CONTACT DERMATITIS

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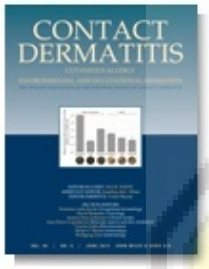
ENVIRONMENTAL AND OCCUPATIONAL DERMATITIS

Contact Dermatitis



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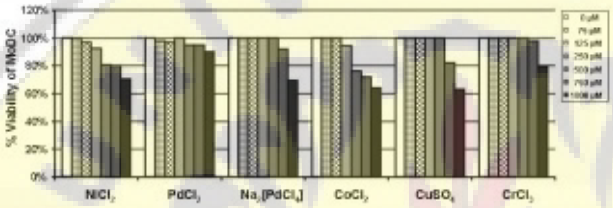

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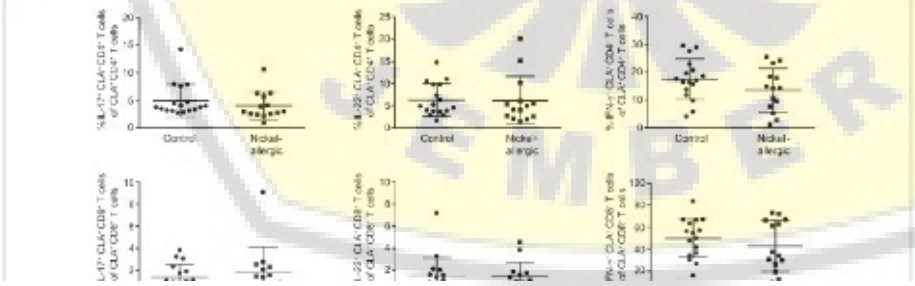

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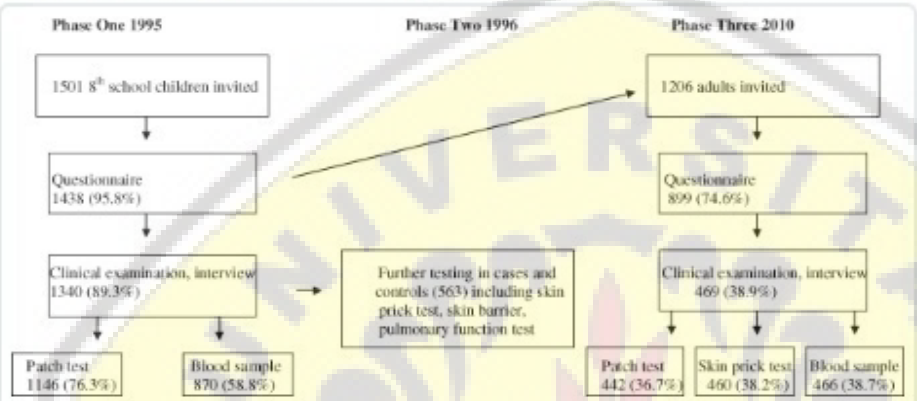

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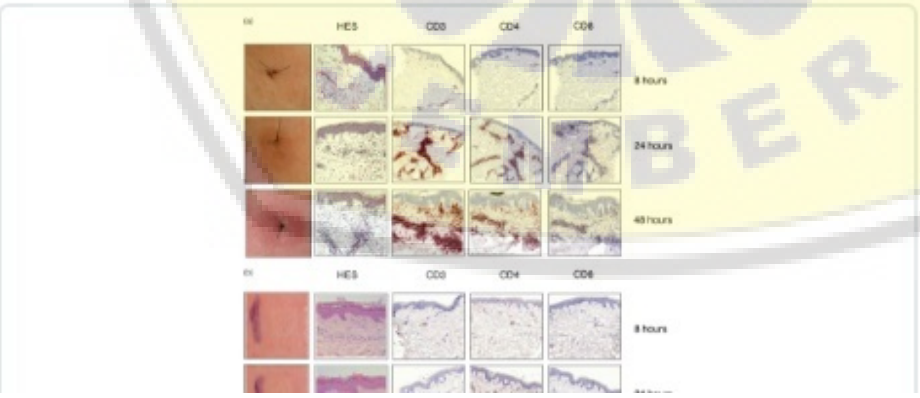

