Innate immune reactivity to dental alloys

Rachmawati, D.

2016

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CHAPTER 4

Dental metal-induced innate reactivity in keratinocytes

Dessy Rachmawati[1,2], Jeroen K. Buskermolen[4], Rik J. Scheper[1], Susan Gibbs[3,4], B. Mary E. von Blomberg[1], Ingrid M.W. van Hoogstraten[1]

[1]Department of Pathology, VU University Medical Center, Amsterdam, Amsterdam, The Netherlands
[2]Department of Biomedical Sciences and Prostodontics, Faculty of Dentistry, University of Jember, Indonesia
[3]Department of Dermatology, VU University Medical Center, Amsterdam, Amsterdam, The Netherlands
[4]Department of Oral Cell Biology, Academic Centre for Dentistry Amsterdam, University of Amsterdam and VU University Amsterdam, The Netherlands

Toxicology in vitro, 2015, Vol.30, pp.325-30

ABSTRACT

Gold, nickel, copper and mercury, i.e. four metals frequently used in dental applications, were explored for their capacity to induce innate immune activation in keratinocytes (KC). Due to their anatomical location the latter epithelial cells are key in primary local irritative responses of skin and mucosa. Fresh foreskin-derived keratinocytes and skin and gingiva KC cell lines were studied for IL-8 release as a most sensitive parameter for NF-kB activation. First, we verified that viral-defense mediating TLR3 is a key innate immune receptor in both skin- and mucosa derived keratinocytes. Second, we found that, in line with our earlier finding that ionized gold can mimic viral dsRNA in triggering TLR3, gold is very effective in KC activation. It would appear that epithelial TLR3 can play a key role in both skin- and mucosa localized irritation reactivities to gold. Subsequently we found that not only gold, but also nickel, copper and mercury salts can activate innate immune reactivity in keratinocytes, although the pathways involved remain unclear. Although current alloys have been optimized for minimal leakage of metal ions, secondary factors such as mechanical friction and acidity may still facilitate such leakage. Subsequently, these metal ions may create local irritation, itching and swelling by triggering innate immune reactions, potentially also facilitating the development of metal specific adaptive immunity.

INTRODUCTION

Metal alloys in dental appliances are located intra-orally for years to decades. During this time period metal components begin to dissolve or corrode with various bio-pathological consequences. The most frequent manifestation of metal-induced complaints is local irritation, potentially facilitating systemic contact hypersensitivity (Martin 2015; Muris et al. 2014; Rachmawati et al. 2015). To study potential direct effects on the outermost cells in skin and mucosa, keratinocytes, we selected four most widely employed dental metals, i.e. gold, mercury, copper and nickel.

Gold alloys are widely used for dental restorations in developed countries 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 an irritant and contact allergen (Ahlgren et al. 2002; Moller 2002). The use of mercury-based amalgam for material fillings in dentistry has declined over recent years due its potential negative health effects. These may vary from autoimmunity (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. Of note, amalgam also contains nickel and copper. Copper has been used for a long time in dentistry as a strengthener and color enhancer in Au-Ag-Cu crowns and bridge alloys (Muris 2015). Copper is an abundant element, poisonous to higher organisms but at lower concentrations an essential trace nutrient to all animal life. Allergic reactivity to copper is usually associated with other metal allergies (notably nickel) potentially resulting from concomitant sensitization or cross-reactivity (Pistoor et al. 1995). Nickel is a major component of stainless steel alloys and is widely used in orthodontics e.g. for brackets and orthodontic retention wires (Milheiro et al. 2012). Actually, these appliances can release distinct amounts of nickel and could also be responsible for extra-oral eczema even in the absence of local reactions. Allergies to nickel are very common and usually associated with exposure to jewelry, piercing, consumer products, and/or medical devices (Thyssen et al. 2009).

