

Epicutaneous exposure to nickel induces nickel allergy in mice via a MyD88-dependent and interleukin-1-dependent pathway

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Summary

Background. Several attempts to establish a model in mice that reflects nickel allergy in humans have been made. Most models use intradermal injection of nickel in combination with adjuvant to induce nickel allergy. However, such models poorly reflect induction of nickel allergy following long-lasting epicutaneous exposure to nickel.

Objective. To develop a mouse model reflecting nickel allergy in humans induced by epicutaneous exposure to nickel, and to investigate the mechanisms involved in such allergic responses.

Methods. Mice were exposed to NiCl₂ on the dorsal side of the ears. Inflammation was evaluated by the swelling and cell infiltration of the ears. T cell responses were determined as numbers of CD4⁺ and CD8⁺ T cells in the draining lymph nodes. Localization of nickel was examined by dimethylglyoxime staining.

Results. Epicutaneous exposure to nickel results in prolonged localization of nickel in the epidermis, and induces nickel allergy in mice. The allergic response to nickel following epicutaneous exposure is MyD88-dependent and interleukin (IL)-1 receptor-dependent, but independent of toll-like receptor (TLR)-4.

Conclusion. This new model for nickel allergy that reflects epicutaneous exposure to nickel in humans shows that nickel allergy is dependent on MyD88 and IL-1 receptor signalling, but independent of TLR4.

Key words: IL-1 signalling; MyD88; nickel allergy; TLR4.

Nickel has been known as a contact sensitizer in humans for the last 130 years (1). Historically, nickel sensitization was induced following epicutaneous exposure to nickel-containing items such as suspenders, clip-on earrings, and blue jeans buttons (2, 3). During the mid-1980s,

ear-piercing became very fashionable, and thereby became a major route for nickel sensitization (4, 5). However, recent studies have indicated that epicutaneous exposure to nickel-containing items remains important, as people are still exposed to nickel in items such as blue jeans buttons and jewellery (6, 7). Nickel allergy is a widespread contact hypersensitivity (CHS) reaction, affecting 10–30% of females and 3–6% of males (8). Nickel allergy is known to be a T cell-mediated response involving both CD4⁺ and CD8⁺ T cells (9). In addition to allergen-induced T cell activation, the early allergen-induced inflammation is also critical for the development of nickel allergy (10). Thus, a recent

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study showed that nickel can induce interleukin (IL)-1 β secretion in macrophages and dendritic cells via the NLRP3–ASC–caspase-1 pathway (11). Apart from this, little is known about the basic mechanisms and signalling pathways required for the development of nickel allergy in humans.

To better understand the immune response to nickel, several attempts have been made to establish mouse models that reflect nickel allergy in humans (12–23). The most powerful reactions against nickel in mice have been obtained when nickel is injected intradermally together with either an irritant or an adjuvant such as lipopolysaccharide (LPS) (12, 17). With the use of intradermal injections of nickel, it was recently shown that nickel directly activates human toll-like receptor (TLR)-4 but not mouse TLR4, and it was suggested that TLR4 is key in nickel allergy (24). Since then, it has become the dogma that TLR4 is the master regulator of nickel-induced inflammation (10, 25, 26). We find this surprising, for the following reasons: (i) nickel is known to be promiscuous in its binding to proteins, and can activate T cells by both peptide-dependent and peptide-independent pathways (27, 28), thus making it unlikely that nickel only activates the innate immune system by binding to TLR4; and (ii) as nickel localizes in the epidermis following exposure of human skin to nickel, and neither freshly isolated human keratinocytes nor Langerhans cells express TLR4, one could question whether nickel and TLR4 actually co-localize during the early response to nickel (29–32).

The aim of this study was to establish a model for nickel allergy in mice based on epicutaneous exposure to nickel, and to elucidate some of the basic signalling pathways required to induce nickel allergy. We succeeded in the establishment of a new mouse model in which epicutaneous exposure to nickel induces nickel allergy, and, by the use of this model, we show that the allergic response to nickel is dependent on MyD88 and IL-1 receptor signalling, but independent of TLR4.

