

DR KANAN BANDO (Orcid ID : 0000-0002-2818-2454)  
DR TOSHINOBU KUROISHI (Orcid ID : 0000-0003-1633-8615)

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## **Interleukin-1 and histamine are essential for inducing nickel-allergy in mice**

**Short running title:** IL-1 and histamine in nickel allergy

Kanan Bando<sup>1</sup>, Toshinobu Kuroishi<sup>2</sup>, Shunji Sugawara<sup>2</sup>, Yasuo Endo<sup>3</sup>

<sup>1</sup>Division of Orthodontics and Dentofacial Orthopedics, Graduate School of Dentistry,  
Tohoku University, 4-1 Seiryomachi, Aoba-ku, Sendai 980-8575, Japan

<sup>2</sup>Division of Oral Immunology, Department of Oral Biology, Graduate School of  
Dentistry, Tohoku University, 4-1 Seiryomachi, Aoba-ku, Sendai 980-8575, Japan

<sup>3</sup>Division of Oral and Maxillofacial Surgery, Graduate School of Dentistry, Tohoku  
University, 4-1 Seiryomachi, Aoba-ku, Sendai 980-8575, Japan

### **Correspondence**

Kanan Bando, Division of Orthodontics and Dentofacial Orthopedics, Graduate School  
of Dentistry, Tohoku University, 4-1 Seiryomachi, Aoba-ku, Sendai 980-8575, Japan.

E-mail: [kananbando@dent.tohoku.ac.jp](mailto:kananbando@dent.tohoku.ac.jp)

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## Abstract

**Background:** We previously reported that (i) lipopolysaccharide (LPS) is a potent adjuvant for inducing Nickel (Ni)-allergy in mice at both the sensitization and elicitation steps, (ii) LPS induces Interleukin-1 (IL-1) and histidine decarboxylase (HDC, the histamine-forming enzyme), and IL-1 induces HDC, (iii) Ni-allergy is induced in mast-cell-deficient, but not IL-1-deficient (IL-1-KO) or HDC-KO mice.

**Objective:** To examine the roles of IL-1 and HDC (or histamine) and their interrelationship during the establishment of Ni-allergy.

**Methods:** Ni (NiCl<sub>2</sub>) 1 mM containing IL-1 $\beta$  and/or histamine was injected intraperitoneally (sensitization step). Ten days later, test substance(s) were intradermally injected into ear-pinnas (elicitation step), and ear swelling was measured.

**Results:** In wild-type mice, Ni+LPS or Ni+IL-1 $\beta$  injection at sensitization step followed by Ni alone at elicitation step induced Ni-allergy. In IL-1-KO, injection of Ni+IL-1 $\beta$  (but not Ni+histamine) was required at *both* sensitization *and* elicitation steps to induce Ni-allergy. In HDC-KO, Ni+IL-1 $\beta$ +histamine at sensitization step followed by Ni+histamine at elicitation step induced Ni-allergy. In histamine-H1-receptor-deficient mice, IL-1 $\beta$  induced HDC, but was ineffective as an adjuvant for inducing Ni-allergy. In wild-type mice, injection into ear-pinnas of Ni 10 mM alone or Ni 1 mM+LPS induced IL-1 $\beta$ , HDC, and a prolonged swelling of ear-pinnas. In non-sensitized mice, injection of IL-1 $\beta$  by itself into ear-pinnas in IL-1-KO mice induced prolonged ear swelling. Ni augmented IL-1 production (both IL-1 $\alpha$  and IL-1 $\beta$ ) and HDC induction in wild-type mice sensitized to Ni.

**Conclusions:** In mice: (i) For inducing Ni-allergy, IL-1 is essential at *both* the sensitization and elicitation steps, and HDC induction is involved in the effect of IL-1, (ii) stimulation of H1 receptor is also essential for inducing Ni-allergy at both sensitization and elicitation steps, and (iii) the “sensitization to Ni” state may be a state where tissues are primed for augmented production of IL-1 $\alpha$  and/or IL-1 $\beta$  in response to Ni. (within 300 words, now 300)

## 1. INTRODUCTION

Nickel (Ni) is the most frequent metal allergen producing contact hypersensitivity (CHS). However, few adequate murine models exist of Ni-induced CHS or Ni-allergy. Indeed, in animal experiments Ni induces Ni-allergy only at very high concentrations. We previously found that bacterial or chemical inflammatory substances can act as adjuvants for the induction of Ni-allergy.<sup>1-5</sup> Among such adjuvants, lipopolysaccharide (LPS), a cell-wall component of Gram-negative bacteria, exhibits the strongest adjuvant activity, and LPS markedly reduces the concentration of NiCl<sub>2</sub> needed to induce Ni-allergy at both the sensitization and elicitation steps (Table 1),<sup>6</sup> and LPS thereby greatly enhances the sensitivity to Ni. However, it is not clear what mechanisms are involved in this sensitization or in the adjuvant effect of LPS, or indeed such effects by other inflammatory substances.

Histamine is a well-known mediator of allergic inflammation. Histamine formation from the amino acid histidine is catalyzed by the enzyme histidine decarboxylase (HDC). Schayer, Kahlson and their coworkers found a marked increase in HDC activity in various tissues of mice or rats under conditions such as inflammatory reactions,<sup>7,8</sup> anaphylaxis,<sup>9</sup> injection of various inflammatory stimulants,<sup>10-12</sup> rapid growth,<sup>13</sup> repair of wounds,<sup>13</sup> and tissue transplantation.<sup>14</sup> They emphasized that “newly formed histamine” via enhanced HDC activity might play important roles under conditions such as those listed above.<sup>12,13,15</sup> At the time of that pioneering research, however, little was known about the newly formed histamine itself, because of the lack of adequate methods to measure it. Moreover, pre-formed, long-lived mRNA had been thought to be responsible for enhancing HDC activity because actinomycin D (inhibitor of RNA synthesis) was either ineffective or actually potentiated HDC activity.<sup>8</sup>

Since then, concerning HDC, the following points have been clarified. (i) Newly formed histamine is detectable in various tissues and blood during the induction of HDC, indicating its release from cells during the induction of HDC.<sup>16,17</sup> (ii) HDC is induced via the formation of short-lived HDC mRNA.<sup>18</sup> (iii) HDC is induced by various inflammatory cytokines including IL-1, TNF, IL-12, and IL-18<sup>19-21</sup> and hematopoietic cytokines (G-CSF, GM-CSF, and IL-3).<sup>22-25</sup> (iv) Various bacterial components induce or promote the induction of HDC.<sup>26-28</sup> (v) HDC induction by LPS or IL-1 is independent of mast cells, and the major HDC-inducing cells are non-mast cells (including macrophages and endothelial cells).<sup>29</sup> (vi) In mice sensitized to an antigen, antigen challenge induces HDC in mast cells and non-mast cells and, in this case, the major HDC-inducing cells are mast cells.<sup>30</sup> Thus, it should be noted that in addition to its release from mast cells and/or basophils, histamine is provided via induction of HDC in other cells.

We have previously reported the following findings. (i) Among exogenous substances, LPS is particularly potent at inducing HDC in mice,<sup>16,26</sup> and C3H/HeJ mice, which lack active receptors for LPS [i.e., Toll-like receptor 4 (TLR4)], are considerably less responsive to LPS with regard to HDC induction<sup>26</sup> and C3H/HeJ mice are insensitive to the adjuvant effect of LPS in Ni-allergy.<sup>1</sup> (ii) Among endogenous substances, IL-1 is particularly potent at inducing HDC in mice<sup>19,24</sup> and Ni-allergy is barely induced at all in mice deficient in IL-1 (IL-1-KO),<sup>1</sup> HDC (HDC-KO),<sup>1,5,31</sup> or histamine H1 receptors (H1R-KO).<sup>31</sup> (iii) Ni-allergy occurs in mast-cell-deficient W/W<sup>v</sup> mice, but not in macrophage-depleted mice.<sup>1</sup>

LPS is a potent (possibly the strongest) inducer of IL-1,<sup>32,33</sup> and IL-1 is primarily associated with innate immunity.<sup>34</sup> Based on these findings and those described above, we earlier hypothesized that IL-1 and histamine may be essential endogenous molecules mediating the adjuvant effect of LPS or other inflammatory substances for the induction of Ni-allergy in mice at either sensitization or elicitation step.<sup>1,5,31</sup> In the present study, on mice,

we examined the roles of IL-1 and HDC (or histamine), and their interrelationship, during the establishment of Ni-allergy or Ni-induced CHS.