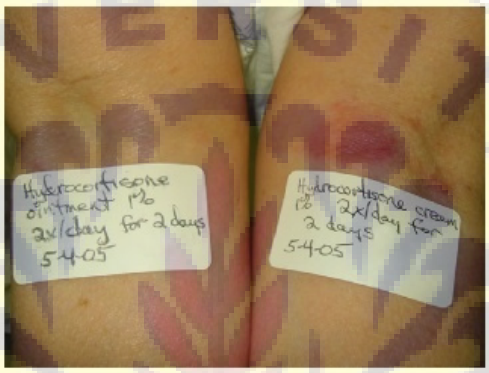

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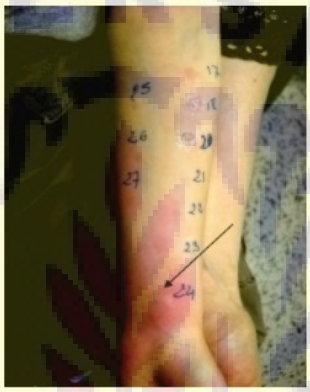

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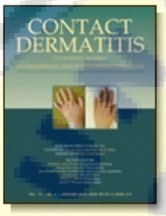

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Transition metal sensing by Toll-like receptor-4: next to nickel, cobalt and palladium are potent human dendritic cell stimulators

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Summary

Background. Nickel was recently identified as a potent activator of dendritic cells through ligating with human Toll-like receptor (TLR)-4.

Objectives. Here, we studied an extended panel of transition metals neighbouring nickel in the periodic table of elements, for their capacity to activate human monocyte-derived dendritic cells (MoDCs).

Methods. The panel included chromium, cobalt, and palladium, all of which are known to be frequent clinical sensitizers. MoDC activation was monitored by assessment of release of the pro-inflammatory mediator interleukin (IL)-8, a major downstream result of TLR ligation.

Results. The data obtained in the present study show that cobalt and palladium also have potent MoDC-activating capacities, whereas copper and zinc, but not iron and chromium, have low but distinct MoDC-activating potential. Involvement of endotoxin contamination in MoDC activation was excluded by *Limulus* assays and consistent stimulation in the presence of polymyxin B. The critical role of TLR4 in nickel-induced, cobalt-induced and palladium-induced activation was confirmed by essentially similar stimulatory patterns obtained in an HEK293 TLR4/MD2 transfectant cell line.

Conclusions. Given the adjuvant role of costimulatory danger signals, the development of contact allergies to the stimulatory metals may be facilitated by signals from direct TLR4 ligation, whereas other metal sensitizers, such as chromium, may rather depend on microbial or tissue-derived cofactors to induce clinical sensitization.

Key words: contact allergy, dendritic cells, TLR4, transition metals.

Metals are among the most notorious clinically known contact sensitizers. This applies in particular to the transition metals nickel, cobalt, and chromium. In dentistry, palladium has also acquired a bad reputation as a frequent cause of oral allergic complaints (1–4).

Recently, it was discovered that nickel ions are able to directly ligate and trigger Toll-like receptor (TLR)-4 on dendritic cells (DCs) (5). Evolutionarily, these receptors developed to respond to bacterial molecular signals, such as lipopolysaccharide (LPS)/endotoxin. Downstream of this signalling pathway, pro-inflammatory mediators are released, such as interleukin (IL-8), IL-1β, and tumour necrosis factor-α (6). These mediators contribute to both rapidly acting innate immune responses and adaptive immunity by driving antigen-induced T cell expansion and cell-mediated immune effector functions and/or recruitment of B cells, leading to antibody generation. Although nickel ions or nickel-binding peptides are almost

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Conflicts of interest: The authors have declared no conflicts.

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invisible to B cell receptors, and thus do not induce nickel-specific antibodies, they may readily trigger specific T cells, as present in healthy individuals. Given the abundance of nickel and the high exposure rates, the direct generation of a strong danger signal by this metal to DCs most likely contributes to this metal being first on the list of clinically relevant contact allergens.

Previously, we and others reported on frequent cross-reactivity between nickel and the neighbouring transition metals copper and palladium at the T cell recognition level (7, 8). Nickel-specific T cell clones were unable to discriminate either between nickel and copper, or between nickel and palladium, indicating close molecular mimicry between these metals. Therefore, we decided to explore to what extent various transition metals, including copper and palladium, also show similar DC-activating and maturing capacities by triggering nuclear factor-κB, leading to downstream IL-8 release. The involvement of direct TLR4 triggering was studied by use of a TLR4/MD2 transfectant cell line. The results show that, similarly to nickel, palladium and cobalt have the capacity to trigger TLR4.

