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A Critical Role for Thymic Stromal Lymphopoietin in Nickel-Induced Allergy in Mice

Meinar Nur Ashrin,^{*,†} Rieko Arakaki,^{*} Akiko Yamada,^{*} Tomoyuki Kondo,^{*} Mie Kurosawa,^{*} Yasusei Kudo,^{*} Megumi Watanabe,[†] Tetsuo Ichikawa,[†] Yoshio Hayashi,^{*} and Naozumi Ishimaru^{*}

Ni is the most frequent cause of contact allergy induced by metals. However, the underlying mechanism of this induction is unknown. Our previous research demonstrates that activation of dendritic cells (DCs) through p38MAPK/MKK6 is required for Ni-induced allergy in mice. In the current study, we investigated the cellular and molecular mechanisms underlying Ni-induced allergy using a mouse model that involves injecting Ni into the ear, with or without Freund's incomplete or complete adjuvants. Nickel had greater potential to cause allergic reactions compared with palladium and gold. Among the proteins expressed at higher levels in mice with Ni-induced allergy, we focused on thymic stromal lymphopoietin (TSLP), which is produced in abundance by keratinocytes. We detected increased expression of the TSLP receptor (TSLPR) in DCs from cervical lymph nodes of mice with Ni-induced allergy, suggesting that DCs in ear tissues were activated through TSLPR signaling induced by keratinocyte-derived TSLP. Furthermore, delayed-type hypersensitivity reactions in mice with Ni-induced allergy were decreased significantly by injection of a *Tslp*-short interfering RNA along with atelocollagen in the ear skin. These results suggest that Ni allergy may be triggered by a TSLP/TSLPR-mediated interaction between epithelial and immune cells. *The Journal of Immunology*, 2014, 192: 000–000.

We continuously encounter materials containing Au, Ag, Hg, Ni, Ti, Cr, and Co in our daily lives. For example, Ni-based alloys are widely used in costume jewelry and dental materials, and Ni frequently causes contact allergy categorized as type IV delayed-type hypersensitivity (DTH), with skin inflammation mediated by hapten-specific T cells (1–5). Approximately 20% of women and 6% of men are sensitized to Ni (6). Symptoms of Ni allergy appear in the oral mucosa and are widely observed in the skin (7–9).

Ni ions (Ni²⁺) released from various alloys are potent allergens or haptens that can trigger inflammation of the skin (10–12). Ni²⁺ penetrates the skin and activates epithelial cells that produce cytokines or chemokines following hyperreactive immune responses involving the activation of APCs and T cells (13–15). APCs, such

as Langerhans cells or dendritic cells (DCs), are induced by certain cytokines, including IL-1 β and TNF- α , which are produced by epithelial cells (16–19). APCs migrate to draining lymph nodes where they present allergens or haptens to naive T cells. Subsequent re-exposure to the same allergen or hapten leads to a hypersensitivity reaction during the effector phase (19). However, the precise molecular mechanisms that mediate interactions between epithelial and immune cells in Ni allergy are unknown.

We reported previously that Ni-activated dermal DCs play an important role in the development of allergic reactions through the activation of p38 MAPK in a model system in which mice are exposed to Ni combined with adjuvants (20). Although Langerhans cells or dermal DCs play fundamental roles in the initial phase of Ni allergy, the mechanisms that regulate dermal APC responses are unknown. Therefore, using this mouse model system, we addressed this question by determining the expression levels of genes involved in allergy. We focused on thymic stromal lymphopoietin (TSLP) and its receptor (TSLPR) because their expression levels were increased significantly in the ear tissues of mice with Ni-induced allergy compared with those of other genes. Moreover, we assessed whether these findings could be used to devise new therapeutic strategies or diagnostic tools to address metal allergies.

Materials and Methods

Mice

Female C57BL/6J mice (8-wk old) were purchased from CLEA Japan (Tokyo, Japan). *Nf- κ B*^{−/−} mice were obtained from The Jackson Laboratory. Mice were provided a standard laboratory diet and water. They were maintained in specific pathogen-free conditions and a temperature-controlled environment under a 12-h light/dark cycle. The Animal Ethics Board of the University of Tokushima approved all procedures.

Induction of DTH using metals

To induce DTH with Ni, 125 μ l 1-mM NiCl₂, with or without 125 μ l IFA (ICN Biomedicals, Aurora, OH), was injected i.p. for initial immunization. Two weeks later, 10 μ l 0.2 mM NiCl₂, with or without 10 μ l CFA (ICN Biomedicals), was injected intradermally into the ear skin using a

^{*}Department of Oral Molecular Pathology, Institute of Health Biosciences, The University of Tokushima Graduate School, Tokushima 770-8504, Japan; and [†]Department of Oral Maxillofacial Prosthodontics, Institute of Health Biosciences, The University of Tokushima Graduate School, Tokushima 770-8504, Japan

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The sequences presented in this article have been submitted to the Gene Expression Omnibus (<http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE52466>) under accession number GSE52466.

Address correspondence and reprint requests to Dr. Naozumi Ishimaru, Department of Oral Molecular Pathology, Institute of Health Biosciences, The University of Tokushima Graduate School, 3-18-15 Kuramotocho, Tokushima 770-8504, Japan. E-mail address: ishimaru@dent.tokushima-u.ac.jp

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Abbreviations used in this article: AD, atopic dermatitis; cLN, cervical lymph node; DC, dendritic cell; DTH, delayed-type hypersensitivity; siRNA, small interfering RNA; TSLP, thymic stromal lymphopoietin; TSLPR, TSLP receptor.

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26-gauge needle (TERUMO, Tokyo, Japan). DTH was determined 48 h after the second challenge by measuring changes in ear thickness using a digital caliper. To induce DTH using other metals, 125 μ l 1 mM PdCl₂ or AuCl₃, together with 125 μ l IFA, was used for the first injection, and 10 μ l 0.2 mM PdCl₂ or AuCl₃, together with 10 μ l CFA, was used for the second injection.