## 2. METHODS

### 2.1. Animals

Wild-type (WT) BALB/c and C57BL/6N mice were purchased from SLC (Shizuoka, Japan). IL-1-KO mice (deficient in both IL-1 $\alpha$  and IL-1 $\beta$ ) were established from original IL-1 $\alpha$ -KO and IL-1 $\beta$ -KO BALB/c mice.<sup>35</sup> HDC-KO and C57BL/6 H1R-KO mice (both C57BL/6) were established as previously described.<sup>36,37</sup> All mice were raised in standard aluminum cages (with a lid made of stainless steel wires) and allowed standard food pellets (LabMR Stock; Nihon Nosan Inc, Yokohama, Japan) and tap water *ad libitum* (the latter from a plastic bottle through a stainless steel tube) in an air-conditioned room at 23  $\pm$  1°C and 55  $\pm$  5% relative humidity with a standard cycle of 12 h light and 12 h dark (lights on at 07:00 a.m.). Female or male mice were used in the various experiments. All animal procedures were approved by the Institutional Animal Care and Use Committee of Tohoku University (approval number: 2018DnA-014). All experiments complied with Regulations for Animal Experiments and Related Activities at Tohoku University.

### 2.2. Reagents

*E. coli* O55:B5 LPS (prepared by phenol extraction) was purchased from Sigma (St. Louis, MO, USA). Recombinant mouse IL-1 $\beta$  was purchased from BioLegend (CA, USA). NiCl<sub>2</sub>·6H<sub>2</sub>O (99.999 %) was purchased from Sigma-Aldrich (St. Louis, MO, U.S.A.). All other reagents were purchased from Wako Pure Chemical Ind. Ltd. (Osaka, Japan), unless otherwise indicated.

### 2.3. Sensitization to Ni and elicitation of Ni-allergy

Unless otherwise indicated, mice (20-23 g female or 23-25 g male) were sensitized to Ni by intraperitoneal (i.p.) injection of a solution containing 1 mM NiCl<sub>2</sub> (Ni) alone or an indicated concentration of a test substance(s). Ten days after the sensitization injection, a solution containing 1 mM Ni alone or an indicated test substance(s) was intradermally (i.d.) injected into ear-pinnas (termed “challenge”), and ear swelling was measured (using a Peacock dial thickness gauge; Ozaki MFG Co. Ltd, Tokyo, Japan), and the induced difference (after vs. before the challenge) was recorded. Throughout this study, a test solution was i.p. injected into a mouse at 0.1 ml/10 g body weight, while 20 µl of a test solution was used for i.d. injection into an ear-pinna.

In some experiments, histamine was i.p. or i.d. injected. Injected histamine is known to be rapidly cleared from the circulation. So, in order to keep the effects of the injected histamine, we tested the following concentrations. The concentration of histamine used for i.p. injection (0.1 ml/10 g body weight) was 180 mM, resulting in 1/10 of the LD<sub>50</sub>. For i.d. injection into ear-pinnas (20 µl/ear), 45 mM histamine was used, the amount of histamine included in such an injection corresponding to about 100-times the histamine normally present within a single ear-pinna (see Section 3.4). These concentrations of histamine did not induce any toxic effects, such as bristling, shock, or swelling.

Incidentally, we showed previously that an epicutaneous injection (Into flanks or ear-pinnas) of a Ni solution containing LPS as an adjuvant is effective at inducing Ni-allergy, and that injections of Ni and LPS via different routes were similarly effective.<sup>1,5</sup> We think that either local or systemic inflammatory stimuli that produce IL-1 may contribute to the establishment of sensitization to Ni. In our previous and present studies, we used i.p. injection at sensitization step.

#### **2.4. Assay of HDC activity**

Tissues (ear and/or spleen) were removed after decapitation, and HDC activity was assayed using our previously described method.<sup>26,29</sup> HDC activity is expressed as nmol of histamine formed during a 1 h period of incubation by the enzyme contained in 1 g (wet weight) of tissue (nmol/h/g).

#### **2.5. Measurement of IL-1 $\beta$ in ear-pinnas and blood serum**

Test materials were injected i.d. into ear-pinnas (20  $\mu$ l/ear) and whole ear-pinnas were removed after the indicate time. In other experiments, test materials were i.p. injected into mice (0.1 ml/10 g body weight of mouse), and their blood was collected directly into test tubes following decapitation. Serum was recovered by centrifugation at 2000  $g$  at 4 °C, then stored at -80 °C until used. Frozen tissue or serum was homogenized in RPMI 1640 solution containing Triton X-100 (5  $\mu$ l/ml), HEPES (10  $\mu$ mol/ml), bovine serum albumin (100  $\mu$ g/ml), gentamicin sulfate (50  $\mu$ g/ml), and proteinase inhibitor cocktail (10  $\mu$ l/ml). The protease inhibitor cocktail contains 4-(2-aminoethyl)benzenesulfonyl fluoride, aprotinin, leupeptin, bestatin, pepstatin A, and E-64. The supernatant obtained by centrifugation (10,000  $g$  for 10 min at 4 °C) of the homogenate was subjected to the measurement of cytokines. IL-1 $\beta$  was measured using ELISA kits from Thermo Scientific (Waltham, MA, USA). The procedures used were essentially the same as those described previously,<sup>38,39</sup> although the ELISA kits and proteinase inhibitor cocktail came from different makers than those used previously.

#### **2.6. Culture of peritoneal cells**

Thioglycol solution (3 %) was i.p. injected into mice (3 ml/mouse). Three days later, peritoneal cells were collected after i.p. injection of cooled saline (5 ml). The cells were cultured (2 x 10<sup>5</sup> cells/well in a 96-well plate) under the presence of LPS (100 ng/ml) or

NiCl<sub>2</sub> (0.5 mM) for 24 hours, and IL-1 $\beta$  in the supernatant was measured.

## **2.7. Data and Statistical analysis**

In this study, each experiment was performed twice, using three male or female mice, and the results confirmed that there are no essential differences between males and females. So, data obtained from experiments using females are presented in the figures, to be consistent with our previous studies. Experimental values are given as the mean  $\pm$  SD. The statistical significance of differences was analyzed using a Bonferroni multiple comparison test after ANOVA with the aid of InStat software (InStat, Scottsdale, AZ, U.S.A.).  $P < 0.05$  was considered to indicate significance.

## **3. RESULTS**

### **3.1. IL-1 $\beta$ acts as an adjuvant for inducing Ni-allergy**

As shown in Table 1, LPS markedly reduces the minimum allergy-inducing concentration of Ni at both sensitization and elicitation steps. We first examined whether IL-1 $\beta$  can exhibit such an adjuvant effect at sensitization step. In this experiment, a 1 mM Ni solution containing various amounts of IL-1 $\beta$  was i.p. injected into mice, and 10 days later, the mice were i.d. challenged into their ear-pinnas with 1 mM Ni. As shown in Fig. 1A, ear swelling did not occur in mice given either 1 mM NiCl<sub>2</sub> alone or 50  $\mu$ g/ml IL-1 $\beta$  alone at sensitization step, while ear swelling did occur in mice given both 1 mM Ni and 50  $\mu$ g/ml IL-1 $\beta$  at sensitization step, indicating that IL-1 $\beta$ , when co-injected with 1 mM Ni, is effective as an adjuvant for inducing Ni-allergy. As shown in Fig. 1B, IL-1 $\beta$  was effective even at 5  $\mu$ g/ml (50  $\mu$ g/kg).