Materials and Methods

Mice

Female C57BL/6 mice were purchased from Taconic (Ry, Denmark). C3H/HeN and C3H/HeJ (TLR4-defective) mice were from Charles River (Germantown, MD, USA). MyD88 knockout mice (33) were backcrossed with C57BL/6 mice. Mice were housed in the specific pathogen-free animal facility at the University of Copenhagen in accordance with national animal protection guidelines

(licence number 2007/501-1357). The mice were between 7 and 9 weeks of age at the onset of experiments.

Allergens

Nickel(II) chloride (NiCl₂) (98%) and 2,4-dinitrofluorobenzene (DNFB) were purchased from Sigma Aldrich (Brøndby, Denmark). White petrolatum (100%) was from the pharmacy (Region Hovedstadens Apoteket, Copenhagen, Denmark), and was the same as used for patch testing of patients in the clinic. NiCl₂ 10% (wt/wt) in white pet. was produced by heating the pet. to 70°C before adding the NiCl₂ to 10%. Subsequently, the NiCl₂ and pet. were mixed to form a homogeneous mixture.

In vivo CHS model

Mice were sensitized for 3 consecutive days with ~100 mg/ear of 10% NiCl₂ in white pet., vehicle alone (white pet.) or 25 μ l of 0.15% DNFB in olive oil/acetone (1:4) on the dorsal side of both ears. Three weeks after sensitization, the mice were challenged twice with 10% NiCl₂ in white pet. or vehicle with a 24-hr interval. The mice were killed 48 hr after the first challenge. In some experiments, the role of IL-1 was investigated by treatment of the mice with the IL-1 receptor (IL-1R) antagonist anakinra (Kineret[®], Amgen Inc., Thousand Oaks, CA, USA), as previously described (34, 35). In these previous studies, different concentrations of anakinra (15 000 μ g versus 200 μ g per intraperitoneal injection) were used, and we chose to use a concentration in between these (34, 35). Consequently, mice were given intraperitoneal injections of either 2500 μ g anakinra in phosphate-buffered saline (PBS) or pure PBS twice daily, starting 16 hr before the first treatment, during both the sensitization and challenge phases. Ear thickness was measured with an engineer's micrometer (Mitutoyo, Tokyo, Japan).

Adoptive transfer experiments

Mice were sensitized for 3 consecutive days (D0–D2) with 10% NiCl₂ in white pet., vehicle (white pet.) or 0.15% DNFB in olive oil/acetone (1:4) on the dorsal side of both ears. On D5, the auricular draining lymph nodes (dLNs) were isolated, and single-cell suspensions were prepared. Lymph node cells (2×10^7) were transferred to naïve mice by intravenous injection. Immediately after the adoptive transfer, the mice were treated with either 10% NiCl₂ in white pet., vehicle or 0.15% DNFB in olive oil/acetone (1:4) on the dorsal side of both ears. The treatment was repeated after 24 hr. Forty-eight hours after the first treatment, the mice were killed and changes in ear thickness were determined.

Flow cytometry

Single-cell suspensions were prepared from the dLN, and the absolute numbers of cells were determined. To study the distribution of T cells, the cell suspensions were incubated with fluorochrome-conjugated anti-CD4 (RM4-5) or anti-CD8a (53-6.7) monoclonal antibody (BD Biosciences, Albertslund, Denmark) for 30 min on ice. After washing, the cells were analysed on a FACSCalibur (BD Biosciences) with CELLQUEST and FLOWJO software, as previously described (36).

Haematoxylin and eosin (H&E) staining

The ears were fixed with 4% paraformaldehyde, and embedded in paraffin. The paraffin-embedded ears were then cut into 5- μ m-thick sections, and stained with H&E, as previously described (37).

