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Semaphorin3A Promotes the Development of Nickel Allergy

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Semaphorin3A Promotes the Development of Nickel Allergy

Abstract

Metal allergy is one of the typical immune disorders encountered during the application of dental/medical materials and has a highly complex pathogenic mechanism. Semaphorin 3A (Sema3A), a member of the semaphorin family, is reported to be involved in various immune disorders. However, its role in metal allergy has not been clarified yet. Herein, we show that Sema3A promoted nickel (Ni) allergy by mediating tumor necrosis factor-alpha (TNF-α) production and mitogen-activated protein kinase (MAPK) activation in keratinocytes. Sema3A was upregulated in $NiCl_2$-stimulated mouse keratinocytes and in Ni allergy-induced mouse ear tissue. TNF-α production and MAPK activation were altered when Sema3A was suppressed in keratinocytes. The specific deletion of Sema3A in keratinocytes alleviated Ni allergy and regulated the macrophage polarization towards an anti-inflammatory direction. Our results demonstrate that Sema3A promotes the development of metal allergy and should be explored as a potential target for the prevention and treatment of metal allergy.

Introduction

Metal allergy, one of the typical disorders of the immune system, is a major challenge in the application of dental/medical materials[1]. It is estimated that up to 17% of women and 3% of men are allergic to Ni and that 1-2% of individuals are allergic to cobalt, chromium, or both[2]. Metal allergy not only causes treatment failure but also affects the skin throughout the body; it has been reported to be associated with lichen planus lesions and palmoplantar pustulosis[3, 4, 5]. An acidic diet, decomposition of food residues, and bacterial metabolism in the oral cavity result in chemical corrosion of metal materials[6]. Corroded and ionized metals tend to cause metal allergy; however, the mechanism is highly complex and many puzzles remain to be solved.

It takes repeated or prolonged exposure to develop a metal allergy. Metal ions released from various alloys that come in contact with the skin could activate epithelial cells, such as keratinocytes, to produce cytokines or chemokines, as well as activate antigen-presenting cells (APCs), such as dendritic cells (DCs) and Langerhans cells. After APCs present antigens to naïve T cells in the draining lymph nodes, the differentiated effector T cells migrate to the peripheral tissues and lead to allergic reactions[7].

Ni, among various metals, is the most common allergen that causes metal allergy[8]. We successfully built a Ni allergy mouse model in our previous study[9, 10, 11]. Using this model, we demonstrated that

the activation of DCs through p38 MAPK/MKK6 is important for the development of Ni allergy in mice[9] and that the thymic stromal lymphopoietin (TSLP)/TSLP receptor-mediated interaction between epithelial and immune cells could trigger Ni allergy[10]. We have also demonstrated that semaphorin 7A, a member of the semaphorin family, enhances inflammation during the effector phase of Ni allergy[11].

Semaphorins are a type of secreted and membrane proteins that were initially recognized as axon guide molecules. Accumulating evidence suggests that semaphorins are involved in the pathogenesis of several immune disorders[12]. Sema3A, a member of the semaphorin family, is known to function as an effective immunomodulator in immune cell migration and regulation[13, 14, 15, 16, 17]. Sema3A downregulates T cell proliferation via the neuropilin-1(Nrp1)-plexin-A4 receptor complex[17] and increases cytokine production in response to toll like receptor (TLR) agonists or bacterial sepsis in mice[18]. Sema3A is also involved in DC transmigration from the periphery to draining lymph nodes[17]. Besides, Sema3A has been reported to be associated with various immune diseases, such as rheumatoid arthritis and systemic lupus erythematosus[19]. It also controls the inflammatory responses in allergic conjunctivitis and allergic rhinitis[20, 21]. Recent studies have suggested that Sema3A could interrupt the itch-scratch cycle by inhibiting the extension and sprouting of C-fiber in the epidermis, thereby alleviating the skin lesions and scratching behavior of NC/Nga mice, a model of atopic dermatitis (AD),

in a dose-dependent manner[22, 23]. Furthermore, Sema3A has been shown to inhibit the migration of $CD4^+$ T cells to inflammatory sites[22]. Considering its role as a potent inhibitor of neurite outgrowth of sensory neurons and inflammatory cell infiltration, Sema3A has been extensively studied for its potential in the treatment of refractory itching skin diseases that exogenous application of Sema3A improves the pathophysiology of AD and psoriasis[24]; however, the association between Sema3A and metal allergy has not yet been reported.

To better clarify the molecular mechanism underlying metal allergy, in this study, we used an Ni allergy mouse model to study the role of Sema3A in Ni allergy. We speculated that Sema3A, being a secretory protein, might function like cytokines or chemokines and may play a crucial role in both intercellular and intracellular communication, thereby influencing the process of metal allergy development and becoming a potential target for the investigation and treatment of metal allergy.

Results

Sema3A is upregulated in keratinocytes when stimulated with $NiCl_2$

The epidermis, which is the outermost layer of the skin, is the first barrier against antigen invasion and plays vital roles in the development of metal allergy[25]. We aimed to determine whether there is

any interaction between Sema3A and keratinocytes, which constitute 90% of the cells of the epidermis, thereby contributing to metal allergy. To investigate Sema3A expression in keratinocytes, we stimulated the Pam2.12 keratinocyte cell line with $NiCl_2$ for different durations. The Sema3A mRNA expression was upregulated in keratinocytes exposed to $NiCl_2$-stimulation over time, although there was no significant difference (Fig. 1a). The staining of keratinocytes with Sema3A antibody showed brightly positive cytoplasmic secretory granules which filled the entire cell cytoplasm. These positive granules were observed to be more strongly expressed on keratinocytes 24 and 48 h after $NiCl_2$ stimulation compared to those in the control group (Fig. 1b). The expression of Sema3A protein was also upregulated and reached a peak 12 h after $NiCl_2$ stimulation by Western blotting (Fig. 1c). 72 h after $NiCl_2$ stimulation, the significantly increased production of Sema3A in the culture medium of Pam2.12 was confirmed by ELISA (Fig. 1d).

Sema3A knockdown in keratinocytes alters MAPK activation and TNF-α expression

To evaluate the functional role of Sema3A in keratinocytes, Pam2.12 cells were transfected with Sema3A siRNA. Without the stimulation of $NiCl_2$, the expression of TNF-α, a known inflammatory cytokine produced by keratinocytes[7], showed no significant difference among groups. However, when

cells were stimulated with $NiCl_2$, the $NiCl_2$-stimulated control and mock group showed higher TNF-α expression than the non-stimulated groups. The expression of TNF-α in the Sema3A-silenced group was significantly inhibited compared to that in the mock group 24 h after $NiCl_2$ stimulation ($p=0.026$ and 0.0014 respectively) (Fig. 2a, b). The cells, 48 h post-siRNA transfection, seemed to recover and exhibited less efficient suppression of TNF-α mRNA than that at 24 h post-siRNA transfection (Fig. 2a). We then checked the expression of TSLP, which is considered a master switch for allergic inflammation and a downstream molecule of TNF-α[26], in $NiCl_2$-stimulated Pam2.12. The protein expression of TSLP was activated in $NiCl_2$-stimulated Pam2.12 and showed a peak 12 h after stimulation (Supplementary Figure 1), consistent with the result of Ashrin in that TSLP production increased in the epidermal keratinocyte COCA cell line stimulated with $NiCl_2$[10]. We also assessed the MAPK activation in Pam2.12. In Sema3A-silenced Pam2.12, the activation of p38 was significantly inhibited 24 h after $NiCl_2$ stimulation compared to that in the control l ($p=0.018$). The suppression effect of siRNA seemed to recover 48 h after stimulation but the protein expression of p38 was still lower than that in the control group (Fig. 2c). These results suggest that Sema3A may play a role in triggering or regulating the process of Ni allergy through mediating p38 kinase activation and TNF-α expression produced by keratinocytes.

