CHAPTER 8

Summary, discussion and conclusions
In this chapter, our findings are summarized and the most relevant findings are highlighted. Different experimental set-ups, as outlined in the respective chapters, were used to address six important questions summarized at the end of Chapter 1. All questions dealt with putative relationships between dental metal exposure and subsequent adverse inflammatory events. While the individual experimental results have been discussed in the previous chapters, our current answers to the six questions will be summarized below.

1. **IS THE CAPACITY OF METALS TO STIMULATE INNATE IMMUNE CELLS, I.C. DENDRITIC CELLS, CORRELATED WITH THEIR SENSITIZING POTENTIAL?**

The development of metal allergy requires several steps, in particular metal exposure, ion release, penetration of skin or mucosa, protein binding, innate triggering and availability of specific T cells (see Chapter 1, Table 5; 5.4.2.). The potential of a metal ion to become allergenic is thus strongly determined by its chemical reactivity whereas in particular its capacity to provide danger signals to the innate immune system seems important. The present study shows that strong skin contact sensitizers such as Ni, and to a lesser extent Au, Pd and Co, indeed provide danger signals to innate immune cells, Ni, Co and Pd via TLR4 and Au most likely via TLR3 triggering (Chapter 2 and 3 resp.). Cr, however, is unexpectedly inactive in the stimulation of innate cells (Chapter 8; Table 1). Of note, patch tests revealed that Cr is a potent skin sensitizer and one of the most common causes for ACD (Thyssen and Menne 2010). E.g. trivalent Cr which is used for leather tanning is a very strong sensitizer, and many individuals develop allergic reactions to Cr in finished leather products such as gloves and shoes (Hansen et al. 2002; Shackelford and Belsito 2002). Our finding that Cr does not trigger innate signalling suggests that sensitization to Cr is facilitated by external co-factors that provide the required danger signals. These may be either bacterial or viral factors or other metals like Ni and Co which are often present together with Cr in metal alloys.

In contrast, some other metals, e.g. Zn and Cu which are known as typical non-sensitizers, showed distinct innate stimulatory activity, in particular in synergy with microbial endotoxin (Chapter 6). Still, these effects were most pronounced at supra-physiological concentrations. Moreover, since Zn and Cu are essential metals in various metabolic processes and abundantly present in body fluids, tolerizing mechanisms may dominate for these metals including lack of specific T cells or abundance of regulatory T cells.

Hg is a special case. Despite its long-known toxicity it has been successfully used in amalgam dental alloys for several decades. Release from Hg ions from the amalgam alloys is apparently extremely small. Intriguingly, most research found no relationship between Hg fillings and symptoms of Hg toxicity. Also allergy to Hg has only been reported in small numbers of
individuals (Garner 2004). Still, we found Hg to show a distinct capacity to elicit danger signals in innate immune cells.

In conclusion, although stronger innate triggering most likely facilitates strong sensitization (e.g. with Ni), the correlation between innate stimulation and sensitization by metals is far from absolute. Despite strong innate triggering, low exposure and/or low frequency of specific T cells may still cause lower sensitization rates (e.g. Au, Hg). On the other hand, low innate triggering may be compensated by high concomitant innate triggering by other alloy-components or micro-organisms (e.g. with Cr).

**Table 1.** Frequencies of metal allergies in dermatology and oral disease patients in relation to innate immune stimulatory capacities

