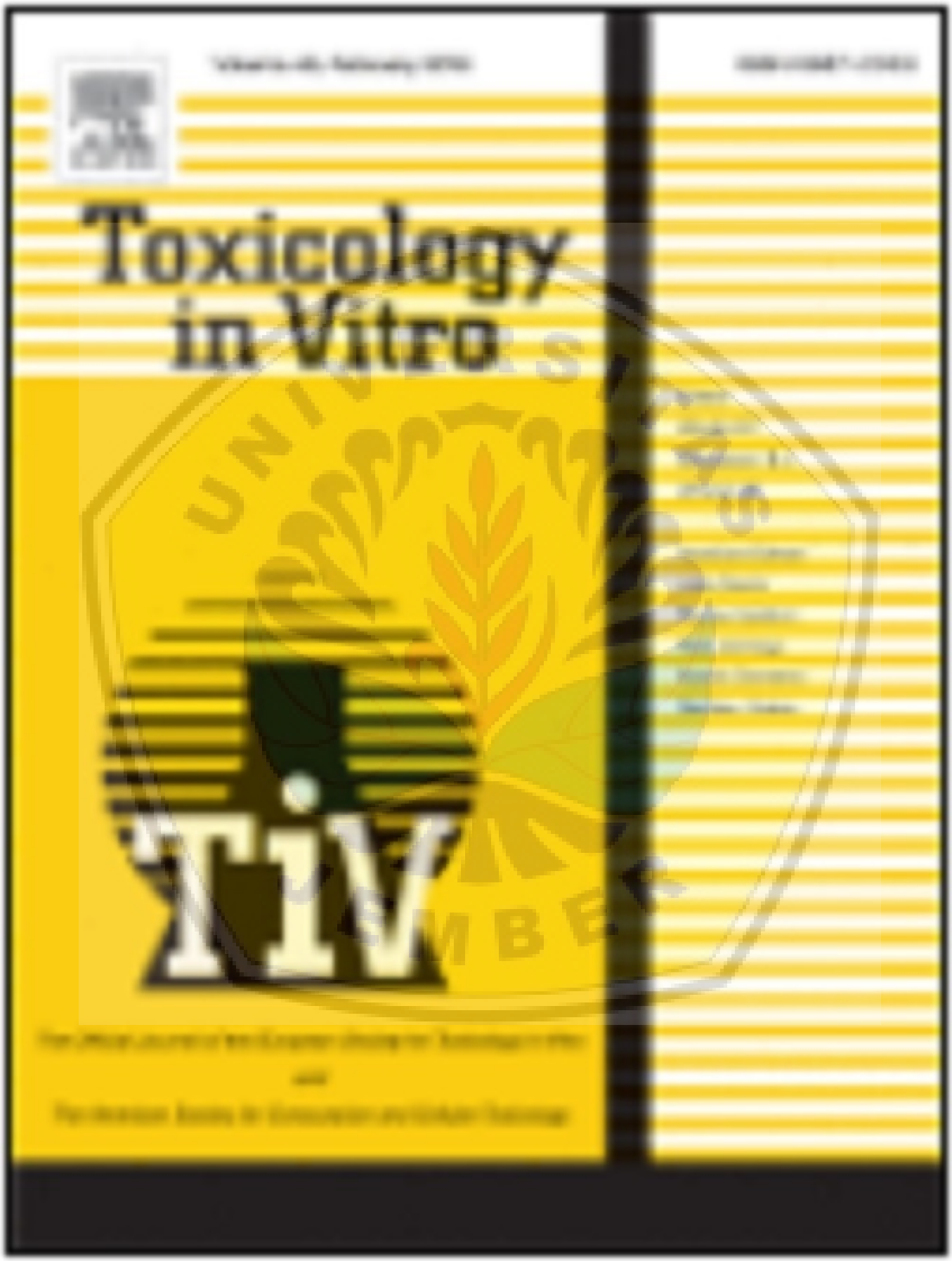

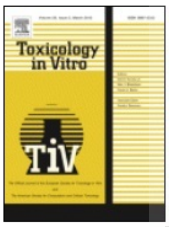

Toxicology in Vitro

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Mechanisms

Effects of CoCr metal wear debris generated from metal-on-metal hip implants and Co ions on human monocyte-like U937 cells Original Research Article
Pages 271-280
Olga M. Posada, Rothwelle J. Tate, M. Helen Grant
Abstract | Close research highlights | Purchase PDF - $35.95 | Supplementary content

Highlights

- Metal debris in combination with Co ions influence cell function.
- Pre-exposure to Co ions seems to sensitise cells to the toxic effects particles.
- Experimental conditions may not allow to discriminate between cytotoxic and cytostatic.

The inflammation and estrogen metabolism impacts of polychlorinated biphenyls on endometrial cancer cells Original Research Article

Pages 308-313

Yajie Chen, Qiansheng Huang, Qionghua Chen, Yi Lin, Xia Sun, Huanteng Zhang, Maobi Zhu, Sijun Dong

Abstract | Close research highlights | Purchase PDF - $35.95 | Supplementary content

Highlights

- Markers for inflammation-endocrine toxicity of PCBs were detected in Ishikawa cells.
- CB126 stimulated the proliferation of Ishikawa whereas CB153 did not.
- PCBs increased the activity of SOD1 and decreased the inflammatory factors.
- The inhibitory effects of PCBs on IL-8 were mediated by ER and AHR receptor.
- CB126 did not impact the metabolism of estradiol.

Use of the ES-D3 cell differentiation assay, combined with the BeWo transport model, to predict relative in vivo developmental toxicity of antifungal compounds Original Research Article

Pages 320-328

Hequn Li, Ivonne M.C.M. Rietjens, Jochem Louisse, Martine Blok, Xinyi Wang, Linda Snijders, Bennard van Ravenzwaay

Abstract | Close research highlights | Purchase PDF - $35.95 | Supplementary content

Highlights

- In vitro developmental toxicity of five antifungal compounds was investigated.
- ES-D3 cell differentiation assay was used to predict developmental toxicity.
- The BeWo transport model was used for the characterization of placental transfer.
- Transport rates across in vitro placental barrier were different among compounds.
- ES-D3 cell differentiation assay, combined with BeWo model, increased predictivity.

Cytotoxicity of monensin, narasin and salinomycin and their interaction with silybin in HepG2, LMH and L6 cell cultures Original Research Article

Pages 337-344

Wojciech Cybulski, Lidia Radko, Wojciech Rzeski

Abstract | Close research highlights | Purchase PDF - $35.95 | Supplementary content

Abstract | Close research highlights | Purchase PDF - $35.95 | Supplementary content

Highlights

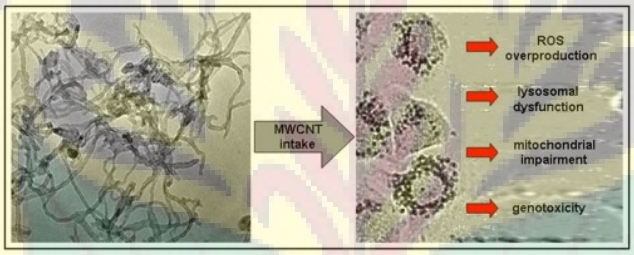
- Assessment of ionophoric polyethers toxicity to hepatoma and myoblast lines; EC_{50}.
- Protective effects of silybin in course of cytotoxic action of ionophoric polyethers.
- Silybin increases viability of all the cells impacted by ionophoric polyethers.

