

Crucial role for human Toll-like receptor 4 in the development of contact allergy to nickel

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Allergies to nickel (Ni²⁺) are the most frequent cause of contact hypersensitivity (CHS) in industrialized countries. The efficient development of CHS requires both a T lymphocyte-specific signal and a proinflammatory signal. Here we show that Ni²⁺ triggered an inflammatory response by directly activating human Toll-like receptor 4 (TLR4). Ni²⁺-induced TLR4 activation was species-specific, as mouse TLR4 could not generate this response. Studies with mutant TLR4 proteins revealed that the non-conserved histidines 456 and 458 of human TLR4 are required for activation by Ni²⁺ but not by the natural ligand lipopolysaccharide. Accordingly, transgenic expression of human TLR4 in TLR4-deficient mice allowed efficient sensitization to Ni²⁺ and elicitation of CHS. Our data implicate site-specific human TLR4 inhibition as a potential strategy for therapeutic intervention in CHS that would not affect vital immune responses.

In Europe, an estimated 65 million people are sensitized to Ni²⁺ and develop allergic contact dermatitis (ACD) upon contact with Ni²⁺-releasing metal alloys such as costume jewelry, body piercings and coins^{1–4}. ACD is based on a contact hypersensitivity (CHS) reaction that involves antigen presentation by dendritic cells (DCs) and the generation of a T lymphocyte response. When sensitized individuals are re-exposed to the contact allergen, the clinically manifest skin disease evolves. Apart from the antigen-specific T cell-activating signal, a second proinflammatory stimulus is required for efficient sensitization^{5–7}. The proinflammatory signal results in the upregulation of cytokines, chemokines and co-stimulatory adhesion molecules primarily in DCs, phagocytes and endothelial cells, all of which contribute to the developing inflammatory response.

Ni²⁺ can directly activate proinflammatory intracellular signal transduction cascades that result in the stimulation of the transcription factor nuclear factor- κ B (NF- κ B) and the mitogen-activated protein (MAP) kinase p38 (refs. 8,9). However, the molecular mechanisms that underlie the generation of the proinflammatory signal are unknown. Gene profiling studies revealed that Ni²⁺ elicits a gene expression pattern reminiscent of that induced by innate immune signals, which is dominated by proinflammatory genes¹⁰. In most cases, the initiation of such signals relies on membrane-bound and intracellular receptors known as pattern recognition receptors (PRRs). The best known classes are the Toll-like receptor (TLR) family, which senses microbial pathogens and endogenous ligands, and the Nod-like receptors, which detect not only microbes but also inorganic agents such as crystalline urates, asbestos, silica and aluminum^{11–13}.

Here we investigated the possible involvement of TLRs in the sensing of Ni²⁺. We show that Ni²⁺ induced an inflammatory response by directly activating human TLR4. Activation required distinct sequence motifs that are present in human but not mouse TLR4. We show that only TLR4-deficient mice expressing transgenic human TLR4 developed CHS to Ni²⁺, whereas animals expressing mouse TLR4 failed to generate CHS. Our data provide a mechanistic explanation of why mice, unlike humans, require co-administration of adjuvants to generate CHS to Ni²⁺. We present a mouse model for Ni²⁺-induced contact allergy that works without obligatory adjuvant co-stimulation.

RESULTS

Ni²⁺ signals through TLR4

Ni²⁺ directly activates proinflammatory intracellular signal transduction cascades, resulting in stimulation of the transcription factor NF- κ B (Fig. 1a). We speculated that the Ni²⁺-induced expression of proinflammatory proteins such as those encoded by the NF- κ B target genes interleukin 8 (*IL8*) and *CCL2* (also known as *MCPI*) might result from the activation of PRRs. To gain insight into the upstream mediators of Ni²⁺-induced NF- κ B signaling, we evaluated its dependency on MyD88, a crucial adaptor molecule that is linked to receptors of the interleukin 1 (IL-1)-TLR family. Depletion of endogenous MyD88 by RNA-mediated interference (RNAi) almost completely abrogated Ni²⁺-induced expression of IL-8 in endothelial cells (Fig. 1b). As described¹⁴, the response to IL-1 β was blocked,

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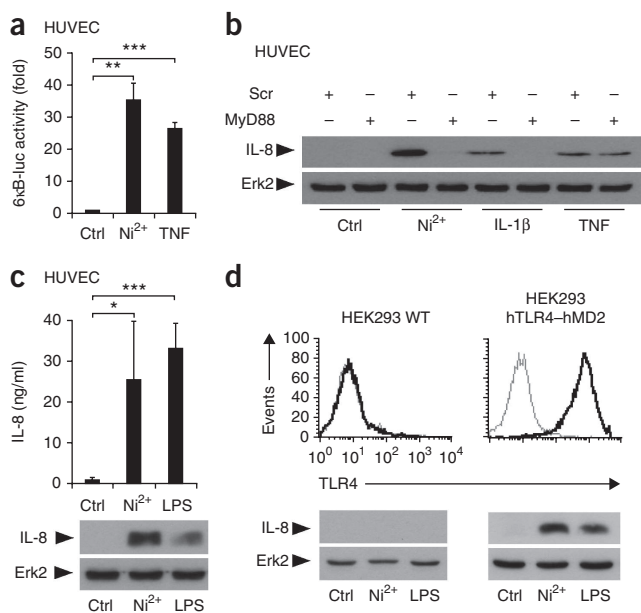