Histology

Ear tissues were fixed with 10% phosphate-buffered formaldehyde (pH 7.2). Paraffin-embedded tissue sections (4 μ m) were stained with H&E.

Flow cytometric analysis

Mononuclear cell preparations (1×10^6 cells) from cervical lymph nodes (cLNs) were reacted with 1 μ g/ml goat polyclonal anti-TSLP Ab (TSLPR; R&D Systems, Minneapolis, MN), which was detected with Alexa Fluor 568-conjugated anti-goat IgG (Invitrogen, Eugene, OR). Cells were analyzed using a FACSCanto (BD Biosciences, San Jose, CA).

Immunohistochemistry

Ear tissues were embedded in tissue-freezing medium (Sakura Finetek USA, Torrance, CA) and rapidly frozen. Frozen sections (6- μ m thick) were mounted on poly-L-lysine-coated glass slides. To determine TSLP expression, the specimens were incubated with anti-mouse TSLP Ab (R&D Systems) and subsequently with Alexa Fluor 568-conjugated anti-goat IgG (Invitrogen). Nuclear DNA was stained with DAPI. Sections were analyzed using a confocal laser scanning microscope (LSM 5 PASCAL; Carl Zeiss, Oberkochen, Germany). Paraffin-embedded sections were stained with anti-TSLP polyclonal Ab (Abcam, Cambridge, MA), and Ag-Ab complexes were detected using an LSAB2 kit containing HRP (Dako, Carpinteria, CA) and 3,3'-diaminobenzidine as substrate. Nuclei were counterstained with hematoxylin.

Quantitative RT-PCR

Total RNA was isolated from the ear tissues of Ni-treated and control mice using ISOGEN (Wako Pure Chemical, Osaka, Japan). Total RNA (500 ng) was reverse transcribed using a PrimeScript II 1st strand cDNA Synthesis Kit (Takara, Kyoto, Japan). This cDNA was used as the PCR template. Transcript levels were measured using a 7300 Real-Time PCR System (Applied Biosystems, Foster City, CA) with SYBR Premix Ex Taq (Takara). The primers were as follows: mouse TSLP, forward 5'-CGA CAG CAT GGT TCT TCT CA-3' and reverse 5'-CGA TTT GCT CGA ACT TAG CC-3'; TLR4, forward 5'-CAG TGG TCA GTG TGA TTG TGG-3' and reverse 5'-TTC CTG GAT GAT GTT GGC AGC-3'; and β -actin, forward 5'-CTC TTT GAT GTC ACG CAC GAT TTC-3' and reverse 5'-GTG GCC GCT CTA GGC ACC AA-3'. Expression levels were normalized to those of β -actin.

Gene-expression analysis

Gene-expression profiling analysis was performed, according to the manufacturer's instructions, using the RT2 Profiler PCR array (SABiosciences, Frederick, MD) related to allergy and asthma (PAMM-067A) that included 84 genes. In brief, total RNA from ear skin tissues was extracted using RNeasy kits (QIAGEN, Valencia, CA) and reverse transcribed. The cDNA was applied in RT2 Profiler PCR array (SABiosciences) plates to detect the expression of genes related to allergy using a 7300 Real-Time PCR System (Applied Biosystems). The PCR array data are available from the Gene Expression Omnibus database under accession number GSE52466 (<http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE52466>).

TSLP ELISA

Inflamed ear tissues of mice with Ni-induced allergy and control mice were punched out (circles of 1.5-mm diameter) and stored at -80°C in extraction buffer containing a protease inhibitor mixture (Sigma-Aldrich) containing 5 mM EDTA in PBS and 2 $\times$ Assay Diluent (BioLegend, San Diego, CA). The ear tissues were homogenized using a TissueLyser (QIAGEN) with 5-mm siliconized glass beads. Homogenized samples were centrifuged at $10,000 \times g$ at 4°C for 30 min, and the supernatants were analyzed for mouse TSLP levels using Mouse TSLP ELISA Max Deluxe Sets (BioLegend).

Culture of mouse keratinocytes (COCA cells)

The mouse epidermal keratinocyte COCA cell line (Health Protection Agency, Salisbury, U.K.) was maintained in epidermal keratinocyte medium CnT-07 (CELLnTEC, Bern, Switzerland). Cells were cultured at 37°C in

humidified air containing 5% CO₂. When the cells were 80% confluent, they were stimulated with 500 or 1000 nM Ni or 1 μ g/ml the TLR 4 agonist LPS (Sigma Aldrich) extracted from the outer membrane of *Escherichia coli* 0111:B4 in DMEM containing 1% FCS for 24 h. For in vitro TLR4 gene knockdown, COCA cells were transfected with TLR4 or control small interfering RNAs (siRNAs; 10 nM) using Lipofectamine RNAiMAX (Life Technologies). TLR4 and negative-control siRNAs (siTrio; cat no. S30C-0126) were purchased from Cosmo Bio (Tokyo, Japan). The sequences of three pairs of oligonucleotides were as follows: sense, 5'-GCA UAG AGG UAG UUC CUA ATT-3'; antisense, 5'-UUA GGA ACU ACC UCU AUG CTT-3'; sense, 5'-CAC AAG AGC CGG AAG GUU ATT-3'; antisense, 5'-UAA CCU UCC GGC UCU UGU GTT-3'; sense, 5'-GAG AAG AGG CUA AGG CCU ATT-3'; and antisense, 5'-UAG GCC UUA GCC UCU UCU CTT-3'.

