Gold allergy: *In vitro* studies using peripheral blood mononuclear cells

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The most exciting phrase to hear in science, the one that heralds new discoveries, is not Eureka! but rather, "hmm.... that's funny...."

Isaac Asimov
Abstract

Positive patch test reactions to gold are commonly seen in dermatology clinics, but it is very unusual for the patients to actually have any clinical symptoms. It is also common with irritant reactions that are not linked to adaptive immunity. Therefore, a deeper understanding of the mechanisms underlying allergic contact dermatitis (ACD) reaction, and the search for a complementing diagnostic tool, is important.

In paper I we included three subject groups; one with morphologically positive patch test reactions to gold sodium thiosulphate (GSTS, the gold salt used in patch testing), one with negative patch tests, and one with irritant reactions to gold. Blood samples were collected and examined regarding the proliferation rate and which cytokines were secreted after culturing with GSTS. We saw that the cultured lymphocytes from the allergic donors proliferated at a significantly higher rate than the two other subject groups, and that the cells secreted cytokines of both Th1 (Interferon (IFN) -γ and Interleukin (IL) -2) and Th2 (IL-13 and IL-10) types. The allergic donors secreted significantly higher levels of IFN-γ, IL-2 and IL-13 than the two other subject groups. Both the negative and irritant subject groups showed suppressed levels of the cytokines as compared with the unstimulated cultures, demonstrating the immunosuppressing effects of gold.

We also examined whether any of the analyzed markers, alone or combined, could be used as an aid for diagnosing ACD to gold. We found that the IFN-γ assay yielded the highest sensitivity (81.8 %) and specificity (82.1 %), and also identified 87.5 % of the irritant group as non-allergic.

In paper II we decided to investigate what cell types and subsets that reacted to the gold stimulation. We analyzed proliferation rate and expression of CD45RA, CD45R0, cutaneous lymphocyte-associated antigen (CLA) and the chemokine receptors CXCR3, CCR4 and CCR10. Similar to what has previously been published about nickel (Ni) allergy, the cells from the gold-allergic subjects that reacted to the GSTS stimulation expressed CD3+CD4+CD45R0+CLA+. However, contrary to findings in studies on Ni-reactive cells, we saw no differences between allergic and non-allergic subjects regarding any of the chemokine
receptors studied.

In conclusion, we found that analysis of IFN-γ might be a useful complement to patch testing, possibly of interest in avoiding the need for repeated tests to rule out irritant reactions. We also saw that the cells that proliferated in response to gold were memory T-cells expressing CD4 and CLA, the marker for skin-homing. However, these cells did not express elevated levels of any of the chemokine receptors analyzed, showing that there are both similarities and differences between the mechanisms for Ni allergy and gold allergy.
Popular Science Summary

Ca 10 % av alla patienter på hudkliniker som gör ett s.k. lapptest, uppvisar en reaktion efter guldkontakt. Trots detta är det mycket ovanligt att patienterna uttrycker någon kliniskt relevant allergi. Samtidigt har nästan alla som har positivt lapptest mot nickel (Ni) kliniska besvär av metallen. Detta gör intresset för fördjupad kunskap och förbättrad eller komplementerande diagnosmetoder stort.

I artikel I undersöks tre patientgrupper: en med positivt lapptest mot guld; en med negativt lapptest; och en grupp med en irritantreaktion mot guld (en tillfällig reaktion som inte är kopplad till allergi). Från patienternas blod renades immunceller, lymfocyter, fram, för att undersöka delningshastighet samt vilka immunologiska markörer, s.k. cytokiner som utsöndrades efter stimulering med guld. Resultaten visade att cellerna från allergiker delade sig mer än celler från de två grupperna, samt utsöndrade högre nivåer av tre av cytokinerna, nämligen Interferon (IFN) –γ, Interlukin (IL) -2 och IL-13. IFN-γ visade sig också vara det cytokin som visade högst potential som diagnostisk markör.

I artikel II undersöks bakomliggande mekanismer mer noggrant. Två patientgrupper samlades in: En grupp med positivt lapptest mot guld som i artikel I visat hög celldelning och höga nivåer av IFN-γ, och en grupp utan guldksärlighet. Från patienternas blod renades lymfocyter fram, och dessa undersöktes sedan med avseende på celldelningshastighet, samt vilken typ av celler som delade sig. Vi fann att i likhet med vad som är känt angående Ni-allergi, så var det T-celler av minnestyp som reagerade på guldet hos allergikerna. Dessa celler uttryckte också en receptor kallad CLA, som får T-celler att vandra till huden. Däremot var de tre andra receptorerna som undersöktes, CXCR3, CCR4 och CCR10, inte uttryckta i någon högre grad hos allergikerna än icke-allergikerna, vilket har visats vid Ni-allergi.

