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Metal ions potentiate microglia responsiveness to endotoxin

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ABSTRACT

Oral metal exposure has been associated with diverse adverse reactions, including neurotoxicity. We showed previously that dentally applied metals activate dendritic cells (MoDC) via TLR4 (Ni, Co, Pd) and TLR3 (Au). It is still unknown whether the low levels of dental metals reaching the brain can trigger local innate cells or prime them to become more responsive.

Here we tested whether dentally applied metals (Cr, Fe, Co, Ni, Cu, Zn, Au, Hg) activate primary human microglia in vitro and, as a model, monocytic THP-1-cells, in high non-toxic as well as near-physiological concentrations. In addition the effects of 'near-physiological' metal exposure on endotoxin (LPS) responsiveness of these cells were evaluated. IL-8 and IL-6 production after 24 h was used as read out.

In high, non-toxic concentrations all transition metals except Cr induced IL-8 and IL-6 production in microglia, with Ni and Co providing the strongest stimulation. When using near-physiological doses (up to 10× the normal plasma concentration), only Zn and Cu induced significant IL-8 production. Of note, the latter metals also markedly potentiated LPS responsiveness of microglia and THP-1 cells.

In conclusion, transition metals activate microglia similar to MoDCs. In near-physiological concentrations Zn and Cu are the most effective mediators of innate immune activation. A clear synergism between innate responses to Zn/Cu and LPS was observed, shedding new light on the possible relation between oral metal exposure and neurotoxicity.

1. Introduction

The use of metal alloys for dental reconstructions in the oral cavity is a contentious issue since local and systemic increased levels of metal ions (Milheiro et al., 2014; Muris et al., 2014) have been associated with neurodegenerative and other neurological diseases such as migraine (Shcherbatykh and Carpenter, 2007; Giacoppo et al., 2014; Dusek et al., 2014; Rosenberg et al., 2013). Little is known about how metal ions act locally and the precise mechanisms by which they contribute to central nervous system disorders have not yet been elucidated. Concentrations of various transition metals were reported to be increased in the cerebrospinal fluid (CSF) from patients with amyotrophic lateral sclerosis, Alzheimer's and Parkinson's disease (Roos et al., 2013; Hozumi et al., 2011). In particular Cu and Zn have been suggested to play important roles in the onset and progression of neurodegeneration (Giacoppo et al., 2014; Singla and Dhawan, 2014). Less information is available regarding the neurotoxic effects of Ni and Au (Pedersen et al., 2014; Kicinski et al., 2015). For Hg neurotoxic effects, in particular those manifested as neuropsychological complaints of patients exposed to amalgam, have been reported (Mutter, 2011; Carocci et al., 2014), although conclusive evidence of negative health effects of amalgam fillings, the most important source of Hg release, is lacking (Roberts and Charlton, 2009).

Pathogenic effects of transition metals could result from their ability to form complexes with peptides and proteins, thereby contributing to for example amyloid depositions, which may subsequently cause

Abbreviations: CD, cluster of differentiation; $CoCl_2$, cobalt (II) chloride; $CrCl_3$, chromium (III) chloride; $CuSO_4$, copper (II) sulphate; CSF, cerebrospinal fluid; CNS, central nervous system; DC, dendritic cell(s); DMEM, Dulbecco's modified Eagles' medium; DMSO, dimethylsulphoxide; EDTA, ethylene-diamine-tetraacetic acid; ELISA, enzyme-linked immunosorbent assay; FACS, fluorescence-activated cell scan; FCS, foetal calf serum; $FeCl_3$, iron (III) chloride; GM-CSF, granulocyte–macrophage colony stimulating factor; $HgCl_2$, mercuric (II) chloride; IL, interleukin; LPS, lipopolysaccharide; MS, multiple sclerosis; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; MoDC, monocyte-derived dendritic cells; MW, molecular weight; $NiCl_2$, nickel (II) chloride; $Na_3Au(S_2O_3)_2.2H_2O$, sodium gold thiosulfate; OD, optical density; PBS, phosphate-buffered saline; RPMI, Roswell Park Memorial Institute; TLR, toll like receptor; $ZnCl_2$, zinc (II) chloride.

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oxidative stress, inflammation and neurotoxicity (Huang et al., 2004; Shcherbatykh and Carpenter, 2007). More recently, several metals, i.e. Zn, Mn and Co, were shown to directly induce the production of inflammatory mediators by microglia, the most important innate immune cells of the CNS (Dusek et al., 2014; Kauppinen et al., 2008; Mou et al., 2012). Moreover, excessive exposure to Mn and Co was shown to potentiate the release of inflammatory mediators induced by endotoxin (Dodd and Filipov, 2011; Mou et al., 2012). Indeed, inflammatory mediators generated by innate immune CNS cells such as microglia are considered the major culprits in neurodegenerative diseases (Lehnardt, 2010; Rosenberger et al., 2014; Amor et al., 2014) and new therapeutic strategies focus on the modulation of the inflammatory response by microglia (Fernandez et al., 2013; Hines et al., 2013).

