

Dose dependence of oral tolerance to nickel

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Abstract

The dose dependence of oral nickel tolerance was analyzed by comparing three different subsets of C57BL/6 mice: Ni^{very low} mice were reared in a nickel-reduced environment, Ni^{low} and Ni^{high} mice were reared in a stainless steel-containing environment and the latter received oral NiCl₂ (10 mM). In spleen and feces, Ni^{very low} mice exhibit significantly lower nickel concentrations than Ni^{low} and Ni^{high} mice. In contrast to Ni^{very low} mice that can be sensitized with a single intradermal administration of NiCl₂ alone, Ni^{low} mice can only be sensitized in the presence of an adjuvant and Ni^{high} mice cannot be sensitized at all. This dose-dependent resistance to nickel sensitization (i.e. Ni^{high} > Ni^{low} > Ni^{very low}) correlates with differences in the number and type of nickel-specific T regulatory (Treg) cells. Adoptive transfer studies into Ni^{very low} recipients showed that Ni^{very low} mice completely lack specific Treg cells whereas Ni^{low} and Ni^{high} mice harbor them, albeit their numbers and/or suppressive strength are much higher in Ni^{high} than Ni^{low} mice. The principal Treg subset in Ni^{low} mice consists of CD4⁺CD25⁺ cells, among which CD4⁺CD25⁺αEβ7⁺ cells are the most effective. In Ni^{high} mice, CD4⁺CD25⁺ Treg cells co-exist with an ensemble of CD8⁺ Treg and CD4⁺CD25[−] suppressor-inducer cells.

Introduction

Nickel is one of the most frequent contact allergens. It is a constituent of various different alloys and, hence, occurs in numerous metal items used in life, from which it can be released in varying amounts in the presence of bodily fluids, including sweat and saliva (1). Nickel ions can generate neoantigens, or signal 1, needed for the activation of nickel-specific T cells. Whether the obligatory co-stimulation (signal 2) required for T cell priming is provided by an adjuvant effect of nickel ions themselves or by a variety of different cofactors subsumed under the term 'danger' (2) remains an unresolved issue. The latter possibility is supported by the clinical experience that nickel allergy preferentially develops after nickel has been in contact with an inflamed or irritated skin, whereas sensitization is unlikely to occur with the intact skin. Consistent with this, the majority of humans fails to become sensitized to nickel despite its ubiquitous occurrence (3), and interestingly, they possess nickel-specific CD4⁺CD25⁺ T regulatory (Treg) cells (4). In fact, even though a reduction in dermal nickel exposure has been shown to

decrease the incidence of newly sensitized nickel allergics (3), other epidemiological observations suggest that nickel hypersensitivity is lowered in the presence of elevated environmental levels of this potential sensitizer (5).

In both humans and experimental animals, most of the bodily absorption of nickel is through oral consumption (6), a route known to obviate the induction of contact hypersensitivity (CHS) for some other allergens and to favor the induction of Treg cells and tolerance (7). Consistent with this, an increased oral nickel uptake rendered experimental animals unresponsive to the induction of nickel allergy (8–10). Moreover, there is solid epidemiological evidence to indicate that in at least some wearers of dental braces, nickel tolerance may be acquired due to the continuous release of low amounts of nickel from such braces (11). Interestingly, however, the amount of nickel released from the braces is negligible when compared with the total daily uptake from food (~0.3 ng^{−1}kg^{−1}day^{−1} versus 2–4 μg^{−1}kg^{−1}day^{−1}) (1, 12, 13). This paradox raises questions about the potential effects

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of different exposure conditions and the dose–response relationship of oral nickel tolerance and nickel-specific Treg cells, respectively. Treg cells specific for nickel have been described as CD4⁺CD25⁺ in non-allergic humans (4). In mice exposed to a high concentration of oral nickel, both CD4⁺ and CD8⁺ T cells are required for the protection from sensitization with NiCl₂ combined with the adjuvant hydrogen peroxide (H₂O₂) (8), whether or not CD4⁺CD25⁺ Treg cells also are involved has not been studied so far.

To further clarify the conditions that allow the development of nickel hypersensitivity or nickel tolerance, we have performed a systematic analysis of the dose dependence required for oral nickel tolerance by comparing three types of C57BL/6 mice which were termed Ni^{high}, Ni^{low} and Ni^{very low} due to their different oral exposure to nickel. Our results indicate that there is a hierarchy Ni^{very low} > Ni^{low} > Ni^{high} mice with respect to the ease of sensitization to nickel and a reverse hierarchy Ni^{high} > Ni^{low} > Ni^{very low} with respect to nickel-specific Treg cells. Moreover, we analyzed to which extent oral nickel tolerance is mediated by CD4⁺CD25⁺ and CD8⁺ Treg cells. Whereas CD4⁺CD25⁺ Treg cells proved capable of preventing sensitization induced by NiCl₂ alone (i.e. without additional adjuvant), an ensemble of CD8⁺ Treg cells and CD4⁺CD25⁺ T cells was needed to prevent the hypersensitivity resulting from the vigorous immunization with NiCl₂/H₂O₂.

Methods

Reagents

NiCl₂·6H₂O (denoted as NiCl₂) and 2,4-dinitrofluorobenzene (DNFB) were purchased from Sigma-Aldrich Chemie GmbH (Steinheim, Germany), and H₂O₂ was obtained from E. Merck (Darmstadt, Germany).

Antibodies

Allophycocyanin (APC)-labeled streptavidin and the following anti-mouse antibodies were purchased from BD Pharmingen (Heidelberg, Germany): APC-anti-CD3ε (145-2C11), PerCP-anti-CD4 (RM4-5), PE- and biotin-anti-CD8β.2 (53-5.8), purified anti-CD16/CD32 (2.4G2), FITC-anti-CD25 (7D4), PE-anti-CD25 (PC61), PE-anti-α_{IEL} chain (M290), PE-anti-I-A/I-E (M5/114.15.2) and FITC-anti-TCRβ chain (H57-597). Anti-CD25 mAb was harvested from hybridoma PC61 (14). The magnetically labeled anti-mouse MHC-II, anti-mouse CD4 and anti-PE microbeads were obtained from Miltenyi Biotec GmbH (Bergisch Gladbach, Germany).

Mice

Ni^{low} mice were specific pathogen-free C57BL/6J mice obtained from Janvier (Le Genest St Isle, France). They were bred and reared in a conventional environment with cage covers and water bottle tips made from nickel-containing stainless steel (grade 304; DIN no. 14301); they received tap water without additional nickel supplementation. Ni^{very low} mice were generated by breeding and rearing Ni^{low} mice for two generations in a nickel-reduced environment using cage covers and water bottle lids made of plastic and glass, respectively. Ni^{high} mice were generated by giving Ni^{low} mice 10 mM NiCl₂ in the drinking water for 4–6 weeks.

All mice received a standard rodent diet (Sniff Spezialdiaeten, Soest, Germany) and were kept in accordance with the animal husbandry, as described in previous papers (8, 15). They were 7–10 weeks old at the onset of experiments. All investigations were carried out using female mice.

Determination of nickel concentration

Nickel concentration in urine, feces and organs of individual animals were determined by atomic absorption spectroscopy using a Zeeman 4100 ZL spectrometer (PerkinElmer, Boston, MA, USA). In the case of Ni^{high} mice, the excretions and bodily organs were obtained 1 day after cessation of a 4-week period of oral nickel administration, and age-matched Ni^{low} and Ni^{very low} mice were used as controls. The animals (three to five mice per group) were bled by heart puncture, and the organs and feces were handled with teflon-coated instruments to avoid contamination by nickel ions released from stainless steel instruments. To further reduce contamination by nickel ions, plastic pipettes and sample collection tubes were washed with 10% NH₄Cl, rinsed with the same aqua bidest as that used for atomic absorption spectroscopy and dried in a heating oven overnight. Samples were prepared for the analysis by high-pressure ashing.

