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Original Article

Effect of Semaphorin7A during the Effector Phase of Nickel Allergy

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Abstract：

Background: Metal allergy is caused by many factors, including cells, cytokines, chemokines, or the environment. Recent studies suggested semaphorin7A (Sema7A), expressed on activated T cells, is crucial in producing inflammation through α1β1 integrin on monocytes and macrophages. However, the role of Sema7A on keratinocytes in metal allergy is still unclear. In this study, we focused on keratinocytes since they are known as an important player for skin immunity, and analyzed the effect of Sema7A expressed on keratinocytes in the development of metal allergy.

Materials and Methods: Mouse keratinocyte line PAM2.12 cells were treated with $NiCl_2$ to analyze the expression of Sema7A. Ni allergy was induced in female C57BL/6J mice (6-8 weeks old) with or without Sema7A suppression to confirm if Sema7A is necessary in producing allergic reactions to $NiCl_2$.

Results: $NiCl_2$ enhanced the expression of Sema7A in a dose and time-dependent manner after 72 hours of stimulation. PAM2.12 produced TNF-α in response to $NiCl_2$, and this secretion was reduced by Sema7A inhibition. In a mouse model, ear thickness, at 48 hours after $NiCl_2$ injection, significantly decreased by Sema7A siRNA administration.

Conclusions: Sema7A is essential in producing an allergic reaction to $NiCl_2$, especially during the effector phase. Since the interaction between Sema7A and α1β1 integrin enhances inflammation in many skin diseases, this interaction may also have possibilities to be a therapeutic target for metal allergy.

Introduction

Various metals are still widely used for dental prostheses in Japan despite the development of CAD-CAM technologies using zirconia or hybrid resins. Hypersensitivity reactions to metals are one of the severe problems for dental treatment, and nickel (Ni) allergy is very frequent among the metal allergies due to its strong antigenicity[1-6)]. Metal allergy is characterized as a delayed-type hypersensitivity, wherein it takes 72 hours in humans or 24-48 hours in mice, for symptoms to develop. Research in recent years elucidated that keratinocytes not only play a role in the barrier mechanism but also play an important role in immune mechanism when in contact with epithelial immune cells such as Langerhans cells, γδT cells, dendritic epidermal T cells (DETCs), resident memory T cells (Trm cells), and monocyte-derived dendritic epidermal cells (IDECs)[7-10)]. Although many clinical and experimental studies suggested that keratinocytes enhance inflammation by releasing cytokines and chemokines such as TNF-α, IL-1β,

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and TSLP in response to damages on the skin during various skin diseases[11-14], the role of keratinocytes is still unclear in the development of metal allergy.

Semaphorins (SEMAs), secreted and membrane-associated proteins, were originally found as axon guidance factors in the nervous system[15]. They are widely expressed on various cells to maintain tissue homeostasis, including the immune system[16, 17]. Sema7A, known as CD108, is a membrane-associated GPI linked protein, which is also identified as an axon outgrowth molecule[18-20]. Although Sema7A expressed on activated T cells is crucial in producing inflammation through α1β1 integrin on monocytes and macrophages[17], the function of Sema7A on keratinocytes has not yet been clarified. Here, we show that Sema7A on keratinocytes regulates the symptoms of Ni allergy in mice.

Materials and methods

Cells and reagents

Mouse keratinocyte cell line PAM2.12 was provided by Dr. S.H.Yuspa (Center for Cancer Research, National Cancer Institute, Bethesda, MD, USA). PAM2.12 cells were cultured in DMEM (low glucose) (Nacalai Tesque, Kyoto, Japan) supplemented with 10% heat-inactivated Fetal Bovine Serum (Biowest, Nuaillé, France) and antibiotic-antimycotic mixed stock solution (Nacalai Tesque). For subsequent experiments, Ni (II) chloride hexahydrate ($NiCl_2$, Wako, Osaka, Japan) was prepared and added to a concentration of 0.1-1000 μM in the media.

Mice

Female C57BL/6J mice (6-8 weeks old) were purchased from Charles River Laboratories Japan (Tokyo, Japan). Mice were provided with a standard laboratory diet and water. All mice were maintained in a specific pathogen-free condition in our animal facility. The Animal Ethics Board of the University of Tokushima approved all procedures.