<table>
<thead>
<tr>
<th>Metal</th>
<th>Frequency of metal allergy in Dermatology patients a)</th>
<th>Frequency of metal allergy in oral disease population b)</th>
<th>Innate stimulatory capacity c)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>DC</td>
<td>Microglia</td>
<td>THP-1</td>
</tr>
<tr>
<td>Ni</td>
<td>17.1 %</td>
<td>19.8 %</td>
<td>+++</td>
</tr>
<tr>
<td>Au</td>
<td>13.5 %</td>
<td>12.8 %</td>
<td>++</td>
</tr>
<tr>
<td>Co</td>
<td>10.3 %</td>
<td>10.2 %</td>
<td>+++</td>
</tr>
<tr>
<td>Cr</td>
<td>6.7 %</td>
<td>13.8 %</td>
<td>-</td>
</tr>
<tr>
<td>Pd</td>
<td>NT d)</td>
<td>9.7 %</td>
<td>+</td>
</tr>
<tr>
<td>Hg</td>
<td>NT</td>
<td>9.7 %</td>
<td>++</td>
</tr>
<tr>
<td>Cu</td>
<td>NT</td>
<td>3.6 %</td>
<td>+</td>
</tr>
<tr>
<td>Zn</td>
<td>NT</td>
<td>0.6 %</td>
<td>+</td>
</tr>
<tr>
<td>Ti</td>
<td>NT</td>
<td>1.1 %</td>
<td>NT</td>
</tr>
</tbody>
</table>

a. Patients suspected for metal allergy visiting a dermatology clinic (Davis et al. 2011)  
   b. Median frequency in oral disease population from studies mentioned in table 4  
   c. Innate cells responses to metals (Rachmawati et al. 2015; Rachmawati et al. 2015; Rachmawati et al. 2013)  
   d. NT: not tested

2. **CAN THE LOW METAL CONCENTRATIONS RELEASED FROM DENTAL ALLOYS STIMULATE INNATE IMMUNE CELLS?**

In the initial experiments with metal salts (Chapters 2, 3 and 4) non-toxic but high concentrations of metals were examined. However, the release of metal ions from dental constructions generally yields much lower systemic concentrations, even though erosion and galvanic or chemical corrosion are reported to result in detectable levels in blood and urine (Matusiewicz 2014).

In the present study we tested dental cast alloys *in vitro* as solid specimens, reflecting the actual situation in the oral cavity (Chapter 5). All of them did induce IL-8 production by
MoDC. Although the direct contact of the cells with the alloys may have contributed to this activation, it is remarkable that also the concentration of metal ions released from these alloys into culture medium in 24 hrs appeared to be high enough to induce innate immune reactions (Chapter 5, figure 1c). The latter finding was most pronounced for the dental alloys that contained Pd-Cu or Au.

These results support the view that exposure to dental alloys can contribute to oral inflammatory symptoms. Repeated or constant exposure to metal alloys at low concentrations over long periods was indeed shown to induce chronic oral lesions (Estlander et al. 2006). Also systemic illnesses as fatigue and autoimmune/inflammatory diseases have been ascribed in some studies to chronic release of metal ions from dental constructions (Stejskal 2014). Whether allergy or an increased inflammatory state due to innate activation plays a major role here, is still not known. Obviously, patients being allergic for metals, as seemed to be the case in the latter study, do react to much lower metal concentrations than non-allergic individuals. Also, the release of metal ions in these patients may have been much higher than expected on the basis of the quality of the alloy due to extreme corrosive environments in the oral cavity. Galvanic corrosion has been established as an important cause of increased metal ion release (Lee et al. 2015). Also the local conditions in the oral cavity, created by a.o. temperature, microbiota, pH, saliva, exogenous agents like tobacco and alcohol, can significantly promote intra-oral corrosion.

Since we were not able to measure the actual metal ion release in the alloy supernatants, we tested additionally (Chapter 6) low, near-physiological concentrations of transition metals (up to 10 fold the normal plasma levels, (Roos et al. 2013) for their innate stimulatory potential. Only Zn and Cu did induce IL-8 production now; of note, both Zn and Cu are present in relatively high concentrations in plasma, and thus in our experiments. The other transition metal salts (Cr, Fe, Co, Ni, Au and Hg) were, however, not stimulatory for innate cells in physiologic concentrations. This is in line with those in vivo studies that failed to provide evidence for real health risks due to dental appliances (Jacobsen and Hensten-Pettersen 2003; Janson et al. 1998; Jensen et al. 2003). Even for amalgam, serious adverse reactions and allergy are rare (Bains et al. 2008; Barregard et al. 1995; Bellinger et al. 2006; Bergdahl et al. 2013). Notably, the oral lichen-oid lesions occasionally seen in non-allergic individuals in the neighborhood of amalgam fillings or other dental reconstructions, are most likely due to an increased release of metal ions and subsequent innate activation and irritation of the mucosa.