Our previous research investigated the stimulatory capacity of dentally applied metals on innate immune cells (human monocyte derived dendritic cells, myelo-monocytic cell lines and TLR transfected cell lines) as assessed by the release of pro-inflammatory mediators including IL-8 (Rachmawati et al., 2013, 2015). In these studies Ni, Co and Pd were shown to induce IL-8 production via TLR4 binding. Au predominantly triggered TLR3, whereas Cu and Hg activated innate cells via thus far unidentified mechanisms. This activation of innate immune responses was, however, observed upon stimulation with relatively high, supra-physiologic, yet non-toxic concentrations of metal salts. Although the release of metal salts from dental restorations are increased due to corrosion for example (Chen et al., 2013; Matusiewicz, 2014), levels in plasma and cerebrospinal fluid (CSF) are generally lower than reported in our previous in vitro studies. Useful data on such physiologic concentrations has recently become available (Roos et al., 2013; Matusiewicz, 2014), but few studies evaluated the immunotoxicity of these concentrations using innate immune cells such as microglia (Mou et al., 2012; Wataha, 2000).

In the present study we focused on the direct response of primary human microglia following exposure to dentally relevant metals (Cr, Fe, Ni, Co, Cu, Zn, Pd, Au and Hg (Al-Hiyasat et al., 2002; Elshahawy et al., 2009; Matusiewicz, 2014)) in maximal non-toxic as well as 'physiological' concentrations. In addition to primary human microglia, the monocytoidr cell line THP-1 was explored as a model for microglia activation (Klegeris et al., 2007; Hendrickx et al., 2014).

Since the concept of microglial priming (i.e. alerting the cells to become more responsive to subsequent stimuli) is widely considered to be an important step in the development of neurodegenerative diseases (Perry and Holmes, 2014) we examined whether metal exposure of microglia and THP-1 cells potentiates their responsiveness to bacterial lipopolysaccharide (LPS), the most prominent microbial stimulatory ligand and relevant in the context of oral infections (Amor et al., 2014).

Here we show that such synergy between exposure to metal and LPS does exist, at least for Cu and Zn indicating that such combinations may contribute to or augment chronic inflammation and neurotoxicity in humans.

2. Materials and methods

2.1. THP-1 cells

THP-1 cells (passage 17; ATCC, Rockville, USA) were cultured in 100 ml flasks (Cellstar Greiner Bio-One) at a density of 1.10^6 cells/ml in RPMI 1640 medium (Biowhittaker, Verviers, Belgium) containing 2 mM L-glutamine (Merck, Darmstadt, Germany), 0.1 mg/ml streptomycin (Invitrogen), 100 IU/ml penicillin (Invitrogen) and 10% heated-inactivated foetal calf serum (FCS; Hyclone, Logan USA). The THP-1 cells were maintained in logarithmic growth by passaging every 3–4 days (1.10^6 cells/ml). For metal exposure the THP-1 cells were seeded in a 96 wells plate (flat bottom, Greiner Bio-One) in a concentration of 5.10^4 cells per well for 24 h with different concentrations of metal salts in a final volume of 200 μl.

2.2. Primary human microglia

Postmortem human brain tissue was obtained according to the protocol of The Netherlands Brain Bank (Amsterdam, The Netherlands), in agreement with the Medical Ethical Committee of the VU University Medical Center (Amsterdam, The Netherlands) (Peferoen et al., 2015). All patients had provided written informed consent for autopsy, the use of their brain tissue and clinical details for research purposes. At autopsy, 10 to 15 g of brain white matter was collected in 30 ml of Dulbecco modified Eagle medium, Ham's nutrient mixture F10 (DMEM; Invitrogen), and 1% (vol/vol) gentamicin (Invitrogen) and stored at 4 °C until further isolation procedures. Microglia were isolated from 10 patients with various neuro-degenerative diseases (Table 1). Isolation procedures were performed as previously described and validated by assessment of the purity of microglia by FACS analysis, showing more than 95% CD68 positive cells, expressing $CD11b^{high}$ and $CD45^{low}$ to distinguish them from macrophages ($CD11^{low}$ and $CD45^{high}$) (Peferoen et al., 2014). After isolation, microglial cells were maintained in 25 ml flasks in DMEM, supplemented with 10% FCS, 1% L-glutamine, 1% β-mercaptoethanol and 1% streptomycin-penicillin. After culturing for 5 days at indicated doses, cells were detached using 0.01% ethylene-diamine-tetraacetic acid (EDTA; Biowhittaker, Verviers, Belgium) in phosphate buffered saline (PBS; Braun, Melsungen, Germany), and a cell scraper (Greiner Bio-One). Cells were counted (CASY®Cell-Counter + analyser system TT; Schärfe System) and seeded in a 96 wells plate (flat bottom, Greiner Bio-One; 5.0×10^4 cells/well). Upon one day of culturing, cells were exposed for 24 h to different concentrations of metal salts in a final volume of 200 μl.