Toxicological assessment of multi-walled carbon nanotubes on A549 human lung epithelial cells Original Research Article

Pages 352-362

Giuseppa Visalli, Maria Paola Bertuccio, Daniela Iannazzo, Anna Piperno, Alessandro Pistone, Angela Di Pietro

Abstract | Close graphical abstract | Research highlights | Purchase PDF - $35.95 | Supplementary content



Innate stimulatory capacity of high molecular weight transition metals Au (gold) and Hg (mercury) Original Research Article

Pages 363-369

Dessy Rachmawati, Inás W.A. Alsalem, Hetty J. Bontkes, Marleen I. Verstege, Sue Gibbs, B.M.E. von Blomberg, Rik J. Scheper, Ingrid M.W. van Hoogstraten

Abstract | Close research highlights | Purchase PDF - $35.95 | Supplementary content

Highlights

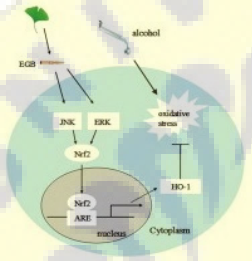
- High molecular weight transitional metals Au and Hg activate innate immunity.
- Gold and mercury induce IL-8 secretion in myelo-monocytic cells.
- Stimulatory capacity of Au could be ascribed to TLR-3 ligation.
- Innate responses may contribute to development of oral and skin allergies.

Transcriptional upregulation centra of HO-1 by EGB via the MAPKs/Nrf2 pathway in mouse C2C12 myoblasts Original Research Article

Pages 380-388

Jianfeng Wang, Li Zhang, Ying Zhang, Meiling Luo, Qiong Wu, Lijun Yu, Haiying Chu

Abstract | Close graphical abstract | Research highlights | Purchase PDF - $35.95 | Supplementary content



Toxicity assessment of aggregated/agglomerated cerium oxide nanoparticles in an *in vitro* 3D airway model: The influence of mucociliary clearance Original Research Article

Pages 389-397

C. Frieke Kuper, Mariska Gröllers-Mulderij, Thérèse Maarschalkerweerd, Nicole M.M. Meulendijks, Astrid Reus, Frédérique van Acker, Esther K. Zondervan-van den Beuken, Mariëlle E.L. Wouters, Sabina Bijlsma, Ingeborg M. Kooter

Abstract | Close research highlights | Purchase PDF - $35.95 | Supplementary content

Highlights

- Testing local defense efficiently predicts local toxicity of particulate matter.
- 3D airway models with mucociliary apparatus can test local mechanical defense.
- Exposure via immersion to cell lines overestimate toxicity of particulate matter.
- Nano CeO_2 has low toxicity for ciliated epithelium, despite expected deposition.
- 3D airway models can help reduce the need for animal models in toxicity assessments.

Toxicity, genotoxicity and proinflammatory effects of amorphous nanosilica in the human intestinal Caco-2 cell line Original Research Article

Pages 398-407

Adeline Tarantini, Rachelle Lanceleur, Annick Mourot, Marie -Thérèse Lavault, Gérald Casterou, Gérard Jarry, Kevin Hogeveen, Valérie Fessard

Abstract | Close research highlights | Purchase PDF - $35.95 | Supplementary content

Highlights

- Toxic effects of silica NPs (15 and 55 nm) were investigated in Caco-2 cells.
- Both NPs were localized within the cytoplasm but did not enter the nucleus.
- 15 nm but not 55 nm silica induced genotoxic and proinflammatory effects.
- Oxidative stress is likely to be involved in the genotoxic mechanism of action.
- SiO_2 NPs may induce potential adverse effects on the intestinal epithelium *in vivo*.

Probing the role of amino acids in oxime-mediated reactivation of nerve agent-inhibited human acetylcholinesterase Original Research Article

Pages 408-414

Carolyn Chambers, Chunyuan Luo, Min Tong, Yerie Yang, Ashima Saxena

Abstract | Close research highlights | Purchase PDF - $35.95 | Supplementary content

Highlights

- Wild-type and mutant human AChEs were inhibited with GA, GB, GF, VX, or VR.
- Reactivation rate constants for 2-PAM, MMB4, HI-6, and HLö-7 were compared.
- Y72, Y124 and W286 were important only in reactivation by bis-pyridinium oximes.
- E202 was important for reactivation by all oximes.
- Human and bovine AChEs display similarity with regard to oxime reactivation.

Review

Toxicogenomics *in vitro* as an alternative tool for safety evaluation of petroleum substances and PAHs with regard to prenatal developmental toxicity Review Article

Pages 299-307

Polyxeni Tsitou, Marjoke Heneweer, Peter J. Boogaard

Abstract | Close research highlights | Purchase PDF - $35.95 | Supplementary content

Highlights

- Toxicogenomics as tool in the assessment of prenatal developmental toxicity of petroleum products.
- Heavy PAH are likely to be responsible for the developmental toxicity of petroleum substances.

In Vitro Toxicology

Volume 21, Issue 6
pp. 977-1212 (September 2007)

Volume 21, Issue 5
pp. 759-976 (August 2007)

Volume 21, Issue 4
pp. 535-758 (June 2007)

Volume 21, Issue 3
pp. 335-534 (April 2007)

Volume 21, Issue 2
pp. 175-334 (March 2007)
In Vitro Cytotoxicity Mechanisms. Proceedings of the 46th ETCS International Meeting and the 3rd International Joint Meeting of AICC and CELLTOX

Volume 21, Issue 1
pp. 1-174 (February 2007)

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Volumes 1 - 10 (1987 - 1996)

- A combination of 3 in vitro tests is proposed to study developmental toxicity of petroleum products.

New Methods and Models

Full Length Articles

In vitro detection of cardiotoxins or neurotoxins affecting ion channels or pumps using beating cardiomyocytes as alternative for animal testing Original Research Article
Pages 281-288
Jonathan Nicolas, Peter J.M. Hendriksen, Laura H.J. de Haan, Rosella Koning, Ivonne M.C.M. Rietjens, Toine F.H. Bovee
Abstract | Close research highlights | Purchase PDF - $35.95 | Supplementary content

Highlights

- We describe a cardiomyocyte-based assay for detection of neurotoxins.
- Cardiomyocytes allow for detection of neurotoxins affecting ion channels or pumps.
- We show that neuro-2a assay may be promising for marine neurotoxin detection.