Induction of Ni allergy

Ni allergy was induced using a method described previously[21-23]. To induce a hypersensitivity reaction to Ni, 25 μl of 1 μmol/ml $NiCl_2$ with 25 μl of Freund's incomplete adjuvant (IFA) (MP Biomedicals, Illkirch, France) was intraperitoneally injected into mice for initial immunization. Two weeks later, mice were administered with intradermal injections to the pinnae with 10 μl of 0.2 μmol/ml $NiCl_2$ with Freund's complete adjuvant (CFA) (MP Biomedicals) using 28 G needles (TERUMO, Tokyo, Japan) for a recall immune response. DTH reactions were determined by measuring the changes in ear thickness at 48 hours after the challenge. Sema7A and control scramble siRNA were obtained from Sigma-Aldrich Japan (Tokyo, Japan). To suppress the function of Sema7A on the mouse ear in the elicitation phase, transfections were carried out 6 hours prior to intradermal injection with $NiCl_2$ using *in vivo*-jetPEI® (Polyplus-transfection®, Illkirch, France) according to the manufacturer's protocol.

Western blot analysis

Sample of PAM2.12 were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with 10% acrylamide gel, transferred onto a poly vinylidene difluoride (PVDF) membrane (Bio-Rad Laboratories, Inc., CA, USA), and the blotted membranes were incubated with antibodies against Semaphorine7A (bs-2702R, 1:750, Bioss Inc., MA, USA), glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (OSG00032W, 1:30000, Osenses, SA, Australia). Immune complexes were detected using horseradish peroxidase (HRP)-conjugated anti-rabbit IgG (#7074, 1:20000, Cell Signaling Technology Inc., MA, USA) and ECL prime (Amersham Bioscience Corp., NJ, USA). For the suppression of Sema7A expression on PAM2.12, transfection was performed 12 hours prior to 10 nM $NiCl_2$ administration with 1 nM Sema7A siRNA using INTERFERin (Polyplus-transfection®) according to the manufacturer's protocol.

Histology and immunohistochemistry

Ear tissues were embedded in tissue-freezing medium O.C.T. Compound (Sakura Finetek Japan Co., Ltd., Tokyo, Japan) and were rapidly frozen. The frozen samples were cut into 8 μm slices using a microtome CM1850 (Leica Biosystems, Wetzlar, Germany), placed on MAS-coated glass slides (Matsunami Glass IND LTD, Osaka, Japan) and fixed with acetone. Blocking One Histo (Nacalai Tesque) was applied for 10 min RT, then washed with PBS 5 min. A 10-minute incubation in 3% hydrogen peroxide was carried out for peroxidase blocking. The incubation of the primary antibody was carried out at 4℃ O/N. The incubation of the secondary antibody was carried out at room temperature for 1 hour. A positive reaction was detected by the ImmPACT DAB substrate (VECTOR LABORATORIES, INC., CA, USA). Following antibody were used: rabbit Sema7A Polyclonal Antibody (1:300, Bioss Inc.), anti-rabbit Alexa Fluor 568 (A-11011, 1:500, Life Technologies Corporation, CA, USA), anti-rabbit IgG HRP-linked Antibody (#7074, 1:500, Cell Signaling Technology Inc.).

Flow cytometry

1 x 10^6 cells were stained with Readidrop™ Propidium Iodide (Bio-Rad Laboratories, Inc., CA, USA) according to the procedure. Cells were analyzed on BD FACSVerse™ (BD

Table 1 Primer sequences for real-time RT-PCR

Target gene	Forward primer	Reverse primer
Semaphorin7A	3'- CGGAAGCAGGAATACAACGG -5'	5‘- GTCAGGGTTGTCTTCTCGGA -3'
β-actin	3'- GGGACTCATCGTACTCCTGCTT -5'	5‘- TCTGGCTCCTAGCACCATGAAGA -3'