TSLP siRNA

TSLP (Custom siSET-3) and control scrambled siRNAs were purchased from Cosmo Bio (Tokyo, Japan). The sequences of the three pairs of oligonucleotides were as follows: 1) TSLP: sense, 5'-CAC AAG AAG UCC AAA ACA UTT-3'; antisense, 5'-AUG UUU UGG ACU UCU UGU GTT-3'; 2) sense, 5'-GGG AGA AAC UCC UGA GAU CTT-3'; antisense, 5'-GAU CUC AGC AGU UUC UCC CTT-3'; 3) sense, 5'-CCU GAC AAA UUC UAA GAU UTT-3'; and antisense, 5'-AAU CUU AGA AUU UGU GAG GTT-3' and 2) control: sense, 5'-AUC GAA UAA CCG UAA CGU UGA-3'; and antisense, 3'-UAG CUU AUU GGC AUU GCA ACU-5'. The siRNAs were injected into the ear skin using an in vivo siRNA transfection local use AteloGene kit (KOKEN, Tokyo, Japan). In brief, 10 or 50 μ M TSLP siRNA was mixed with AteloGene, rotated gently at 4°C for 20 min, and centrifuged at $10,000 \times g$ at 4°C for 1 min. According to a published protocol for inducing Ni allergy (20), this solution was injected s.c. into ear pinnae 3 d before the second challenge.

Western blotting

Proteins purified from CD11c⁺ cells isolated from cLNs using an anti-CD11c mAb (eBioscience) and magnetic beads (Dyna) were subjected to SDS-PAGE using a 10% acrylamide gel, the proteins were transferred onto a polyvinylidene fluoride membrane, and the electroblotted membranes were incubated with anti-p38 and phospho-p38 mAbs (Cell Signaling Technology, Danvers, MA). Immune complexes were detected using HRP-conjugated anti-mouse IgG (Bio-Rad Laboratories) and ECL Plus reagents (Amersham Bioscience, Piscataway, NJ).

Statistical analysis

Results are expressed as the mean $\pm$ SD. Statistical comparisons were performed using the Student *t* test. The *p* values < 0.05 were considered statistically significant.

Results

Analysis of immune responses induced by Ni, Pd, and Au

DTH to Ni, Pd, and Au was induced using the protocol described in *Materials and Methods* (Fig. 1A). The mean ear thickness of mice injected with Pd or Au was slightly increased compared with that of control mice (Fig. 1B). In contrast, the mean ear thickness of mice injected with Ni was significantly increased compared with that of controls (Fig. 1B). Redness and swelling were observed in the ear skin of mice injected with Ni + IFA or Ni + CFA but not in that of controls (Fig. 1C). Further, histological examination revealed edema, congestion, and infiltration of inflammatory cells only in the ear tissues of mice injected with Ni + IFA and Ni + CFA (Fig. 1D). These results show that Ni, but not Pd or Au, induced allergic reactions. However, skin lesions induced by Ni did not ulcerate or erode. We attribute this to the concentration of CFA used.

Allergy-related gene-transcription profiling of mice with Ni allergy

We used PCR array analysis to investigate the expression of genes involved in allergy and asthma in ear tissues of mice with Ni-induced allergy compared with those of controls. The list of genes encoding cytokines, chemokines and their receptors, transcription

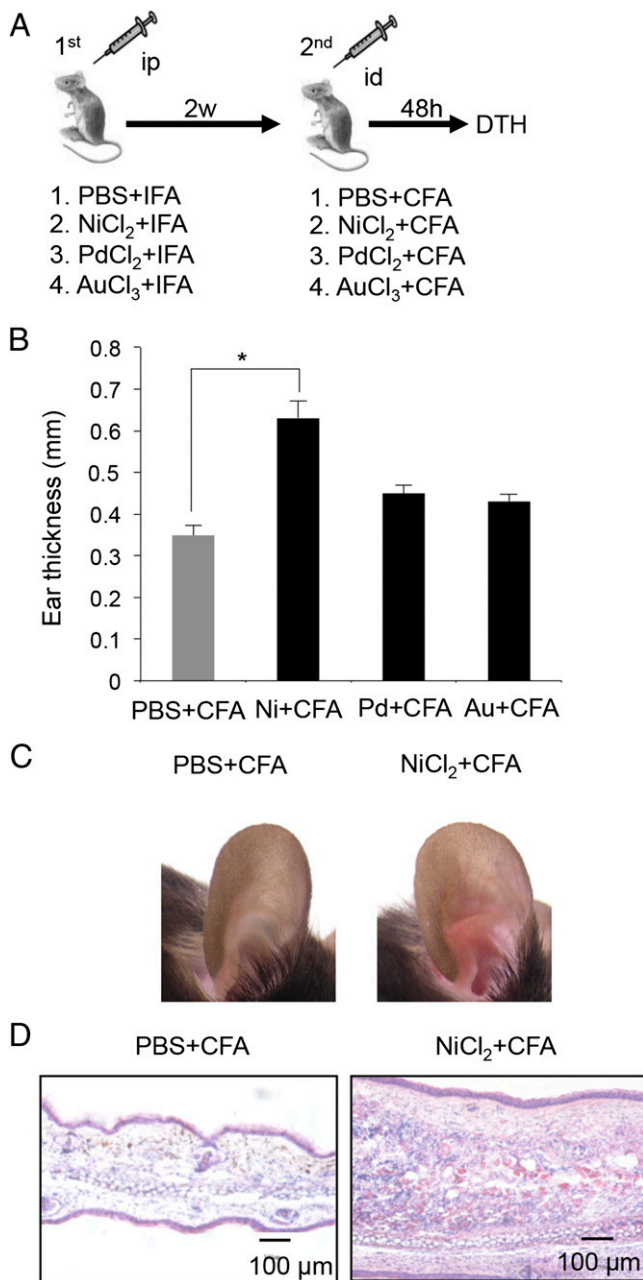


FIGURE 1. Comparison of immune responses to Ni, Pd, and Au. **(A)** Protocol for generating metal allergy models. Mice were treated with metal salts and IFA or CFA, as described in *Materials and Methods*. **(B)** DTH was determined by measuring ear thicknesses 48 h after the second challenge. Data are mean $\pm$ SD for four mice/group using data from one of three independent experiments. **(C)** Representative photographs of inflammatory lesions in the ear tissues of control and NiCl₂ + CFA-injected mice. **(D)** Histology of inflammatory lesions in ear tissues. Representative photomicrographs (H&E stain) of four mice/group are shown as data from three independent experiments. * $p < 0.05$.

