Cytokine responses in metal-induced allergic contact dermatitis: Relationship to in vivo responses and implication for in vitro diagnosis

Jacob Taku Minang

Stockholm, 2005
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SUMMARY

Transition metals such as nickel (Ni), cobalt (Co), palladium (Pd), chromium (Cr) and gold (Au) are widely used as alloys in jewelry and biomaterials such as orthodontic and orthopaedic appliances. These metals also cause cell-mediated allergic contact dermatitis (ACD) reactions in a significant proportion of the population upon prolonged direct exposure. The immune mechanisms underlying the response to these metals are not yet well defined. In the studies described in this thesis we therefore investigated the profile of cytokine responses to various metal ions in vitro and the relationship with the ACD reaction in vivo. In the first study, we investigated the relationship between the profile and magnitude of Ni^{2+}-induced cytokine responses in vitro and the degree of in vivo reactivity to Ni^{2+}. PBMC from Ni^{2+}-reactive (ACD) and non-reactive control subjects were cultured with or without NiCl₂. The numbers of IL-4-, IL-5- and IL-13-producing cells and the concentrations of IFN-γ, IL-10 and IL-13 produced were analysed by ELISpot and ELISA respectively. Ni^{2+} elicited a mixed Th1- and Th2-type cytokine profile in PBMC from ACD subjects with a positive correlation observed between the levels of the elicited cytokines and the degree of patch test reactivity. Hence, suggesting an involvement of both Th1- and Th2-type cytokines in ACD to Ni^{2+} and a direct association between the magnitude of the Ni^{2+}-induced cytokine response overall and the in vivo reactivity to Ni^{2+}. The impact of the regulatory cytokine IL-10 on Ni^{2+}-induced Th1- and Th2-type cytokine responses in human PBMC was investigated in the next study. PBMC from blood donors with a history of Ni^{2+} reactivity and non-reactive control donors were stimulated with Ni^{2+} ex vivo with or without addition of human recombinant IL-10 (rIL-10) or neutralising mAb to IL-10. Depletion/enrichment experiments were performed to phenotype the Ni^{2+}-specific cytokine producing cells. Exogenous rIL-10 significantly down-regulated the production of all cytokines but with a more pronounced effect on IFN-γ. IL-10 neutralisation, on the other hand, enhanced the levels of Ni^{2+}-induced IFN-γ only. Ni^{2+}-specific cytokine-producing cells in PBMC were found to be predominantly CD4^{+} T cells. Thus, IL-10 may play a regulatory role in vivo by counteracting the ACD reactions mediated by CD4^{+} T cells producing Th1-type cytokines. In the third study, we investigated the relationship between in vivo patch test reactivity to a number of metals (Ni, Co, Pd, Cr and Au) included in the standard and/or dental patch test series and in vitro responses to the metals in question. PBMC from metal patch test positive and negative control subjects were stimulated with a panel of eight metal salts and cytokine responses analysed by ELISpot and/or ELISA. A mixed Th1- (IL-2 and/or IFN-γ) and Th2-type (IL-4 and/or IL-13) cytokine profile was observed in PBMC from most metal allergic subjects upon in vitro stimulation with the metal(s) to which the subject was patch test positive. Our data suggest that other metals included in the standard and dental patch test series, just like Ni^{2+}, induce a mixed Th1- and Th2-type cytokine profile in PBMC from ACD subjects in vitro. We further developed a simplified ELISpot protocol utilising plates precoated with capture monoclonal antibodies (mAb) and subsequent detection in one step using enzyme-labelled mAb, for enumerating the frequency of allergen (Ni^{2+})-specific cytokine producing cells. This was compared with a regular ELISpot protocol, with an overnight incubation for capture mAb adsorption and detection with biotinylated mAb followed by enzyme-labelled streptavidin. PBMC from Ni^{2+}-reactive and non-reactive subjects were incubated with or without NiCl₂ and the enumeration of cells producing IFN-γ, IL-4 or IL-13 by the two protocols were compared. PBMC from Ni^{2+}-reactive subjects showed significantly higher Ni^{2+}-induced IL-4 and IL-13 responses and the number of antigen-specific cytokine-producing cells determined by the two ELISpot protocols correlated well. In a nutshell, our data point to the potential use of in vitro cytokine assays as diagnostic tools in distinguishing ACD subjects sensitised to different metals and non-sensitised subjects.
“Without love, benevolence becomes egotism”

- Dr. Martin Luther King Jr. (1929-1968)

“People like you and I, though mortal of course like everyone else, do not grow old no matter how long we live…[We] never cease to stand like curious children before the great mystery into which we were born.”


To my dear mum, Minang Margaret Atih
This thesis is based on the following original papers, which are referred to in the text by their Roman numerals.

**Paper I.**


**Paper II.**


**Paper III.**


**Paper IV.**


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<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>ACD</td>
<td>Allergic contact dermatitis</td>
</tr>
<tr>
<td>ALP</td>
<td>Alkaline phosphatase</td>
</tr>
<tr>
<td>APC</td>
<td>Antigen-presenting cell</td>
</tr>
<tr>
<td>Au$^{1+/3+}$</td>
<td>Gold ions</td>
</tr>
<tr>
<td>BcR</td>
<td>B-cell receptor</td>
</tr>
<tr>
<td>CD</td>
<td>Cluster of differentiation</td>
</tr>
<tr>
<td>CLA</td>
<td>Cutaneous lymphocyte antigen</td>
</tr>
<tr>
<td>Co$^{2+}$</td>
<td>Cobalt ion</td>
</tr>
<tr>
<td>Cr$^{3+/6+}$</td>
<td>Chromium ions</td>
</tr>
<tr>
<td>CTL</td>
<td>Cytotoxic T-lymphocyte</td>
</tr>
<tr>
<td>DC</td>
<td>Dendritic cell</td>
</tr>
<tr>
<td>DTH</td>
<td>Delayed-type hypersensitivity</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>ELISpot</td>
<td>Enzyme-linked immunospot assay</td>
</tr>
<tr>
<td>FACS</td>
<td>Fluorescent activated cell sorted</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
</tr>
<tr>
<td>FCS</td>
<td>Fetal calf serum</td>
</tr>
<tr>
<td>FSC</td>
<td>Forward scatter</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>Granulocyte monocyte colony stimulating factor</td>
</tr>
<tr>
<td>IFN</td>
<td>Interferon</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>KC</td>
<td>Keratinocyte</td>
</tr>
<tr>
<td>LC</td>
<td>Langerhans cells</td>
</tr>
<tr>
<td>LPS</td>
<td>Lypopolysaccharide</td>
</tr>
<tr>
<td>LTT</td>
<td>Lymphocyte-transformation test</td>
</tr>
<tr>
<td>mAbs</td>
<td>Monoclonal antibodies</td>
</tr>
<tr>
<td>MHC</td>
<td>Major Histocompatibility Complex</td>
</tr>
<tr>
<td>Ni$^{2+}$</td>
<td>Nickel ion</td>
</tr>
<tr>
<td>NK</td>
<td>Natural killer cell</td>
</tr>
<tr>
<td>PAMP</td>
<td>Pathogen-associated molecular patterns</td>
</tr>
<tr>
<td>PBMC</td>
<td>Peripheral blood mononuclear cells</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>Pd$^{2+}$</td>
<td>Palladium ion</td>
</tr>
<tr>
<td>PHA</td>
<td>Phytohaemagglutinin</td>
</tr>
<tr>
<td>pNPP</td>
<td>para-nitro-phenyl phosphate</td>
</tr>
<tr>
<td>PPD</td>
<td>Purified protein derivative</td>
</tr>
<tr>
<td>PRR</td>
<td>Pattern-recognition receptor</td>
</tr>
<tr>
<td>PVDF</td>
<td>Polyvinylidifluoride</td>
</tr>
<tr>
<td>SA</td>
<td>Strepavidin</td>
</tr>
<tr>
<td>SSC</td>
<td>Side scatter</td>
</tr>
<tr>
<td>Tc</td>
<td>T-cytotoxic cell</td>
</tr>
<tr>
<td>TcR</td>
<td>T-cell receptor</td>
</tr>
<tr>
<td>TGF</td>
<td>Transforming growth factor</td>
</tr>
<tr>
<td>Th</td>
<td>T-helper cell</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll-like receptor</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumour necrosis factor</td>
</tr>
<tr>
<td>Tr</td>
<td>T-regulatory cell</td>
</tr>
<tr>
<td>TT</td>
<td>Tetanus toxoid</td>
</tr>
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</table>
BRIEF OVERVIEW OF THE IMMUNE SYSTEM

The immune system

The human immune system is a truly amazing constellation of responses to attacks from outside the body. It has many facets, a number of which can change to optimize the response to these unwanted intrusions. The immune system has a series of dual natures, the most important of which is self/non-self recognition. The others are: natural/adaptive (innate/acquired), cell-mediated/humoral and primary/secondary immunity. Functional integration of the immune system is accomplished mainly by cell-to-cell communication that relies on cell adhesion molecules that act in concert with a number of small soluble molecules. Every immune system cell is equipped to synthesize and release a variety of these small molecules, mostly cytokines, which travel to other cells (both immune and non-immune) and stimulate those cells to become either more active (up-regulated) or less active (down-regulated).

Innate immunity

Innate immunity refers to antigen non-specific defense mechanisms that a host uses immediately or within several hours after exposure to an antigen. This is the immunity one is born with and is the initial response by which the body eliminates microbes and prevents infection. Innate immune responses involve; anatomical barriers (skin and mucosa), physiological barriers (temperature, pH), molecules (complement proteins, acute phase proteins, antimicrobial peptides and cytokines), cells that release inflammatory mediators (basophils, mast cells, and eosinophils), phagocytic cells (neutrophils, monocytes, and macrophages) and natural killer cells (NK cells).

Cells of the innate immune system use proteins, pattern-recognition receptors referred to as PRR’s (e.g Toll-Like Receptors; TLRs), encoded in an organism's germ line to detect potentially dangerous substances. These cells are thought to recognise particular highly conserved pathogen-associated molecular patterns or PAMP’s (e.g carbohydrate structures such as lipopolysaccharides; LPS) present on many different microorganisms (reviewed in Franc et al., 1999; Teixeira et al., 2002). New findings show that the innate immune system, once thought to be the unnecessary vestigial tail of ancient antimicrobial systems that have been made redundant by the evolution of acquired immunity, is the cornerstone of the body's ability to fight infection. In many aspects, the innate immune response is also a prerequisite for an efficient induction of the specific (acquired) immune response. Following activation the
innate system induces key costimulatory molecules on antigen presenting cells (APCs), which are essential for antigen-driven clonal expansion of T and B cells (Akira et al., 2001; Pasare and Medzhitov, 2004; Hedges et al., 2005; Xu et al., 2005). Dendritic cells (DCs) activated by innate stimuli and loaded with foreign antigen travel to regional lymph nodes to activate the acquired-immune system. Subsequently, the activated acquired-immune cells move into tissues, where the innate immune system sets-off the danger signal (Matzinger, 1994). The chemokine system is an essential regulator of this dendritic cell and lymphocyte trafficking, which is necessary to turn an innate immune response into an adaptive response (Luster, 2002).

**Acquired immunity**

Acquired (adaptive) immunity includes humoral immunity (antibody-mediated) and cell-mediated immunity. Acquired immunity involves; APCs such as macrophages and DCs, the activation and proliferation of antigen-specific T and B-lymphocytes and the production of antibody molecules, cytotoxic T-lymphocytes (CTLs), and cytokines. T and B-lymphocytes possess specific receptors that arise from complex gene recombination reactions giving rise to a broad spectrum of antigen specificities that is a hallmark of the adaptive immune system. The T-cell receptor (TcR) recognizes only epitopes on antigens processed by APCs and presented in the context of the major histocompatibility complex (MHC) molecules (reviewed in Sebzda et al., 1999; Inaba and Inaba, 2005). Self/non-self recognition is achieved by having every cell displaying a marker based on the MHC complex (Doherty and Zinkernagel, 1975). Any cell not displaying this marker is treated as non-self and attacked (Oberg et al., 2004). The B-cell receptor (BcR), in contrast can recognise specific epitopes on whole antigens (Takata et al., 1995). The adaptive immune system has the capacity to “store” instructions obtained from the innate immune system upon first encounter with a foreign body by changing from a naïve to an appropriate memory phenotype (reviewed in José et al., 1999). This property, that distinguishes the adaptive immune system from the innate, is referred to as immunological memory.

**T lymphocyte subsets**

Immature T lymphocytes differentiate from pluripotent stem cells in the bone marrow and migrate to the thymus where their maturation occurs. Surface expression of the cluster of differentiation molecule, CD3, is a unique feature of T cells. T cells are further classified by the type of TcR they express on their surface, having either α/β or γ/δ combination (Miescher
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et al., 1988). The α/β TcR expressing T cells only recognize antigens as peptide fragments presented as a complex with MHC antigens on APCs (Schwartz et al., 1976; Ball and Stastny, 1984, Braciale et al., 1987). There are two distinct subpopulations of T cells within the α/β TcR expressing lymphocytes defined based on their surface expression of either CD4 or CD8 in combination with the accessory marker CD3 (de Gast et al., 1985). The CD4+ T cells, designated T-helper (Th) cells, recognize peptides, which are bound to MHC class II molecules. CD8+ T cells, referred to as T-cytotoxic (Tc) cells recognize antigens bound to MHC class I molecules, displayed as peptide-MHC class I complexes on the surface of APCs (Van Seventer et al., 1986). Antigen recognition by γ/δ TcR expressing T cells does not require processing and is not MHC restricted (reviewed in Chien et al., 1996).

The Th1/Th2 concept

The Th1/Th2 hypothesis emerged in the late 1980s, stemming from observations in mice of two distinct populations of CD4+ T-helper cells differing in cytokine secretion patterns and other functions upon activation (Mosmann et al., 1986). The concept subsequently was applied to human immunity (Mosmann and Coffman, 1989). Both the Th1 and Th2 subsets are produced from a non-committed population of precursor (naïve) T cells (Lafaille, 1998). The Th1/Th2 concept rests largely on a dichotomy of cytokine profiles, with Th1 cells described as mainly IFN-γ, IL-2 and TNF-β producers while Th2 cells are IL-4, IL-5 and IL-13 producers (McKenzie et al., 1993; Wang et al., 1994; Thomas and Kemeny, 1998). However, as with other immune cells, the array of cytokines produced by the Th1 and Th2 cells varies greatly and is influenced by a large number of experimental variables, as well as the danger from artefacts (Romagnani, G., 2000). The differences in cytokine profiles between Th1 and Th2 responses described in earlier studies relied heavily on mice and cultured cells. However, more recent research involving human subjects has proved much of this earlier work to be highly simplistic or otherwise inaccurate (Reviewed in Dent, 2002). Currently Th1 cells are referred to as cells that produce far more IFN-γ and IL-2 than do Th2 cells, while the Th2 cells produce far more IL-4 (and perhaps IL-5) than do the Th1 cells. CD8+ T cells (Tc cells) have also been suggested to, depending on the type of antigen and cytokine milieu, differentiate into cells which produce IFN-γ, but not IL-4 (Tc1), and cells that produce IL-4, but not IFN-γ (Tc2) (Mosmann and Sad, 1996; Kemeny et al., 1999). A third population of cells, Th0 and Tc0, has been described as lymphocytes exhibiting an unrestricted profile of cytokines; i.e., they produce both Th1 and Th2-type cytokines.
There are two key features of the Th1/Th2 hypothesis; firstly, each cell subset produces cytokines that serve as growth factors of that subset (autocrine effects) and secondly, cytokines produced by the two subsets cross-regulate each other's development and activity either by blocking polarised maturation of the opposite cell type or by blocking its receptor functions (Abbas et al., 1996). Whereas commitment to the Th1 phenotype is reversible, that to the Th2 phenotype appears to be final, since efforts to reverse such differentiated cells have not been successful (Rogge et al., 1997; Lafaille, 1998). Recently, it has been suggested that CD4+ T-helper cells polarisation could be indicative of a more profound polarisation of the immune system as a whole. That is, a kind of type 1/type 2 polarisation already begins with those cells having the primary contact with antigens, including the DCs, monocytes and macrophages, and other APCs (Moser and Murphy, 2000; Fujimura et al., 2004; Aktas et al., 2005). These APCs likely polarise into type 1 and type 2 cells in response to the type of antigen experience, and then subsequently bias the polarization of the T-helper population functionally "downstream" from them.