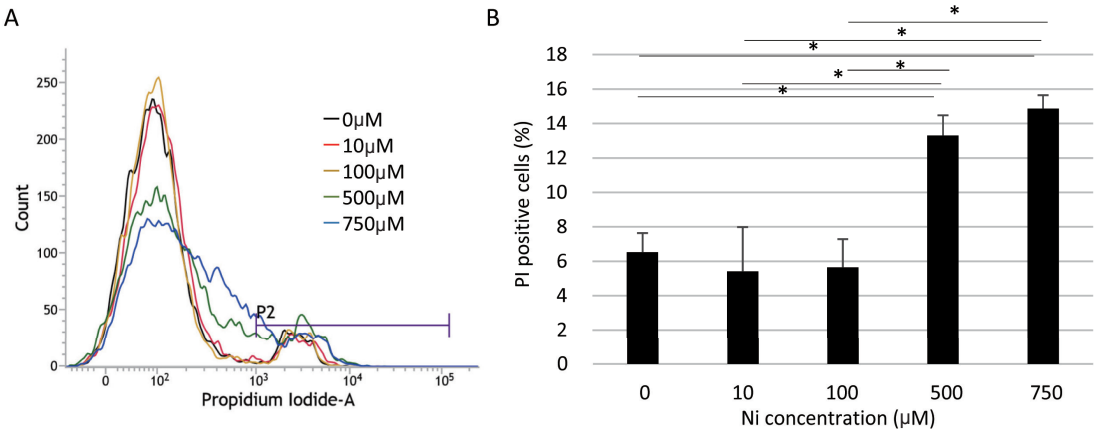

Fig. 1 Effect of $NiCl_2$ on PAM2.12
(A) FCM analysis of cell viability on PAM2.12 at 48-hour stimulation with different concentration of $NiCl_2$.
(B) Dead cell/ Total cell rates are shown on the graph. At 48 hours of observation, no apparent cell death was observed when the concentration of $NiCl_2$ was 100 μM or less. Data are shown as mean ± SD and are representative of three independent experiments and analyzed using one-way ANOVA Bonferroni post hock test. Values of $P < 0.05$ were considered statistically significant (*$p < 0.05$)

Biosciences, CA, USA).

ELISA

1 x 10^4 PAM2.12 cells per well were seeded in a 96-well dish. Twenty-four hours later, PAM2.12 was stimulated with 10 μM $NiCl_2$. To suppress Sema7A expression, transfection was performed with 1 nM Sema7A siRNA using INTERFERin (Polyplus-transfection®) according to the manufacturer's protocol. Culture supernatant was collected at 24 hours and 48 hours from Ni stimulation. Mouse TNF-alpha Quantikine ELISA Kit (MTA00B, R&D Systems, MN, USA) was used to measure the concentration of TNF-α in the supernatant.

Quantitative real-time PCR reactions and primers

Total RNA was extracted using TRIzol (Thermo Fisher Scientific, MA, USA) according to the manufacturer's protocol. First-strand cDNA was synthesized with 500 ng RNA by Prime Script (Takara Bio Inc, Shiga, Japan). Real-time PCR was performed with TB-Green (Takara Bio Inc) on the ABI7300 Real-time PCR System (Applied Biosystems, MA, USA). The primer sequences are listed in Table 1.

Statistical analysis

Results are expressed as the mean ± SD. Statistical comparisons were performed using Bonferroni post hock test or Student's T-test, and p values < 0.05 were considered statistically significant.

Results

Expression of Sema7A in keratinocytes was enhanced by $NiCl_2$

To investigate the effect of Ni on keratinocytes, we used $NiCl_2$ as a metal antigen, which is highly antigenic and has been well-established in mouse models. During cell culture, concentrations of $NiCl_2$ below 100 μM did not affect cell viability (Fig. 1AB), consistent with studies performed on the human keratinocyte cell line HaCaT[24]. Enhancement of Sema7A mRNA and protein expressed in PAM2.12 stimulated with 10 μM $NiCl_2$ were observed by quantitative real-time PCR, Western blot analysis, and immunohistochemistry (Fig. 2A, B, C). Sema7A mRNA reached the highest expression at 48 hours of stimulation (Fig. 2A), and protein was increased in a time-dependent manner (Fig. 2B). The expression