Nickel staining of mouse ears

Mice were anaesthetized with hypnorm/midazolam, and were treated on both ears either with epicutaneous application of 10% NiCl₂ in white pet., or with intradermal injection of 50 μ l of 10 mM NiCl₂ in PBS. As controls, mice treated with epicutaneous application of white pet. or with intradermal injection of 50 μ l of PBS were included. The mice were killed at either 30 min or 20 hr after the treatment. The ears were excised, embedded in OCT, and snap frozen in liquid nitrogen. The ears were subsequently sectioned into 30- μ m-thick sections, stained with dimethylglyoxime for 15 min, and then stained with haematoxylin for 1 min.

IL-1 β quantitative polymerase chain reaction (PCR)

The ears were disrupted in a PreCellys24 homogenizer (Bertin Technologies, Aix-en-Provence, France), and total RNA was extracted with an RNeasy kit (Qiagen, Copenhagen, Denmark). For reverse transcription, we used TaqMan reverse transcription reagents (Applied Biosystems, Foster City, CA, USA). IL-1 β mRNA expression was detected with TaqMan Gene Expression Assays (Assay ID: Mm00434228_m1), with the glyceraldehyde-3-phosphate dehydrogenase gene (Mm99999915_g1) as the control housekeeping gene. Real-time PCR reactions were conducted on an Mx3005P Real-Time PCR machine (Agilent Technologies, Santa Clara, CA, USA), with the following conditions: 50°C for 2 min, 95°C for 10 min, and 40 PCR cycles at 95°C for 15 seconds and 60°C for 1 min.

Statistical analysis

The unpaired Student's *t*-test was used to test for statistical significance. *p*-Values of < 0.05 were considered to be statistically significant.

Results

Development of adjuvant-free nickel allergy in mice

In humans, nickel allergy can develop following long-lasting epicutaneous exposure to nickel (38), and we therefore speculated whether nickel allergy might be induced in mice with a similar exposure regimen. To test this hypothesis, we sensitized C57BL/6 mice to nickel and challenged them with nickel by repeated exposure of the ears to nickel. Exposure to 5% NiSO₄ or 5% NiCl₂ in pet. induced no or very weak responses, as measured by changes in ear-swelling (data not shown). However, by increasing the concentration of NiCl₂ to 10%, we found that sensitization caused local skin inflammation as measured by ear-swelling (Fig. 1a). Challenge with nickel in nickel-sensitized mice caused marked erythema and ear-swelling (Fig. 1a), and also resulted in increased numbers of both CD4⁺ and CD8⁺ T cells in the dLN (Fig. 1b,c). It could be suspected that the vehicle pet. by itself induces skin irritation which bypass the need for adjuvant; however, no differences in ear thickness or T cell infiltration in the dLN were seen between untreated mice and mice treated with pet. (Fig. 1d and data not shown). Furthermore, no differences in ear thickness were seen between mice sensitized to nickel and challenged with pet. and mice both sensitized to pet. and challenged with pet. (data not shown). Finally, we found that the response to nickel could be adoptively transferred to naïve mice (Fig. 1e,f), underlining the fact that epicutaneous exposure to nickel activates nickel-specific T cells.

Epicutaneous exposure to nickel leads to prolonged localization of nickel in the epidermis

The use of different modes of exposure to nickel might explain why we, in contrast to others, were able to induce nickel allergy without the use of adjuvant (12, 17, 24). Studies investigating the localization of nickel in human skin after patch testing have shown that nickel is primarily located in the epidermis (32). It is therefore likely that localization of nickel in the skin following epicutaneous exposure is different from its localization in models where nickel is injected into the skin. To investigate this, we exposed mice to 10% NiCl₂ epicutaneously or to 10 mM NiCl₂ by intradermal injection, as these represent the modes of exposure to nickel in our model and previous models, respectively (12, 17, 24). After 30 min and 20 hr, nickel localization was determined by dimethylglyoxime staining. In agreement with studies in human skin (32), nickel was localized in the epidermis 30 min after epicutaneous exposure to nickel whereas no staining was seen in vehicle treated ears (Fig. 2a,b) and was still