**The Th1-type cytokines**

APCs secreting IL-12 upon uptake and processing of type-1 promoting (type-1 biased) antigens promptly migrate to nearby lymph nodes. As this cytokine increases in concentration it begins to influence naïve T cells to eventually become Th1 cells (Verhasselt et al., 1997; Sparwasser et al., 1998; Hessle et al., 2000). NK cells also respond to the IL-12 environment and proceed to release IFN-γ, which reinforces the APC's production of IL-12 and also helps to drive the naïve T-cell commitment process (Hart et al., 2005; Walzer et al., 2005). As the T cells attain maturity, Th1 cells also produce IFN-γ, which (together with the NK cells) stimulates the APC and naïve T cells to polarise into more Th1 cells, in a self-reinforcing "autocrine" loop. Th1-type cytokines are mainly produced in response to intracellular pathogens, cell wall antigens or other smaller fragments of the organism, and trigger cell-mediated immunity and/or production of opsonising antibodies (Table 1). Th1 cells are hypothesised to lead the attack against viral and bacterial antigens through the classic delayed-type hypersensitivity (DTH) reaction (Newport et al., 1996; Lienhardt et al., 2002). Th1 cells are also important in the fight against cancer cells (Sato et al., 1998). On the negative side, the Th1 pathway is often portrayed as being the more aggressive of the two, and apparently, when it is over reactive, can generate organ-specific autoimmune diseases.
(e.g., arthritis, multiple sclerosis, type 1 diabetes) (Tang et al., 1998; Pakala et al., 1997; Schulze-Koops et al., 2001)

The Th2-type cytokines
The emergence of Th2 cells, like the Th1 cells, is also dependent on their cytokine environment. Their maturation is likely initiated by the cytokine IL-6 (Croft and Swain, 1992; Rincon et al., 1997) produced by APCs (monocytes and macrophages), endothelial cells, fibroblasts and mast cells (Bradding et al., 1993; Derocq et al., 1994). IL-4 released by NK cells, mast cells and eosinophils, also participates in driving Th2-cell maturation (Schmitt et al., 1990; Marcinkiewicz and Chain, 1993). As the Th2 cells mature they also produce IL-4, which together with the other participating cell types generate an autocrine loop to the naïve T cells to make more Th2 cells (Chen et al., 2004) (Table 1). The Th2 pathway is thought to be primarily involved in the triggering of IgE-mediated (immediate) hypersensitivity disorders, including allergic asthma, eczema, hay fever, and urticaria (Singh et al., 1999) and also in protection against helminthic infections (Coffman and von der Weid, 1997; reviewed in Dent, 2002).

T-regulatory cells (Tr1 and Tr2/Th3)
T-regulatory (Tr) cells were first described in the early 1970s based on their induction of tolerance in other lymphocyte populations (Gershon and Kondo, 1970). However, failure to define their exact phenotype led to controversy in the 1980s about their existence (Möller, 1988). Tr cells have recently become more prominent (Mason, 2001; McGuirk and Mills 2002; Chen et al., 2003) and have been shown to intervene and to block either Th1 or Th2 activities or both (reviewed in Kidd, 2003). CD4+CD25+ T lymphocytes have been suggested to be the main cell population with immunoregulatory properties (Shevach, 2002; Nishimura et al., 2004; Ruprecht et al., 2005). The CD4+CD25+ Tr cells comprise 5-10% of the total peripheral T-cell pool (McGuirk and Mills, 2002) and constitutively express the CD25 marker (IL-2Rα), already upon exit from the thymus (Sakaguchi et al., 1995). These cells are thus referred to as “natural” Tr cells (Trn) (Table 1). Trn cells have been shown to possess a contact-dependent cytokine-independent mechanism of immunosuppression (Fontenot et al., 2003; Khattri et al., 2003). A second population of CD4+ Tr cells, generated in the periphery, has been described (Baecher-Allan et al., 2001). This Tr subset has a cytokine-dependent mechanism of action and is subdivided into Type 1 Tr (Tr1) and Type 2 Tr (Tr2) cells based on their cytokine expression profiles. Tr1 cells secrete high levels of IL-10 and low-to-
moderate levels of TGF-β, while Tr2 cells primarily secrete TGF-β (Groux et al., 1997; Gorelik and Flavell, 2002). Tr2 cells were previously named T helper-3 (Th3) cells (Weiner, 2001) but later renamed as Tr2 cells to highlight their suppressor function (reviewed in Horwitz et al., 2003). CD8+ T cells with regulatory properties have also been described (Gilliet and Liu, 2002). Unlike CD8+ effector cells, CD8+ Tr cells lack cytotoxic activity and produce IL-10 (Tr1) and/or TGF-β (Tr2) (Rich et al., 1995; Dhodapkar and Steinman, 2002). Functional studies indicate that Tr1 cells and other Tr populations may help to terminate Th1-related inflammatory responses to pathogens, tumors, and alloantigens (Fukaura et al., 1996; Groux et al., 1997; reviewed in Gorelik and Flavell, 2002; Ruprecht et al., 2005).

**B lymphocytes and IgE class switching**

B-lymphocytes mature in the bone marrow and, unlike T lymphocytes, are able to recognise soluble antigens without the need for processing (Takata et al., 1995). Antigen recognition by naïve immunocompetent B cells is via surface expressed immunoglobulins mainly of the IgM and IgD classes. Activated B cells differentiate into plasma or memory B cells in highly organised compartments of secondary lymphode organs (i.e. lymph nodes and spleen) during which time, depending on the antigen and cytokine milieu, class switching occurs in the immunoglobulin genes to give rise to other classes; IgG, IgE or IgA (reviewed in Coffman et al., 1993) which, display the same antigen specificity but have different biological functions.

IgE has been shown to be the main mediator of type I allergies (see section on ‘Hypersensitivity/Allergic reactions’). The switch to the IgE antibody class is induced by IL-4 and IL-13 (Del Prete et al., 1988; Punnonen et al., 1993) (see section on ‘Cytokines investigated in this study’). Measurement of total serum IgE antibody levels is used as a marker of the atopic status of an individual although determination of allergen specific IgE is considered a more reliable indicator of atopic allergy (Kasaian et al., 1995; Di Lorenzo et al., 1997; Di Gioacchino et al., 2000; Jackola et al., 2004). IgE has also been shown to play a role in the clearance of many parasitic infections (Coffman and von der Weid, 1997; reviewed in Dent, 2002). There are a few reports of detection of nickel (Ni2+) specific IgE antibodies in serum samples from some Ni2+ allergic subjects (Shirakawa et al., 1992; Estlander et al., 1993); however, the relevance of these antibodies to the skin inflammatory response seen in allergic contact dermatitis (ACD) is not known.
**Table 1.** General properties of CD4^+^ and CD8^+^ T regulatory and helper subsets

<table>
<thead>
<tr>
<th>T cell subset</th>
<th>Predominant cytokine secreted</th>
<th>Response to antigen^a^</th>
<th>Response to IL-2 or IL-15^b^</th>
</tr>
</thead>
<tbody>
<tr>
<td>Regulatory/suppressor</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD4^+^ CD25^+^ Trm</td>
<td>None</td>
<td>Unresponsive</td>
<td>Proliferate</td>
</tr>
<tr>
<td>CD4^+^ Tr1*</td>
<td>IL-10</td>
<td>Unresponsive</td>
<td>Proliferate</td>
</tr>
<tr>
<td>CD4^+^ Tr2/Th3*</td>
<td>TGF-β</td>
<td>Unresponsive</td>
<td>Proliferate</td>
</tr>
<tr>
<td>CD8^+^ Tr1 or Tr2*</td>
<td>IL-10 or TGF-β</td>
<td>Hyporesponsive</td>
<td>Proliferate</td>
</tr>
<tr>
<td>Helper/Cytotoxic</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD4^+^ Th1</td>
<td>IL-2, IFN-γ</td>
<td>Proliferate</td>
<td>Proliferate</td>
</tr>
<tr>
<td>CD4^+^ Th2</td>
<td>IL-4, IL-5^c^, IL-13</td>
<td>Proliferate</td>
<td>Proliferate</td>
</tr>
<tr>
<td>CD8^+^ Tc1</td>
<td>IFN-γ</td>
<td>Proliferate</td>
<td>Proliferate</td>
</tr>
<tr>
<td>CD8^+^ Tc2</td>
<td>IL-4</td>
<td>Proliferate</td>
<td>Proliferate</td>
</tr>
</tbody>
</table>

^a^CD4^+^ or CD8^+^ Tr1 or Tr2 subsets can also display CD25 following activation.  
^b^Restimulation of T cells specific for ovalbumin or peptide 110–119 of influenza HA with the same antigen.  
^c^Both cytokines use the shared common cytokine receptor gamma chain (γ c) and both function as T cell growth and survival factors.  

**HYPERSENSITIVITY/ALLERGIC REACTIONS**

The term ‘allergy’ was first used by von Pirquet, to denote both host-protective and potentially host-injurious immune responses (Bendiner, 1981). Today, hypersensitivity is defined as an exaggerated reaction towards a stimulus, whereas allergy is a more specific hypersensitivity reaction caused by an immunological sensitisation.

**Classification of hypersensitivity/allergic reactions**

Hypersensitivity reactions are generally divided into four types depending on the effector mechanism involved in the response (Reviewed in Rajan, 2003) (Table 2). The type I hypersensitivity response is very rapid (within minutes or hours of re-exposure to the allergen) and is mainly IgE antibody mediated with allergen-specific IgE receptor bearing mast cells and eosinophils playing a significant role (Siroux *et al.*, 2004) (see also section below on ‘atopic contact dermatitis’). The clinical manifestation of type II and III hypersensitivity responses, just like the type I response, appear within a short time upon subsequent exposure to the allergen but unlike the type I response, the IgG antibody class plays a major effector role; either as opsonising antibodies promoting target cell lysis or in complexes with allergen resulting in complement-mediated lysis of underlying epithelia (Skokowa *et al.*, 2005). The type IV response, also referred to as the DTH response, on the other hand takes several days to
appear upon re-exposure to the sensitising agent and is cell-mediated (Silvennoinen-Kassinen et al., 1992; Shah et al., 1998; Grabbe and Schwarz, 1998).

The more important allergic disorders include bronchial asthma, perennial rhinoconjunctivitis or seasonal rhinoconjunctivitis (hay fever), urticaria, extrinsic allergic alveolitis (farmer’s lung, bird keeper’s lung), food allergies, acute generalised allergic reactions (anaphylactic shock), allergic drug reactions, atopic dermatitis and contact dermatitis.

**Table 2.** The Gell-Coombs classification of hypersensitivity/allergic reactions

<table>
<thead>
<tr>
<th>Gell-Coombs scheme</th>
<th>Characteristics</th>
<th>Effector mechanism</th>
<th>Manifestations</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Type I</strong></td>
<td>IgE mediated</td>
<td>IgE-dependent activation of mast cells and basophils (Th2-cell dependent)</td>
<td>Atopic allergy, anaphylaxis, hay fever, asthma</td>
</tr>
<tr>
<td></td>
<td>Immediate reaction</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Type II</strong></td>
<td>Antibody mediated</td>
<td>Antibodies to microbial antigens, C5a release</td>
<td>Cytotoxic reactions, thrombocytic purpura</td>
</tr>
<tr>
<td></td>
<td>Rapid reaction</td>
<td>Leukocyte infiltration</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Antibody-antigen complexes activating the complement system</td>
<td>Immune complex diseases, vasculitis</td>
</tr>
<tr>
<td><strong>Type III</strong></td>
<td>Antibody mediated</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Rapid reaction</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Type IV</strong></td>
<td>Cell mediated</td>
<td>T-cell mediated (type 1 T cells implicated)</td>
<td>Allergic contact dermatitis</td>
</tr>
<tr>
<td></td>
<td>Delayed type reaction</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>


**CONTACT DERMATITIS**

Contact dermatitis is defined as an inflammatory skin reaction subsequent to direct contact with noxious agents in the environment. This phenomenon was first described as early as the first century A.D. where some individuals were reported to experience severe itching when cutting pine trees (reviewed in Rosenberg, 1955). Depending on the offending agent and/or the mechanism of the reaction (immediate or delayed, allergic or non-allergic), three main types of contact dermatitis reactions have been described viz; allergic contact dermatitis (ACD), atopic contact dermatitis and irritant contact dermatitis.

**Allergic contact dermatitis**

ACD results from a specific immunological reaction (type IV allergy or DTH) (Grabbe and Schwarz, 1998) and primarily affects the hands, head/face and feet in adults (Schnuch et al.,
Cytokine responses in metal-induced ACD

1998; Shah et al., 1998; West et al., 1998). Common contact allergens include organic hapten molecules found in plant products (e.g. urushiol from the North American poison ivy) (Kalish et al., 1994), drug metabolites, perfume fragrances, rubber, plastics, additives and preservatives (e.g. methylisothiazolinones) (Gruverberger, 1997; Schnuch et al., 1997) as well as metal ions such as nickel, chromium, cobalt, gold and palladium (Gawkrodger et al., 2000; Thierse et al., 2005) (Table 3).

Table 3. Some important allergens included in the European standard series and mean prevalence of positive reactions to these allergens among subjects with eczematous reactions.