In vitro safety assessment of food ingredients in canine renal proximal tubule cells Original Research Article
Pages 289-298
J. Koči, B. Jeffery, J.E. Riviere, N.A. Monteiro-Riviere
Abstract | Close research highlights | Purchase PDF - $35.95 | Supplementary content

Highlights

- *In vivo* kidney injury markers (e.g. KIM-1) are not applicable to an *in vitro* model
- Volatile ingredients (CINA, CBO, LGO) studied are more toxic than the nephrotoxicant 4-AP
- Feasibility of extrapolating data from canine cells for human toxicity studies
- Lemongrass oil (LGO) induces high levels of oxidative stress represented by GSTA3
- Gene relationship and TEM analyses predict the LGO-induced cellular response

In vitro re-expression of the aryl hydrocarbon receptor (*Ahr*) in cultured *Ahr*-deficient mouse antral follicles partially restores the phenotype to that of cultured wild-type mouse follicles Original Research Article
Pages 329-336
A. Ziv-Gal, L. Gao, B.N. Karman, J.A. Flaws
Abstract | Close research highlights | Purchase PDF - $35.95 | Supplementary content

Highlights

- Growth in *Ahr* re-expressed mouse antral follicles is partially restored to the levels of wild-type follicles.
- *Ahr* re-expression partially restored expression of factors in the AHR signaling pathway to levels in wild-type follicles.
- *Ahr* re-expressed mouse antral follicles partially respond to TCDD in a manner similar to wild-type follicles.

Differential immunomodulatory responses to nine polycyclic aromatic hydrocarbons applied by passive dosing Original Research Article

Pages 345-351

Gertie J. Oostingh, Kilian E.C. Smith, Ulrike Tischler, Isabella Radauer-Preiml, Philipp Mayer

Abstract | Close research highlights | Purchase PDF - $35.95 | Supplementary content

Highlights

- Passive dosing allows testing hydrophobic chemicals in *in vitro* cell systems.
- Passive dosing provides defined and controlled exposure concentrations.
- Immunomodulatory responses of bronchial epithelial cells differ between PAHs.
- Differences in dose–response patterns suggest mechanistic differences.
- Dose–response testing is of great importance in immunotoxicity testing.

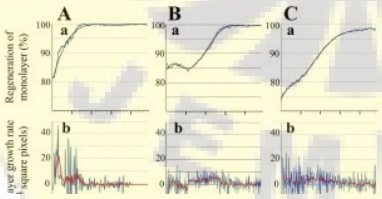
Antibiotics delay *in vitro* human stem cell regrowth Original Research Article

Pages 370-379

Melinda Turani, Gaspar Banfalvi, Agota Peter, Krisztina Kukoricza, Gabor Kiraly, Laszlo Talas, Bence Tanczos, Balazs Dezso, Gabor Nagy, Adam Kemeny-Beke

Abstract | Close graphical abstract | Research highlights | Purchase PDF - $35.95 | Supplementary content

Characteristic regeneration of monolayer surface upon antibiotic treatment of limbal stem cells as a function of time. A. Control scratch model to mimic the regeneration of stem cell monolayer. B. Regeneration of monolayer after treatment with 0.5 mg/ml chloramphenicol. C. Regeneration of scratched monolayer upon treatment with 0.1 mg/ml rifampicin. Lower panels: oscillations in growth rate indicating cellular surface motion changes.



Short Communications

Liposomes and MTT cell viability assay: An incompatible affair
Pages 314-319
Fabrizio Angius, Alice Floris
Abstract | Close research highlights | Purchase PDF - $35.95 | Supplementary content

Highlights

- Liposomes delivering drugs could produce inconsistent values of MTT absorbance.
- Empty-liposomes interfere, per se, on MTT assay by its lipidic nature.
- MTT assay may be inappropriated in studies involving liposome treatments.
- Additional method to evaluate cytotoxic effects should accompanied the in vitro MTT assay.

Letter to the Editor

Short commentary to "Human *in vivo* database now on ACuteTox home page" [Toxicol. *In Vitro* 27 (2013) 2350–2351]
Page 415
Pilar Prieto, Agnieszka Kinsner-Ovaskainen
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Innate stimulatory capacity of high molecular weight transition metals Au (gold) and Hg (mercury)

Dessy Rachmawati [a,b,*], Inás W.A. Alsalem [a], Hetty J. Bontkes [a], Marleen I. Verstege [c], Sue Gibbs [d], B.M.E. von Blomberg [a], Rik J. Scheper [a], Ingrid M.W. van Hoogstraten [a]

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ABSTRACT

Nickel, cobalt and palladium ions can induce an innate immune response by triggering Toll-like receptor (TLR)-4 which is present on dendritic cells (DC). Here we studied mechanisms of action for DC immunotoxicity to gold and mercury. Next to gold ($Na_3Au(S_2O_3)_2 \cdot 2H_2O$) and mercury ($HgCl_2$), nickel ($NiCl_2$) was included as a positive control. MoDC activation was assessed by release of the pro-inflammatory mediator IL-8. Also PBMC were studied, and THP-1 cells were used as a substitution for DC for evaluation of cytokines and chemokines, as well as phenotypic, alterations in response to gold and mercury. Our results showed that both $Na_3Au(S_2O_3)_2 \cdot 2H_2O$ and $HgCl_2$ induce substantial release of IL-8, but not IL-6, CCL2 or IL-10, from MoDc, PBMC, or THP-1 cells. Also gold and, to a lesser extent mercury, caused modest dendritic cell maturation as detected by increased membrane expression of CD40 and CD80. Both metals thus show innate immune response capacities, although to a lower extent than reported earlier for $NiCl_2$, $CoCl_2$ and $Na_2[PdCl_4]$. Importantly, the gold-induced response could be ascribed to TLR3 rather than TLR4 triggering, whereas the nature of the innate mercury response remains to be clarified. In conclusion both gold and mercury can induce innate immune responses, which for gold could be ascribed to TLR3 dependent signalling. These responses are likely to contribute to adaptive immune responses to these metals, as reflected by skin and mucosal allergies.

1. Introduction

Metals are amongst the most notorious contact sensitizers clinically known. This counts in particular for the low-molecular weight transition metals nickel, cobalt and palladium. Still, in dentistry also mercury and gold have obtained a poor reputation as frequent causes for oral allergic complaints (Moller, 2002, 2010; Roberts and Charlton, 2009; Evrard and Parent, 2010). The latter metals are located next to each other in the periodic table as high molecular weight transition elements.

Cast gold alloys are the material of choice for most dental restorations in developed countries, like in Europe, United States and Japan. These alloys have been found to be ideal for these applications because of their corrosion resistance and biocompatibility. Next to their use in oral applications, gold-based alloys are also widely used in skin appliances, e.g. by jewellers for ear rings and piercing studs. These frequent uses of metallic gold are complemented by the medical use of gold salts for the local treatment of chronic inflammations such as in rheumatoid arthritis. Still, despite its biocompatibility, or even immunosuppressive capacities, gold has also obtained some disrepute as a contact allergen (Moller, 2002; Ahlgren et al., 2002).