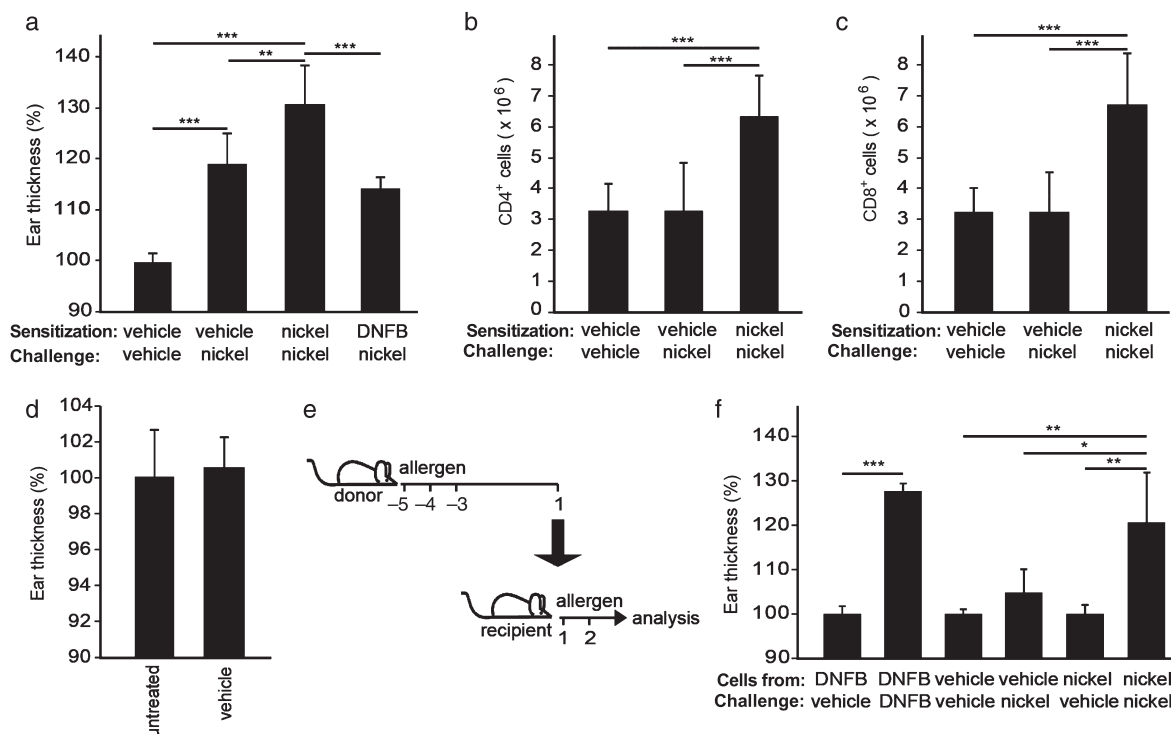


Fig. 1. Nickel allergy in C57BL/6 mice. (a–c) Mice were sensitized with 10% NiCl₂, 0.15% dinitrofluorobenzene (DNFB) or vehicle (white petrolatum) on both ears for 3 consecutive days. After a minimum of 21 days, mice were challenged with 10% NiCl₂ or vehicle on the ears. The challenge was repeated after 24 hr, and the responses were determined after an additional 24 hr. Shown are the relative ear thickness normalized to vehicle-treated ears (a) and the total numbers of CD4⁺ (b) and CD8⁺ cells (c) in the draining lymph node of one representative experiment out of three independent experiments with 8 mice in each group. (d) Mice were either treated with vehicle on both ears for 3 consecutive days (D0–D2) or left untreated. The relative ear thickness normalized to untreated ears was determined on D5. Shown is one representative experiment out of two independent experiments with 4 mice in each group. (e) Schematic model for the adaptive transfer experiments. (f) Relative ear thickness following exposure of the ears to vehicle, nickel or DNFB in naïve mice that had received lymph node cells from mice sensitized with vehicle, nickel, or DNFB, as indicated. Shown is one representative experiment out of two independent experiments with 4 mice in each group. Statistical significance was evaluated with Student's unpaired two-tailed *t*-test (**p* < 0.05, ***p* < 0.01, ****p* < 0.001). Data are shown as mean ± standard deviation.

detectable in the epidermis 20 hr after exposure (Fig. 2c). In contrast, nickel was localized in both the epidermis and dermis 30 min after intradermal injection again no nickel staining was seen in vehicle treated ears (Fig. 2d,e), and was hardly detectable in the skin 20 hr after injection (Fig. 2f). In summary, the mode of exposure to nickel seems to be crucial for the spatiotemporal distribution of nickel in the skin.

