原著論文

# 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 to produce 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 NiCl2 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 to produce allergic reactions to NiCl2.

Results: NiCl2 enhanced the expression of Sema7A in a dose and time-dependent manner after 72 hours of stimulation. PAM 2.12 produced TNF-α in response to NiCl2, and this secretion was reduced by Sema7A inhibition. In a mouse model, ear thickness, at 48 hours after NiCl2 injection, was significantly decreased by Sema7A siRNA administration.

Conclusions: Sema7A is essential to produce an allergic reaction to NiCl2, 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 by 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 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

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that keratinocytes enhance inflammation by releasing cytokines and chemokines such as TNF-α, IL-1β, 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 to produce 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 NiCl2 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 NiCl2 with Freund's complete adjuvant (CFA) (MP Biomedicals, Illkirch, France) using 28 G needles (TERUMO, Tokyo, Japan) for a recall immune response. DTH reactions were determined by measuring the changes in ear thickness at 24 or 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). To suppress Sema7A expression, transfection was performed with 1 nM Sema7A siRNA using INTERFERin (Polyplus-transfection®, Illkirch, France) 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 10min RT, then wash 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., MA, USA), Purified rat anti-mouse CD3 Antibody (1:300, BioLegend, Inc., CA ,USA), anti-rat Alexa Fluor 488 (A-21208, 1:500, Life Technologies Corporation, CA, USA), 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., MA, USA).

**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 Biosciences, CA, USA).

**ELISA**

1x$10^4$ PAM2.12 cells per well were seeded in a 96-well dish. Twenty-

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' |

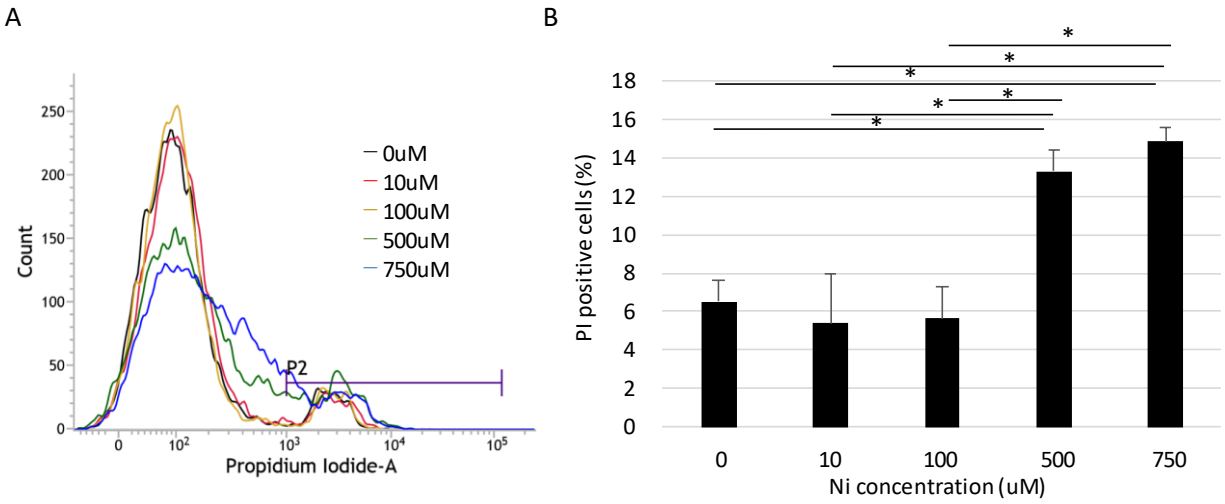


Fig. 1 Effect of $NiCl_2$ on PAM2.12.
(A) FCM analysis of cell viability on PAM2.12 at 48-hour stimulation with different concentration of $NiCl_2$.
(B) Dead cell/ Total cell rates are shown on the graph. At 48 hours of observation, no apparent cell death was observed when the concentration of $NiCl_2$ was 100 uM 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 (*$p<0.05$)

four hours later, PAM2.12 was stimulated with 10 μM NiCl2. To suppress Sema7A expression, transfection was performed with 1 nM Sema7A siRNA using INTERFERin (Polyplus-transfection®, Illkirch, France) 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-αin 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, Shiga, Japan) 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 $NiCl_2$.**

T To investigate the effect of Ni on keratinocytes, we used $NiCl_2$ as a metal antigen, which is high 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 NiCl2 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 stimulation (Fig. 2A), and