Mercury-based amalgam has been used for material fillings in dentistry for over a century. Its popularity for this application, however, has declined over recent years due its potential negative

Abbreviations: ACD, allergic contact dermatitis; DC, dendritic cell(s); DMSO, dimethylsulphoxide; FACS, fluorescence-activated cell sorting applied; $FeCl_3$, iron(III) chloride; HEK, human embryonic kidney; GM-CSF, granulocyte-macrophage colony stimulating factor; $HgCl_2$, mercuric chloride; iDC, immature dendritic cell(s); IL, interleukin; LPS, lipopolysaccharide; MTT, (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; LTA, lipoteichoic acid; MoDC, monocyte-derived dendritic cells; MW, molecular weight; $NiCl_2$, nickel (II) chloride; $Na_3Au(S_2O_3)_2 \cdot 2H_2O$, sodium gold thiosulfate; PBS, phosphate-buffered saline; PBMC, peripheral blood mononuclear cell; TLR, toll like receptor.

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0887-2333/

health effects. These may vary from autoimmune phenomena (Nielsen and Hultman, 2002; Pigatto and Guzzi, 2010; Rowley and Monestier, 2005) to neurological problems (Kern et al., 2012; Mutter, 2011). Yet, negative reports are still scarce or disputed, and certainly in developing countries amalgam fillings are still widely being used nowadays.

The focus of the present investigation was to analyze whether gold and mercury might induce innate immune responses. First dendritic cells DC generated from culturing peripheral blood monocytes were used. DC are central between innate and adaptive immune responses, and were found to be highly sensitive in revealing innate stimulatory capacities of the low-molecular weight transitional metals nickel, cobalt and palladium (Rachmawati et al., 2013; Raghavan et al., 2012; Schmidt et al., 2010; Toebak et al., 2006). Upon capturing antigens, DC begin to mature, and migrate to draining lymph nodes via afferent lymphatic vessels. This process is orchestrated by pro-inflammatory mediators, released from skin cells and DC, such as IL-8, IL-6, IL-1β and TNFα (Martin et al., 2011; Miyazawa et al., 2007; Toebak et al., 2006). Furthermore, it was studied whether observed responses could also be detected in freshly prepared PBMC, as well as in monocytic leukaemia cell line, THP-1 cells.

Innate stimulatory capacities were assessed by cytokine/chemokine levels (IL-8, CCL2, IL-6) and phenotypic alterations as detected by flow cytometric (FACS) analyses (CD40 and CD80). Following up on our previous report on nickel, cobalt and palladium-induced signalling (Rachmawati et al., 2013), TLR-2, 3 and 4 dependency of the gold and mercury-induced signalling was studied using HEK transfectant cell lines.

2. Materials and methods

2.1. Metal chemicals

As metal allergens the following chemicals were used: nickel (II) chloride hexahydrate ($NiCl_2{\cdot}6H_2O$), sodium gold thiosulfate $Na_3Au(S_2O_3)_2{\cdot}2H_2O$; Chemo technique Diagnostics, Vellinge, Sweden), mercuric chloride ($HgCl_2$; Riedel-de Haën, Seelze, Germany). LPS was obtained from Escherichia coli 055:B5 (Sigma, St. Louis, MO, USA). $NiCl_2$, $Na_3Au(S_2O_3)_2{\cdot}2H_2O$, $HgCl_2$, LPS were dissolved in H_2O as stock solutions and further diluted with culture medium just before use.

2.2. Peripheral blood mononuclear cells (PBMC)

PBMC were isolated from 40 to 50 ml freshly drawn peripheral venous blood of at least 4 different healthy donors without known metal allergies by Ficoll (Lymphoprep, Fresenius KabiNorge AS) density gradient centrifugation. Culture medium was Iscove's modified Dulbecco's medium (IMDM, Biowhittaker, Verviers, Belgium) with 10% heat-inactivated fetal calf serum (FCS, Hyclone, Logan USA), 1% penicillin streptomycin, 1% L-glutamine and 1% β-mercaptoethanol (2ME).

2.3. MoDC culture

MoDC were generated from freshly prepared PBMC. After 2 h incubation of the PBMC in a humidified incubator, we removed the non-adherent cells by aspiration and washed once very gentle with 5 ml PBS. MoDC were generated as previously described (Bontkes et al., 2002). Briefly, adherent monocytes were 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% 2ME, 1000 U/ml granulocyte–macrophage colony stimulating factor (GM-CSF Novartis, The Netherlands) and 20 ng/ml IL-4 (R&D systems lot AG270911A). After 6–7 days, immature dendritic cells (iDC) were harvested and plated in 96 well flat tissue culture plates (Cellstar Greiner Bio-One) at approximately 5×10^4 cells per well.

2.4. THP-1 cells

A vial of cell passage 17 of THP-1 cells (ATCC, Rockville, USA) was kept at −80 °C until thawing. The cells were cultured in 100 ml culture flasks at a density of 10^6 cells/10 ml of RPMI 1640 containing 2 mM L-glutamine, 0.1 mg/ml streptomycin, 100 IU/ml penicillin, supplemented with 10% heated-inactivated FCS.

2.5. Metal toxicity experiments

In order to design appropriate concentration ranges of metals, the maximal non-toxic concentration was determined by the MTT reduction test (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide). Approximately 100 µl of cells (5×10^4/well) were plated in 96 well culture plates and exposed to increasing concentrations of the metals. After 24 h incubation supernatants were removed and 50 µl of MTT solution (5 mg/ml) was added per well. MTT solution was prepared freshly and dissolved with H_2O, filtered through a 0.22-µm filter. The plates were incubated in the dark at 37 °C. After 2–3 h of incubation, 50 µl DMSO (Merck, Darmstad, Germany) was added to each well and after shaking, the solution was measured using an ELISA reader at OD (optical density) 490 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) × 100%.

2.6. Metals and LPS exposure

PBMC, iDC, THP-1 (5×10^4 cells/well) were exposed to LPS (100 ng/ml) and metals at concentrations between 0 and 750 µM. Plates were incubated at 37 °C in 5% humidified CO_2. After 24 h, supernatants were collected and stored at −20 °C 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-QCL™ bulk kit, Lonza).

For metal exposures, cells were exposed to $Na_3Au(S_2O_3)_2{\cdot}2H_2O$, $HgCl_2$ and $NiCl_2$ at 4 different serial dilutions, 750 µM (for $HgCl_2$ - nM) as the highest concentration. Total volume in each well was 200 µl. iDC and PBMC (5×10^4 cells/well), supernatants were collected after 16–24 h and kept at −20 °C until cytokine/chemokine assessment (see below). Cell viability was tested in the cell pellet in the same plate (see below).

To explore involvement of mitogen-actived protein kinase (MAPK) pathways, notably with the p38 MAPK inhibitor SB203580, cells were cultured as above, but with and without addition of 20 µM SB203580 (InvivoGen, San Diego, USA) as from one hour at 37 °C before starting metal and ligand exposures.