Sensitization of mice

Mice were primed as described previously (8) with minor modifications. In the case of nickel, Ni^{low} mice were injected intradermally (i.d.) into both flanks (50 µl each) with either sterile, pyrogen-free saline, 10 mM NiCl₂ in saline or 10 mM NiCl₂ in saline containing 1% H₂O₂. When priming Ni^{very low} mice, however, 50 µl of either 1 mM NiCl₂ in saline or saline containing 1% H₂O₂ was used. In the case of DNFB, mice were primed by painting 0.5% (w/v) DNFB onto shaved flanks (25 µl each); DNFB was solved in a 4:1 (v/v) mixture of acetone and olive oil, respectively.

Challenge for recall and ear-swelling test

Ten days after priming, mice were challenged for recall by injecting 50 µl of 10 mM NiCl₂ in sterile, pyrogen-free saline into the pinna of each ear or by applying 50 µl of 0.2% DNFB onto each ear. Forty-eight hours after challenge with NiCl₂ and 24 h after challenge with DNFB, delayed-type hypersensitivity (DTH) reactions were determined by measuring the increment in ear thickness compared with pre-challenge values. For determination of pre-challenge values, mice were anesthetized; for measurement post-challenge, they were sacrificed by asphyxiation with CO₂. Measurements were performed in a blind fashion using a micrometer (Oditest D 1000 gauge, The Dyer Co., Lancaster, PA, USA). The data shown represent individual experiments comprising groups of five to six mice each, the mean ear-swelling response is expressed in units of mm × 10^{−2} ± SEM.

Adoptive T cell transfer

In all transfer experiments, Ni^{very low} mice (five to six animals per group) were used as recipients. Suspensions of the indicated type and number cell were injected intravenously (i.v.) into recipient mice (150 µl per mouse). To study the ability of transferred T cells in preventing subsequent sensitization

to nickel, recipients were primed i.d. 1 day after adoptive transfer; 10 days after priming, they were challenged for recall at the ears, and 48 h after recall, their ear-swelling response was measured. This experimental approach was used for all experiments except that shown in Fig. 4B in which the capacity of transferred T cells to suppress the elicitation of hypersensitivity in nickel-sensitized mice was studied. Here, recipients were primed 10 days prior to transfer; 1 day after transfer, they were challenged at the ears and 48 h later ear-swelling responses were determined.

Cell enrichment and sorting

To enrich donor T cells, single-cell suspensions of erythrocyte-free splenocytes were prepared in complete RPMI medium (PAA Laboratories, Coelbe, Germany) containing 10% FCS and passed through nylon wool columns. Eluted T cells were then further purified by either depletion of MHC-II⁺ cells to obtain total T cells or depletion of MHC-II⁺ cells followed by positive selection of CD4⁺ or CD8⁺ T cells using MACS microbeads labeled with the respective mAbs (Miltenyi Biotec GmbH). The sorted T cells were contaminated with <1% MHC-II⁺ cells; the sorted CD4⁺ and CD8⁺ T cells contained <1% CD8⁺ and <2% CD4⁺ T cells, respectively. In some experiments, the sorted CD8⁺ and CD4⁺ T cells were recombined in a 1:1 ratio prior to injection into the recipients.

To obtain CD4⁺ $\alpha_E\beta_7^-$ T cells, sorted CD4⁺ T cells were incubated with PE-labeled mAb against α_E integrin (CD103), followed by anti-PE microbeads. After depletion of $\alpha_E\beta_7^+$ T cells, the remaining contamination by such cells in the CD4⁺CD25⁺ T cell subset was ~4%.

Flow cytometry

For phenotyping T cells, single-cell suspensions were prepared in PBS and pre-incubated with anti-CD16/CD32 mAb (Fc block), before staining with the respective antibodies. For the determination of total CD4⁺CD25⁺ T cells (Fig. 8A) and the population of $\alpha_E\beta_7^+$ T cells among CD4⁺CD25⁺ T cells (Fig. 8B), 15 and 13 mice were individually analyzed. Flow cytometry was performed using a FACSCalibur (BD Biosciences, Heidelberg, Germany), and the results were analyzed with CellQuest software.

In vivo depletion of CD25⁺ cells

Hybridoma PC61 was grown to over-confluent levels in DMEM medium (PAA Laboratories) supplemented with 10% ultra-low IgG FCS (PAN Biotech GmbH, Aidenbach, Germany). Anti-CD25 mAb was then obtained using a Hitrap Protein G column with an ÄKTA prime (Amersham Pharmacia Biotech, Uppsala, Sweden). After concentrating the antibody molecules using centrprep (Millipore GmbH, Eschborn, Germany), the concentration levels were determined by standard protein assays. Purified anti-CD25 mAb (0.25 mg per mouse) was injected intra-peritoneally (i.p.) into either Ni^{high} or Ni^{low} mice, and 3 days later the mice were sacrificed and their spleen cells isolated, as described above.

Statistical analysis

Statistical significance of results was determined by analysis of variance (ANOVA) followed by Newman-Keuls test. One-

way ANOVA tests were performed using GraphPad Prism (GraphPad software, San Diego, CA, USA).

Results

Analysis of nickel concentration in tissues and excretions from Ni^{very low}, Ni^{low} and Ni^{high} mice

Concentrations of nickel found in the tissues and excretions from Ni^{high} mice obtained directly after oral NiCl₂ treatment were stopped far exceeded those found in Ni^{low} and Ni^{very low} mice. When comparing the latter two mouse subsets, statistically significant differences were only found in the feces and spleen. In both cases, the values measured in Ni^{low} mice exceeded those in the Ni^{very low} mice.

Unlike Ni^{low} mice, Ni^{very low} mice can be sensitized by NiCl₂ alone

Differences in the susceptibility of Ni^{high} and Ni^{low} mice to become sensitized toward nickel have already been reported (8, 16, 17). Here, we asked whether or not there is a corresponding difference between Ni^{low} and Ni^{very low} mice. To increase skin inflammation and irritation, we used intradermal rather than epicutaneous application to induce hypersensitivity to nickel (15). In confirmation with earlier results (8, 16, 17), a re-challenge in the ears with NiCl₂ via intradermal application resulted in only background ear swelling in Ni^{low} mice primed with NiCl₂ alone, but elicited significantly higher responses in Ni^{low} mice sensitized with NiCl₂/H₂O₂ (Fig. 2, bar 1 versus bar 2). In marked contrast, the Ni^{very low} mice could be sensitized with NiCl₂/H₂O₂ (Fig. 2, bar 7) and NiCl₂ alone (Fig. 2, bar 6). In either case, the immune response of Ni^{very low} mice was nickel specific, because these nickel-primed mice failed to elicit an ear-swelling response when recalled with DNFB (Fig. 2, bars 9 and 10). Moreover, DNFB-immunized mice showed high ear-swelling responses when recalled with DNFB (Fig. 2, bar 11), but not NiCl₂ (Fig. 2, bar 8).

