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The elicitation step of nickel allergy is promoted in mice by microbe-related substances, including some from oral bacteria

Ling Huang^{a,b,1}, Masayuki Kinbara^{a,1}, Hiromi Funayama^{a,b,c}, Haruhiko Takada^b, Shunji Sugawara^a, Yasuo Endo^{a,*}

^a Department of Oral Molecular Regulation, Graduate School of Dentistry, Tohoku University, 4-1 Seiryomachi, Aoba-ku, Sendai 980-8575, Japan

^b Department of Oral Microbiology, Graduate School of Dentistry, Tohoku University, 4-1 Seiryomachi, Aoba-ku, Sendai 980-8575, Japan

^c Department of Pediatric Dentistry, Tsurumi University School of Dental Medicine, 2-1-3 Tsurumi, Tsurumi-ku, Yokohama 230-8501, Japan

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ABSTRACT

Microbial components activate the host's innate immunity via interactions with molecules including TLRs and NODs. We previously reported that in mice (i) *Escherichia coli* lipopolysaccharide (LPS; TLR4 agonist) promotes Ni-allergy even in T-cell-deficient mice, (ii) *E. coli* LPS reduces the minimum allergy-inducing concentrations of Ni at both the sensitization and elicitation steps, and (iii) various microbe-related substances promote sensitization to Ni. Here, we examined the effects of microbe-related substances at the elicitation step. Mice (except for TLR4-mutated C3H/HeJ mice) were sensitized to Ni by intraperitoneal injection of NiCl₂ + *E. coli* LPS. Ten days later their ear-pinnas were challenged with 1 μM NiCl₂ with or without a test substance. Although NiCl₂ alone at this concentration does not induce Ni-allergy, its combination with the following substances induced Ni-allergy in BALB/c mice: LPS preparations from oral Gram-negative bacteria (*Prevotella intermedia* and *Porphyromonas gingivalis*), a mannan preparation from a fungus (*Saccharomyces cerevisiae*), and synthetic NOD2 and TLR2 agonists. The effect of the mannan preparation was small in C3H/HeJ mice (sensitized with NiCl₂ + the *P. intermedia* preparation). The *P. intermedia* preparation promoted Ni-allergy in C3H/HeJ and nude mice, but not in mice deficient in either TLR2 or histidine decarboxylase. Intragingival injection of the *P. intermedia* preparation and later challenge with NiCl₂ alone to ear-pinnas also promoted Ni-allergy. These results indicate that (i) in Ni-allergy, a microbial milieu or innate immunity is important at the elicitation step, too, and (ii) some oral bacteria may promote Ni-allergy via TLR2-stimulant(s) production.

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1. Introduction

Nickel (Ni) is a component of many types of alloys, and these may be said to flood our modern environment. In addition, Ni-containing alloys are widely used as implants in replacement surgery, dentures, orthodontic wires, and various other devices. Consequently, we are exposed to Ni through a variety of routes, and indeed Ni is the most frequent contact allergen among the metals [1,2].

It is hard to induce Ni-allergy in mice [3], but Artic et al. [4] succeeded in sensitizing mice to Ni so as to induce reproducible Ni-allergy. In their method, a mixture of NiCl₂ and H₂O₂ is given to C57BL/6 mice at 20 mM and 0.6 M, respectively. However, we suspected that actual Ni-allergy (in humans) might be caused by a much lower concentration of Ni because the Ni ions released from alloys in the environments in which

they are commonly employed (see above) may be at far below the mM level. In addition, we would expect such a high concentration of H₂O₂ to be highly toxic.

Lipopolysaccharide (LPS; an outer cell-wall component of Gram-negative bacteria) stimulates innate immunity via Toll-like receptor (TLR) 4, and TLRs have been suggested to modify adaptive immunity [5]. Moreover, LPS can activate dendritic cells to undergo a maturation process [6–8]. For example, LPS-induced stimulation of the TLR4 on dendritic cells blocks the suppressive effect of regulatory T-cells [9]. These reports prompted us to examine the effects of LPS on the establishment of Ni-allergy in mice, and we have already reported that in mice (i) *Escherichia coli* LPS promotes Ni-allergy at both the sensitization and elicitation steps [10], (ii) *E. coli* LPS reduces the minimum allergy-inducing concentrations of Ni (Ni-MAICs) at these two steps (especially at the latter) [11], and (iii) various microbe-related substances promote Ni-allergy at the sensitization step [12]. Interestingly, such Ni(+) LPS allergy occurs even in T cell-deficient mice, but it is minimal or weak in mice that lack TLR4, IL-1, or the histamine-forming enzyme, histidine decarboxylase (HDC) [10]. In addition, it is especially notable that *E. coli* LPS reduces the Ni-MAIC to as low as 1×10^{-12} M at

* Corresponding author at: Department of Molecular Regulation, Graduate School of Dentistry, Tohoku University, 4-1 Seiryomachi, Aoba-ku, Sendai 980-8575, Japan. Fax: +81 22 717 8322.

E-mail address: endo@dent.tohoku.ac.jp (Y. Endo).

¹ These authors contributed equally to this work.

the elicitation step [11], although the Ni-MAIC at this step is around 1×10^{-5} M without LPS (i.e., 1×10^{-6} M NiCl₂ by itself did not induce allergic responses). Thus, the magnitude of the Ni-MAIC depends on the presence of LPS, and we supposed that other microbial substances might also reduce the Ni-MAIC. Hence, in the present study, we examined whether 1×10^{-6} M (i.e., 1 μ M) NiCl₂ can induce allergic responses when combined with a microbial substance.

LPS is a powerful and typical stimulator of innate immunity. In the innate immune system, in addition to LPS a variety of components of microorganisms are recognized by receptors such as TLRs [13,14] and nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs) [15,16]. In the present study, we examined some microbial components and synthetic agonists of TLR2 and NOD2 for their effects on Ni-allergy at the elicitation step because this step may be the actual target for therapies aimed at combating metal allergies. For this purpose, mice (except for TLR4-mutated C3H/HeJ mice and their control C3H/HeN mice) were sensitized to Ni by intraperitoneal injection of NiCl₂ + *E. coli* LPS. Ten days later their ear-pinnas were challenged with 1 μ M NiCl₂ with or without a test substance. As described above, NiCl₂ by itself at this concentration does not induce allergic ear-swelling [11]. In the present study, we also tested LPS preparations from oral bacteria, because *Prevotella intermedia* and *Porphyromonas gingivalis*, which were formerly classified as belonging to the genus *Bacteroides*, are the dominant Gram-negative bacteria in the periodontal pocket in patients with periodontitis, and indeed are considered to be the major pathogens responsible for periodontal diseases [17,18].