<table>
<thead>
<tr>
<th>Allergen name</th>
<th>Combined prevalencea (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nickel 5%</td>
<td>18.6 (11.1-26)</td>
</tr>
<tr>
<td>Fragrance mix 5%</td>
<td>10.7 (5.4-16)</td>
</tr>
<tr>
<td>Myroxylon pereirae 25%</td>
<td>6.7 (3.5-9.9)</td>
</tr>
<tr>
<td>Cobalt chloride 1%</td>
<td>5.8 (3.1-8.4)</td>
</tr>
<tr>
<td>Colophonium 20%</td>
<td>5.2 (3.2-6.9)</td>
</tr>
<tr>
<td>Thiuram mix 1%</td>
<td>3.5 (2.8-4.2)</td>
</tr>
<tr>
<td>Wool alcohols 30%</td>
<td>3.3 (1.3-5.3)</td>
</tr>
<tr>
<td>p-Phenylenediamine 1%</td>
<td>3.0 (2.1-3.9)</td>
</tr>
<tr>
<td>Neomycin 20%</td>
<td>2.9 (2.0-3.9)</td>
</tr>
<tr>
<td>MCI &amp; MI b 0.01% aq</td>
<td>2.4 (0.8-4.0)</td>
</tr>
<tr>
<td>Formaldehyde 1% aq</td>
<td>2.1 (1.2-3.0)</td>
</tr>
<tr>
<td>Potassium dichromate 0.5%</td>
<td>2.1 (1.1-3.1)</td>
</tr>
<tr>
<td>Caine mix IIIc 10%</td>
<td>1.5 (1.0-2.0)</td>
</tr>
<tr>
<td>Quaternium 15 1%</td>
<td>1.3 (0.1-2.6)</td>
</tr>
<tr>
<td>Epoxy resin 1%</td>
<td>1.2 (0.1-2.3)</td>
</tr>
<tr>
<td>Mercapto mix 1%</td>
<td>1.1 (0-2.4)</td>
</tr>
<tr>
<td>Sesquiterpene lactone mix 0.1%</td>
<td>1.1 (0-2.1)</td>
</tr>
<tr>
<td>Parabens mix 16%</td>
<td>1.1 (0-2.3)</td>
</tr>
<tr>
<td>PTBP formaldehyde resin 1%</td>
<td>1.0 (0-3.0)</td>
</tr>
<tr>
<td>Mercaptobenzothiazole 2%</td>
<td>0.8 (0.3-1.22)</td>
</tr>
</tbody>
</table>

In petrolatum unless otherwise indicated. a Combined prevalence (95% confidence interval) of positive reactions to 20 most important contact allergens included in the European standard series based on data from seven test centres in the United Kingdom (UK). b methylchloroisothiazolinone and methylisothiazoline; c benzocaine 5%; cinchocaine 2.5%; amethyocaine 2.5%. Modified from Britton et al., (2003).
The risk for developing ACD is related to the exposure concentration (dose per unit area) and the inherent allergic potency of the individual chemical (Boukhman and Maibach, 2001). Results from a number of epidemiological studies suggest that ACD is common, with 20-25% of the European population reported to be sensitised to one or more contact allergens (Brasch and Geier, 1997; Schnuch et al., 1997; Meding et al., 2001; Meding and Jarvholm, 2002). It is worth noting that allergic contact dermatitis can be prevented upon appropriate diagnosis and avoidance of the offending agent as illustrated by the important examples of ACD to nickel and chromium (Lachapelle et al., 1980; Avnstorp, 1989; Jensen et al., 2002).

Atopic contact dermatitis
Atopic contact dermatitis refers to an immediate-type (IgE-mediated) eczematous allergic contact reaction in an atopic person (Hannuksela, 1980). The terms ‘atopic dermatitis’, ‘immediate contact reaction’, ‘contact urticaria’ or ‘contact urticaria syndrome’ have been used interchangeably in the literature to describe different manifestations of atopic dermatitis (De Waard-van et al., 1998; Aalto-Korte and Makinen-Kiljunen, 2003). The inflammatory response in atopic contact dermatitis appears within minutes to an hour after contact with an eliciting substance and usually disappears within a few hours. The reaction could be described as immunologic or non-immunologic depending on whether prior sensitisation to the offending agent is required. Sensitisation most commonly occurs via the respiratory or gastrointestinal tracts, but can also occur through the skin, as is the case of latex and some foods (Guillet et al., 2005). Allergen-specific IgE antibodies, produced during primary contact with the offending agent, bind to high-affinity Fcε receptors on the surface of mast cells and basophils. Subsequent exposure to the same sensitising agent (allergen) and interaction between the allergen and allergen-specific IgE antibodies on mast cells and basophils leads to cross-linking of the high-affinity Fcε receptors and the release of a panel of pharmacologically active mediators (Di Lorenzo et al., 1997; reviewed in Jackola et al., 2004).

Atopic dermatitis (or atopic eczema) is characterised by skin infiltration of T lymphocytes expressing a Th2-type profile (IL4, IL-5 or IL-13) of cytokines (Herrick et al., 2003). The Th2-type cytokine, IL-4, plays a central role in the antibody class switch to IgE production (See ‘IL-4’ under ‘cytokines investigated in this study’). Itching, tingling or burning accompanied by erythema are the weakest types of reactions. A local wheal and flare suggest contact urticarial reaction. Generalised urticaria is less common but has been reported in cases
of contact with agents eliciting immunologic IgE-mediated contact urticaria (Reviewed in Wuthrich, 1998). In addition to local skin symptoms, other organs are occasionally involved giving rise to conjunctivitis, rhinitis, an asthmatic attack or anaphylactic shock (reviewed in Harvel et al., 1994).

**Irritant contact dermatitis**

Irritant contact dermatitis refers to an eczematous reaction in the skin of external origin where, in contrast to allergic contact dermatitis, no obvious eliciting allergens can be identified (see section on ‘allergic contact dermatitis’) (Bryld et al., 2003; Duarte et al., 2003). Depending on the nature of the irritant, the body region that is exposed or the duration of exposure, the irritant contact dermatitis response can be referred to as; subjective, chronic or due to chemical burns (Tupker, 2003; Smith et al., 2004; Pedersen et al., 2005). In the past, the pathogenesis of irritant contact dermatitis was thought to be non-immunological (Malten et al., 1979), however, it is now generally accepted that the immune system plays a vital role in the induction and maintenance of irritant-induced skin reactions (Wakem et al., 2000; Levin and Maibach, 2002; Lisby and Hauser, 2002). T lymphocytes expressing the CD4⁺ phenotype have been shown to be the major skin infiltrating lymphocyte population in irritant contact dermatitis (Willis et al., 1993; Kondo et al., 1996). These skin infiltrating CD4⁺ T cells secrete mainly IFN-γ and IL-2 (Hoefakker et al., 1995; Kondo et al., 1996). Noteworthy however, in contrast to allergic skin reactions, no immunological memory seems to be involved in eliciting irritant contact dermatitis (Moed et al., 2004a) and the development of irritant skin reactions does not require prior sensitisation (see preceding section on ‘allergic contact dermatitis’).

**Hapten immune system interaction in allergic contact dermatitis**

Haptens are small organic molecules or metal ions that are non-immunogenic by themselves and must be able to react to or bind to proteins (larger carrier molecules) to become contact allergens (Landsteiner and Jacobs, 1935). Thousands of potential contact sensitizers exist. However, parameters such as whether the hapten is released from compound mixtures, whether people are exposed, if it permeates the skin or if it has a strong sensitising capacity, narrow down the number of interesting haptens to a few dozens of high relevance (Wahlberg, 2001). The interaction between a hapten-carrier complex and the immune system and the ensuing inflammatory response can be divided into two phases. There is a ‘sensitization (induction) phase’ which describes the reactions (generally asymptomatic) following primary
application of the hapten to the skin and then an ‘elicitation (effector) phase’ during which the clinical symptoms of ACD are manifested upon re-exposure to the hapten.

The Sensitisation phase
Following primary application to the skin, DCs, mainly epidermal LCs that reside in normal skin in a ‘resting’ functional state, take up the hapten and process it (Fig. 1). Hapten application also results in activation of KCs, which together with LCs and other skin-cell types secrete inflammatory cytokines such as IL-1β, TNF-α, IL-6, IL-12 and GM-CSF (Enk and Katz, 1992b; Kimber and Cumberbatch, 1992; Kaplan et al., 1992).

Figure 1. Sensitisation phase of contact hypersensitivity. Topical application of contact allergen induces cytokine secretion by keratinocytes (KCs), langerhans cells (LCs) and other skin cell types. The secreted cytokines activate resting LCs and promote the migration of antigen (Ag)-carrying LCs towards regional lymph nodes (step 1). In the lymph node, LCs establish contact with naïve T cells (T naïve) (step 2) and activate them via expression of Ag-MHC complexes along with costimulatory and adhesion molecules (step 3). Primed T cells (T sens) alter their migration pathways and begin to recirculate through peripheral tissues (step 4). Modified from Grabbe and Schwarz, (1998) with permission from Elsevier.

These cytokines activate more LCs and upregulate their expression of cell-surface molecules (e.g MHC class I and II, B7, CCR7 etc) and secretion of cytokines and chemokines (e.g. IL-8) (Enk et al., 1993). Activated antigen-carrying LCs migrate via afferent lymphatic vessels to the paracortical zone of regional draining lymph nodes where they establish contact with and activate T cells (Sozzani et al., 1995). Antigen-specific activation alters the migration pathways of primed T cells through increased expression of skin homing markers such as cutaneous lymphocyte antigen (CLA) leading to their recirculation through peripheral tissues.
(Barker et al., 1991). Other APCs, such as dermal DCs have also been implicated in priming of naïve T cells after epicutaneous contact with hapten (Morikawa et al., 1992).

**The elicitation phase**

Re-exposure to a hapten leads to the same initial direct effects on the skin as primary hapten contact during sensitization (i.e. LC activation, proinflammatory effects). Activated LCs release cytokines that activate endothelial cells and upregulate their expression of adhesion molecules with a resultant attraction of leukocytes to the site of hapten application (Fig. 2).

![Figure 2. Elicitation phase of contact hypersensitivity (CHS). Secondary hapten application on the skin leads to the activation of Langerhans cells (LCs) and subsequent secretion of proinflammatory cytokines (step 1). The released cytokines upregulate the expression of adhesion molecules on LCs and activate endothelial cells (step 2) with a resultant attraction of leukocytes to the site of hapten application (step 3). Among these are primed T cells (T\textsubscript{sen}), which are activated upon antigen (Ag) presentation either by resident cells or by infiltrating APCs (step 4). Ag-specific T-cell activation (step 5) induces mediator release by hapten-specific T cells (step 6), which amplifies the inflammatory process leading to further accumulation of infiltrating cells (step 7), resulting in clinically manifest allergic contact dermatitis (ACD). The time between application of allergen and elicitation of ACD is approximately 24-72 h. Modified from Grabbe and Schwarz, (1998) with permission from Elsevier.

Among these are primed T cells, which are activated upon antigen presentation either by resident cells or by infiltrating APCs and/or LCs (Ishii et al., 1994; Ishii et al., 1995). Activated hapten-specific T cells induce mediator release (Ptak et al., 1991a). This amplifies the response by generating an inflammatory process that leads to further accumulation of infiltrating cells including neutrophils, mononuclear cells and antigen-nonspecific but MHC-restricted late acting CD3\textsuperscript{+}CD4\textsuperscript{+}CD8\textsuperscript{+} T cells (Ptak et al., 1991b; Moed et al., 2004b). These cells are thought to be responsible for the eczematous reaction resulting in clinically manifest ACD. \(\gamma\delta\) TcR expressing cells have also been shown to play a role in the elicitation of contact
hypersensitivity (Askenase et al., 1995). The time between application of allergen and elicitation of ACD is approximately 24-72 h.

**RELATED BACKGROUND**

**Biochemistry of transition (heavy) metals**

Transition metals represent a block of elements (~50 metals) in the periodic table that show great chemical similarities within a given period as well as within a given vertical group. This contrasts to the representative elements whose chemistry changes markedly across a given period as the number of valence electrons changes, with chemical similarities occurring mainly within vertical groups (Zumdahl, 1997). This difference occurs because the last electrons added for transition metals are inner (d or f) electrons. This inner d and f electrons cannot participate easily in bonding, as can the valence s and p electrons of the representative elements. In forming ionic compounds with nonmetals, the transition metals exhibit two typical characteristics; 1) more than one oxidation state is often found (e.g Cr^{3+} and Cr^{6+}), 2) the cations are often complex ions i.e. species where the transition metal ion is surrounded by two or more ligands. For example, the compound [Co(NH$_3$)$_6$]Cl$_3$ contains the [Co(NH$_3$)$_6$]$^{3+}$ cation and Cl$^-$ anions where NH$_3$ serves as the ligand. When dissolved in water, the solid behaves like any ionic solid; the cations and anions are assumed to separate and move about independently.

Transition metals turn to produce geometrically highly defined coordination complexes with four or six electron-donators such as nitrogen or oxygen in amino acid side chains of appropriate proteins or peptides (Fausto da Silva and Williams, 2001; Zhang and Wilcox 2002). For example, a complex of Co$^{2+}$ ions has a typical tetrahedral arrangement, Ni$^{2+}$ and Pd$^{2+}$ a square planar tetra-coordinated arrangement (Fig. 3) and Cr$^{3+}$ a six-ligand octahedral arrangement.

**Transition metals and allergic contact dermatitis**

Although several transition metal ions are known to play a vital role in living organisms as well as in industry, a number of transition metals and their compounds also present important occupational and health hazards (Waalkes, 1995; Garner, 2004). Some of these metals are well known contact allergens capable of inducing a delayed type hypersensitivity response in susceptible individuals upon prolonged direct exposure.
The number of ligands and the geometry of the coordination complexes, formed between the metal ions and the electron rich atoms in amino acid side chains of some proteins or peptides, seem to be the major factors determining the allergenicity of these metals as well as their cross reactivities (Lepoittevin, 2001). Transition metal ions in this context serve as haptens, with high immunogenic potential, only when in complex with cellular or matrix proteins of the skin (Walton, 1983; Gawkrodger et al., 1986; de Fine Olivarius and Menne, 1992).

Unlike the irreversible convalent bonds formed by classical haptens and their protein carriers, the coordination complexes formed by transition metal ions (‘non-classical’ haptens), are reversible and allow for the exchange of the allergenic metal ions between different acceptor sites (Thierse et al., 2005). One metal may have allergenic properties at two or more oxidation states e.g. while Cr$^{6+}$ has been assumed to be the major form of chromium involved in ACD and is used for patch testing, Cr$^{3+}$ has also recently been shown to elicit ACD (Hansen et al., 2003). The distribution in nature, uses in industry and oxidation state relevant to sensitisation for each of the metals investigated in the studies reported in this thesis are reviewed below.

Ni$^{2+}$ is a component of many different types of alloys including white gold, German silver, nickel plating, monel solder, gold plating and stainless steel. Its presence in such a wide variety of products makes it an especially common contactant difficult to avoid (Liden, 1992). Ni$^{2+}$ is the single most common cause of ACD (see Table 3) (Hansen et al., 2003; reviewed in Garner, 2004). Jewelry is the main source of Au$^{1+}$ exposure (often seen as dermatitis at the site of jewelry contact e.g. earlobes, fingers) but amalgams used in gold dental restoration are a major cause of sensitisation (Vamnes et al., 2000). Co$^{2+}$ is found in abundance in our
environment and used with e.g. Ni\textsuperscript{2+} in various alloys but also found in pigments and paints (Liden and Wahlberg, 1994). Cr\textsuperscript{6+} is present in cement but also found in metal alloys with e.g. Co\textsuperscript{2+} and Ni\textsuperscript{2+} (Liden and Wahlberg, 1994). Pd\textsuperscript{2+} on the other hand, is a precious metal used in the telecommunications industry, dental alloys, high temperature solders and jewelry. White gold may contain up to 20% Pd\textsuperscript{2+}, and dental alloys may contain up to 10% Pd\textsuperscript{2+} (Vincenzi, 1995).