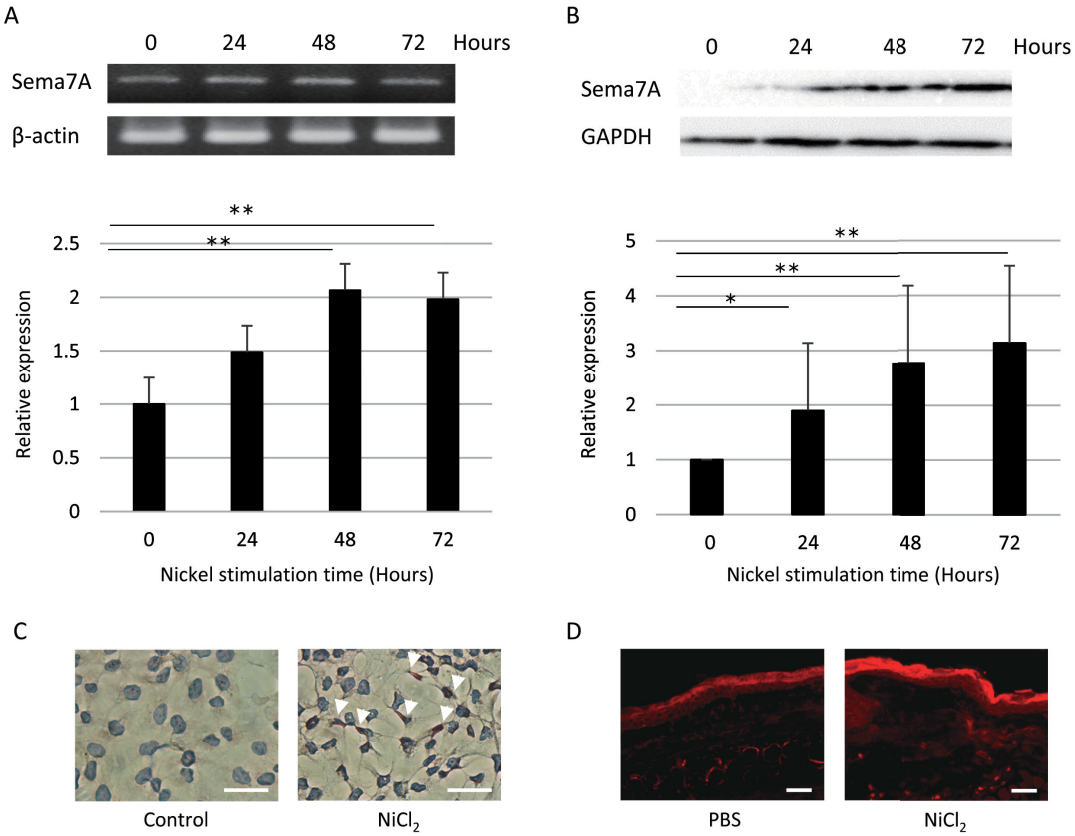

Fig. 2 Sema7A Expression on PAM2.12 with $NiCl_2$

(A and B) Sema7A mRNA and protein expression level was significantly increased from 48 to 72 hours after stimulation by $NiCl_2$. All data are shown as mean ± SD and are representative of at least three independent experiments and analyzed using one-way ANOVA Bonferroni post hock test. Values of $P < 0.05$ were considered statistically significant (*$p < 0.05$, **$p < 0.01$).

(C) Immunohistochemistry of Sema7A expressed on PAM2.12 at 48 hours-stimulation with $NiCl_2$. *in vitro*. Scale bar, 50 μm

(D) Immunofluorescence images of Sema7A (red) in mouse ear skin. Sema7A expressed in outer layer of epithelial tissue. Scale bar, 20 μm

of Sema7A was mainly in epithelial cells. Furthermore, expression in those cells were enhanced at 48 hours after Ni stimulation. (Fig. 2D).

Inhibition of Sema7A reduced TNF-α production in keratinocytes

Then, we examined the effect of Sema7A inhibition on PAM2.12 by siRNA administration. Figure 3A shows the effect of gene silencing. The production of TNF-α, which is a typical inflammatory cytokine secreted from keratinocytes in response to stimulation, was confirmed by ELISA. The amount of TNF-α production significantly increased 24 hours after Ni stimulation compared with the control and was reduced by Sema7A suppression on PAM2.12 (Fig. 3B)

Silencing of Sema7A suppressed ear swelling in nickel allergy mouse model

Since gene silencing of Sema7A on PAM2.12 reduced cytokine production, we evaluated this effect of inhibition on the development of Ni allergy in mice. The Ni allergy mouse model was prepared following the protocol previously described[23]. Compared with the Ni allergy group, Sema7A expression was found to be suppressed in keratinocytes and subepithelial cells by Sema7A siRNA administration, which was intradermaly injected into the pinnae 6 hours before intradermal Ni injection for elicitation (Fig. 4A). Ear thickness 48 hours after Ni injection was significantly reduced by Sema7A siRNA administration (Fig. 4B, C, D). This result indicates that the suppression of Sema7A during the elicitation phase reduced inflammation in Ni allergy mouse model.