Sema3A is enhanced in Ni allergy-induced mouse ear tissue

To investigate Sema3A expression *in vivo*, we induced Ni allergy in mice as described previously[9] (Fig. 3a). After 48 h of Ni re-challenge, delayed-type hypersensitivity (DTH) reaction, characterized by severe swelling and redness in the ear tissue, was confirmed in the allergy-induced group (Fig. 3b). Hematoxylin and eosin (H&E) staining showed increased thickness and more infiltrated cells in the allergy-induced ear tissue (Fig. 3c). Ear thickness was significantly increased 24, 48, and 72 h after $NiCl_2$ injection compared to that in the control group (Fig. 3e). Sema3A expression was found to be mainly located in the epidermis layer and expressed more strongly than that in the control (Fig. 3d). Consistently, the western blotting results of Sema3A showed significantly stronger expression in the Ni allergy group than in the control ($p=0.000024$) (Fig. 3f). We also investigated some inflammatory cytokines and chemokines associated with skin inflammation. The upregulation of IL-17, L-23, CCL20, and CXCL1 mRNA was observed in Ni allergy-induced mouse ear tissues (Fig. 3g). Since Sema3A expression is strongly enhanced in Ni allergy-induced mouse ear tissue than in the control, we speculate that Sema3A may be positively correlated with the development of Ni allergy.

The number of monocytes, macrophages, and DCs increases in Ni allergy-induced mouse ear tissue

Results of flow cytometry showed that the number of $CD11b^+$ cells increased markedly in the Ni allergy-induced groups (Fig. 4a, d). Among them, the number of $CD11b^+CD115^+$ monocytes increased in the Ni allergy-induced ear tissue compared to that in the control group (Fig. 4a, b). Following immunofluorescence staining of frozen sections, an increased number of monocytes was observed in the Ni allergy-induced ear tissue (Fig. 4c). Similarly, an increase in the number of $CD11b^+F4/80^+$ macrophages was observed in the Ni allergy-induced group compared to that in the control group ($p=0.006$) (Fig. 4d-f). The number of $CD11c^+$MHC class II^+ DCs was found to be increased in the Ni allergy-induced group, and the immunofluorescence staining of epidermal sheet showed coincident results ($p=0.002$) (Fig. 4g-i). These results indicate the involvement of monocytes, macrophages, and DCs in the development of Ni allergy.

Sema3A-specific deletion in keratinocytes reduces Ni allergy

Sema3A is reported to mainly localize in the keratin14-positive keratinocytes in the stratum basale of the human epidermis[27]. Therefore, to investigate the role of Sema3A in Ni allergy *in vivo*, we crossed

129 Sema3A$^{fl/fl}$ mice with keratin5 (K5)-Cre mice using a cre/loxp system to knockout Sema3A in K5,
130 which is a similar protein and partner of keratin14. The DTH reaction was confirmed in Sema3A$^{fl/fl}$
131 mice, similar to that in C57BL/6J mice; therefore, we used Sema3A$^{fl/fl}$ mice as a control group in the
132 confirmation of Ni allergy development in Sema3A conditional knockout (cKO) mice. The results
133 showed that the development of Ni allergy and inflammatory reactions was alleviated in Sema3A cKO
134 mice compared to that in the control mice (Fig. 5a). After 24 h Ni elicitation, the ear thickness of the
135 control mice was significantly increased in the Ni allergy-induced ear tissue than in the control
136 ($p=0.000015$), while the ear thickness of Sema3A cKO mice just increased slightly, and not to the
137 degree of the control group (Fig. 5b). After 48 h, the ears of the control mouse remained to be red and
138 swollen; however, the ears of Sema3A cKO mice almost recovered to normal (Fig. 5a, b). The ratio of
139 Ni allergy-induced ear thickness to the control ear thickness, which represents the degree of thickening
140 of ears, was significantly lower in the Sema3A cKO group ($p=0.018$ and 0.001 respectively) than in
141 the control group 24 h and 48 h post-Ni re-challenge (Fig. 5b). In other words, when inducing Ni
142 allergy, the ear swelling in Sema3A cKO mice was less severe than that in the control mice. In the
143 H&E staining of Ni allergy-induced ear tissue of Sema3A cKO mice, less severe edema and fewer
144 infiltrated inflammatory cells were observed than in the control mice (Fig. 5c). To determine the genes,

cytokines, and chemokines associated with Sema3A deletion in keratinocytes, we further investigated the expression levels of TNF-α, IL-1β, IL-23, and CXCL1, which were found to be significantly enhanced in the Ni allergy-induced control mouse ear tissue but blunted in the Sema3A cKO group. The upregulation of CCL20 seemed to be unaffected with the deletion of Sema3A (Fig. 5d). These results indicate that Sema3A probably promotes the development of Ni allergy.

Sema3A-specific deletion in keratinocytes increases M2/M1 macrophage polarization

To examine the change in immune cell profiles during Ni allergy development in Sema3A cKO mice, flow cytometry was performed. An increased number of $CD11b^{+}F4/80^{+}$ macrophages was detected in both control and Sema3A cKO Ni allergy-induced groups, and there was no apparent difference between them (Fig. 6a). CD206 was used to further determine M1 and M2 macrophages. The analysis revealed that the proportion of $CD206^{+}CD11b^{+}F4/80^{+}$ M2-like macrophages increased significantly in the Sema3A cKO Ni allergy-induced group (Fig. 6a, b). The ratio of $CD206^{+}CD11b^{+}F4/80^{+}$M2-like macrophages to $CD206^{-}CD11b^{+}F4/80^{+}$ M1-like macrophages was significantly increased in the Sema3A cKO Ni allergy-induced group compared to that in the control group ($p=0.014$) (Fig. 6c). This result indicates that the alleviated Ni allergy in Sema3A cKO mice might be related to the polarization

of macrophage towards the M2 phenotype, which produces anti-inflammatory cytokines that contribute to tissue healing.