Materials and Methods

Metal chemicals

As metal allergens, the following chemicals were used: nickel sulfate hexahydrate ($NiSO_4$; Merck, Darmstadt, Germany), nickel(II) chloride hexahydrate ($NiCl_2.6H_2O$), chromium(III) chloride hexahydrate ($CrCl_3.6H_2O$), potassium dichromate ($K_2Cr_2O_7$), cobalt(II) chloride hexahydrate ($CoCl_2.6H_2O$), copper(II) sulfate ($CuSO_4$), iron(III) chloride ($FeCl_3$), zinc chloride ($ZnCl_2$) (all from Fluka/Riedel de Haën, Seelze, Germany), sodium tetrachloropalladate(II) ($Na_2[PdCl_4]$; Sigma-Aldrich Chemie GmbH, Steinheim, Germany), and palladium chloride ($PdCl_2$; Sigma). LPS was obtained from *Escherichia coli* 055:B5 (Sigma-Aldrich, St. Louis, MO, USA). $CrCl_3$, $K_2Cr_2O_7$, $FeCl_3$, $CoCl_2$, $NiSO_4$, $NiCl_2$, $CuSO_4$, $ZnCl_2$, $Na_2[PdCl_4]$ and LPS were dissolved in Iscove's modified Dulbecco's medium (IMDM) (Biowhittaker, Verviers, Belgium) containing 10% heat-inactivated fetal calf serum (FCS) (Hyclone, Logan, UT, USA).

Peripheral blood mononuclear cell (PBMC) isolation and culture of monocyte-derived DCs (MoDCs)

PBMCs were isolated from 40–50 ml of freshly drawn peripheral venous blood of 6 different healthy donors without known metal allergies by Ficoll (Lymphoprep; Fresenius Kabi Norge AS, Oslo, Norway) density gradient centrifugation. The cells were counted with a CASY cell counter (Schärfe System, TT-2-BA-1007, Rutlingen Germany) and trypan blue. MoDCs were generated as previously described (9). Briefly, adherent monocytes were then cultured for 6–7 days in the humidified incubator in 10 ml of IMDM supplemented with 10% FCS, 1% penicillin/streptomycin, 1% L-glutamine, 1% β-mercaptoethanol, 1000 U/ml granulocyte–macrophage colony-stimulating factor (Novartis, The Netherlands), and 20 ng/ml IL-4 (lot AG270911A; R&D Systems Europe, Abingdon, UK). After 6–7 days, immature DCs (iDCs) were harvested and plated in 96-well flat tissue culture plates (Cellstar; Greiner bio-one, Frickenhausen, Germany) at approximately 5×10^4 cells per well.

Metal toxicity experiments

In order to determine appropriate concentration ranges of metals, cytotoxicities were measured with the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (MTT) reduction test. Approximately 100 µl of cells (5×10^4/ml) were plated in 96-well culture plates and exposed to increasing concentrations of the metals. After 24 hr of incubation, culture medium was removed, and 50 µl of MTT solution (5 mg/ml) was added per well. The MTT solution was prepared fresh, dissolved in water, and filtered through a 0.22-µm filter. The plates were incubated in the dark at 37°C. After 2–3 hr of incubation, 50 µl of dimethylsulfoxide (Merck, Darmstadt, Germany) was added to each well, and, after shaking, the solution was measured with an enzyme-linked immunosorbent assay (ELISA) reader at an optical density (OD) of 450 nm. The OD of the cells in the absence of metal was considered to be 100%. The viabilities of exposed cells were determined with the formula: (OD experimental sample/OD of control cells) ×100%.

Metals and LPS exposure

LPS was tested at 15 and 50 ng/ml. The total volume in each well was 200 µl. iDCs (5×10^4 cells per well) were exposed to metals at concentrations between 0 and 750 µM. Plates were incubated at 37°C in 5% humidified CO_2. After 24 hr, supernatants were collected and stored at −20°C until measurement. Where indicated, polymyxin B sulfate (25 µg/ml; Sigma-Aldrich) was mixed with the metal salt solutions in the culture wells before iDCs were added, to exclude possible involvement of endotoxin in metal-induced DC stimulation (10, 11). Complementary checks for LPS contamination were carried out with *Limulus* amebocyte lysate (LAL) assays (Kinetic-QCL™ bulk kit; Lonza, Cologne, Germany).