Figure 1 Activation of primary cells by the contact allergen Ni²⁺ requires MyD88–human TLR4. **(a)** Fold average luciferase activation of a 6κB luciferase (6κB-luc) reporter transfected into primary human umbilical vein endothelial cells (HUVECs). Cells were stimulated for 16 h with Ni²⁺ (1.5 mM), TNF (2 ng ml⁻¹) or medium (Ctrl). **(b)** Immunoblot of IL-8 expression upon stimulation with Ni²⁺, IL-1β and TNF in HUVEC transfected with siRNA for human MyD88 (MyD88) or a scrambled siRNA control (Scr). **(c)** IL-8 production determined by ELISA of supernatants (top) or immunoblot of cell lysates (bottom) in HUVECs stimulated with Ni²⁺ or LPS (*Escherichia coli* O26:B6; 1 μg ml⁻¹). **(d)** Immunoblot of IL-8 expression (lower) and flow cytometric analysis of surface human TLR4 expression (upper, bold lines) in comparison to isotype staining (thin lines) in wild-type HEK293 cells and HEK293 cells expressing human TLR4–human MD2 (hTLR4–hMD2) after stimulation with Ni²⁺ or LPS. Cells were incubated with the specified stimuli for 8 h unless indicated otherwise. Bar diagrams in **a** and **c** represent means ± s.d. of three independent experiments. Immunoblots and flow cytometry data in **b–d** are representative of three independent experiments. **P* < 0.05, ***P* < 0.01, ****P* < 0.001; unpaired *t*-test.

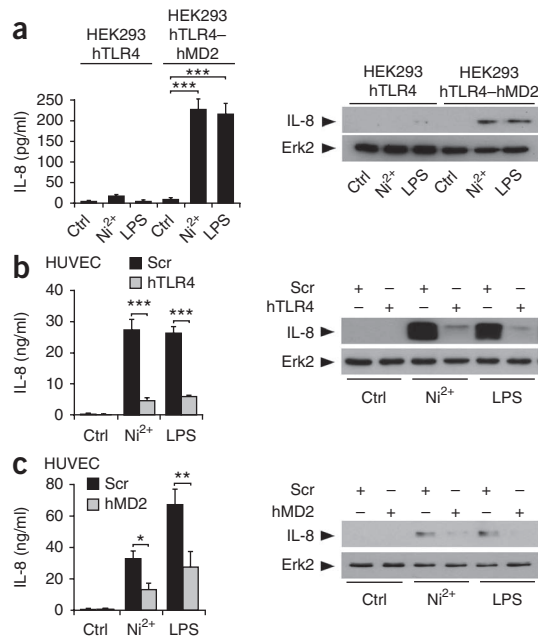
but tumor necrosis factor (TNF)-dependent IL-8 expression was unaffected (**Fig. 1b**). Likewise, blockade of endogenous IL-1 receptor-associated kinase-1 (IRAK1) by expression of a dominant negative mutant (IRAK1-DD) resulted in almost complete inhibition of Ni²⁺- and IL-1β-induced expression of CCL2 but not of the TNF-mediated response (**Supplementary Fig. 1**). Depletion of IRAK1 by retroviral expression of small hairpin RNA produced similar results (data not shown). These data indicate that Ni²⁺ induces NF-κB-dependent gene expression through MyD88 and IRAK1. Having excluded autocrine stimulation by IL-1 (ref. 8), we tested whether TLRs were involved in the Ni²⁺-induced response. The TLR4 agonist lipopolysaccharide (LPS) induced IL-8 in endothelial cells (**Fig. 1c**). To evaluate whether TLR4 can mediate the Ni²⁺-induced proinflammatory responses, we stably expressed human TLR4 and its co-receptor human MD2 in human embryonic kidney cells (HEK293), which do not express endogenous TLRs. Whereas the parental HEK293 cells did not respond to Ni²⁺ and LPS, cells that expressed human TLR4–MD2 responded to both stimuli (**Fig. 1d**). Expression of other TLRs in HEK293 cells did not allow them to be activated by Ni²⁺ (**Supplementary Fig. 2a**). These observations indicate that Ni²⁺-induced expression of the NF-κB target gene IL-8 requires human TLR4–MD2. We then investigated whether expression of human TLR4 alone is sufficient to mediate recognition of the Ni²⁺ signal. In the absence of MD2, human TLR4 expression alone failed to induce IL-8 upon stimulation with LPS or Ni²⁺ (**Fig. 2a**). Depletion of endogenous endothelial human TLR4 (**Fig. 2b**) or

human MD2 (**Fig. 2c**) in human primary endothelial cells, which were transfected with validated small interfering RNA (siRNA) against human TLR4 or human MD2, led to the inhibition of Ni²⁺- and LPS-mediated expression of IL-8. Potential contamination of Ni²⁺ by LPS was excluded by *limulus* assays (data not shown) as well as by addition of polymyxin B sulfate, which blocked activation by LPS but not by Ni²⁺ (**Supplementary Fig. 2b**). Together, these data show that Ni²⁺-induced proinflammatory gene expression is independent of LPS but requires both human TLR4 and human MD2.

Ni²⁺ activates human TLR4 but not mouse TLR4

Next, we investigated whether Ni²⁺ could also induce proinflammatory gene expression in other primary cells. Ni²⁺ caused the upregulation of the NF-κB target gene *TNF* in human buffy coat monocytes (**Fig. 3a**) and fibroblasts (data not shown). Mouse cells such as bone marrow-derived macrophages (**Fig. 3a**) or Raw264.7 macrophages (data not shown) did not produce TNF when exposed to Ni²⁺. The inability of mouse cells to respond to Ni²⁺ was not due to a lack of mouse TLR4 or mouse MD2, as stimulation with LPS readily triggered induction of TNF (**Fig. 3a**).

Figure 2 Proinflammatory gene expression by Ni²⁺ requires human TLR4 and its co-receptor human MD2. **(a)** Left, ELISA of supernatants and right, immunoblot of cell lysates show Ni²⁺- and LPS-induced production of IL-8 in HEK293 cells stably expressing human TLR4 (hTLR4) alone or with human MD2 (hMD2). **(b,c)** Analysis of Ni²⁺- and LPS-induced IL-8 synthesis in human primary endothelial cells transfected with siRNA against either human TLR4 (**b**) or human MD2 (**c**) or scrambled siRNA (Scr) as control. Left, ELISA of supernatants or right, western blot analysis of cell lysates show IL-8 production. Bar diagrams represent means ± s.d. of three independent experiments. Western blots are representative of three (**a**) or two (**b,c**) independent experiments. Cells were stimulated for 8 h with the indicated stimuli. **P* < 0.05, ***P* < 0.01, ****P* < 0.001; unpaired *t*-test.