### 3.2. IL-1 $\beta$ induces HDC

LPS is a potent inducer of HDC.<sup>16</sup> Indeed, intravenously injected LPS, even at 1  $\mu$ g/kg body weight, induces HDC within a few hours in various tissues (e.g. spleen, liver, and lung).<sup>16</sup> I.p. injection of LPS (20  $\mu$ g/kg) also induces HDC in various tissues.<sup>24</sup> Therefore, we examined whether i.p. injected IL-1 $\beta$  can induce HDC in ear pinnas. As shown in Fig. 2, IL-1 $\beta$  induced HDC in spleen, as well as ear pinnas, even at 0.04  $\mu$ g/ml (0.4  $\mu$ g/kg), and 1 mM Ni had no significant effect on the IL-1 $\beta$ -induced HDC activity. Thus, we supposed that IL-1 $\beta$  might exert its adjuvant effect via an induction of HDC. In the following sections, we examined this hypothesis by using IL-1-KO and H1R-KO mice.

### 3.3. In IL-1-KO mice, IL-1 $\beta$ induces HDC and is effective as an adjuvant in the induction of Ni-allergy

IL-1-KO mice are non- or low-responder mice as regards the Ni-allergy induced by Ni + LPS,<sup>1</sup> and LPS and IL-1 are potent inducers of HDC.<sup>16,19</sup> Thus, we examined whether co-injection of IL-1 $\beta$  and 1 mM Ni might induce HDC and Ni-allergy in IL-1-KO mice. As shown in Fig. 3A, i.p. injection of 4  $\mu$ g/ml IL-1 $\beta$  induced HDC in spleen and ear-pinnae similarly between WT and IL-1-KO mice. As shown in Fig. 3B, co-injection of 1 mM NiCl<sub>2</sub> and 4  $\mu$ g/ml IL-1 $\beta$  at sensitization step followed by co-injection of 1 mM Ni and 2.5  $\mu$ g/ml IL-1 $\beta$  at elicitation step induced Ni-allergy in IL-1-KO mice. However, co-injection of Ni and histamine 45 mM, or injection of Ni alone, at elicitation step was ineffective at inducing Ni-allergy in IL-1-KO mice. These results suggest that for Ni-allergy to be induced, IL-1 is required as an adjuvant at *both* the sensitization step *and* the elicitation step (i.e., not just at one of them), supporting our previous study.<sup>5</sup> As IL-1 has the ability to induce HDC, we examined the relationship between the adjuvant effect of IL-1 and the induction of HDC (i.e., newly formed histamine), as described in the following sections.

### **3.4. IL-1 $\beta$ by itself, but not histamine, has the ability to induce allergy-like prolonged ear swelling**

The ear-pinna is the tissue containing the highest amount of histamine (ca. 500 nmol/g) in mice.<sup>30</sup> The elevation of blood histamine by its release from mast cells after an antigen challenge in mice previously sensitized to the antigen is rapid, but its duration is short (ending within 3 hours of the antigen challenge).<sup>30</sup> In contrast, the elevated blood histamine that occurs via induction of HDC after i.p. injection of LPS is slow but long-lasting (more than 3 hours).<sup>29</sup> Thus, in the experiment shown in Fig. 3B, in order to keep a high level of histamine within ear pinnas, 20  $\mu$ l of a high-concentration histamine solution (45 mM) was injected into each ear (i.e., 900 nmol histamine/ear pinna), i.e., about 100-fold greater than the histamine normally contained within ear pinnas. It should be noted that there was no detectable difference in ear swelling between Ni 1 mM alone and Ni 1 mM + histamine 45 mM, indicating that histamine by itself does not induce allergy-like (long-lasting) ear swelling. However, unexpectedly and importantly, we found that 1 mM Ni + 2.5  $\mu$ g/ml IL-1 $\beta$  induced a prolonged (albeit small magnitude) ear swelling in non-sensitized IL-1-KO mice (Fig. 3B), and IL-1 $\beta$  alone also induced such a swelling (data not shown), indicating that IL-1 $\beta$  by itself has the ability to induce allergy-like prolonged ear swelling.

### **3.5. The adjuvant activity of IL-1 $\beta$ is mediated by H1R**

As described above, IL-1 $\beta$  is effective as an adjuvant in the induction of Ni-allergy via induction of HDC, i.e., via formation of new histamine. Histamine H1 receptors are known to be involved in inflammatory reactions. Thus, we examined whether IL-1 $\beta$  + Ni at sensitization step can induce Ni-allergy in H1R-KO mice. As shown in Fig. 4A, IL-1 $\beta$  induced HDC similarly between WT and H1R-KO mice. However, IL-1 $\beta$  was not effective as

an adjuvant for inducing Ni-allergy in H1R-KO mice (Fig. 4B). These results indicate that the adjuvant effect of IL-1 $\beta$  in the induction of Ni-allergy is mediated by stimulation of H1R by the histamine newly formed via induction of HDC.

### **3.6. IL-1 $\beta$ may exhibit its adjuvant activity via an additional effect(s) other than HDC induction**

Next, we examined whether histamine itself might be effective as an adjuvant for inducing Ni-allergy. Incidentally, the LD<sub>50</sub> of i.p. injected histamine is reported to be around 2,020 mg (18 mmol/kg) in mice.<sup>40</sup> So, in the present experiment, histamine was i.p. injected at a dose of 1.8 mmol/kg (1/10 of LD<sub>50</sub>) (i.e., 180 mM histamine solution was i.p. injected at 0.1 ml/10 g body weight) into WT mice, and these mice showed no detectable toxic signs (such as bristling or shock). Such i.p. injection of histamine, either alone or in combination with 1 mM Ni, did not increase serum IL-1 $\beta$  (Fig. 5A) and was not effective as an adjuvant at sensitization step for inducing Ni-allergy (Fig. 5B). These results indicate that histamine alone is not sufficient as an adjuvant for inducing Ni-allergy, i.e., IL-1 $\beta$  may exhibit its adjuvant activity via an additional effect(s) as well as by HDC induction.

### **3.7. In HDC-KO mice, Ni + IL-1 $\beta$ + histamine at sensitization step and Ni + histamine (*without* IL-1) at elicitation step induces Ni-allergy**

Ni-allergy is not induced in HDC-KO mice<sup>1,5,31</sup>. In the present study, IL-1 $\beta$  exhibited an LPS-like adjuvant effect (see above), leading us to suppose that the adjuvant effect of IL-1 $\beta$  may be (at least partly) mediated by histamine newly formed via induction of HDC. Hence, we tried to induce Ni-allergy in HDC-KO mice by using combinations of Ni, IL-1 $\beta$ , and/or histamine at both sensitization and elicitation steps. In this experiment, 180 mM histamine was used at sensitization step as in Fig. 5 (where it was ineffective as an adjuvant), and 45

mM histamine at elicitation step as in Fig. 3 (where it did not augment the inflammatory effect of Ni). As shown in Fig. 6, in HDC-KO mice treated at sensitization step with Ni + IL-1 $\beta$  + histamine (but not Ni alone or Ni + IL-1 $\beta$ ), challenge with Ni + histamine at elicitation step *without* IL-1 $\beta$  did induce Ni-allergy.

### **3.8. Ni by itself has the potential to induce IL-1 $\beta$ and HDC, effects that are augmented by LPS and lead to ear-swelling**

**(i) Systemic effects of Ni and/or LPS** As described above, induction of IL-1 and release of histamine are required as adjuvants for producing Ni-allergy at both sensitization and elicitation steps, and the adjuvant effect of LPS may be due to its ability to induce IL-1 and HDC. However, as shown in Table 1, Ni alone, albeit at a high concentration (i.e., 10 mM), can sensitize mice to Ni, suggesting that Ni by itself may exhibit an adjuvant effect even if only at high concentrations, possibly via induction of IL-1 and HDC. Thus, we examined the effect of Ni 10 mM. As shown in Fig. 7A, i.p. injected 10 mM Ni significantly increased IL-1 $\beta$  in serum and induced HDC in spleen. However, the effects of 1 mM Ni were very slight, if any. These results indicate that i.p. injected Ni by itself can, at a high concentration, have an adjuvant effect via systemic inductions of IL-1 $\beta$  and HDC in various tissues. Interestingly, combined i.p. injection of 1 mM Ni with LPS augmented the inductions of IL-1 $\beta$  and HDC.