Discussion

Sema3A is the member of the semaphorin family known to function as an axonal repulsion factor through interaction with the plexinA/Nrp1 receptor[12]. The significance of sema3A in regulating immune-mediated inflammation has been widely reported, as an example, the expression level of Sema3A in the epidermis of patients with AD and psoriasis was remarkably decreased compared to that in healthy volunteers[28] and in the epidermis of AD patients, epidermal hyperinnervation and high levels of nerve growth factor (NGF) were observed[29]. Since Sema3A is known to inhibit the intraepidermal extension of peripheral nerve and has been reported to abolish the growth promoting effect of NGF on sensory afferents in adult rat spinal cord and mouse embryonic neurons[30, 31, 32], the epidermal innervation in AD and psoriasis is likely to be regulated by a balance of NGF and Sema3A, and the decreased Sema3A in the epidermis of these patients could result from increased NGF. In our study, the expression of Sema3A was increased in the inflammatory site of the skin of metal allergy mouse models, suggesting that Sema3A may be involved in the pathogenesis of both diseases through

177 different mechanisms. Previous studies have demonstrated that activated keratinocytes can release
178 certain proinflammatory cytokines such as IL-1β and TNF-α, TSLP, and chemokines in response to
179 pathogens, injury and wound healing as well as cancers[7, 33, 34, 35]. In addition, recent studies on the
180 effect of Sema3A on immune response have reported its association with TNF-α and MAPK pathway.
181 In our results, both TNF-α and p38 kinase expressions were inhibited when Sema3A was suppressed
182 in keratinocytes. In previous studies, significantly decreased TNF-α expression in microglial cells in
183 response to TLR4 stimulation was observed with the knockdown of the receptor of PlexinA1, which
184 is a receptor of Sema3A[36, 37]. These results reflect the possibility that the production of TNF-α on
185 keratinocytes stimulated with $NiCl_2$ depends on Sema3A secretion.
186 On the other hand, keratinocytes stimulated with $NiCl_2$ resulted in activation of p38, and inhibition
187 of Sema3A with siRNA inhibited activation of p38. It is known that p38 promotes the upregulation of
188 IL-1β and TNF-α, which are strongly involved in skin inflammation[38, 39]. In addition, studies using
189 itch-deficient ($Itch^{-/-}$) mice showed enhancement of phosphorylation of p38α in skin lesions, and
190 proinflammatory cytokines such as TNF-α and IL-1β were increased in the skin of $Itch^{-/-}$ mice.
191 Moreover, that inhibition of p38 significantly suppresses inflammation in the skin of $Itch^{-/-}$ mice[40].
192 Therefore, our results suggest that inhibition of Sema3A expression may have suppressed TNF-α

193 secretion by inhibiting p38 in keratinocytes.

194 Keratinocytes, which make up the majority of epidermal cells, are the first cells to have access to
195 metal ions coming from the outside. When keratinocytes come in contact with metal ions, they secrete
196 abundant Sema3A and TNF-α. TNF-α has been reported to promote tissue repair and enhance
197 keratinocyte motility and attachment[41, 42]. It also regulates innate immunity and inflammation by
198 inducing chemokines that attract neutrophils, macrophages, and skin-specific memory T-cells[43]. In our
199 study, we observed an increase in the number of monocytes and macrophages in the Ni allergy ear
200 tissue by flow cytometry analysis, which implicates TNF-α produced by keratinocytes in the
201 pathogenesis of Ni allergy by attracting the immune cells via chemokines such as CXCL1 to initiate
202 the process of inflammation. CXCL1 is known as keratinocyte-derived chemokines and is expressed
203 on macrophages, neutrophils, and epithelial cells and activates the ERK pathway during the
204 inflammatory response and contributes to the inflammatory process[44]. In our study, when CXCL1
205 expression was upregulated in allergic skin and downregulated when Sema3A was inhibited in the ear
206 skin using siRNA or in the cKO mice at the onset of allergy, CXCL1 was reduced and TNF-α
207 expression was also suppressed. Altogether, Sema3A promotes the secretion of TNF-α and attracts
208 immune cells via CXCL1, which is expressed as a result, and induces local allergic reactions.

In recent years, polarized macrophages have acquired significant attention. M1 macrophages are reported to promote the process of inflammation, while M2 macrophages suppress inflammation[45]. Therefore, the balance of M1/M2 macrophage activity plays a significant role in physiology and pathology. In our study, Sema3A cKO mice were protected against Ni allergy. Furthermore, a high M2/M1 ratio, which represents the anti-inflammatory responses, was observed in the Sema3A cKO allergy-induced group, while blunted upregulation of inflammatory cytokines/chemokines TNF-α, IL-1β, IL-23, and CXCL1 was detected. It has been widely accepted that M1 macrophages could be typically induced by TNF-α[46, 47], and there are reports that reduction in TNF-α mRNA could result in enhanced M2 mRNA expression[48], TNF-α deletion could inhibit M1 macrophage polarization[49]. Therefore, we speculate that the increased M2/M1 ratio in Ni allergy-induced Sema3A cKO mice in our study might be caused by the reduced TNF-α production in keratinocytes due to Sema3A-specific deletion in keratinocytes. The production of cytokine/chemokines produced by M1 macrophages, such as TNF-α, IL-23, and CXCL1, was hindered, thus reducing the inflammation and impeding the process of Ni allergy. In some studies, recombinant Sema3A protein was reported to affect the proinflammatory state of macrophages and increase the expression of M1-markers, such as inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2), with the stimulation of lipopolysaccharide (LPS)[50].

225 Therefore, there is also a possibility that the deletion of Sema3A directly affects the polarization of
226 M1 macrophages.

227 Intradermal injection or ointment allocation of Sema3A were found to inhibit scratching behavior
228 to improve AD-like symptoms compared to controls. Moreover, the nerve fiber number in epidermis,
229 inflammatory cell infiltration, and the cytokine production were reduced in Sema3A-treated skin
230 lesion[22, 23]. Decreased Sema3A concentration may lead to the occurrence or enhancement of itching in
231 AD patients and NC/Nga mice[51, 52]. In the studies above, exogenous Sema3A was applied to the lesions
232 during inflammation, which is different from our study as we blocked endogenous Sema3A before the
233 initiation of inflammation, which reduced the Ni allergy reaction successfully in the initial phase. It is
234 presumed that exogenous Sema3A, in addition to inhibiting the elongation of epidermal nerve fibers,
235 could downregulate endogenous Sema3A mRNA expression in keratinocytes through autocrine signal
236 transduction, and finally inhibit the occurrence of inflammation by mediating the expression of TNF-
237 α. Further confirmation, such as quantitative RT-PCR or western blotting of endogenous Sema3A
238 mRNA expression upon exogenous Sema3A, is required in this regard.

239 In conclusion, through this study, we clarified that Sema3A could promote the development process
240 of Ni allergy by mediating the activation of MAPK and the production of TNF-α. The specific deletion

241 of Sema3A in keratinocytes results in reduced TNF-α expression, which regulates the macrophage
242 M1:M2 balance towards an anti-inflammatory direction, resulting in alleviated Ni allergy. It is
243 conceivable that Sema3A has the potential to be further developed to provide new ideas and therapeutic
244 targets for metal allergy.
245
246 **Methods**
247 Mice
248 Female C57BL/6J mice (8-week-old) were purchased from Charles River Laboratories, Inc.
249 (Kanagawa, Japan). K5-cre[53] mice (STOCK-Tg(K5-Cre) Jt) were obtained from CARD R-BASE,
250 Kumamoto University. K5-Cre-specificity was determined following the PCR protocol described
251 previously[54]. Sema3A$^{fl/fl}$ mice (ICR.Cg-Sema3a<tm1.2Tyag>/TyagRbrc, RBRC01106) were provided
252 by RIKEN BRC through the National BioResource Project of the MEXT/AMED, Japan. The
253 genotyping of Sema3A$^{fl/fl}$-specificity was performed according to the previous study[55]. Sema3A$^{fl/fl}$
254 conditional mice were crossed with K5-Cre mice to generate Sema3A cKO mice. Wild-type mice of
255 the same genetic background strains were used as control animals. All mice used were aged 6-8 weeks.
256 All mice were maintained under specific pathogen-free conditions and fed with autoclaved diet and

water in the animal facilities at Tokushima University, they were treated in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. All experimental procedures were approved by IACUC (No. T2019-51) and Institute for Genome Research (No.30-46) of Tokushima University.