2.3. Metal salts and LPS exposure

Microglia and THP-1 cells were exposed to maximum non-toxic concentrations of metal salts (Rachmawati et al., 2013, 2015), or exposed to physiological concentrations of metal salts. For stock solutions, metal salts obtained as analytical grade metal salts, purchased from Fluka/Riedel de Haen, Seelze, Germany except for $(Na_3Au(S_2O_3)_2.2H_2O$ were purchased from Chemotechnique Diagnostics, Vellinge, Sweden, were dissolved in distilled water and further dilutions were made in culture medium prior to cell culture. In the first experiment supra-physiologic concentrations were examined, i.e. for $CrCl_3$, $NiCl_2$, $CoCl_2$, $CuSO_4$, $ZnCl_2$, $Na_2(PdCl_4)$, $Na_3Au(S_2O_3)_2$: 750, 500 and 250 μM and for $HgCl_2$ 750, 500 and 250 nM. $FeCl_3$ was not tested in these high concentrations because of low solubility. In the second study physiological doses were tested of $CrCl_3$, $FeCl_3$, $NiCl_2$, $CoCl_2$, $CuSO_4$, $ZnCl_2$, $Na_3Au(S_2O_3)$ and $HgCl_2$. These concentrations were based on the maximum plasma concentrations of metals as determined by Roos and colleagues (Roos et al., 2013) (Table 2). In addition, 3-fold ('3× phys') and 10-fold ('10× phys') of these concentrations were used to mimic possible levels obtained locally i.e. orally. To mimic exposure to multiple metals as may be expected in the oral cavity or in plasma a 'metal mix' of 6–8 metal salts were made as 10×, 3× and 1× physiological plasma concentration (Table 2).

Supernatants were collected at 24 h after metal exposure. To evaluate the impact of metal exposure on LPS responsiveness, microglia and THP-1 cells were exposed to 'physiological' concentrations of metal salts or metal mixes in the absence or presence of LPS (50 ng/ml E.coli 055:B5; Sigma Aldrich, St. Louis, MO, USA).

2.4. MTT assay

To determine the viability of the microglia and THP-1 cells after 24 h of metal salt exposure, MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide; Sigma, St. Louis, MO, USA) assays were performed. MTT was freshly prepared by dissolving 7.5 mg/ml in water and filtering it through a 0.22 μm filter prior to addition of 50 μl MTT solution to each well. The 96-wells plate with THP-1 cells and

Table 1
Patient characteristics of microglia samples.

Sample	Age	Gender	Diagnosis	Cause of death	Post mortem delay (hours: minutes)
1[a]	57	m	Multiple system atrophy	Euthanasia	5: 45
2[a]	69	f	Alzheimer disease	Ileus	5: 45
3[a]	71	m	Huntington's disease	Euthanasia	4: 25
4[a]	86	m	Parkinson's disease	Euthanasia	4:10
5[a]	65	m	Fronto temporal dementia	Cachexia	5: 00
6[a]	73	f	Multiple system atrophy	General deterioration	2: 20
7[a]	57	m	Multiple sclerosis	Sepsis	10:15
8[a]	47	m	Fronto temporal dementia	Pneumonia	5: 25
9[a]	89	f	Alzheimer disease	Peritonitis	4: 30
10[b]	66	m	Multiple sclerosis	Euthanasia	10: 55

[a] Samples 1–9 were tested with high, non-toxic concentrations of metal salts (Table 3).
[b] Sample 10 was tested with physiologic (low) concentrations of metals (Figs. 1a, b, 2a).

MTT solution was incubated in the dark at 37 °C for 2–3 h. After incubation, 50 μl of DMSO (dimethylsulfoxide, Merck, Darmstad, Germany) was added to each well and the plate put on a shaker for 10–15 min at room temperature. An enzyme-linked immunosorbent assay (ELISA) reader with an optical density (OD) of 570 nm was used to determine the percentage viability of the cells i.e. OD of exposed cells/OD of unexposed cells) × 100%.

2.5. IL-8 and IL-6 evaluation

Interleukin-8 (IL-8) and IL-6 levels in the microglia and THP-1 culture supernatants were measured using ELISA kits (PeliKine kits (Sanquin, Amsterdam, The Netherlands) in 96-wells plates (Nunc maxisorp microtitre plates; Nalge Nunc International, Roskilde Denmark) according to the manufacturer's instructions. The absorbance was measured at 450 nm using an ELISA reader (BioTek, ELx808). A standard curve was used to measure the concentration of IL-8 and IL-6 with a lower detection limit of 15.4 pg/ml and 11.5 pg/ml respectively. The supernatants of the THP-1 cells were diluted 5 and 50 times and of the microglia cells 100 and 500 times before testing.