Molecular and cellular mechanisms underlying metal-induced allergic contact dermatitis

A number of models have been proposed to explain metal-protein interactions underlying the transport and delivery of metal ions (non-peptide hapten) to APC and their interactions with HLA and TcRs. In the first model referred to as processing-independent presentation, a metal ion (Ni\textsuperscript{2+} in this case) either directly interacts with endogenous or exogenous peptides and the resulting Ni\textsuperscript{2+}-peptide complex then binds to MHC molecules (Romagnoli et al., 1991) or on the other hand, Ni\textsuperscript{2+} may complex directly to an MHC-bound peptide or to the MHC molecule itself thus circumventing processing (Fig. 4, left panel) (van den Broeke et al., 1999). The Ni\textsuperscript{2+}-MHC/peptide complexes result in conformational changes of these proteins that may create new epitopes (neoantigens), which may be recognized by T cells (Romagnoli et al., 1991).

In the second model referred to as processing-dependent presentation, Ni\textsuperscript{2+} is thought to form coordination bonds with membrane-bound or soluble proteins, principally human serum albumin (HSA), on the skin via cysteine or histidine residues (Sadler et al., 1994). The Ni\textsuperscript{2+}-carrier protein complex is then taken up by epidermal APCs (mainly LCs), processed and presented to T cells as Ni\textsuperscript{2+}-peptide complexes on MHC molecules (Fig. 4, right panel) (Moulon et al., 1995).

A third model has recently been proposed (Thierse et al., 2004; reviewed in Thierse et al., 2005). Here the metal, Ni\textsuperscript{2+}, is suggested to bind to the protein HSA (a known shuttling molecule for Ni\textsuperscript{2+}), and the presence of the HSA-Ni\textsuperscript{2+} complex in the vicinity of transient contacts between TcR and APC-exposed HLA molecules is thought to facilitate a specific transfer of Ni\textsuperscript{2+} from the protein (HSA) to high-affinity coordination sites created at the TcR/HLA-interface.
Cytokine responses in metal-induced ACD

Figure 4. Mechanism of metal ion (e.g. Ni\textsuperscript{2+}) presentation. Two pathways of metal ion presentation are outlined: A) To become a complete antigen, the metal ion binds directly to a MHC-bound peptide (processing-independent presentation). B) The metal ion forms coordinative bonds to cysteine or histidine residues of soluble or membrane-bound proteins. The modified proteins are taken up by antigen-presenting cells (APC), and are processed and presented to T cells as metal-peptide complexes on MHC molecules. Modified from Büdinger and Hertl, (2000) with permission from Blackwell Publishing.

Despite evidence showing HLA restriction of Ni\textsuperscript{2+} recognition by Ni\textsuperscript{2+}-specific T cells, Ni\textsuperscript{2+}-induced ACD has not been associated with the distribution of any HLA alleles (Emtestam et al., 1993; Moulon et al., 1998). However, a preference for certain TcR-V\beta elements by both skin- and blood derived Ni\textsuperscript{2+}-reactive T cells has been demonstrated (Werfel et al., 1997; Cederbrant et al., 2003).

Cross reactivity versus co-sensitisation in metal-induced allergic contact dermatitis

Santucci et al., reported more frequent associated patch test positive reactions among transition metals than between these metals and other substances normally included in the standard patch test series (see ‘in vivo diagnosis of allergic contact dermatitis’) (Santucci et al., 1996). This may be due to concurrent exposure to different metals (co-sensitisation) or concomitant responses of T cell clones (cross reactivity) resulting from similar chemical properties of the metals and the consequent interactions inside the skin. Furthermore, sensitisation to one metal ion has been suggested to increase the chances of being sensitised to additional metals (Brasch et al., 2001).

Coordination complexes formed by Ni\textsuperscript{2+} and Pd\textsuperscript{2+} ions display marked spatial (geometric) conformational similarity as oppose to Co\textsuperscript{2+} or Cr\textsuperscript{3+} (see ‘Biochemistry of transition (heavy) metals’). The antigen determinants created by either metal ions with certain matrix or cellular proteins in the skin could be very similar and hence potentially interact with the same set of
specific TcR/MHC complex. Indeed, Moulon et al., demonstrated cross-reactivity between some Ni^{2+}-specific T cell clones and Pd^{2+} but not Co^{2+} or Cr^{3+} (Moulon et al., 1995). Later studies also showed patch test reactivity to Pd^{2+} to be almost exclusively found in subjects with reactivity to Ni^{2+} (Gawkrodger et al., 2000). A strong Ni^{2+} response may thus be indicative of potential Pd^{2+} reactivity. Subjects sensitised to one metal that induces a cell-mediated response cross-reactive to other metals, would thus be generally assumed to stand the risk of a relapse of contact dermatitis when exposed to the cross reacting metals.

Associated patch test reactivity to other transition metals has also been suggested to be due to co-sensitisation (concurrent exposure). Wahlberg and co-worker, using the repeated open application tests (ROATs) in guinea pigs, showed that animals induced by Co^{2+} reacted in the patch test and ROATs with Co^{2+} but not Ni^{2+}. Those induced with Ni^{2+} also reacted in patch testing to Ni^{2+} but not to Co^{2+} and in the ROATs to Ni^{2+} and less to Co^{2+} (Wahlberg and Liden, 2000). A significant number of subjects with Cr^{3+} and almost all with Co^{2+} reactivity have been shown to also react with Ni^{2+} in the in vivo patch test (Hegewald et al., 2005; Gawkrodger et al., 2000). It is worth noting that, isolated Co^{2+} reactivity is rare and reportedly linked, in some instances, to possible Ni^{2+} contamination of Co^{2+} patch test material (Eedy et al., 1991; Lisi et al., 2003).

Immune cells in metal-induced allergic contact dermatitis

Antigen presenting cells

The skin is the site of immediate contact with agents capable of inducing an ACD reaction. The epidermal LC, originally described in 1868 by Paul Langerhans, is the only cell type in normal epidermis that exhibits all accessory cell functions. LCs form a contiguous network within the epidermis and constitute 2%-5% of the total epidermal cell population (reviewed in Teunissen, 1992). LCs originate from CD34\(^+\) bone marrow progenitors that enter the epidermis via the blood stream (Dieu et al., 1998). Their numbers in the epidermis is maintained by local proliferation (Czernielewski and Demarchez, 1987). LCs express high levels of molecules mediating antigen presentation (e.g MHC Class I and II, CD1), as well as cellular adhesion and costimulatory molecules (e.g CD54, CD80 and CD86) (reviewed in Romani and Schuler, 1992). Thus, LCs represent the 'professional' APCs in the skin (Nestle and Burg, 1999) and play a pivotal role in the induction of cutaneous immune responses to infectious agents as well as contact sensitizers (Kimber et al., 1998).
Epidermal KCs, fibroblasts, and infiltrating mononuclear cells (e.g. macrophages that mature from blood monocytes) can upregulate their expression of MHC class I or class II molecules in the presence of LC derived IL-1β or TNF-α (Kondo and Sauder, 1995) and can hence serve as ‘non’ professional’ APC (Enk and Katz 1992b). Haptenised KCs produce IL-1β, TNF-α and lymphocyte-attracting chemokines, like CXCL10 (IP-10) (Flier et al., 1999) thus amplifying the ACD reactivity in the epidermis (Sterry et al., 1991).

**CD4+ T cells**
T cells expressing CD4 molecules recognize hapten in complex with MHC class II molecules (Van Seventer et al., 1986). CD4+ T cells have been shown to have a clear preponderance in cutaneous infiltrates during skin inflammatory responses induced by allergen contact. Hence this T cell lymphocyte subset is mostly associated with the effector phase of the ACD reaction (Moed et al., 2004b). Recently, a lot of attention has been focused on CD4+ CD25+ T cells, defined as Tr cells, suggested to be critical for the outcome of the ACD response in humans (Cavani et al., 1998; Cavani et al., 2000; Sebastiani et al., 2001; Cavani et al., 2003; Moed et al., 2005).

**CD8+ T cells**
CD8+ T cells recognize hapten in complex with MHC class I molecules (Van Seventer et al., 1986). Lipophilic haptens are generally associated with MHC class I molecules due to their ability to directly penetrate into LC, conjugate with cytoplasmic proteins and be processed along the ‘endogenous’ processing route (Kalish et al., 1994). However, metal (e.g. Ni2+)-specific CD8+ T cell clones have been successfully generated both from skin and peripheral blood of contact allergic subjects (Cavani et al., 1998, Moulon et al., 1998; Traidl et al., 2000). Thus, this suggests a possible mechanism of MHC class I presentation even for hydrophilic metal ions. CD8+ T cells have been demonstrated in epidermal mononuclear cell infiltrates during an ongoing skin inflammatory response to contact sensitisers (Zanni et al., 1998). CD8+ T cells are thought to mediate skin inflammation through killing of hapten-bearing target cells (Moulon et al., 1998; Traidl et al., 2000).

**γδ T cells**
In humans, less than 5% of T cells bear the γδ heterodimer, and the percentage of γδ T cells in the lymphoid organs of mice has been reported to range from 1% to 3% (Miescher et al., 1988). Surprisingly, γδ TcR expressing cells appear to represent a major T-cell population in
the skin, intestinal epithelium, and pulmonary epithelium (Pawankar et al., 1996). The localization of γδ T cells at epithelial sites, suggests that they are especially suited to combat epidermal or intestinal antigens and thus form a surveillance system that monitors the external milieu of the epithelial cells (Reviewed in Kabelitz et al., 2005). γδ TcR expressing cells have been shown to play a role in the elicitation of contact hypersensitivity (Askenase et al., 1995) probably in a non-antigen-specific and non-MHC-restricted manner (Ptak et al., 1991b; Dieli et al., 1998).

Cytokines investigated in this study
In the present study we investigated metal-induced cytokine-expression profiles representing different polarisations of the immune response; Th1- (IL-2 and IFN-γ), Th2-type (IL-4, IL-5 and IL-13) and T-regulatory (IL-10) (Table 1).

IFN-γ
IFN-γ, the key Th1-type cytokine, is involved in the induction or upregulation of cell adhesion molecules (Dustin et al., 1988) and exerts inflammatory effects mainly through effects on macrophages (Arenzana-Seisdedos et al., 1985). IFN-γ has been shown to be important in protection against mycobacterial infections (Newport et al., 1996) through the induction of tumour necrosis factor (TNF), NO\(^{-}\) and \(H_2O_2\). The latter involved in the killing of the intracellularly living bacteria. Several reports point to a critical role for IFN-γ in the induction and elicitation of metal-induced ACD (Kapsenberg et al., 1992; Traidl et al., 2000).

IL-2
IL-2, produced by activated CD4\(^{+}\) T helper cells (Carter and Swain, 1997) has been shown to be a growth and survival factor for antigen primed CD4\(^{+}\) and CD8\(^{+}\) T cells as well as NK cells (Horwitz et al., 2003). IL-2 has been described as both a Th0- and a Th1-type cytokine (Mosmann and Sad, 1996; O’Garra, 1998) due to its pleiotropic growth promoting properties on activated Th1-, Th2-, Tc1- and Tc2-cells. Increased production of IL-2 has been reported in ex vivo stimulated PBMC cultures from subjects allergic to Ni\(^{2+}\) but not control non-
allergics (Falsafi-Amin et al., 2000; Jakobson et al., 2002; Lindemann et al., 2003) suggesting a role, also for this cytokine in the ACD response to metals.

**IL-4**

IL-4 is the signature cytokine for Th2-type immune responses. It has been implicated in a broad spectrum of biological responses which include; regulation of the differentiation of naïve CD4\(^+\) T cells into a Th2 phenotype (O’Garra, 1998; Chen et al., 2004) and control of humoral immune responses by regulating switching in B cells from IgM/G to IgE and IgG4, in humans (Del Prete et al., 1988; Punnonen et al., 1993). IL-4 is a key cytokine in the development of IgE-mediated allergic inflammation (Steinke and Borish, 2001). IL-4 has been shown to cause erythema and induration when released in the skin (Asherson et al., 1996; Rowe and Bunker, 1998) suggesting a role for IL-4 in the inflammatory response to haptens by sensitised individuals upon subsequent exposure to the offending metal hapten.

**IL-5**

IL-5, considered to be a Th2-type cytokine, has been shown to play a role in the differentiation and maturation of cells involved in IgE-mediated allergic reactions, such as mast cells and eosinophils. IL-5 also inhibits certain macrophage functions (Sanderson, 1992) and acts as a growth factor for IgA-producing B cells (Sonoda et al., 1989). Rustemeyer et al. recently demonstrated elevated levels of IL-5 in PBMC cultures from Ni\(^{2+}\) allergic but not control subjects after stimulation with Ni\(^{2+}\) (Jakobson et al., 2002; Rustemeyer et al., 2004). However, the relationship between this and the inflammatory response in vivo is not yet known.

**IL-10**

IL-10, formerly described as a Th2-type cytokine that functions by down regulating Th1-cell activity, has been shown to be produced in comparable amounts by other cell types such as monocytes, macrophages, DCs, mast cells and keratinocytes (Enk and Katz, 1992a; Moser and Murphy, 2000). IL-10 displays immunomodulatory effects on both Th1 and Th2 cells (Bettelli et al., 1998) by inhibiting the production of IL-1\(\alpha\), IL-1\(\beta\), IL-6, IL-8, IL-12 and TNF-\(\alpha\), as well as its own production, and by down regulating the expression of co-stimulatory molecules required for appropriate antigen presentation (de Waal et al., 1991;
IL-10 has been shown to be produced by activated keratinocytes during the induction (sensitisation) phase of ACD and induce clonal anergy in hapten-specific T cells via effects on langerhans cells (LC) (Enk et al., 1993) and keratinocytes (KC) (Curiel-Lewandrowski et al., 2003). IL-10, produced by Ni\(^{2+}\)-specific CD4\(^+\)CD25\(^+\) Tr cell clones, has also been shown to inhibit the ability of DCs to stimulate Ni\(^{2+}\)-specific Th1 and Tc1 responses (Cavani et al., 2000).

**IL-13**

IL-13, like IL-4, has been described as a Th2-type cytokine, which promotes switching in B cells from IgM/G to IgE (the effector molecule in IgE-mediated allergy) and IgG\(_4\) in humans (Zurawski and de Vries, 1994; Punnonen et al., 1997). IL-13 has been shown to exert anti-inflammatory functions on monocytes and macrophages (McKenzie et al., 1993). Significantly higher IL-13 production has been demonstrated in cultures of PBMC from subjects allergic to metal (Ni\(^{2+}\)) or organic (methylisothiazolinones) haptens but not control subjects (Jakobson et al., 2002; Masjedi et al., 2003). However, similar to IL-5, the relationship between the magnitude of the IL-13 response *in vitro* and the ACD reaction *in vivo*, in terms of the patch test reactivity remains to be elucidated.

**In vivo diagnosis of allergic contact dermatitis**

The present diagnosis of ACD relies on the patch test first described by Jadassohn in 1895 (reviewed in Lachapelle, 2001). The patch test is a provocation test where the skin is exposed to a panel of haptens and the ensuing reaction is graded. The patch test is performed on normal skin on the back and involves the administration of a standard series of the most common contact allergens, which commonly include approximately 25-30 organic compounds as well as metal salts (Brasch and Geier, 1997) (see Table 3). There are specially designed patch test panels ‘tailor-made’ for individuals in different occupations/professions or with different types of dermatological problems. For example, for assessment of ACD in subjects with lichenoid reactions in the mouth, a dental series of haptens is often used and this panel includes, in addition to Ni\(^{2+}\), Co\(^{2+}\) and Cr\(^{6+}\), various acrylates and metals such as Au\(^{1+}\), Pd\(^{2+}\) and mercury (Hg\(^{2+}\)). Small volumes of a defined concentration of the allergen in a suitable vehicle (usually 5% petrolatum) are applied epicutaneously and then covered with specific strips. The strips are removed 48 h after application and the results read, scored and recorded on days 2 and 4 or days 3 and 6-7. Patch test results are read based on a scoring system recommended by the International Contact Dermatitis Research Group (ICDRG)
Cytokine responses in metal-induced ACD (Table 4) (Wahlberg, 2001). Positive reactions are defined as the appearance of erythema and oedema and with stronger reactions also papules and vesicles. Conclusive clinical diagnosis of ACD is based not only on patch testing but also the history of the patient, assessment of exposure as well as clinical examination.