The innate immune response is the first line of host defense against exogenous toxic threats, including metals and microorganisms. Recognition of microbial factors involves signaling through specific receptors, of which the TLRs represent a major group. TLRs recognize various so-called pathogen-associated molecular patterns (PAMPs) conserved in microorganisms, including triacylated lipoproteins (TLR1/2 agonist), diacylated lipoproteins

(TLR2/6 agonist), double-stranded RNA (TLR3 agonist), LPS (TLR4 agonist), flagellin (TLR5 agonist), single-stranded RNA (TLR7, TLR8 agonist) and CpG motifs in DNA (TLR9 agonist) (Cloonan and Choi 2012). Following pathogen binding, TLRs mediate activation of innate immune responses through modulation of inflammatory gene expression by immune cells. This may subsequently facilitate adaptive immune responses involving pathogen-specific T and/or B cells. Whereas TLR receptors are abundantly present on dendritic cells, primary contacts with exogenous microorganisms are with epithelial keratinocytes. Also keratinocytes express TLRs, thus providing a first layer of defense in skin and mucosa (Baker et al. 2003; Mempel et al. 2003). Indeed, exposure of KC to microbial constituents known as TLR ligands led to activation of the NFkB pathway as revealed by release of a.o. IL-8 (Lebre et al. 2007). Still, no consensus exists on which TLR members show functional expression on KC. Some studies report functional expression of at least 4 members, i.e. TLR3, 4, 5 and 9 (Lebre et al. 2007; Flacher et al. 2006; Olaru and Jensen 2010) whereas other data suggest only substantial expression of TLR3 (Kollisch et al. 2005; Oosterhoff et al. 2013). After the discovery that nickel and cobalt metal ions could associate with TLR4 thus activating downstream signaling (Schmidt et al. 2010), we recently reported that palladium ions show the same capacity (Rachmawati et al. 2013). Subsequently we found that gold-ions could trigger TLR3, whereas mercury and copper also activated IL-8 release, the latter metal potentially via TLR5 (Rachmawati et al. 2015). In these studies next to TLR-transfected cells, various immune cells were used, including PBMC, MoDC and THP-1 cells.

In the present study, we decided to explore the potential of distinct metals, frequently used in dental applications, to activate the innate immune pathway in keratinocytes. Due to their anatomical location and critical role in skin and mucosal inflammatory and immunological reactions, these epithelial cells are key in primary local responsiveness. Next to fresh foreskin-derived keratinocytes, skin and gingiva KC cell lines were used. IL-8 was selected as a read-out while this chemokine is the most abundantly produced inflammatory cytokine (Coquette et al. 2003; Toebak et al. 2006). Most importantly, NF-kB signalling as revealed by IL-8 release highlights not only signalling via TLR but also other major innate immune receptor pathways, such as NLR, CLR, RLR (Caruso et al. 2014; Kawamura et al. 2014)(. and EGFR (Frankart et al. 2012).
We first set out to identify most prominent TLR receptors on KC. Then, with TLR3 surfacing as the major receptor we verified that Au, as frequently used in dental and skin contexts, would not only directly stimulate DC but also keratinocytes. Secondly, we hypothesized that other dental relevant transition metals, in particular Ni, Hg and Cu might also directly activate the TLR-NF-kB-IL8 pathway in keratinocytes: via TLR4 (Ni) or unknown (Cu, Hg).

MATERIALS AND METHODS

Metal Chemicals

As metal allergens the following chemicals were used: nickel (II) chloride hexahydrate ($NiCl_2.6H_2O$), sodium gold thiosulfate ($Na_3Au(S_2O_3)_2.2H_2O$; Chemotechnique Diagnostics, Vellinge, Sweden), Copper sulphate ($CuSO_4$), mercuric chloride ($HgCl_{2;}$ Riedel-de Haën, Seelze, Germany). LPS was obtained from Escherichia coli 055:B5 (Sigma, St Louis, MO, USA). $Na_3Au(S_2O_3)_2.2H_2O$, $HgCl_2$, $CuSO_4$ and $NiCl_2$ were dissolved in H_2O as stock solutions and further diluted with culture medium just before use.

Primary keratinocytes

Human foreskin was obtained from healthy donors. The VU University medical centre approved all experiments. The study was conducted according to the Declaration of Helsinki Principles. Epithelial keratinocytes (KC) were isolated from healthy skin essentially as described earlier (Kroeze et al. 2012). In brief, KC were cultured in KC medium (Dulbecco's Modified Eagle Medium (DMEM) (Lonza, Basel, Switzerland)/Ham's F-12 (Gibco, Grand Island, USA) (3:1) containing 1% UltroserG (BioSepra S.A. Cergy-Saint-Christophe, France), 1% penicillin-streptomycin (Gibco), 1 µmol/l hydrocortisone, 1µmol/l isoproterenol, 0.1µmol/l insulin containing 2ng/ml keratinocyte growth factor (KGF) at 37°C, 7.5% $CO_{2.}$ Cultures were passaged when 90% confluent, using 0.5mM EDTA/0.05% trypsin (Gibco) and used for experiments at passage 2.