The response to nickel is dependent on MyD88 but independent of TLR4

Having established a model for nickel allergy induced by epicutaneous exposure to nickel, we next studied the basic mechanisms involved in nickel allergy induction. By using mice deficient for the intracellular adaptor molecule MyD88, which is required for downstream signalling of all TLRs except for TLR3, we found that MyD88 is essential

for nickel-induced skin inflammation as measured by ear-swelling (Fig. 3a). This observation is in good agreement with a previous study showing that CHS caused by DNFB is MyD88-dependent (39). It has recently been suggested that nickel allergy cannot be induced in mice without the use of adjuvant or irritants, as mouse TLR4 is presumably unable to bind nickel directly (24). As we were able to induce nickel allergy in mice without the use of adjuvant or irritants, and as TLR4 has been shown to be involved in the induction of CHS in mice, we wanted to examine the role of mouse TLR4 in our model for nickel allergy (40). To investigate this, we sensitized C3H/HeN and C3H/HeJ mice, which have functional and defective TLR4 signalling, respectively (41), to nickel. Upon challenge with nickel, similar ear-swelling and cellular infiltration of the ears was seen in C3H/HeN and C3H/HeJ mice (Fig. 3b,c). Furthermore, this observation excluded the

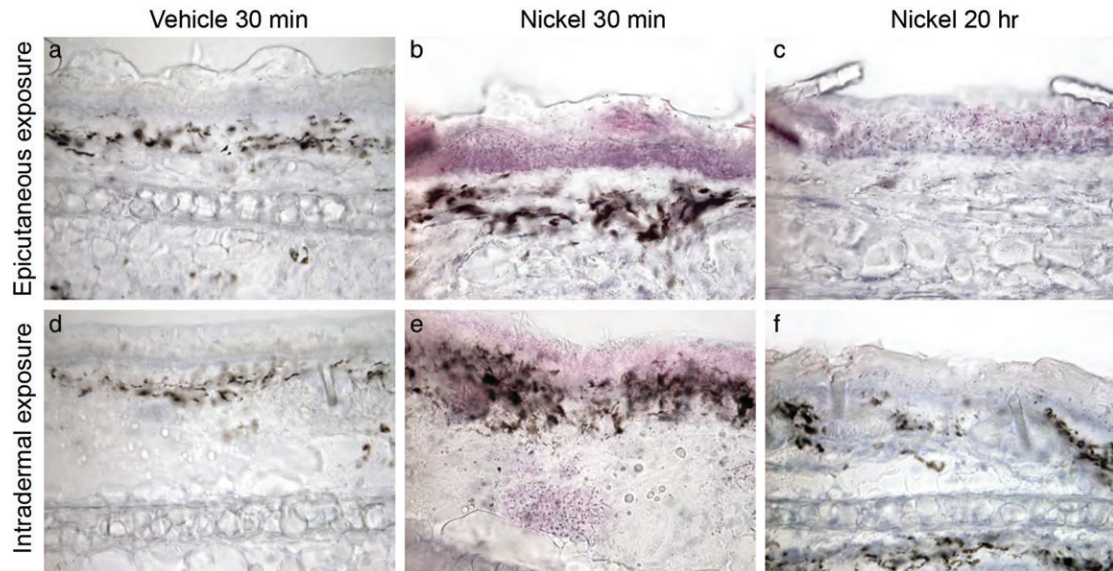


Fig. 2. Epicutaneous exposure to nickel leads to prolonged nickel localization in the epidermis. Mice were treated epicutaneously with vehicle or 10% NiCl_2 , or with intradermal injection of vehicle or 10 mM NiCl_2 . The ears were removed and snap frozen in liquid nitrogen 30 min or 20 hr after the treatment, and cryosections were stained for nickel with dimethylglyoxime (pink) followed by haematoxylin. (a) Epicutaneous vehicle treatment 30 min. (b) Epicutaneous nickel treatment 30 min. (c) Epicutaneous nickel treatment 20 hr. (d) Intradermal vehicle injection 30 min. (e) Intradermal nickel injection 30 min. (f) Intradermal nickel injection 20 hr. Scale bar: 30 μm .