In conclusion, this study provides additional evidence that exposure to dental alloys does not pose a serious health risk to patients, unless under extreme corrosive conditions or in metal allergic individuals. Of note, as outlined in Chapters 5 and 6, also concomitant bacterial infections may lower the concentration threshold for innate responsiveness to metals.
3. DOES CO-STIMULATION WITH MICROBIAL MOLECULES, E.G. ENDOTOXIN, AFFECT METAL-INDUCED INNATE RESPONSES?

Dental metal constructions in the oral cavity constantly interact with saliva and get colonized by micro-organisms. Bacteria inhabit all structures of the oral cavity and easily adhere to alloys. It is widely known that microorganisms influence corrosion of metals immersed in an aqueous environment. This insight was supported by results showing that Streptococcus mutans-treated base-metal dental alloys released strongly increased levels of metal ions (McGinley et al. 2013). The exacerbated release of metal-ions from the nickel-based and cobalt-chromium-based dental casting alloys was thought to result from the pH reduction during S. mutans growth. Leakage was monitored by ICP mass spectrometry and measurements of cellular toxicity following exposure to cultured keratinocytes.

Our studies with monocyte-derived dendritic cells (Chapter 5) and microglia or THP-1 cells (Chapter 6) as targets and IL-8 release as read-out, showed that synergy between microbial and metal factors could further augment innate immune signaling. We found that innate responses to dental alloys and metal salts were strongly potentiated in the presence of the bacterial component LPS/endotoxin. This held true for most alloys tested, but was particularly prominent for Au and Pd-Cu alloys, and Zn and Cu when tested as metal salts. It is important to realize that many more microbial products activate innate immune responses and that such activation can facilitate the development of adoptive immune responses such as contact allergy. Actually, for this reason TLR ligands have become important immune adjuvants in vaccination approaches (Toussi and Massari 2014). In conclusion, metal ions leached from different types of solid metal alloys may, in combination with bacterial products such as LPS, promote the development of chronic inflammation, neurotoxicity and allergies in humans. These findings warrant further studies involving characterization of bacterial infections in individuals with metal-related complaints.

4. ARE NOT ONLY DENDRITIC CELLS BUT ALSO EPITHELIAL CELLS FROM SKIN OR ORAL MUCOSA SHOWING INNATE IMMUNE ACTIVATION UPON METAL EXPOSURE?

In skin and mucosa, keratinocytes are abundantly present and these cells are the first to encounter metal ions that penetrate from outside. Results from studies by Lebre and Olaru (Lebre et al. 2007; Olaru and Jensen 2010). indicated that, upon exposure to sensitizers, not only dendritic cells but also KC secrete chemotactic factors, including IL-8 as a most sensitive read-out for innate immune signalling involving NF-kB activation In our studies we thus took advantage of the fact that IL-8 release could be used not only for dendritic cells but also for KC as a sensitive marker for innate activation. IL-8 is a prominent chemokine that attracts various immune cells to the exposed skin area thereby strengthening innate and adoptive immune responses (Frankart et al. 2012). Our results as presented in Chapter 4 confirm the view that KC play a distinct role in innate immune reactivity to major dental metals. Still, in
contrast to some earlier reports (Lebre et al. 2007), when testing both skin and mucosa-derived KC with a panel of TLR ligands, we only observed functionality of TLR-3. Indeed, Au was very effective in triggering KC, which was in line with our earlier finding using TLR transfectant cell lines, showing that Au utilizes this receptor for innate signalling (Chapter 3). The potent innate immune reactivity-stimulating capacity of Au may contribute to its well-known irritant capacity. Importantly, although we could not demonstrate functional presence of other TLR receptors on KC, also the three other dental metals tested, i.e. Ni, Cu and Hg, showed innate activation of KC. For the potent TLR activating metal Ni this might relate to relatively low expression of TLR4 on KC (Lebre et al. 2007) which we did not pick up in our functional TLR-ligand assay. But for Cu and Hg further studies are warranted to reveal whether other innate pathways leading to IL-8 release are involved, possibly involving TLR-receptors not studied here. In addition, future research may address potential synergies between epithelial and mucosal or dermal layers in which all three major cell types KCs, DCs and fibroblasts are combined. Such synergies have been noted earlier for local release of IL-1beta and TNF-alpha (Griffiths et al. 2005) and various inflammatory and growth factors (Spiekstra et al. 2009).