2. Materials and methods

2.1. Animals

C3H/HeN, C3H/HeJ, and nude BALB/c mice (6 weeks old) were purchased from SLC Japan (Shizuoka, Japan). TLR2-knockout (KO) mice (C57BL/6 background) were provided by Prof. Akira Shizuo [19]. HDC-KO mice (C57BL/6 background) were established by Ohtsu et al. [20]. BALB/c, C57BL/6, and HDC-KO mice were raised in our laboratory. In the present study, female mice (7–8 weeks old) were used for all experiments. They were kept under standard conditions [i.e., under a light (07:00–19:00)–dark (19:00–07:00) cycle at a controlled temperature (23 ± 1 °C)]. The mice were raised in standard aluminum cages (with a lid made of stainless steel wires), with water (supplied by the water service of our city) being accessible ad libitum from a plastic bottle through a stainless steel tube. All experiments complied with the *Guidelines for Care and Use of Laboratory Animals in Tohoku University*. Detailed experimental protocols are described in the section relating to each experiment. In our previous study [10], we made specific attempts to prevent the mice having contact with Ni outside the experiment itself. However, in the present study we made no such attempt.

2.2. Reagents

First-grade NiCl₂ (purity >95%) was purchased from Wako Pure Chemical Ind. (Osaka, Japan). LPS from *E. coli* O55:B5 prepared by the method of Westphal and Jann [21] (phenol–water extraction) was purchased from Sigma (St. Louis, MO, USA). *P. gingivalis* 381 LPS was prepared by the method of Westphal and Jann [21], while *P. intermedia* ATCC 25611 LPS was prepared by the method of Galanos et al. [22] (phenol–chloroform–petroleum ether extraction), as reported previously [23]. A mannan preparation from *Saccharomyces cerevisiae* was purchased from Sigma (St. Louis, MO, USA). Pam₃Cys-SKKKK-HCl (Pam3; synthetic TLR2 agonist) was from Calbiochem (San Diego, USA). *N*-Acetylmuramyl-L-alanyl-D-isoglutamine (MDP; a synthetic version of a cell-wall component of bacteria) was purchased from the Protein Research

Foundation Peptide Institute (Osaka, Japan). NiCl₂ and the test samples described above were dissolved in sterile saline.

2.3. Sensitization to Ni

2.3.1. BALB/c, C57BL/6, TLR2-KO, BALB/c nude, and HDC-KO mice

A mixture of NiCl₂ and *E. coli* LPS, when it is injected either intraperitoneally or intradermally into flanks, easily sensitizes mice to Ni [10]. Intradermal injection of *E. coli* LPS into flanks together with intraperitoneal injection of *E. coli* LPS is also effective [10]. In these methods, *E. coli* LPS is effective when it is injected 0–3 h before NiCl₂, but not when it is injected after NiCl₂ [11]. In the present study, therefore, a solution containing 0.5 mM NiCl₂ and *E. coli* LPS (0.5 μ g/mL) was injected intraperitoneally (0.1 mL/10 g body weight).

2.3.2. C3H/HeJ mice and their control C3HHeN mice

C3H/HeJ mice have mutated dysfunctional TLR4 [24,25]. We recently found that the *P. intermedia* preparation is effective at promoting Ni-allergy via stimulation of TLR2 [12]. Therefore, for sensitizing C3H/HeJ mice and their control C3HHeN mice to Ni, a solution containing both 0.5 mM NiCl₂ and *P. intermedia* preparation (0.5 μ g/mL) was injected intraperitoneally (0.1 mL/10 g body weight).

2.4. Provocation of Ni-allergy

As previously reported, NiCl₂ by itself at 1 μ M does not induce allergic ear-swelling [11]. However, *E. coli* LPS markedly reduces the minimum allergy-inducing concentrations of Ni at both the sensitization and elicitation steps, especially at the elicitation step [11]. Therefore, 10 days later, to examine such effects of various microbe-related substances, ear-pinnas (left and/or right) were intradermally challenged with a solution (20 μ L/ear, near the root of the ear) containing 1 μ M (or less) NiCl₂ and/or a test substance (indicated in each experiment) after anesthesia with ethyl ether. Ear swelling was measured at a site 4–6 mm distant from the challenge site at the indicated times (see text) using a Peacock dial thickness gauge (Ozaki Mfg. Co. Ltd., Tokyo, Japan), and the induced difference (after vs. before the challenge) was recorded.

2.5. Intragingival, intraperitoneal, or intravenous injection of a test sample

In some experiments, a test sample was given by an intragingival, intraperitoneal, or intravenous injection. Intragingival injection was performed in the manner described previously [26]. Briefly, a 31-G needle (external diameter 0.26 mm, tip angle 22°; Hamilton) was encased in auto-polymerizing resin before being attached to a microsyringe (Hamilton). A portion of mucobuccal fold near the lower right first molar (M1) of each mouse (not anesthetized) was pierced by the needle almost at a right angle to the alveolar bone, the needle being stopped when it reached the bone. LPS solution (10 μ L/mouse) was then injected slowly. In the case of intraperitoneal or intravenous (via a tail vein) injection, 0.2 mL of a test sample was given to each mouse.

2.6. Statistical analysis

Experimental values are given as the mean $\pm$ standard deviation (SD). The statistical significance of differences was assessed using either a Student's unpaired *t*-test (for comparing two means) or a Bonferroni multiple-comparison test after ANOVA with the aid of InStat software. In each test, *P* values less than 0.05 were considered to indicate significance.

3. Results

3.1. Effects of LPS preparations from *E. coli* and oral bacteria (*P. intermedia* and *P. gingivalis*)

First, we tested the effects of LPS preparations from *E. coli* and two oral bacteria (*P. intermedia* and *P. gingivalis*). As shown in Fig. 1A–C, 1 μM NiCl_2 alone, 0.5 $\mu\text{g/mL}$ *E. coli* LPS alone, 5 $\mu\text{g/mL}$ *P. gingivalis* LPS alone, and 50 $\mu\text{g/mL}$ *P. intermedia* LPS alone caused transient, slight ear-swelling that peaked within 24 h of their injection. Although the data are not shown, similar ear-swelling, in terms of extent and time course, occurred after saline injection, too. These results indicate that such small, transient swelling is largely due to the injection itself and that the inflammatory effects of these LPS preparations are very weak, although Fig. 1C suggests that 50 $\mu\text{g/mL}$ *P. gingivalis* LPS may be transiently inflammatory. Thus, to reduce complexity, the saline injection-induced swelling was omitted in some figures. Combined injection of 1 μM NiCl_2 with any one of the LPS preparations at the indicated concentration induced a marked ear-swelling that peaked at 48 h after the injection. These effects were maximal at 0.05 $\mu\text{g/mL}$ *E. coli* LPS and 0.5 $\mu\text{g/mL}$ *P. intermedia* LPS, while a similar degree of ear-swelling was induced by *P. gingivalis* LPS at 50 $\mu\text{g/mL}$.