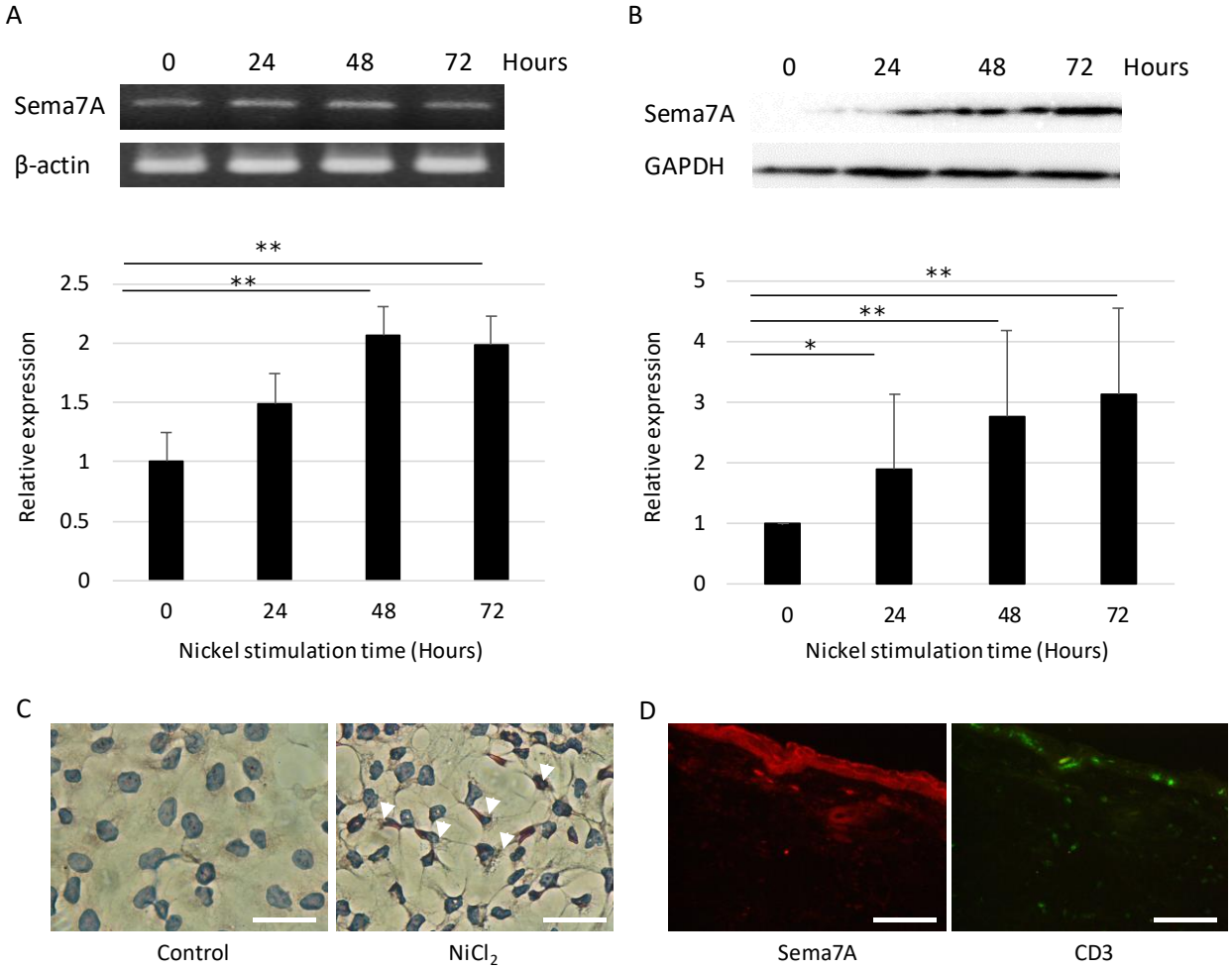


Fig. 2 Sema7A Expression on PAM2.12 with $NiCl_2$

(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, 50μm. (D) Immunofluorescence images of Sema7A (red) and CD3 (green) in mouse ear skin. Sema7A expressed in outer layer of epithelial tissue. CD3 positive cells were seen in the outer layer of epithelial tissue looks like dendritic epidermal T cells (DETC). Scale bar, 100μm