2.7. Assessment of TLR 2, 3 and 4 signalling with HEK293 transfectant cells

Human Embryonic Kidney (HEK) 293 TLR 2, 3 and TLR4/MD2 cells were cultured in T75 flasks in DMEM, 1% Glutamine, 1% pen/strep, 0.5 µg/ml G418 (Sigma–Aldrich, St. Louis, MO, USA) and harvested upon confluence. Wild type HEK293 and HEK293 cells stably expressing human TLR2, TLR3 or TLR4-MD2, were a

kind gift from D.T. Golenbock (MA, 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 1×10^5 cells/well in 100 μl medium in 96-wells flat bottom plates. After allowing cells to adhere for 1.5–2 h 100 μl of metal salt solution was added to give final concentrations of 0, 250 and 500 μM (for $HgCl_2$ nM). As a positive control $NiCl_2$ was used. Cells were incubated for 24 h at 37 °C, and supernatants were harvested for IL-8 ELISA.

2.8. Flow cytometry

After 48 h exposure to metals, MoDC were washed in FACS medium (PBS containing 1% BSA). Expression of CD40 and CD80 was analysed by flow cytometry. Cell staining was performed using PE and APC-labelled monoclonal antibodies: mouse anti-human-CD40-PE and anti-human-CD80-APC (IgG_1 Pharmingen, B&D systems). Isotype controls assessing non-specific binding were monoclonal mouse IgG_1 APC and IgG_1 PE (Pharmingen, B&D systems). Cells were stained with antibodies for 30 min in the dark at room temperature, then washed. Flow cytometry was performed with BD-FACS Calibur and analysed using Cell Quest software. Mean fluorescence index (MFI) was calculated by formula: mean fluorescence intensity of DC stimulated with metals/mean fluorescence non-stimulated cells. The relative MFI was defined as fold increase over isotype control.

2.9. Cytokine/chemokine production

IL-4, IL-6, IL-8, IL-10, and IFNg production was measured by Enzyme-linked immunosorbent assay (ELISA) with Peli-Kine ELISA kits (Sanquin, Amsterdam, The Netherlands) and for CCL2 by human CCL2 (MCP-1) ELISA ready set-Go (eBioscience, San Diego, USA) using 96-well microtiter plates (Nunc maxisorp microtiter plates, Nalge Nunc international), as per the manufacturer's instructions. Absorbance was measured at 450 nm. The amount of cytokine/chemokine in the supernatant was assessed by using standard curves (lower detection limit of IL-4: 20.5 pg/ml; IL-6: 11.5 pg/ml; IL-8: 15.4 pg/ml; IL-10: 7.7 pg/ml; IFNg: 19.2 pg/ml; CCL2: 31.3 pg/ml). Data are presented in picograms or nanograms per ml.

2.10. Data analysis

The statistical significance of the effects of various metals on the secretion of IL-8 was analysed by using one way ANOVA and Kruskall–Wallis test (non parametric ANOVA), with statistic program GraphPad Prism Software version 6.0 (San Diego, CA, USA). $P \leqslant 0.05$ was considered statistically significant. All data are presented as mean ± SD.

3. Results

3.1. Cytotoxic effects of gold and mercury

To study potential immunostimulatory effects of gold and mercury on mononuclear and blood cells (MoDC, PBMC and THP-1 cells), maximal non-toxic concentrations were determined first. Cytotoxicity experiments were carried out using MTT reduction assays as a read-out. Cells were exposed for 24 h to increasing dosages of $Na_3Au(S_2O_3)_2$, $HgCl_2$ and $NiCl_2$ and concentration ranges between 0 and 750 μM were found to be appropriate for gold and nickel, whereas mercury required a 1000-fold lower dose range (0–750 nM). Of note, THP-1 cells were found to be slightly more sensitive to gold and nickel exposure than both primary cell types MoDC and PBMC (Fig. 1).

3.2. Gold and mercury induced MoDC activation as detected by cytokine/chemokine secretion

Subsequently, gold thiosulfate and mercury chloride were studied for their capacity to activate MoDC, as reflected by IL-8, CCL2 and IL-6 release. As a positive control nickel chloride was included as it is known to activate MoDC through direct TLR4 triggering. Although less impressive than nickel, gold also induces distinct IL-8 release at marginally toxic dosages, whereas mercury shows similar release, but at a lower level (Fig. 2). In contrast to nickel, however, the stimulatory capacities of gold and mercury were not reflected by an appreciable increase in levels of other, less abundantly produced inflammatory (IL-6 or CCL2) or anti-inflammatory (IL-10) mediators.

3.3. PBMC

In order to further confirm the innate stimulatory capacities for gold and mercury, using the same approach, unseparated peripheral blood mononuclear cells (PBMC) were also studied. Robust production of IL-8 by PBMC could be observed for $NiCl_2$ exposure, albeit at a lower level than for MoDC. Again gold, and to a lesser extent mercury, showed distinct IL-8 release by PBMC (Fig. 3). Production profiles for IL-6 were less clear, but supported the innate stimulatory capacities of gold and mercury. Additional ELISA's carried out to detect CCL2, IFNg and IL-4 in PBMC supernatants did not reveal relevant signals for these cytokines. (data not shown).

3.4. THP1

To explore applicability of THP-1 cell line cells for studying metal-induced innate signalling, essentially similar experiments were carried out with these cells. Supporting the earlier data for MoDC and PBMC, not only $NiCl_2$ induced a dose-dependent increase in IL-8 secretion, but also both gold and mercury. Both high MW transition metals showed remarkably high levels of IL-8 release (Fig. 3). Strongest responses were observed for mercury, which also induced appreciable IL-6 release in these cells.

3.5. Phenotypic analysis of metal-induced DC maturation

MoDC were exposed to the metal salts at maximally non-toxic dosages, i.e. 500 μM (nickel and gold), or 500 nM (mercury), whereas LPS was tested at 100 ng/ml. Exposure to LPS and $NiCl_2$ resulted in small but distinct increases in CD80 expression, whereas both heavy weight transitional metals only induced marginal increases in maturation markers which did not reach significance, except for gold-induced CD40 expression (Fig. 4). In our hands THP1 cells were even less responsive in this regard, although again hints of both gold and mercury-induced CD40 expression could be observed.

3.6. Gold and mercury induced IL-8 secretion in HEK293 WT and TLR2, 3 and 4 transfectant cells

Further experiments were carried out to investigate whether, like for nickel, cobalt and palladium, TLR4 signalling might play a role in innate cell signalling to the high-molecular weight transitional metals. Besides the TLR4-transfected HEK293 TLR4/MD2 cell line, two additional HEK293 cell lines, transfected with TLR2 and TLR3 respectively, were tested. Next to gold and mercury salts, nickel and LPS were used as positive controls for TLR4-mediated cell activation. Lipoteichoic acid (LTA) and poly-IC were added as positive controls to the metal panel for testing TLR2 and 3 transfectants, respectively. Again, IL-8 release was utilized as read-out for down-stream signalling (Rachmawati et al., 2013).