3.2. Effects of a mannan preparation, Pam3, and MDP

Next, we tested the effects of a preparation of mannan (a component of the fungus *S. cerevisiae*), Pam3 (a synthetic TLR2 agonist), and MDP (a synthetic bacterial peptidoglycan-related compound that stimulates intracellular NOD2).

The inflammatory effect of the mannan preparation itself was very weak if any at 50 $\mu\text{g/mL}$ or less (Fig. 2A). However, combined injection of 1 μM NiCl_2 with the mannan preparation at 5 and 50 $\mu\text{g/mL}$ induced marked ear-swelling in a dose-related manner.

The inflammatory effects of Pam3 at 50 $\mu\text{g/mL}$ and MDP at 500 $\mu\text{g/mL}$ were weak by themselves (Fig. 2B and C). However, combined injection of 1 μM NiCl_2 with Pam3 or MDP at these concentrations or less induced marked ear-swelling in a dose-related manner.

3.3. Effects of mannan preparation in TLR2-KO mice and TLR4-mutated C3H/HeJ mice

As described above, in the induction of Ni-allergy, 50 $\mu\text{g/mL}$ mannan preparation was effective in BALB/c mice. Thus, we tested whether this preparation can promote Ni-allergy in TLR2-KO mice and TLR4-mutated C3H/HeJ mice. The control mice for TLR2-KO mice and C3H/HeJ mice are wild-type (WT) C57BL/6 mice and C3H/HeN mice, respectively. In these experiments, we used 500 $\mu\text{g/mL}$ mannan as an adjuvant to sensitize these mice to Ni, because preliminary experiments suggested that the adjuvant effect of 50 $\mu\text{g/mL}$ mannan was insufficient for the above WT mice. The inflammatory (ear-swelling) effects of the mannan preparation itself in these mice were very weak, even at 500 $\mu\text{g/mL}$, and similar to that induced in BALB/c mice by 50 $\mu\text{g/mL}$ mannan (Fig. 2A) (data not shown).

As shown in Fig. 3A, the extent of the ear-swelling induced by combined injection of 1 μM NiCl_2 with the mannan preparation was very similar between TLR2-KO and control WT mice, indicating that TLR2 may not be important for the Ni-allergy-promoting effect of the mannan preparation.

Next, we examined whether this mannan preparation can promote Ni-allergy in C3H/HeJ mice. Before doing this experiment, we determined the effective and ineffective concentrations of NiCl_2 with regard to the induction of Ni-allergy in C3H/HeJ and C3H/HeN mice. It should be noted that as reported previously [10], *E. coli* LPS is not effective at sensitizing C3H/HeJ mice to Ni. However, we recently found that the LPS preparation from *P. intermedia* can sensitize C3H/HeJ mice [12]. Thus, in the present study, these mice were

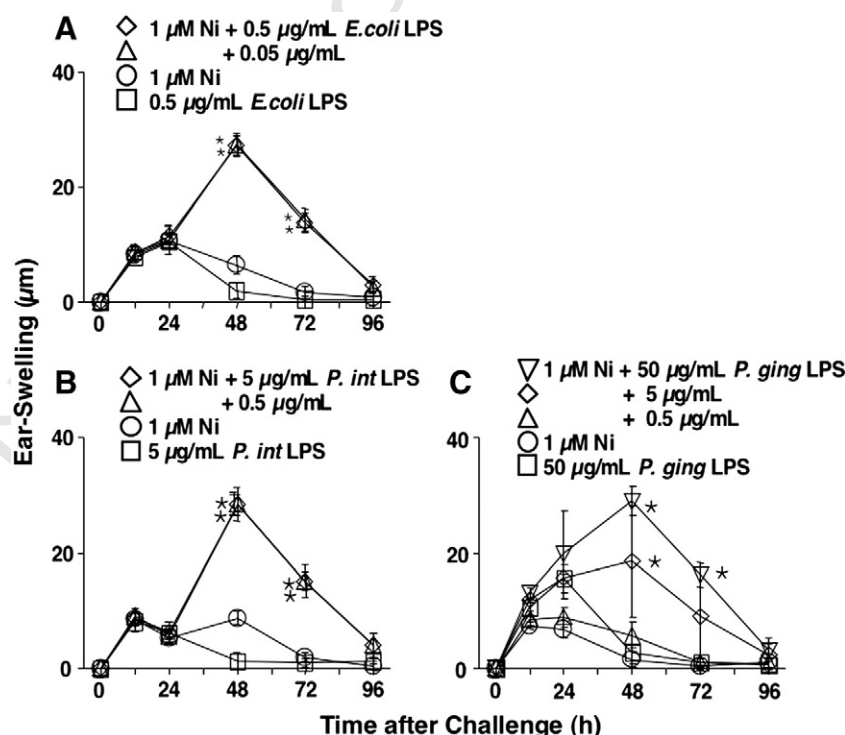


Fig. 1. Effects of LPS preparations. Ear skins of Ni-sensitized BALB/c mice were challenged intradermally with a solution containing 1 μM NiCl_2 and/or an LPS preparation from *E. coli* (A), *P. intermedia* (B), or *P. gingivalis* (C). The concentrations of these LPS preparations are indicated in the keys. Saline, test samples alone, and 1 μM NiCl_2 alone served as controls. Each value is the mean $\pm$ SD from 6 ears (in 3 mice). * P < 0.01 vs. controls (Ni alone and LPS alone). To reduce complexity, data for saline were omitted because there were no significant differences from those for Ni alone and LPS alone. It should also be noted that in the experiments shown in A and B, the results were identical between 1 μM NiCl_2 combined with LPS at either of two concentrations with a 10-fold difference.