NCTC 2544 cells

Skin-derived NCTC 2544 keratinocytes (Institute Zooprofilattico di Brescia, Brescia, Italy) were used at passage 37. The cells were grown in 75 cm^2 culture flasks and maintained in RPMI containing 2mM L-glutamine, 2% pen/strep, supplemented with 10% heated-inactivated fetal calf serum. $1.25x10^6$ cells were cultured per flask (10 ml culture medium) for 3 days at 37C in 5% $CO_{2.}$ NCTCC 2544 cells were reported earlier to show different responses to irritants and contact allergens (Corsini et al. 2013).

Gingival keratinocyte cell line

The gingiva keratinocyte cell line OKG4 cells (Lindberg and Rheinwald 1990) was provided by J.G. Rheinwald, Harvard Skin Disease Research Centre, Boston, MA, USA, and cultured in KC medium exactly as described above for primary keratinocytes. Cells were used for experiments at passage 39-41.

TLR ligand exposure

Primary KC, NCTC 2544 and OKG4 cells ($4x10^5$ cells/well) were cultured in 6 well plates and exposed to TLR ligands : TLR3 (poly Ic, working concentration: 1 & 10 µg/ml); TLR4 (LPS: 50 & 100 ng/ml); TLR5 (Flagellin: 10 & 100 ng/ml); TLR7/8 (Loxoribine: 1 & 5 mM); TLR9 (CpG: 1

& 10 µg/ml). Total volume in each well was 2 ml. Cells ($4x10^5$ cells/well), supernatants were collected after 24 hours and kept at -20°C until IL-8 assessment.

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). 2 mL of cells ($4x10^5$/well) were plated in 6 well culture plates and exposed to increasing concentrations of the metals. After 24 hours incubation supernatants were removed and 1 ml of MTT solution (7.5 mg/ml) was added per well. MTT solution was prepared freshly and dissolved with PBS. The plates were incubated in the dark at 37˚C. After 2-3 hours of incubation, 1ml DMSO (Merck, Darmstad, Germany) was added to each well and after shaking, the solution was measured using an ELISA reader at optical density (OD) 490 nm. The viability 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%. The MTT analysis was performed in 96-well plates, essentially as described in detail previously (Gibbs et al. 2013).

Metal exposure

Primary KC and NCTC 2544 ($4x10^5$ cells/well) were exposed to metals (Na3Au(S2O3)2.2H2O, CuSO4, HgCl2 and NiCl2) at concentrations between 0 and 3 mM (0 – 3 nM for HgCl2). Plates were incubated at 37˚C in 5% humidified CO_2. After 24 hours, supernatants were collected and stored at -20˚C until measurement. Complementary checks for LPS contamination were carried out with the *Limulus amebocyte lysate* (LAL) assays (Kinetic-QCL™ bulk kit, Lonza). For metal exposure, PKC and NCTC 2544 cells were exposed to $Na_3Au(S_2O_3)_2.2H_2O$, $CuSO_4$, $HgCl_2$ and $NiCl_2$ at 6 different serial dilutions, 3 mM (for $HgCl_2$ µM) as the highest concentration. Total volume in each well was 2 ml. Cells ($4x10^5$ cells/well), supernatants were collected after 16-24 hours and kept at -20°C until cytokine/chemokine assessment (see below).

Cytokine/chemokine production

IL-8 production was measured by Enzyme-linked immunosorbent assay (ELISA) with Peli-Kine ELISA kits (Sanquin, Amsterdam, The Netherlands) 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 IL-8 in the supernatant was assessed by using standard curves (lower detection limit of IL-8: 15.4 pg/ml).

Data analysis

Data are presented as the mean IL-8 production of 4 independent experiments ± SD, except for $HgCl_2$ in PKC n = 3 independent experiments. 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≤ 0.05 was considered statistically significant.

RESULTS

First, to explore functional expression of TLRs on human keratinocytes, a panel of microbial ligands was tested for their capacity to induce IL-8 release in primary keratinocytes (PKC). As presented in Figure 1a (upper panel), only the TLR3 ligand poly- I:C induced substantial IL-8 release, whereas LPS (TLR4 ligand), Flagellin (TLR5 ligand), loxoribine (TLR7 and 8 ligand), and CpG (TLR9 ligand) failed to produce detectable signalling in PKC. In subsequent experiments, essentially similar results were obtained with the skin-derived NCTC and gingiva-derived keratinocyte cell lines (OKG4 cells) (Fig 1b and 1c).