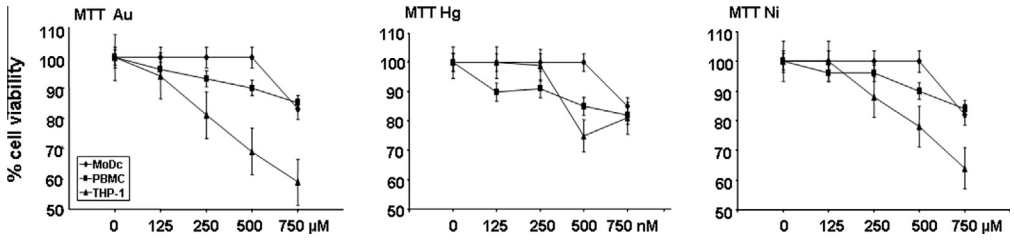

Fig. 1. Cell viabilities after metal salt exposure. Cell viability (%) relative to vehicle exposure is given after culturing MoDC, PBMC or THP-1 cells for 24 h with increasing concentrations of gold thiosulfate and nickel chloride (in µM) or mercury chloride (in nM). Cell viabilities were assessed as outlined in Materials and Methods. Graphs represent means ± SD for 3 independent experiments.

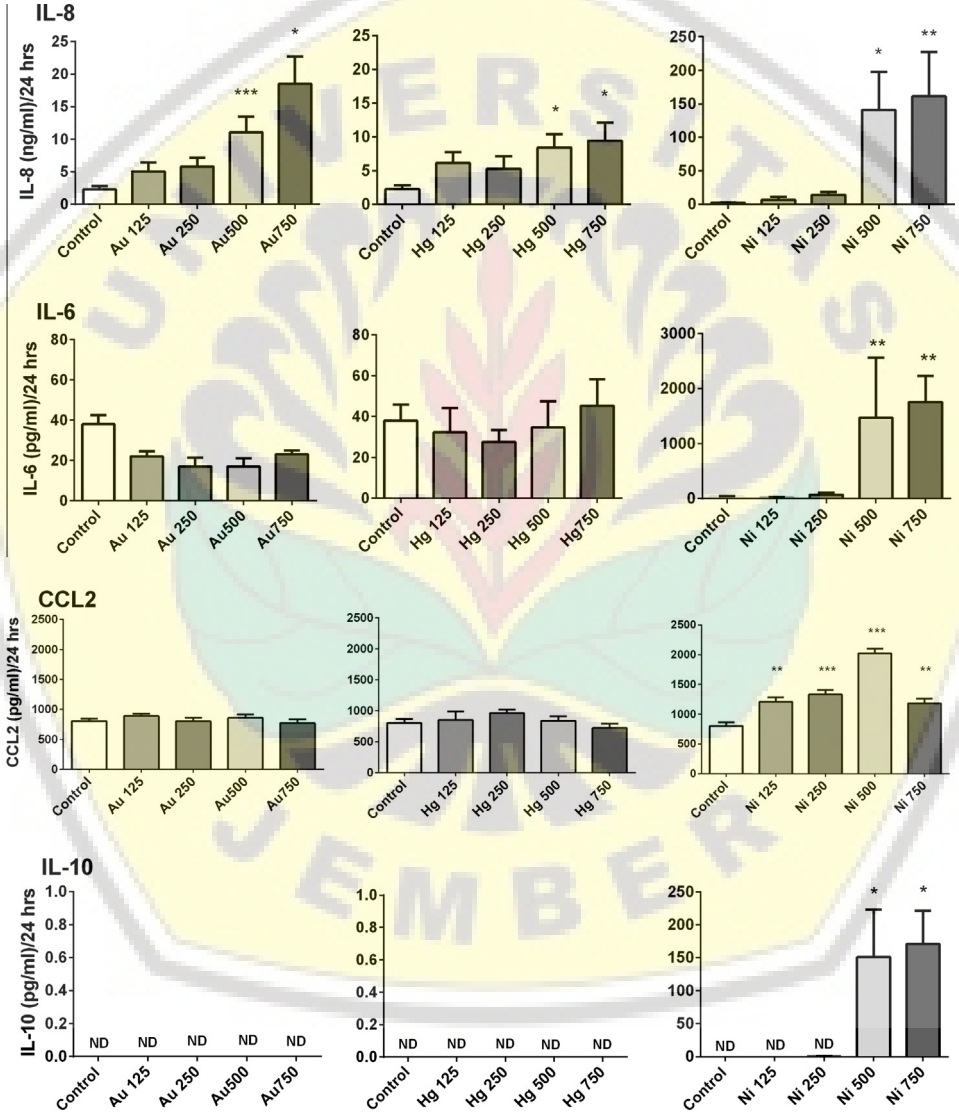

Fig. 2. IL-8, IL-6, CCL2 and IL-10 production after metal exposure of MoDCs. Immature MoDCs were exposed to increasing concentrations of gold thiosulfate and nickel chloride (in µM) or mercury chloride (in nM) for 24 h. Bars represent mean ± SD from six independent experiments (n = 3 donors). Asterisks specify statistically significant (One way ANOVA and Kruskall–Wallis test (non parametric ANOVA)) differences in production of IL-8, IL-6, CCL2 and IL-10 at the given metal concentration as compared to the control medium (open bar): $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***). ND = not detectable.

As shown in Fig. 5, in contrast to nickel, neither gold nor mercury induced activation signals in the TLR4/MD2 transfectant. Also TLR2-mediated signalling was not involved, as was clear from testing the same panel in TLR2 transfectant cells, that did respond well to the positive control LTA. Interestingly, gold was strongly active in the TLR3 transfectant cells, which also showed specific reactivity to poly-IC as the internal positive control. The respective ligand-induced effects were clearly due to the presence of TLR4/MD2,