Ni^{low} T cells suppress both the induction and elicitation of nickel hypersensitivity in Ni^{very low} recipients immunized with NiCl₂ alone

Ni^{high} mice proved resistant to sensitization not only with NiCl₂ alone but also with NiCl₂/H₂O₂; moreover, their T cells were anergic upon re-stimulation with NiCl₂ *in vitro* and able to transfer nickel tolerance to Ni^{low} recipients. In contrast, Ni^{low} mice were susceptible to sensitization with NiCl₂/H₂O₂, but not to sensitization with NiCl₂ alone (8, 15). Thus, we asked whether Ni^{low} mice possessed nickel-specific Treg cells which provided this partial resistance to nickel sensitization. To test this, T cells isolated from Ni^{high}, Ni^{low} or Ni^{very low} donors were transferred into naive Ni^{very low} recipients which were subsequently immunized, challenged and tested (Fig. 3A). Indeed, Ni^{low} donors provided nickel-specific Treg cells to the Ni^{very low} recipients that protected the latter from being sensitized by NiCl₂ alone (Fig. 3A, bar 5 versus bar 1). Interestingly, these Ni^{low} T cells failed to protect Ni^{very low} recipients that were immunized with NiCl₂/H₂O₂ (Fig. 3A, bar 6), indicating that the latter way of immunization against nickel is more difficult to suppress than the former. In contrast,

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transferred $\text{Ni}^{\text{very low}}$ T cells completely failed to suppress the induction of nickel-induced hypersensitivity (Fig. 3A, bars 1 and 2). As expected, Ni^{high} T cells not only prevented the sensitization induced with NiCl_2 alone (shown in Fig. 6E, bar 3) but also that induced with $\text{NiCl}_2/\text{H}_2\text{O}_2$ (Fig. 3A, bar 3). The suppression exerted by the transferred Ni^{low} T cells was nickel specific (Fig. 3A, bar 7 versus 9); the specificity of Treg cells from Ni^{high} mice has been demonstrated elsewhere (8).

Knowing that Treg cells from Ni^{high} and Ni^{low} mice can completely or partially prevent the post-transfer sensitization toward nickel, respectively (Fig. 3A), we asked whether they were also able to inhibit the elicitation of nickel hypersensitivity in already sensitized mice. Therefore, T cells from Ni^{high} , Ni^{low} or $\text{Ni}^{\text{very low}}$ donors were transferred into $\text{Ni}^{\text{very low}}$ recipients that were sensitized 10 days before transfer (Fig. 3B). In contrast to non-suppressive $\text{Ni}^{\text{very low}}$ T cells, Ni^{high} T cells profoundly suppressed the elicitation of nickel hypersensitivity and this was irrespective of how hypersensitivity was induced (Fig. 3B, bars 4 and 5). Ni^{low} T cells were equally able to suppress nickel hypersensitivity in $\text{Ni}^{\text{very low}}$ recipients sensitized with NiCl_2 alone (Fig. 3B, bar 7). In recipients immunized with $\text{NiCl}_2/\text{H}_2\text{O}_2$, donor Ni^{low} T cells demonstrated an intermediate suppressive capacity (Fig. 3B, bar 8). From the results shown in Fig. 3, we can conclude that the increase in oral nickel uptake correlates with the suppressive capacity of the transferred T cells.

Origin of T cell and sensitization determine the minimal number of T cells required for transferring nickel unresponsiveness to $\text{Ni}^{\text{very low}}$ mice

In Fig. 3, $\text{Ni}^{\text{very low}}$ recipients received 10^7 enriched donor T cells. However, previous studies using Ni^{low} recipients demonstrated that tolerance could be transferred with only 10^2 Ni^{high} donor T cells (8). Consequently, we wondered how few donor T cells were actually required for the transfer of unresponsiveness to $\text{Ni}^{\text{very low}}$ recipients. To establish this minimal number, we performed dose-response experiments using Ni^{high} and Ni^{low} donor T cells. Following immunization with NiCl_2 alone, it became apparent that 10^3 Ni^{high} T cells were sufficient to tolerize $\text{Ni}^{\text{very low}}$ recipients (Fig. 4A, bar 4). Interestingly, 10^4 T cells from the same donors were required when recipients were immunized with $\text{NiCl}_2/\text{H}_2\text{O}_2$ (Fig. 4B, bar 5). Furthermore, whereas 10^3 Ni^{high} T cells sufficed to transfer the tolerance to $\text{Ni}^{\text{very low}}$ recipients subsequently immunized with NiCl_2 alone (Fig. 4A, bar 4), as many as 10^6 Ni^{low} T cells were required to achieve the same effect (Fig. 4C, bar 7). Clearly, the number and/or type of nickel-specific Treg cells generated within Ni^{high} mice are superior to those from Ni^{low} mice.

Differential suppressive capacity of CD4^+ and CD8^+ T cells from Ni^{high} and Ni^{low} donors

Previously, we reported that both CD4^+ and CD8^+ Ni^{high} donor T cells were required for a successful transfer of tolerance into Ni^{low} recipients that were subsequently immunized with $\text{NiCl}_2/\text{H}_2\text{O}_2$ (8) (Fig. 5A, bar 8). We have now used the same experimental approach in order to compare the effects of CD4^+ and CD8^+ T cell sub-populations obtained from Ni^{low}

donors. As anticipated (cf. Fig. 3), T cell subsets from $\text{Ni}^{\text{very low}}$ donors, or combinations thereof, were unable to prevent the sensitization at any of the cell numbers transferred (Fig. 5A–C). In contrast to CD8^+ T cells, CD4^+ T cells from Ni^{high} (10^3) and Ni^{low} (10^6) donors were able to prevent post-transfer sensitization by NiCl_2 alone (bar 4 in Fig. 5B and C, respectively). In contrast, when the recipients were immunized with $\text{NiCl}_2/\text{H}_2\text{O}_2$, a transfer of even 10^4 Ni^{high} CD4^+ T cells still failed to prevent their sensitization (Fig. 5A, bar 4). In fact, CD8^+ T cells from these donors were devoid of any suppressive capacity when transferred alone (bar 6 in Fig. 5A–C). These results confirmed our previous findings that the sensitization induced by NiCl_2 alone can be more readily suppressed than that induced by $\text{NiCl}_2/\text{H}_2\text{O}_2$ (Figs 3–5) and that Ni^{high} CD4^+ T cells alone are unable to prevent sensitization with $\text{NiCl}_2/\text{H}_2\text{O}_2$ (8).

Although we have previously shown that 10^2 antigen-presenting cells from Ni^{high} donor mice are sufficient in transferring tolerance to Ni^{low} recipients (8), this contaminating population is not responsible for the prevention of hypersensitivity in $\text{Ni}^{\text{very low}}$ recipients upon transfer of 10^4 CD4^+ / CD8^+ T cells from Ni^{high} mice (Fig. 5A, bar 8). This conclusion is drawn from the results obtained from the transfer of the individual T cell subsets (10^4) which were unable to prevent hypersensitivity (Fig. 5A, bars 4 and 8).

Taken together, the data shown in Fig. 5 demonstrate that nickel tolerance, as induced by adoptive cell transfer into $\text{Ni}^{\text{very low}}$ recipients, is a fine blend between the mode of recipient immunization (NiCl_2 alone or with H_2O_2), the amount of oral nickel exposure of the T cell donor and the transferred T cell subsets (CD8^+ and/or CD4^+).

Suppressive activity of $\text{CD4}^+\text{CD25}^+$ Treg cells

Next, we studied whether the protection from transferred donor CD4^+ T cells (bar 4 of Fig. 5B and C) was provided by the $\text{CD4}^+\text{CD25}^+$ population (18). Thus, Ni^{high} (Fig. 6A) and Ni^{low} (data not shown) donors were depleted of CD25^+ T cells by injection of anti- CD25 mAb. Based on the information obtained earlier, we transferred either 10^3 CD4^+ Ni^{high} T cells (Fig. 6B), 10^4 unsorted Ni^{high} T cells (Fig. 6C), 10^3 unsorted Ni^{high} T cells (Fig. 6E) or 10^6 CD4^+ Ni^{low} T cells (Fig. 6D); the $\text{Ni}^{\text{very low}}$ recipients were immunized after transfer with either $\text{NiCl}_2/\text{H}_2\text{O}_2$ (Fig. 6C) or NiCl_2 alone (Fig. 6B, D and E). Indeed, the cell types and numbers specified sufficed to transfer nickel tolerance when the donor mice had not been depleted of CD25^+ cells prior to transfer (bar 3 of Fig. 6B–E). However, when the CD4^+ T cells from either Ni^{high} or Ni^{low} donors were depleted of CD25^+ cells, their capacity to prevent the sensitization by NiCl_2 alone was alleviated (bar 2 in Fig. 6B and D). Therefore, Ni^{high} and Ni^{low} mice possess $\text{CD4}^+\text{CD25}^+$ Treg cells which are able to prevent nickel hypersensitivity induced by NiCl_2 in $\text{Ni}^{\text{very low}}$ recipients.