2.6. Flow cytometry

After 48 h of metal exposure or medium control THP-1 cells were washed in PBS containing 1% BSA. Cells were collected and 5.10^5 cells/ml stained to examine expression of CD40, CD80 and CD86. Briefly cells were incubated for 30 min in the dark at room temperature, with PE and APC-labelled mouse monoclonal antibodies: anti-human-CD40-PE, anti-human-CD80-PE and anti-human-CD86-APC (IgG_1, Becton Dickinson (BD) Pharmingen). Isotype controls assessing non-specific binding were monoclonal mouse IgG_1-APC and IgG_1-PE (Becton Dickinson (BD) Pharmingen). Flow cytometry was performed with BD-FACS Calibur and analysed using Cell Quest software.

Table 2
Physiologic metal concentrations and metal salts used.

Metal	Physiologic concentration of metal[a] (in μg/l, for Hg ng/l)		Metal salt used in vitro	Concentration used in vitro[b] '1× phys'[c]
	plasma	CSF		(in μM, for Hg in nM)
^{52}Cr	0.72	1.65	$CrCl_3.6H_2O$	0.014
^{56}Fe	1227	374	$FeCl_3$	21.9
^{59}Co	0.29	0.22	$CoCl_2.6H_2O$	0.005
^{59}Ni	12.99	18.4	$NiCl_2.6H_2O$	0.2
^{64}Cu	1912	35.3	$CuSO_4$	31.1
^{65}Zn	898	249	$ZnCl_2$	13.7
^{197}Au	0.23	0.01	$Na_3Au(S_2O_3)_2.2H_2O$	0.001
^{201}Hg	0.91	1.19	$HgCl_2$	0.005

[a] Maximal physiologic concentrations in plasma and CSF of controls according to (Roos et al., 2013).
[b] Based on maximal physiologic concentrations in plasma.
[c] 1× phys stands for the physiologic concentration of metals tested (Roos et al., 2013).

2.7. Data analysis

The statistical significance of the effects of various concentrations of metal salts on the cytokine secretion was analysed by using Kruskall–Wallis (nonparametric, one way ANOVA) and data presented as median and interquartile range (25th to 75th percentile), using Graphpad software (San Diego, California, USA). $P \leq 0.05$ was considered to be statistically significant.

3. Results

3.1. Metal salts induce microglia activation as detected by IL-8 secretion

To study the potential immunostimulatory effects of metal salts on human microglia, high, yet non-toxic concentrations were determined by examining the cell viability (≥80%) after 24 h of incubation with the metal (data not shown). This revealed that the concentrations were suitable for a panel of 8 metal salts: 0–750 μM for $CrCl_3$, $CoCl_2$, $NiCl_2$, $CuSO_4$, $ZnCl_2$, $Na_2(PdCl_4)$, $Na_3Au(S_2O_3)_2$ and a 1000-fold lower dose range (0–750 nM) for $HgCl_2$ (data not shown).

Human microglia samples from 9 donors were exposed to non-toxic concentrations of metals salts or to LPS as positive control, and IL-8 and IL-6 secretion determined in the supernatants after 24 h. The production of IL-6 paralleled that of IL-8 in all patients, except case 2, in which IL-6 levels were higher. Since no pattern emerged when considering the cytokine pattern per metal salt only the IL-8 data are given in Table 3. As expected all microglia samples reacted to LPS (Perry and Holmes, 2014). The responses to the other metals showed high variability. In agreement with our previous data no clear associations with the clinical background of the patients from which the microglia were derived were noted (Perry and Holmes, 2014). Despite a high baseline and high LPS induced production of IL-8 by microglia sample 6 responsiveness to the metal salts was not detected.

3.2. Microglia and THP-1 responses to near-physiologic metal concentrations

Since the metal concentrations in plasma and central nervous system tissue are expected to be much lower than those tested in the initial microglia experiments (Tables 2 and 3), additional exposure studies were performed with 'physiological' metal concentrations as established for plasma by Roos and colleagues (Roos et al., 2013). The metals were either given as a mix of 8 metals in 1-, 3- and 10-fold physiologic concentrations or as individual metal salts in a 10-fold physiologic concentration.

In addition, due to the low yield of human microglia, the monocytoid cell line THP-1 was used to examine the effects in repeated exposure experiments. THP-1 cells have been used previously for metal exposure

Table 3
IL-8 production[a)] by human microglia upon exposure to high, non-toxic concentrations of metal salts.