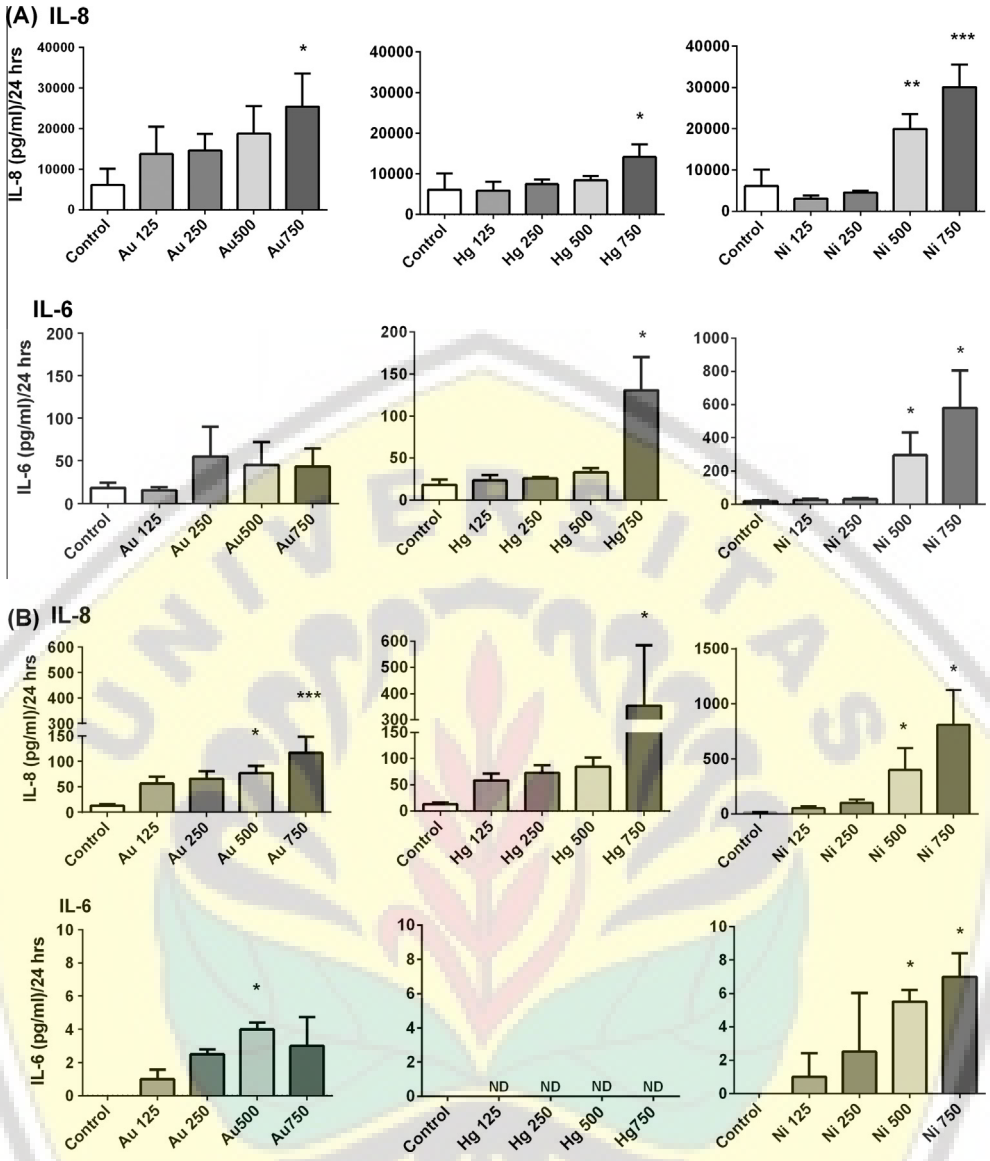

Fig. 3. IL-8 and IL-6 production after metal exposure of PBMC and THP-1. PBMC (A) and THP-1 cells (B) were exposed to increasing concentrations of gold thiosulfate and nickel chloride (in μM) or mercury chloride (in nM) for 24 h. Bars represent mean ± SD from three independent experiments (for PBMC $n = 3$ donors). Asterisks specify statistically significant (one way ANOVA and Kruskall–Wallis test (non parametric ANOVA)) differences in production of IL-8, IL-6 by cells at the given metal concentration as compared to the control medium (open bar): $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***). ND = not detectable.

TLR 3 and TLR2 receptors, since none of the metals, nor the control ligands induced responses in wild type, non transfectant HEK293 cells (Fig. 5).

4. Discussion

Inflammation is a protective response by the body to ensure removal of detrimental stimuli, as well as a healing process for repairing damaged tissue (Medzhitov, 2008). Inflammation is caused by various factors such as microbial infection, tissue injury and exposure to toxic materials. The primary contributor to acute inflammation is innate immune reactivity, which can also recruit adaptive immune responses. Whereas parenchymal and stromal cells, such as epithelial cells, endothelial cells and fibroblasts, contribute to innate immunity, pivotal roles are played by innate immune cells including macrophages and DC. These cells express high levels of germline-encoded pattern recognition receptors (PRRs) which are responsible for sensing the presence of microorganisms. The recognized structures are often conserved among microbial species, and called pathogen associated molecular patterns (PAMPs). The most prominent family of PRRs comprises transmembrane proteins, the Toll-like receptors (TLRs). Recent evidence indicates that one of this family member, i.e. TLR4 is also triggered by distinct low-molecular weight transition metals, such as nickel, cobalt and palladium (Schmidt et al., 2010; Raghavan et al., 2012; Rachmawati et al., 2013). This finding has shed new light on clinical and experimental experiences with these metals showing remarkable activities in inducing innate as well as adaptive immune responses. Here, we considered that some other, high molecular weight, transition metals are widely used in dental applications, i.e. gold and mercury, but also gained serious disrepute from immunotoxic capacities. The present study, therefore, was set up to further explore the capacities of the latter metals to activate TLR-mediated cell signalling.

As reported earlier, TLR-signalling in DC is most readily detected by pro-inflammatory cytokine/chemokine (notably IL-8, and to a lesser extent CCL2 and IL-6) release, next to augmented

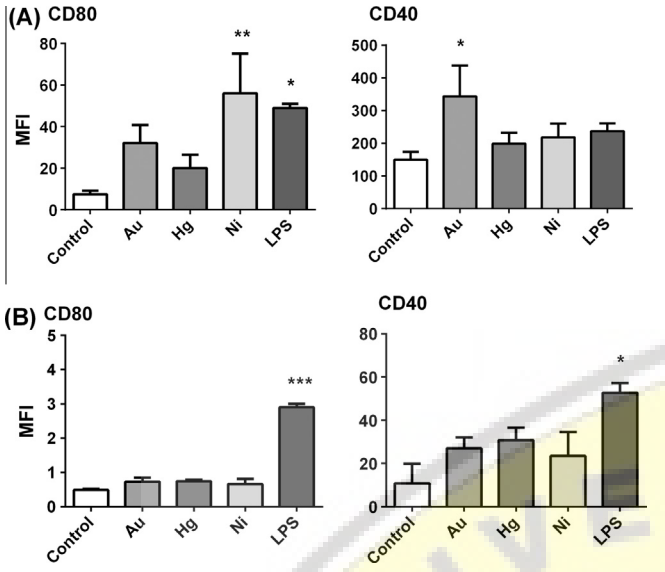

Fig. 4. Phenotypic maturation after metal exposure of MoDC or THP-1 cells. MoDC (A) or THP-1 cells (B) were exposed for 24 h to LPS, gold thiosulfate and nickel chloride (500 μM) or mercury chloride (500 nM) as indicated. Bars represent mean ± SD from three independent experiments (for MoDC: $n = 3$ donors). Asterisks specify statistically significant (one way ANOVA and Kruskall–Wallis test (non parametric ANOVA)). MFI was calculated by mean fluorescence intensity of DC stimulated with metals as compared to mean fluorescence non-stimulated cells (open bar): $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$.