Slutsatserna är att de celler som delar sig hos allergiker efter guldstimulering är av minnes-T typ som är destinerade för huden. Till skillnad från Ni-allergi, så uttrycker de här cellerna inte receptorerna CXCR3, CCR4 och CCR10 i någon högre grad. Cellerna utsöndrar både typ 1-markörer (IFN-γ, IL-2) och typ 2-markörer (IL-13), vilket stödjer vissa teorier om Ni, medan andra hävdar att Ni-stimulerade celler endast uttrycker typ-1 markörer.
Original Publications

I

Christiansen J*, Färm G, Eid-Forest R, Anderson C, Cederbrant K, Hultman P.

Interferon-gamma secreted from peripheral blood mononuclear cells as a possible diagnostic marker for allergic contact dermatitis.


II

Clifford J, Anderson C, Cederbrant K, Hultman P.

T-cells expressing CD4, CD45RO and CLA from gold-allergic but not healthy subjects react to gold sodium thiosulphate in vitro.

Manuscript.

*The Author’s maiden name was Christiansen.
**Abbreviations**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>ACD</td>
<td>Allergic contact dermatitis</td>
</tr>
<tr>
<td>AP-1</td>
<td>Activator Protein 1</td>
</tr>
<tr>
<td>APC</td>
<td>Antigen presenting cell</td>
</tr>
<tr>
<td>Au</td>
<td>Gold</td>
</tr>
<tr>
<td>CD45R0-EF</td>
<td>CD45R0-Enriched fraction</td>
</tr>
<tr>
<td>CD45RA-EF</td>
<td>CD45RA-Enriched fraction</td>
</tr>
<tr>
<td>CLA</td>
<td>Cutaneous lymphocyte-associated antigen</td>
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<tr>
<td>CPM</td>
<td>Counts per minute</td>
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<tr>
<td>DC</td>
<td>Dendritic cell</td>
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<tr>
<td>EPI-</td>
<td>Epicutaneous patch test negative</td>
</tr>
<tr>
<td>EPI+</td>
<td>Epicutaneous patch test positive</td>
</tr>
<tr>
<td>EPI-IR</td>
<td>Epicutaneous patch test irritant reaction</td>
</tr>
<tr>
<td>FasL</td>
<td>Fas Ligand</td>
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<tr>
<td>GSTM</td>
<td>Gold sodium thiomalate</td>
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<tr>
<td>GSTS</td>
<td>Gold sodium thiosulphate</td>
</tr>
<tr>
<td>Hg</td>
<td>Mercury</td>
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<tr>
<td>HMGB1</td>
<td>High mobility group box chromosomal protein</td>
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<tr>
<td>IFN</td>
<td>Interferon</td>
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<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>IR</td>
<td>Irritant Reaction</td>
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<tr>
<td>LTT</td>
<td>Lymphocyte transformation test</td>
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<tr>
<td>MFI</td>
<td>Mean fluorescence intensity</td>
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<tr>
<td>MHC I/II</td>
<td>Major Histocompatibility Complex Class I/II</td>
</tr>
<tr>
<td>NFκB</td>
<td>Nuclear Factor κB</td>
</tr>
<tr>
<td>Ni</td>
<td>Nickel</td>
</tr>
<tr>
<td>NK</td>
<td>Natural Killer cell</td>
</tr>
<tr>
<td>PBMC</td>
<td>Peripheral blood mononuclear cells</td>
</tr>
<tr>
<td>PI</td>
<td>Propidium Iodide</td>
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<tr>
<td>PRR</td>
<td>Pattern recognition receptor</td>
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<tr>
<td>RA</td>
<td>Rheumatoid Arthritis</td>
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<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>SI</td>
<td>Stimulation index</td>
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<tr>
<td>TCR</td>
<td>T-cell receptor</td>
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<tr>
<td>TGF</td>
<td>Tumour growth factor</td>
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<tr>
<td>Th1/Th2</td>
<td>T helper 1/2</td>
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<td>TLR</td>
<td>Toll-like receptor</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumour necrosis factor</td>
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<tr>
<td>Treg</td>
<td>Regulatory T-cell</td>
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Introduction

_T-cells and T-cell memory_

_T-cell production and maturation_

T-cells are a cell type that is heavily involved in the immune response. The cells are produced in the thymus, from progenitor stem cells that have migrated there from the bone marrow (reviewed in (1)). To become mature T-cells, the progenitor cells go through several control steps of different gene rearrangements, and the cells that fail to rearrange their $\alpha$-chain- and $\beta$-chain genes are induced to undergo apoptosis (2). Among the surviving thymocytes, cells with low-affinity T-cell receptors (TCRs) and TCRs recognising self-peptides are stopped from further development.