factors, and IgE; genes expressed in mast cells, eosinophils, NK cells, and alternatively activated macrophages; and genes expressed during asthma is available at the Gene Expression Omnibus Web site under accession number GSE52466 (<http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE52466>). Upregulated genes were defined as those with a ≥ 3 -fold higher expression in mice with Ni-induced allergy compared with controls: *Tslp*, *Ear5*, *Cd40lg*, *Csf2*, *Tnfrsf4*, *Chi3l1*, *Kit*, *Crlf2* (*Tslpr*), *Il18*, *Cml1*, *Ccl12*, *Ccl22*, *Tpsb2*, and *Il13* (Supplemental Table I). In the following section,

we focus on the role of TSLP and its receptor, TSLPR, because their transcript levels were significantly upregulated (TSLP: 7.02; TSLPR: 3.38).

TSLP expression in ear tissues of mice with Ni-induced allergy

Tslp mRNA expression in the ear tissues of mice with Ni-induced allergy and control mice was determined using quantitative RT-PCR. In mice with Ni-induced allergy, *Tslp* mRNA expression was significantly increased compared with CFA-injected or naive mice

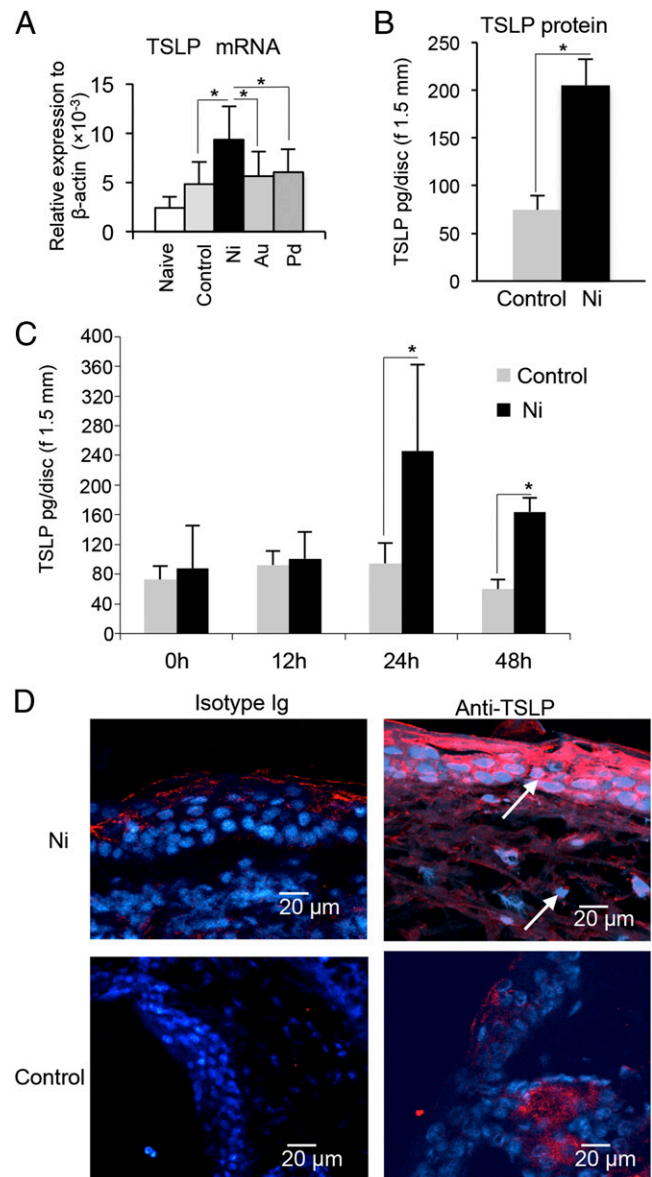


FIGURE 2. TSLP expression in ear tissues of mice with Ni allergy. **(A)** Quantitative RT-PCR analysis of *Tslp* mRNA expression in ear tissues of naive mice and those injected with PBS + CFA (control), Ni (Ni + CFA), Au (Au + CFA), or Pd (Pd + CFA) 24 h after the second treatment. Data are mean $\pm$ SD for 10 mice/group. **(B)** ELISA of TSLP levels in ear tissue samples (1.5-mm diameter) from mice with Ni allergy and controls 48 h after the second challenge. Data are mean $\pm$ SD for five mice/group. **(C)** ELISA of TSLP levels in ear tissue samples from mice with Ni allergy 0–48 h after the second treatment. Data are mean $\pm$ SD for five mice/group. **(D)** TSLP expression in the allergic lesions was detected using immunofluorescence assays. Results are representative of four mice/group. Alexa Fluor 568-conjugated anti-goat IgG was used as the secondary Ab. Cell nuclei were stained with DAPI. Arrows indicate TSLP expression. The results represent three independent experiments. * $p < 0.05$.

(Fig. 2A). In contrast, injection of Au or Pd had no significant differential effect (Fig. 2A). The *Tslp* mRNA expression of Ni-injected mice was significantly higher than that of Au- or Pd-injected mice (Fig. 2A). Furthermore, ELISAs showed that TSLP levels in ear tissues of mice with Ni-induced allergy were significantly increased compared with those of controls (Fig. 2B). Furthermore, we determined the changes in TSLP levels after a second injection of Ni and observed that they reached a peak after 24 h. The level decreased slightly after 48 h, but it was significantly higher than that in controls (Fig. 2C). To determine the identities of cells that produced TSLP, we used immunofluorescence staining to determine TSLP expression levels in allergic lesions. TSLP immunofluorescence staining was intense in epithelial cells and weak in immune cells in stromal tissues. However, a small amount of TSLP was detected in ear tissues of control mice (Fig. 2D). Using immunohistochemistry, we detected higher TSLP expression in the epithelium of ear tissues of mice with Ni-induced allergy (CFA + Ni) compared with control (CFA) mice, whereas lower TSLP expression was observed in the ear tissue of naive mice (Supplemental Fig. 1).