Microglia sample	1	2	3	4	5	6	7	8	9	10	% of responders[b]	Mean response[c]
Medium control[d]	7500	8400	12,500	10,000	650	27,728	7570	8406	8570	9660		
LPS[a]	+++	+++	+	+++	+++	+++	+	++	+++	NT	100%	2.4
$CoCl_2$[a]	+	+++	++	+	++	−	++	+/−	++	NT	83%	1.5
$NiCl_2$[a]	NT	−	++	+	++	−	++	+	++	NT	75%	1.3
$CuSO_4$[a]	+	−	−	+	+	NT	+	NT	NT	NT	67%	0.7
$ZnCl_2$[a]	NT	+/−	−	+	+	−	+	+	+/−	NT	63%	0.6
$Na_2(PdCl_4)$[a]	+/−	+/−	−	+/−	++	NT	++	NT	NT	NT	58%	0.9
$Na_3Au(S_2O_3)_2$[a]	NT	−	−	+/−	+	−	+++	NT	+/−	NT	50%	0.7
$HgCl_2$[a]	NT	−	−	+/−	++	−	+	+	+	NT	56%	0.7

NT: not tested.
[a] IL-8 levels by microglia to LPS (50 ng/ml) or metal salts (maximum of dose response curve 250–750 μM, for $HgCl_2$ 250–750 nM) as fold increases compared to the baseline (medium control) and categorized as >10 fold: +++; 4–10 fold: ++; 1.5–4 fold: +; 1–1.5 fold: ± and <1 fold: −.
[b] 'Responders' showed an increased IL-8 production (at least +) upon metal exposure. Borderline responses were considered as ½.
[c] Mean response is given by the mean number of + 's per sample.
[d] Baseline IL-8 production in culture medium in 24 h, given in pg/ml.

studies (Rachmawati et al., 2015; Wataha et al., 2000) and provide a useful model to examine microglia activation (Klegeris et al., 2007). In our hands THP-1 cells reacted to metal exposure comparable to ex vivo human cells as MoDC and microglia, although IL-8 production was generally lower in the THP-1 cells.

As shown in Fig. 1a microglia (donor 10) seemed to react in a dose dependent manner to the physiological metal mix, the response being most pronounced to Zn and Cu (Fig. 1b). Since the availability of fresh microglia was limited, more extensive studies were performed in the THP-1 model. These confirmed the findings in donor 10, showing a dose dependent IL-8 production upon stimulation with physiologic metal concentrations, reaching significance for the 10 fold concentration (Fig. 1c). When specifying the response for the individual metals, only Zn and Cu induced significant IL-8 production (Fig. 1d).

3.3. Near-physiologic metal-mixes potentiate LPS responsiveness of microglia and THP-1

To evaluate whether physiologic metal concentrations could potentiate responsiveness of microglia to LPS, LPS responses were evaluated in the presence of 1-, 3- and 10-fold physiologic metal concentrations. Fig. 2a shows that LPS induced IL-8 production by microglia from