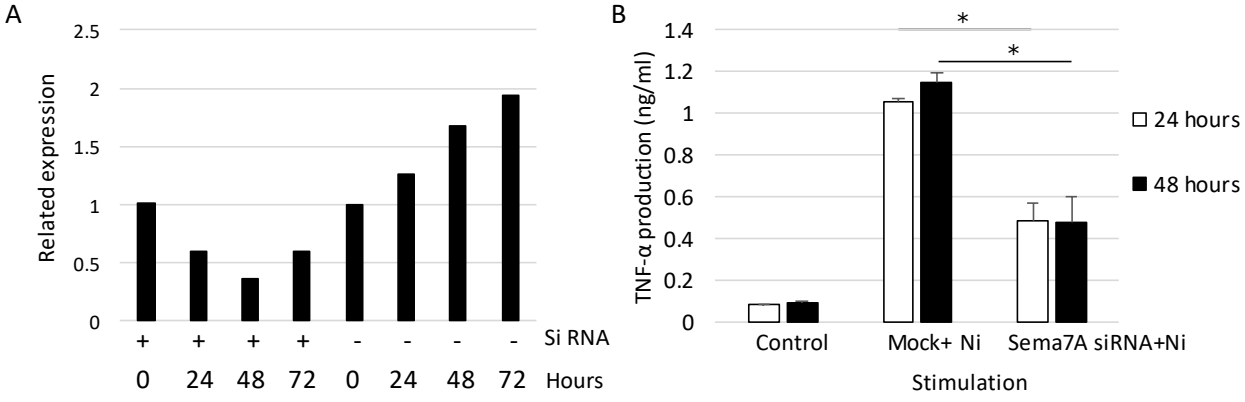


Fig. 3 Effect of Sema7A inhibition in PAM2.12 stimulated by $NiCl_2$.

(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

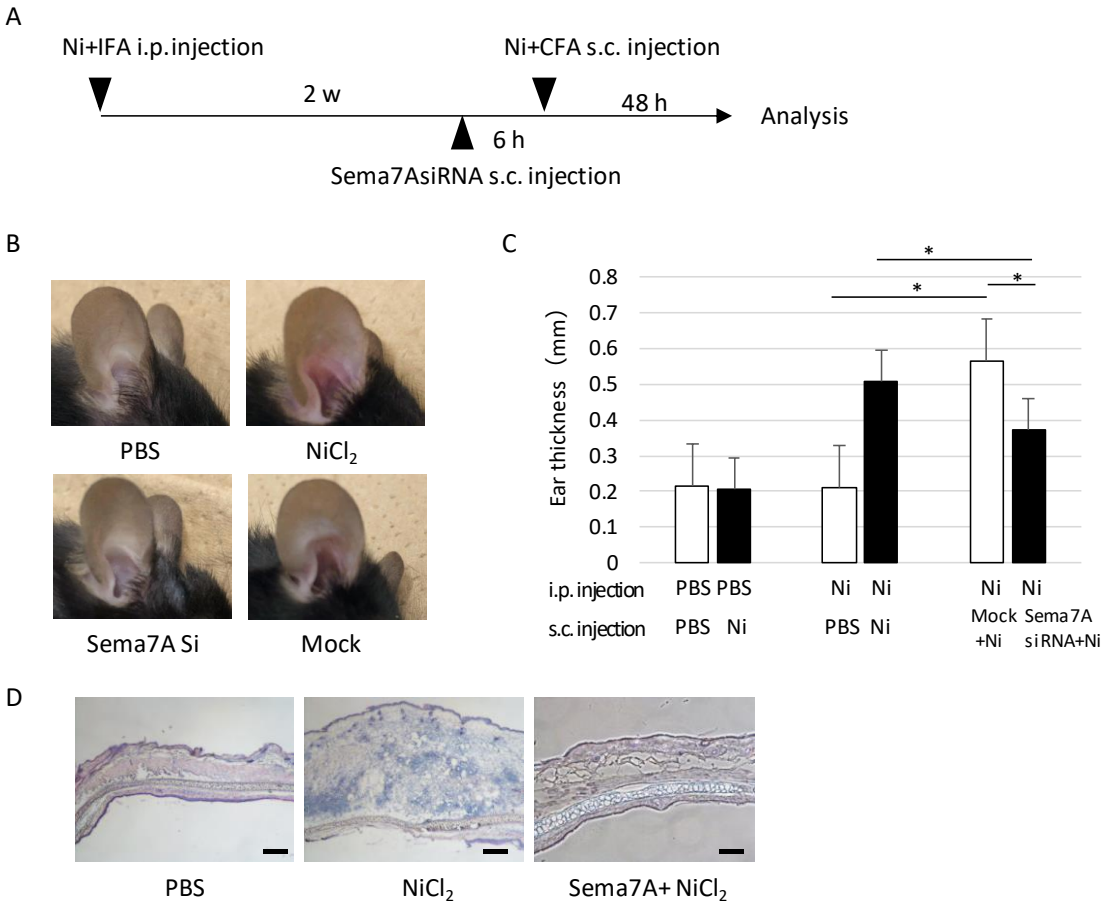


Fig. 4 Effect of Sema7A inhibition in Ni allergy mouse model.
(A) Schedule for mouse ear swelling test (MEST) with Sema7A inhibition. (B and C) Sema7A siRNA treated ear showed smaller swelling than conventional Ni allergic skin and mock siRNA treated ear skin. *p<0.05 (D) H&E stain images of mouse ear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

