CHAPTER 2

TRANSITION METAL SENSING BY TOLL-LIKE RECEPTOR-4: NEXT TO NICKEL, COBALT AND PALLADIUM ARE POTENT HUMAN DENDRITIC CELL STIMULATORS

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ABSTRACT

Background: Nickel was recently identified as a potent activator of dendritic cells (DC) through ligation with human TLR4.

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 (MoDC).

Methods: The panel included chromium, cobalt, copper and palladium, all known as frequent clinical sensitizers. MoDC activation was monitored by assessment of release of the pro-inflammatory mediator IL-8, a major downstream result from TLR ligation.

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

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

Metals are amongst the most notorious contact sensizers clinically known. This counts in particular for the transition metals nickel, cobalt and chromium. In dentistry also palladium has obtained a bad reputation as a frequent cause of oral allergic complaints (Uter et al. 2009; Peiser et al. 2012; Muris et al. 2009; Faurschou et al. 2011).

Recently it was discovered that nickel ions are able to directly ligate and trigger Toll-like receptor-4 (TLR4) on dendritic cells (DC) (Schmidt et al. 2010). Evolutionarily, these receptors developed to respond to bacterial molecular signals, such as LPS/endotoxin. Downstream of this signalling pathway pro-inflammatory mediators are released, such as IL-8, IL-1β and TNFα (Martin et al. 2011). These mediators contribute to both rapidly-acting innate immune responses and adaptive immunity in 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 virtually invisible for 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 high exposure rates, the direct generation of a strong danger signal by this metal to DC most likely contributes to the first place of this metal on the list of clinically relevant contact allergens.

Previously, we and others reported on frequent cross-reactivities between nickel and the neighbouring transition metals copper and palladium at the T cell recognition level (Pistoor et al. 1995; Moulon et al. 1995). 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 NF-κB leading to downstream IL-8 release. Involvement of direct TLR4 triggering was studied using a TLR4-MD2 transfectant cell line. The results show that, similar to nickel, especially palladium and cobalt have the capacity to trigger TLR4.

MATERIALS AND METHODS

Metal Chemicals

As metal allergens the following chemicals were used: nickel sulphate hexahydrate (NiSO₄, Merck, Darmstadt, Germany), nickel (II) chloride hexahydrate (NiCl₂·6H₂O), chromium (III) chloride hexahydrate (CrCl₃·6H₂O), potassium dichromate (K₂Cr₂O₇), cobalt (II) chloride hexahydrate (CoCl₂·6H₂O), copper (II) sulfate (CuSO₄), iron (III) chloride (FeCl₃), zinc chloride (ZnCl₂) (all from Fluka/Riedel de Haën, Seelze, Germany), sodium tetrachloro-palladate (II) (Na₂[PdCl₄], Sigma), palladium chloride (PdCl₂, Sigma-Aldrich Chemie GmbH, Steinheim,
Peripheral blood mononuclear cell (PBMC) isolation and culture of MoDC

PBMCs were isolated from 40-50 ml freshly drawn peripheral venous blood of 6 different healthy donors without known metal allergies by Ficoll (Lymphoprep, Fresenius KabiNorge 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. MoDC were generated as previously described (Bontkes et al. 2002). Briefly, adherent monocytes were then cultured for 6-7 days in the humidified incubator in 10 ml IMDM medium supplemented with 10% FCS, 1% penicillin-streptomycin, 1% L-glutamine, and 1% β-mercaptoethanol, 1000 U/ml granulocyte-macrophage colony stimulating factor (GM-CSF Novartis, The Netherlands) and 20 ng/ml IL-4 (lot AG270911A: R&D systems Europe, Abingdon, UK). After 6-7 days, immature dendritic cells (iDCs) were harvested and plated in 96 well flat tissue culture plates (Cellstar Greiner Bio-One, Frickenhausen, Germany) at approximately 5x10^4 cells per well.