Reagents

Nickel(II) Chloride was obtained from Wako Pure Chemical Corporation. Freund's incomplete adjuvant (IFA) and complete adjuvant (CFA) were from MP Biomedicals. The primary antibodies specific for Sema3A were from Bioss Antibodies (for Western blotting) and Abcam (for immunohistochemistry/immunocytochemistry), respectively. Antibodies specific for phospho-p38 (Thr180/Tyr182) and p38 were obtained from Cell Signaling Technology. Antibody specific for mouse TSLP was from Abcam. Rabbit antibody to GAPDH was obtained from Osenses. Alexa Fluor 488 anti-mouse CD11c, Alexa Fluor 488 anti-mouse CD11b, PE anti-mouse CD115, PE anti-mouse F4/80, PE anti-mouse I-A/I-E and Alexa Fluor 647 anti-mouse CD206 (MMR) were purchased from BioLegend. Horseradish peroxidase (HRP)-conjugated anti-rabbit secondary antibody were obtained from Cell Signaling. Anti-rabbit Alexa Fluor 488 secondary antibody was from Abcam.

Induction of Ni Allergy

Ni allergy was induced on mouse ears as described previously[9, 10, 11]. In brief, 25µl 1mM $NiCl_2$ with 25µl IFA was intraperitoneally injected to mice for initial immunization. After two weeks, 10µl 0.2mM $NiCl_2$ with 10µl CFA was intradermally injected into the ear skin as a second challenge. For negative control, PBS with CFA was injected. 48 h later, DTH reaction was observed and confirmed by measuring the ear thickness of mice.

Histology and immunofluorescence microscopy

Frozen sections (4-8µm) were stained with H&E. Sections for immunofluorescence staining were fixed with pre-cooled acetone (4 ℃) for 10 minutes. After this, the slides were rinsed with PBS and blocked with 5% goat serum for 30 minutes. A 1:300 dilution of primary antibodies or fluorescently-conjugated antibodies was applied to the sections and incubated in a dark humidified chamber overnight at 4°C, subsequently followed by incubation with anti-rabbit Alexa 488 Fluor secondary antibody at room temperature for 1 h. The nuclei were stained with DAPI solution (0.1 µg/ml; Cell Signaling Technology, Danvers, MA, USA). Immunofluorescence images were captured using

Keyence all-in-one fluorescence-microscope BZ-X800 (magnification, ×200; ×400; ×1000; KEYENCE CORPORATION, Tokyo, Japan).

Epidermal sheet preparation

Epidermal sheets were prepared using a modification of previously-reported method[56, 57]. In brief, the ears were peeled into halves and put dermis-side down floating on a dish of cold ammonium thiocyanate. After incubated 13 minutes at 37 ℃, the epidermal sheets were peeled with small tweezers. The epidermis flats were washed with PBS and fixed with cold acetone, then used for the following immunofluorescence staining like frozen sections.

Flow cytometry

The single-cell suspensions of mouse ears were prepared based on the method described previously[58, 59]. Briefly, the ears were split and cut, incubated with a solution of RPMI containing 1mg/ml DNase I (NIPPON GENE, Tokyo, Japan) and 1mg/ml collagenase (Worthington Biochemical, Lakewood, NJ, USA) 90 min at 37 ℃. 1 x 10^6 cells of homogeneous cell suspension were stained with Alexa Fluor 488 anti-mouse CD11c, Alexa Fluor 488 anti-mouse CD11b, PE anti-mouse CD115, PE anti-mouse

F4/80, PE anti-mouse I-A/I-E Alexa Fluor 647 anti-mouse CD206 according to the recommended concentration. Cells were analyzed on BD FACSVerse™ (BDBiosciences, San Jose, CA, USA).

Cell culture and siRNA transfection

Mouse keratinocyte cell line Pam2.12 was kindly provided by Dr. S. H. Yuspa (National Cancer Institute, Bethesda)[60]. The cells were cultured in DMEM (nacalai tesque, Kyoto, Japan) supplemented with 10% FBS and 1% penicillin/streptomycin/amphotericin B in a humidified atmosphere of 5% CO_2 at 37°C. Pam2.12 cells were stimulated with 250μM $NiCl_2$ for 0, 6, 12, 24, 48, 72 h before analysis (The concentration was decided according to previous study[11]). The Sema3A esiRNA was obtained from Sigma-Aldrich (Tokyo, Japan). Pam2.12 cells were transfected with 50nM of Sema3A siRNA using INTERFERin (Polyplus transfection, Illkirch, France) in half-decreased-volume serum free DMEM, after 4 h, complete DMEM was added to restore the usual culture volume. Medium was changed 24h after transfection and 250μM $NiCl_2$ was added to the cells for another 24 and 48 h.

Immunocytochemistry

Pam2.12 cells were seeded in dish with glass coverslips in the bottom. 300μM $NiCl_2$ was added

when cells grew to 40% confluency. After $NiCl_2$ stimulation for 24 or 48 h, coverslips were removed and washed with PBS, followed by fixation with 4% paraformaldehyde at room temperature for 10 minutes. Then the coverslips were incubated in 0.25% Triton X-100 in PBS at room temperature for another 10 minutes and blocked in 3% BSA for 30 minutes. Finally, they were stained with immunofluorescence like frozen sections.

Quantitative RT-PCR

The total RNA of Pam2.12 and the total RNA of mouse ears were extracted. cDNA was made using PrimeScript™ RT reagent Kit (TaKaRa Biotech, Shiga, Japan). TaKaRa Ex Taq® DNA Polymerase (TaKaRa Biotech, Shiga, Japan) was used for preliminary experiment to confirm expressions. qPCR was performed using TB Green® Premix Ex Taq™ II (TaKaRa Biotech, Shiga, Japan) in a total volume of 15μl with ABI7300 Real-time PCR System (Applied Biosystems, MA, USA) according to manufacturer instructions. The polymerase was initially activated at 95℃ for 30 seconds, then the PCR was run in 40 cycles of denaturation at 95℃ for 5 seconds and annealing/extension at 60℃ for 31 seconds. The sequences for primers were as follows: Sema3a, forward: 5'-GAAGAGCCCTTATGATCCCAAAC-3'; reverse: 5'-AGATAGCGAAGTCCCGTCCC-3'. Tnf-

alpha, forward: 5’-ATGGCCTCCCTCTCATCAGT-3’; reverse: 5’-CTTGGTGGTTTGCTACGACG-3’. Il1b, forward: 5’-GCTGAAAGCTCTCCACCTCA-3’; reverse: 5’-AGGCCACAGGTATTTTGTCG-3’. Il17a, forward: 5’-GCTCCAGAAGGCCCTCAG-3’; reverse: 5’-CTTTCCCTCCGCATTGACA-3’. Il23, forward: 5’-CCAGCAGCTCTCTCGGAATC-3’; reverse: 5’-TCATATGTCCCGCTGGTGC-3’. Cxcl1, forward: 5’-CCGAAGTCATAGCCACACTCAA-3’; reverse: 5’-GCAGTCTGTCTTCTTTCTCCGTTA-3’. Ccl20, forward: 5’-GTACTGCTGGCTCACCTCTG-3’; reverse: 5’-CTTCATCGGCCATCTGTCTTGTG-3’. Actb, forward: 5’-TCTGGCTCCTAGCACCATGAAGA-3’; reverse: 5’-GGGACTCATCGTACTCCTGCTTG-3’. Gene expression was normalized to levels of β-actin.