The cells that leave the thymus for the blood stream are mature naïve T-cells expressing either CD4 or CD8 on their surface (2). The T-cells then circulate through the blood stream and lymphatic system where they are available for antigen presenting cells. Fig. 1 shows a schematic description of the development of T-cells.

_T-cell activation and antigen presentation_

When a potential pathogen enters the body, it is quickly engulfed by various phagocytosing cells, such as macrophages or dendritic cells (DCs), residing at the various body linings (3). The phagocytosing cell becomes activated, and migrates to a lymph node, where it can present the processed antigen to T-cells. These antigen-bearing cells are collectively termed antigen-presenting cells (APCs).

The phagocytosing cells have several methods for recognising non-self substances in the body. The main recognition method is through various receptors recognising foreign patterns, so called pattern recognition receptors (PRRs). The main receptor type for this is the family of Toll-like receptors (TLRs) (4). When these receptors bind structures that
are foreign to the body, the phagocytosing cells become activated (fig. 2), and engulfs
the foreign body.

<table>
<thead>
<tr>
<th>Localisation</th>
<th>T-cell type</th>
<th>Surface molecules</th>
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<tbody>
<tr>
<td>Bone marrow</td>
<td>Stem cell</td>
<td></td>
</tr>
<tr>
<td>Thymus</td>
<td>Early progenitor cell</td>
<td>CD44</td>
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<td></td>
<td>Late thymocyte</td>
<td>CD4, CD8, CD3</td>
</tr>
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</table>

Figure 1. Schematic diagram of the differentiation and maturation of T-cells, from stem cells to active T-cells. Adapted from Janeway (5).

Activated macrophages engulf foreign exogenic pathogens and digest them in the lysosomes, which are acidic compartments in the cell where pathogens are degraded into short peptide sequences (6, 7). These peptides are then transported to the macrophage cell surface, where they are displayed on Major Histocompatibility Complex (MHC), primarily on the class II receptors. The macrophage also starts to secrete cytokines, such as Interleukin (IL) -1β, IL-12, IL-6 and Tumour Necrosis Factor (TNF) -α, which are proinflammatory, as well as chemokines such as IL-8, which acts as a chemoattractant to attract other immune cells to the site of infection (8). Neutrophils already circulating in
the blood stream arrive first, and when they engulf the pathogens, also secrete chemokines. This will help to increase the chemokine gradient to the site. The activated macrophage then receives signals from the active lysosomes to start migrating into the lymphatic system to a lymph node, where it can activate T-cells (3).

Figure 2. The process of activating T-cells. In A, invading pathogens are met by resident macrophages (yellow) and DC (blue). These cells recognise pathogen patterns with their TLR (black), bind and engulf the pathogens, and degrade them intracellularly. The degraded peptides are then displayed on the MHC molecules (green) on the cell surface, and the cells start to secrete cytokines (TNF-α, IL-1β, IL-12) and chemokines (IL-8) to attract other lymphocytes. The activated phagocytic cells (here only the DC is shown) migrate in the lymphatic system to the nearest lymph node (B), where they can meet circulating T-cells. When a T-cell recognises the antigen, displayed on the DC surface, with their TCR (black double ovals), antigen presentation takes place. The T-cell then begins to proliferate (C), to produce armed effector cells. These cells then migrate back to the inflamed tissues (D), where they secrete cytokines (IFN-γ, IL-2) to further activate the residing B-cells, cytotoxic T-cells or phagocytic cells (here, only a macrophage is shown). DC: Dendritic cell. TLR: Toll-like receptor. MHC: Major Histocompatibility Complex. TNF: Tumour Necrosis Factor. IL: Interleukin. TCR: T-cell receptor. IFN: Interferon.
Mature naïve T-cells express the TCR on their surface, which is a receptor that recognizes MHC I or II (1). However, the receptor will only activate the T-cell if the MHC displays a peptide for which the T-cell is specific (3). When the activated macrophage enters the lymph node, it can encounter a circulating T-cell. The macrophage then acts as an APC, and the T-cell becomes activated. It enters the bloodstream from the lymph node, and starts to express chemokine receptors which will help the cell to migrate towards the gradient of chemokines.