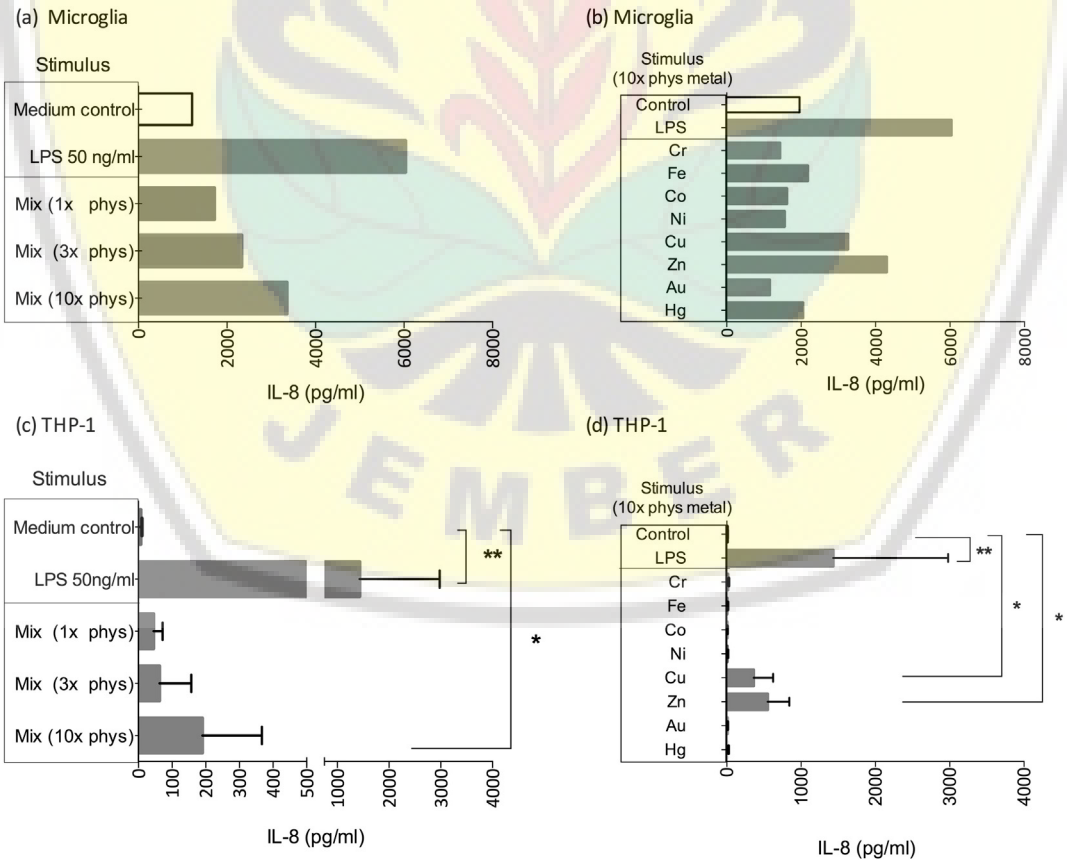

Fig. 1. Microglia and THP-1 responses to physiologic metal concentrations. Microglia from donor 10 (a) and THP-1 cells (c) were exposed to increasing concentrations of physiologic metal mixes (1×, 3×, 10× phys) and LPS 50 ng/ml for 24 h. Additionally microglia from donor 10 (b) and THP-1 cells (d) were exposed to the individual metal salts present in the metal mix (10× phys). The production of IL-8 is given in pg per ml. Bars represent median and interquartile range 25th to 75th percentile from seven independent experiments with the THP-1 (for microglia n = 1 donor, sample 10). Asterisks indicate significantly increased IL-8 production (Kruskall–Wallis, nonparametric-one way ANOVA) as compared to the medium control (open bar): $p < 0.05$ (*), $p < 0.01$ (**).

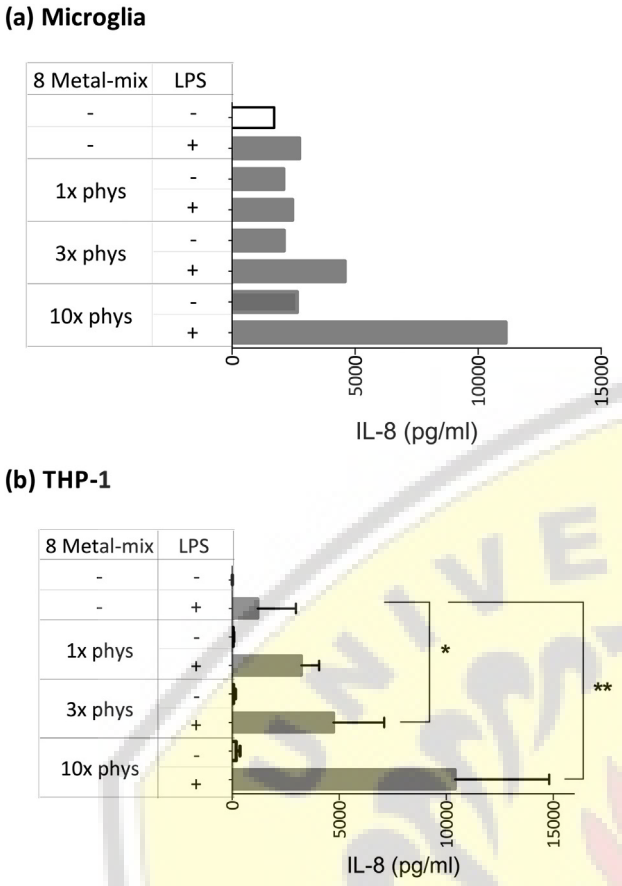

Fig. 2. Effects of physiologic metal salt exposure on LPS responsiveness of microglia and THP-1. Effects of physiologic metal salt concentrations (1×, 3×, 10× phys) on LPS induced IL-8 production by microglia from donor 10 (a) and THP-1 (b) were evaluated. Bars represent median and interquartile range 25th to 75th percentile from five independent experiments (for microglia n = 1 donor, sample 10). Asterisks indicate significantly increased responses to LPS with mean: 8.5-fold higher than the LPS only (Kruskall–Wallis, nonparametric-one way ANOVA) in the presence of physiologic metal mix as compared to the LPS response in the absence of metal salts: $p < 0.05$ (*), $p < 0.01$ (**).

donor 10 is considerably higher in the presence of a 10-fold physiological metal mix, suggesting synergy of the stimuli. This putative synergy was confirmed using LPS responsiveness of THP-1 cells. Significant potentiation of the LPS induced IL-8 production was observed for both 3- and 10-fold physiologic metal concentrations (Fig. 2b; $p < 0.05$ and $p < 0.01$ resp.). In addition to IL-8 production, phenotypic maturation of THP-1 was evaluated in 3 independent experiments. Expression of CD80 and CD86 was not increased upon LPS and/or metal stimulation. However LPS induced CD40 expression was consistently up-regulated in the presence of the physiological metal mix in all experiments (mean fluorescence intensities of a representative experiment: medium control: 79.8; metal mix 3-fold phys: 86.6; LPS 50 ng/ml: 159.0; metal mix 3-fold phys + LPS 50 ng/ml: 286.0). These results are in line with the above shown synergy between LPS and metal stimuli.