A different picture emerged when unsorted Ni^{high} T cells, instead of CD4^+ Ni^{high} T cells, were depleted of CD25^+ T cells and then transferred. In this case, the depletion of CD25^+ cells failed to alleviate the suppressive capacity and this was regardless of whether the recipients were immunized with $\text{NiCl}_2/\text{H}_2\text{O}_2$ or NiCl_2 alone (bar 2 in Fig. 6C and E). It should be realized here that the unsorted Ni^{high} T cells

depleted of CD25⁺ cells contained CD4⁺CD25[−] and CD8⁺ T cells. As shown above, the nickel hypersensitivity in recipients immunized with either NiCl₂/H₂O₂ or NiCl₂ alone could not be prevented by the sole transfer of Ni^{high} CD8⁺ T cells (bar 6 of Fig. 5A and B), but required the co-transfer of Ni^{high} CD4⁺ T cells (Fig. 5A, bar 8). Therefore, it is conceivable that the maintenance of suppressor activity by unsorted CD25[−] Ni^{high} T cells (bar 2 in Fig. 6C and E) was due to the preservation within that population of CD8⁺ Treg cells and that these cells received help from CD4⁺CD25[−] T cells acting as so-called T suppressor-inducer cells.

Suppressive activity of CD4⁺CD25⁺αEβ₇⁺ Treg cells

CD4⁺CD25⁺ T cells expressing the integrin αE chain, a component of the homing receptor αEβ₇⁺ integrin or CD103, were reported to have a somewhat stronger suppressive capacity than CD4⁺CD25⁺αEβ₇[−] or CD4⁺CD25[−]αEβ₇⁺ T cells (19). To determine whether CD4⁺CD25⁺αEβ₇⁺ T cells were the main population that can suppress nickel hypersensitivity, CD4⁺ or CD4⁺αEβ₇[−] T cells from Ni^{high} or Ni^{low} donor mice were transferred into Ni^{very low} recipients. Confirming the results described above (Figs 5 and 6), CD4⁺ T cells from both Ni^{low} and Ni^{high} donors completely suppressed nickel hypersensitivity induced by NiCl₂ alone (Fig. 7, bars 2 and 4). When αEβ₇⁺ cells from these CD4⁺ T cells were depleted, however, the suppression partially disappeared (Fig. 7, bars 3 and 5). Together with the results shown in Fig. 6, it becomes apparent that the suppression of nickel hypersensitivity induced by NiCl₂ alone was provided by CD4⁺CD25⁺ T cells from Ni^{high} and Ni^{low} mice and that within that population the CD4⁺CD25⁺αEβ₇⁺ T cells have the most suppressive capacity.

Oral nickel uptake dose dependently enhances the percentage of splenic CD4⁺CD25⁺αEβ₇⁺ T cells

As far as immunization with NiCl₂ alone is concerned, the data presented here indicate that a 1000 times more Ni^{low} than Ni^{high} CD4⁺ donor T cells are required to prevent sensitization (Fig. 4A and C). In addition, the major sub-populations involved are CD4⁺CD25⁺ and CD4⁺CD25⁺αEβ₇⁺ T cells (Figs 6B, D and 7), two phenotypes associated with Treg cells. Thus, we asked whether the differential suppressive capacity of Ni^{high} and Ni^{low} CD4⁺ T cells could be due to a higher frequency of these Treg sub-populations in Ni^{high}

than Ni^{low} mice. In order to test this, we analyzed the spleens of uninjected Ni^{high}, Ni^{low} and Ni^{very low} mice for the frequencies of CD4⁺CD25⁺ and CD4⁺CD25⁺αEβ₇⁺ T cells. As shown in Fig. 8 (left panel), an increased oral uptake of nickel (cf. Fig. 1) resulted in an increased percentage of splenic CD4⁺CD25⁺ T cells. Similarly, there were also significant increases with nickel intake in the percentage of αEβ₇⁺ cells among the CD4⁺CD25⁺ T cells of Ni^{high}, Ni^{low} and Ni^{very low} mice (Fig. 8, right panel). Together, the results in Figs 7 and 8 suggest that the stronger suppressive capacity of Ni^{high} CD4⁺ T cells was due to the higher frequency of CD4⁺CD25⁺αEβ₇⁺ Treg cells observed in these animals.

Discussion

With our three types of nickel-exposed C57BL/6 mice, we have established that there is a clear-cut inverse dose-response relationship between the amount of oral nickel uptake and the ease in which mice can be sensitized to nickel. To summarize, whereas Ni^{high} mice could not be sensitized to nickel at all, Ni^{very low} mice could be sensitized even with NiCl₂ alone and Ni^{low} mice with NiCl₂/H₂O₂. This hierarchy of Ni^{very low} > Ni^{low} > Ni^{high} mice with respect to the ease of sensitization was mirror imaged by the hierarchy of Ni^{high} > Ni^{low} > Ni^{very low} with regard to the number and the suppressive capacity of nickel-specific Treg cells detected in these mice.

Since Ni^{low} mice received the same food and drinking water as Ni^{very low} mice, the only additional nickel ions accessible to the former were those released from their stainless steel cage covers (by saliva and gnawing) and drinking bottle lids (through water contact and licking). This elevated oral nickel uptake of Ni^{low} mice was verified by their small but significantly higher nickel concentration in the spleen and feces. This background level of nickel is enough to induce a state of partial tolerance which protects Ni^{low} mice from being sensitized by nickel ions alone, albeit not by nickel ions combined with the adjuvant H₂O₂ (or other adjuvants) (15; Fig. 2). A similar scenario exists in humans: continuous exposure to nickel in the oral cavity (from orthodontic braces) was found to correlate with a decreased incidence of nickel allergy (20). A possible explanation for this partial nickel tolerance of Ni^{low} mice and wearers of

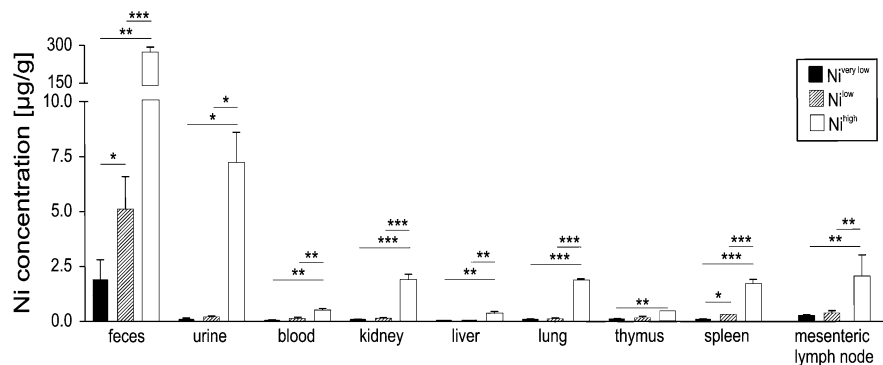


Fig. 1. Nickel concentrations in excretions and organs of Ni^{very low}, Ni^{low} and Ni^{high} mice. Results represent one of two experiments yielding very similar results. In this and the following figures, asterisks denote a significant statistical difference (**P* ≤ 0.05, ***P* ≤ 0.01 and ****P* ≤ 0.001).