When the T-cell becomes activated, it starts to proliferate to produce active, armed T-cells. These cells then start to express various chemokine receptors that induce the cell to migrate towards the chemokine gradient to the site of infection (9, 10). Different chemokines and other adhesion molecules are expressed at different locales in the body. For instance, epithelial cells in the skin express the ligand for the cutaneous lymphocyte-associated antigen (CLA), and T-cells expressing CLA are thus directed to the skin (11). Other, similar specific ligands exist in the airway system and the gut mucosa as well (12, 13).

Depending on what kind of pathogen has invaded the body, the T-cell will react in different ways (1, 14-16). If it is an intracellular pathogen or a virus, the Th1 branch of the immune system will be activated, with activated cytotoxic CD8+ T-cells and aggressive macrophages as effector cells. If it’s a soluble antigen, the Th2 branch will instead be activated, with plasma cells and antibodies as a response. These two branches of the immune system secrete different sets of cytokines (see below), that also inhibit the other immune type.

**T-cell subsets**

There are two major kinds of T-cells: T helper cells (Th-cells), which express the co-receptor CD4 that recognises MHC II, and cytotoxic T-cells, expressing the co-receptor CD8 that recognise MHC I (1). CD4+ T-cells also exist in different varieties, which are
classified according to what cytokines they secrete (17). Th1 cells, which are mainly focussed on viral combat and the cellular branch of the adaptive immune system, secrete mainly Interferon (IFN) -γ and IL-2 (14-16, 18). This activates primarily macrophages, which in turn secrete TNF-α, IL-6 and IL-1β, and CD8+ T-cells that help kill infected cells (8). The cytotoxic CD8+ T-cells help to kill infected cells using different cytotoxic enzymes, as well as death-inducing receptors (including the Fas – Fas Ligand (FasL) system) (19).

Th2, which mainly activate the adaptive immune response involving antibodies, secrete mainly IL-4, IL-5, IL-10 and IL-13, which in turn primarily activates B-cells (14-16, 20, 21). Regulatory T-cells (Treg), that regulate the immune responses, express the receptor CD25 and secrete mainly Tumour growth factor (TGF) –β, IL-4 and IL-10, to help contain infections and stop immune reactions (22).

Recently, another Th subset has been defined, called Th17 (reviewed extensively in (23)). This subset secretes a distinct selection of cytokines, including IL-1b, IL-6, IL-21, IL-22, IL-23, and of course IL-17. The functions of Th17 cells are still being explored, but it seems that the Th17 response results in a massive inflammation when infections are not completely cleared by either Th1 or Th2 reactions (24).

**T-cell memory and the CD45R receptor**

T-cells express a receptor on their surface called the CD45R (25, 26). This receptor exists in several different isoforms, and these are expressed differently depending on the maturity level of the T-cell. Naïve T-cells express the CD45RA isoform, but upon activation, this receptor is gradually down-regulated, and the differently spliced isoform CD45R0 is expressed instead (26). This has proven to be a relatively good marker for naïve or activated T-cells. However, the activated T-cells that turn into memory cells continue to express the CD45R0 isoform, and circulating T-cells expressing this isoform in healthy individuals are therefore considered to be memory T-cells. It has been shown
that the expression of the different isoforms is cyclic, at least \textit{in vitro}, and re-activated memory cells (memory cells encountering their antigen a second time) can express both isoforms simultaneously (27, 28).

\textbf{Contact allergy}

\textbf{The four types of allergy in brief}

There are different types of allergic reactions, but the most common classification system was described by Coombs and Gell in 1963 ((29), reviewed in (30)). This system divides allergic reactions into four different groups, depending on the mechanisms involved (Table 1). Typical for allergic reactions is that they are all mediated chiefly by the adaptive branch of the immune system.

Type I reactions are the immediate reactions that are mediated by a rapid release of IgE (30). Typical examples of this reaction are hay fever (allergic rhinitis and/or conjunctivitis) and asthma. Type II reactions are mediated by the humoral cytotoxic cells, and involve IgM and IgG antibodies. A typical example of this is drug-induced cytopenia, where the patients suffer from almost immediate rashes after ingestion of drugs such as penicillin (30, 31). Type III reaction is mediated by immune complexes, where the IgG and IgM antibodies target soluble antigens and form complexes. These complexes then activate mast cells and other leukocytes, and 4-6 hours after antigen introduction, the patients suffer from the reaction (30, 32). Depending on where in the body the reaction occurs, different symptoms are expressed, but the more common ones includes allergic alveolitis in the lungs, or vasculitis in blood vessel walls.