Assessment of TLR4 signalling with TLR4 transfectant cells

HEK293 TLR4/MD2 cells were cultured in T75 flasks in Dulbecco's modified Eagle's minimal essential medium, 1% glutamine, 1% penicillin/streptomycin, and 0.5 μg/ml G418, and harvested upon confluence. Wild-type HEK293 and HEK293 cells stably expressing human TLR4 and MD2 were a gift from D. T. Golenbock (University of Massachusetts Medical School, USA) to Y. van Kooyk/M. Verstege. Cells were split twice weekly until they were ready for use (12). Cells were plated at 1×10^5 cells per well in 100 μl of medium in 96-well flat-bottomed plates. After cells had been allowed to adhere for 1.5–2 hr, 100 μl of metal salt solution was added, to give final concentrations of 0, 250 and 500 μM. Cells were incubated for 24 hr at 37°C, and supernatants were harvested for IL-8 ELISA.

Cytokine production

IL-8 production was measured with an ELISA, with a PeliKine ELISA kit for human IL-8 (Sanquin, Amsterdam, The Netherlands) and 96-well microtitre plates (Nunc maxisorp microtitre plates; Nalge Nunc International, Roskilde, Denmark), according to the manufacturer's instructions. Absorbance was measured at 450 nm. The amount of IL-8 in the supernatant was assessed by using a standard curve of IL-8 (lower detection limit: 15.4 pg/ml). Generally, supernatants were diluted 25, 250 and 1000 times before testing. Data are presented as IL-8 production in picograms or nanograms per millilitre.

Data analysis

The statistical significance of the effects of various metals on the secretion of IL-8 was analysed by using a paired two-tailed Wilcoxon test, with the computer program Medcalc Software (Mariakerke, Belgium). $P \leq 0.05$ was considered to be statistically significant. All data are presented as median [P25–75] and mean ± standard error of the mean.

Results

Cytotoxic effects of selected transition metals on MoDCs

The transition metals that were to be studied for their potential stimulatory effects on DCs were first tested to define the testing range and maximal non-toxic concentrations. Cytotoxicity experiments were carried out with MTT reduction assays as a read-out. After exposure of MoDCs for 24 hr to increasing dosages of $CrCl_3$, $FeCl_3$, $CoCl_2$, $NiCl_2$, $CuSO_4$ and $Na_2[PdCl_4]$, concentration ranges between 0 and 750 μM were found to be appropriate. Cobalt and copper salts showed the highest toxicities, but, up to 500 μM, no viability reductions exceeding 25% were observed for any metal of the panel (Fig. 1).

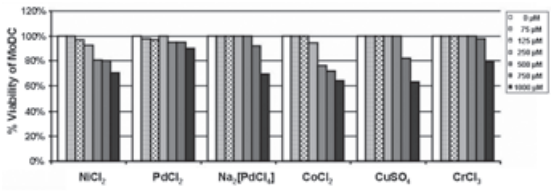

Fig. 1. Viability of monocyte-derived dendritic cells in the presence of increasing metal concentrations. Cell viabilities were assessed with the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay, as outlined in Materials and Methods. The results shown are from one representative experiment out of four.

Transition metal-induced MoDC activation as detected by IL-8 secretion

Subsequently, the selected transition metals were studied for their capacity to activate MoDCs, as detected by IL-8 release. As positive controls, nickel chloride and LPS were included, as both compounds have previously been shown to activate MoDCs through direct TLR4 triggering. Dose responses are given for all metals tested (Fig. 2). The results show that, next to $NiCl_2$, $CoCl_2$ stands out for its strong capacity to activate MoDCs. Copper, zinc and palladium salts induced much lower, but still significant, levels of IL-8, whereas $CrCl_3$ and $FeCl_3$ only induced non-significant levels of IL-8 at the highest test concentrations.

Transition metal-induced MoDC activation is not caused by contamination with LPS

To exclude the possible involvement of endotoxin contamination in transition metal-induced MoDC activation, metal panel experiments were repeated with or without the LPS inhibitor polymyxin B added to the cultures. Polymyxin B is a positively charged polypeptide, and is active as an antibiotic for gram-negative bacteria. Its binding to LPS/endotoxin can also be exploited to clear endotoxin contamination. Dose–response data are presented in Fig. 3, and show that, as expected, addition of polymyxin B caused a reduction of at least 90% in LPS induced IL-8 release. In contrast, none of the metal-induced responses was affected by endotoxin clearance by the addition of polymyxin B, supporting the view that metal-induced DC activation is an intrinsic property of the metals. Complementary negative LAL assay results confirmed the exclusion of a role for LPS in these findings (data not shown).