Western blotting analysis

$NiCl_2$-stimulated Pam2.12 cells were washed in cold PBS before lysis in 60μl of RIPA buffer (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan) containing 1:100 EZBlock™ Protease Inhibitor Cocktail (BioVision, San Francisco, CA, USA). Tissue extracts were prepared by homogenizing mouse ear tissue in lysis buffer. Proteins were separated in a 10% SDS-PAGE acrylamide gel for Pam2.12, transferred onto a polyvinylidene fluoride (PVDF) membrane.

353 Membranes were incubated with primary antibodies against Sema3A, TSLP, phosohorylated-p38
354 MAPK at 4°C overnight. Total p38 MAPK and GAPDH were incubated at room temperature for 1 h.
355 All the primary antibodies were used at a dilution 1:1000. After washing with TBST (0.05M Tris-HCl,
356 0.15M NaCl, pH7.6), a 1:20000 dilution of HRP-conjugated anti-mouse secondary antibody was
357 applied to the membrane for 1 h at room temperature. Membranes were detected with ECL Prime
358 reagents (GE healthcare, Chicago, IL, USA) using ChemiDoc XRS (Bio-Rad, Hercules, CA, USA).
359
360 ELISA
361 Pam2.12 cells were seeded in 96-well dish and stimulated with 250μM $NiCl_2$ for 0, 12, 24, 48, 72 h
362 before analysis of Sema3A production in medium using a mouse Sema3A ELISA kit (Signalway
363 Antibody, College Park, MD, USA). Pam2.12 cells seeded in 96-well dish was performed with
364 Sema3A siRNA transfection using INTERFERin as described above to suppress Sema3A expression.
365 Culture supernatant 24 h after $NiCl_2$-stimulation was collected and stored at -20°C until analysis. The
366 TNF-α production was measured using a mouse TNF-α ELISA (eBioscience, San Diego, CA, USA),
367 according to the manufacturer instructions.
368

Statistical analysis

All experiments were repeated at least three times with similar results. Experimental values are given as means ± SD. The statistical difference was determined by One-Way ANOVA with LSD (Least Significant Difference), Tukey's HSD (honestly significant difference) test or Two independent samples t test. Statistical significance is presented in the following manner: *p＜0.05, **p＜0.01, ***p ＜0.001.

Data availability

The authors declare that the data supporting the findings of this study are available within the paper and from the authors upon reasonable requests. Source data are provided with this paper.

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516

517 **Competing Interests**

518 Disclosures: The authors declare no competing interests exist.

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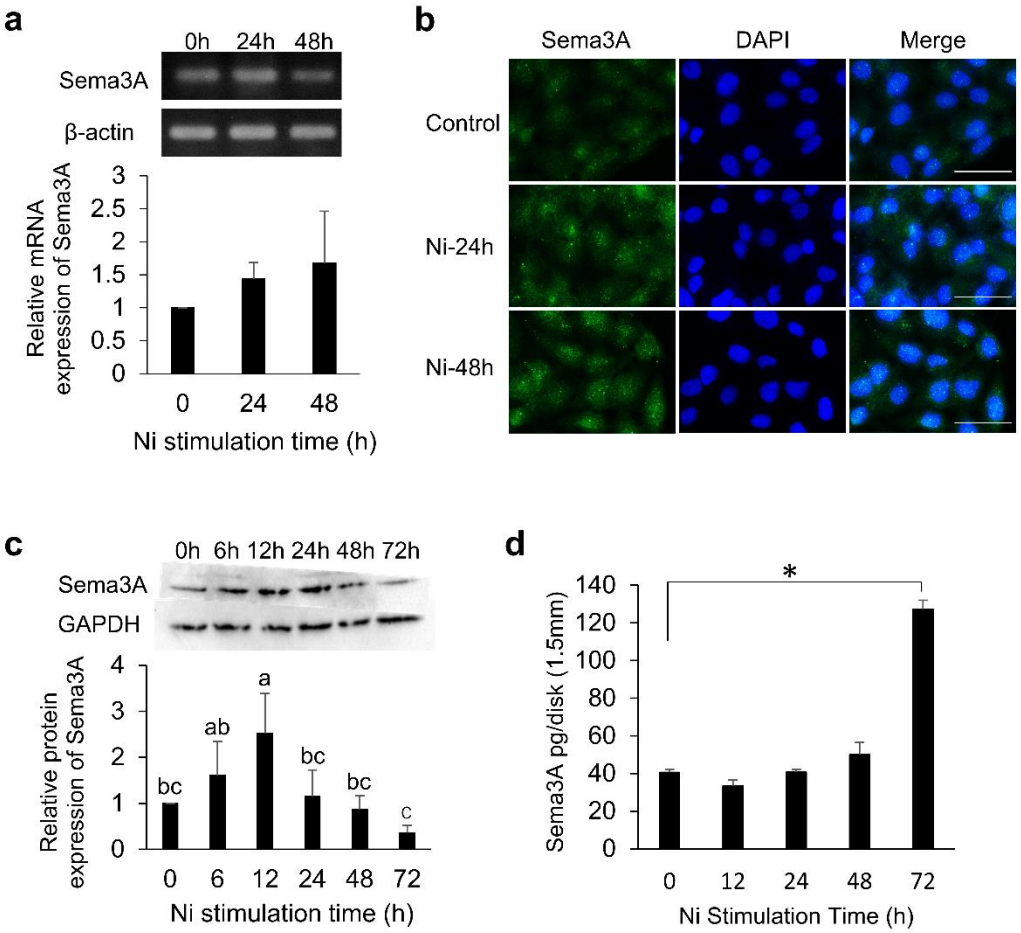
Figures