Table 4. The scoring system for patch test reactions recommended by the International Contact Dermatitis Research Group (ICDRG)

<table>
<thead>
<tr>
<th>Score</th>
<th>Definition</th>
<th>Clinical manifestations</th>
</tr>
</thead>
<tbody>
<tr>
<td>+</td>
<td>Weak positive reaction</td>
<td>Erythema, infiltration, possible papules</td>
</tr>
<tr>
<td>++</td>
<td>Strong positive reaction:</td>
<td>Erythema, infiltration, papules, possible vesicles</td>
</tr>
<tr>
<td>+++</td>
<td>Extreme positive reaction:</td>
<td>Intense erythema and infiltration and coalescing vesicles</td>
</tr>
<tr>
<td>-</td>
<td>Negative reaction†</td>
<td></td>
</tr>
<tr>
<td>IR</td>
<td>Irritant reactions of different types.</td>
<td>Discrete patchy erythema or homogenous erythema without infiltration, patchy follicular erythema*</td>
</tr>
<tr>
<td>?</td>
<td>Doubtful reaction</td>
<td>Faint macular or homogenous erythema, no infiltration</td>
</tr>
</tbody>
</table>

†Patients who show negative reaction to the allergens may be sensitive to substances other than those tested. Testing with complementary substances may be indicated.
*Petechiae and follicular pustules are usually irritant and do not normally indicate allergy. It is sometimes very difficult to distinguish between an irritant reaction and a very weak positive reaction. Retesting maybe necessary to certify contact allergy. Modified from Wahlberg, 2001.

Apart from the potential risk for the patient to be sensitised due to direct skin exposure to the contact allergens, a number of problems have been associated with the in vivo patch test. The use of certain drugs, especially corticosteriods, or exposure to UV radiation has been reported to result in negative patch test reactions in subjects with a well-documented history of ACD (Bruze, 1986; reviewed in Wahlberg, 2001). Patch test results have also been shown to fluctuate in women with different results obtained at different times during their menstrual cycle (Hindsen et al., 1999). Most intriguing, complete discordant reactions have been reported when subjects are patch tested at the same time point with the same contact allergen preparation on opposite sides of the upper back (Bourke et al., 1999). Furthermore, interpretation of patch test results, especially with regards to discriminating between a negative, weak (+) or doubtful (?) reaction, is very difficult and tends to be subjective and depends on the observer’s experience (reviewed in Wahlberg, 2001). Frequent reports of
false-positive and false-negative reactions are a direct consequence of some or all of these drawbacks. There is therefore a need for reliable in vitro diagnostic test methods.

**In vitro diagnosis of allergic contact dermatitis**

A number of studies has been carried out aimed at developing in vitro assays that can replace or complement the patch test. The lymphocyte transformation test (LTT) exploits the proliferative responses of T cells upon in vitro activation with allergens (Nordlind, 1984; Masjedi et al., 2003). However, the LTT has been shown to have a low specificity, i.e. proliferative responses also reported in individuals with negative patch test reactions to specific haptens and no history of ACD (von Blomberg-van der Flier et al., 1987; Lisby et al., 1999). Results from recent studies aimed at characterising immune responses to metal and organic haptens by cytokine profiling, suggest that the measurement of cytokine production in response to contact allergens could be utilized as a diagnostic tool (Jakobson et al., 2002; Masjedi et al., 2003; Rustemeyer et al., 2004).

**Cytokine Detection Assays**

The enzyme-linked immunosorbent assay (ELISA) and the enzyme-linked immunospot (ELISpot) assay were used to define the cytokine expression profiles in metal-induced ACD in the studies reported in this thesis. The ELISA assay is very useful in determining the amount (concentration) of cytokines produced as a result of an immune response. The ELISA was first described by Engvall and Perlmann (1972) and employs high protein binding microtitre plates commonly in a 96-well format. There is a first step of adsorption of capture monoclonal antibodies (mAb) specific to an epitope of the cytokine of interest. The plates are subsequently incubated with supernatants from cells cultured in the presence or absence of defined antigens. Serial dilutions of recombinant human cytokines are assayed in parallel to obtain a standard curve. Thereafter the plates are incubated with biotinylated mAb specific to a second epitope on the captured cytokines followed by enzyme-labelled streptavidin (SA). The plates are then incubated with a substrate that forms a coloured soluble product. The colour intensity is read and standard plots drawn using the concentrations of the recombinant human cytokines from which the concentrations of the samples are extrapolated.

The ELISpot assay was first described as a method to determine the number of antibody-producing B cells (Czerkinsky et al., 1983; Sedgwick and Holt, 1983). The technique was later modified (Czerkinsky et al., 1988) for measurement of the frequency of antigen-specific
cytokine-producing cells and has become a useful method when elucidating immune responses at the single cell level. The principle is similar to that of the capture ELISA. Just as for ELISA, the assay involves a first step of adsorption of capture mAb to 96-well ELISpot plates utilising membranes as the solid phase for attachment of the capture mAb. However, instead of culture supernatants being used in the subsequent step, cells are incubated for varying periods (depending on the cell population, type of stimuli or cytokine investigated) in the plates and the mAb captures the cytokine produced. Detection of the captured cytokine is subsequently achieved by incubation of a biotinylated mAb followed by enzyme-labelled SA. Spots are generated by enzymatic cleavage of a substrate that yields an insoluble precipitate. The spots, a footprint of the locally accumulated cytokine produced by one cell, can later be counted using a dissection microscope or an image-analysis system. Since the ELISpot assay detects single cytokine-producing cells, and thus is dependent only on a locally high concentration of cytokine, it is possible to identify low frequencies of e.g. antigen-specific T cells that together produce cytokine levels below the detection level of a regular capture ELISA (Tanguay and Killion 1994; Ewen et al., 2001; Ekerfelt et al., 2002; Masjedi et al., 2003).
THE PRESENT STUDY

OBJECTIVES

The present study was undertaken with the overall objective of defining the relationship between *in vivo* responses in terms of patch test reactivity and *in vitro* responses to metals known to cause ACD in terms of cytokine production and to evaluate the potential of the cytokine detection assays, ELISpot and ELISA, for diagnosis of metal-induced ACD. The study had as specific objectives:

- To relate the *in vitro* reactivity to the *in vivo* reactivity to Ni$^{2+}$ in order to assess if subjects with different degrees of allergic reactions differ in their cytokine response profile and/or magnitude [I]
- To investigate the role of IL-10 in the regulation of cytokine responses in ACD to Ni$^{2+}$ [II]
- To define the profile and magnitude of cytokine responses to other common metal contact allergens *in vitro* [III]
- To optimise the ELISpot assay for diagnostic purposes [IV]
METHODOLOGY
More detailed descriptions of the methods used in this thesis are found in the accompanying papers (I-IV). Below is a brief description of the subjects, the reagents and the methods used to obtain the results discussed in this thesis.

Study subjects:
Forty subjects participated in the study described in paper I. These included 30 female subjects with a positive patch test to NiSO₄ (i.e. 10 subjects each with +3, +2 or +1 patch test reactivity) and 10 healthy volunteers (control subjects; 9 female and 1 male) with no history of contact allergy and a negative patch test result to NiSO₄. The study reported in paper III included 31 (28 female and 3 male) subjects with a positive patch test to one or more metal haptens and five healthy volunteers (control subjects; 4 female and 1 male) with no history of contact allergy and a negative patch test result to metals included in the standard as well as dental patch test series. The metal-allergic individuals and their matched controls were recruited from the Department of Dermatology and Venereology at Karolinska University Hospital in Stockholm. Both studies were approved by the Ethics committee at the Karolinska Hospital, Stockholm, Sweden (Ethical permission Dnr: 00-238).

Regular blood donors (n=40) registered at the Karolinska University Hospital, Stockholm, Sweden; either with unknown allergic status or with a clinical history of Ni²⁺-allergy, as stated by the subjects, provided buffy coats used in the studies reported in papers II and IV. Subjects included in the studies gave their informed consent to participation.

Diagnosis of contact allergy by the in vivo patch test (papers I and III):
The subjects were either patch tested with a standard patch test series including the metals nickel (NiSO₄; 5% in petrolatum), cobalt (CoCl₂: 1 % in petrolatum) and chromium (K₂Cr₂O₇: 0.5 % in petrolatum) (papers I and III) or with a dental series including the additional metals palladium (PdCl₂: 2 % in petrolatum) and gold (Na₃(Au(S₂O₃)₂); 2 % in petrolatum) (paper III). The metal haptens in petrolatum were applied by FinnChambers (Epitest Ltd Oy, Tuusula, Finland) epicutaneously on the backs of the subjects for 48 h. The patches were removed and the reaction read and scored using the scoring system recommended by the International Contact Dermatitis Research Group (ICDRG) (Brasch and Geier, 1997) (see ‘Table 4’ p30 on the ICDRG scoring system).
Blood sample collection and processing:
Whole blood samples were obtained from metal-allergic and healthy control subjects by venepuncture and collected in sterile heparinized glass vials and plasma samples separated from the whole blood [papers I and III]. Buffy coats were obtained from regular blood donors by centrifugation to remove the erythrocyte rich fraction (papers II and IV). PBMC were separated from the whole blood (papers I and III) or buffy coats (papers II and IV) by density-gradient centrifugation over Ficoll-Paque and kept frozen in liquid nitrogen until use.

IgE measurement (paper I):
Total plasma IgE levels (reference range 1.6-122 kU L⁻¹) were measured with the Pharmacia ImmunoCAP kit (Pharmacia Diagnostics, Uppsala, Sweden).

Measurement of cytokine levels in cell supernatants by ELISA (papers I, II and III):
The capture ELISA assay was used to determine the levels of cytokines in supernatants from cell cultures with or without antigen stimulation.

Evaluation of the detection sensitivity of one-step and two-step reagents in capture ELISA (paper IV):
Mouse anti-human IL-4, IL-13 and IFN-γ detection mAbs directly conjugated to the enzyme alkaline phosphatase (ALP) were developed for use in a simplified ELISpot assay for detecting allergen-induced cytokine production. The sensitivity of these directly conjugated mAbs was compared to that using the same mAbs conjugated to biotin requiring a second incubation step with a SA-ALP conjugate in ELISA. Briefly, ELISA plates were coated overnight with mouse anti-human capture mAbs [82.4 (IL-4), IL13-I (IL-13) and 1-D1K (IFN-γ)] (Mabtech, Nacka, Sweden) and subsequently with serial dilutions (1000 pg/ml to 2.0 pg/ml) of recombinant human IL-4, IL-13 (R&D Systems, Minneapolis, Minn., USA) or IFN-γ (Bender MedSystems GmbH, Vienna, Austria). The detection mAbs [12.1 (IL-4), IL13-II (IL-13) or 7-B-6-1 (IFN-γ) (Mabtech)] were either directly conjugated to ALP (one-step reagent) or biotinylated (two-step reagent). Plates incubated with the one-step reagents were incubated directly with the substrate [para-nitro-phenyl phosphate (pNPP)] whereas those with the two-step reagent needed an extra incubation step with SA-ALP prior to incubation with pNPP. The concentration of recombinant cytokine required to give an absorbance value of 2.0 for either system was compared as an indicator for the detection sensitivity.
Enumeration of cytokine-producing cells by ELISpot:
ELISpot assays were performed using either polyvinyl difluoride (PVDF) plates (MAIPS4510; Millipore Corp, Bedford, MA, U.S.A) pre-coated with capture mAbs (Mabtech, Stockholm, Sweden) [paper I and IV] or PVDF plates (ELIIP10SSP: Millipore Corp, Bedford, MA, U.S.A) coated with the capture mAbs overnight (a day before the assay) [papers II, III and IV]. Cell suspensions in culture medium containing antigens at defined concentrations were applied in triplicates to the plates; unstimulated cells were included in each plate to assess spontaneous production. The cytokines captured were detected using matched secondary mAbs either directly conjugated to ALP [paper IV] (Fig 5) or biotinylated with subsequent addition of SA-ALP [papers I, II, III and IV]. Plates were developed by incubation with bromo-chloro-indolyl phosphate/nitro blue tetrazolium-Plus substrate (Moss, Inc., Pasadena, MD, U.S.A) and spots analysed as described previously (Masjedi et al., 2003).

Determination of optimal concentration of metal salts for stimulation of PBMC (paper III).
The potential of different concentrations of a panel of metal salts to induce non-specific cytokine production or to exert toxic effects on PBMC in vitro i.e. suppression of mitogen-induced cytokine production, were assessed using the standard ELISpot assay. The ELISpot assay was set up using PBMC from non-allergic subjects with or without addition of the different metal salts and the frequency of IL-2-, IL-4- or IL-13-producing cells determined. A metal salt was defined as mitogenic at any given concentration if it induced a three-fold increase in the spontaneous production of all three cytokines (i.e an increment of at least 10 spots per 2.5x10^5 input cells over spontaneous production) at the said concentration. The toxicity of the metal salts was evaluated by adding PHA (PHA; 2 µg/ml) to parallel cultures and any metal salt concentration resulting in ≥ 25 % reduction in PHA-induced cytokine production was defined as toxic. Optimal concentrations for each metal salt (i.e concentrations that would induce optimal cytokine production with no mitogenic or toxic effects, as defined above) were determined using standard IL-2, IL-4 and IL-13 ELISpot assays. However, unlike above, PBMC (3.0x10^5 input cells/well) from metal allergic subjects were used.

Phenotypic characterisation of Ni^{2+}-specific cytokine producing cells using depletion experiments (paper II):
To determine the phenotype of Ni^{2+}-specific cytokine-producing cells, different cell populations were depleted or enriched in PBMC from Ni^{2+} reactive donors and the frequency
of Ni\textsuperscript{2+}-specific IL-4 producing cells and the levels of IFN-\(\gamma\) production determined. Briefly, 2.0 \times 10^7 PBMC in sterile 2% FBS/PBS were incubated with 4.0 \times 10^7 anti-human CD3, CD4 or CD8 (Dynal Biotech, Oslo, Norway) mAb coated beads. Cytokine production in cultures of the different cell fractions (whole PBMC, CD4\textsuperscript{+} or CD8\textsuperscript{+} enriched or CD3\textsuperscript{−}, CD4\textsuperscript{+} or CD8\textsuperscript{+} depleted) with or without 50 \(\mu\)M NiCl\textsubscript{2}.6H\textsubscript{2}O (Merck) or PHA (2 \(\mu\)g/ml; Orion Diagnostics) was determined by ELISpot or ELISA as described above. As an additional control for cell viability, the CD3 depleted cell fractions were stimulated separately with LPS (100 pg/ml; Sigma Aldrich, St Louis, MO, USA) or anti-human CD3 mAb (100 ng/ml; Mabtech) and IL-6, IL-10 and IFN-\(\gamma\) levels measured by ELISA in supernatants of 2 day cultures.