3.4. Potentiation of LPS responsiveness by physiologic metal mix is due to Cu and Zn

Based on the findings in Fig. 1, Zn and Cu are the most likely candidates in the metal mix to account for the potentiation of LPS responsiveness in microglia and THP-1. In support of this, metal mixes (10-fold physiological) with and without Zn and Cu, as well as Zn and Cu alone were tested for their capacity to increase LPS responsiveness in THP-1 cells. As can be seen in Fig. 3, indeed mixtures with Zn and Cu induced significantly ($p < 0.05$) higher LPS induced IL-8 production (mean: 8.5-fold higher than the LPS only) than a mixture of the other 6 metals (Mean: 1.5-fold higher than the LPS only).

4. Discussion

The findings obtained in this study may have important implications for understanding the role of metal exposure in the pathogenesis of several brain diseases, since increased neuroinflammation due to activated microglia is of major concern in several; neurodegenerative diseases (Amor et al., 2014) as well as chronic fatigue syndrome, and migraine (Rosenberg et al., 2013; Stejskal, 2014).

It is well-established that human microglia are activated by proinflammatory agents, including IFN-γ or by bacterial products such as LPS (Lehnardt, 2010). Indeed such activation regimens have been used to identify different phenotypes of microglia (Peferoen et al., 2014). We now report that, as hypothesised, microglia in vitro also react to Ni, Co and Pd, metals that have previously been shown to activate innate immune responses via TLR4 (Rachmawati et al., 2013). Several studies have elegantly shown that microglia express TLRs 1–9 (Rosenberger et al., 2014; Lehnardt, 2010; Jack et al., 2005; Lee et al., 2013). It was expected therefore that Au, known to activate TLR3 (Rachmawati et al., 2015), would also activate microglia. However, Au showed a clear positive response only in one microglia sample, obtained from a patient with multiple sclerosis (MS). Whether this is a phenomenon that is restricted to MS patients requires further study. Also reactivity to the other transition metals was not consistent. Although not the aim of this study, we examined the possible relationship between innate responsiveness to metal exposure and clinical characteristics, but no clear association was found. That microglia isolated from aged brains of patients with neurodegenerative diseases are already in an activated state in vivo may cause on one hand reduced sensitivity for subsequent TLR triggers in vitro due to endotoxin tolerance (Biswas and Lopez-Collazo, 2009) or on the other hand increased responsiveness due to priming (Perry and Holmes, 2014).

In the initial microglia experiments (Table 3) high yet non-toxic concentrations of metals were examined. However, the release of metal ions from dental implants and reconstructions yield much lower systemic concentrations, even though erosion and galvanic or chemical corrosion are reported to lead to detectable metal concentrations in blood or urine (Matusiewicz, 2014). In vivo, low metal concentrations, as released from dental reconstructions induced systemic immunological consequences, *e.g.* the induction of metal specific tolerance, both in experimental models and man (van Hoogstraten et al., 1992). Little is known, however, about the effects of low metal concentrations on innate immune cells within the oral cavity or in brain tissue. For dental metals such concentrations are still ill-defined yet the release of metal ions from dental alloys has been clearly shown in different body compartments (Bocca et al., 2006; Giacoppo et al., 2014; Hozumi et al., 2011; Kodaira et al., 2013; Roos et al., 2013; Chen et al., 2013; Michalke and Nischwitz, 2010). The concentrations of metal used in the current study reflect the maximal plasma concentrations found by Roos and colleagues. However, given the variable reference values we also tested the metal salts in 3- and 10-fold higher concentrations.

Physiological metal concentrations, applied as a mixture did not significantly activate THP-1 cells except when 10-fold concentrations were used. In contrast, LPS responsiveness was potentiated with a 3-fold physiologic dose. That the responses to combined stimulation, *i.e.* with metal mix and LPS, were higher than obtained by individual metal salts, suggests a synergy between LPS and the metal-mix in stimulating microglia and THP-1. Although in our experiments stimuli were given simultaneously, the results are in line with the idea that metal ions may prime microglia in vivo, resulting in hyperactive or altered state of innate immune cells indicating an increased risk for chronic inflammation and neurotoxicity in some individuals.