**(ii) Local effects of Ni and/or LPS** Fig. 7B shows the local effects induced by Ni (1 or 10 mM) and/or LPS (1 mg/ml) when i.d. injected into ear pinnae in WT mice. The local effects of Ni and/or LPS on IL-1 $\beta$  and HDC were, as a pattern, broadly similar to the systemic effects shown in Fig. 7A. Interestingly, Ni 10 mM alone or a combination of Ni 1 mM with 1 mg/ml LPS induced ear swelling. LPS 1 mg/ml alone induced increases in IL-1 $\beta$  and HDC activity that were comparable to those induced by 10 mM Ni (Fig. 7B). However, the ear

swelling induced by 1 mg/ml LPS was far smaller than that induced by Ni 10 mM or Ni 1 mM + LPS 1 mg/ml (see Discussion for explanation). It should be noted that a value suggesting a significant amount of IL-1 $\beta$  was detected even in the ears of IL-1-KO mice. This suggests that the values for IL-1 $\beta$  obtained using the present method may include a significant amount of non-IL-1 $\beta$  substances, which might interfere with our ability to detect a small increase in IL-1 $\beta$  (see Section 4.2).

**(iii) *In vitro* effects of Ni and/or LPS** As shown in Fig. 7C, Ni itself did not stimulate the production of IL-1 $\beta$  *in vitro* by peritoneal cells, although it markedly augmented the LPS-induced production of IL-1 $\beta$ .

Collectively, the findings shown in Fig. 7 may be summarized as follows. (i) Ni by itself has the potential to induce IL-1 $\beta$  and HDC (although the present method is limited in its ability to detect their levels). (ii) The combined presence of Ni and LPS leads to augmented inductions of IL-1 $\beta$  and HDC. (iii) Such augmented inductions of IL-1 $\beta$  and HDC may lead to ear swelling.

### **3.9. Inductions of IL-1 (both $\alpha$ and $\beta$ ) and HDC are augmented in mice sensitized to Ni**

Lastly, we examined the effects of Ni on the inductions of IL-1 $\alpha$ , IL-1 $\beta$ , and HDC in mice (C57BL/6) sensitized to Ni (Fig. 8). Although IL-1 $\beta$  tended to increase, no increase in IL-1 $\alpha$  or in HDC activity was induced by 1 mM Ni in mice pretreated with 1 mM Ni + LPS (i.e., Ni-sensitized mice). However, 5 mM Ni significantly increased all of IL-1 $\alpha$ , IL-1 $\beta$ , and HDC in mice sensitized to Ni, but not in non-sensitized mice. These results indicate that the Ni-induced productions of IL-1 $\alpha$  and/or IL-1 $\beta$  and induction of HDC may be augmented in mice sensitized to Ni.

## 4. DISCUSSION

### 4.1. Summary of the findings

We previously reported that (i) LPS is a potent adjuvant for inducing Ni-allergy in mice at both sensitization and elicitation steps, (ii) Ni-allergy is not induced in IL-1-KO mice or HDC-KO mice, although Ni-allergy is induced in mast-cell-deficient mice, and (iii) LPS induces IL-1 and HDC, and IL-1 induces HDC. In the present study, we found the following. (a) By supplying IL-1 $\beta$  and/or histamine to IL-1-KO mice and HDC-KO mice, respectively, Ni-allergy can be induced in these mice (Table 2). (b) IL-1 $\beta$ , when co-injected with 1 mM Ni, is effective as an adjuvant for inducing Ni-allergy, and the adjuvant effect of IL-1 $\beta$  is mediated via an induction of HDC (i.e., newly formed histamine) and subsequent stimulation of histamine H1R at both sensitization and elicitation steps. (c) IL-1 exhibits its adjuvant effect via additional actions as well as HDC induction (i.e., histamine alone is not sufficient for inducing Ni-allergy) (Table 2). (d) Ni by itself, at a high concentration (10 mM), exhibits activities inducing IL-1 $\beta$  and HDC, and combined injection of 1 mM Ni and LPS augments such inductions of IL-1 $\beta$  and HDC (both systemically and locally) (Fig. 7). (e) The productions of IL-1 $\alpha$  and IL-1 $\beta$  by Ni are augmented in mice sensitized to Ni (Fig. 8). (f) When i.d. injected into the ear pinnae of mice not sensitized to Ni, IL-1 $\beta$  (2.5  $\mu$ g/ml) (Fig. 3) or LPS (1 mg/ml) (Fig. 7B) by themselves induce ear swelling, albeit of small magnitude, while a high concentration of Ni (10 mM) alone or Ni (1 mM) + LPS (1 mg/ml) causes marked ear swelling (Fig. 7B). We now discuss these findings.

### 4.2. Adjuvant effect of IL-1

Like LPS, resin monomers and nitrogen-containing bisphosphonates exhibit adjuvant effects in the induction of Ni-allergy,<sup>3,5</sup> and they also stimulate productions of IL-1 $\alpha$  and IL-1 $\beta$ ,<sup>5,33,41</sup> although they have no adjuvant effects either in IL-1-KO mice or in macrophage-depleted

mice.<sup>5</sup> In addition, cell-transfer experiments in IL-1-KO mice have indicated that both sensitization and elicitation steps depend on IL-1.<sup>5</sup> In mouse models of the CHS induced by organic haptens such as dinitrofluorobenzene (DNFB) and trinitrochlorobenzene (TNCB), IL-1 has been shown to be involved in sensitization step. Enk and Katz (1992)<sup>42</sup> reported that painting skins with DNFB stimulates a rapid production of IL-1 $\beta$  mRNA in Langerhans cells, this being the fastest of the tested cytokine mRNAs (within 15 min after the application of DNFB). Shornick et al. (1996)<sup>43</sup> reported that “IL-1 $\beta$ ”-KO (not IL-1-KO) mice showed defective CHS responses to topically applied TNCB, and this defect in IL-1 $\beta$ -KO mice could be overcome either by application of very high doses of TNCB or by local intradermal injection of recombinant IL-1 $\beta$  immediately before the TNCB application, this being similar to the findings concerning Ni-allergy made in the present study. Recently, we reported a mouse model of CHS to hydroquinone (a widespread chemical in our environment) in which hydroquinone stimulated the productions of IL-1 $\alpha$  and IL-1 $\beta$ , and the CHS to hydroquinone was not induced in IL-1-KO mice.<sup>44</sup> These findings, collectively, strongly support the idea that IL-1 is essential for the establishment of the CHS to either metals or organic haptens.

In the present study, the ear swelling induced by LPS 1 mg/ml alone was very small, although there were increases in IL-1 $\beta$  and HDC activity (Fig. 7B). It should be noted that the IL-1 $\beta$  antibody used in the method for measuring IL-1 $\beta$  cannot distinguish mature IL-1 $\beta$  (active) from pro-IL-1 $\beta$  (inactive). For that reason, we speculate that the LPS-induced IL-1 $\beta$  may be largely inactive pro-IL-1 $\beta$ . We intend to examine this in a future study.