The cell depletions were confirmed by flow cytometry using phycoerythrin (PE)-conjugated anti-human CD3, CD4 or CD8 mAb (Becton and Dickinson) staining as described previously (Zuber et al., 2005) with saponin permeabilisation excluded from the protocol.

**Intracellular cytokine measurement by flow cytometry** (paper II):

PBMC, 6 \times 10^6 cells/ml, in culture medium (Gibco BRL) with or without either NiCl\textsubscript{2}.6H\textsubscript{2}O (50 \(\mu\)M; Merck, Darmstadt, Germany) or purified protein derivative (PPD; 5 \(\mu\)g/ml) (Orion Diagnostics, Trosa, Sweden), were incubated in 24 well (0.5ml/well) flat-bottom tissue culture plates (Costar Corp., Cambridge, MA, USA) at 37\textdegree c in a humidified atmosphere of 5% CO\textsubscript{2} for 3 days. Six hours before the end of the cultures, 20 ng/ml of phorbol myristate acetate (PMA) plus 1 \(\mu\)M ionomycin (Sigma Immunochemicals, St. Louis, MO, USA) were added to the cultures. In order to promote the accumulation of de-novo synthesised cytokines in the Golgi apparatus of the synthesising cell, Brefeldin A (BFA) (Sigma) at a 10 \(\mu\)g/ml final concentration was added to the cell cultures at the same time point as PMA-ionomycin. After stimulation, cells were processed for intracytoplasmic cytokine analysis as described previously (Zuber et al., 2005). Briefly, to simultaneously stain the cells with directly conjugated anti-cytokine and surface marker antibodies, 50 \(\mu\)l solutions of PE-labelled antibodies to either human IL-4, IL-10 (both from Becton and Dickinson, Franklin Lakes, NJ, USA) or IFN-\(\gamma\) (Mabtech) together with FITC labelled antibodies to either human CD4 (Mabtech) or CD8 (Becton and Dickinson) in saponin buffer were added to the cell suspensions and the plates incubated at RT for 1 h. The labelled cells were washed once with PBS containing 0.5% FCS, resuspended in 0.3 ml of the same buffer and immediately analyzed by flow cytometry in a FACScalibur\textsuperscript{®} (Becton and Dickinson) equipped with the CellQuest\textsuperscript{®} software. List mode data for 50,000 events in a “live gate” mode were acquired.
Single-cell cytokine production was evaluated after forward scatter (FSC) and side scatter (SSC) gating on lymphocytes.

**Statistical methods:**
Cytokine responses between groups of subjects displaying different patch test reactivity or controls [I and III] or between blood donors whose PBMC were reactive or not with Ni\(^{2+}\) [II and IV] were compared using the Mann-Whitney U-test. The Wilcoxon matched pairs test was used to compare responses by PBMC determined by the regular and a simplified ELISpot protocol [IV]; the same test was use to compare the change in the Ni\(^{2+}\)-induced cytokine responses by Ni\(^{2+}\)-reactive PBMC in the presence or absence of human recombinant IL-10 (rhIL-10) or anti-human-IL-10 (αh-IL-10) mAb respectively [II]. To evaluate the relationship between observed parameters, correlations were computed using the Spearman rank-order correlation coefficient r_s [I, II and IV]. A multivariate correlation analysis was performed to test for associations between multiple parameters (patch test, cytokine and IgE levels) [I]. For comparison of antigen-specific responses, values with background (spontaneous production) subtracted were used. A p-value < 0.05 was considered to be statistically significant. All tests were performed using the software STATISTICA 5.1 (StatSoft, Tulsa, OK, USA).
RESULTS AND DISCUSSION

Ni\textsuperscript{2+} induces a mixed cytokine response profile in PBMC from Ni\textsuperscript{2+}-sensitised subjects and this correlates with the in vivo patch test score (paper I):

We investigated, in the first study, if there is an association between specific cytokine profiles or the magnitude of the cytokines induced by Ni\textsuperscript{2+} in vitro and the severity of allergic reactivity in vivo. The in vivo reactivity to Ni\textsuperscript{2+}, defined by the subject’s patch test reactivity to NiSO\textsubscript{4}, was graded as +1 (weak), +2 (moderate) and +3 (strong) according to recommendations by the ICDRG (Wahlberg, 2001). The frequency of Ni\textsuperscript{2+}-specific cytokine producing cells and the levels of Ni\textsuperscript{2+}-induced cytokine production in PBMC from Ni\textsuperscript{2+}-allergic and control non-allergic subjects was determined by ELISpot and ELISA, respectively.

Our results showed significantly higher levels of Ni\textsuperscript{2+}-induced production of all cytokines [Th1- (IFN-\(\gamma\)), Th2-type (IL-4, IL-5 and IL-13) and T regulatory (IL-10)] in subjects patch test positive to NiSO\textsubscript{4} as compared to the controls. Most notably, there was a significant correlation between the degree of patch test reactivity [+1 (weak), +2 (moderate) or +3 (strong)] and the magnitude of the Ni\textsuperscript{2+}-induced production of all the cytokines.

Earlier studies investigating the cytokines implicated in ACD to Ni\textsuperscript{2+} suggested a major role for Th1-type cytokines in the ensuing detrimental inflammatory response (Sinigalia et al., 1985; Silvennoinen-Kassinen et al., 1991; Kapsenberg et al., 1992). Data from more recent reports suggest a mixed cytokine profile elicited by Ni\textsuperscript{2+} in PBMC from Ni\textsuperscript{2+}-allergic subjects (Falsafi-Amin et al., 2000; Jakobson et al., 2002; Lindemann et al., 2003). Our observation of concommittant induction of Th1-, Th2- and Tr-type cytokines by Ni\textsuperscript{2+} in PBMC from Ni\textsuperscript{2+}-sensitised subjects is thus in agreement with these recent reports. However, unique to our study, is the fact that we further categorised the Ni\textsuperscript{2+}-allergic subjects based on their different patch test reactivities (+1, +2, +3 or controls). We observed a positive correlation between the magnitude of the cytokine responses in vitro and the degree of reactivity in the in vivo patch test. Thus, Ni\textsuperscript{2+} induces a similar profile of cytokine responses in subjects with different degrees of in vivo reactivity to Ni\textsuperscript{2+} but the magnitude of the cytokine response is positively correlated with the degree of in vivo reactivity.
Noteworthy, however, despite the correlations observed between patch-test reactivity and cytokines representing both Th1- and Th2-type responses, we observed an exclusive Th2-type cytokine response to Ni$^{2+}$ in several of the Ni$^{2+}$-allergic subjects. Thus, in the context of contemporary descriptions of Ni$^{2+}$-induced ACD, our results suggest that Th2-type cytokines may have simultaneous down-regulatory effects by counterbalancing the deleterious Th1 responses during the DTH response, characteristic of ACD. On the other hand, Th2-type cytokines may exacerbate the proinflammatory responses in ACD already set off by Th1-type cytokine responses or in fact may have a direct role in the pathogenesis of ACD independent of Th1-type cytokines as is the case in atopic allergies (Parronchi et al., 1992; McHugh et al., 1994; Asherson et al., 1996; Rowe and Bunker, 1998; Liu et al., 2003).

To investigate if there existed any relationship between atopy and the exclusive Th2-type Ni$^{2+}$-induced cytokine response profile observed in a number of the Ni$^{2+}$-allergic subjects, total plasma IgE levels were measured. Interestingly, the IgE levels of the Ni$^{2+}$–allergic subjects were found to correlate negatively with the Ni$^{2+}$-induced IFN-γ responses ($p=0.026$, $r_s=-0.46$, $n=23$) of these subjects. The total level of plasma IgE is a crude, but nevertheless relevant correlate for the atopic status of an individual (Kasaian et al., 1995; Di Lorenzo et al., 1997; Di Gioacchino et al., 2000; Jackola et al., 2004). Lower proliferative as well as IL-2 responses to Ni$^{2+}$ have been reported in PBMC from subjects with ACD to Ni$^{2+}$ with concomitant atopic dermatitis but not in ACD subjects with no atopic dermatitis symptoms (Buchvald et al., 2004). It is, thus, possible that the atopic status of a subject can influence their cytokine response to contact allergens. However, due to the small number of Ni$^{2+}$-allergic subjects in this study that displayed high IgE levels, a possible relationship between atopy and IFN-γ responses to Ni$^{2+}$ can be best assessed in studies specifically designed with this question in mind.

**Diagnostic potential of in vitro cytokine responses to Ni$^{2+}$ in relation to the in vivo patch test (paper I):**

Our finding of a higher magnitude of Ni$^{2+}$-induced cytokine production by PBMC from Ni$^{2+}$-allergic compared to control subjects and the correlation to the *in vivo* patch test score suggests a diagnostic value for these cytokine assays. We therefore attempted a definition of positive and negative responses to Ni$^{2+}$ by setting cut off values based on the responses displayed by control subjects. The cut off criteria included both the cytokine increment (i.e cytokine production in cultures with Ni$^{2+}$ minus the unstimulated or spontaneous production)
and the cytokine ratio or index (i.e. ratio of cytokine production in cultures with Ni\textsuperscript{2+} and the unstimulated or spontaneous production).

Using these cut off criteria, the number of patch positive subjects responding in one or more of the assays was 23/30 (paper I). Eighteen out of the 20 subjects in the two groups with a more clearly defined allergy to Ni\textsuperscript{2+} [strong (+3) or moderate (+2) patch test reactivity and a clinical history of Ni\textsuperscript{2+} allergy] in contrast to only 5/10 of the weakly (+1) reactive subjects were positive according to the cytokine assays. All control subjects were negative. Most notably, the highest number of positive responses was found for the IL-4 ELISpot (n=19), IL-13 ELISA (n=19) and IL-13 ELISpot (n=17) assays, respectively. This, thus, suggests a better diagnostic value for the Th2-type (IL-4 and IL-13) cytokine assays as \textit{in vitro} correlates for an allergic reaction to Ni\textsuperscript{2+} as opposed to IFN-\(\gamma\). This would be useful especially for diagnosing ACD in Ni\textsuperscript{2+}-allergic subjects with concurrent atopis symptoms especially since diminished Th1-type cytokine responses to Ni\textsuperscript{2+} have been shown in this category of subjects (Buchvald et al., 2004). Our findings are in line with recent results by Rustemeyer et al. reporting better discrimination between Ni\textsuperscript{2+}-sensitised and nonsensitised subjects using IL-5, a Th2-type cytokine, but not the Th1-type cytokine, IFN-\(\gamma\) (Rustemeyer et al., 2004). Worthy of mention is the fact that, in the aforementioned study, IL-13 was not measured and IL-4 was used instead as a supplement in combination with IL-7 in seven days PBMC cultures to boost the levels of Ni\textsuperscript{2+}-induced IL-5 production.

The cytokine ELISpot assay has been shown to have potential applications as a diagnostic tool for infectious diseases such as \textit{Mycobacterium tuberculosis} infection (Lalvani et al., 2001a; Lalvani et al., 2001b). Our data put cytokine assays into focus as potential diagnostic tools for contact allergy to Ni\textsuperscript{2+} that could replace or complement the currently used \textit{in vivo} patch test. While the magnitude of the IL-4 and IL-13 ELISpot responses was in accordance with those reported by Jakobson et al. (2002) it was much higher than that reported by Lindemann et al. (2003) wherein detectable but still low levels of Ni\textsuperscript{2+}-induced IL-4 measured by ELISpot could only be obtained after pre-incubation with Ni\textsuperscript{2+} for two days. The use of nitrocellulose membrane plates instead of PVDF plates for the ELISpot assay (Schielen et al., 1995) may be one of several possible explanations for this discrepancy. The Lindemann study also identified IL-2 as an additional cytokine of interest for measurement of Ni\textsuperscript{2+}-induced responses by ELISpot.
With regard to the correlation between the *in vivo* patch test reactivity and the magnitude of the *in vitro* cytokine responses to Ni$_{2+}$, it is useful to highlight the uncertainties involved when describing subjects with weak (+1) as oppose to moderate (+2) or strong (+3) positive responses in the patch test. The reliability/reproducibility of a patch test score of +1 was evaluated by performing a second patch test on some of the subjects with a historic patch test score of +1 (unpublished data). This group included seven of the ten subjects with a historic patch test reactivity score of +1; the difference in time between patch test 1 and 2 was between 1-3 years and in one case 5 years. Four of these subjects did not respond to Ni$_{2+}$ *in vitro* according to the cut off criteria described above. Strikingly, only one of these subjects had a positive test score (+1) in the second test. The other six were scored as negative (Table 5).

**Table 5.** Discordance between patch tests, clinical history and *in vitro* cytokine test in +1 subjects

<table>
<thead>
<tr>
<th>Subject</th>
<th>Patch Test</th>
<th>Repeated Patch test</th>
<th>History of Ni$_2^+$ allergy</th>
<th>IL-4 ELISpot</th>
<th>IL-13 ELISA</th>
</tr>
</thead>
<tbody>
<tr>
<td>13</td>
<td>+1</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
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<tr>
<td>19</td>
<td>+1</td>
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<tr>
<td>21</td>
<td>+1</td>
<td>NDa)</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>22</td>
<td>+1</td>
<td>-</td>
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<tr>
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<tr>
<td>41</td>
<td>+1</td>
<td>ND</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

a) Not done.

Most reports of false positive or irritative reactions recorded as positive or vice versa at different times, mostly involve patch test responses described as weakly positive (+1) (Bourke *et al.*, 1999; Hindsen *et al.*, 1999; Wahlberg, 2001). In line with this, we observed a poor concordance between patch test results, history of allergy to Ni$_2^+$ and *in vitro* reactivity to Ni$_2^+$ in the group of subjects with +1 reactivity in the patch test. Thus, the variability of the patch
test results makes it difficult to compare patch test and \textit{in vitro} test results for patch test positive subjects with a score of +1.

\textbf{The regulatory cytokine, IL-10, downregulates both Th1- and Th2-type cytokine responses in Ni\textsuperscript{2+}-induced ACD (paper II):}

A key finding in the first study was our observation of elevated levels of Ni\textsuperscript{2+}-induced production of the regulatory cytokine IL-10 in the Ni\textsuperscript{2+}-allergics which, just like the Th1- and Th2-type cytokine responses, correlated with the degree of reactivity of the subjects in the \textit{in vivo} patch test. This is in line with another report (Cedebrant \textit{et al.}, 2003). However, Rustemeyer \textit{et al.} recently showed elevated levels of Ni\textsuperscript{2+}-induced IL-10 mainly in tolerised subjects (Rustemeyer \textit{et al.}, 2003). We therefore tried to define the role of IL-10 in the ACD response to Ni\textsuperscript{2+} in relation to its effects on other cytokines.