Specification of the metal ions that stimulate microglia and THP-1 in near-physiologic doses revealed that only Zn and Cu accounted for the

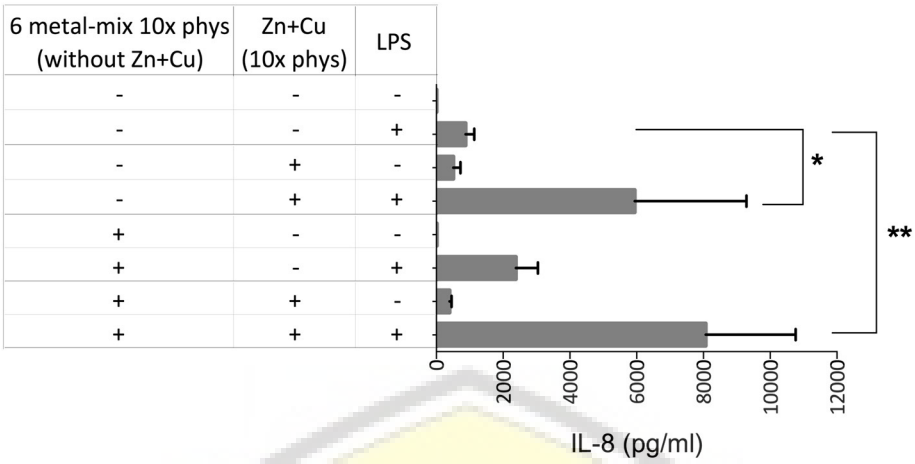

Fig. 3. Only Zn and Cu in the metal mix account for the potentiation of LPS responsiveness of THP-1. Effects of physiologic metal salt mixes (10× phys), with and without Zn and Cu, as indicated in the figure, on LPS (50 ng/ml) induced IL-8 production by THP-1 were evaluated after 24 h of culture. Bars represent median and interquartile range 25th to 75th percentile of at least 3 experiments. Asterisks indicate significantly increased responses to LPS (Kruskall–Wallis, nonparametric-one way ANOVA) in the presence of physiologic metal mix as compared to the LPS response in the absence of metal salts: $p < 0.05$ (*), $p < 0.01$ (**).

microglial activation and for the observed synergy with LPS, whereas the metal mix without Zn and Cu did not induce detectable activation, not even in a 10-fold physiological concentration. Of note, Zn and Cu, both metals with important physiological functions, show to be in much higher levels in plasma — and thus in our in vitro stimulation experiments — than the other transition metals (Table 2). Although it is unlikely that dental alloys containing Cu (present in Pd based alloys and amalgam) or Zn (present in Au based alloys) will substantially add to these relatively high systemic levels (Al-Hiyasat et al., 2002; Elshahawy et al., 2013; Mikulewicz et al., 2014), disturbances in homeostatic control of Zn and Cu should be considered as potential risk factors for chronic brain inflammation.

The effects of pairwise TLR activation in microglia for neuroinflammation and degeneration, recently reviewed by Rosenberger and colleagues (Rosenberger et al., 2014), may clarify the synergism between Zn/Cu and LPS. Despite indications that Zn can trigger TLR4 in some models, other routes of cell activation are considered dominant in its pro-inflammatory effects (Tsou et al., 2013). This might explain the synergistic effect with the TLR4 stimulator LPS. Likewise Cu is known to stimulate innate cells via still ill defined routes, possibly involving TLR5 (Rachmawati et al., 2013), which is expressed on both microglia and astrocytes (Bernardino et al., 2008; Lee et al., 2013). Despite extensive evaluation of distinct pairwise TLR activation, little or no data on TLR5 is given (Rosenberger et al., 2014). Nevertheless, from our study it is clear that synergistic effects with LPS occur when levels of Zn as well as Cu increase more than 3 times due to excessive exposure and/or a disrupted homeostasis.

With respect to oral exposure to Ni and Co, the strongest innate stimulators, also for microglia, pathogenic signals resulting in increased neurotoxicity might be expected. These metals are, however, present in such low levels in the circulation, that they are unlikely to directly activate innate cells, nor increase their LPS responsiveness, unless there is an extreme corrosive environment. Even 10-fold physiological concentrations did not induce detectable activation whether or not in synergy with LPS. Likewise oral exposure to Cr, Fe, Au and Hg would be relatively safe with respect to untoward innate triggering of microglial cells. Still, one should realize that, depending on integrity of the blood–brain barrier, distinct transporter molecules can facilitate metal uptake in the brain, which in conjunction with reduced actual efflux may cause immunotoxic accumulation in the central nervous tissue (Dusek et al., 2014; Mutter, 2011; Yokel, 2006).

In conclusion, the present study shows that transitional metals in supra-physiologic, non-toxic concentrations can activate microglia similar to that shown for MoDCs, with Ni and Co giving the strongest stimulation. Near-physiologic concentrations do however, not activate microglia, except for Zn and Cu. Since Zn and Cu also potentiate the inflammatory response to LPS, excessive exposure potentially causing dyshomeostasis of these metals should be avoided.

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