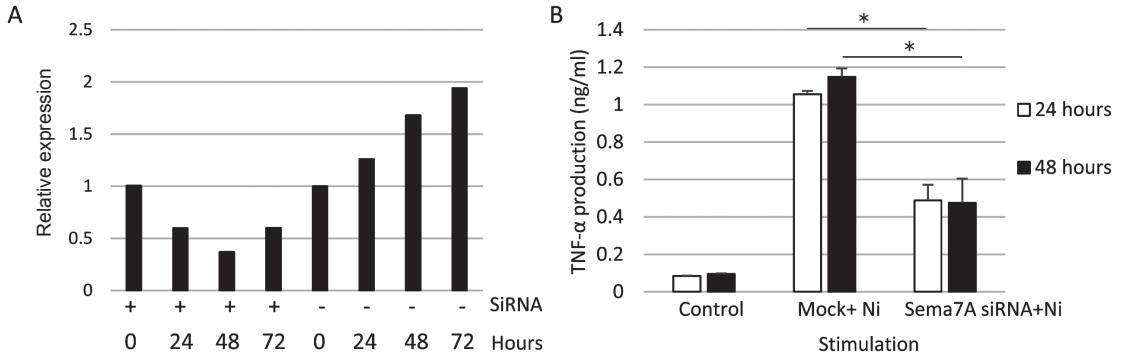

Fig. 3 Effect of Sema7A inhibition in PAM2.12 stimulated by $NiCl_2$
(A) Inhibitory effect on Sema7A protein expression in PAM2.12 with siRNA.
(B) Inhibition of Sema7A significantly reduced TNF-α production in PAM2.12 compared with mock-control.
*$p < 0.05$