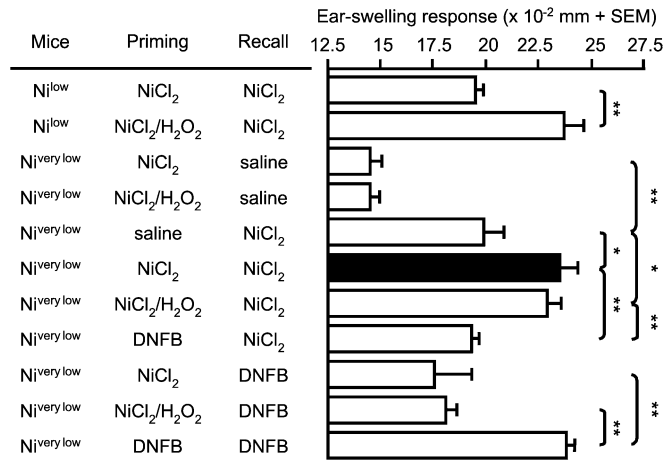


Fig. 2. Unlike Ni^{low} mice, Ni^{very low} mice can be sensitized by exposure to nickel ions alone (i.e. without the need for co-administration of the adjuvant H₂O₂). Ni^{low} and Ni^{very low} mice were primed with saline, NiCl₂ alone or NiCl₂ in H₂O₂, as indicated, and control groups were painted with DNFB. They were challenged for recall, as indicated, and their ear-swelling responses determined. The data shown represent one of three independent experiments showing comparable results. In this and the following figures, any reference to bar numbers in the text refers to the order of the bars read from top to bottom. Additionally, black bars indicate the critical experimental groups.

orthodontic braces could be that, as in hamsters (21), the mucosa of the cheek pouch of mice and men is an immunologically privileged site.

The use of Ni^{very low} mice as recipients of adoptively transferred T cells enabled us to compare the suppressive capacity of nickel-specific T cells obtained from either Ni^{high} or Ni^{low} donors. Due to the following two criteria, we conclude that unsorted T cells from Ni^{high} donors contain more potent nickel-specific Treg cells than those from Ni^{low} mice. First, the required number of unsorted Ni^{high} T cells to prevent induction of nickel hypersensitivity is much lower than that of Ni^{low} donors. Second, the capacity of unsorted Ni^{high} Treg cells to prevent the subsequent induction of nickel hypersensitivity and to preclude its elicitation in pre-sensitized recipients functioned independently from the type of immunization (i.e. NiCl₂ alone or with H₂O₂) of the latter. In contrast, the suppressive capacity of Ni^{low} Treg cells depended on the mode of recipient sensitization. Unlike Ni^{high} and Ni^{low} mice, the Ni^{very low} mice were found to completely lack nickel-specific Treg cells. Interestingly, regulatory T cells from Ni^{high} donor mice were able to suppress both primary and secondary responses to nickel (Fig. 3A, bar 3 and Fig. B, bar 5). In contrast, Ni^{low} Treg cells that were unable to suppress the subsequent immunization with NiCl₂/H₂O₂ (Fig. 3A, bar 6) were also unable to inhibit a secondary response after sensitization with NiCl₂/H₂O₂ (Fig. 3B, bar 7). Collectively, these data support the notion that nickel-specific regulatory T cells are not only able to suppress the immunization within a time frame of 10 days but also secondary immune responses within 2 days upon adoptive transfer.

The suppressive capacity of CD4⁺CD25⁺ Treg cells has been widely established (18). We demonstrate here that

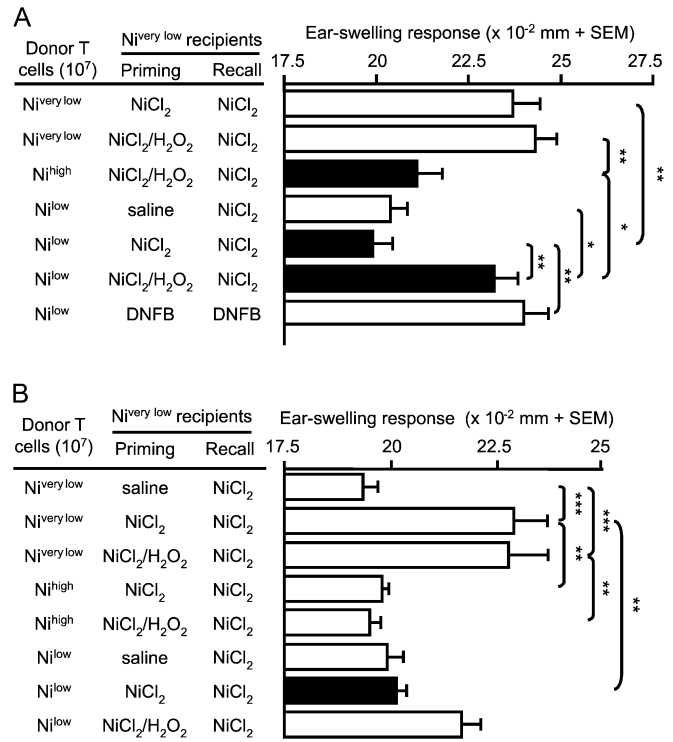


Fig. 3. Adoptively transferred Ni^{low} T cells suppress the nickel hypersensitivity induced by immunization with NiCl₂ alone, whereas the suppressive capacity of Ni^{high} T cells is not confined by the mode of recipient immunization. Splenic T cells (10⁷ per recipient) from Ni^{high}, Ni^{low} or Ni^{very low} donors were transferred into Ni^{very low} recipients. (A) Showing the effect of donor T cells on recipients that were primed and challenged for recall after the cell transfer. (B) Showing the effect of donor T cells on recipients that were primed before the transfer and challenged for recall thereafter. Data represent one of four (A) or two (B) independent experiments showing comparable results.

while donor Treg cells from Ni^{high} or Ni^{low} mice could prevent hypersensitivity induced by NiCl₂ alone, CD4⁺CD25⁺ T cells failed to do so. However, the CD4⁺CD25⁺ Treg cell-containing CD4⁺ T cells failed to prevent the hypersensitivity inducible by NiCl₂/H₂O₂. Thus, vigorous immunization can break the incomplete tolerant state provided by CD4⁺CD25⁺ Treg cells. This conclusion correlates to findings made *in vitro* showing that the suppressive activity of CD4⁺CD25⁺ Treg cells can be overruled by providing a more potent signal 2 (22, 23).