#### **4.3. Adjuvant effect of HDC induction or free histamine formed via HDC induction**

Mast cells are well-known providers of histamine, and the present study suggests that histamine is essential as an adjuvant for Ni-allergy. This could be of particular interest for an understanding of how mast cells are involved in contact hypersensitivity. However, the roles

of mast cells in hypersensitivity are controversial (including both positive and negative regulations), possibly because of the use of SCF or *Kit* mutant mice for such studies on mast cells. Dudeck et al. (2011)<sup>47</sup> pointed out that *Kit* mutant W/W<sup>v</sup> mice develop various immunologically unusual features -- such as macrocytic anemia, neutropenia, decreased  $\gamma\delta$  T cells, and a high rate of spontaneous dermatitis -- because *Kit* is also expressed in cells other than the mast cell lineage (in particular, hematopoietic stem cells and precursor cells of most lineages). In our Ni-allergy model induced by LPS + Ni, allergic ear-swelling also occurs in W/W<sup>v</sup> and is markedly prolonged in these mice,<sup>1</sup> although allergic swelling in our model can be strongly reduced by prior treatment with a histamine H1-receptor antagonist (pyrilamine).<sup>31</sup> Interestingly, such a prolonged ear-swelling also occurs in DNFB-induced contact hypersensitivity in W/W<sup>v</sup> mice.<sup>47</sup> Based on the results obtained from experiments using mast cell-deficient mice produced by mast cell-specific gene inactivation, Dudeck et al. (2011)<sup>47</sup> concluded that (i) the CHS induced by organic haptens (including DNFB) depends on mast cells, (ii) an early innate inflammatory response to haptens (i.e., inflammatory effects of haptens themselves) is essential for the induction of CHS, and (iii) the early innate inflammatory response to haptens is dependent on mast cell-derived histamine, and consequently the DNFB-induced CHS is reduced by pyrilamine. Surprisingly, however, it should be noted that in mice in which 90 % or more of mast cells have been constitutively depleted, allergic ear-swelling still occurs, although its magnitude is about 50 % of that seen in control mice.<sup>47</sup> As described in the above section, organic haptens can stimulate a rapid production of IL-1, and we have shown that IL-1 is a potent inducer of HDC. Thus, we suppose that (i) organic haptens can induce HDC even in mast cell-deficient mice, and (ii) in addition to mast cell-derived histamine, the free histamine newly produced via induction of HDC is also involved in the mediation of organic hapten-induced CHS. Therefore, IL-1-induced HDC induction may be a prerequisite for the establishment of CHS in response

to either organic haptens or Ni.

In the present study, we showed that histamine did not induce IL-1 $\beta$ , and that histamine itself was not effective as an adjuvant in the induction of Ni-allergy (Fig. 5). In IL-1-KO mice, Ni + IL-1 $\beta$  (but not Ni + histamine) was effective at both sensitization and elicitation steps for inducing Ni-allergy (Fig. 3). In HDC-KO mice, Ni + IL-1 $\beta$  + histamine at sensitization step and Ni + histamine at elicitation step induced Ni-allergy (Fig. 6). These results indicate that both IL-1 and histamine are involved at *both* sensitization and elicitation steps in the induction of Ni-allergy. This implies that the adjuvant effect of IL-1 is due not only to an induction of HDC, but also to additional effects. At present, although we have no data to indicate what such additional effects might be, we speculate that IL-1 may have effects such as increased expressions of TLR4 and/or Ni-binding proteins (such as CXCL4<sup>45</sup> and heat shock protein 90 $\beta$ <sup>46</sup>), and activation of immune competent cells, which may lead to augmented production of IL-1 $\alpha$  and/or IL-1 $\beta$ .

#### **4.4. Comparison between mice and humans**

In 2007, we reported that TLR4 (the receptor for LPS) is important in the induction of Ni-allergy in mice<sup>1</sup>. Later, Schmidt et al. (2010)<sup>48</sup> reported that (i) Ni binds to human TLR4 but not to murine TLR4, because their TLR4 structures are different (due to an amino-acid displacement), and (ii) Ni-allergy can be induced by Ni alone (i.e., without the addition of any adjuvant) in mice in which human TLR4 is expressed. These findings *might* be thought to suggest that the mechanism underlying Ni-allergy is different between mice and humans. However, those findings may also indicate that TLR4 is essential not only in mice but also in humans. Moreover, it should be noted that in the study by Schmidt et al., a high concentration of Ni (10 mM) was used at both the sensitization and elicitation steps to stimulate TLR4 (compare to the concentrations of Ni shown in Table 1), suggesting that the ability of Ni to

stimulate TLR4 is much weaker than that of LPS (i.e., Ni stimulates TLR4 only at high concentrations). Thus, stimulation of TLR4 by LPS may be important as an adjuvant effect in humans, too, and thus the mechanism underlying the induction of Ni-allergy may be essentially the same between mice and humans. If so, mouse models may provide data that are directly applicable to the development of strategies against human Ni-allergy.

#### **4.5. Conclusion**

On the basis of the above results and discussion, we conclude that (i) IL-1 is essential for inducing Ni-allergy at *both* the sensitization step *and* the elicitation step, and HDC induction is involved in the effect of IL-1, (ii) stimulation of H1 receptors is also essential for inducing Ni-allergy at both sensitization and elicitation steps, and (iii) the state described as “sensitization to Ni” may be a state in which tissues are primed for augmented production of IL-1 $\alpha$  and/or IL-1 $\beta$  in response to Ni (Fig. 9).

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#### **CONFLICT OF INTEREST**

The authors declare no conflict of interest.

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## Figure legends

**Figure 1. IL-1 $\beta$  acts as an adjuvant for inducing Ni-allergy in WT mice.** Sensitization and challenge were carried out as indicated in WT C57BL/6 mice. Each value is the mean  $\pm$  SD (n = 6 ears). \**P* < 0.05 vs. Ni 1mM alone.

**Figure 2. IL-1 $\beta$  induces HDC in WT mice.** Saline or the indicated concentration of Ni and/or IL-1 $\beta$  was i.p. injected into WT C57BL/6 and, 4 h later, spleen and ears were subjected to measurement of HDC activity. Each value is the mean  $\pm$  SD (n = 3 spleens or 6 ears). \**P* < 0.05.

**Figure 3. In IL-1-KO mice, injected IL-1 $\beta$  induces HDC and is effective for inducing Ni-allergy.** (A) Saline or the indicated concentration of IL-1 $\beta$  was i.p. injected into IL-1-KO Balb/c or WT Balb/c mice. Then, 4 h later, the spleen and ears were subjected to measurement of HDC activity. Each value is the mean  $\pm$  SD (n = 3 spleens or 6 ears). \**P* < 0.05. (B) Sensitization and challenge were carried out in IL-1-KO mice as indicated. Each value is the mean  $\pm$  SD (n = 6 ears). \**P* < 0.05 vs. (a).

**Figure 4. In H1R-KO mice, IL-1 $\beta$  induces HDC, but it is not effective for inducing Ni-allergy.** (A) Saline or the indicated concentration of IL-1 $\beta$  was i.p. injected into WT C57BL/6 or H1R-KO C57BL/6 mice, and, 4 h later, the spleen and ears were subjected to measurement of HDC activity. Each value is the mean  $\pm$  SD (n = 3 spleens or 6 ears). \**P* < 0.05. (B) Sensitization and challenge were carried out as indicated. Each value is the mean  $\pm$  SD (n = 6 ears). \**P* < 0.05 vs. same time in WT.

**Figure 5. In WT mice, injected histamine is not effective as an adjuvant for inducing Ni-allergy.** (A) The indicated concentration of Ni and/or histamine was i.p. injected into WT C57BL/6 mice and, 4 h later, the blood was subjected to measurement of IL-1 $\beta$ . (B) The sensitization and elicitation were carried out as indicated. Each value is the mean  $\pm$  SD (n = 3 serum samples or 6 ears).

**Figure 6. In HDC-KO mice, co-injected histamine and IL-1 $\beta$  act as adjuvants for inducing Ni-allergy.** Sensitization and challenge were carried out as indicated. Each value is the mean  $\pm$  SD (n = 6 ears). \**P* < 0.05 vs. Ni 1mM alone.

**Figure 7. *In vivo* (i.p. or i.d.) and *in vitro* effects of Ni on IL-1 $\beta$  and HDC and effects of LPS in WT C57BL/6 mice.** (A) Indicated concentrations of Ni and/or LPS were i.p. injected into WT mice, and 4 h later IL-1 $\beta$  and HDC activities were measured. (B) Indicated concentrations of Ni and/or LPS were i.d. injected into ear pinnas of WT mice. Then, 24 h later, IL-1 $\beta$ , HDC activity, and ear swelling were measured. Each value is the mean  $\pm$  SD (n = 3 serum samples, 3 spleens, or 6 ears). (C) Peritoneal cells were treated as described in Methods. The results were expressed as the mean  $\pm$  SD of a single experiment performed in triplicate. \* $P$  < 0.05. Note: For reference, basal levels of IL-1 $\beta$  in the serum and ear of IL-1-KO mice are shown in A and B.