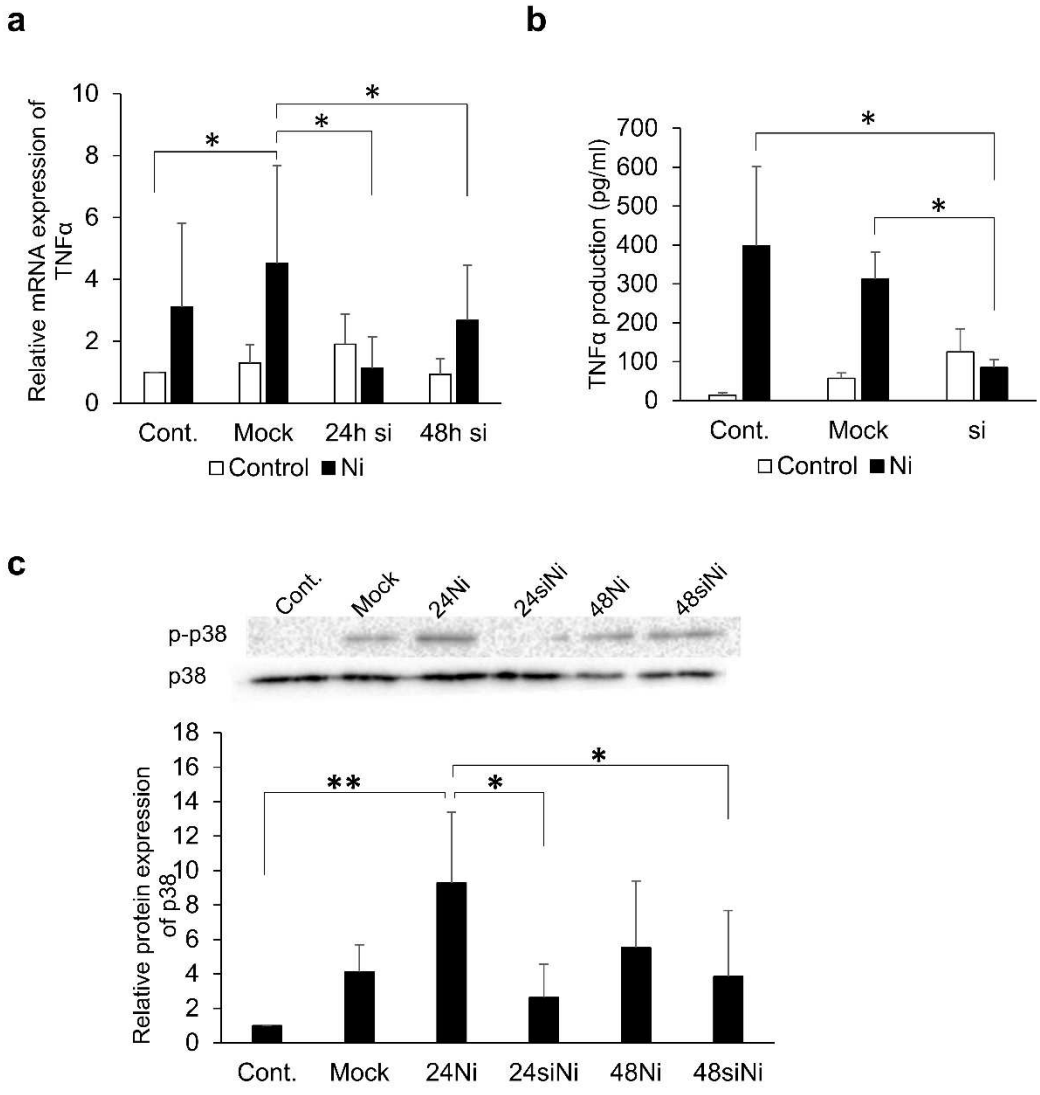
Figure 1. Sema3A expression.



a Sma3A was expressed on $NiCl_2$-stimulated Pam2.12 and the mRNA expression increased after $NiCl_2$ stimulation. **b** Immunocytochemistry of Sema3A expressed in Pam2.12 24 h and 48 h after $NiCl_2$ stimulation. Scale bar, 50μm. **c** Upregulated Sema3A in $NiCl_2$-stimulated Pam2.12 was analyzed by Western blotting analysis. GAPDH was used as the internal control. **d** Upregulated Sema3A production in $NiCl_2$-stimulated Pam2.12 was analyzed by ELISA. Data are shown as mean ± SD and are

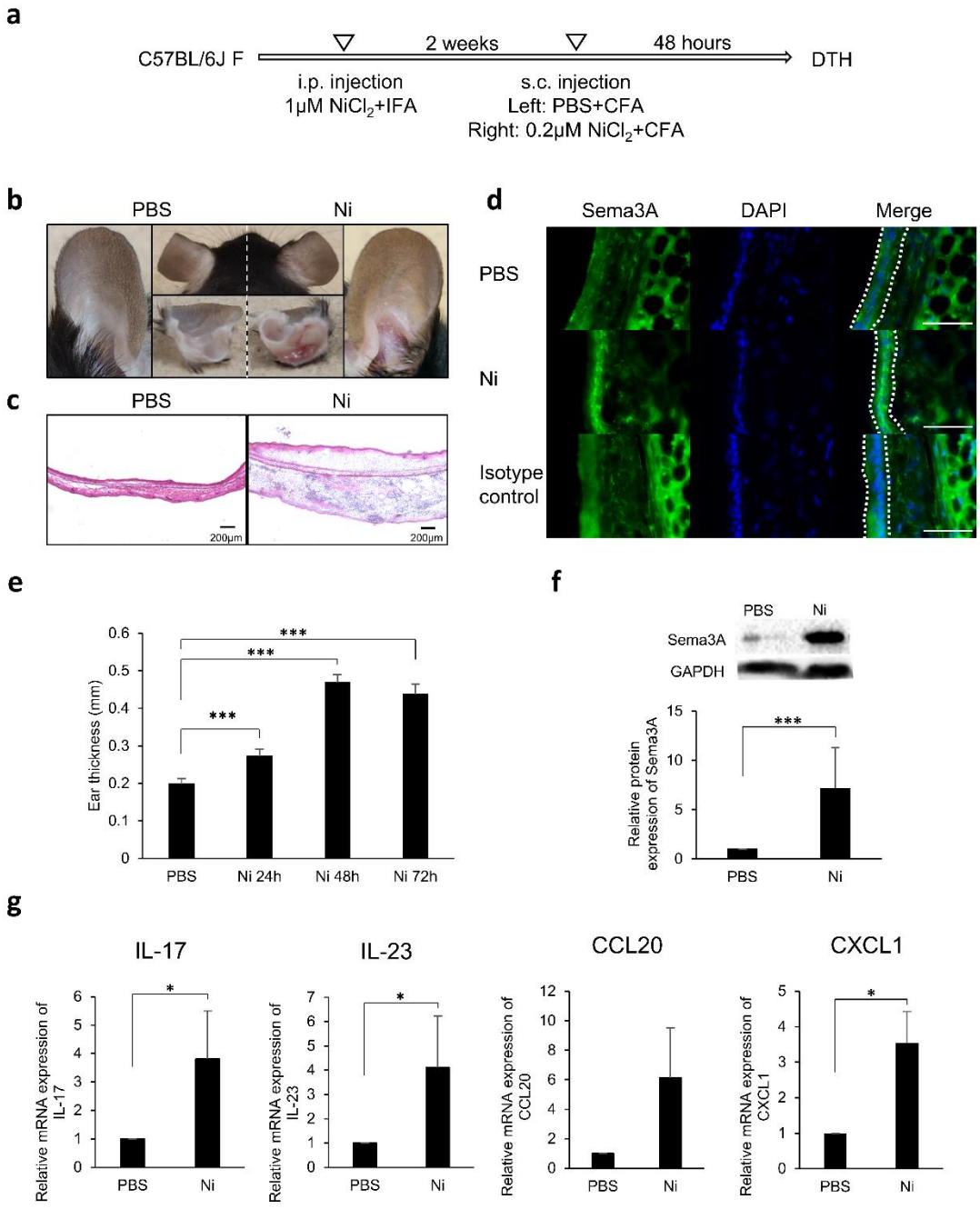
representative of at least three independent experiments. Different letters on bars indicate significant differences. *p<0.05; One-Way ANOVA with LSD. Source data and p values are provided as a Source Data file.

Figure 2. Effect of Sema3A siRNA.



a TNFα mRNA expression in Sema3A siRNA-treated Pam2.12 after $NiCl_2$ stimulation was analyzed using quantitative RT-PCR. Gene expression was normalized to levels of β-actin. 24h si/48h si represents Sema3A siRNA-treated groups with or without 24/48 h-$NiCl_2$ stimulation. **b** TNFα protein production in Sema3A siRNA-treated Pam2.12 after $NiCl_2$ stimulation was analyzed by ELISA. The samples with or without $NiCl_2$ stimulation were collected after 24 h. **c** Inhibited p38 phosphorylation in Sema3A siRNA-treated Pam2.12 after $NiCl_2$ stimulation was analyzed by Western blotting analysis. GAPDH was used as the internal control. 24Ni/48Ni represents the groups with 24/48 h-$NiCl_2$ stimulation. 24siNi/48siNi represents the Sema3A siRNA-treated groups with 24/48 h-$NiCl_2$ stimulation. Data are shown as mean ± SD and are representative of at least three independent experiments. *$p<0.05$, **$p<0.01$; One-Way ANOVA with LSD. Source data and p values are provided as a Source Data file.