Although CD4⁺ T cells (containing CD4⁺CD25⁺ Treg cells) from both Ni^{high} and Ni^{low} mice prevented nickel hypersensitivity induced by NiCl₂ alone, the number of effective CD4⁺ T cells required from Ni^{low} mice was 1000 times higher than that needed from Ni^{high} donors. Interestingly, when analyzing the percentage of CD25⁺ cells among CD4⁺ T cells and the percentage of α E β ₇⁺ cells among CD4⁺CD25⁺ T cells, we found a distinct hierarchy of Ni^{high} > Ni^{low} > Ni^{very low} with regard to the frequencies of total CD4⁺CD25⁺ and CD4⁺CD25⁺ α E β ₇⁺ T cells. CD4⁺CD25⁺ α E β ₇⁺ T cells studied *in vitro* have been reported to have a stronger suppressive capacity than CD4⁺CD25⁺ α E β ₇⁻ T cells (19, 24), and *in vivo* α E β ₇ expression is important for CD4⁺CD25⁺ Treg cells to

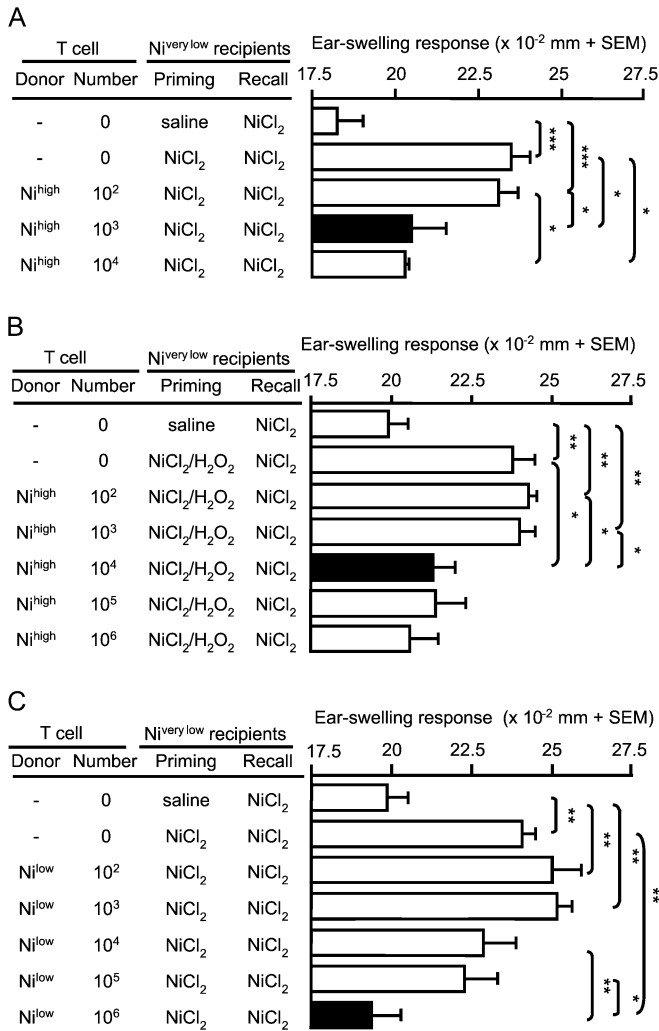


Fig. 4. The minimal number of T cells required for preventing post-transfer sensitization of Ni^{very low} recipients is determined by both the type of T cell donor and the mode of recipient immunization. Indicated numbers of unsorted T cells from Ni^{high} (A and B) and Ni^{low} (C) donor mice were transferred and the recipients primed and challenged, as indicated. Saline-injected Ni^{very low} mice served as negative control groups throughout. (A) Demonstrates the number of Ni^{high} donor T cells required to prevent sensitization after priming with NiCl₂ alone. (B) Shows the number of Ni^{high} donor T cells required to prevent sensitization after priming with NiCl₂/H₂O₂. (C) Depicts the number of Ni^{low} donor T cells required to prevent sensitization after priming with NiCl₂ alone. Data represent one of two independent experiments showing comparable results.

enter into the inflamed tissue and suppress activated T cells (25). In our experimental system, CD4⁺CD25⁺αEβ₇⁺ T cells were more effective than CD4⁺CD25⁺αEβ₇⁻ T cells in preventing nickel hypersensitivity in Ni^{very low} mice. Additional studies are needed to clarify whether the enhanced suppressive capacity of CD4⁺CD25⁺αEβ₇⁺ T cells is due to a superior suppressive ability on a single-cell level, a better homing potential or a combination of the two. Consistent with published data (19, 24), those CD4⁺CD25⁺ T cells that fail to express αEβ₇ probably also show some degree of suppressive capacity because we observed that removal of

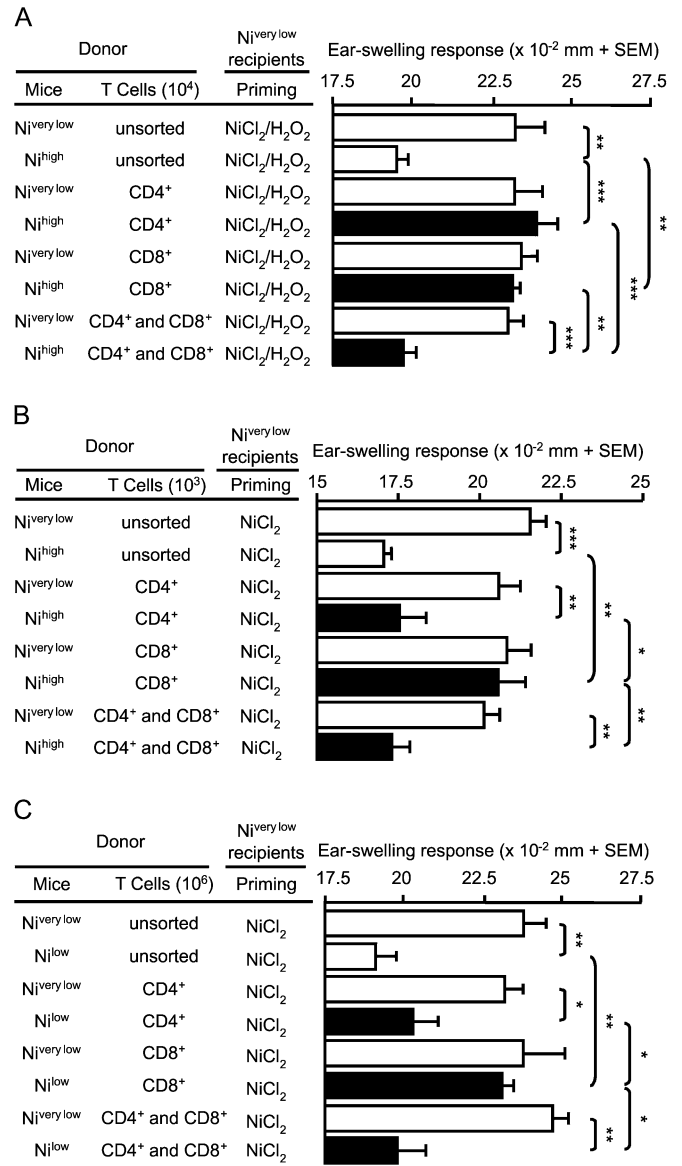


Fig. 5. Both Ni^{high} and Ni^{low} CD4⁺ T cells prevent nickel hypersensitivity induced by NiCl₂ alone, but combined CD4⁺ and CD8⁺ T cells from Ni^{high} donors are needed to prevent the hypersensitivity induced by NiCl₂/H₂O₂. (A–C) T cells from Ni^{very low}, Ni^{low} or Ni^{high} donor mice were transferred, as indicated. They were transferred as unsorted cells (bars 1 and 2), sorted CD4⁺ T cells (bars 3 and 4), sorted CD8⁺ cells (bars 5 and 6) or as an ensemble of sorted CD4⁺ and CD8⁺ T cells that were recombined at a 1:1 ratio prior to injection to yield the number of transferred T cells indicated (bars 7 and 8). In (A) 10⁴ T cells were transferred and recipients primed with NiCl₂/H₂O₂ after transfer, while in (B) 10³ and in (C) 10⁶ T cells were transferred and recipients primed with NiCl₂ alone. All recipients were challenged with NiCl₂, and their ear-swelling response was determined. Data represent one of two independent experiments showing comparable results.

αEβ₇⁺ T cells from CD4⁺ T cells only partially alleviated the suppression.