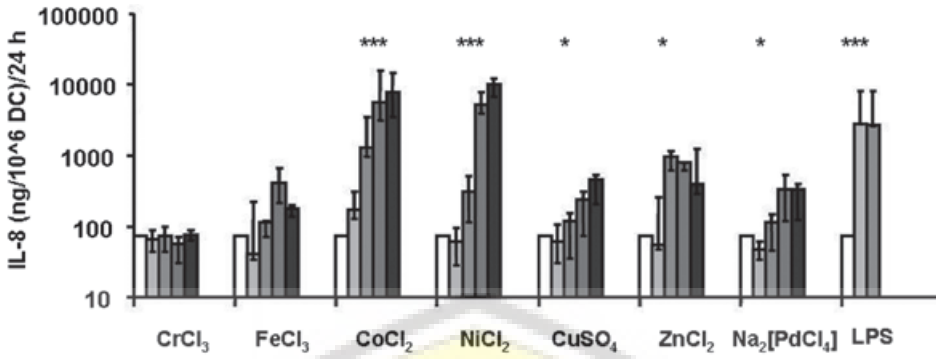

Fig. 2. Profile of interleukin (IL)-8 secretion after exposure of immature monocyte-derived dendritic cells to selected transition metal salts for 24 hr. Supernatants were harvested after exposure to medium only (open bars) or exposure to increasing concentrations of metal salts (from left to right: 125, 250, 500 and 750 μM). Metals are arranged in the graph following their order of appearance in the periodic table of elements. The production of IL-8 is given as median [P 25–75] for at least 6 donors in three different individual experiments. For statistical analysis, the 500 μM values were compared with the medium control (Wilcoxon paired test: $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$). DC, dendritic cell; LPS, lipopolysaccharide.

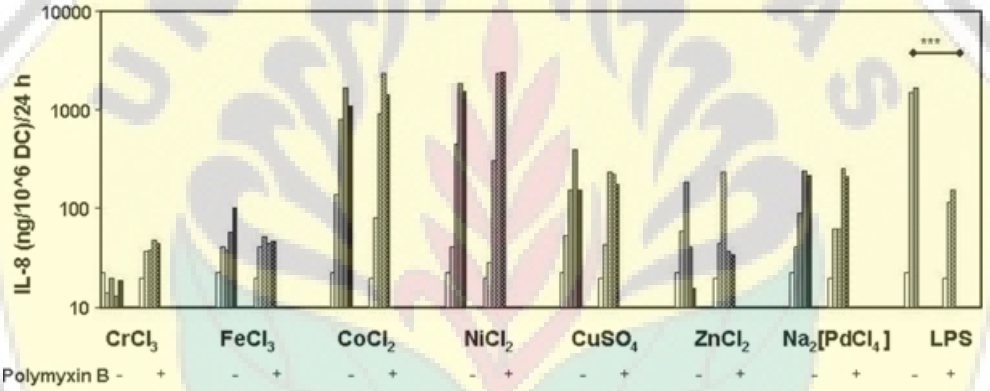

Fig. 3. Profile of interleukin (IL)-8 secretion after exposure of monocyte-derived dendritic cells to culture medium only (open bar for each bar cluster) or increasing concentrations of metals (from left to right: 125, 250, 500 and 750 μM) for 24 hr, with or without polymyxin B sulfate. Representative results are shown from three independent experiments (note logarithmic scale). For lipopolysaccharide (LPS), Wilcoxon paired test: $^{***}p < 0.001$. DC, dendritic cell.

Salt effects in transition metal-induced MoDC activation

The capacity of metal ions to act as stimulatory ligands in MoDC activation is assumed to depend on the anions that are set free from the dissolved salts. With $NiCl_2$ and $NiSO_4$ being used in both *in vitro* and clinical *in vivo* studies on nickel allergy, we checked whether both salts were similarly effective in direct MoDC stimulation. This did indeed appear to be the case, as results from six independent experiments showed similar IL-8 production induced by both nickel salts (data not shown).