PBMC used in this study were from blood donors with a stated history of ACD to Ni\textsuperscript{2+} and a confirmed \textit{in vitro} responsiveness to Ni\textsuperscript{2+} (ACD PBMC) and donors with unknown allergic status but a confirmed non-responsiveness to Ni\textsuperscript{2+} \textit{in vitro} (control PBMC). \textit{In vitro} responsiveness was determined using cut off definitions for IL-4 and IL-13 ELISpot as defined in the first study. The levels of IL-13, IL-10 and IFN-\gamma production and the number of IL-4-, IL-13- and IFN-\gamma-producing cells after Ni\textsuperscript{2+} stimulation were assessed by ELISA and ELISpot, respectively in the presence or absence of a concentration range of rIL-10. As expected, we observed significantly higher and correlated Th1-, Th2-type and regulatory (IL-10) cytokine responses to Ni\textsuperscript{2+} in ACD PBMC as compared to control PBMC. Furthermore, addition of rIL-10 to ACD PBMC reduced the levels of Ni\textsuperscript{2+}-induced IL-13 and IFN-\gamma; rIL-10 had the most potent down regulatory effect on Ni\textsuperscript{2+}-induced IFN-\gamma levels with a significant reduction seen even at the lowest concentration of rIL-10 (0.2 ng/ml), which better reflects the endogenous levels of IL-10 induced in response to Ni\textsuperscript{2+}.

To assess if also the Ni\textsuperscript{2+}-induced endogenous IL-10 exerts a similar suppressive effect on cytokine responses to Ni\textsuperscript{2+} as seen with rIL-10, we measured Ni\textsuperscript{2+}-induced cytokine production in PBMC cultures with or without \textit{α}-hIL-10 mAb (10 \textmu g/ml). If Ni\textsuperscript{2+}-induced IL-10 indeed suppresses the production of other cytokines, we reasoned, then Ni\textsuperscript{2+}-induced cytokine responses would be enhanced upon its neutralisation. Whereas we observed a significant increase in the levels of IFN-\gamma in response to Ni\textsuperscript{2+} in the presence of mAb to IL-10,
there was no net effect on the IL-13 levels or number of IL-4-, IL-13- or IFN-γ-producing cells upon addition of the α-hIL-10 mAb. Furthermore, a greater enhancing effect of IL-10 neutralisation on the levels of production of IFN-γ was seen in donors with high (increment above background >30 pg/ml) IL-10 production. It is worth mentioning that, the already low or absent production of cytokines in response to Ni²⁺ by control PBMC was not affected by addition of mAb to IL-10.

Cavani and co-workers had showed previously that IL-10 produced by Ni²⁺-specific regulatory T cell clones markedly diminished the capacity of monocytes to stimulate Ni²⁺-specific type 1 T cell clones (Cavani et al., 2000). Our data confirms a key regulatory role for IL-10 in the immune reactivity to Ni²⁺. Noteworthy, is the fact that we used ex vivo stimulated PBMC, which more closely reflect the in vivo situation. A number of studies have reported a protective role for IL-10 in IgE-mediated allergic immune responses (Akdis et al., 1998; Bellinghusen et al., 2001; Royer et al., 2001). Akdis et al., showed an increase of both Th1-(IFN-γ) and Th2-type (IL-13) allergen-specific responses in PBMC from healthy subjects upon neutralisation of endogenous IL-10 elicited by an atopic allergen (Akdis et al., 1998). Although we also found a pleiotropic downregulatory effect on IL-4, IL-13 and IFN-γ responses to Ni²⁺ by PBMC after adding rIL-10, only the IFN-γ production levels increased significantly upon IL-10 neutralisation. This apparent difference in the number and type of cytokines affected by neutralisation of endogenous IL-10 in Ni²⁺-induced ACD compared to atopy has a bearing on the distinct cytokine profiles elicited in atopic and non-atopic as opposed to ACD and non-ACD subjects. Allergens causing atopic allergy would generally elicit a mixed (Th1-, Th2- and regulatory type) cytokine response in both atopic and non-atopic subjects (Akdis et al., 2004), with a preponderance of the Th2 responses in the atopics and the regulatory (IL-10) type responses in non-atopics. In ACD to Ni²⁺, elevated Ni²⁺-induced IL-10 responses tend to coincide with increased Th1- and Th2-type responses in allergic subjects but little or no responses in healthy controls. With respect to immune responses to M. tuberculosis, a microbe known to induce a classical Th1-type (IFN-γ) mediated DTH response (Lalvani et al., 2001a; Lalvani et al., 2001b), endogenous IL-10 neutralisation has been shown to induce strong CD8⁺ cytotoxic T cell-mediated lytic activity together with an increased expression of costimulatory molecules on target cells (De la Barrera et al., 2004). Endogenous IL-10 neutralisation may result in similar enhanced effector
activity on T cells involved in ACD to Ni\(^{2+}\) consequent to the observed increase in the levels of Ni\(^{2+}\)-induced IFN-\(\gamma\) production.

The significant reduction both in the number of cytokine producing cells and the levels of the secreted cytokines upon addition of rIL-10 may suggest a direct suppressive effect of IL-10 on individual cytokine producing cells. IL-10 has been shown to directly suppress T cell activation by binding to a CD28-associated IL-10 receptor on T cells thereby inhibiting tyrosine phosphorylation of this key costimulatory molecule (Akdis et al., 2000). IL-10 neutralisation on the other hand, seems to boost cytokine production per cell without any major effect on spontaneous (non-specific) production; hence our observation of elevated levels of Ni\(^{2+}\)-induced IFN-\(\gamma\) production in the presence of \(\alpha\)-hIL-10 mAb without any corresponding increase in the number of IFN-\(\gamma\)-producing cells.

**CD4\(^+\) T cells are the lymphocyte population in PBMC solely responsible for IL-4 and IFN-\(\gamma\) production after Ni\(^{2+}\) stimulation** (paper II):

In light of our demonstration of a downregulatory effect of IL-10 on Ni\(^{2+}\)-induced production of both Th1- and Th2-type cytokines coupled with reports of a direct suppressive effect of IL-10 on T cells (Akdis et al., 2000), we next investigated the phenotype of the Ni\(^{2+}\)-reactive cells in PBMC i.e. the cells likely affected by the immunomodulatory effects of IL-10.

To achieve this aim, PBMC were enriched for CD4\(^+\) or CD8\(^+\) or depleted of CD3\(^+\), CD4\(^+\) or CD8\(^+\) cells and the frequency of IL-4 producing cells and the levels of IFN-\(\gamma\) produced upon Ni\(^{2+}\) stimulation were determined in these different cell fractions. Cell depletion was confirmed by flow cytometry and 89-99\% of the CD3\(^+\), CD4\(^+\) or CD8\(^+\) cells were depleted using our protocol with the best purity obtained following CD4\(^+\) cell depletion. Depletion of the cells expressing the pan T cell marker, CD3 (\(\alpha/\beta\) and \(\gamma/\delta\) TcR expressing T cells) (Miescher et al., 1988), completely abrogated both IL-4 and IFN-\(\gamma\) production after Ni\(^{2+}\) stimulation. Depletion of the Th cell fraction only (i.e. CD4\(^+\) T cell) (De Gast et al., 1985) had a similar effect on IL-4 and IFN-\(\gamma\) production. On the contrary, depletion of the CD8\(^+\) cell fraction (T cytotoxic/effector cells) (Van Seventer et al., 1986) had a positive effect on the magnitude of the Ni\(^{2+}\)-induced production of both cytokines. Significant levels of Ni\(^{2+}\)-induced production of both cytokines were found in the CD4\(^+\) enriched cell fraction after substraction of the spontaneous background. These were however generally lower than the
levels seen in the whole PBMC (data not shown). There was no detectable Ni\textsuperscript{2+}-induced production of either cytokine above background in the CD8\textsuperscript{+} enriched cell fraction.

Previous studies involving long-term cultured T-cell lines or clones have attributed Th1- and Th2-type cytokine responses to Ni\textsuperscript{2+} to both CD4\textsuperscript{+} and CD8\textsuperscript{+} T cells (Moulon et al., 1998; Traidl et al., 2000). However, Moed et al., using cells directly isolated from PBMC, recently showed that only CD4\textsuperscript{+} CLA\textsuperscript{+} CD45RO\textsuperscript{+} and not CD8\textsuperscript{+} T cells proliferate and produce both Th1- (IFN-\gamma) and Th2-type (IL-5) cytokines in response to Ni\textsuperscript{2+} (Moed et al., 2004b). In this study, we investigated the effect of depletion of the CD3\textsuperscript{+}, CD4\textsuperscript{+} or CD8\textsuperscript{+} populations from PBMC, a more direct way of confirming the role of these cells, on Ni\textsuperscript{2+}-induced Th1- and Th2-type cytokine production. Our results suggest that CD4\textsuperscript{+} T cells may be the cell population mainly responsible for production of both IL-4 and IFN-\gamma in response to Ni\textsuperscript{2+} stimulation.

Analysis of Ni\textsuperscript{2+}-induced IL-10 production following depletion of different lymphocyte fractions, unlike IL-4 and IFN-\gamma measurements, yielded inconclusive results. We obtained decreased levels of IL-10 after depletion of either CD4\textsuperscript{+} or CD8\textsuperscript{+} cells in some instances but in most instances no significant effect was observed. Earlier studies by other workers, using cell clones, suggested that CD4\textsuperscript{+} CD25\textsuperscript{+} T regulatory cells are responsible for the bulk of antigen-induced IL-10 production (Cavani et al., 2000; Sebastiani et al., 2001). The mixed results we obtained could be due to the generally low levels of Ni\textsuperscript{2+}-induced IL-10 production (around 30 pg/ml or less) seen in most donors in this study. On the other hand, IL-10 production has been demonstrated in a wide variety of cell types, besides T cells (Enk and Katz, 1992a; Royer et al., 2001). This could account for the lack of any clear observable differences in the levels of Ni\textsuperscript{2+}-induced IL-10 after depleting the T (CD3\textsuperscript{+}, CD4\textsuperscript{+} or CD8\textsuperscript{+}) cell fractions.

In a nutshell, our data confirms and extends previous results by us and others showing that PBMC from subjects with ACD to Ni\textsuperscript{2+} produce IL-10 upon Ni\textsuperscript{2+} stimulation and that the magnitude of the Ni\textsuperscript{2+}-induced IL-10 correlated positively with Th1- and Th2-type responses. Further, endogenous IL-10 levels resulting from Ni\textsuperscript{2+} stimulation have a major down-regulatory effect on the IFN-\gamma producing capacity of Ni\textsuperscript{2+}-specific CD4\textsuperscript{+} T cells.
Transitional metals commonly used in jewelry, metal alloys in dentistry and orthopaedics, induce significant cytokine production in PBMC from contact allergic subjects (paper III):

We as well as others have shown that Ni\(^{2+}\) is capable of inducing significant production of cytokines (both Th1- and Th2-type) in ex vivo stimulated PBMC from subjects with ACD to Ni\(^{2+}\) but not control non-allergics (Jakobson et al., 2002; Lindemann et al., 2003; Rustemeyer et al., 2004). However, there is very little information regarding the cytokine profiles induced by other transition (heavy) metals also known to cause ACD. Knowledge on the profile and the magnitude of the cytokines induced by other metals, apart from further elucidating the immune mechanisms involved in metal-induced ACD, could be potentially exploited in developing in vitro methods for the diagnosis of ACD. We therefore, investigated the profile and magnitude of cytokine responses to a panel of metals in PBMC from subjects with a history of ACD and confirmed in vivo patch test reactivity to one or several metals. The frequency of IL-2-, IL-4- and IL-13-producing cells and the levels of production of IL-2, IL-13 and IFN-\(\gamma\) in PBMC stimulated with a panel of metal salts was measured by ELISpot and ELISA, respectively.

In agreement with our previous results (paper I), Ni\(^{2+}\) induced significantly higher cytokine responses in PBMC from Ni\(^{2+}\) patch test reactive subjects compared to the non-allergic controls; a mixed Th1-/Th2-type response was seen in most of these subjects. Use of either nickel salts (NiCl\(_2\) or NiSO\(_4\)) yielded a similar magnitude and profile of cytokine responses. Furthermore, both Ni\(^{2+}\) salts induced cytokine production in PBMC from non-allergic control subjects (non-specific cytokine-inducing effects) at concentrations greater than or equal to 100 \(\mu\)M. Both chromium salts (CrCl\(_3/\)Cr\(^{3+}\) or K\(_2\)Cr\(_2\)O\(_7/\)Cr\(^{6+}\)) induced significant cytokine [Th1- (IL-2) and/or Th2-type (IL-4 and IL-13)] production in PBMC from Cr\(^{6+}\) patch test positive subjects but not in the non-allergic controls. Cr\(^{3+}\) tended to induce responses of higher magnitude. Furthermore, we also showed that Cr\(^{6+}\) but not Cr\(^{3+}\) is toxic at very low concentrations. Low concentrations of Cr\(^{6+}\) have previously been reported to induce cell death or inhibition of cytokine production in vitro and also cause skin irritation (Reinhardt et al., 1985; Li and Wang, 2002). Moreover, Hansen et al., reported induction of eczema at low concentrations of Cr\(^{6+}\) but not Cr\(^{3+}\) (Hansen et al., 2003). Thus, our data shows that Cr\(^{3+}\) just like Cr\(^{6+}\) induces significant cytokine production in vitro in PBMC from chromium patch test positive subjects and suggests that the salt with Cr\(^{3+}\) may be preferable for use in stimulation of PBMC for in vitro cytokine analysis. Just under half of the CoCl\(_2\) (Co\(^{2+}\)) patch test positive subjects responded to Co\(^{2+}\) with significant cytokine production to Co\(^{2+}\) in vitro. PBMC
Cytokine responses in metal-induced ACD

responses to Co\(^{2+}\) stimulation generally involved production of either IL-2 alone or the complete profile of cytokines (IL-4, IL-13 and IFN-\(\gamma\)). Co\(^{2+}\) has been shown to elicit irritative and/or doubtful reactions in the patch test (Garner et al., 2004). This inherent irritative property may account for some of the false positive results that have been reported. Thus \textit{in vitro} cytokine assays, which measure immunological memory, may be better at confirming a true allergic reaction as well as discriminating this from any false positive reaction seen with the patch test.

Besides our observation of positive \textit{in vitro} response to Pd\(^{2+}\) with production of at least one cytokine by PBMC from all subjects patch test positive to Pd\(^{2+}\) in the dental series, Pd\(^{2+}\) also induced significant cytokine production in PBMC from some subjects tested only with the standard series (excluding Pd\(^{2+}\) and Au\(^{+}\)). This suggests that the prevalence of ACD to Pd\(^{2+}\) may be considerably higher than assumed since most population/prevalence studies are based on patch testing with the standard series (Brasch et al., 2001; Bryld et al., 2003; Hegewald et al., 2005). Similar to Ni\(^{2+}\), Pd\(^{2+}\) induced non-specific cytokine production at concentrations greater than 100 \(\mu\)M. PBMC from gold patch test positive subjects showed significantly higher levels of IL-13 and IFN-\(\gamma\) responses to either salt of the metal (Na\(_3\)(Au(S\(_2\)O\(_3\))\(_2\)/Au\(^{+}\) or HAtCl\(_4\)/Au\(^{3+}\)) \textit{in vitro} compared to the non-allergic controls. Au\(^{+}\) and Au\(^{3+}\) had toxic effects on mitogen (PHA)-activated PBMC at concentrations above 50 \(\mu\)M and 70 \(\mu\)M, respectively. However, despite the observed toxicity of Au\(^{+}\) at lower concentrations compared to Au\(^{3+}\), PBMC stimulation with Au\(^{+}\), at concentrations below 50 \(\mu\)M, resulted in responses of higher magnitude with a better distinction between subjects patch test positive to Au\(^{+}\) and non-allergic controls.