It is well established that oral administration of antigens favors tolerance induction, include CD4⁺CD25⁺ Treg cell differentiation (26). These CD4⁺CD25⁺ Treg cells rapidly divided preferentially in gut lymphoid tissue (27). Multiple

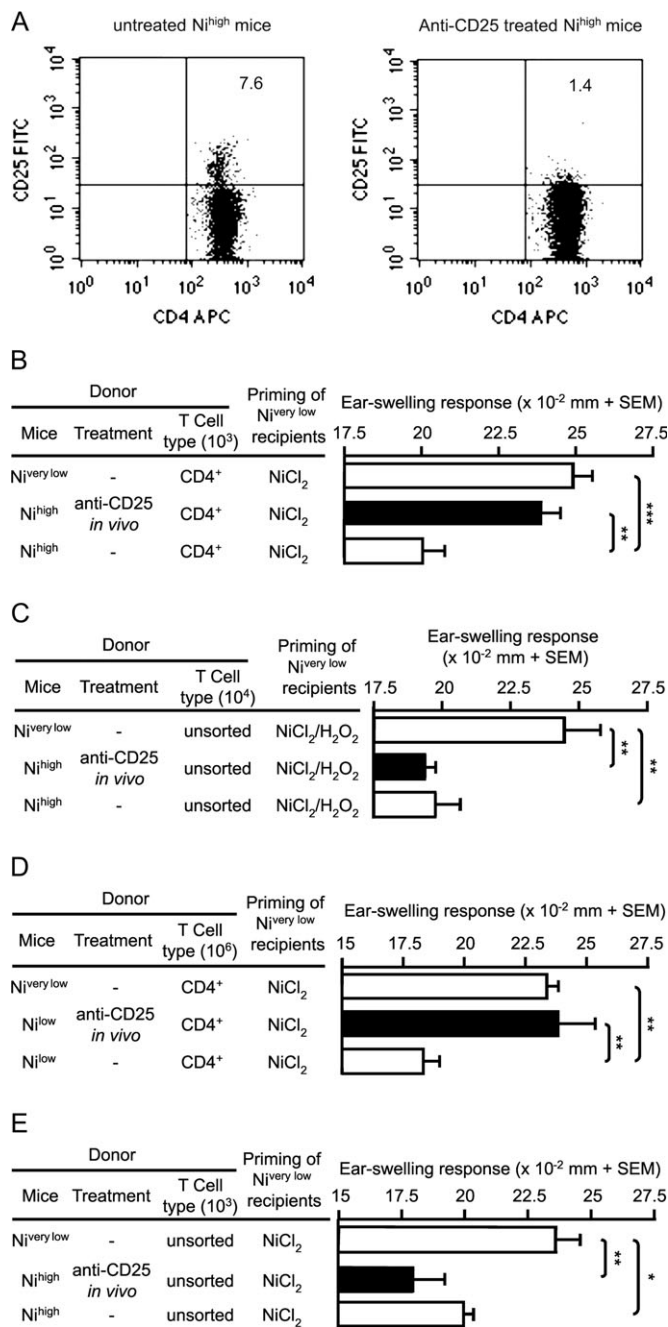


Fig. 6. Whereas CD4⁺CD25⁺ T cells from nickel-exposed donors are able to suppress $NiCl_2$ alone-induced sensitization, both CD4⁺CD25⁻ and CD8⁺ T cells are required to prevent sensitization by $NiCl_2/H_2O_2$. (A) Indicating the percentage of CD4⁺CD25⁺ T cells in the spleen of untreated Ni^{high} mice (left panel) and Ni^{high} mice that had received an i.p. injection of anti-CD25 mAb (right panel). (B–E) Ni^{low} and Ni^{high} were injected with anti-CD25 mAb or left untreated, as indicated. Specified numbers and T cell subsets from the indicated donor mice were then transferred into $Ni^{very\ low}$ recipients. Equivalent numbers and cell types obtained from $Ni^{very\ low}$ donor mice served as non-suppressive control cells. After recipients were primed, as indicated, and challenged with $NiCl_2$, their ear-swelling response was determined. Data represent one of two independent experiments showing comparable results.

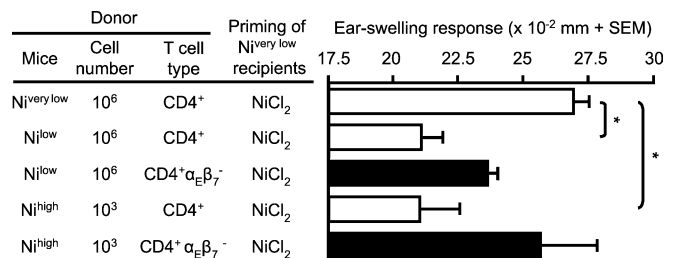


Fig. 7. CD4⁺CD25⁺ $\alpha_E\beta_7^+$ T cells from Ni^{high} and Ni^{low} donors can prevent the sensitization induced by $NiCl_2$ alone. Indicated numbers and subsets of T cells were obtained from $Ni^{very\ low}$, Ni^{low} or Ni^{high} donors and transferred as either sorted CD4⁺ cells (bars 1, 2 and 4) or sorted CD4⁺ $\alpha_E\beta_7^-$ cells (bars 3 and 5). After the recipients were primed and challenged with $NiCl_2$, their ear-swelling response was determined. Data represent one of three independent experiments showing comparable results.

mechanisms have been proposed to explain oral tolerance and Treg cell induction. One of them is that the intestinal mucosa has high basal levels of IL-4, IL-10 and transforming growth factor- β (TGF- β) (28), and short after oral antigen uptake, expression of these cytokines up-regulated (29). These cytokines, especially TGF- β , play a central role in oral tolerance; it not only be able to convert naive peripheral CD4⁺CD25⁻ T cells to CD4⁺CD25⁺ Treg cells via induction of FoxP3 (30) but also act as master regulators of most of mechanisms triggered by antigen feeding (31). Within CD4⁺CD25⁺ Treg cells, CD4⁺CD25⁺ $\alpha_E\beta_7^-$ cells showed naive-like phenotype, CD4⁺CD25⁺ $\alpha_E\beta_7^+$ represents effector/memory-like phenotype and might be induced or expanded in response to antigenic stimulation (19). Interestingly, the expression of $\alpha_E\beta_7$ on CD4⁺CD25⁺ Treg cells is positively regulated by TGF- β (25). Therefore, TGF- β influences both the number of CD4⁺CD25⁺ Treg cells and CD4⁺CD25⁺ $\alpha_E\beta_7^+$ Treg cells. Although no data about TGF- β up-regulation after oral uptake of nickel exist, it has been reported that i.p. injection of nickel enhances the TGF- β level in rat serum (32); thus, it is conceivable that oral administration of nickel may enhance the percentages of CD4⁺CD25⁺ in CD4⁺ T cells and CD4⁺CD25⁺ $\alpha_E\beta_7^+$ T in CD4⁺CD25⁺ Treg cells.

Furthermore, nickel ions have remarkable chemical versatility, which enables them to generate a great variety of different metal-protein complexes or neoantigens, as discussed in detail elsewhere (20). Since none of the animals was primed and the only difference between the three mouse subsets was their exposure to nickel, one can indirectly conceive that an increasing nickel concentration in the body is associated with an increase in the variety of different neoantigens induced by nickel ions and, consequently, different nickel-reactive T cell clones and Treg cells. In correlation with this, the percentage of CD25⁺ cells among CD4⁺ T cells and the percentage of $\alpha_E\beta_7^+$ cells among CD4⁺CD25⁺ T cells were both significantly higher in Ni^{high} mice when compared with Ni^{low} mice (Fig. 8). Therefore, a higher exposure to nickel may lead to a more diverse nickel-reactive T cell repertoire and thus account for the differences in the strengths of the differentiated Treg cell populations (including the CD8⁺ T cells in Ni^{high} mice) in preventing hypersensitivity responses.