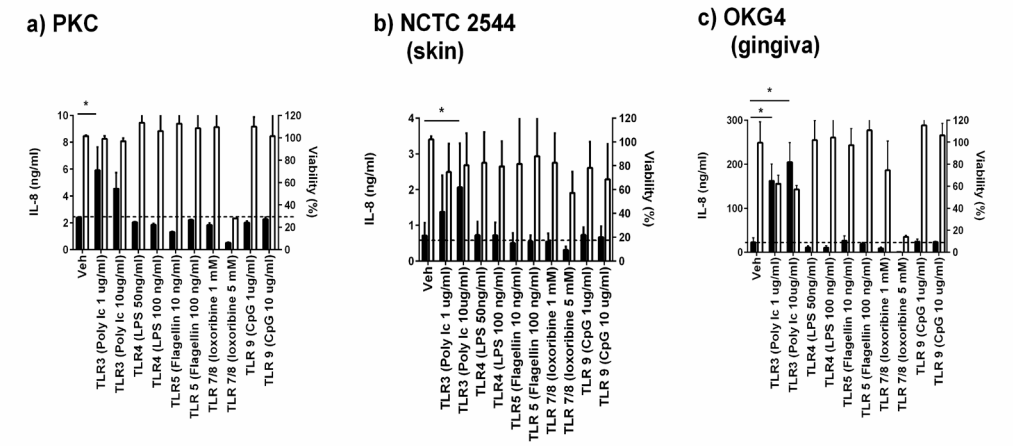

Figure 1. a) primary keratinocytes (PKC; 3 donors), b) skin-derived keratinocyte cell line cells (NCTC 2544) and c) gingiva-derived keratinocyte cell line cells (OKG4) were exposed to working doses of TLR ligands for 24 hours. Values are mean ± SD of 3 independent experiments. IL-8 secretion by stimulated cells is compared to that of vehicle-stimulated cells. Black bars represent IL-8 production (ng/ml); white bars represent viability of the cells (%). Asterisks indicate a statistical significant increase in secretion compared to respective vehicle controls: p ≤ 0.05 (*).

These results then could be utilized for testing our first hypothesis, i.e. that Au, as frequently used in dental and skin contexts, does not only directly stimulate immune effector cells such as DC, but also human keratinocytes. As shown in Figure 2 (upper panels), both freshly prepared PKC and NCTC cell line cells showed indeed strong IL-8 release when exposed to $Na_3Au(S_2O_3)_2.2H_2O$ in a marginal to intermediate toxic dose-range. These results are in line with our earlier finding that Au is able to induce TLR3 triggering.

Subsequently, when testing the extended panel of dental metals in marginal to intermediate toxic dose ranges, it appeared that also mercury (of note: at 1000-fold lower concentrations

than the other metals) and copper elicited substantial IL-8 release in both fresh KC and NCTC cells. Of note, copper showed the most prominent signal at the lowest, marginal toxic dose tested. On the other hand, less prominent IL-8 release by keratinocytes was observed after exposure to nickel-ions. Thus, despite different response patterns, all four dental metals tested showed distinct innate immune activation of keratinocytes.

DISCUSSION

Whereas earlier studies reported on the presence of multiple TLRs on keratinocytes (Mempel et al. 2003; Lebre et al. 2007), the present ligand-stimulation data highlight the high functional expression of TLR3 on these cells. Actually, a similar prominent role of this particular TLR on KC had been noted in passing by others (Oosterhoff et al. 2013; Kollisch et al. 2005). It would appear that recognition of viral intruders is of key importance in epithelial defenses against microorganisms, since TLR3 recognizes double-stranded RNA (dsRNA) which is found during the replication cycle of most viruses (Miller 2008). To this end, TLR3 is primarily located intracellularly, and for downstream signaling dependent on TRIF rather than MyD88 adaptor molecules, which are used by most other TLRs. Utilization of the TRIF pathway by TLR3 results in the activation of both NFκB and MAP kinases in a similar manner as the MyD88 pathway, and translates into IL-8 release. Moreover, in contrast to MyD88, TRIF activates interferon (IFN)-regulatory factors promoting the production of type I interferon (i.e. IFNα and IFNβ) (O'Neill and Bowie 2007). These type I interferon responses are critical in the immune response against viruses. It should be noted that we observed the prominent expression of TLR3 in both primary KC, and skin- and mucosa derived KC cell lines. Still, variable expression and potentially increased roles of other TLRs may depend on secondary differentiation and activation events.