possibility that the response to nickel was attributable to LPS contamination of the nickel solutions, as C3H/HeJ mice have defective LPS signalling (41). Thus, we can conclude that MyD88 is critically important, whereas TLR4 is dispensable, for the development of nickel allergy following epicutaneous exposure to nickel in mice.

IL-1 is essential for the response to nickel

MyD88 acts not only downstream of TLR signalling but also downstream of IL-1R signalling (33), and previous studies have suggested that CHS responses are dependent on the inflammasome and IL-1 β (34, 42–44). Furthermore, it has recently been shown that nickel can induce IL-1 β secretion via the NLRP3–ASC–caspase-1 pathway in macrophages and dendritic cells (11). Thus, the unresponsiveness to nickel in MyD88-deficient mice might be caused by defective IL-1R signalling. To investigate this, we first examined whether IL-1 β is induced in the skin following epicutaneous exposure to nickel in mice. We found that nickel caused a sixfold increase in the level of IL-1 β mRNA (Fig. 4a). To further investigate the role of IL-1, mice were treated with the IL-1R antagonist anakinra during sensitization and challenge. Treatment with anakinra inhibited nickel-induced ear-swelling (Fig. 4b). This indicates that IL-1R signalling is required for induction of nickel allergy, and might explain the dependency of the allergic response to nickel on MyD88.

Discussion

In this study, we have shown that nickel allergy can be induced in mice following epicutaneous exposure to nickel. Macroscopically, the allergic response in the mice resembled contact allergy to nickel in humans with erythema and swelling. The response was allergen-specific and mediated by T cells, as shown by adoptive transfer experiments. We found that epicutaneous and intradermal exposure to nickel lead to different spatiotemporal distributions of nickel in the skin, with epicutaneous exposure leading to prolonged nickel localization in the epidermis, and intradermal injection leading to shorter and more diffuse nickel localization in the skin. Furthermore, we found that the response to nickel was dependent on MyD88 and IL-1R signalling, but independent of TLR4.

For > 30 years, scientists have tried to create a model for nickel allergy that reflects nickel allergy in humans (12–24). Most models used until now have been based on intradermal injections of nickel together with either an irritant or an adjuvant (12, 17). The reason why we could induce nickel allergy in this study without the use of irritants or adjuvants might be that nickel is removed much faster from the skin after intradermal injection than following epicutaneous application. Furthermore, in addition to the route of exposure, it is likely that the concentration of nickel plays an important role in nickel