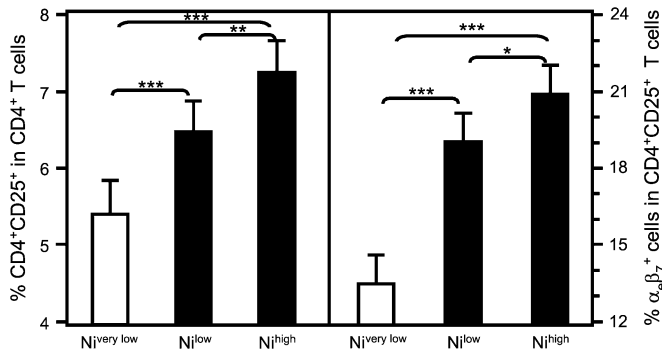


Fig. 8. Increasing oral nickel uptake augments both the percentage of CD25⁺ cells within the CD4⁺ T cell population and the percentage of $\alpha_E\beta_7$ ⁺ T cells among CD4⁺CD25⁺ T cells. Splenic CD4⁺ and CD4⁺CD25⁺ T cells from the indicated mouse subsets were gated, and the percentages of CD25⁺ cells within CD4⁺ T cells (left panel) and $\alpha_E\beta_7$ ⁺ T cells among CD4⁺CD25⁺ T cells (right panel) were determined.

Interestingly, nickel-specific CD8⁺ Treg cells could be identified in Ni^{high} mice. A possible explanation for this could be that only in the Ni^{high} mice the self-proteins bound to nickel reach a high enough concentration so that sufficient numbers of nickel-induced neoantigens are presented to CD8⁺ T cells (33, 34). In order to be cross-presented by MHC-I molecules, extracellular antigens have to be presented at a high concentration (34–36).

By blocking CD25 on Treg cells, we could compare the suppressive power of CD4⁺CD25⁺ and CD8⁺ Treg cells in response to nickel. Even without CD4⁺CD25⁺ Treg cells, the remaining T cell population in Ni^{high} mice contained powerful suppressor T cells since they were also able to prevent sensitization induced by NiCl₂/H₂O₂. Whether this superior suppressive capacity of CD8⁺ Treg cells is due to a higher frequency or a stronger suppressive ability on a single-cell level when compared with CD4⁺CD25⁺ Treg cells is unknown. Nevertheless, transfer of Ni^{high} CD8⁺ Treg cells alone cannot prevent sensitization to NiCl₂/H₂O₂. For this, both CD4⁺ and CD8⁺ T cells have to be co-transferred. Indeed, synergism between suppressor-inducer CD4⁺ T cells and CD8⁺ suppressor-effector T cells has been observed in other models (37–42), but the mode of interaction of these cells remains poorly understood. As an example, when tolerance to trinitrophenol (TNP) was induced by i.v. injection of TNP-coupled syngeneic splenocytes, the tolerance transfer by CD8⁺ T suppressor-effector cells required either the co-transfer of TNP-specific CD4⁺ T suppressor-inducers or treatment of recipient mice with agonistic anti-CD40 mAb (43). These findings suggest that the CD8⁺ T suppressor-effector cells acquire some kind of help from activated CD4⁺ T suppressor-inducers which act through their CD40 ligand. Consistent with this, after the transfer of unsorted Ni^{high} T cells (containing both CD4⁺ and CD8⁺ T cells) to Ni^{low} recipients, vigorous recipient immunization with NiCl₂/H₂O₂ is required for the activation of CD4⁺ T cells and subsequent infectious spread of tolerance from donor cells to cells of the recipient (8).

CD4⁺ suppressor-inducer cells that cooperate with CD8⁺ suppressor-effector T cells in the suppression of DTH responses were characterized in detail in the model of anterior

chamber-induced immune deviation (ACAID). Tolerance induction in that model (38, 41, 44) shows a remarkable number of similarities with that of Ni^{high} mice (16, 45). In ACAID, the CD4⁺ Treg suppressor-inducer cells were unequivocally identified as CD25⁺ T cells (41). We found that a population of Ni^{high} T cells, which comprised both CD8⁺ T cells and CD4⁺CD25⁺ T cells, was able to prevent the sensitization from NiCl₂/H₂O₂, whereas neither CD4⁺ T cells alone nor CD8⁺ T cells alone was capable of doing so. While this might suggest that Ni^{high} CD4⁺CD25⁺ T cells acted as suppressor-inducers, we did not explicitly show that Ni^{high} CD4⁺CD25⁺ T cells were unable to do so. We cannot rule out that the CD4⁺CD25⁺ T cells transferred along with CD8⁺ T cells switched their phenotype from CD25⁺ to CD25⁺ in the post-transfer period, in which the recipient mice were immunized with NiCl₂/H₂O₂ before being challenged 9 days later. Another unresolved question is why it takes the ensemble of CD8⁺ suppressor-effector and CD4⁺ suppressor-inducer T cells in order to prevent sensitization from NiCl₂/H₂O₂, whereas CD4⁺CD25⁺ Treg cells are unable to do so but suffice to prevent the sensitization induced by NiCl₂ alone.

With regard to the daily amount of nickel per kilogram body weight that is taken up from food and drinking water (12), there appears to be no fundamental difference between humans and Ni^{low} mice, and conceivably, the same is true for the nickel-specific CD4⁺CD25⁺ Treg cells described in non-allergic humans (4) and the Ni^{low} mice studied here. In these animals, the protection provided by CD4⁺CD25⁺ Treg cells is overcome when nickel ions are encountered in the context of danger, such as H₂O₂ which mimics inflammation. If we extrapolate from the Ni^{low} mice to humans, nickel-specific CD4⁺CD25⁺ Treg cells could account for the lack of sensitization when humans encounter nickel ions on the intact skin, that is, in the absence of danger. However, when human exposure to nickel occurs in the context of danger, such as ear piercing or irritated skin, CD4⁺CD25⁺ Treg cells may be less sufficient at protecting from sensitization. Furthermore, we hypothesize that if the oral uptake of nickel in humans were as low as that of our Ni^{very low} mice, humans might lack nickel-specific Treg cells altogether; thus, an increase in the incidence of nickel allergy would ensue.

By definition, the hypersensitivity induced in our study is a DTH rather than CHS reaction. Although CHS is one type of DTH response, it differs from other forms of DTH responses. While the effector T cells in DTH response are CD4 T cells, those in CHS response are mainly CD8 T cells, although in some instance can also be CD4 T cells. Furthermore, in DTH responses, CD4 T cells act as effector T cells whereas they exert a regulatory function in CHS responses (46, 47). In this respect, our nickel model well fits the standard of a DTH response since Ni^{low} mice sensitized by injection of NiCl₂/H₂O₂ harbor CD4 effector cells (15, X. Wu *et al.*, unpublished data). By this standard, nickel allergy in humans is also not a pure CHS response since nickel-reactive T cells in human peripheral blood are mainly of the CD4⁺ subtype (48–50). Actually, when we further study the cause of nickel allergy in human, one can find that nickel allergy is really not pure CHS response because it can be caused by either contact nickel at irritated skin or 'intra-dermal' contact such as ear piercing, the latter is typical DTH response.

Therefore, our mouse model is a useful animal model to study nickel allergy and tolerance with relevance to human nickel allergy.

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Abbreviations

ACAIID	anterior chamber-induced immune deviation
ANOVA	analysis of variance
APC	allophycocyanin
CHS	contact hypersensitivity
DNFB	2,4-dinitrofluorobenzene
DTH	delayed-type hypersensitivity
H ₂ O ₂	hydrogen peroxide
i.d.	intradermally
i.p.	intra-peritoneally
i.v.	intravenously
TGF-β	transforming growth factor-β
Treg	T regulatory

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