMD was investigated by ELISA and flow cytometry. Histograms show fluorescence intensities for the markers within viable CD4⁺ gated population (left).

the reduced cytokine expression is due to the impaired downstream signaling events of TCR activation by ndG3-TMD, the level of CD69 and CD25 on the cell surface and the secretion of IL-2 into the culture medium was examined after stimulation of CD4⁺ naïve T cells with anti-CD3/CD28 in the presence of ndG3-TMD. Hph-1 fused enhanced green fluorescent protein (hEGFP) was used as a nuclear transducible protein control for this functional assay [19]. The induced expression of CD69 and CD25, and IL-2 secretion was not affected by ndG3-TMD (Fig. 2E and F). Therefore, the decrease in Th2 cytokine secretion was mediated by ndG3-TMD functioning as a competitive inhibitor of endogenous GATA3-mediated transcriptional activity.

3.3. Effect of ndG3-TMD on T cell subset differentiation and function

When naïve T cells are exposed to Th2-skewing conditions, the IL-4 signaling pathway phosphorylates the cytoplasmic signal transducer and activator of transcription 6 (STAT6), which translocates into the nucleus and binds to the GATA3 promoter region. GATA3 in Th2-differentiating cells autoactivates its expression by a feedback loop for lineage maintenance [23]. To determine whether ndG3-TMD suppresses Th2 differentiation by interrupting this process, naïve CD4⁺ T cells cultured in Th2-polarizing condition were analyzed for GATA3 and IL-4 expression by flow cytometry. Cells activated with anti-CD3/CD28 for the same period without

cytokines were used as a negative control. ndG3-TMD treatment reduced the expression of GATA3 and the proportion of CD4⁺IL-4⁺ T cells in a concentration-dependent manner (Fig. 3A–C). In addition, ndG3-TMD inhibited the secretion of IL-5 and IL-13 from Th2-differentiating cells (Fig. 3D).

GATA3 is widely known for its essential role in the bifurcation of Th1/Th2 lineage commitment as a negative regulator for Th1-related transcription [24]. Recent studies have also demonstrated that GATA3 controls multiple pathways involved in other T cell subset functions or differentiation [25]. To determine whether ndG3-TMD exerts functional regulation on Th1, Th17, or Treg cell differentiation, mouse naïve CD4⁺ T cells treated with ndG3-TMD were first differentiated into Th1 cells. T-bet and IFN- γ expression in the differentiated Th1 cells was analyzed by flow cytometry. Unexpectedly, Th1 cells treated with ndG3-TMD showed a slight decrease in both T-bet and IFN- γ expression (Fig. 3E–G). It was previously reported that GATA3 suppresses IFN- γ production by physically interacting with runt-related transcription factor 3 (RUNX3) using the ZFNs [26]. As the ndG3-TMD contains two ZFNs, it is possible that intranuclear delivery of ndG3-TMD would inhibit the expression of IFN- γ by taking RUNX3 away from T-bet/RUNX3 complex, which in turn contributed to the decrease in T-bet expression. Next, the level of TGF- β -induced Th17 cell and iTreg cell differentiation following ndG3-TMD treatment was evaluated by the expression of its specific transcription factor. Unlike in Th1