Overall, the results show that other metals included in the standard and/or dental series of the \textit{in vivo} patch test, just like Ni\(^{2+}\), are capable of inducing specific cytokine production \textit{in vitro}. Furthermore, our data point to potential future applications of the \textit{in vitro} cytokine assays, as complementary or confirmatory tools, in the diagnosis of ACD.

\textbf{In vitro evidence of in vivo cross-reactivity and/or co-sensitisation patterns} (paper III):

A significant number of subjects showed positive reactions in the \textit{in vivo} patch test to more than one metal in both the standard and dental series. We therefore investigated if PBMC from these subjects responded \textit{in vitro} to all the metals to which they were reactive in the patch test.
Approximately 45% (4/9) and 75% (9/12) of those patch test positive to Cr\(^{6+}\) and Co\(^{2+}\), respectively, were also reactive to Ni\(^{2+}\) in the in vivo patch test. Interestingly, in vitro cytokine responses were induced by Ni\(^{2+}\) stimulation of PBMC from a higher percentage of these subjects i.e about 90% (8/9) and 85% (10/12) for the Cr\(^{6+}\) and Co\(^{2+}\) patch test positive subjects, respectively. Some of the subjects that were positive for both Ni\(^{2+}\) and Co\(^{2+}\) in the in vivo patch test showed positive in vitro cytokine responses only to Ni\(^{2+}\). Furthermore, all Co\(^{2+}\) reactive subjects with concurrent Ni\(^{2+}\) patch test reactivity showed a positive in vitro response also to Pd\(^{2+}\) with in vitro responses to Ni\(^{2+}\) and Pd\(^{2+}\) seen even in one Co\(^{2+}\) patch test positive subject that failed to respond to Co\(^{2+}\) in vitro; a few Cr\(^{6+}\) reactive subjects also responded to Au\(^{1+}/Au^{3+}\), Co\(^{2+}\) and Pd\(^{2+}\). There have been reports of concurrent Co\(^{2+}/Cr^{6+}\), Co\(^{2+}/Ni^{2+}\), Cr\(^{6+}/Ni^{2+}\) or Ni\(^{2+}/Co^{2+}/Cr^{6+}\) responses in the in vivo patch test (Gawkrodger et al., 2000; Uter et al., 2004; Hegewald et al., 2005). Our results therefore confirm, in vitro, the reported in vivo reactivities observed with these metals but also suggest that there may exist low levels of subclinical Ni\(^{2+}\)-cross reactivity in subjects, patch test positive to metals other than Ni\(^{2+}\). Some authors have even suggested that some cases of isolated Co\(^{2+}\) reactivity in the in vivo patch test may be as a result of Ni\(^{2+}\) contamination of Co\(^{2+}\) patch test material (Rystedt, 1979; Eedy et al., 1991; Lisi et al., 2003).

Less than half of the Au\(^{6+}\) reactive subjects showed a positive reaction to other metals in the in vivo patch test (1/10 to Co\(^{2+}\) or Cr\(^{2+}\) and 3/10 to Ni\(^{2+}\) or Pd\(^{2+}\)). While, a significant number of subjects that reacted to other metals, but Au\(^{6+}\), in the in vivo patch test tended to also react to Au\(^{1+}/Au^{3+}\) in vitro, most subjects that were exclusively patch test positive to Au\(^{2+}\) responded only to Au\(^{1+}/Au^{3+}\) in vitro. All except one of the Pd\(^{2+}\) patch test positive subjects were also positive to Ni\(^{2+}\). Strikingly, all the Pd\(^{2+}\) patch test positive subject showed a strong positive reaction to Ni\(^{2+}\) in vitro. In a few instances, Pd\(^{2+}\) reactive subjects showed a positive reaction to Au\(^{1+}/Au^{3+}\) or Co\(^{2+}\). A number of Pd\(^{2+}\) patch test negative subjects (i.e those tested with the dental series) reacted to Pd\(^{2+}\) in vitro; all subjects that reacted with Pd\(^{2+}\) in vitro also reacted with Ni\(^{2+}\) in vitro. Moulon et al., showed that Ni\(^{2+}\)-specific T cell clones cross-react with Pd\(^{2+}\) but not Co\(^{2+}\) or Cr\(^{2+}\) (Moulon et al., 1995). In addition, other studies have suggested an overlap in responses to Ni\(^{2+}\) and Pd\(^{2+}\) in the in vivo patch test with Pd\(^{2+}\) reactivity found almost entirely in subjects with an already confirmed reactivity to Ni\(^{2+}\) (Santucci et al., 1996; Gawkrodger et al., 2000).
In conclusion therefore, apart from the potential of discriminating between metal-sensitized and non-sensitized subjects, *in vitro* cytokine assays could be useful in identifying subjects with multiple metal sensitisations. Furthermore, with respect to Pd$^{2+}$ reactivity, our data suggests that a strong Ni$^{2+}$ response *in vitro* may be indicative of Pd$^{2+}$ reactivity.

**Optimisation of a simplified ELISpot assay for enumeration of allergen-specific cytokine-producing cells** (paper IV):

The above results together with data from previous studies by our group (Jakobson *et al*., 2002), using the ELISpot assay point to a potential use of the cytokine ELISpot as a tool to discriminate between Ni$^{2+}$-sensitised and non-sensitised subjects. In paper IV, we aimed at evaluating a simplified ELISpot protocol for use in the detection of Ni$^{2+}$-specific cytokine producing cells.

The simplified assay consisted of precoated plates and one-step (ALP-conjugated mAb) detection reagents as opposed to overnight adsorption of capture mAb and two-step (biotinylated mAb followed by SA-ALP) detection reagents in the conventional assay. To validate the simplified assay, we compared the sensitivities of the one-step and two-step detection reagents in parallel in a capture ELISA. The one-step detection system displayed a higher sensitivity than the two-step detection system with approximately 1.8 and 1.4 times lower concentrations of recombinant cytokine needed to obtain absorbance values of 2.0 for IL-4 and IL-13 respectively. On the other hand, detection with the one-step detection system required approximately 2.3 times higher concentration of the recombinant cytokine to yield the same absorbance value as that obtained in the two-step detection system for IFN-$\gamma$.

Although, in order to increase the detection sensitivity, the use of enzyme-labelled detection antibodies in sandwich-based immunoassays have to a large extent been replaced by the use of two-step detection systems (Porstmann and Kiessig, 1992; Ahlborg and Paulie, 2002), the detection sensitivity of the one-step and two-step detection systems investigated in this study are quite comparable.

To further investigate the usefulness of the simplified compared with the regular ELISpot protocol, the two protocols were used in parallel to analyse the number of antigen (Ni$^{2+}$, TT or PPD) or mitogen (PHA)–induced IL-4-, IL-13- and IFN-$\gamma$-producing PBMC from blood donors with or without a history of ACD to Ni$^{2+}$. Using both protocols, significantly higher Ni$^{2+}$-induced IL-4 and IL-13 responses in PBMC from the subjects with a history of ACD to
Ni$^{2+}$ (p=0.005 and p=0.02 for the simplified and regular IL-4 ELISpot and p=0.009 and p=0.04 for the corresponding IL-13 ELISpot, respectively) were seen. There were no significant differences between the two protocols in the detection of cells producing IL-4, IL-13 or IFN-γ after activation with any of the three antigens. A highly significant correlation was found between the two ELISpot protocols when the number of cytokine-producing cells in response to all three antigens was compared for all the subjects (Fig. 6).

Several studies have highlighted the value of the cytokine ELISpot as a powerful tool to monitor and measure antigen-specific T-cell responses (Czerkinsky et al., 1988; Smith et al., 2001) in experimental research and recently also for possible diagnostic applications (Lalvani et al., 2001a; Lalvani et al., 2001b). PBMC from Ni$^{2+}$-reactive subjects have been shown, using the cytokine ELISpot, to produce significantly higher levels of both Th1 and Th2 cytokines in response to Ni$^{2+}$ compared to non-allergic subjects (Jakobson et al., 2002; Lindemann et al. 2003). The observation in the present study of Ni$^{2+}$-induced IL-4 and IL-13 responses only in PBMC from blood donors with a history of contact allergy to Ni$^{2+}$ further supports the use of the ELISpot assay as an alternative diagnostic test.

The number of cells spontaneously producing cytokine was found to be higher using the regular ELISpot, especially for IFN-γ. This may suggest a higher detection capacity by the regular protocol. However, the amount of spontaneously secreted cytokines is generally low as opposed to antigen-induced cytokines (Ekerfelt et al., 2002; Mäkitalo et al., 2002). Thus, a decreased detection of spontaneously producing cells combined with a comparable detection of antigen-specific spots could be an advantage when distinguishing between antigen-specific responses in responder and non-responder individuals since a better signal-to-noise (antigen-specific/spontaneous) ratio can be obtained.

The development of a simplified ELISpot assay with an overall reduction of work and incubation steps, apart from reducing the assay time makes it possible to use one batch of precoated plates throughout a study, thus eliminating one source of inter-assay variation. The simplified ELISpot assay protocol, compared to the regular assay format, would facilitate larger clinical as well as experimental studies without any loss of sensitivity.
Ni\textsuperscript{2+}-sensitised and non-sensitised subjects respond to recall antigens with a similar cytokine profile (In progress and paper IV):

We have therefore established that subjects with ACD to Ni\textsuperscript{2+} but not control non-allergic subjects show elevated Th1-, Th2- and regulatory cytokine responses to Ni\textsuperscript{2+}. However, whether this points to a tendency for subjects with ACD to Ni\textsuperscript{2+} to manifest a generalised proinflammatory response to recall antigens has not been elucidated. We therefore investigated if PBMC from subjects with a history of ACD and a confirmed \textit{in vitro} reactivity to Ni\textsuperscript{2+} displayed a tendency of responding with a particular cytokine pattern also to other stimuli such as tetanus toxoid (TT) and purified protein derivative (PPD) from \textit{M. tuberculosis} or the mitogen PHA.

Whereas TT elicited production of IFN-\gamma, IL-13 as well as IL-4 at similar levels, PPD elicited a higher number of cells producing IFN-\gamma than IL-13 and few IL-4-producing cells. However, unlike Ni\textsuperscript{2+}, TT and PPD induced significant cytokine responses in both subjects with a history of ACD to Ni\textsuperscript{2+} and the controls with no significant difference between the ACD subjects, as a group, and the controls. Strikingly, when the subjects with ACD to Ni\textsuperscript{2+} were subdivided based on their patch test reactivity score (+3, +2 and +1) the TT-induced response was generally the highest in the +1 group and lowest in +3 group (Fig. 7). Our observation of a positive correlation between the patch test reactivity score and the magnitude of the \textit{in vitro} response to Ni\textsuperscript{2+} implies a generally higher frequency of Ni\textsuperscript{2+}-specific memory T cells in ACD subjects with a patch test score of +3 compared to those with +2, +1 or the controls. We therefore speculated that an increase in the number of Ni\textsuperscript{2+}-specific memory T cells due to a more recent sensitisation and/or repeated exposure to Ni\textsuperscript{2+} results in a decrease in the net proportion of memory T cells specific to recall antigens to which subjects were immunised decades ago. In the absence of recent boosting or exposure to the pathogen a drop in the frequency of circulating memory T cells specific to recall antigens would compensate for the increase in the proportion of the Ni\textsuperscript{2+}-specific memory T-cell pool; a sort of requirement for immunological homeostasis (reviewed in Seder and Ahmed, 2003). This is not unreasonable given the mean age of the subjects that participated in our studies (mostly women above 40 years of age).

Although we observed individual variations in the response to PHA, there was no general tendency that the PHA-induced responses differed between subjects with ACD to Ni\textsuperscript{2+} and the controls.
These preliminary findings therefore show that subjects with ACD to Ni\textsuperscript{2+} are not prone to elevated proinflammatory (both Th1- and Th2-type) cytokine responses to every antigen. However, the degree of \textit{in vivo} reactivity to Ni\textsuperscript{2+} may affect the balance of the memory T cell pool specific to recall antigens arising from old vaccination regimens. However, more studies using a larger pool of antigens commonly used in vaccination regimens are needed to further pursue this phenomenon.
GENERAL CONCLUSIONS AND PERSPECTIVES

The following general conclusions can be drawn from the results discussed in this review and in the individual articles that follow;

- Subjects with different degrees of patch test reactivity to Ni\(^{2+}\) display a similar mixed Th1/Th2-type cytokine response to Ni\(^{2+}\) \textit{in vitro}. However, the magnitude rather than the profile of the cytokine response is predictive of the outcome of the allergic reactivity \textit{in vivo} [paper I].

- Ni\(^{2+}\) induces elevated levels of IL-10 that correlate with the Th1- and Th2-type cytokine responses in subjects with ACD to Ni\(^{2+}\) and endogenous IL-10 levels resulting from Ni\(^{2+}\) stimulation have a downregulatory effect on Th1-type (IFN-\(\gamma\)) responses by CD4\(^+\) T cells [paper II].

- Other transition metals included in the standard and/or dental series of the \textit{in vivo} patch test, similar to Ni\(^{2+}\), also elicit a mixed Th1/Th2-type cytokine response \textit{in vitro} in PBMC from metal allergic subjects [paper III].

- From a diagnostic point of view, our data show that \textit{in vitro} cytokine assays can be used to determine the reactivity of ACD patients not only reactive with Ni\(^{2+}\) but also with other metals such as Au\(^{1+}/Au^{3+}\), Co\(^{2+}\), Cr\(^{3+}/Cr^{6+}\) and Pd\(^{2+}\) [papers I, III and IV]. However, to further improve the usefulness of the ELISpot technique in the diagnosing of ACD it would be relevant to;

  1) Evaluate methods of enhancing the cytokine responses in weak responders using different mAbs that inhibit regulatory cytokines or activate costimulatory molecules.

  2) Investigate similarities in cytokine responses to organic lipophilic and hydrophilic contact allergens to ensure that an agreed-upon \textit{in vitro} test would cover all or most of the present series of compounds included in the patch test.
Figure Legends

Figure 5
The simplified and regular ELISPOT assays. There are only three major steps in the simplified assay compared to five in the regular assay format. This saves time as well as reduces inter-assay variation since one batch of pre-coated plates could be used for one large study (clinical trial or experimental study).

Figure 6
Highly significant correlation between the simplified and regular ELISPOT assay formats. Cytokine responses (IL-4, IL-13 and IFN-γ) to all three antigens tested (Ni²⁺, TT and PPD) by PBMC from all 14 blood donors were pooled and analysed by the Spearman rank-order correlation coefficient rs. All analyses were done using values with background (spontaneous responses) subtracted (i.e. 14 blood donors x 3 cytokines x 3 antigens = 126 data points).

Figure 7
Nickel (Ni²⁺)-allergic and non-allergic subjects display a similar cytokine profile in response to a recall antigen. Peripheral blood mononuclear cells (PBMC), 2.5 x 10⁵ cells/well, from Ni²⁺-allergic subjects with different patch test scores (+3, +2 and +1; n=10 per group) and non-allergic subjects (n=10) were cultured with or without Ni²⁺ or tetanus toxiod (TT). The number of IL-4 (A), IL-5 (B) and IL-13 (C) producing cells were determined by ELISPOT. Results shown are the means ± SD for each group. Statistical differences between the groups following a Mann-Whitney U test are depicted with asterisks (*p<0.05; ** p<0.01; ***p<0.001).
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