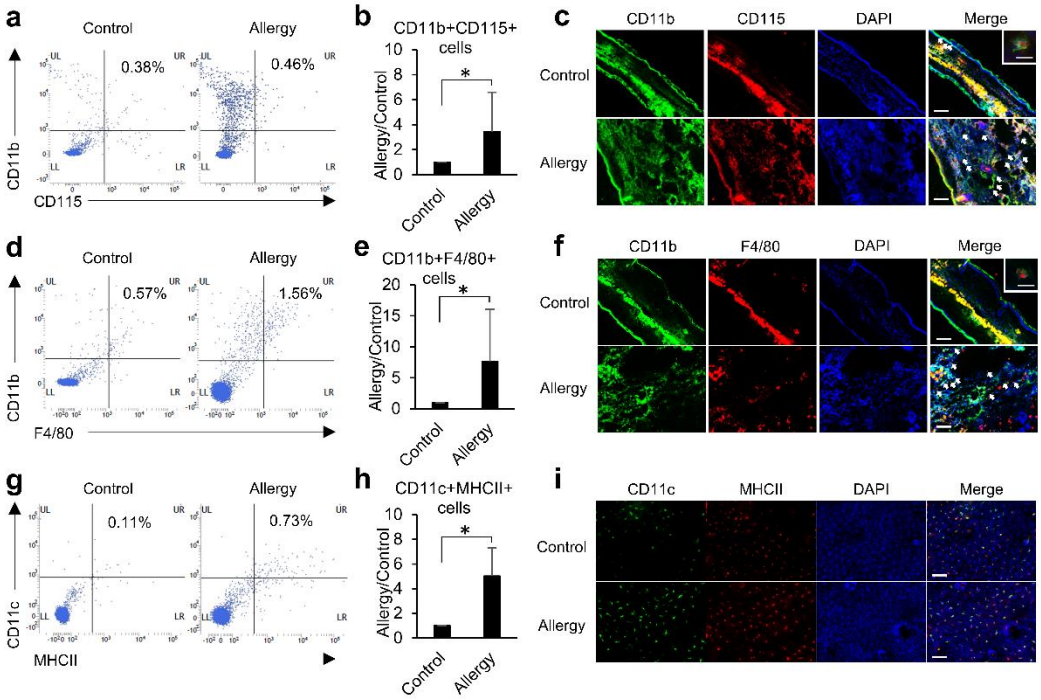
Figure 3. Sema3A and cytokine/chemokine expression in allergy site.



a Protocol for inducing Ni allergy in mouse ear tissue. **b** Representative photos of the Ni allergy-induced mouse ear and the control ear. Photos show the ventral side (right and left), dorsal side (middle upper) and cross-section (middle lower). **c** Histological images of Ni allergy-induced mouse ear tissue

and control ear tissue. Scale bar, 200μm. **d** Sema3A expression in the control and Ni allergy-induced ear tissue was stained using immunofluorescence assays. Staining without Anti-rabbit Fluor Alexa 488 secondary antibody was set for isotype control. Dashed line shows the epidermis zone. Scale bar, 50μm. **e** DTH was determined by ear thickness measuring after Ni re-challenge. Data are shown as mean ± SD of 5 mice/group. **f** Upregulated Sema3A expression in Ni allergy-induced mouse ear tissue was analyzed by Western blotting analysis. GAPDH was used as the internal control. **g** The mRNA expression of inflammatory cytokine/chemokine in Ni allergy-induced mouse ear tissue were analyzed using quantitative RT-PCR. Gene expression was normalized to levels of β-actin. Data are shown as mean ± SD and are representative of at least three independent experiments. *$p<0.05$, ***$p<0.001$; Tukey's test for **e** and Two independent samples t test for **f** and **g**. Source data and p values are provided as a Source Data file.

Figure 4. Cell population in allergy site.



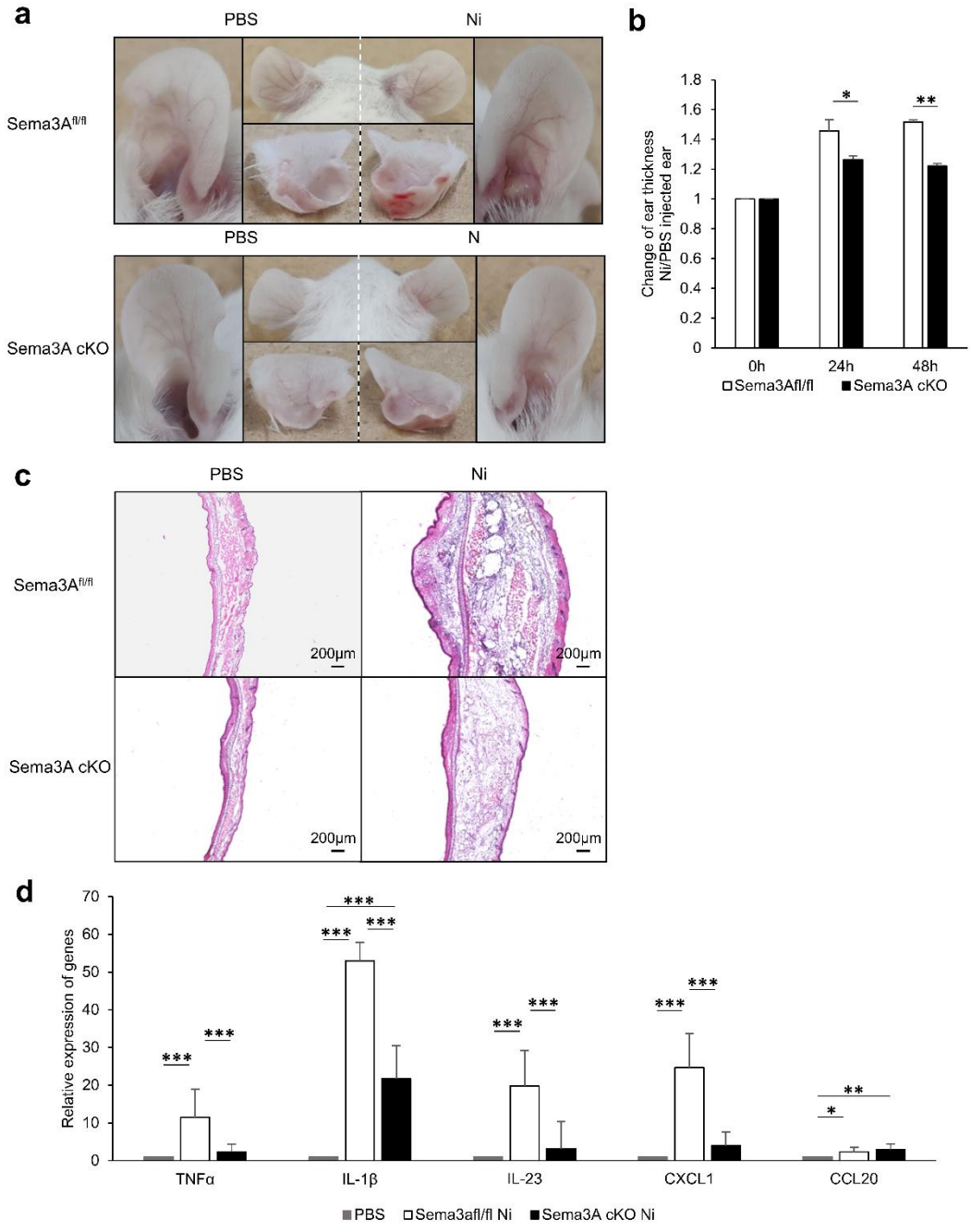
a, d, g The population of $CD11b^{+}CD115^{+}$ monocytes (**a**), $CD11b^{+}F4/80^{+}$ macrophages (**d**), and $CD11c^{+}MHCII^{+}$ DCs (**g**) in mouse ear tissue was analyzed by flow cytometry. **b, e, h** The number of $CD11b^{+}CD115^{+}$ monocytes (**b**), CD11b+F4/80+ macrophages (**e**), and $CD11c^{+}MHCII^{+}$ DCs (**h**) in the Ni allergy-induced mouse ear tissue was increased. Data are shown as mean ± SD of 3 mice/group and are representative of four independent experiments. **c, f** $CD11b^{+}CD115^{+}$ monocytes (**c**) and $CD11b^{+}F4/80^{+}$ macrophages (**f**) in the control and Ni allergy-induced ear tissue were stained using immunofluorescence assays. Arrows indicate double-positive cell location. Scale bar, 100μm. Higher magnification images of $CD11b^{+}CD115^{+}$ monocyte and $CD11b^{+}F4/80^{+}$ macrophage are shown in the upper right of the control group images. Scale bar, 10μm. **i** $CD11c^{+}MHCII^{+}$ DCs in the epidermal sheet