The prominent role of TLR3 did allow for verification of KC responsiveness to gold-ions, as postulated from our earlier findings (Rachmawati et al. 2015). Indeed, substantial IL-8 production was observed at low to intermediate levels of Au(1+) ions, as generated from dissolving Na3Au(S2O3)2.2H2O. Most likely, the potent innate immune reactivity-stimulating capacity of gold plays a role in its irritant capacity. Although this particular gold compound is still considered one of the least irritating salts and widely used for testing skin allergy to gold, it still may cause the development of mouth sores, e.g. when used for treating rheumatoid arthritis (Taukumova et al. 1999). Also, gold crowns can elicit local redness and pain, and a positive relationship between presence and amount of dental gold alloys and contact allergy to gold has been reported (Ahlgren et al. 2002). The present results do confirm our hypothesis that TLR3 can play a key role in these effects.

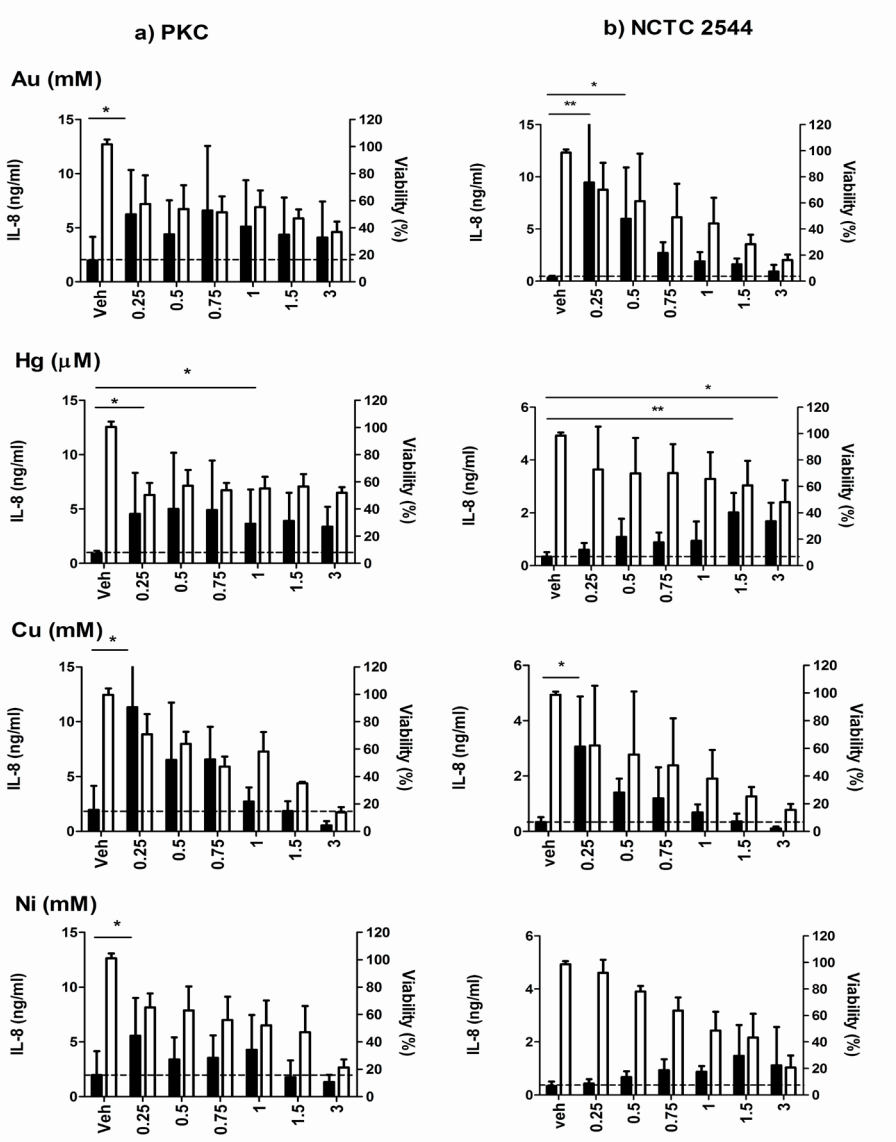

Figure 2. a) primary keratinocytes (PKC)and b) keratinocyte cell line cells (NCTC 2544) were exposed to 6 doses of metal salts, for 24 hours. Culture supernatants were analyzed for IL-8 by ELISA. Values are mean ± SD of 4 independent experiments except for HgCl2 in PKC in 3 independent experiments. IL-8 secretion by stimulated cells is compared to that of vehicle-stimulated cells. Black bars represent IL-8 production (ng/ml); white bars represent viability of the cells (%). Asterisks indicate a statistical significant increase in secretion compared to respective vehicle controls, *p ≤ 0.05, **p ≤ 0.005.

As to the other dental metals tested, i.e. nickel, copper and mercury, the present results show that they are capable of directly triggering innate immune reactivity in KC, as revealed

by IL-8 release, but the involvement of TLR triggering remains as yet unclear. Because nickel-responsiveness of immune effector cells, notably dendritic cells, is known to associate with productive TLR4 binding, the low responsiveness of KC to nickel fits with relatively low TLR4 expression on these cells. In contrast, copper shows strongly positive responses in KC, which paralleled the earlier observed high responsiveness of wild-type human embryonal kidney HEK293 cells (Rachmawati et al. 2013). Of note, the latter cells are notoriously low in expressing TLRs (Hornung et al. 2002), which actually facilitates their use in studying roles of transfected TLR molecules (Liu et al. 2013), but do express low levels of TLR5 (Kollisch et al. 2005). However flagellin, a TLR5 ligand, did not stimulate KC. The strong innate signaling by copper ions may therefore rather utilize alternative pattern recognition receptors, e.g. C-type lectin receptors (CLR), retinoic-acid inducible gene (RIG)-I-like receptors (RLR) or NOD-like receptors (NLR). Actually, the same reasoning holds true for mercury and the obtained results justify follow-up with more in-depth studies, e.g. with transfected cell line panels and testing a wide array of inflammatory cytokines, into the actual pathways involved in mercury, copper and nickel responses.