$PdCl_2$ is commonly used for skin testing for palladium allergies, as are observed frequently in dental patients. Recently, we obtained evidence from clinical skin test studies and from *in vitro* lymphocyte proliferation testing that the use of this salt is not optimal (4, 13, 14), probably because it generates multimolecular complexes leading to low free ion concentrations. In contrast, the related tetrachloride salt, $Na_2[PdCl_4]$, was found to be much more effective in this regard. For this reason, we included palladium as the tetrachloride from the onset. When we compared both salts for their capacity to activate MoDCs, the latter salt, as expected, caused markedly increased IL-8 release (Fig. 4).

Finally, given the clinical relevance of chromium allergies and the lack of noticeable activation by $CrCl_3$, we also compared both $CrCl_3$ and $K_2Cr_2O_7$ for their respective MoDC stimulatory activities. In fact, the latter salt showed no superior activity as compared with $CrCl_3$ (data not shown).

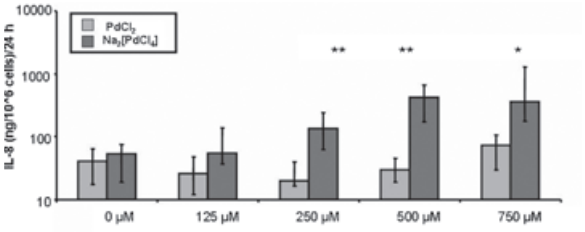

Fig. 4. Interleukin (IL)-8 production after exposure of monocyte-derived dendritic cells (MoDCs) to $PdCl_2$ or $Na_2[PdCl_4]$. Immature MoDCs were exposed to increasing concentrations of both palladium salts for 24 hr. Open bar: MoDCs with culture medium. Light grey bar: $PdCl_2$. Dark grey bar: $Na_2[PdCl_4]$. Values are median [P 25–75] from six independent experiments (n = 3 donors). Asterisks specify statistically significant differences in the production of IL-8 between the two salts (Wilcoxon, paired test: $^{*}p < 0.05$, $^{**}p < 0.01$). DC, dendritic cell.

Induction of IL-8 secretion in HEK293 TLR4/MD2 transfectant cells by different metals

In earlier studies, the remarkable MoDC-activating capacity of nickel could be explained by its unique binding to distinct histidine residues in TLR4 receptor molecules. We therefore decided to study whether and to what extent the stimulatory capacity of cobalt, and, to a lesser extent, of palladium, copper, and zinc, also might relate to direct TLR4 triggering. To this end, the TLR4-transfected HEK293 TLR4/MD2 cell line was used, which also allowed for IL-8 release as the primary read-out for downstream signalling (12). Indeed, next to the primary positive control LPS, nickel induced a strong activational signal in this transfectant cell line, which was almost equalled by cobalt. Again, $CrCl_3$ and $FeCl_3$ were negative, and zinc and copper showed marginal and weak signs of activation respectively. Interestingly, palladium was similarly active in the TLR4 transfectant as both positive controls, LPS and nickel (Fig. 5). In order to verify that the observed effects were caused by the presence of TLR4/MD2, the experiments were extended with wild-type, non-transfectant HEK293 cells. Indeed, none of the metals induced detectable IL-8 release, whereas, surprisingly, copper caused a robust response that was consistently higher than in the TLR4/MD2 transfectant cells (Fig. 6).

Discussion

With the recent unmasking of nickel as a potent stimulatory TLR4 ligand, greater understanding was obtained regarding its unique position as the most prominent contact sensitizer in all continents (5). However, within the panel of transition metals of the periodic table, nickel is not unique as a sensitizer. In fact, neighbouring elements are also notorious for their sensitizing capacities, such as cobalt, palladium and, to a lesser extent, copper. Cobalt is often associated with nickel, both in nature and in products such as metal alloys, ceramics, and paints. Palladium exposure is more rare, but palladium is frequently used in dental appliances. Copper is an abundant element, and is poisonous to higher organisms, but, at lower concentrations, is an essential trace nutrient for all animal life. For all three metals, allergic contact hypersensitivity can develop. Previously, we reported that this may relate to molecular cross-reactivity with nickel at the T cell-recognition level. However, the low clinical relevance of copper allergies may be explained by its presence as an essential nutrient potentially leading to peripheral tolerance of copper-specific or nickel–copper cross-reactive clones. Cross-reactivity might also occur at the primary level of sensitization, that is, in activation of dendritic, allergen-presenting cells. Indeed, the results of this study show that, like nickel, cobalt can activate MoDCs, and palladium, copper, and zinc, but not iron and chromium, showed low but distinct MoDC-activating potential. Essentially similar results were obtained in the HEK293 TLR4/MD2 transfectant cell line, but for copper (see below), confirming a critical role of TLR4 in this process. The involvement of endotoxin contamination in MoDC activation was excluded by LAL assays and consistent stimulation in the presence of polymyxin B.