Metal toxicity experiments

In order to design appropriate concentration ranges of metals, cytotoxicities were measured by the MTT reduction test (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide). Approximately 100 µl of cells (5x10^4/ml) were plated in 96 well culture plate and exposed to increasing concentrations of the metals. After 24 hours incubation culture medium was removed and 50 µl of MTT solution (5mg/ml) was added per well. MTT solution was prepared freshly and dissolved with H2O, filtered through a 0.22-µm filter. The plates were incubated in the dark at 37°C. After 2-3 hours of incubation, 50 µl DMSO (Merck, Darmstad, Germany) was added to each well and after shaking, the solution was measured using ELISA reader at OD (optical density) 450 nm. The OD of the cells in the absence of metal was considered as 100%. Viabilities of exposed cells were determined by the formula: OD experimental sample/OD of control cells) x 100%.

Metals and LPS exposure

LPS was tested at 15 and 50 ng/ml. Total volume in each well was 200 µl. iDC(5x10^4 cells/well) were exposed to metals at concentrations between 0 and 750 µM. Plates were incubated at 37°C in 5% humidified CO2. After 24 hours, supernatants were collected and stored at -20° until measurement. Where indicated polymyxin B sulphate (25 µg/ml; Sigma-Aldrich, Inc St Louis USA) 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 (Loutet et al. 2011; Roelofs et al. 2006). Complementary checks for LPS contamination were
carried out with the *Limulus amebocyte lysate* (LAL) assays (Kinetic-QCLTM bulk kit, Lonza, Cologne, Germany).

**Assessment of TLR4 signalling with TLR4 transfectant cells**

Human Embryonic Kidney (HEK) 293 TLR4/MD2 cells were cultured in T75 flasks in DMEM, 1% Glutamine, 1% pen/strep, 0.5 μg/ml G418 and harvested upon confluence. Wild type HEK293 and HEK293 cells stably expressing human TLR4 and MD2 were a kind gift from D.T. Golenbock (University of Massachusetts Medical School, USA) to Y. van Kooyk/M. Verstege. Cells were split twice a week until ready for use (Kuijf et al. 2010). Cells were plated at 1x10⁵ cells/well in 100 μl medium in a 96-wells flat bottom plates. After allowing cells to adhere for 1.5 to 2 hours, 100 μl of metal salt solution was added to give final concentrations of 0, 250 and 500 μM. Cells were incubated for 24 hours at 37°C, and supernatants were harvested for IL-8 ELISA.

**Cytokine production**

IL-8 production was measured by Enzyme-linked immunosorbent assay (ELISA) with a Peli-Kine ELISA kit for human IL-8 (Sanquin, Amsterdam, The Netherlands) using 96-well microtiter plates (Nunc maxisorp microtiter plates, Nalge Nunc International, Roskilde, Denmark), as per 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 ml.

**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 computer program MedCalc (Mariakerke, Belgium). P≤ 0.05 was considered statistically significant. All data are presented as median (P25-75) and mean ± standard error of the mean.

**RESULTS**

**Cytotoxic effects of selected transitional metals on MoDCs**

Transition metals to be studied for their potential stimulatory effects on DC were first tested to define the testing range and maximal non-toxic concentrations. Cytotoxicity experiments were carried out using MTT reduction assays as a read-out. After exposing MoDC for 24 hrs to increasing dosages of CrCl₃, FeCl₃, CoCl₂, NiCl₂, CuSO₄ and Na₂[PdCl₄], concentration ranges between 0 – 750 μM were found to be appropriate. Cobalt and copper salts showed highest toxicities, but up to 500 μM no viability reductions were observed exceeding 25% for any metal of the panel (Figure 1).
Transition metal–induced MoDC activation as detected by IL-8 secretion

Subsequently, the selected transition metals were studied for their capacity to activate MoDC, as detected by IL-8 release. As positive controls, nickel chloride and LPS were included, since both compounds have been shown earlier to activate MoDC through direct TLR4 triggering. Dose-responses are given for all metals tested (Figure 2). The results show that, next to NiCl₂, CoCl₂ stands out for its strong capacity to activate MoDC. Next, copper, zinc and palladium salts induced much lower, but still significant levels of IL-8, whereas CrCl₃ and FeCl₃ only induced non-significant levels of IL-8 at the highest test concentrations.