In conclusion, viral-defense mediating TLR3 is a key innate immune receptor on both skin- and mucosa derived keratinocytes. Since ionized gold can mimic viral dsRNA in triggering TLR3, epithelial TLR3 is likely to play a key role in both skin- and mucosa localized irritation reactivities to gold. Actually, it is surprising that gold is a strong innate activator of both DC and KC, whereas clinical irritation/sensitization for gold is infrequent. Most likely its nobility assures very low generation and leakage of gold-ions from dental appliances. Still, distinct patients may suffer from increased leakage by several factors acting alone or in concert: mechanical friction, interfering effects of other metals, and acid or bacterial effects, etc. For the base metal nickel similar factors may play a role albeit with different intensities: while monocyte/DC stimulatory power is strong, KC stimulatory power is weak at most. But, for nickel as a base metal ionization is certainly easier than for gold. Fortunately current alloy compositions show very low nickel-release (Senkutvan et al. 2014) and complaints concerning oral nickel appliances are rare despite their widespread use in orthodontic treatments. Of note, the high frequency of skin allergy (allergic contact dermatitis) to nickel may not add much to the infrequent oral nickel-problems given the fact that the causative effector T cells are strongly biased towards skin-migration rather than influx into mucosal surfaces (Geginat et al. 2014; Islam and Luster 2012).

Next to gold and nickel, also copper and mercury can activate innate immune reactivity in keratinocytes. Since the latter base metals are also widely used as components of metal alloys for restorative treatments, the respective appliances are clearly well-designed to prevent untoward leakage of metal ions. Still, also for these metals secondary factors may facilitate this leakage, e.g. mechanical friction or food and fluid consumption, and individual-related factors, ultimately triggering local innate immunity with irritation, itching

and swelling. Since the four metals tested can also be presented by dendritic cells to trigger specific T cell responses, innate stimulation may incidentally also generate adaptive immune responses. Whether this ultimately leads to systemic tolerance or allergic reactivity may depend on other cofactors, notably concomitant microbial pressure by oral bacteria (de Kivit S et al. 2014).

ACKNOWLEDGEMENTS

This study was supported by The Ministry of Research, Technology and Higher Education of The Republic of Indonesia. We also thank Sander Spiekstra and Ilona Kosten, Dept of Dermatology, VUmc Amsterdam, for their help with primary keratinocyte cell culturing.

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