As well as studying distinct metals, we also tested different salts. Given the use of nickel chloride and sulfate salts for both *in vivo* and *in vitro* studies, we expected there to be no difference in DC stimulatory activity, and this was found to be the case. In contrast, for palladium, complex formation and related solubility issues have been described. As compared with the nickel salts, the widely used skin test salt $PdCl_2$ is nearly insoluble in water, and, if it dissolves, it forms oligonucleotide or polynucleotide molecules (4, 15). In contrast, the very soluble $Na_2[PdCl_4]$, which also contains bivalent palladium, was found to be highly suitable for both *in vivo* (skin testing) and *in vitro* assays for the detection of palladium allergy. The present results confirmed our hypothesis that the tetrapalladate salt is distinctly more effective than the regular chloride (13, 14). It is of note that the marked stimulatory activity of palladium was most visible when the TLR4 transfectant cell line was used. Whether the lower reactivity of cultured DCs might result from additional interactions with other unidentified receptors, causing suppression of signalling in those cells, warrants further investigation. With regard to chromium, surprisingly, neither the dichromate nor chloride salts

caused discernable activation. Some positive stimulation observed for the trichloride in earlier experiments might have been caused by endotoxin contamination, which was not checked at the time (16). As costimulatory danger signals, such as those created by TLR4 triggering, are assumed to function as adjuvants in promoting the development of contact allergies, the conclusion seems warranted that clinical chromium sensitization may rather depend on external, for example bacterial, cofactors. Alternatively, internal cofactors acting as TLR ligands may contribute, such as hyaluronic acid fragments created by oxidative breakdown (6).

Importantly, cobalt was found to almost equal nickel in its DC-activating capacity, in line with earlier findings obtained with an IL-6 release assay (17). Although, at this stage, it is unknown whether other TLR receptors, or immune sensors of the NLR, RLR or CLR series (18), may contribute to the direct stimulatory activity of cobalt, the effect could certainly be attributed to direct TLR4 triggering, as shown by its similar efficacy in the TLR4/MD2 transfectant cell line. A very recent report by Raghavan et al. (19) confirms this view, and makes clear that, in contrast to LPS, both nickel and cobalt can induce TLR4 dimerization and signalling independently of MD2. Interestingly, only incidental cross-reactivity between nickel and cobalt has been observed at the T cell clonal level (7). Nevertheless, apparently both metals share the capacity to productively ligate with TLR4. For nickel, this binding was shown to depend on binding to two histidine residues of the TLR4 molecule (5). Given

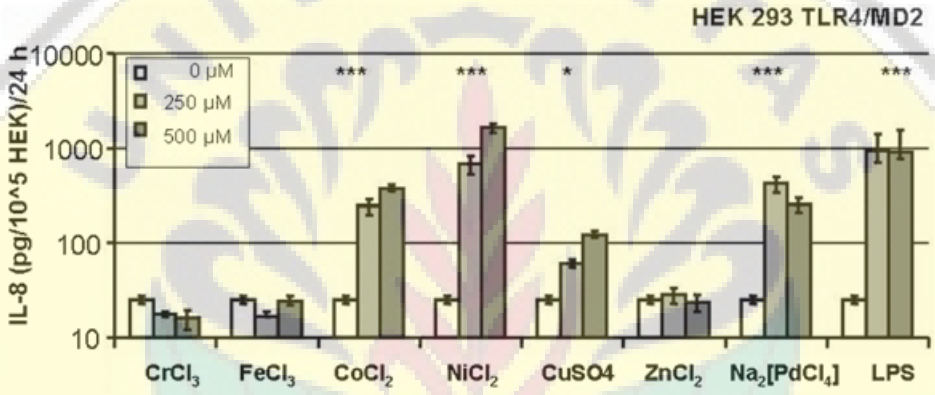

Fig. 5. Profile of interleukin (IL)-8 secretion after exposure of HEK293 TLR4/MD2 transfectant cells to a panel of transition metals. Cells were stimulated by two concentrations of metal (250 and 500 µM) and lipopolysaccharide (LPS) (50 and 100 ng/ml) (light and dark grey bars, respectively), and harvested after 24 hr. Representative results are shown from three independent experiments (values are mean ± standard error of the mean). Open bar: HEK293 TLR4/MD2 with culture medium. For statistical analysis, the highest dose values were compared with the medium control (Wilcoxon paired test: $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$).