**Figure 8. Effects of i.d. injected Ni on IL-1 ( $\alpha$  and  $\beta$ ) production and HDC induction in ear-pinnae of WT mice sensitized or non-sensitized to Ni.** Saline or Ni 1 mM containing LPS 1  $\mu$ g/ml was i.p. injected into C57BL/6 mice, and 10 days later Ni (1 or 5 mM) was i.d. injected into their ear-pinnae. IL-1 ( $\alpha$  and  $\beta$ ) and HDC activities were measured 48 h after the challenge. Each value is the mean  $\pm$  SD (n = 6 ears). \* $P$  < 0.05. Note: For reference, basal levels of IL-1 $\beta$  and IL-1 $\alpha$  in the ear of IL-1-KO mice are shown in A and B.

**Figure 9. Roles of IL-1 and HDC in inducing Ni-allergy.** Inflammatory stimuli associated with infection or exposure to chemicals induce IL-1 $\alpha$  and/or IL-1 $\beta$  (IL-1 $\alpha/\beta$ ) and HDC (the induced IL-1 $\alpha/\beta$  by itself also induces HDC). The histamine (H) newly formed by the HDC induction stimulates H1R. Under the presence of IL-1 $\alpha/\beta$  and the newly formed histamine, sensitization to Ni is established. It is supposed that “sensitization to Ni” is the primed state for inducing a large amount of IL-1 (and thus HDC), and their augmented production after the second exposure to Ni results in allergic inflammation. As described in the text, it should be noted that (i) at a high concentration, Ni by itself induces IL-1 and HDC [in humans, Ni

reportedly acts as an agonist of TLR4 (see Ref. 48)], (ii) the presence of an inflammatory substance(s) augments the induction of IL-1 $\alpha$ / $\beta$  and HDC and reduces the allergy-inducing concentration of Ni (Table 1), and (iii) the adjuvant effect of IL-1 $\alpha$ / $\beta$  is not due only to the induction of HDC, but also to other unidentified effect(s).

**Table 1. Summary of the effects of LPS on the minimum allergy-inducing concentration of NiCl<sub>2</sub> (Ni) for inducing Ni-allergy in Balb/c mice** [derived from results published by Kinbara et al., 2011] [(Ref. 6)].

| Concentrations of solutions containing LPS and/or Ni |            |                       |            |
|------------------------------------------------------|------------|-----------------------|------------|
| At sensitization step*                               |            | At elicitation step** |            |
| LPS ( $\mu$ g/ml)                                    | Ni         | LPS ( $\mu$ g/ml)     | Ni         |
| 0                                                    | 10 mM      | 0                     | 10 $\mu$ M |
| 1                                                    | 1 mM       | 0.01                  | 2 nM       |
| 10                                                   | 10 $\mu$ M | 0.1                   | 20 pM      |
| 50                                                   | 2 $\mu$ M  | 1                     | 2 pM       |

\* Mice were challenged by 5 mM Ni to induce Ni-allergy

\*\*Mice were sensitized to Ni by a solution containing Ni 0.5 mM and LPS 0.5  $\mu$ g/ml.

In the experiments shown in this table, a solution containing Ni alone or Ni plus LPS (Ni-LPS solution) was injected at sensitization and elicitation steps. At sensitization step, 250  $\mu$ l of a Ni or Ni-LPS solution was i.p. injected. At elicitation step (10 days after the injection for sensitization), 20  $\mu$ l of a Ni or Ni-LPS solution was i.d. injected into each ear-pinna. The table shows that, for example, without using LPS, 10 mM and 10  $\mu$ M of Ni is needed at sensitization and elicitation steps, respectively, while at sensitization step, if 1  $\mu$ g/ml LPS is included, 1 mM Ni is sufficient for sensitizing mice to Ni.

Note:

- (i) LPS acts as an adjuvant at markedly low concentrations at elicitation step.
- (ii) Concentrations of Ni and LPS used at sensitization step do not affect the concentrations of LPS and Ni required at elicitation step.
- (iii) Reported concentrations of Ni in humans who have contact with metals are 0.1-1 mM in sweat, 0.01 ~ 1  $\mu$ M in saliva, and ~1  $\mu$ M in blood (see Ref. 6).

**Table 2. Summary of the effects of IL-1 $\beta$  and histamine (H) on the induction of Ni-allergy in mice.**

Test solutions were i.p. injected (sensitization), and 10 days later test solutions were i.d. injected into ear-pinnas (challenge). In these experiments, 1 mM Ni was used for both the sensitization and challenge.

| Mice    |         | Sensitization                          | Challenge                          | Ni-allergy           |
|---------|---------|----------------------------------------|------------------------------------|----------------------|
| WT      | Balb/c  | <b>Ni + LPS*</b>                       | <b>Ni alone</b>                    | <b>Yes</b> (Table 1) |
|         | C57BL/6 | <b>Ni + LPS*</b>                       | <b>Ni alone</b>                    | <b>Yes</b> (Ref. 31) |
|         |         | Ni alone                               | Ni alone                           | No (Fig. 1)          |
|         |         | <b>Ni + IL-1<math>\beta</math></b>     | <b>Ni alone</b>                    | <b>Yes</b> (Fig. 1)  |
|         |         | Ni + H                                 | Ni alone                           | No (Fig. 5)          |
| IL-1-KO | Balb/c  | Ni + IL-1 $\beta$                      | Ni alone                           | No (Fig. 3)          |
|         |         | Ni + IL-1 $\beta$                      | Ni + H                             | No (Fig. 3)          |
|         |         | <b>Ni + IL-1<math>\beta</math></b>     | <b>Ni + IL-1<math>\beta</math></b> | <b>Yes</b> (Fig. 3)  |
| HDC-KO  | C57BL/6 | Ni alone                               | Ni alone                           | No (Fig. 6)          |
|         |         | Ni + IL-1 $\beta$                      | Ni alone                           | No (Fig. 6)          |
|         |         | Ni + IL-1 $\beta$ + H                  | Ni alone                           | No (Fig. 6)          |
|         |         | <b>Ni + IL-1<math>\beta</math> + H</b> | <b>Ni + H</b>                      | <b>Yes</b> (Fig. 6)  |
| H1R-KO  | C57BL/6 | Ni + IL-1 $\beta$                      | Ni + IL-1 $\beta$                  | No (Fig. 4)          |

\*LPS concentrations are 1 or 0.5  $\mu$ g/ml.

Note: (i) HDC is induced in either IL-1-KO or H1R-KO mice by IL-1 $\beta$  (Figs. 3 and 4), and (ii) Ni + IL-1 $\beta$  induces allergy-like prolonged ear swelling in non-sensitized IL-1-KO mice (Fig. 3).