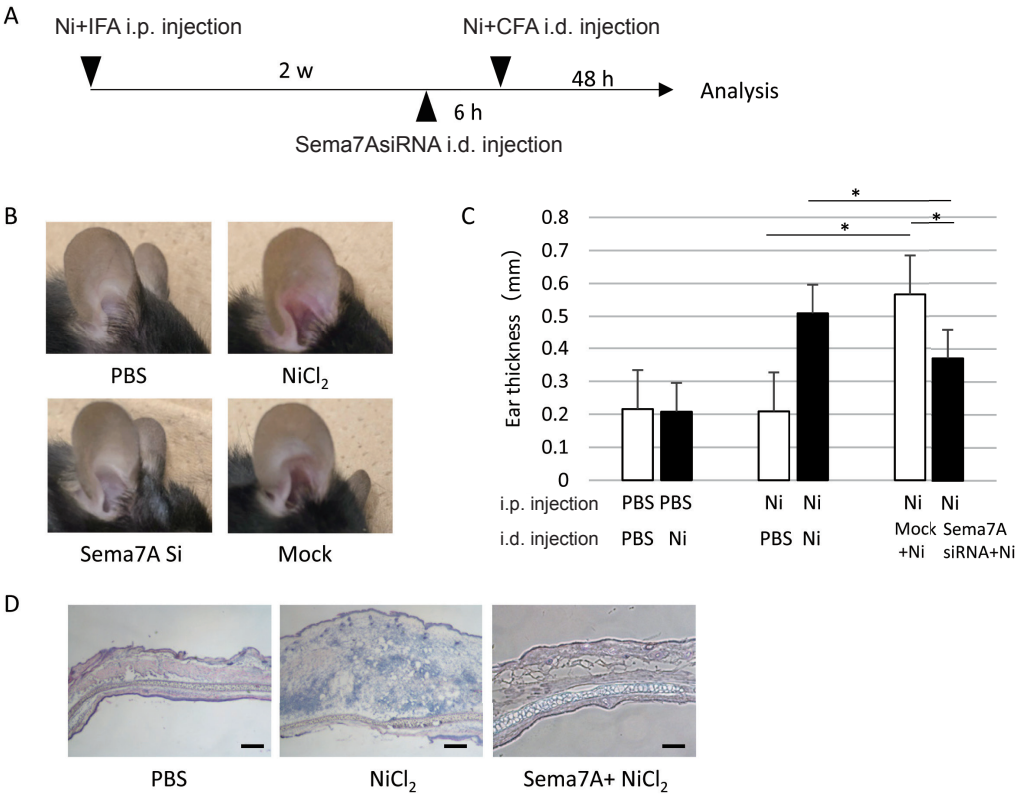

Fig. 4 Effect of Sema7A inhibition in Ni allergy mouse model
(A) Schedule for mouse ear swelling test (MEST) with Sema7A inhibition.
(B and C) Sema7A siRNA treated ear showed smaller swelling than conventional Ni allergic skin and mock siRNA treated ear skin. *$p < 0.05$
(D) H&E stain images of mouse ear skin. Lymphocytic infiltration was clearly decreased in subepithelial tissue by Sema7A inhibition. Scale bar, 100 μm

Discussion

The skin acts as the first barrier for the host defense by separating the body from the outside environment. Since it is directly exposed to various stressors such as bacteria, toxins, and pathogens, an intact skin immune function is essential for protecting the body. The skin immune system is supported by the cooperation of various cells, such as Langerhans cells (LCs), DETC, γδT cells[25, 26], and keratinocytes. The outer

layer of the skin is made of keratinocytes, which play a key role in regulating immune homeostasis and skin inflammation. Once keratinocytes are damaged or come in contact with pathogens, they produce TNF-α to induce the migration of LCs to the draining lymph nodes, after which they pressent antigens to T cells. T cells express cutaneous leukocyte antigen (CLA) on their surface and start migrating to the skin by being attracted by E-selectin, which is expressed in the endothelial cells in the inflamed area. In addition, TNF-α and IL-1β produced by damaged keratinocytes, induce CCL-27 in neighboring keratinocytes, and CCL-27 also attracts CLA positive T cells. Thus, keratinocytes orchestrate skin immunity.

In this study, we demonstrated that the expression of Sema7A was enhanced by $NiCl_2$, and suppression of Sema7A decreased the symptoms of Ni allergy.

Sema7A is expressed in various myeloid and lymphoid cells involved in immune responses. It is reported that Sema7A expressed on activated T cells binds to macrophages through α1β1 integrin to promote inflammation by increasing the production of proinflammatory cytokines such as TNF-α. Sema7A also induces monocytes to migrate to the inflamed part. Keratinocytes express Sema7A to initiate T cell-mediated inflammatory responses[27]. Actually, Sema7A knock-out mice show resistance to contact hypersensitivity (CHS) induced by DNFB because of the reduced effector immune responses, especially to impair the priming function of T cells[17].

In disordered skin such as psoriasis, various inflammatory cytokines are produced by several cells, such as Trm cells, LCs, and macrophages. Particularly, IFN-γ, TNF-α, and TGF-β increase expression of Sema7A on keratinocytes. Since activated keratinocytes secrete TNF-α and TGF-β, they could affect keratinocytes to upregulate the expression of Sema7A in an autocrine manner. In psoriasis patients, monocyte-derived dendritic epidermal cells (IDECs) exist in the epidermis of lesional skin[28], and they can be activated by Sema7A expressed on keratinocytes through β1 integrin to enhance inflammation in skin[27]. As recent studies suggested that psoriasis might be related to metal allergy[29, 30], our result of decreased allergic reactions to $NiCl_2$ may be related with the mechanism of psoriasis pathology. However, The injection of siRNA may affect other cells in the skin except for keratinocytes, so that using conditional Sema7A knock-out in keratinocytes should be required for a more detailed study.

In conclusion, Sema7A is associated with the development of Ni allergy, which may be crucial during the effector phase of allergic reactions. Interaction between Sema7A and α1β1 integrin has possibilities to be a therapeutic target for allergic diseases.

Acknowledgment

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Conflicts of Interest

The authors declare no conflict of interest.

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