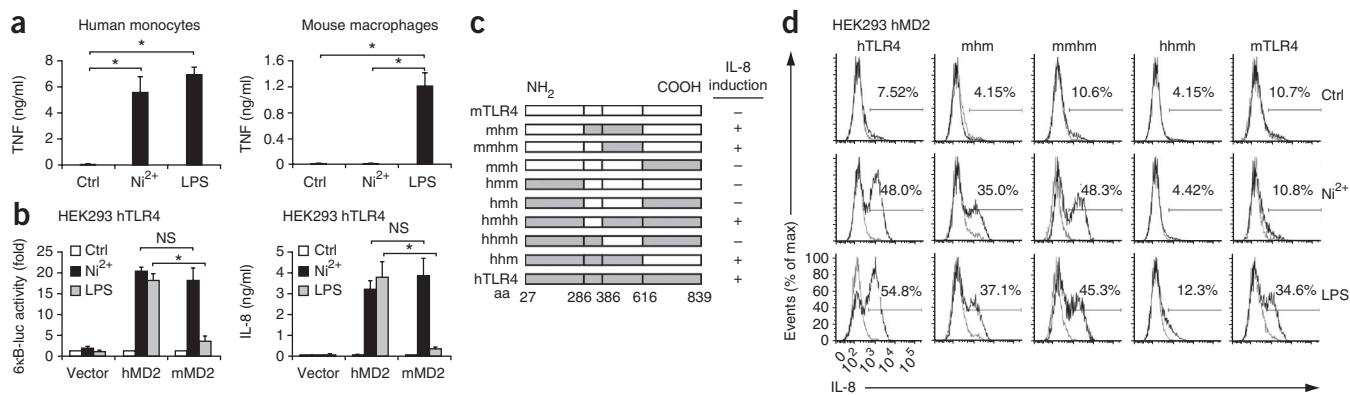
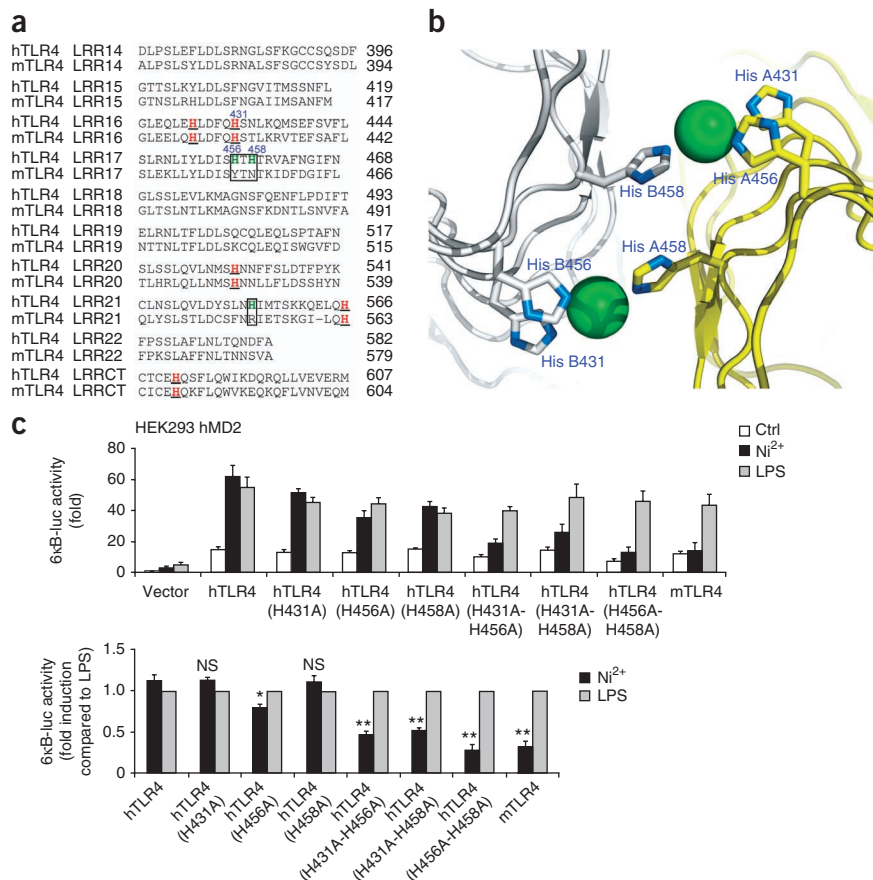


Figure 3 Ni²⁺-induced gene expression requires sequence motifs that are present in human TLR4 but not in mouse TLR4. **(a)** ELISA showing TNF release from primary human monocytes (left) or bone marrow macrophages from C57Bl/6 wild-type mice (right) exposed to Ni²⁺ or LPS (*Salmonella minnesota* R595; 1 μg ml⁻¹) for 8 h. **(b)** Activation of a 6kb-luc reporter (left) and IL-8 release (ELISA, right) from human TLR4 (hTLR4)-expressing HEK293 cells transfected with human MD2 (hMD2), mouse MD2 (mMD2) or empty vector and stimulated with Ni²⁺ or LPS. Data in **a** and **b** represent means ± s.d. of three independent experiments. **P* < 0.001; NS, not significant; unpaired *t*-test. **(c)** ELISA of IL-8 release (–, <2-fold increase; +, ≥2-fold increase) in stable HEK293-human MD2 cells transfected with hemagglutinin (HA)-tagged human TLR4, HA-mouse TLR4 (mTLR4) or the indicated chimeric HA-TLR4 constructs and exposed to Ni²⁺ or LPS (as above) for 8 h. Data are representative of four independent experiments. Gray boxes, human sequence parts; open boxes, mouse sequence parts. **(d)** Flow cytometry of intracellular IL-8 expressed by the cells used in **c**. Transfection was monitored by immunostaining for hemagglutinin and gating on the hemagglutinin-positive population. Black lines, IL-8 staining; gray lines, isotype-staining; data are representative of three independent experiments. Numbers denote the percentage of IL-8 expressing cells in the hemagglutinin-gated population.