**Fig. 1**

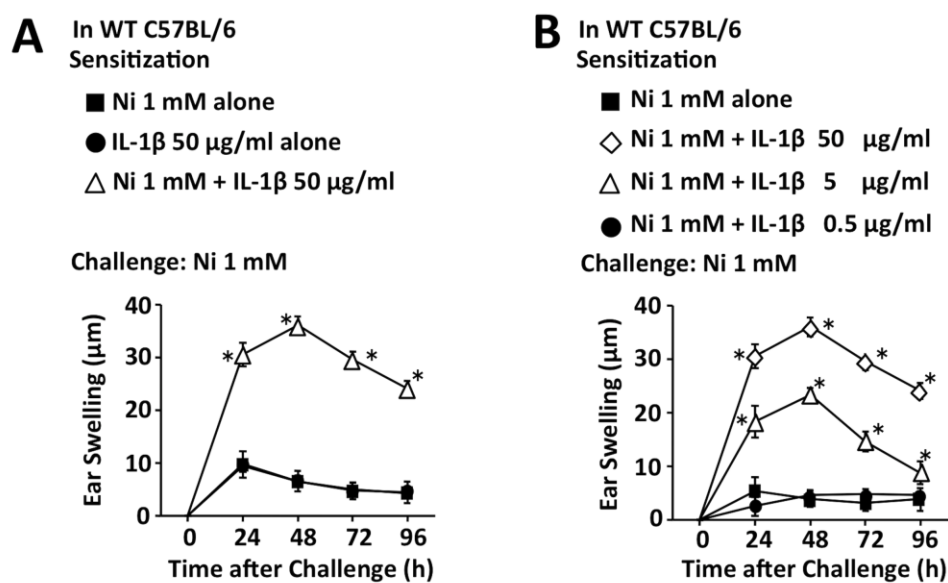


**Fig. 2**

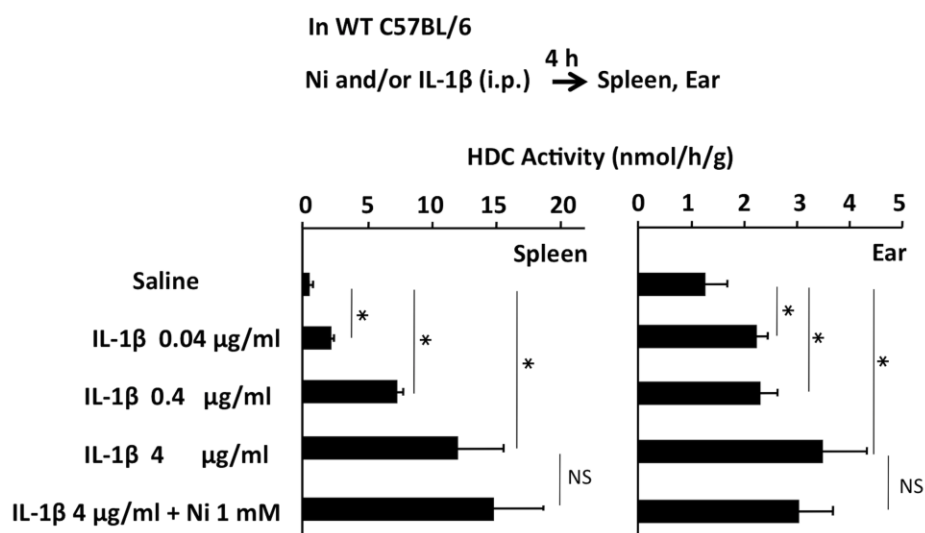
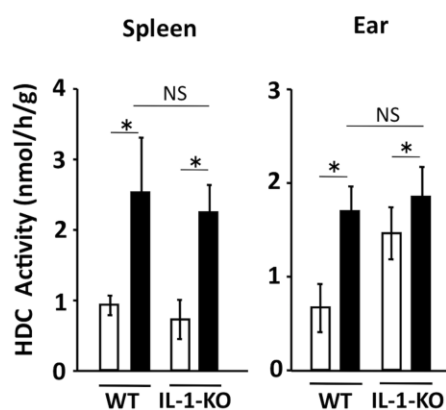


**Fig. 3**

**A** In WT Balb/c and IL-1-KO Balb/c

IL-1 $\beta$  4  $\mu$ g/ml (i.p.)  $\xrightarrow{4\text{ h}}$  Spleen, Ear

□ Saline    ■ IL-1 $\beta$



**B** In IL-1-KO Balb/c

**Sensitization**  
 Ni 1 mM + IL-1 $\beta$  4  $\mu$ g/ml  $\rightarrow$

**Challenge**  
 ◇ Ni 1 mM  
 △ Ni + Histamine 45 mM  
 ○ Ni + IL-1 $\beta$  2.5  $\mu$ g/ml  
 Saline  $\rightarrow$  □ Ni + IL-1 $\beta$  2.5  $\mu$ g/ml

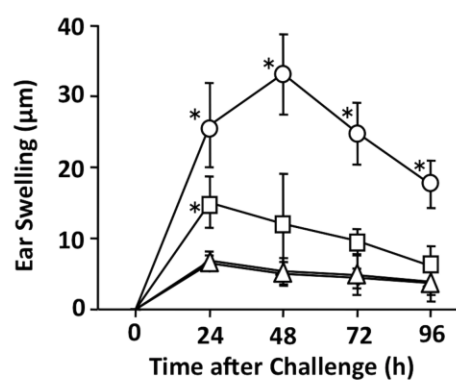
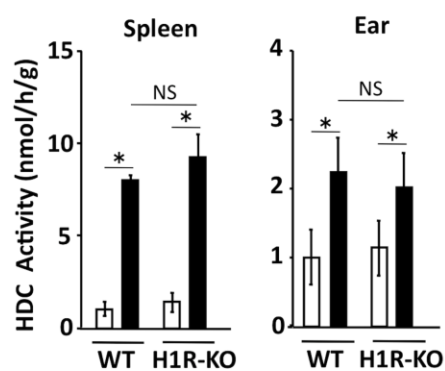