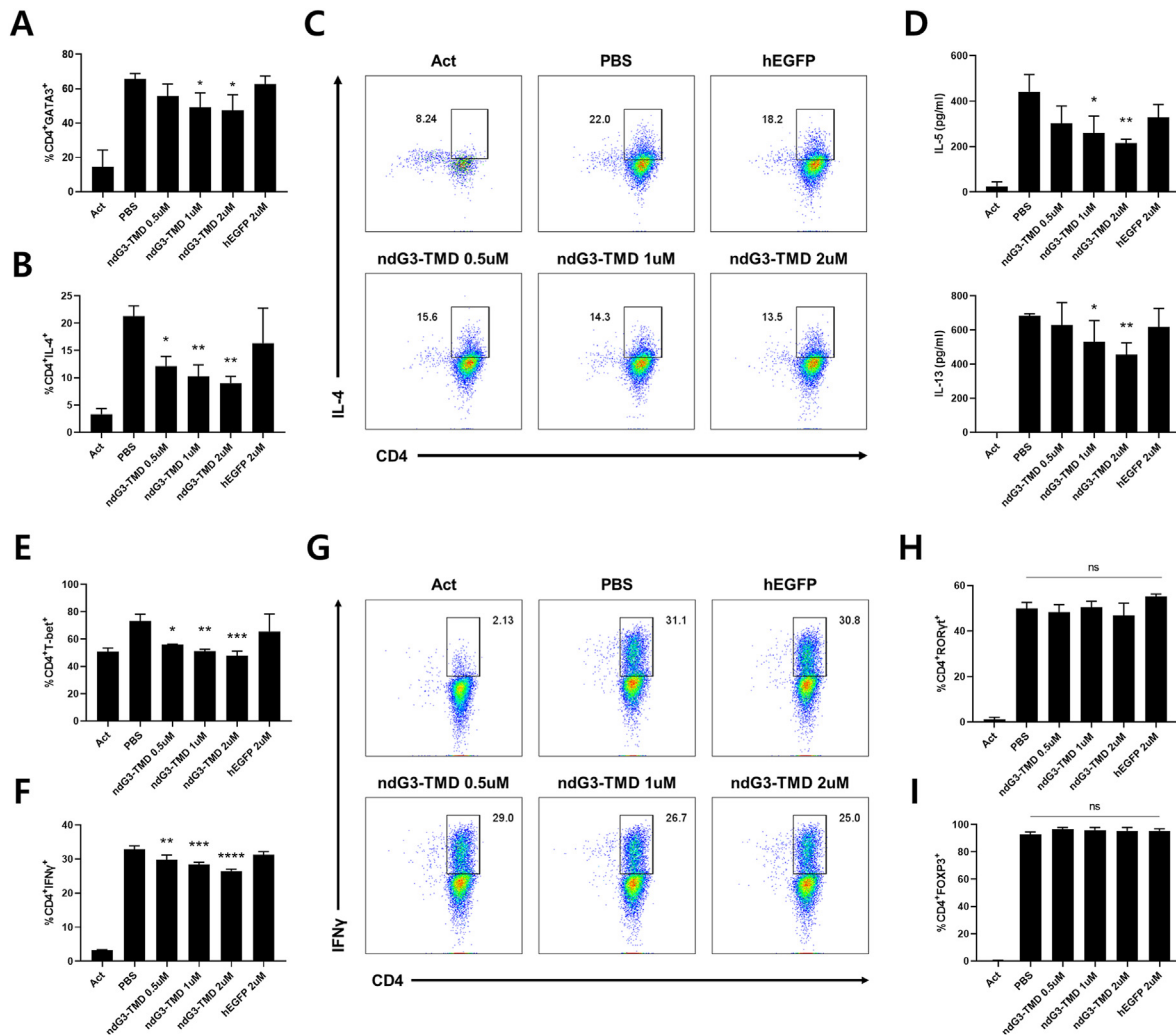


Fig. 3. Effect of ndG3-TMD on naive CD4⁺ T cell differentiation into various T cell subsets

The level of Th2 differentiation in the presence of ndG3-TMD was examined by intracellular (A) GATA3 and (B–C) IL-4 expression using flow cytometry. (D) Th2 cytokine levels in the Th2 culture medium were measured by ELISA. Effect of ndG3-TMD on differentiation of naive CD4⁺ T cells into other subsets was examined by flow cytometry or ELISA. The proportions of (E) CD4⁺T-bet⁺ and (F–G) CD4⁺IFN-γ⁺ populations in the differentiated Th1 cells were analyzed by flow cytometry. Differentiation of Th17 or iTreg following ndG3-TMD treatment was assessed by flow cytometry for (H) CD4⁺ROR-γt⁺ or (I) CD4⁺FOXP3⁺ populations.

differentiation, ndG3-TMD did not affect the expression of ROR-γt and FOXP3 in Th17 and iTreg cells, respectively. (Fig. 3H and I). These results demonstrate that ndG3-TMD possesses a specific inhibitory potential toward Th2 cells in the immune microenvironment through the functional modulation of endogenous GATA3.

3.4. Alleviation of Th2-mediated allergic airway inflammation by intranasal delivery of ndG3-TMD

One of the critical considerations in developing a treatment for respiratory allergic diseases, including rhinitis and asthma, is to enable topical administration to minimize systemic side effects caused by repeated use [27]. An OVA-induced asthma mouse model was used to evaluate the therapeutic potential of ndG3-TMD in Th2-mediated inflammation *in vivo*. The intranasal treatment schedule of the asthma mouse model with ndG3-TMD was performed as shown in Fig. 4A, and the severity of asthma was assessed by measuring airway hypersensitivity (AHR) to methacholine (MCh) on day 25 via the enhanced pause (Penh) method. While the untreated OVA mice exhibited severe AHR with increasing MCh concentration (5–100 mg/ml), a significant

reduction of AHR to MCh was observed in ndG3-TMD-treated mice (Fig. 4B). After the measurement of AHR, the mice were sacrificed for further analysis of *in vivo* immunological status.