To elucidate whether the difference in response to Ni²⁺ was due to species-specific differences in MD2, we transfected HEK293 cells expressing human TLR4 with either human MD2 or mouse MD2. Irrespective of the origin of the transfected MD2 co-receptor, human TLR4 could mediate Ni²⁺ signals (Fig. 3b), indicating

that the differences between the Ni²⁺ responses of mice and humans might depend on sequence variations in TLR4. We predicted that non-conserved sequence motifs of human TLR4 were probably required for recognition of Ni²⁺ signals. To test our hypothesis, we transfected HEK293 cells stably expressing human MD2 with

Figure 4 Non-conserved histidines in human TLR4 (hTLR4) provide a potential binding site for Ni²⁺. **(a)** Protein sequence alignment of human TLR4 and mouse TLR4 (mTLR4) (aa 371–604) covering LRRs 14–22 in the C-terminal domain of the TLR4 β-sheet¹⁶. Conserved histidine residues (H) are shown underlined and in red; non-conserved histidines boxed. **(b)** Model of the potential double metal-binding site in human TLR4 showing close-up view from the top along the twofold symmetry axis. The model is based on the crystal structure of the human TLR4–human MD2 (hTLR4–hMD2) complex (PDB entry 3FXI) with LPS omitted. The side chains of the histidine residues H431, H456 and H458 (sticks with nitrogen colored blue) were repositioned with appropriate side chain rotamers, followed by addition of Ni²⁺ ions (green spheres) to yield a pair of metal-binding sites with a geometry similar to that seen in an engineered serine protease/inhibitor complex²⁵ (PDB entry 1SLW). **(c)** Luciferase activity of a 6kb-luc reporter transfected into HEK293 cells expressing human MD2 and the indicated TLR4 constructs. Cells were stimulated for 8 h with Ni²⁺, LPS or medium as control (Ctrl). Luciferase activity is presented as fold stimulation (top) or as Ni²⁺ stimulation in relation to LPS (arbitrarily set to 1, bottom). Data represent means ± s.d. of three independent experiments. **P* < 0.01, ***P* < 0.001; NS, not significant; unpaired *t*-test.



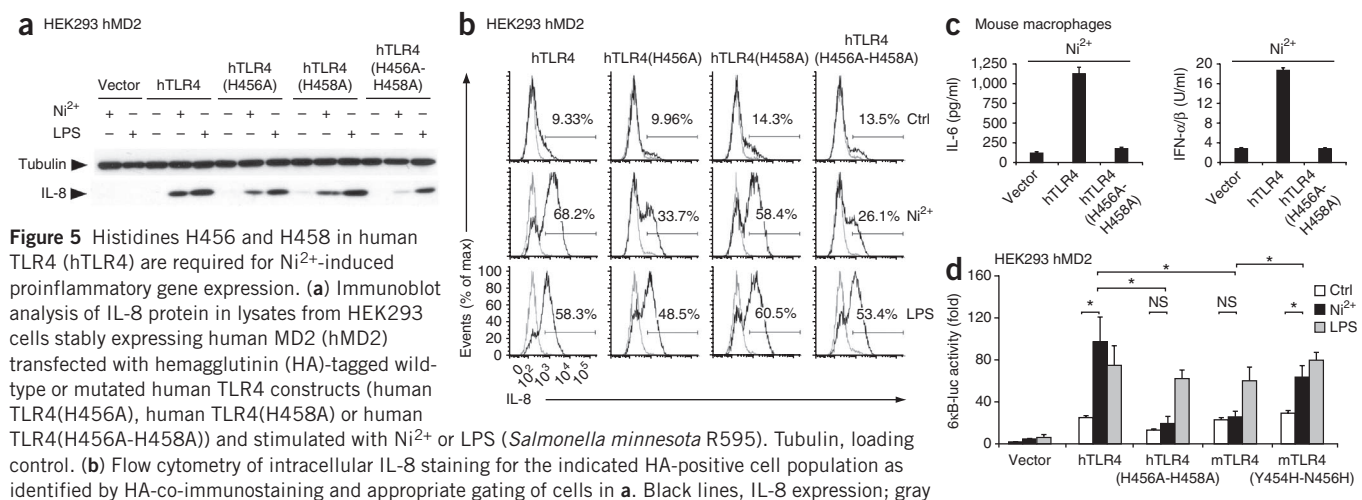


Figure 5 Histidines H456 and H458 in human TLR4 (hTLR4) are required for Ni²⁺-induced proinflammatory gene expression. **(a)** Immunoblot analysis of IL-8 protein in lysates from HEK293 cells stably expressing human MD2 (hMD2) transfected with hemagglutinin (HA)-tagged wild-type or mutated human TLR4 constructs (human TLR4(H456A), human TLR4(H458A) or human TLR4(H456A-H458A)) and stimulated with Ni²⁺ or LPS (*Salmonella minnesota* R595). Tubulin, loading control. **(b)** Flow cytometry of intracellular IL-8 staining for the indicated HA-positive cell population as identified by HA-co-immunostaining and appropriate gating of cells in **a**. Black lines, IL-8 expression; gray lines, isotype controls. For statistical analysis of flow cytometry data see **Supplementary Figure 4a**. Data in **a** and **b** are representative of three independent experiments and represent means \pm s.d. **(c)** IL-6 (left) or IFN- α/β (right) cytokine production of Ni²⁺-stimulated (for 6 h) mouse macrophages retrovirally infected to stably express wild-type human TLR4, human TLR4(H456A-H458A) or empty vector as control. Data represent means \pm s.d. of two independent experiments. **(d)** Luciferase activity of a transfected 6 \times B-luc reporter in HEK293-human MD2 cells transfected with human TLR4, human TLR4(H456A-H458A), mouse TLR4 or mouse TLR4(Y454H-N456H). Cells were stimulated with Ni²⁺, LPS or medium as control for 8 h. Data represent means \pm s.d. of three independent experiments. **P* < 0.01; NS, not significant; unpaired *t*-test.