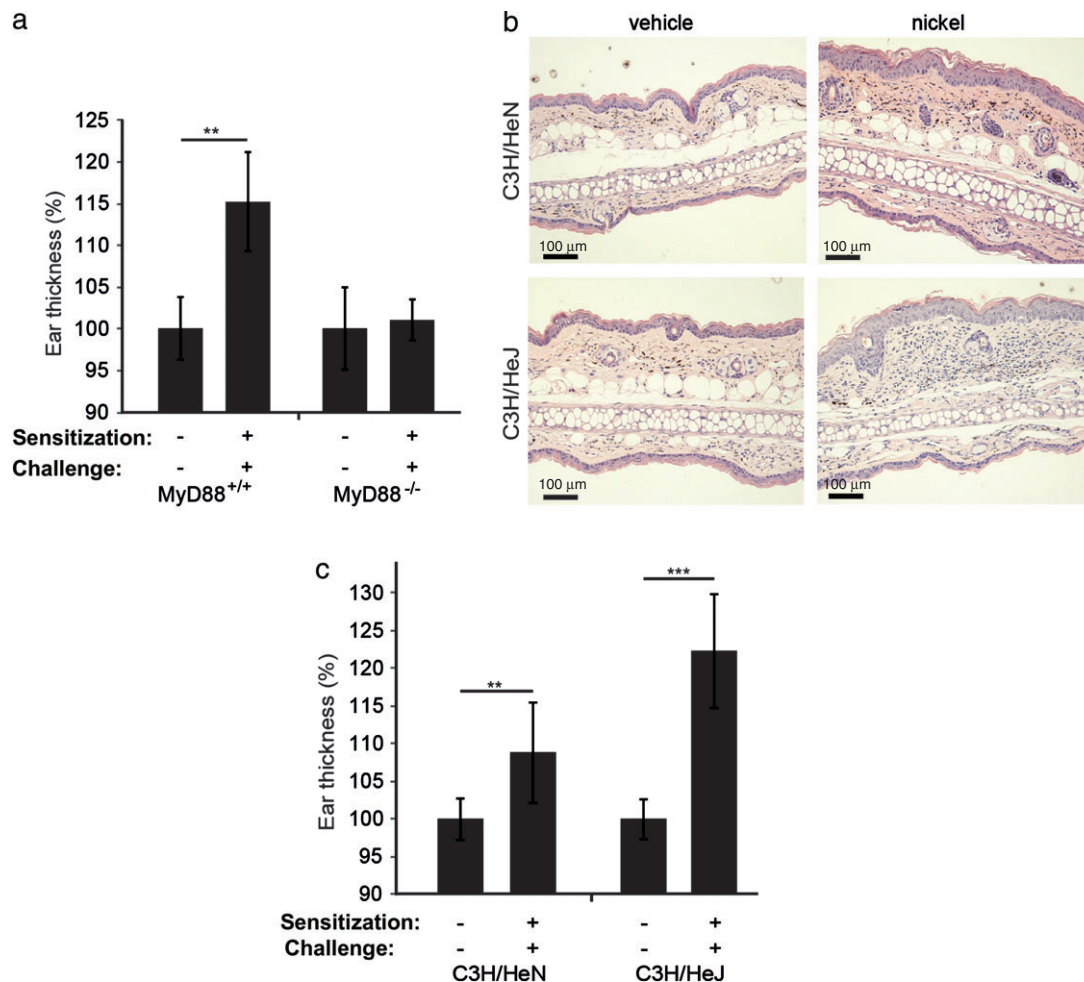


Fig. 3. Nickel allergy is dependent on MyD88 but independent of toll-like receptor-4 (TLR4). MyD88^{+/+}, MyD88^{-/-}, C3H/HeN and C3H/HeJ (TLR4-defective) mice were sensitized with either 10% NiCl₂ or vehicle for 3 consecutive days on both ears. Mice were challenged after a minimum of 21 days for 2 consecutive days on both ears. (a) Relative ear thickness of MyD88^{+/+} and MyD88^{-/-} mice normalized to vehicle-treated ears, shown as mean $\pm$ standard deviation (SD) of one representative experiment out of two independent experiments with 4 mice in each group. (b) Haematoxylin and eosin staining of ear samples collected from C3H/HeN (top) and C3H/HeJ (bottom) mice 48 hr after the first challenge. Original magnification: $\times 200$. (c) Relative ear thickness of C3H/HeN and C3H/HeJ (TLR4-defective) mice, shown as mean $\pm$ SD of one representative experiment out of three independent experiments with 8 mice in each group. Statistical significance was evaluated with Student's unpaired two-tailed *t*-test (***p* < 0.01, ****p* < 0.001).

sensitization. In this study, mice were exposed to 10% NiCl₂ epicutaneously or to 10 mM NiCl₂ by intradermal injection, which makes it difficult to compare the nickel concentrations used in the two modes of exposure (12, 17, 24). Finally, we show that 10% NiCl₂ in pet. has an irritant effect, as seen by the ear-swelling induced in naïve mice following exposure to nickel, which is in good agreement with human studies showing that NiCl₂ has a stronger irritant effect than NiSO₄ (45). This irritant effect most likely potentiates the ability of NiCl₂ to induce sensitization and the ensuing allergic response against nickel.