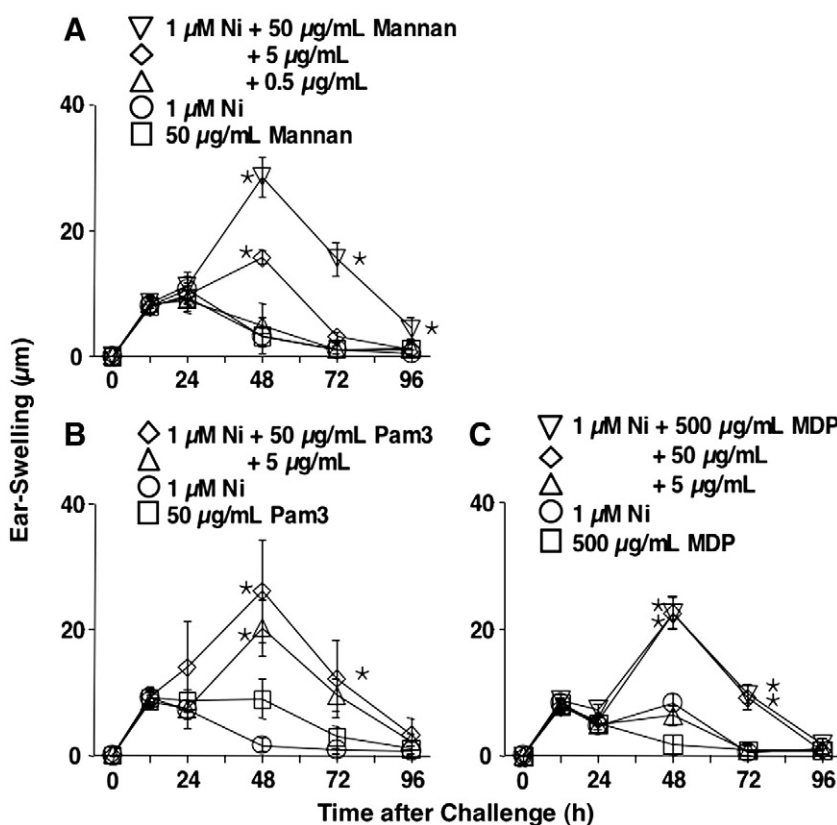


Fig. 2. Effects of a preparation of mannans, Pam3, and MDP. Ear skins of Ni-sensitized BALB/c mice were challenged intradermally with a solution containing 1 μM NiCl_2 and/or a mannans preparation from *S. cerevisiae* (A), Pam3 (B), or MDP (C). The concentrations of the various test samples are indicated in the keys. Saline, test samples alone, and 1 μM NiCl_2 alone served as controls. Each value is the mean $\pm$ SD from 6 ears (in 3 mice). * P < 0.01 vs. controls (Ni alone and the test substance alone). To reduce complexity, data for saline were omitted.

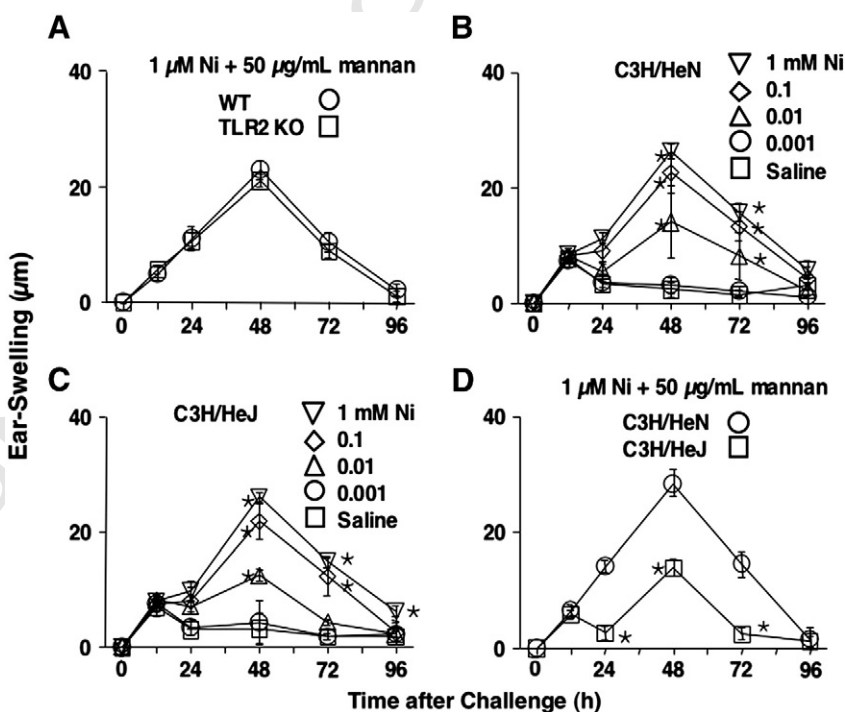


Fig. 3. Effects of a mannans preparation in TLR2-KO and TLR4-mutated C3H/HeJ mice. As described in Materials and methods, TLR2-KO mice and their control wild-type (WT) C57BL/6 mice were sensitized to Ni by intraperitoneal injection of NiCl_2 + *E. coli* LPS, while C3H/HeJ mice and their control C3H/HeN mice were sensitized to Ni by intraperitoneal injection of a solution containing NiCl_2 + *P. intermedia* LPS preparation. (A) Ear pinnas of sensitized TLR2-KO and WT mice were challenged with a solution containing both 1 μM NiCl_2 and a mannans preparation (500 $\mu\text{g/mL}$). (B and C) Saline or NiCl_2 alone (at the indicated concentration) was challenged to ear pinnas of sensitized C3H/HeJ and C3H/HeN. Each value is the mean $\pm$ SD from 6 ears (in 3 mice). * P < 0.01 vs. saline. (D) A solution containing both 1 μM NiCl_2 and a mannans preparation (500 $\mu\text{g/mL}$) was challenged to ear pinnas of sensitized C3H/HeJ and C3H/HeN. Each value is the mean $\pm$ SD from 6 ears (in 3 mice). * P < 0.01 vs. C3H/HeN.

sensitized by intraperitoneal injection of a solution containing 0.5 mM NiCl₂ and 0.5 µg/mL *P. intermedia* preparation (see [Materials and methods](#)). As shown in [Fig. 3B](#) and [C](#), the ear skins of sensitized mice were challenged with various concentration of NiCl₂ alone, and it was confirmed that 0.01 mM or more NiCl₂ induces Ni-allergy, whereas 0.001 mM (i.e. 1 µM) is insufficient, indicating that as in other strains of mice, 1 µM NiCl₂ alone does not induce Ni-allergy in C3H/HeJ and C3H/HeN mice. However, as shown in [Fig. 3D](#), a combined challenge with 1 µM NiCl₂ plus the mannan preparation induced significantly greater ear-swelling in C3H/HeN mice than in C3H/HeJ mice (both mice being sensitized to Ni with NiCl₂ + *P. intermedia* LPS preparation). This indicates that TLR4 may be important for the Ni-allergy-promoting effect of the mannan preparation, although we cannot rule out the commercial mannan preparation being contaminated with LPS stimulant(s).