hemagglutinin-tagged wild-type constructs for either human TLR4 or mouse TLR4. We also studied hemagglutinin-tagged chimeric TLR4 with distinct single or multiple domain exchanges of human TLR4 and mouse TLR4 (ref. 15). Cells that expressed human TLR4 and human MD2 responded strongly to Ni²⁺, whereas cells expressing mouse TLR4 and human MD2 did not synthesize IL-8 when exposed to Ni²⁺ (**Fig. 3c,d**). Moreover, we found that the human sequence from amino acid position 369 to 616 is essential for the induction of NF- κ B-dependent gene expression by Ni²⁺ (**Fig. 3c,d**). The differences did not arise from expression differences because co-staining with a hemagglutinin-tag-specific antibody confirmed that human TLR4 and the non-responsive constructs were expressed equally efficiently (**Supplementary Fig. 3** and data not shown).

Histidine residues mediate response to Ni²⁺

Analysis of the species-dependent amino acid variations in this region covering the leucine-rich repeats (LRR) 14 to 22 showed three non-conserved histidine residues that were potentially suitable for Ni²⁺ binding (**Fig. 4a**). The crystal structure of the human TLR4-human MD2 complex, which forms a homodimer in the presence of LPS¹⁶, revealed the presence of two of the non-conserved histidines, H456 and H458, in close proximity to the conserved histidine residue H431. In a proposed dimer these residues are predicted to form a cluster of six histidine residues at the twofold symmetry axis of the two human TLR4 monomers (**Fig. 4b**). This arrangement suggests several potential cross-linking binding sites for Ni²⁺ ions. Structural modeling of putative metal binding sites showed that particularly the imidazole groups of H456 and H458 were in optimal distance for interaction with a Ni²⁺ ion, whereas the ones of H431 were further apart (**Fig. 4b**).

To study the requirements for histidine residues H431, H456 and H458, we generated various single or double alanine mutants and transfected them into HEK293 cells stably expressing human MD2. Individual removal of the histidine side chains at positions 431 or 458 had no effect on Ni²⁺-induced NF- κ B activation in luciferase assays, whereas mutation of H456 partially diminished the response to Ni²⁺ (**Fig. 4c**). By contrast, expression of double mutants efficiently suppressed Ni²⁺-regulated NF- κ B-dependent gene expression in all

combinations, with the strongest inhibition occurring upon mutation of the two human-specific histidines H456 and H458 (**Fig. 4c**). Double mutation of H456 and H458 considerably decreased Ni²⁺-induced IL-8 production in various readouts including western blot (**Fig. 5a**), flow cytometry (**Fig. 5b** and **Supplementary Fig. 4a**) and enzyme-linked immunosorbent assay (ELISA; **Supplementary Fig. 4b**), whereas single substitution of H456 or H458 had comparably mild or no effects, respectively. In addition, stable expression of wild-type human TLR4 in mouse macrophages conferred responsiveness to Ni²⁺, whereas expression of the human TLR4 (H456A-H458A) double mutant almost completely abolished Ni²⁺-induced cytokine expression (**Fig. 5c**). Notably, all mutants still showed LPS-induced cytokine induction (**Figs. 4c** and **5a,b** and **Supplementary Fig. 4a,b**), indicating that LPS and Ni²⁺ have different sequence requirements for activation. This was also supported by blocking experiments using the anti-human TLR4 HTA-125 antibody, which neutralized LPS-induced but not Ni²⁺-induced activation of human TLR4 (**Supplementary Fig. 4c**).

We finally explored whether introduction of histidines at the corresponding positions of mouse TLR4 was sufficient to mimic the response of human TLR4. Expression of a mouse TLR4 (Y454H-N456H) double mutant in HEK cells conferred responsiveness to Ni²⁺ (**Fig. 5d**). These data suggest that unique sequence motifs of human TLR4 dictate the species difference for recognition of Ni²⁺ by human and mouse cells. On the basis of our mutagenesis data and the known crystal structure of the human TLR4-human MD2 dimer complex, we propose a molecular model wherein Ni²⁺ cross-links the two receptor monomers through specific histidine side chains, triggering formation of a dimer that structurally resembles the one induced by LPS¹⁶ (**Fig. 4b** and **Supplementary Fig. 5**).

Expression of human TLR4 transfers Ni²⁺ sensitivity to mouse

Previous attempts to establish a functional mouse model for Ni²⁺-induced CHS have failed because mice have a low natural susceptibility to this allergen; only co-administration of irritants, complete Freund's adjuvant or LPS made it possible to develop Ni²⁺ allergy in mice^{17,18}. We generated transgenic mice expressing human TLR4 and explored whether the introduction of *TLR4* could confer Ni²⁺ susceptibility