TSLPR expression in CD11c⁺ DCs from cLNs of mice with Ni-induced allergy

The results of immunofluorescence analysis suggested that epithelial cells may produce TSLP during an allergic reaction to Ni. Because TSLPR is expressed in a variety of cells, including DCs (21), we hypothesized that TSLP produced by epithelial cells in response to Ni may engage the TSLPR to induce DC activation or their migration into the cLNs. Therefore, we analyzed TSLPR expression in DCs from the cLNs of mice. Flow cytometry showed that TSLPR expression increased significantly in CD11c⁺ DCs from cLNs of mice with Ni-induced allergy (Fig. 3A, 3B).

TSLP production by keratinocytes after Ni treatment

We subsequently determined whether the COCA epithelial cell line secreted TSLP in the presence of Ni (Fig. 4). TSLP production increased as a function of Ni concentration (Fig. 4A). Ni triggers DTH by directly activating human TLR4 (22). Therefore, we

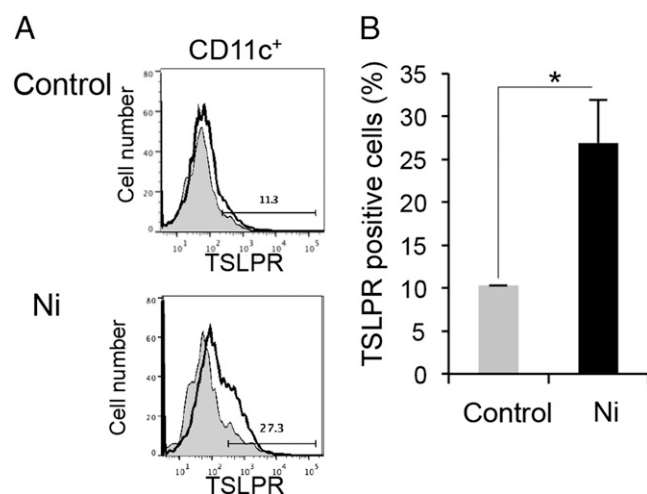


FIGURE 3. TSLPR expression by CD11c⁺ DCs from cLNs of mice with Ni-induced allergy. **(A)** Flow cytometric analysis of TSLPR expression by CD11c⁺ DCs of control mice (CFA-injected) and mice with Ni-induced allergy. Bold lines represent staining of cells with a TSLPR Ab. Shaded graphs represent the control. Representative results are shown. **(B)** TSLPR expression by CD11c⁺ DCs in cLNs of mice with Ni-induced allergy and controls (CFA injected). Data are mean $\pm$ SD for five mice/group. The results represent three independent experiments. * $p < 0.05$.

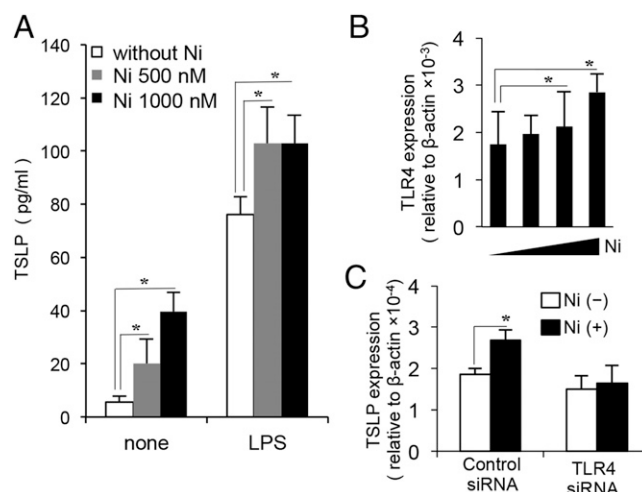


FIGURE 4. Analysis of TSLP signaling through TLR4 in keratinocytes treated with Ni. **(A)** TSLP production by COCA cells after Ni treatment. The mouse epidermal keratinocyte COCA cell line was stimulated with Ni, LPS, or Ni + LPS for 24 h. TSLP production in culture supernatants was determined using ELISA. LPS (1 μ g/ml) was used as a TLR4 agonist. **(B)** TLR4 mRNA expression of COCA cells cultured with Ni (0, 250, 500, and 1000 nM) was quantified using real-time RT-PCR. **(C)** COCA cells were transfected with TLR4 or control siRNA (10 nM), and 24 h later, the cells were stimulated with Ni for an additional 24 h. TSLP mRNA was analyzed using real-time RT-PCR. Data are mean $\pm$ SD of triplicate assays. The results represent three independent experiments. * $p < 0.05$.

examined the effects of the TLR4 agonist LPS on TSLP production by COCA cells. Ni added together with LPS synergistically increased TSLP production by COCA cells (Fig. 4A), as well as by the human keratinocyte HaCaT cell line (data not shown). Taken together, these results suggest that Ni enhances TSLP production by keratinocytes together with other stimulators, such as TLR agonists. We subsequently investigated whether Ni controls TLR4 expression by keratinocytes and observed that TLR4 mRNA expression by COCA cells was increased by the addition of Ni (Fig. 4B). Furthermore, knockdown of TLR4 gene expression using an siRNA inhibited Ni-induced TSLP expression in COCA cells (Fig. 4C, Supplemental Fig. 2). These findings suggest that the control of TSLP expression by keratinocytes treated with Ni is partially dependent on TLR4 signaling.