Transition metal-induced MoDC activation is not due to contamination with LPS

To exclude possible involvement of endotoxin contamination in the 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, active as an antibiotic for gram-negative bacteria. Its binding properties to LPS/endotoxin can also be exploited to clear endotoxin contamination. Dose-response data are presented in Fig 3 showing that, as expected, addition of polymyxin B causes at least 90% reduction of 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 was intrinsic to metal-properties. Complementary negative LAL assays confirmed the exclusion of a role for LPS in these findings (data not shown).
Figure 2. Profile of IL-8 secretion after exposure of immature MoDCs to selected transition metal salts for 24 hours. Supernatants were harvested after exposure to medium only (open bars) or increasing concentrations of metal salts (grey till black bars 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 by median (P 25-75) for at least 6 donors in 3 different individual experiments. For statistical analysis the 500μM values were compared to the medium control (Wilcoxon paired *p<0.05, **p<0.01 and ***p<0.001).

Figure 3. Profile of IL-8 secretion after exposure of MoDCs 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 hours with or without polymyxin B sulphate. Representative results are shown from 3 independent experiments (please note logarithmic scale). For LPS Wilcoxon paired test: ***p<0.001.

Salt-effects in transition metal-induced MoDCs activation.

The capacity of metal-ions to act as stimulatory ligands in MoDC activation, is assumed to depend on the anions set free from the dissolved salts. With NiCl₂ and NiSO₄ 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 6 independent experiments showed similar IL-8 production induced by both nickel salts (data not shown).

PdCl\textsubscript{2} is commonly used for skin testing palladium allergies, as are observed frequently in dental patients. Recently, we obtained evidence from clinical skin test studies as well as from \textit{in vitro} lymphocyte proliferation testing, that the use of this salt is not optimal (Muris et al. 2012; Muris et al. 2009), probably because it generates multi-molecular complexes leading to low free ionic concentrations. In contrast, the related tetrachloride salt, Na\textsubscript{2}[PdCl\textsubscript{4}] was found to be much more effective in this regard. For this reason, we had included palladium as the tetrachloride from the onset. When comparing both salts in their capacity to activate MoDC, as expected the latter salt showed markedly increased IL-8 release (Figure 4).

Finally, given the clinical relevance of chromium allergies and the lack of noticeable activation by CrCl\textsubscript{3}, we also compared both CrCl\textsubscript{3} and K\textsubscript{2}Cr\textsubscript{2}O\textsubscript{7} for their respective MoDC stimulatory activities. In fact the latter salt showed no superior activity as compared to CrCl\textsubscript{3} (data not shown).

**Figure 4.** IL-8 production after exposure of MoDCs with PdCl\textsubscript{2} or Na\textsubscript{2}[PdCl\textsubscript{4}]. Immature MoDCs were exposed to increasing concentrations of both palladium salts for 24 hours. Open bar: MoDCs with culture medium; grey bar: PdCl\textsubscript{2}; black grey bar: Na\textsubscript{2}[PdCl\textsubscript{4}]. Values are median [P 25 - 75] from six independent experiments (n = 3 donors). Asterisks specify statistically significant differences in production of IL-8 between the two salts (Wilcoxon, paired test: *p<0.05, **p<0.01)

**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 down-stream signalling. 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, CrCl₃ and FeCl₃ were negative, and copper and zinc showed marginal signs of activation. Interestingly, palladium was similarly active in the TLR4 transfectants both positive controls, LPS and nickel (Figure 5). In order to verify that the observed effects were due to 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 showed a robust response which was consistently higher than in the TLR4/MD2 transfectant cells (Figure 6).

**DISCUSSION**

With the recent unmasking of nickel as a potent stimulatory TLR4 ligand, better understanding was generated on its unique position as number one contact sensitizer in all continents (Schmidt et al. 2010). Still, 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 frequently used in dental appliances. Copper is an abundant element, poisonous to higher organisms but at lower concentrations an essential trace nutrient to all animal life. For all three metals allergic contact hypersensitivity can develop. Earlier we reported that this may relate to molecular cross-reactivities with nickel at the T cell-recognition level. Still, 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. Anyhow, cross-reactivities might also occur at the primary level of sensitization, i.e. in activation of dendritic, allergen-presenting cells. Indeed, the results of this study show that, like nickel, also cobalt can activate MoDC, whereas 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. Involvement of endotoxin contamination in MoDC activation was excluded by LAL assays and consistent stimulation also in the presence of polymyxin B.