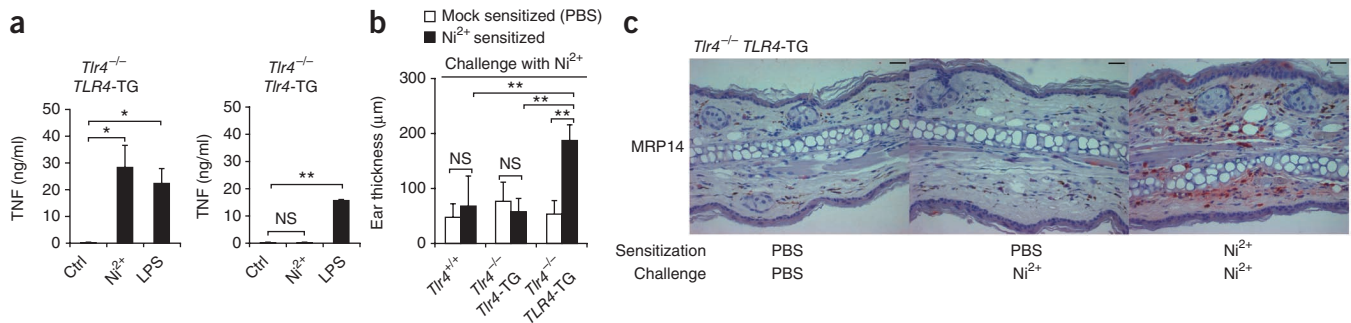


Figure 6 Transgenic expression of human TLR4 in TLR4-deficient mice confers susceptibility to Ni^{2+} and allows induction of contact hypersensitivity. (a) Comparative ELISA analysis of TNF production induced by Ni^{2+} or LPS (*Salmonella minnesota* R595) treatment of macrophages from transgenic mice expressing human TLR4 (*Tlr4*^{-/-} TLR4-TG left) or mouse TLR4 (*Tlr4*^{-/-} Tlr4-TG right) on a C57BL/10ScCr background. Data represent means \pm s.d. of three independent experiments. (b) Ear thickness measurements of wild-type and mouse *Tlr4*-TG or human TLR4-TG mice intracutaneously sensitized at day 0 with Ni^{2+} or PBS (mock) at the abdominal wall and challenged with Ni^{2+} on both ears at day 11. Measurements were taken 24 h after challenge. Data represent means \pm s.d. of three to four animals per group and are representative of four independent experiments. (c) Histological immunostaining for MRP14 shows monocyte and neutrophil infiltration in ear skin sections from human TLR4-TG mice sensitized and challenged with Ni^{2+} or PBS (mock) 24 h after challenge. Data are representative of two independent experiments. * $P < 0.01$, ** $P < 0.001$; NS, not significant; unpaired *t*-test.

and enable mice to develop CHS to Ni^{2+} . We introduced the human *TLR4* gene into C57BL/10ScCr mice, which bear an inactivating natural deletion of the *Tlr4* gene¹⁹. Animals from the resulting mouse line (*Tlr4*^{-/-} TLR4-TG) showed cell-specific expression and organ distribution of human TLR4, typical for mouse TLR4 in wild-type mice, and showed a mild overexpression in relevant cells such as monocytes and macrophages that was comparable to previously described *Tlr4*^{-/-} mouse *Tlr4* transgenic (*Tlr4*^{-/-} Tlr4-TG) mice²⁰ (Supplementary Fig. 6 and unpublished data). Bone marrow-derived macrophages from *Tlr4*^{-/-} TLR4-TG mice produced substantial amounts of TNF (Fig. 6a), IL-1 β , IL-6 and IL-10 (Supplementary Fig. 7a) in response to LPS and Ni^{2+} . By contrast, macrophages from control *Tlr4*^{-/-} mice expressing transgenic mouse TLR4 (ref. 20) produced these cytokines when stimulated with LPS but not with Ni^{2+} (Fig. 6a and Supplementary Fig. 7a). These effects were not due to higher expression of human TLR4 than of mouse TLR4 in the transgenic animals, as control staining of splenic macrophages and DCs from TLR4-deficient mice expressing the transgenes showed similar if not lower expression of human TLR4 (Supplementary Fig. 6b). Other Ni^{2+} -mediated responses such as phosphorylation of p38 MAP kinase and interferon regulatory factor 3 (IRF3) and synthesis of interferon (IFN) α/β were seen only in cells obtained from *Tlr4*^{-/-} TLR4-TG mice but not from *Tlr4*^{-/-} Tlr4-TG mice (Supplementary Fig. 7b,c). Thus, Ni^{2+} triggered multiple proinflammatory signals downstream of human TLR4 even when expressed in mouse cells.

We then tested the ability of Ni^{2+} to induce ACD in a mouse model of CHS. *Tlr4*^{-/-} TLR4-TG or *Tlr4*^{-/-} Tlr4-TG mice were sensitized to Ni^{2+} (or PBS as control) and challenged with the contact allergen 11 days later. Twenty-four hours after elicitation, ear thickness was determined as the classical measure of a mouse CHS reaction (Fig. 6b). *Tlr4*^{-/-} TLR4-TG mice sensitized to Ni^{2+} developed significant ear swelling upon elicitation with Ni^{2+} , whereas mock-sensitized animals or *Tlr4*^{-/-} Tlr4-TG mice did not. After sensitization to Ni^{2+} , *Tlr4*^{-/-} TLR4-TG mice consistently showed considerable leukocyte infiltration into the skin at sites of allergen challenge (Fig. 6c). By contrast, mock-treated animals did not respond.

We have shown that human TLR4 is a crucial receptor for Ni^{2+} that allows the generation of a proinflammatory signal (Supplementary Fig. 8) and have described a mouse model of contact allergy to this abundant allergen.

DISCUSSION

Our study describes the molecular basis of inflammatory co-stimulation for a contact allergen and identifies Ni^{2+} as an inorganic activator of the TLR system. In contrast to other contact allergens such as 2,4,6-trinitrochlorobenzene, oxazolone and fluorescein isothiocyanate (FITC), which seem to interact indirectly with TLR2 and TLR4 signaling^{7,21}, Ni^{2+} directly triggers PRR signaling. As a consequence, NF- κ B-, p38- and IRF3-dependent signal transduction cascades are activated that result in the expression of multiple proinflammatory genes. Accordingly, our observation explains why Ni^{2+} , but not other contact allergens, directly triggers NF- κ B-dependent activation of human DCs²².