Therapeutic effects of TSLP siRNA on Ni-induced allergic reactions

Our findings that Ni induced the secretion of TSLP by keratinocytes led us to test a possible therapeutic strategy by targeting a putative pathogenic gene. Using our model system to induce Ni allergy, we determined the effect of injecting a TSLP siRNA together with atelocollagen into the ear skin 3 d before the second Ni injection and evaluated DTH by measuring ear thicknesses after 48 h (Fig. 5A). Mice treated with TSLP siRNA exhibited significantly decreased ear skin thickness as a function of siRNA dose compared with controls (Fig. 5B). Moreover, the levels of TSLP protein and mRNA expression were decreased in mice injected with TSLP siRNA (Fig. 5C, Supplemental Fig. 3). These results suggest that TSLP siRNA may provide an effective treatment for Ni allergy.

Molecular mechanism underlying Ni allergy

p38/MKK6 activation in DCs plays a key role in the induction of Ni allergy (20). Therefore, we determined whether p38/MKK6 was phosphorylated in DCs of mice with Ni-induced allergy. Western blotting revealed increased p38 phosphorylation in CD11⁺ DCs of cLNs from mice with Ni-induced allergy (Fig. 6A).

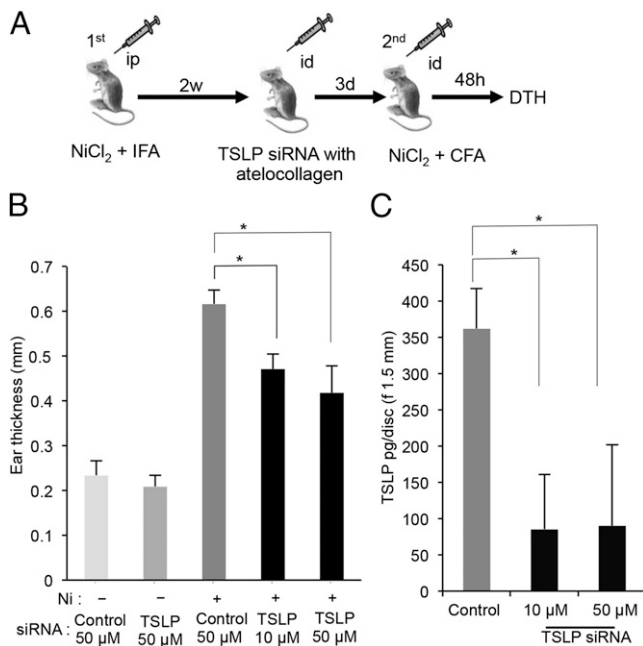


FIGURE 5. Therapeutic effects of TSLP siRNA on allergic reactions to Ni. **(A)** TSLP siRNA plus atelocollagen were injected s.c. into the ear skin 3 d before the second challenge. **(B)** Measurements of ear thickness of allergic mice treated with siRNAs. Data are mean $\pm$ SD for five mice/group. **(C)** TSLP expression in ear tissue samples (1.5-mm diameter) from allergic mice treated with siRNAs was determined using ELISA. Data are mean $\pm$ SD for five mice/group, * p < 0.05.

Furthermore, we determined whether Ni treatment contributed to NF- κ B activation or IL-1 β expression in vivo. When we compared DTH (ear thickness) induced by Ni + CFA in *Nf- κ B1*^{-/-} mice with that of wild-type mice, there was no significant difference (Fig. 6B). These results demonstrate that increased TSLP production by keratinocytes is independent of NF- κ B signaling.

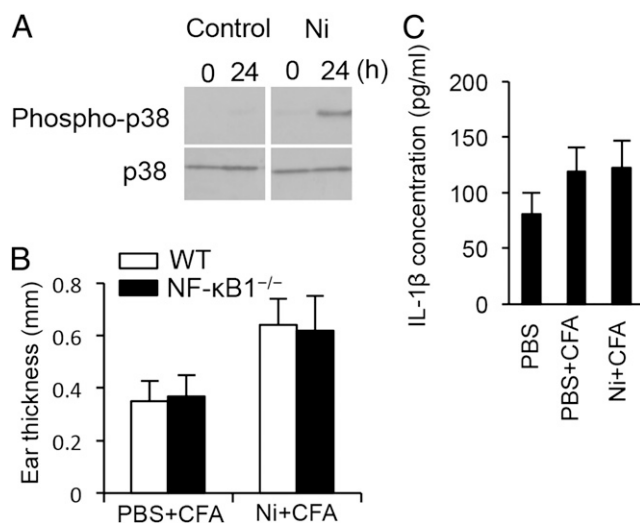


FIGURE 6. Molecular mechanism of Ni allergy. **(A)** Upregulated p38 phosphorylation in cLN tissues from allergic mice was detected using Western blotting. The total p38 expression level served as a loading control. The results represent five mice/group. **(B)** Ni allergy was induced in wild-type and *Nf- κ B1*^{-/-} mice. DTH was determined by measuring ear thicknesses 48 h after the second treatment with Ni. Data are mean $\pm$ SD for five mice/group. **(C)** The concentrations of IL-1 β in sera from naive, control, and allergic mice were determined using ELISA. Data are mean $\pm$ SD for five mice/group.

Moreover, IL-1 β concentration in the sera of mice with Ni-induced allergy was not increased compared with that in controls (Fig. 6C).

Discussion

Contact hypersensitivity to Ni is the most common cause of contact dermatitis. Although Ni is widely used in dentistry and fabricating jewelry, its potential to induce allergic reactions is unknown. To investigate the mechanisms underlying allergic reactions to Ni, we used an established animal model of Ni allergy in this study (20). Thus, DTH was induced in C57BL/6J mice by systemically and locally injecting NiCl₂ with IFA and NiCl₂ with CFA, respectively. Induction of Ni allergy requires an adjuvant, such as LPS (23, 24). These studies show that using CFA during the second injection influences the severity of Ni-induced DTH, suggesting that the adjuvant plays a significant role in triggering or enhancing an allergic reaction to Ni. Our model of Ni allergy differs from others, because CFA is administered together with the second injection of Ni (20), and we reproduced this finding in this study. In particular, a small (20–30%), but statistically significant, increase in ear-skin thickness was observed when NiCl₂ alone was used for the second injection (20). Further, histological analysis showed only slight inflammation of ear tissue (20).