protein was increased in a time-dependent manner (Fig. 2B). The expression of Sema7A was expressed in keratinocytes mainly. Furthermore, those cells were localized on the outer layer of the epithelial tissue 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 was 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 metal 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 described23). Compared with the Ni allergy group, Sema7A expression was found to be suppressed in keratinocytes and subepithelial cells by Sema7A siRNA administration which was subcutaneously injected into the pinnae 6 hours before intradermal Ni injection for elicitation (Fig. 4A). Ear thickness of 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.

## Discussion

The skin act 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, γδ T cells[25,26], and keratinocytes. The outer layer of the skin is made by 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 reduced effector immune responses, especially to impair 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 decrease allergic reactions to $NiCl_2$, may be concerned with the mechanism of psoriasis pathology. However, our experiment of siRNA injection may include suppression of various cells in the skin, 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 for the effector phase of allergic reactions. Interaction between Sema7A and α1β1 integrin has possibilities to be a therapeutic target for allergic diseases.


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## Conflicts of Interest

The authors declare no conflict of interest.

## References


1) Büdinger L and Hertl M. Immunologic mechanisms in hypersensitivity reactions to metal ions: an overview. Allergy 55, 108-115 (2000)
2) Schnuch A, Aberer W, Agathos M, Becker D, Brasch J, Elsner P, Frosch P.J, Fuchs T, Geier J, Hillen U, Löffler H, Mahler V, Richter G, Szliska C and f.t.G.C.D.R. Group. Patch testing with contact allergens. JDDG: Journal der Deutschen Dermatologischen Gesellschaft 6, 770-775 (2008)
3) Hannuksela M. Epicutaneous testing. Allergy 34, 5-10 (1979)
4) Nielsen NH and Menné T. The relationship between IgE-mediated and cell-mediated hypersensitivities in an unselected Danish population: The Glostrup Allergy Study. British Journal of Dermatology 134, 669-672 (1996)
5) Thyssen JP, Linneberg A, Menné T and Johansen JD. The epidemiology of contact allergy in the general population – prevalence and main findings. Contact Dermatitis 57, 287-299 (2007)
6) Dotterud LK and Smith-Sivertsen T. Allergic contact sensitization in the general adult population: a population-based study from Northern Norway. Contact Dermatitis 56, 10-15 (2007)
7) Chambers ES, Vukmanovic-Stejic M. Skin barrier immunity and ageing. Immunology, doi:10.1111/imm.13152.
8) Salmon JK, Armstrong CA and Ansel JC. The skin as an immune organ. The Western Journal of Medicine 160, 146-152 (1994)
9) Tsepkolenko A, Tsepkolenko V, Dash S, Mishra A, Bader A, Melerzanov A and Giri S. The regenerative potential of skin and the immune system. Clinical, Cosmetic and Investigational Dermatology 12, 519-532 (2019)
10) Klicznik MM, Szenes-Nagy AB, Campbell DJ and Gratz IK. Taking the lead - how keratinocytes orchestrate skin T cell immunity. Immunology Letters 200, 43-51 (2018)
11) Saito M, Arakaki R, Yamada A, Tsunematsu T, Kudo Y and Ishimaru N. Molecular Mechanisms of Nickel Allergy. International Journal of Molecular Sciences 17, 202-209 (2016)
12) Ashrin MN, Arakaki R, Yamada A, Kondo T, Kurosawa M, Kudo Y, Watanabe M, Ichikawa T, Hayashi Y and Ishimaru N. A critical role for thymic stromal lymphopoietin in nickel-induced allergy

in mice. Journal of immunology 192, 4025-4031 (2014)

13) Curtis A, Morton J, Balafa C, MacNeil S, Gawkrodger DJ, Warren ND and Evans GS: The effects of nickel and chromium on human keratinocytes: differences in viability, cell associated metal and IL-1alpha release. Toxicology in vitro 21, 809-819 (2007)