3.4. Effects of *P. intermedia* LPS preparation in nude mice and HDC-KO mice

Previously, we reported (a) that NiCl₂ (0.5 mM) + *E. coli* LPS (0.5 µg/mL) can also sensitize athymic (nude) BALB/c mice, but not HDC-KO mice, to Ni, and (b) that challenge with 5 mM NiCl₂ alone induces Ni-allergy in nude mice, but not in HDC-KO mice [10]. In the present study, we examined whether challenge with 1 µM NiCl₂ together with the *P. intermedia* LPS preparation can induce Ni-allergy in nude mice, and whether HDC-KO mice are resistant to such promotion of Ni-allergy. In the above mice [treated 10 days earlier with an intraperitoneal injection of NiCl₂ (0.5 mM) + *E. coli* LPS (0.5 µg/mL)], the inflammatory effects of 1 µM NiCl₂ alone and of 50 µg/mL *P. intermedia* LPS preparation alone were very small ([Fig. 4A](#)). However, challenge with 1 µM NiCl₂ plus the *P. intermedia* LPS preparation induced a marked ear-swelling in nude mice ([Fig. 4A](#)), but not in HDC-KO mice ([Fig. 4B](#)), indicating that (i) as in other strains of mice, the *P. intermedia* LPS preparation promotes Ni-allergy in nude mice, and (ii) HDC-KO mice are resistant to Ni-allergy even if the Ni is combined with the *P. intermedia* LPS preparation.

3.5. Effects of intralingual injection of *P. intermedia* LPS preparation

As described above ([Fig. 1B](#)), the LPS preparation from *P. intermedia* exhibited a Ni-allergy-promoting effect approaching that of *E. coli* LPS in potency. This led us to speculate that the oral bacterium *P. intermedia*, which is thought to be a pathogen of periodontal diseases, might be an important risk factor in the promotion of Ni-allergy. We therefore examined whether intralingual injection of this LPS preparation might have a Ni-allergy-promoting effect. To this end, we compared the effects of the *P. intermedia* LPS preparation among the intralingual, intraperitoneal,

and intravenous routes. However, to avoid the effect of tissue damage caused by repeated injection into the same tissue, we did not perform intradermal injection of LPS preparations into ear skins. We previously reported that *E. coli* LPS, injected intraperitoneally, was effective when injected simultaneously or 3 h before the NiCl₂ injection [11]. However, it was not effective when injected after NiCl₂ injection [11]. Thus, in the following experiments, the *P. intermedia* LPS preparation was given (to mice that had been sensitized 10 days previously with *E. coli* LPS plus NiCl₂) at 3 h before the challenge with NiCl₂.

First, we compared the effects of intraperitoneal and intralingual injections of the same dose of the *P. intermedia* LPS preparation (20 µg/mouse; given 3 h before NiCl₂ injection into ear-pinnas). In these experiments, NiCl₂ was injected at 0.01–1 µM. As shown in [Fig. 5A](#), 1 µM NiCl₂ by itself induced a mild ear-swelling (see S → Ni in [Fig. 5A](#)), and the *P. intermedia* preparation augmented this swelling in a manner that was dependent on the concentration of NiCl₂. In this experiment, for comparison, the ear skins of one group of mice were directly challenged using a mixture containing the LPS preparation (100 µg/mL) and NiCl₂ (1 µM) (20 µL/ear; i.e., 2 µg of the LPS preparation/ear) (filled diamond symbol in [Fig. 5A](#)). This direct injection of the LPS preparation into ear skins induced a significantly greater allergic response at 24 and 48 h than its intraperitoneal injection. Interestingly, intralingual injection of the *P. intermedia* preparation was also effective at augmenting the allergic response to Ni, and this effect, too, was dependent on the concentration of NiCl₂ ([Fig. 5B](#)). This augmenting effect seen upon intralingual injection appeared to be greater than that induced by intraperitoneal injection (compare [Fig. 5A](#) and [B](#) at a given Ni-concentration).

Next, we compared the effects of various concentrations of the *P. intermedia* preparation between intravenous and intralingual injections. In this experiment, the three doses of the *P. intermedia* preparation were the same between the two injection routes (0.2, 2, or 20 µg/mouse), and the *P. intermedia* preparation was injected 3 h before the challenge injection of 1 mM of NiCl₂ into ear skins. As shown in [Fig. 6A](#) and [B](#), intravenous and intralingual injections had quantitatively similar augmenting effects on the allergic responses, in each case in a manner that was dependent on the concentration of the *P. intermedia* preparation.

3.6. Effects of LPS preparations in TLR2-KO mice

It is generally believed that the effects of LPS are exerted via TLR4 [13]. However, there are reports suggesting that LPS prepared from *P. gingivalis* has TLR2-agonistic activities [27,28]. Hence, we decided to examine whether TLR2 is involved in the Ni-allergy-promoting effect of the LPS preparations used in the present study. In the following experiments, we used the C57BL/6 strain as control wild-type (WT)

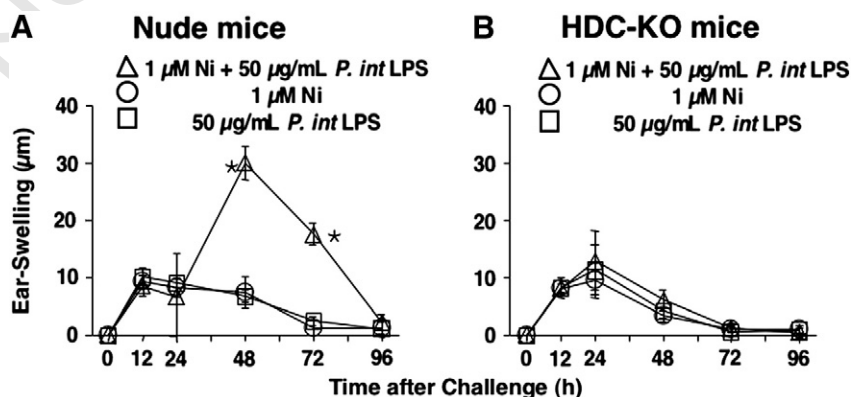


Fig. 4. Effects of a *P. intermedia* LPS preparation in nude (T-cell-deficient) mice and HDC-KO mice. BALB/c nude mice and HDC-KO mice (C57BL/6 background) were sensitized to Ni by intraperitoneal injection of NiCl₂ + *E. coli* LPS (see [Materials and methods](#)). A solution containing 1 µM NiCl₂ and/or *P. intermedia* LPS preparation (50 µg/mL) was challenged to ear pinnas of the sensitized mice. Each value is the mean ± SD from 6 ears (in 3 mice). *P < 0.01 vs. NiCl₂ alone and *P. intermedia* LPS preparation alone.