Therefore, we attempted to establish a model for more severe Ni allergy using adjuvants, because some patients present with erosion and infection of lesions in the skin and oral mucosa, suggesting that the development of metal allergy may be exacerbated by other factors, such as bacterial infection. Although Ni is the most frequent cause of metal allergy (2, 5), the precise mechanisms underlying Ni-specific reactions remain unknown. In a previous report (20) using an Ni allergy model, we demonstrated that the activation of DCs through p38MAPK/MKK6 is involved in the induction of Ni allergy.

In the current study, we observed that the allergic reaction to Ni was more intense compared with other metals, such as Au and Pd. We subsequently searched for genes that were differentially expressed in allergic mice. We succeeded in showing that the genes encoding TSLP and TSLPR were expressed at considerably higher levels in allergic mice. TSLP, which is produced by keratinocytes, the tonsil-crypt epithelium, and bronchial epithelial cells, mediates allergic inflammatory reactions in patients with asthma and atopic dermatitis (AD) (25, 26). The TSLPR complex comprises the TSLP receptor and IL-7R α (27–30).

In the current study, we show that the COCA keratinocyte cell line produced TSLP after treatment with Ni and that Ni synergistically enhanced TSLP production when added to these cells together with the TLR4 agonist LPS. Murine, but not human, TLR4 does not respond to nickel stimulation (22). In contrast, LPS is an important inducer of Ni allergy, and LPS-enhanced Ni allergy is not observed in TLR4-mutant mice (24). Moreover, TLR4 expressed by keratinocytes regulates wound healing through CCL5 expression (31). Therefore, we speculate that TLR4 produced by keratinocytes may play a key role in Ni allergy, although the precise signaling pathway remains unclear. Keratinocytes produce danger signal-induced cytokines or chemokines in the skin (29). Consistent with these in vitro results, we observed that an Ab against TSLP intensely stained keratinocytes present in lesions of mice with Ni-induced allergy. These results show that TSLP produced by epithelial cells is a factor in the pathogenesis of Ni allergy. TSLP initiates a wide variety of responses in several cell types and contributes to the activation and maturation of B cells, DCs, and T cells (30). Thus, it is reasonable to conclude that TSLP contributes to DC activation through p38/MKK6 to induce Ni allergy.

This conclusion is supported by our demonstration that TSLP mRNA and protein levels in the skin of mice with Ni-induced allergy were significantly increased compared with those of control mice. TSLP is expressed by keratinocytes in acute and chronic skin lesions of patients with AD but not in uninvolved skin of patients with AD (32). TSLP production in skin lesions is restricted to epidermal keratinocytes in the suprabasal layer and is absent in the undifferentiated basal layer (33). In Ni allergy, TSLP produced by keratinocytes may play an important role in inducing DTH. Moreover, nonhematopoietic cells, such as skin keratinocytes, epithelial cells, smooth muscle cells, and lung fibroblasts, express high amounts of TSLP when cultured in growth medium, suggesting that these cells are capable of producing TSLP (33).

Human TSLP potently activates human CD11c⁺ DCs but does not directly affect B cells, T cells, NK cells, neutrophils, or mast cells (29). Further, DCs are activated through TSLPR in response to TSLP, which is expressed by epithelial cells in AD lesions (34). TSLPR is expressed by several immune cell types, including DCs, T cells, B cells, mast cells, NK cells, and monocytes, as well as in heart, skeletal muscle, kidney, and liver tissues (35). In the current study, TSLPR expression was increased in CD11c⁺ DCs from cLNs of mice with Ni-induced allergy.

DC activation is a critical step in the pathogenesis of allergic inflammation (20). TSLP is highly expressed by keratinocytes in AD lesions, and TSLP-stimulated DCs prime naive CD4⁺ T cells to produce IL-4, IL-13, IL-5, and TNF- α (33). Our hypothesis derived from the present results proposes that TSLP-stimulated DCs migrate into draining lymph nodes, that these DCs express TSLPR more intensely in mice with Ni-induced allergy, and that allergen-specific T cell proliferation and differentiation into Th1 cells are induced in the regional lymph nodes in response to the allergic reaction to Ni. This hypothesis is supported by findings that T cells infiltrate skin tissue of mice with Ni-induced allergy, similar to the findings reported for patients with AD (36). Moreover, skin-homing T cells from AD lesions produce Th2 cytokines (37). The results of the current study suggest that TSLP production by epithelial cells increased in response to Ni and stimulated DC maturation, activation, and migration into cLNs, where DCs enhance Ni-specific T cell responses that result in DTH through a complex mechanism.

In this study, we demonstrate for the first time, to our knowledge, that the pathogenesis of Ni allergy proceeds through a complex mechanism involving a TSLP/TSLPR-mediated interaction between the local immune system and epithelial tissues in response to Ni. Moreover, injecting siRNA, which targeted *Tslp* mRNA, mitigated symptoms in mice with Ni-induced allergy. Downregulation of Ni-induced TSLP production by epithelial cells may inhibit the early steps of the immune response through DC functions. This suggests that a new therapeutic strategy using TSLP and its receptor may be applicable to Ni allergy.

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Disclosures

The authors have no financial conflicts of interest.