583 of mouse ears were analyzed using immunofluorescence assays. Scale bar, 100μm. *p<0.05. Two

584 independent samples t test. Source data and p values are provided as a Source Data file.

585

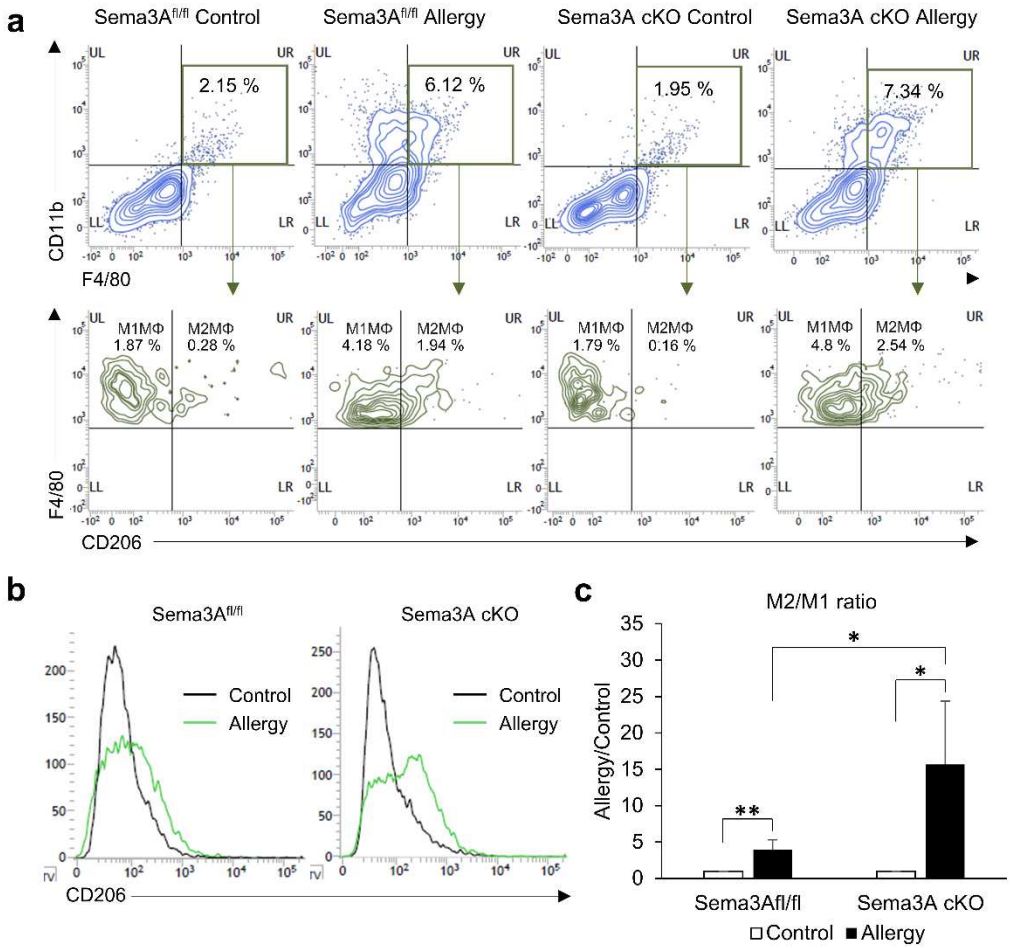
586 **Figure 5. Allergy symptoms in Sema3A cKO mouse.**



587

a Representative photos of the Ni allergy-induced ear and control ear of Sema3A$^{fl/fl}$ and Sema3A cKO groups. Photos show the ventral side (right and left), dorsal side (middle upper) and cross-section (middle lower). **b** DTH was determined by ear thickness measuring after Ni re-challenge. The ratio of allergy/control ear thickness of Sema3A$^{fl/fl}$ and Sema3A cKO mice is shown to present the change of ear thickness. Data are shown as mean ± SD of 3 mice/group. **c** Histological images of the Ni allergy-induced mouse ear tissue and control ear tissue of Sema3A$^{fl/fl}$ and Sema3A cKO groups. Scale bar, 200μm. **d** The mRNA expressions of related cytokines/chemokines in Ni allergy-induced mouse ear tissue of Sema3A$^{fl/fl}$ and Sema3A cKO groups were analyzed using quantitative RT-PCR. Gene expression was normalized to levels of β-actin. *$p<0.05$, **$p<0.01$, ***$p<0.001$. Two independent samples t test for **b**, and One-Way ANOVA with LSD for **d**. Source data and p values are provided as a Source Data file.

Figure 6. Cell population in allergy site in cKO mouse.



a The population of $CD11b^{+}F4/80^{+}$ macrophages in $Sema3A^{fl/fl}$ and Sema3A cKO mouse ears was analyzed by flow cytometry. MΦ represents for macrophage. M1-like MΦ was defined as $CD206^{-}$ $CD11b^{+}F4/80^{+}$ cells and M2-like MΦ was defined as $CD206^{+}CD11b^{+}F4/80^{+}$ cells. **b** Representative flow cytometry analysis of $CD206^{+}$ cells in $Sema3A^{fl/fl}$ and Sema3A cKO mouse ears. Black lines represent the control groups and green lines represent the Ni allergy groups. **c** M2/M1 ratio was increased in Ni allergy-induced Sema3A cKO mouse ear compared to that in $Sema3A^{fl/fl}$ mice. Data

612 are shown as mean ± SD and are representative of four independent experiments. *p<0.05, **p<0.01.

613 Two independent samples t test and One-Way ANOVA with LSD. Source data and p values are

614 provided as a Source Data file.

Supplementary Files

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