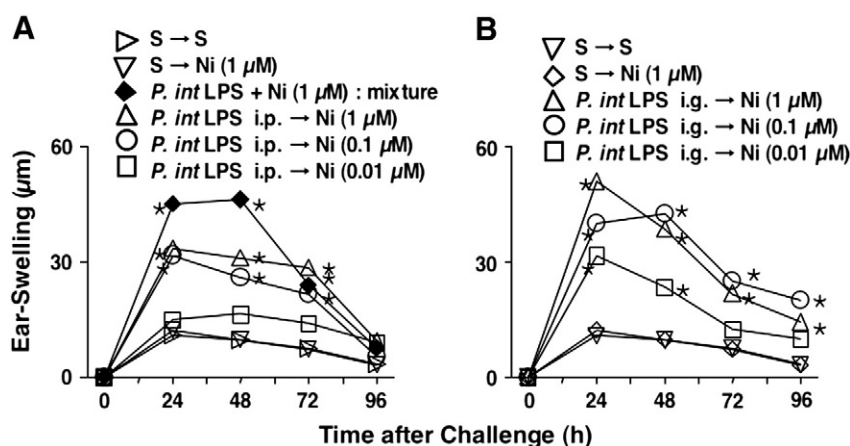


Fig. 5. Effects of intraperitoneal injection of a *P. intermedia* LPS preparation on the allergic responses induced by various concentrations of NiCl₂. In each of the experiments illustrated by panels (A) and (B), the dose of the *P. intermedia* LPS preparation was the same (20 μg/mouse). Thus, 100 μg/mL (A) or 2 mg/mL (B) of the LPS preparation was injected intraperitoneally [0.2 mL/mouse; (A)] or intragastrically [10 μL/mouse; (B)] into Ni-sensitized BALB/c mice. In each case, 3 h later one of three concentrations of NiCl₂ (0.01, 0.1, or 1 μM) was challenged to the ear skins of the mice (20 μL/mouse). It should be noted that in this experiment, for comparison, the ear skins of one group of mice (filled diamond symbol in Fig. 5A) were directly challenged using a mixture containing the LPS preparation (100 μg/mL) and NiCl₂ (1 μM) (20 μL/ear; i.e., 2 μg of the LPS preparation/ear). It should also be noted that saline (S) → 1 μM NiCl₂ induced a mild degree of ear-swelling. Each value is the mean ± SD from 6 ears (in 3 mice). **P* < 0.01 vs. S → S and S → 1 mM NiCl₂. To reduce complexity, mean values are shown without standard deviations.

mice as described above. These mice were sensitized to Ni in the same way as the BALB/c mice (see **Materials and methods**), and 10 days later a solution containing 1 μM NiCl₂ and an LPS preparation (0.5 μg/mL for *E. coli* LPS, 50 μg/mL for *P. intermedia* and *P. gingivalis* LPS) was challenged intradermally to the ear skins of the mice (20 μL/ear).

As shown in Fig. 7A, the allergic response induced by the NiCl₂ + *E. coli* LPS preparation was similar between WT and TLR2-KO mice, indicating that the Ni-allergy-augmenting effect of the *E. coli* LPS preparation is not due to stimulation of TLR2. However, the allergic response induced by NiCl₂ + an LPS preparation from either *P. intermedia* or *P. gingivalis* was markedly weaker in TLR2-KO mice than in control WT mice (Fig. 7B and C). These results suggest that as regards the LPS preparations from these two oral bacteria, each of which augments the allergic response to Ni in WT mice, the major components exert their effects via stimulation of TLR2.

4. Discussion

The findings made in the present study may be summarized as follows. Although NiCl₂ alone at 1 μM does not induce Ni-allergy in BALB/c mice (sensitized with NiCl₂ + *E. coli* LPS), combined challenge

with NiCl₂ plus one of the following substances did induce Ni-allergy: LPS preparations from oral Gram-negative bacteria (*P. intermedia* and *P. gingivalis*), a mannan preparation from a fungus (*S. cerevisiae*), and synthetic NOD2 and TLR2 agonists. The effect of the mannan preparation was small in C3H/HeJ mice (sensitized with NiCl₂ + the *P. intermedia* preparation). The *P. intermedia* preparation promoted Ni-allergy in C3H/HeJ and nude mice, but not in mice deficient in either TLR2 or HDC. Intragastric injection of the *P. intermedia* preparation and later challenge to ear-pinnas with NiCl₂ alone also promoted Ni-allergy, and this effect appeared to be more powerful than that seen upon intraperitoneal injection, but similar to that seen upon intravenous injection. In the following paragraphs, we discuss these findings.

The approximate minimal effective concentrations of the test-sample solutions used for challenging ear skins are summarized in Table 1. In addition to preparations of LPS and mannan, synthetic agonists of TLR2 (Pam3) and NOD2 (MDP) promoted Ni-allergy. Despite NOD2 being located within cells, rather than on the cell-surface [15], MDP exhibited promoting effects at 50 μg/mL, suggesting that MDP may exert such an effect at a much lower concentration within cells. These findings suggest that a variety of bacterial components can promote Ni-allergy at the elicitation step, and seem