The substantially lower levels of serum IgE and immune cell number were detected in the bronchoalveolar lavage (BAL) fluids of ndG3-TMD-treated mice when compared to the untreated OVA mice (Fig. 4C and D). In particular, the airway infiltration of eosinophils and neutrophils, which play a critical role in airway hypersensitivity, as well as macrophages and lymphocytes significantly decreased in ndG3-TMD-treated mice (Fig. 4D). Histological analysis also confirmed the reduced inflammatory cell infiltration in the peribronchial tissue and pulmonary airway damage upon ndG3-TMD treatment. Furthermore, ndG3-TMD-treated mice exhibited fewer mucus-secreting goblet cells around the bronchial airway (Fig. 4E). To examine whether the functions of Th2 cells in an OVA-induced asthma model were changed by ndG3-TMD treatment, the splenocytes isolated from the asthma mice were stimulated with anti-CD3/CD28 mAb *ex vivo*, and the level of Th2-cytokine secretion was analyzed by ELISA (Fig. 4F). Consistent with the *in vivo* results above, IL-4 and IL-5 concentrations in the culture medium were significantly lowered in the ndG3-TMD group. These results

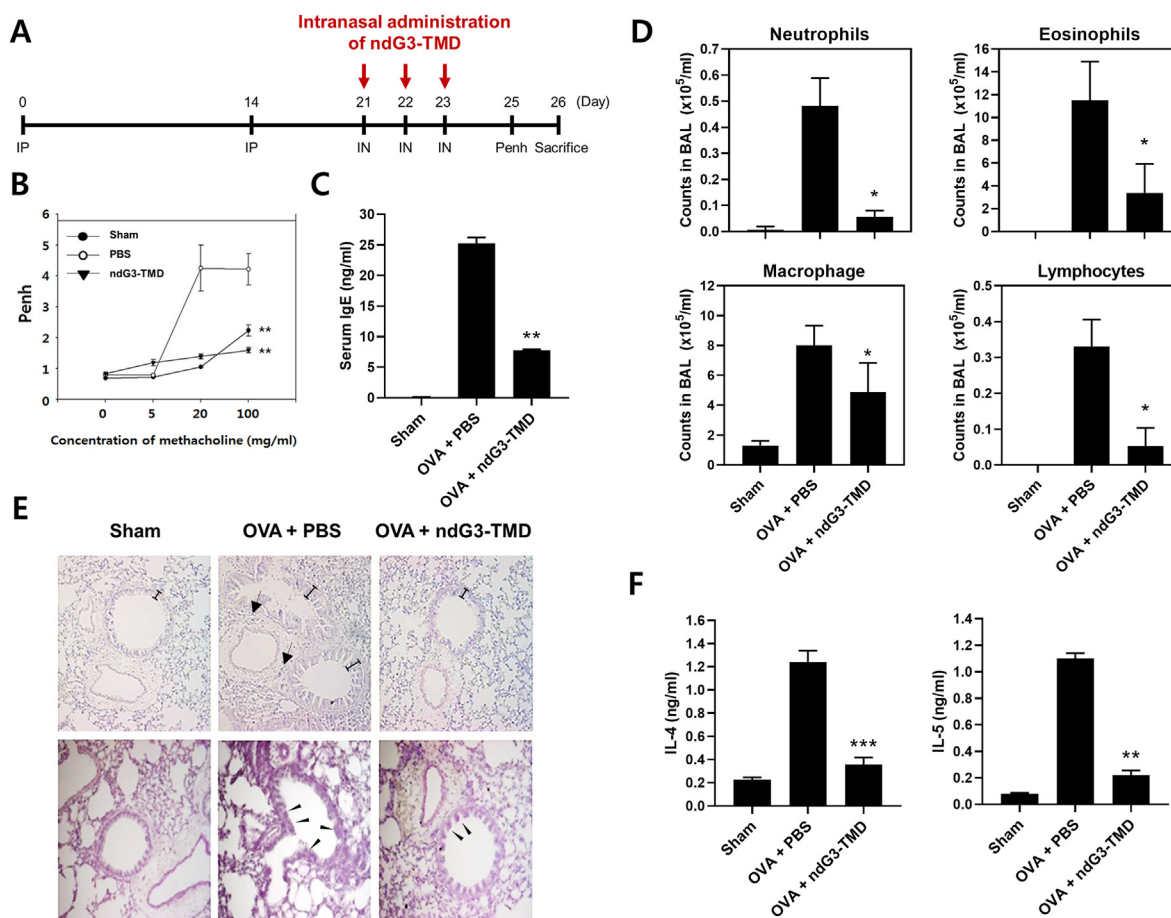


Fig. 4. Therapeutic potential of ndG3-TMD on OVA-induced mouse model of asthma.