14) Sebastiani S, Albanesi C, Nasorri F, Girolomoni G and Cavani A. Nickel-specific CD4(+) and CD8(+) T cells display distinct migratory responses to chemokines produced during allergic contact dermatitis. The Journal of investigative dermatology 118, 1052-1058 (2002)

15) Kolodkin A, Matthes DJ and Goodman CS. The Sema genes encode a family of transmembrane and secreted growth cone guidance molecules. Cell 75, 1389-1399 (1993)

16) Kikutani H and Kumanogoh A. Semas in interactions between T cells and antigen-presenting cells. Nature Reviews Immunology 3, 159-167 (2003)

17) Suzuki K, Okuno T, Yamamoto M, Pasterkamp RJ, Takegahara N, Takamatsu H, Kitao T, Takagi J, Rennert PD, Kolodkin AL, Kumanogoh A and Kikutani H. Sema7A initiates T-cell-mediated inflammatory responses through alpha1beta1 integrin. Nature 446, 680-684 (2007)

18) Xu X, Ng S, Wu ZL, Nguyen D, Homburger S, Seidel-Dugan C, Ebens A and Luo Y. Human Sema K1 Is Glycosylphosphatidylinositol-linked and Defines a New Subfamily of Viral-related Semas. The Journal of Biological Chemistry 273, 22428-22434(1998)

19) Lange C, Liehr T, Goen M, Gebhart E, Fleckenstein B and Ensser A. New eukaryotic Semas with close homology to Semas of DNA viruses. Genomics 51, 340-350(1998)

20) Pasterkamp RJ, Peschon JJ, Spriggs MK and Kolodkin AL. Sema7A promotes axon outgrowth through integrins and MAPKs. Nature 424, 398-405 (2003)

21) Artik S, Vultée CV, Gleichmann E, Schwarz T and Griem P. Nickel Allergy in Mice: Enhanced Sensitization Capacity of Nickel at Higher Oxidation States. The Journal of Immunology 163, 1143-1152 (1999)

22) Nakae S, Naruse-Nakajima1 C, Sudo K, Horai R, Asano M and Iwakura Y. IL-1α, but not IL-1β, is required for contact-allergen-specific T cell activation during the sensitization phase in contact hypersensitivity. International Immunology 13, 1471-1478 (2001)

23) Watanabe M, Ishimaru N, Ashrin MN, Arakaki R, Yamada A, Ichikawa T and Hayashi Y. A Novel DC Therapy with Manipulation of MKK6 Gene on Nickel Allergy in Mice. PLOS ONE 6, e19017 (2011).

24) Ermolli M, Menne C, Pozzi G, Serra MA and Clerici LA. Nickel, cobalt and chromium-induced cytotoxicity and intracellular accumulation in human hacat keratinocytes. Toxicology 159, 23-31 (2001)

25) Havran WL, Chien YH and Allison JP. Recognition of selfantigens by skin-derived T cells with invariant gamma delta antigen receptors. Science 252, 1430-1432 (1991)

26) Toulon A, Breton L, Taylor KR, Tenenhaus M, Bhavsar D, Lanigan C, Rudolph R, Jameson J and Havran WL. A role for human skin–resident T cells in wound healing. Journal of Experimental Medicine 206, 743-750 (2009)

27) Kamata M, Tada Y, Uratsuji H, Kawashima T, Asano Y, Sugaya M, Kadono T, Tamaki K and Sato S. Sema7A on keratinocytes induces interleukin-8 production by monocytes. Journal of Dermatological Science 62, 176-182 (2011)

28) Wollenberg A, Kraft S, Hanau D and Bieber T. Immunomorphological and ultrastructural characterization of Langerhans cells and a novel, inflammatory dendritic epidermal cell (IDEC) population in lesional skin of atopic eczema. Journal of Investigative Dermatology 106, 446-453 (1996)

29) Quaranta M, Eyerich S, Knapp B, Nasorri F, Scarponi C, Mattii M, Garzorz N, Harlfinger AT,Jaeger T, Grosber M, Pennino D, Mempel M, Schnopp C, Theis FJ, Albanesi C, Cavani A, Schmidt-Weber CB, Ring J and Eyerich K. Allergic contact dermatitis in psoriasis patients: typical, delayed, and non-interacting. PLoS One 9, e101814 (2014)

30) Rasool F, Akhtar S, Hassan I, Zeerak S, Mubashir S and Sheikh G. Common Contact Allergens in Patients with Palmoplantar and Scalp Psoriasis and Impact of their Avoidance on Dermatology Life Quality Index: A Hospital-Based Study. Indian Journal of Dermatology 63, 160-164 (2018)