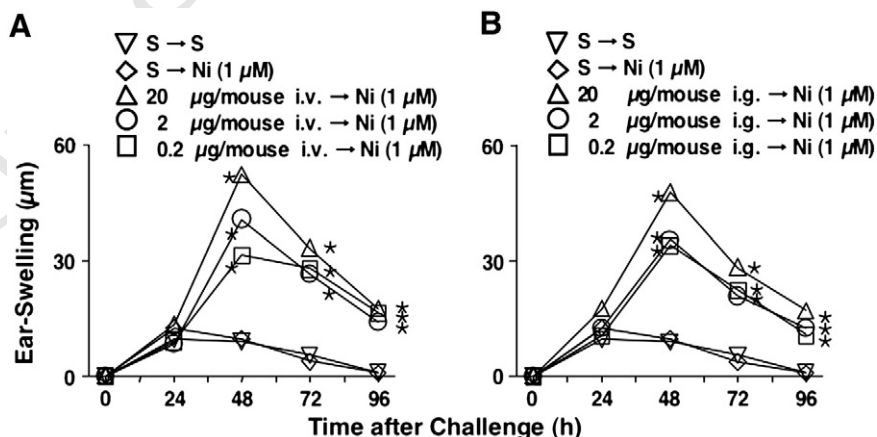


Fig. 6. Effects of intragastric injection of various doses of the *P. intermedia* LPS preparation on the allergic response induced by 1 μM NiCl₂. In the experiments illustrated by panels (A) and (B), saline (S) or various concentrations of *P. intermedia* LPS preparation were injected intravenously (i.v.) [0.2 mL/mouse; (A)] or intragastrically (i.g.) [10 μL/mouse; (B)] into Ni-sensitized mice. In each case, 3 h later 1 mM of NiCl₂ was challenged to the ear skins of the mice (20 μL/mouse). It should be noted that the doses of the *P. intermedia* preparation were the same regardless of whether the intravenous or intragastric route was used (0.2, 2, or 20 μg/mouse). Each value is the mean ± SD from 6 ears (in 3 mice). **P* < 0.01 vs. S → S and S → 1 μM NiCl₂. To reduce complexity, mean values are shown without standard deviations.

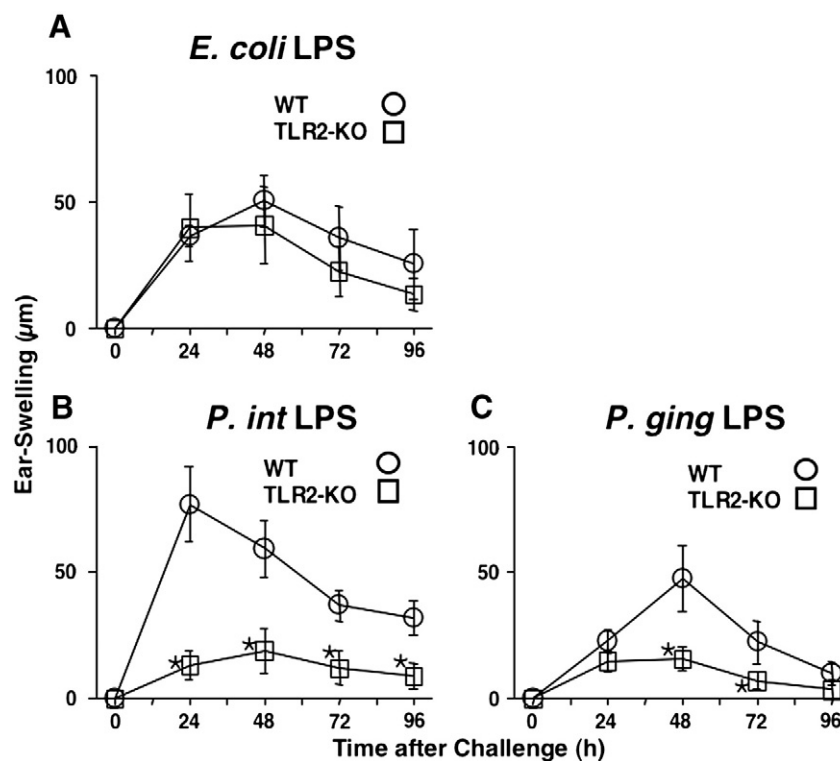


Fig. 7. Effects of LPS preparations from *E. coli* (a), *P. intermedia* (b), and *P. gingivalis* (c) in TLR2-KO mice. TLR2-KO and wild-type control C57BL/6 mice (WT) were sensitized as described in Materials and methods. Ten days later, a mixture containing 1 μ M NiCl₂ and an LPS preparation (0.5 μ g/mL for *E. coli* LPS, 50 μ g/mL for *P. intermedia* and *P. gingivalis* LPS) was challenged intradermally to the ear skins of the mice (20 μ L/ear). The ear-swelling effects induced in TLR2-KO and WT mice by 1 μ M NiCl₂ alone were similar to that induced in BALB/c mice by 1 μ M NiCl₂ (Fig. 6a). So, these data were omitted for simplicity. Each value is the mean $\pm$ SD from 6 or 8 ears (in 3 or 4 mice). **P* < 0.01 vs. WT mice.

strongly to support the idea that innate immune systems are critically important in the development of Ni-allergy in mice.

Hashimoto et al. [29] showed that an LPS preparation of *P. gingivalis* is inevitably contaminated with a lipoprotein that potently stimulates TLR2. It has also been proposed that a lipid A structure in *P. gingivalis* LPS is capable of activating TLR2 as well as TLR4 [28]. In this context, we previously showed that the *P. intermedia* LPS preparation used in the present study was almost devoid of mitogenic and cytokine-production activities *in vitro* in cells isolated from C3H/HeJ mice [23], indicating that these *in vitro* activities are due to the LPS itself. On the other hand, Ogawa et al. [30], who chemically synthesized a counterpart with the proposed lipid A structure of *P. gingivalis* LPS, clearly demonstrated that the lipid A structure was completely devoid of TLR2-agonistic activity, and that the activity of the LPS preparation from *P. gingivalis* was largely due to contamination with a lipoprotein that stimulates TLR2. Incidentally, the protein contamination in the *P. intermedia* LPS used in this study is at most 5.2 μ g/mg of preparation [31]. Thus, if the TLR2-stimulating activity of the present *P. intermedia* LPS preparation is due to a contaminating (lipo)protein(s), this contaminant must have a very powerful TLR2-stimulating activity. Black-pigmented bacteria, such as *P. gingivalis*

and *P. intermedia*, are dominant Gram-negative bacteria in the periodontal pocket of patients with periodontitis, and are considered to be major pathogens. Therefore, it would be very interesting to know the identity of the powerful TLR2-agonist(s) contained within the LPS preparations derived from oral bacteria.