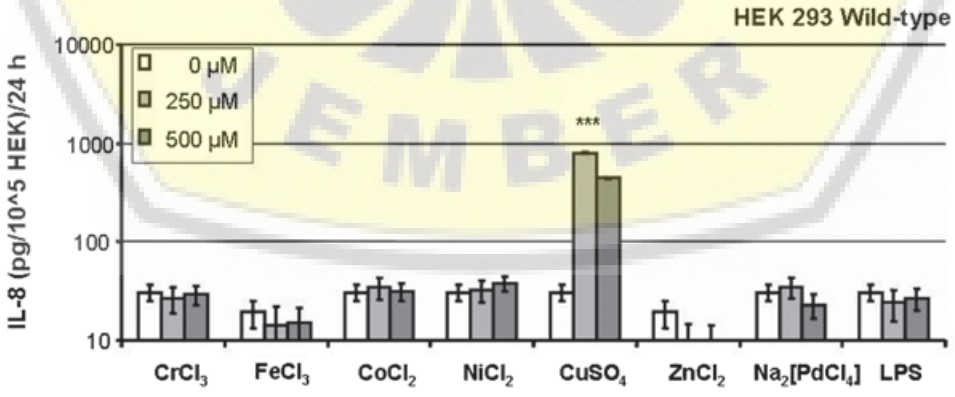

Fig. 6. Interleukin (IL-8) secretion after exposure of wild-type HEK293 cells to a panel of transition metals and lipopolysaccharide (LPS). Wild-type HEK293 cells were exposed to metals (250 and 500 µM) and LPS (50 and 100 ng/ml) (light and dark grey bars, respectively) for 24 hr. Values are means of two independent experiments (mean ± standard error of the mean). Open bar: HEK293 wild type with culture medium. For statistical analysis, the highest dose values were compared with the medium control (Wilcoxon paired test: $^{***}p < 0.001$).

the similar physical properties of cobalt, including two electrons in its outer shell, this metal most likely also allows for coordination binding to histidine-imidazole groups. Indeed, like nickel-based chelating resins, cobalt-based resins are in widespread laboratory use for the purification of histidine-tagged proteins. Moreover, within the list of top sensitizers in humans, cobalt (5.3%) is the next highest after nickel (17.6%). As it has similar intrinsic adjuvant properties, cobalt's lower clinical relevance may relate to its lower level of release from skin-contacting alloys, lower frequencies of cognate T cells, and/or more stringent silencing of metal-reactive T cells by regulatory T cells (6). The last of these mechanisms is favoured by chronic exposure, and has long been shown to be operative in nickel allergy (20), but is still unexplored in cobalt allergy.

Low but distinct DC activation was found for copper, but this could not be ascribed to TLR4 signalling, as the TLR4-negative HEK293 wild-type cells showed even higher IL-8 release. Further experiments to check the involvement of an unidentified native receptor on human HEK293 cells are warranted. Like copper, zinc is a very common element, and is a vital element for human health. Zinc ions are known to play pleiotropic roles in DCs, leading to activation or inhibition, for example through direct interaction with protein kinase C or IRAK-1, respectively, of TLR signalling pathways (21). In line with these mutually counteracting effects, only modest effects of this metal were seen. Despite its abundance, zinc allergy is not a clinically frequent or robust finding. Actually, the same holds true for iron, for which we could not find any direct danger signalling capacity, and which is also not known as a potential contact allergen.

In aggregate, evidence was obtained for a more widespread capacity of transition metals to trigger the TLR4 signalling pathway. The resulting generation of pro-inflammatory mediators such as IL-8 creates so-called danger signals, which clinically can contribute to local inflammation and to the expansion of metal-specific T cells, if present. The results obtained shed further light on the development of clinical contact allergies, and may also contribute to the development of novel treatments, such as those based on interfering with distinct immune sensor pathways.

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