As well as with studying distinct metals we also tested different salts. Given the usage of both nickel chloride and sulphate salts for both in vivo and in vitro studies, we anticipated no difference in DC stimulatory activity, which in fact was found to be the case. In contrast, for palladium complex-formation and related solubility issues have been described. Compared
to the nickel salts, the widely used skin test salt PdCl$_2$ is nearly insoluble in water, and if it dissolves it forms oligo- or polynucleotide molecules (Muris et al. 2009; Muris et al. 2012). In contrast, the well soluble Na$_2$[PdCl$_4$], also containing 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 was distinctly more effective than the regular chloride (Muris et al. 2012; Muris et al. 2008). Of note, the marked stimulatory activity of palladium was most visible from using the TLR4 transfectant cell line. Whether the lower reactivity of cultured DC might result from additional interaction with other unidentified receptors causing a suppressive role in 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 due to endotoxin contamination, which was not checked at the time (Toebak et al. 2006). As costimulatory danger signals, such as 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 co-factors. Alternatively, internal cofactors acting as TLR ligands may contribute, like hyaluronic acid fragments created by oxidative breakdown (Martin et al. 2011).

Importantly, cobalt was found to almost equal nickel in its DC activating capacity, in line with earlier findings using an IL-6 release assay (Antonios et al. 2009). Although at this stage it is unknown whether other TLR receptors, or immune sensors of the NLR, RLR or CLR series (Bax et al. 2011), 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 (Raghavan et al. 2012) confirms this view, and made clear that, in contrast to LPS, both nickel and cobalt can induce TLR4 dimerization and signalling independent from MD2. Interestingly, only incidental cross-reactivity between nickel and cobalt has been observed at the T cell clonal level (Pistoor et al. 1995). 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 (Schmidt et al. 2010). Given the similar physical properties of cobalt, including 2 electrons in its outer shell, this metal most likely also allows for coordination binding to histidine-imidazole groups. Indeed, like nickel-based chelating resins, also cobalt-based resins are in widespread laboratory use for purification of histidine-tagged proteins. Moreover, within the list of top-sensitizers in man, after nickel (17.6%), cobalt (5.3%) is the first metal to appear high on the list. With similar intrinsic adjuvanticity, cobalt’s lower clinical relevance may relate to its lower 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. The latter mechanism is favoured by chronic exposure and has long
been shown to be operative in nickel allergy (Cavani 2008), but is still unexplored in cobalt allergy.

Figure 5. Profile of IL-8 secretion after exposure of HEK293 TLR4/MD2 transfectant cells to panel of transition metals. Cells were stimulated by two concentrations of metals (250 and 500 μM) and LPS (50 and 100 ng/ml), light and dark grey bars respectively, and harvested after 24 hrs. Figure shows representative result from 3 independent experiments (values are mean ± SEM). Open bar = HEK293 TLR4/MD2 with culture medium; For statistical analysis the highest dose values were compared to the medium control (Wilcoxon paired **p<0.01 and ***p<0.001).

Figure 6. IL-8 secretion after exposure of Wild-Type HEK 293 cells to transition metal panel and 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 hours. Values are means of two independent experiments (mean ± SEM). Open bar: HEK293 Wild type with culture medium. For statistical analysis the highest dose values were compared to the medium control (Wilcoxon paired ***p<0.001).

Low but distinct DC activation was found for copper which, however, could not be ascribed to TLR4 signaling since the TLR4 negative HEK293 Wild type cells showed an even higher IL-8
release. Further experiments to check involvement of an unidentified native receptor on human HEK293 cells are warranted. Like copper, zinc is a very common element and another vital element for human health. Zinc ions are known to play pleiotropic roles in dendritic cells, leading to activation or inhibition, e.g. through direct interaction with protein kinase C or IRAK-1 respectively, of TLR signalling pathways (Haase and Rink 2007). In line with these mutually counteracting effects, here 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 discern any direct danger signalling capacity, and which metal 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 like 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 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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