5. **CAN METAL-EXPOSURE INDUCE INNATE IMMUNE REACTIVITY IN BRAIN CELLS, I.C. MICROGLIA, AND THUS CONTRIBUTE TO NEUROTOXICITY?**

Metals have been speculated to cause neurotoxic symptoms such as headache and disorientation and to be involved in the pathogenesis of neuro-degenerative diseases. Amalgam is in particular debated for its safety. Attempts to link its usage with neurodiseases were reinforced by the finding that elemental Hg is lipid soluble and can cross the brain-blood barrier. (Park and Zheng 2012). Moreover its half-life time in the brain is estimated to last from several years to decades (Mutter 2011). Also the level of other transition metals was found to accumulate in CSF from neurologic patients (Roos et al. 2013). However, little evidence for immune mediated effects of dental alloys on brain cells has been reported so far. Since chronic activation of innate immune responses is a common finding in various neuro-degenerative diseases (Amor et al. 2014) we set out to evaluate the innate stimulatory effects of dental metals.

We found that transition metals in high supra-physiologic concentrations can activate brain microglia, with Ni and Co giving the strongest stimulation (Chapter 6). However, in low physiologic concentrations, even up to 10 fold the levels found in normal blood plasma, only Zn and Cu induced detectable IL-8 production. Therefore, the commonly used high quality alloys are considered to be relatively safe with respect to neurotoxicity. Still, one should realize that, depending on possible corrosive conditions in the oral cavity, as well as on the integrity of the blood-brain barrier and the actual efflux from the brain, metals may accumulate in the central nervous system, thereby reaching incidentally neurotoxic levels.
In addition, we found that bacterial endotoxin could potentiate the innate stimulatory potential of Zn and Cu. Therefore also low grade infections may contribute to the neurotoxic potential of metals.

In conclusion, we now have clear evidence that microglia can respond to metal exposure with IL-8 production and thus contribute to an elevated state of inflammation in the brain. Actual in situ concentrations of metals are, however, unlikely to reach these neurotoxic levels.

6. SHOULD ORAL METAL EXPOSURE BE CONSIDERED A NOTEWORTHY RISK FACTOR FOR AUTOIMMUNE DISEASE?

Chronic exposure to transition metals is associated with several adverse reactions, of which hypersensitivity reactions are most common (Lawrence and McCabe, Jr. 2002; Vas and Monestier 2008; Muris et al. 2015). The relation between metal exposure and the development of autoimmunity is, however, currently less clear, despite many epidemiological studies in particular on Hg and Au (Bains et al. 2008; Stejskal 2014; Elshahawy et al. 2013).

This study (Chapter 7) focused on clinical and serological parameters for autoimmune disease (AID) in relation to oral metal exposure in non-allergic and metal allergic individuals, as well as in patients with oral lesions attributed to dental restorations. Unexpectedly, we found a significant correlation between oral Ni exposure and the presence of clinical AID in a limited, age corrected group, whereas oral Pd, Au or Hg contacts were not associated with any of the clinical or serological autoimmune phenomena tested. The association became even stronger when only gingival Ni exposure was evaluated. The group was, however relatively small (n=74 in total) and metal exposure turned out to be age related, requiring further group size reduction.