It has been shown that LPS increases the response to nickel following intradermal exposure to nickel (14, 17). Interestingly, a recent study suggested that nickel allergy could only be induced in mice in combination with either an irritant or adjuvant, as mouse TLR4 lacks the nickel-binding histidines found in human TLR4 (24). However, the fact that we could induce comparable responses against nickel in TLR4-intact C3H/HeN mice and TLR4-defective, and thereby LPS-unresponsive, C3H/HeJ mice excluded the possibility that the nickel responses induced in our study were attributable to LPS contamination (41). Thus, our data suggest that nickel, at least in mice,

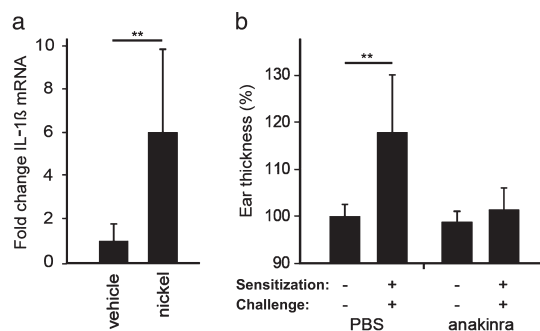


Fig. 4. Nickel induces interleukin (IL)-1 β production in the skin, and the response to nickel is dependent on IL-1 receptor signalling. (a) Relative levels of IL-1 β in ears treated twice with vehicle or 10% NiCl₂ with a 24-hr interval. The expression levels of IL-1 β 24 hr after the last treatment were determined by quantitative polymerase chain reaction. Shown is the fold change in IL-1 β mRNA of mice treated with nickel as compared with mice treated with vehicle of one representative experiment out of two independent experiments with 8 mice in each group. (b) Mice were sensitized with either 10% NiCl₂ or vehicle, and challenged after a minimum of 21 days. During both the sensitization and challenge phases, mice received intraperitoneal injections of either phosphate-buffered saline (PBS) or 2500 μ g of anakinra in PBS (twice daily), starting 16 hr before vehicle/nickel treatment. Shown is the relative change in ear thickness of one representative experiment out of two independent experiments with 8 mice in each group. Statistical significance was evaluated with Student's unpaired two-tailed *t*-test (***p* < 0.01). Data are shown as mean $\pm$ SD.

can activate the innate immune response by additional mechanisms other than via TLR4. This was further supported by the dependency of the response to nickel on MyD88 and IL-1R signalling. As nickel is localized in the epidermis following exposure of human skin to nickel, and as TLR4 is normally not expressed on the cells in the human epidermis, this further support the idea that TLR4 is not the only mechanism whereby nickel can activate the innate immune response in humans (29–32). However, the roles of the exposure route, nickel concentration and TLR4 in the response to nickel in humans need further investigation.

Previously, the epidermis was considered to be merely a physical barrier against pathogens, but it is now clear that cells resident in the epidermis play an important role in shaping immune responses in the skin. Our results suggest that the immune response in CHS depends on the route of allergen delivery. From this, it could be speculated that cells in the outer layers of the skin are potent activators of immune responses, and that they might use different mechanisms than cells in the deeper layers of the skin to activate the immune system. This idea is supported by the observation that stronger protective immunity is induced when mice are vaccinated epicutaneously against poxvirus through scarified skin than when the antigen is delivered by intradermal injection (46), and by the observation that epicutaneous sensitization with ovalbumin induced a robust local and systemic Th17 immune response, whereas intraperitoneal antigen application did not induce a Th17 response (47). Taken together, these studies support the central role of the epidermis not just in local but also in systemic immune responses.

In conclusion, we have established a model for nickel allergy that reflects nickel allergy in humans following epicutaneous exposure to nickel (38). We have shown that the route of exposure is central for the spatiotemporal localization of nickel in the skin, and that nickel allergy is dependent on MyD88 and IL-1R signalling, but independent of TLR4. Finally, our results suggest that anakinra or other drugs that block IL-1/IL-1R signalling might be used in the treatment of severe cases of nickel allergy.

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