We found that Ni^{2+} -induced proinflammatory responses are species-specific and require non-conserved histidines that are found in human TLR4 but not mouse TLR4. As a result, we failed to induce proinflammatory responses and CHS in *Tlr4*^{-/-} Tlr4-TG mice but readily could induce these responses in *Tlr4*^{-/-} TLR4-TG mice. The species dependency of Ni^{2+} -induced TLR4 activation explains why current mouse models for Ni^{2+} -induced contact allergy require additional co-stimulatory agents or adjuvants^{17,18}. Our *Tlr4*^{-/-} TLR4-TG mice provide a mouse model that allows the study of Ni^{2+} -induced CHS by Ni^{2+} treatment alone, which more closely resembles the human situation. Such an adjuvant-free model should facilitate further research on the mechanisms of Ni^{2+} -induced contact allergy without the obscuring effects of additives.

On the basis of our mutagenesis data and the published crystal structure of the human TLR4-human MD2 complex, we propose a model of activation by Ni^{2+} . Although neither the human TLR4 H431-H456-H458 triplet nor the histidine doublet formed by the closely spaced H458 residues at the interface of two TLR4 monomers have the proper distances and side chain geometry to form a high-affinity binding site²³, minor local conformational adjustments allow an interdigitated arrangement of the histidine side chains from both human TLR4 monomers and, owing to the increased structural flexibility in this mode of interaction, lead to two putative chelating sites for Ni^{2+} at the interface. This model can nicely explain the complex role of the individual histidine residues that became apparent from the mutagenesis studies: elimination of a single histidine at any of the positions 431, 456 or 458 alone had little or no effect on Ni^{2+} responsiveness, whereas substitution of any pair of histidine residues by alanine led to a loss of receptor activation. The proposed $(\text{Ni}^{2+})_2$ -(human TLR4)₂ complex can be

modeled without hampering the gross dimeric receptor quaternary structure that is induced by LPS¹⁶. The crystallographic analysis of the LPS–human TLR4–human MD2 complex has shown that many pre-existing hydrophobic and hydrophilic interactions contribute to the dimerization interaction between the individual human TLR4–human MD2 moieties. As inorganic complex formation, especially between Ni²⁺ and imidazole groups, involves strong chemical bonds, it can be envisaged that Ni²⁺-mediated cross-linking of the two receptor monomers as described above leads to a structurally very similar dimer that can trigger signal transduction. As a consequence, proinflammatory gene expression is induced and delivers the co-stimulatory signal ('signal 2') that is required for efficient sensitization and elicitation of contact allergy⁷. The mechanism outlined here fundamentally differs from the interaction of the major house dust mite allergen Der p 2 with TLR4 (ref. 24). Unlike Ni²⁺, which acts independently of LPS, Der p 2 functionally mimics MD2 and requires the presence of LPS to trigger TLR4 signaling. It therefore acts as sensitizer for LPS, whereas Ni²⁺ is a direct activator of TLR4 signaling. Our identification of a unique Ni²⁺-responsive region in human TLR4, which is distinct from its LPS-binding domain, implies that it might be possible to specifically interfere with Ni²⁺-induced TLR4 signaling without affecting LPS responses. Thus, future strategies for prophylaxis and treatment of contact allergy to Ni²⁺ may no longer need to rely on currently used topical immunosuppressants. In light of the species differences in TLR4 responses, our study furthermore implies that assessments of the allergenic and toxic potential of chemicals and other environmental compounds in animal models might not predict their properties in humans and should be interpreted with care.

METHODS

Methods and any associated references are available in the online version of the paper at <http://www.nature.com/natureimmunology/>.

Accession codes. HUGO: *TLR4*, U88880; *LY96 (MD2)*, AB018549 and NM_015364; *IRAK1*, L76191; MGI: *Thr4*, 96824; *Ly96 (Md2)*, 1341909.

Note: Supplementary information is available on the Nature Immunology website.

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AUTHOR CONTRIBUTIONS

M.S., B.R., V.M., T.V., G.F., S.T., S.K., C.K., P.J.N., S.F.M. and M.A.F. performed the experiments. M.S., C.G., J.R., A.S., S.F.M., M.A.F. and M.G. designed the experiments and analyzed the data. M.G., M.S., S.F.M., A.S. and M.A.F. wrote the paper. M.G. managed the project and had overall responsibility for data interpretation and writing the manuscript. All authors discussed and commented on the manuscript.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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ONLINE METHODS

Cells and cell culture. Primary HUVECs, monocytes isolated from buffy coats, HEK293 wild-type cells or HEK293 cells stably expressing human TLR4 and/or human MD2 (InvivoGen) were cultured as described^{10,26,27}. Blood monocytes, bone marrow-derived macrophages, DCs, splenic macrophages and myeloid DCs from various mouse strains were prepared, cultured and characterized by flow cytometry as described^{21,28–30}.

Reagents. NiCl₂·6H₂O (referred to as Ni²⁺) was purchased from Merck. Endotoxin contaminations were excluded by *limulus* amoebocyte lysate assay (BioWhittaker) and blocking experiments using polymyxin B sulfate (Sigma). LPS from *Salmonella minnesota* R595 was purchased from Alexis, LPS from *E. coli* 055:B5 from Sigma.

Plasmids and transient gene transfer. IRAK1-DD encoding bp 83–544 of the human *IRAK1* gene³¹ and acting as dominant-negative mutant was transfected into HUVECs using nucleofection (Amaxa). Expression vectors for human and mouse MD2 were provided by M. Muroi³². Chimeric hemagglutinin-tagged *TLR4* receptor genes in which coding sequences for distinct domains of human TLR4 and mouse TLR4 were exchanged were provided by A.M. Hajjar¹⁵. Plasmids were transiently transfected into HEK293 cells according to standard protocols. The composition of expressed receptors was: human TLR4, aa 27–839; mouse TLR4, aa 26–835; hhm, human TLR4 aa 27–616 and mouse TLR4 aa 614–835; mmh, mouse TLR4 aa 26–613 and human TLR4 aa 617–839; hmh, human TLR4 aa 27–286, mouse TLR4 aa 286–613 and human TLR4 aa 617–839; hmm, human TLR4 aa 27–286, mouse TLR4 aa 286–35; hmhh, human TLR4 aa 27–286, mouse TLR4 aa 285–366 and human TLR4 aa 369–839; hhmh, human TLR4 aa 27–368, mouse TLR4 aa 367–618 and human TLR4 aa 617–839.