Various innate immune receptors, including TLR2 and TLR4, are involved in the host's immune responses to fungal infections [32,33]. Interestingly, TLR2- and TLR4-mediated signals have been suggested to induce anti-inflammatory and pro-inflammatory responses, respectively, in fungal infections [33]. Tada et al. [34] first demonstrated that mannan fractions prepared from *S. cerevisiae* as well as from *Candida albicans* (a common inhabitant of the oral cavity) activated TLR4, but not TLR2, in human monocytic cells. The TLR-usage of fungal mannan, however, is still controversial. In this study, we clearly demonstrated TLR4-mediated (but not TLR2-mediated) augmentation by fungal mannan of the allergic reaction at the elicitation step (Fig. 3). Our evidence suggests that the receptors via which the mannan and *P. intermedia* preparations exerted their effects were, for each preparation, the same between the sensitization and elicitation steps: viz. TLR4 for mannan, but TLR2 for *P. intermedia* (the present results and those in ref. [12]). Taken together, the above findings suggest that fungal mannan (including that from oral fungi) may also be involved in the pathogenesis of metal allergy.

As described in the Introduction, Ni(+ LPS) allergy occurs even in T-cell-deficient nude mice [10]. As shown in Fig. 7, the present *E. coli* LPS preparation exerts its promoting effect on Ni-allergy via TLR4. As described above, other LPS preparations promote murine Ni-allergy via TLR2 stimulation, and such promotion also occurred in nude mice. These results suggest that in the absence of T-cells and/or during co-stimulation of innate immunity by more than one infectious agent, another type(s) of cells have the potential to induce or promote Ni-allergy. It is also likely that Ni-allergy in mice involves a mechanism different from that involved in the contact hypersensitivity induced by organic haptens, which form irreversible complexes via covalent bonds

Table 1

Approximate minimum effective concentrations (MEC) of substances or preparations tested for their promotion of Ni-allergy in BALB/c mice at the elicitation step.

Tested substances or preparations and their properties			MEC (μ g/mL) ^a
<i>E. coli</i> LPS	TLR4 ligand		<0.05
<i>P. intermedia</i> LPS	Including a potent TLR2 ligand(s)		<0.5
<i>P. gingivalis</i> LPS	Including a potent TLR2 ligand(s)		$\geq$ 5
<i>S. cerevisiae</i> mannan	TLR2 and/or TLR4 ligand		>5
Pam3	Synthetic TLR2 ligand		$\geq$ 5
MDP	Synthetic NOD2 ligand		$\geq$ 5

^a Concentrations of test samples in the solutions used for challenging ear skins, each solution containing 1 μ M NiCl₂.

with the host's protein molecules. In any event, it remains to be clarified what kinds of cells are involved in murine Ni-allergy.

Unlike classical haptens, metal ions may form geometrically highly defined, but reversible, coordination complexes with partner molecules, and so the host may recognize metal ions in complicated ways [35]. As yet, the partner molecules in the host ("self-proteins") that confer allergenicity on metals have not been identified. That being so, it is difficult to discuss the mechanisms that might underlie the augmenting effects of microbial materials on Ni-allergy, or on metal-allergies in general. However, we previously found that macrophages, IL-1, and histamine are involved in the induction of Ni(+LPS)-allergy in mice [10]. In the present study, promotion of Ni-allergy by the *P. intermedia* preparation was also shown to be dependent on HDC (the histamine-forming enzyme). We previously found that various microbial and related substances—including LPS preparations from *P. intermedia* and *P. gingivalis*—by themselves or cooperatively can induce HDC in mice [26,36–40]. We have also found (a) that histamine amplifies the immune response in gingival fibroblasts [41], and (b) that deficiency of biotin (a vitamin), which leads to an upregulation of TNF and IL-1 β production in murine macrophages, promotes or exacerbates Ni-allergy in mice [42,43]. On the basis of these findings, we may, albeit speculatively, list the following possibilities. These microbial materials may exert their promoting effects on Ni-allergy via stimulation of (i) macrophages, (ii) IL-1 production, (iii) histamine synthesis, and/or (iv) the production of "self-proteins". We also speculate that infection may lead to local biotin-deficiency, and that this leads in turn to upregulation of TNF and/or IL-1.

In real life, we are exposed to Ni through various routes. Among these routes, the skin and the mouth may be very important, because fungi and bacteria are almost always resident there. In addition, through daily oral hygiene procedures or dental surgical procedures, exposure to oral bacteria may occur repeatedly or frequently, especially in patients with periodontitis [44]. Thus, oral bacteria may frequently exert systemic effects. Indeed, intragingival injection into mice of small doses of *P. intermedia* LPS induces inflammatory reactions in extra-oral as well as in oral tissues [26]. In the present study, intragingival injection of *P. intermedia* LPS seemed to be more effective than its intraperitoneal injection. A potent hepatitis-inducing effect of an LPS upon intragingival injection has also been reported previously in mice [26]. Since various alloys are widely used in the oral cavity, the present findings suggest that oral bacteria and fungi may have an important role to play in the development of allergic inflammation in response to metals, including Ni.

We previously reported that (i) TLR4 stimulation promoted Ni-allergy at both the sensitization and elicitation steps [10,11] and (ii) various microbe-related substances (including TLR2-, TLR3-, and NOD2-stimulants and inducers of IL-1) promote Ni-allergy at the sensitization step [12]. In the present study, it was shown that stimulation of various microbe-recognizing receptors (TLR2, TLR4, or NOD1) at the elicitation step can also promote Ni-allergy. It is notable that NOD2 stimulation primes mice to augment the *E. coli* LPS-stimulated induction of HDC and production of IL-1 [45,46]. In addition, *P. intermedia* LPS preparation promotes allergic responses in mice to ovalbumin at both the sensitization and elicitation steps [47]. These findings suggest that stimulation of various receptors that recognize microbes may individually or mutually promote the host's responses to metal antigens and protein antigens, thereby causing or exacerbating allergic immune reactions (we tentatively call this "the aggravation effect"). This idea is in contrast to the so-called "hygiene hypothesis" that suggests that reduced frequency of infections in early infancy (by clean surroundings or by frequent use of antibiotics) may interfere with the maturation of Th1 immunity, leading to an enhancement of Th2 immune responses (i.e., allergic disorders) [48–50]. Concerning periodontitis, it is reported that there is an inverse association between periodontitis and respiratory allergies [51]. Thus, we suppose that in real-life, these "hygiene" and

"aggravation" effects could be present together, and unidentified factors (such as age, duration of infection, timing of exposure to allergens and infection, etc.) might be involved in determining which predominates at a given time.

In conclusion, the present results suggest that (i) a microbial milieu (e.g., in the oral cavity or on the skin) and/or innate immunity may play important roles in Ni-allergy, (ii) LPS preparations derived from oral bacteria may include a component(s) that strongly stimulates TLR2, and (iii) better oral and skin hygiene may prevent and/or reduce Ni-allergy.

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