(A) Schematic protocol for asthma induction and ndG3-TMD treatment through intranasal pathway. Various disease scales of asthma were analyzed following the ndG3-TMD treatment. (B) Airway hyperresponsiveness was measured by enhanced pause to estimate disease severity in OVA-induced asthma mice treated with ndG3-TMD at day 25. (C) IgE levels in the blood serum of asthma mice were measured by ELISA. (D) Bronchial infiltration of immune cells (neutrophils, eosinophils, macrophages, and lymphocytes) was examined by Diff-Quik staining of BAL fluid. (E) Lung sections stained with H&E and PAS show airway infiltrated immune cells (arrow) and mucus-producing cells (arrowhead), respectively. (F) IL-4 and IL-5 production by splenocytes isolated from asthma mice. The cells were stimulated with anti-CD3/CD28 for 3 days and the culture medium was examined by ELISA.

demonstrate that ndG3-TMD inhibited Th2 cell differentiation and functions triggered by the OVA challenge, thus preventing the activation of respiratory immune cells and mucus secretion. In conclusion, the intranasal administration of ndG3-TMD effectively modulates the activity of endogenous GATA3 in the asthma-inducing environment, thereby inhibiting the Th2-mediated inflammation in the airway.

4. Discussion

Induced by inhaled allergens in our daily environment, Th2 cells play a central role in the pathogenesis of asthma. They induce IgE production by plasma cells, sequential sensitization of granulocytes, and mucus secretion by airway goblet cells, leading to airway thickening and hyperresponsiveness [28]. The clinical implication of Th2 and its key transcription factor GATA3 in asthma and their functional characterization using mAb specific to IL-4, IL-5, or IL-13, or corticosteroid have been elucidated *in vitro* and in asthma animal models. It has been suggested that the functional regulation or conversion of the asthmatic microenvironment by targeting the essential transcription factor would be the novel therapeutic strategy instead of simply targeting Th2 cytokines. In this regard, the development of the therapeutic reagent to

modulate the interactomic network of endogenous GATA3 has yet to be attempted. In this study, ndG3-TMD, a fusion protein between Hph-1-PTD and TMD of GATA3, was used as a new therapeutic entity because of two therapeutic advantages: (1) Hph-1-PTD can transduce the GATA3-TMD into the nucleus of the immune cell effectively and function as a transcription modulator of the endogenous GATA3 *in vitro* and *in vivo*. (2) ndG3-TMD can be delivered into the asthma animal model through the intranasal pathway without systemic exposure.

ndG3-TMD was effectively delivered to the nucleus within a short period and remained stable inside the cells without any cellular cytotoxicity. In the reporter gene assays where luciferase expression is driven by IL-4, IFN- γ , or IL-17A promoter, ndG3-TMD functions explicitly as a competitive inhibitor for GATA3-mediated transcription, thus reducing the production of Th2 cytokines and GATA3 expression without affecting the early and late signaling events for T cell activation. Therefore, ndG3-TMD is a competitive transcription modulator for the endogenous GATA3. Unexpectedly, the differentiation of naïve T cells into Th1 cells in the presence of ndG3-TMD showed a slightly decrease in both T-bet and IFN- γ expression. It has been known that under the Th1 cell differentiation condition, the protein complex between the increased level of T-bet and RUNX3 can induce the expression of the genes

responsible for the Th1-specific microenvironment, and RUNX3 interacts with the zinc-finger motif of GATA3. Therefore, it is possible that ndG3-TMD with two zinc-finger motifs of GATA3 may inhibit the T-bet/RUNX3 complex formation, leading to the reduced expression of Th1-type cytokine genes.

The therapeutic potential of ndG3-TMD was tested in the OVA-induced asthma animal model through intranasal administration. ndG3-TMD treatment showed immediate protection from airway challenge of allergens in asthma mice. Various symptoms, including airway hypersensitivity due to the functions of activated Th2 cell, were significantly reduced in ndG3-TMD-treated mice. Specific inhibition of GATA3-mediated transcription by ndG3-TMD is the unique therapeutic regime for the modulation of the Th2-related autoimmune microenvironment. Especially, intranasal administration of ndG3-TMD is expected to reduce the systemic side effects, which are the therapeutic limitation associated with conventional immunosuppressants such as corticosteroids.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests. Su-Hyeon Lee reports financial support was provided by Good T Cells, Inc. Sang-Kyou Lee reports financial support was provided by Good T Cells, Inc. Jung-Ho Kim reports was provided by Good T Cells, Inc. Sang-Kyou Lee has patent pending to Sang-Kyou Lee.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.bbrc.2022.11.095>.

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