Site-directed mutagenesis. Mutant TLR4 receptors with single- or double-point mutations were generated by site-directed mutagenesis (Quikchange II site-directed mutagenesis kit, Stratagene) and verified by sequencing: human TLR4(H431A), human TLR4(H456A), human TLR4(H458A), human TLR4(H456A-H458A), human TLR4(H431A-H456A), human TLR4(H431A-H458A), mouse TLR4(Y454H-N456H).

Retroviral gene transfer. Selected human TLR4 constructs were cloned into the pMYS-IRES-GFP retroviral vector and transiently transfected into PlatE packaging cells. Clarified and filtered supernatants containing retroviral particles were used directly to infect mouse macrophages in the presence of polybrene.

RNA interference. HUVECs were transfected with validated siRNA from Qiagen (siRNAs against human MyD88 (SI000300909), human TLR4 (SI100151004), human MD2 (SI03246446) or scrambled siRNA) at a final concentration of 200 nM using Oligofectamine (Invitrogen Life Technologies).

Promoter reporter gene studies. HUVECs were transiently transfected with 6kB-luc and ubiquitin-dependent *Renilla* luciferase as described¹⁰. Luciferase activity was measured using the DualGlo Luciferase Assay System (Promega). 6kB-dependent luciferase activity was normalized to the luminescence generated by the *Renilla* luciferase control reporter and expressed as fold stimulation.

Flow cytometry. For analysis of TLR4, CD14, CD11c and F4/80 surface expression and intracellular detection of chemokines CCL2 and IL-8, cells were immunostained using appropriate fluorochrome-labeled antibodies^{21,30,33}. Successful transfection of HEK293 cells with HA-TLR4 constructs was monitored by staining with an anti-hemagglutinin primary antibody (3F10, Roche) and successful transfection of HUVEC with the IRAK1-DD construct was controlled by co-transfected green fluorescent protein (GFP). Readout molecules were analyzed after gating on hemagglutinin-positive or GFP-positive cells.

Detection of cytokines. Cell culture supernatants were assayed for human IL-8 or TNF and mouse IL-6 or IL-10 using ELISA kits from BD Biosciences or eBioscience. For detection of mouse IL-1 β , macrophage lysates were analyzed by ELISA (eBioscience). Mouse TNF and IFN- α/β were determined using bioassays as described³⁰.

Western blot. Cells were lysed in E1A lysis buffer, Laemmli or RIPA buffer and proteins detected by western blot as described³⁴ using antibodies against

IL-8 (BD Biosciences), phospho-p38 or phospho-IRF3 (Cell Signaling). Equal loading was controlled by labeling with rabbit antisera against Erk2, p38 (Santa Cruz Biotechnology) or α -tubulin (Sigma).

Generation of human TLR4 transgenic mice. Mice expressing human TLR4 on a *Tlr4*^{-/-} background were constructed by pronuclear injection of fertilized eggs derived from the C57BL/10ScCr line¹⁹ with the *TLR4* BAC clone 152C16 described by and obtained from A. Poltorak and B. Beutler³⁵. Bacterial vector sequences were removed before injection. The BAC clone contains ~100 kb of human genomic sequences beginning in the region between 14 and 30 kb upstream of the major transcriptional start site of *TLR4*, extending through the 12-kb genomic region of *TLR4* containing all exons and ending in a region between 41 and 91 kb downstream of the *TLR4* polyA site. Expression of the transgene in mice was monitored by RT-PCR using total RNA extracted from various organs (data not shown). Surface expression of human TLR4 on macrophages, Mac-1⁺ blood leukocytes and B lymphocytes but not T lymphocytes from transgenic mice was confirmed by flow cytometry. The introduction of the human TLR4 transgene conferred LPS sensitivity to C57BL/10ScCr mice as verified by cytokine production (TNF, IL-6) following LPS treatment of mice *in vivo* or mouse macrophages *in vitro* and mitogenic activity after treatment of splenic B lymphocytes *in vitro* (data not shown).

***In vivo* CHS model.** Naturally TLR4-deficient C57BL/10ScCr mice¹⁹ and TLR4-deficient C57BL/10ScCr mice transgenically expressing either mouse TLR4²⁰ or human TLR4 were bred under specific pathogen-free (SPF) conditions at the Max-Planck-Institute for Immunobiology and used at 6–10 weeks of age. For induction of contact hypersensitivity, 50 μ l of NiCl₂ (10 mM) was injected intracutaneously at two sites of the abdominal wall; control mice received solvent only. Challenge was performed on day 11 by intracutaneous injection of 50 μ l NiCl₂ (10 mM) into the dorsal side of the ear pinna of both ears after isoflurane anesthesia. Ear measurement was done before and 24 h after ear challenge using an engineer's micrometer. All animal experiments were performed in accordance with institutional, state and federal guidelines on animal welfare. The animal experiments were approved by the Regierungspräsidium Freiburg and supervised by the animal protection representatives of the Max-Planck-Institute and the University Medical Center Freiburg.

Immunohistochemistry. Leukocyte infiltration to the ear skin of mice was studied by immunostaining of paraffin-embedded skin samples using an antiserum against MRP14 (also known as S100A9) that detects neutrophils and monocytes²⁹.

Molecular modeling. Molecular modeling and graphics preparation were performed using PyMOL software³⁶.

Statistics. Statistical analysis was performed using unpaired Student's *t* test. Differences at *P* < 0.05 were considered as statistically significant.

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