expression of distinct surface molecules reflecting maturation and adaptive immune stimulatory capacity (CD80 and CD40) (Toebak et al., 2006). In particular IL-8 is abundantly produced, which actually led to its early identification as a major interleukin, whereas the costimulatory molecules are pivotal in providing help to adaptive immune cells (Munroe, 2009). The present study confirms the release of IL-8 as a most sensitive read-out for innate immune signalling, whereas the most remarkable finding was that gold salt was very effective in this regard which, using TLR transfectant cell lines, could be ascribed to triggering TLR3. The gold salt used for testing was gold-thiosulphate, which contains gold ions at their lowest oxidation state, thus representing the first ions generated upon oxidation of metallic gold. This salt is also used for skin testing gold contact allergy, as well as used for anti-rheumatoid arthritis treatment. Actually, the latter feature of gold, i.e. immunosuppression, would appear contradictory to its innate immune stimulatory activity, which has intrigued clinicians and (immuno) toxicologists for many years (Buckley et al., 2011; Merchant, 1998). Gold (I) salts (auranofin, sanocrysin) may exert immunosuppressive actions through inhibiting IkappaB kinase activation and/or through pro-apoptotic activities (Jeon et al., 2003; Kim et al., 2004). Gold-induced release of the immunosuppressive mediator IL-10 may not play a role in this feature, as observed here. Still, pro-inflammatory activity can be clearly exerted through direct triggering of TLR3, and increased release of downstream mediators. The net outcome of these seemingly contradictory effects may depend on ill-defined secondary factors. At any rate, the latter pro-inflammatory activity can be expected to contribute to the frequent development of adaptive immune responses to gold (Martin et al., 2011). How sufficient gold ions reach the intracellular locale of TLR3, i.e. lysosomal surfaces, for triggering is as yet unclear. Alternatively, small numbers of extracellular TLR3 receptor molecules suffice for this purpose. Since gold (I) can be further oxidized inside phagocyte lysosomal compartments and resulting gold (III) represents a major hapten in gold allergy (Goebel et al., 1995), these effects may act synergistically in sensitization. Exposure to gold salt indeed also led to augmented expression of co-stimulatory molecules, although this effect was less pronounced than observed with nickel. Anyhow, the gold paradox is not unique, since also e.g. steroids are known to exert both immunosuppressive and immunostimulatory effects (Baeck and Goossens, 2012).

While mercury does not present a paradox, its toxicities are undisputed. It is a non-essential metal in the human body, whereas it is ubiquitously distributed in the environment. Certainly because of its superior mechanical features and wide availability, the usage of mercury in dental amalgams has found its way across all continents. But, because of its toxicity and the appearance of competitive dental composite materials, the use of dental amalgams has now become extinct in affluent societies. Major toxic activities include its irreversible reactivity with selenium, an essential dietary element required by selenoenzymes (Reeves and Hoffmann, 2009). These enzymes prevent and reverse oxidative damage in

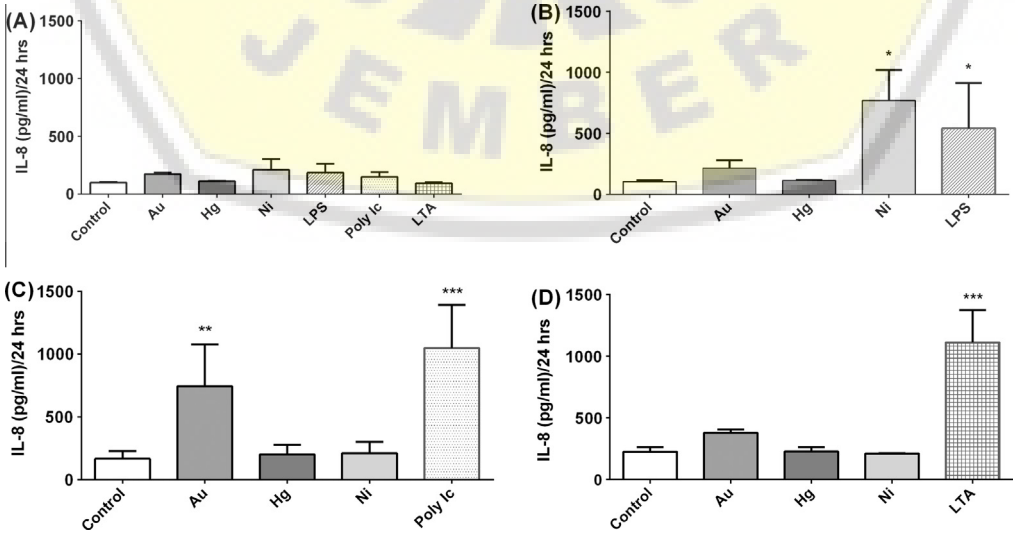

Fig. 5. IL-8 production after metal exposure of HEK WT (A), TLR4/MD2 (B), TLR 3 (C) and TLR 2 (D) transfectant cells. HEK293 WT and transfectant cells were exposed for 24 h to LPS (50 ng/ml), LTA (50 ng/ml), poly-IC (50 ng/ml), gold thiosulfate and nickel chloride (500 μM) or mercury chloride (500 nM) (grey bars) or culture medium as indicated. Results are shown from three independent experiments (values are mean ± SD). For statistical analysis, the highest dose values were compared with the medium control (one way ANOVA and Kruskall–Wallis test (non parametric ANOVA): $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$).

cells, e.g. in the brain and endocrine organs. Here, we observed an hitherto not reported effect of mercury, i.e. stimulation of innate immunity. Given our dental focus, we did not include organic mercury in these experiments. Like with gold, for mercury salt this triggering of innate immunity was most clearly revealed by IL-8 release. Using the available limited panel of TLR transfectants we could not identify the triggering receptor(s) involved.

In fact, these might also belong to any of the alternative PRR families, i.e. C-type lectin receptors (CLR), retinoic-acid inducible gene (RIG)-I-like receptors (RLR) or NOD-like receptors (NLR). Preliminary experiments on the pathways involved, using the p38 MAPK blocker SB203580, resulted in strong (70–80%) suppression of both gold and mercury-induced IL-8 production by THP-1 cells [data not shown], indicating that the innate signalling by mercury, like for gold, involves p38 MAPK phosphorylation. Anyhow, the innate triggering capacity of mercury as revealed in the present experiments is likely to contribute to its irritant properties, causing e.g. pustular lesions, and to its sensitizing capacity (Mutter, 2011), as well as to its putative role in the induction of autoimmunity (Nielsen and Hultman, 2002).

5. Conclusions

To conclude, the present study adds the high molecular weight transitional metals gold and mercury to the panel of metals showing distinct innate immune stimulatory capacities. For gold, evidence was obtained for a role of TLR3 in activation of mononuclear cells. This activation was robust, and could be detected in freshly prepared monocyte-derived DC, as well as in unseparated PBMC and in monocytoid cell line THP1 cells. Of course, clinical relevance of these findings relates to the local release of the metal ions tested, i.e. Au (I) and Hg (II), from alloys or amalgams. Application of alloys and amalgams with lowest release of such ions would reduce the immunotoxic risks as outlined. For gold, appropriate alloys have been identified, whereas for amalgams continued use for dental applications seems contra-indicated.

Conflicts of Interest

The authors declare that there are no conflicts of interest.

Transparency Document

The Transparency document associated with this article can be found in the online version.

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