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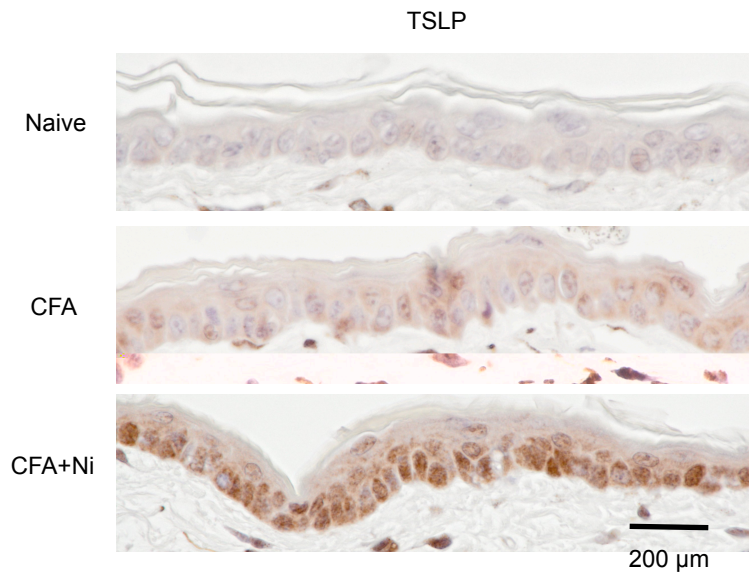
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Supplementary Table 1 Allergy-related gene profiling in the ear tissue of Ni allergy model by RT2 Profiler PCR array

Gene	Fold difference	Gene	Fold difference	Gene	Fold difference	Gene	Fold difference
Tslp	7.02	Adrb2	2.36	Il9	1.61	Cyslrl	1.11
Ear5	5.08	Rorc	2.35	Postn	1.60	Ear11	1.06
Cd40lg	4.11	Il13ra1	2.28	Satb1	1.59	Foxp3	1.00
Csf2	4.03	Il2ra	2.27	Clca3	1.54	Mrc1	0.95
Tnfrsf4	3.76	Cpa3	2.26	Il10	1.53	Pmch	0.86
Chi3l1	3.65	Itga4	2.13	Ccr4	1.52	Il12a	0.85
Kit	3.51	Ccl17	2.12	Icos	1.45	Ccl24	0.82
Crlf2	3.38	Il13ra2	2.09	Adam33	1.45	Gata3	0.71
Il18	3.38	Il25	2.06	Stat6	1.45	Il1rl1	0.64
Cma1	3.33	Pparg	2.06	Il3ra	1.45	Csf3r	0.53
Ccl12	3.14	Retnlg	2.04	Ifngr2	1.39	Alox5	ND
Tpsb2	3.07	Ccr8	2.03	Tgfb1	1.39	Ccl11	ND
Ccl22	3.02	Ms4a2	2.00	Il4	1.38	Ccr3	ND
Il13	3.02	Arg1	1.98	Ccl4	1.37	Chia	ND
Ccl5	2.88	Ifng	1.96	Kitl	1.34	Epx	ND
Areg	2.86	Il4ra	1.96	Il5ra	1.33	Il17rb	ND
Fcer1a	2.86	Il5	1.90	Gpr44	1.29	Il21	ND
Il3	2.61	Il33	1.89	Stat5a	1.22	Ltb4r1	ND
Ccl26	2.56	Mmp9	1.66	Maf	1.20	Pdcd1	ND
Tbx21	2.52	Muc5ac	1.66	Il12b	1.20	Prg2	ND
Ets1	2.48	Il17a	1.66	Bcl6	1.17	Tnfrsf4	ND

Up-regulated or down-regulated genes in the ear tissue of Ni allergy model compared with control mice were listed with 3 mice in each group. The PCR-array data were approved by GEO (Accession number: GSE52466, Website: <http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE52466>). ND, not detectable.

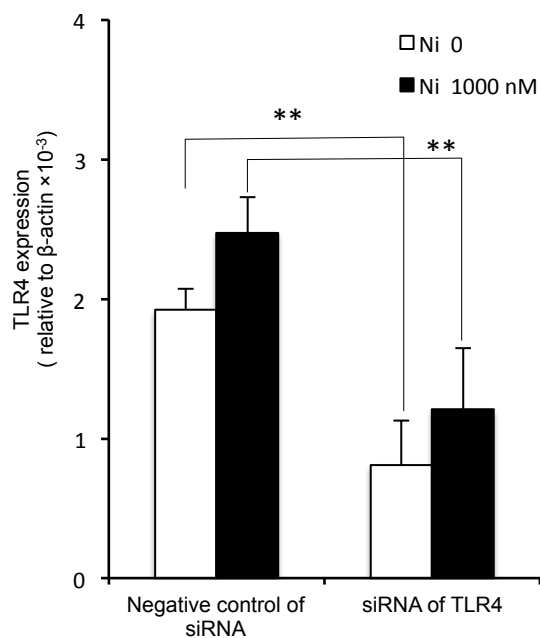
Supplementary Figure 1



Supplementary Figure 1.

Immunohistochemical analysis of TSLP was performed using of the ear tissues from PBS (naïve), CFA, and CFA+Ni-injected mice after the first immunization with IFA+Ni. Results are representative of five mice in each group.

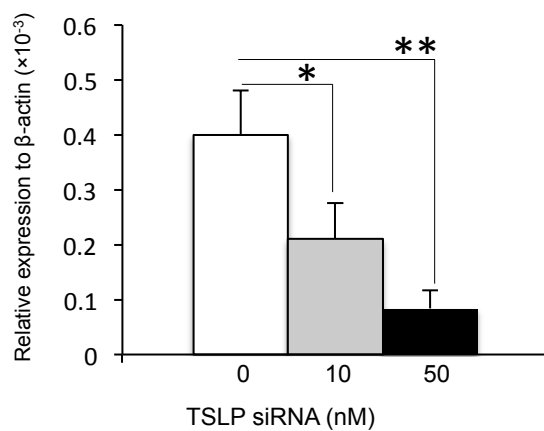
Supplementary Figure 2



Supplementary Figure 3.

The efficiency of TLR4 siRNA on knockdown of TLR4 gene in COCA cells was analyzed by real-time RT-PCR. Results are expressed as mean $\pm$ SD for 3 mice in each group. * $P < 0.05$, ** $P < 0.005$.

Supplementary Figure 3



Supplementary Figure 2.

The *in vivo* efficiency of the injection of TSLP siRNA on knockdown of TSLP gene was analyzed by real-time RT-PCR. Results are expressed as mean $\pm$ SD for 3 mice in each group. * $P < 0.05$, ** $P < 0.005$.