Together our findings do not support the hypothesis that chronic Au, Pd and Hg exposure would induce autoantibodies or autoimmune disease. Still, Ni exposure was surprisingly associated with the presence of AID. Although the mechanisms of metal induced autoimmunity are still not completely understood, in addition to metal hypersensitivity (Stejskal 2014) and interference with T cell regulation and selection (Pollard et al. 2010), innate stimulation via TLRs has been suggested to play an important role (Pollard and Kono 2013). Indeed, Ni turned out to provide the strongest innate signal to dendritic cells, via TLR4. Further investigations in larger, age matched groups are therefore needed to verify these preliminary findings.

In conclusion, the results of this study support the view that oral exposure to Ni, but not to Pd, Au or Hg, may facilitate the development of AID. Therefore, further investigations into a possible role of transition metals, in particular Ni, in the development of AID are warranted. At this stage, no conclusive answer can be given to the question whether oral metal exposure should be considered as a major risk factor for autoimmune disease.
Final remarks

The studies presented in this thesis shed a new light on the triggering of innate immunity by frequently used dental metals. Like Ni, most dentally relevant neighbouring elements in the periodic table, such as Co, Au, Hg, Zn, Pd, Cu and Cr, display distinct MoDC activating capacities. Still, adverse events are remarkably rare. Whereas vast numbers of peer-reviewed publications on toxic and immunological responses to dental alloys have appeared, reported frequencies of adverse events remained relatively low. Many explanations may be given for this notable fact: minimal concentrations of metal ions released from currently available alloys, substantial flushing and buffering capacity of saliva, increased vascularity of oral mucosa compared to skin (Tosti et al. 1997), lower numbers of Langerhans cells and T lymphocytes in mucosa and their skewing towards immune suppression (Kosten et al. 2015) etc. Of note, saliva may not only promote corrosion (Chaturvedi 2009; Matusiewicz 2014) but saliva glycoproteins may also mediate immune inhibition in the oral cavity (Bass et al. 1993; Sfondrini et al. 2010).

Nevertheless, also small risks deserve attention and further reduction wherever possible. The risk of clinical use of metal alloys in restorative dentistry involves not only patients but also dentists and dental technicians (Rustemeyer and Frosch 1996). Inflammatory reactions and allergy may become manifest in various ways (Chapter 1 table 3). Dentists, technicians and patients should work together to identify possible relationships between the history of exposure to particular metals or dental alloy constructions and the onset and type of symptoms. Extensive medical records should be kept in the patient’s chart in particular for patients with metal sensitivity. When allergies are considered, dentists should refer patients to an experienced allergologist for a more comprehensive evaluation. Patch tests and if possible blood-tests are recommended for suspected metal-sensitivity patients when restorations are planned. The dentist should also be very careful in selecting the type of alloys for individual patients and may personally check for absence of undesired release of metal ions, like nickel by using the dimethylglyoxime test (Thyssen et al. 2010). Dentists should have up-to-date knowledge on the components of dental alloys and their possible biological effects in order to be able to inform patients properly. They should read instructions and stickers supplied by metal alloy manufacturers. To ensure the safety of patients, dentists and dental technicians, alloy manufacturers should provide clear information on the composition of alloys produced and must measure corrosion rates to reach standards to gain ISO and/or ADA approval and accreditation. This is particularly relevant for those metals with relatively high health risks, notably Ni, Co, Au, Hg, Zn, Pd, Cu and Cr. These health risks are primarily local toxicities. Whether and to what extent Ni exposure might contribute to the development of systemic autoimmune diseases needs further studies. In short, the use of metal alloys in dentistry as well as in numerous other applications will never be avoided. Metal alloys cannot always be replaced by other materials such as methacrylates since
their characteristics are essential for distinct clinical requirements. Results from this study indicate that dental metal alloys may initiate local and systemic immune reactivities. Still, we should emphasize that careful production of dental metal alloys and appropriate clinical monitoring all contribute to their safe use in oral applications.
REFERENCES