**Fig. 4**

**A** In WT C57BL/6 and H1R-KO C57BL/6

IL-1 $\beta$  4  $\mu$ g/ml (i.p.)  $\rightarrow$  4 h Spleen, Ear

□ Saline ■ IL-1 $\beta$



**B** In WT C57BL/6 and H1R-KO C57BL/6

Sensitization: Ni 1 mM + IL-1 $\beta$  4  $\mu$ g/ml

Challenge: Ni 1 mM

■ WT ○ H1R-KO

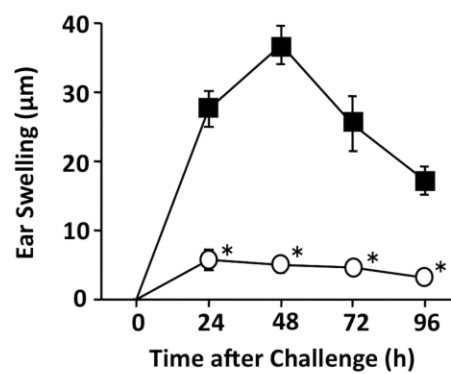


**Fig. 5**

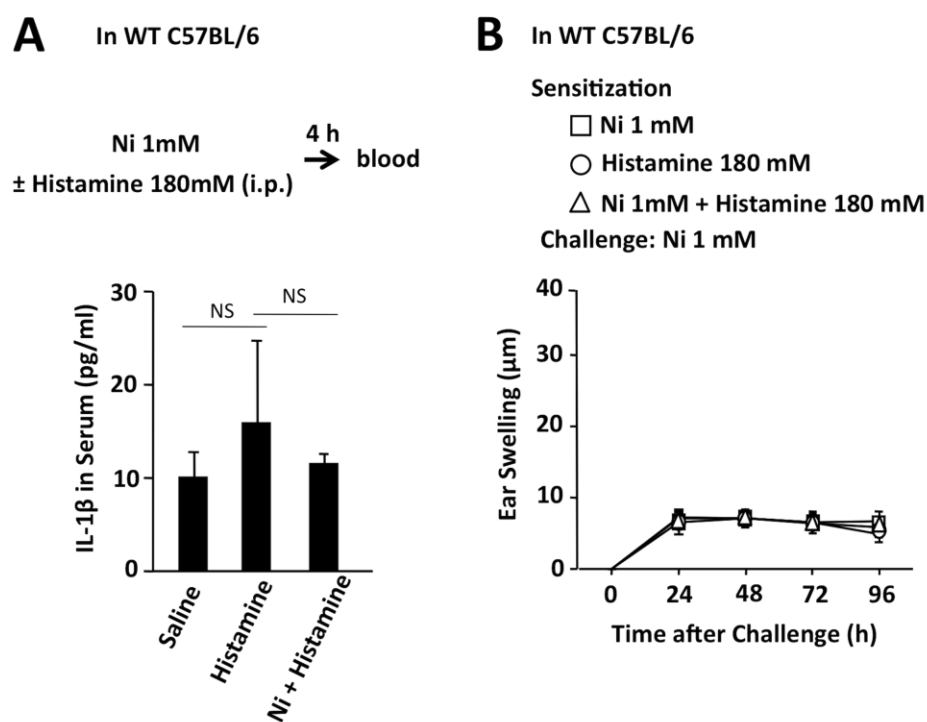


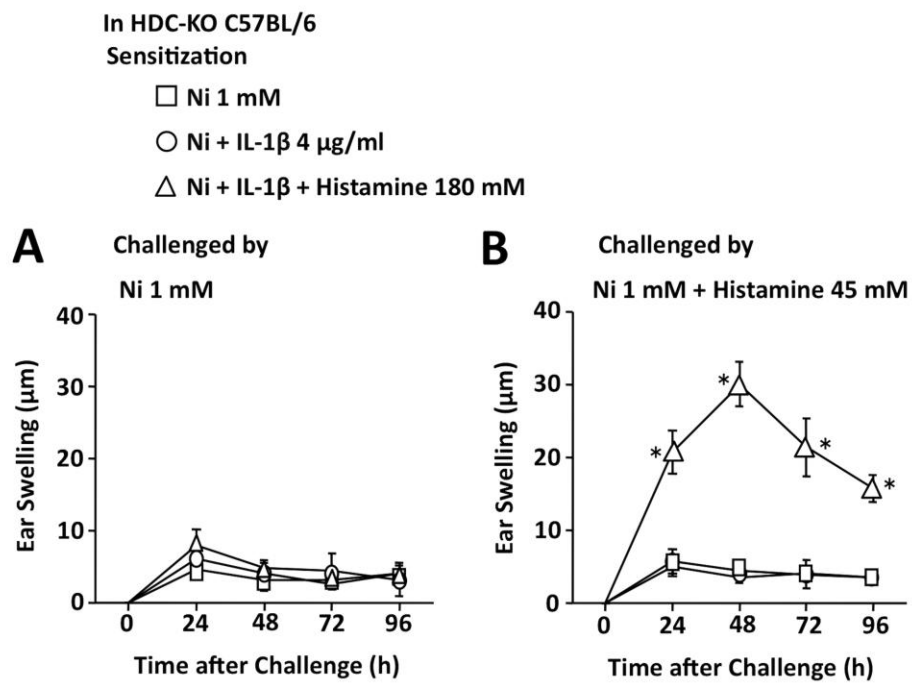
**Fig. 6**

**Fig. 7**

In WT C57BL/6 mice

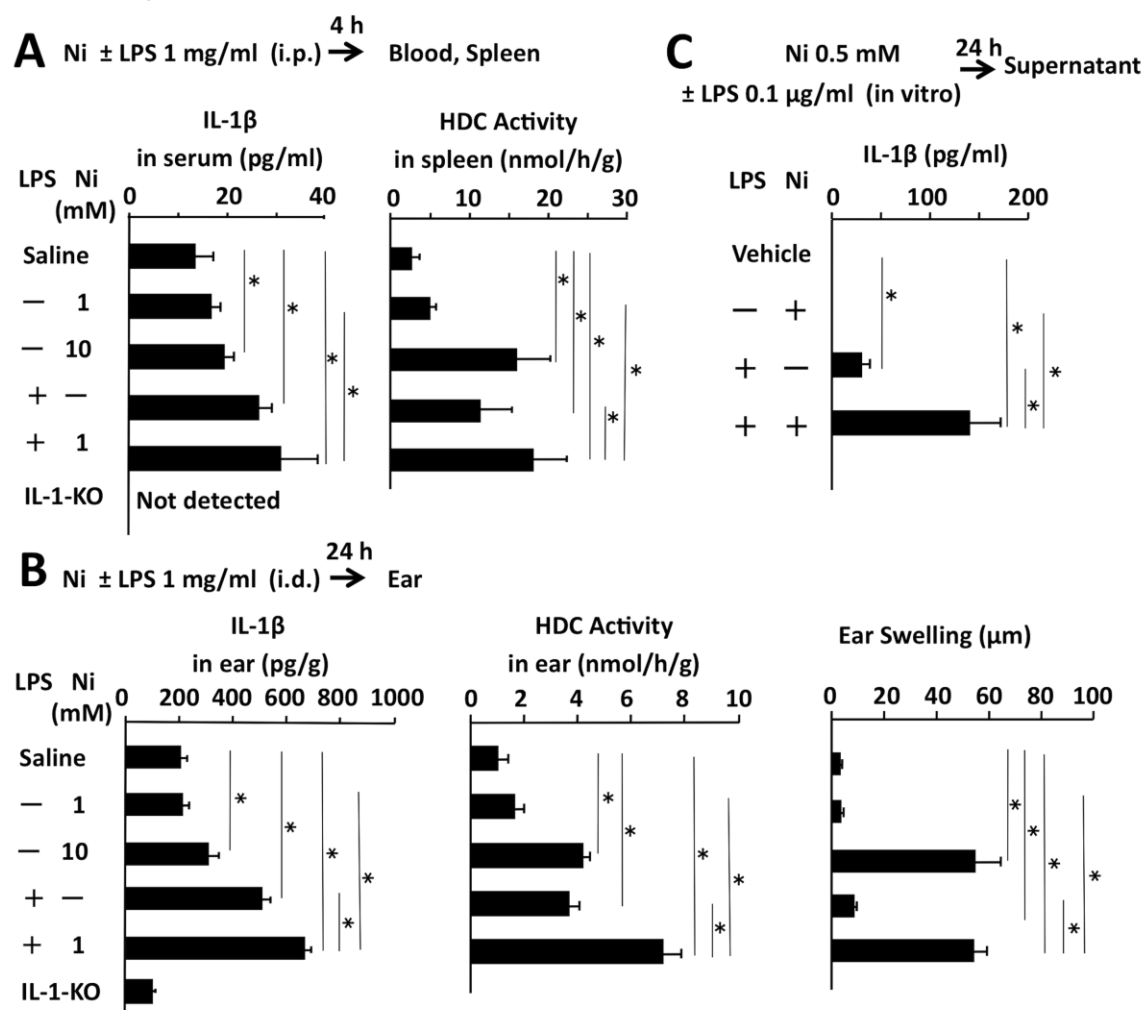


**Fig. 8**

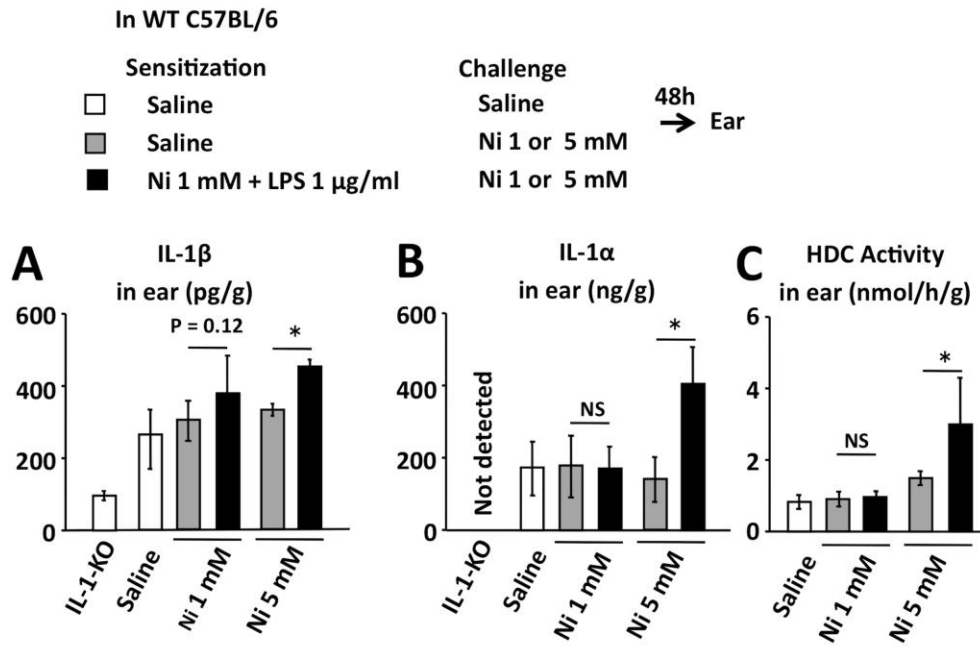


Fig. 9