Finally, there is a fourth type of reaction termed the Type IV hypersensitivity reaction, and one typical example of this is the tuberculin reaction (30, 33). This reaction is antibody independent, and relies on phagocytic and cytotoxic T-cells, as well as CD4$^+$ T-cells. Typically, symptoms do not occur until 24-48 hours after antigen exposure, and
this type of reaction occurs almost exclusively in the skin. Allergic contact dermatitis (ACD) is classed as a Type IV reaction.

Table 1. The four main types of allergic reaction, described by Coombs and Gell.

<table>
<thead>
<tr>
<th>Antibody-mediated reactions</th>
<th>Cell-mediated reactions</th>
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<tr>
<td><strong>Type I</strong></td>
<td><strong>Type II</strong></td>
</tr>
<tr>
<td><strong>Mechanism</strong></td>
<td>Immediate Ig-E mediated</td>
</tr>
<tr>
<td><strong>Symptoms</strong></td>
<td>Allergic rhinitis</td>
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<td></td>
<td>Bronchial asthma</td>
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**Allergic Contact Dermatitis**

Contact allergens normally occur as low molecular weight, soluble compounds, called haptons, which can freely enter the skin (34-39). There are several ways that haptons can interact with the immune system. Some compounds can react pharmacologically, thereby activating the immune system (40). Other substances are classed as pro-haptons, which need to be metabolized before they can cause an allergic reaction. The way that is most probable regarding gold is that the Au ions bind covalently directly to proteins, thereby changing the protein structure (40, 41). This makes the “new” haptenated protein a target for DC, which then engulf the protein and displays it on their MHC II molecules (36). During the sensitization phase, the DC becomes active and migrates to the local lymph node where it presents the haptenated protein to T-helper cells. The CD4+ T-cell then starts to proliferate and activate CD8+ cells, as well as generating hapten-specific memory cells (reviewed in (35). This reaction can take up to 15 days, and is usually asymptomatic.
When the allergen is encountered a second time, the memory cells react faster, and an elicitation phase takes place (fig. 3) (42, 43). Cytokines secreted from activated DCs, including IL-1β and TNF-α, promptly activates surrounding cells, including keratinocytes and residing lymphocytes (36, 44). Cells of a Th1 type secrete IFN-γ and IL-2, which also activates keratinocytes (KC). These active KC also secrete cytokines and chemokines, and recruit more cells from the circulation (45). Active CD8+ cytotoxic T-cells target the activated KCs, and induce apoptosis through Fas-FasL interaction. There is also evidence that Natural Killer cells (NK-cells) are involved in the pathogenic mechanism, as they have been shown to infiltrate skin during the elicitation phase of ACD (46). The damaged KCs are the central feature of the reaction, but other cells in the epidermis and dermis are also involved. The reaction is then controlled by Tregs secreting TGF-β, IL-10 and IL-4 (47).

The symptoms of ACD are classically erythema, papules and vesicles, but the appearance can also include weeping and crusted to lichenified, scaling rashes, depending on the extent and timing of the exposure (48). Since the reaction can take up to 5 days to develop, detailed attention to the clinical history together with extensive patch testing is needed, to elucidate whether a particular allergen caused the rash. Since the visual symptoms can be similar to several other skin dermatoses, including atopic dermatitis, fungal infections, and a long list of less common inflammatory disorders, these need to be ruled out in the diagnostic process as well (48).

The distribution of the rash is dependent on the type of exposure, and sometimes this can be an aid in diagnosis (49). For example, allergy towards cosmetics and hair dyes frequently result in rashes where these are applied (face and hands).
Figure 3. Schematic of the Type IV hypersensitivity reaction. When an allergen (red dots) is encountered and enters the skin, it is bound to protein (olive lines) and engulfed by DC (blue cells) expressing PRRs. The DC then migrates to the lymph node and activates T-cells. If the antigen is present for a sufficiently long time, a rash develops even after the sensitization phase, when the activated T-cells arrive at the exposed site. If the antigen has been encountered before, there are circulating and skin-residing memory T-cells (pink) that immediately recognise the antigen-MHC complex. The activated T-cell then proliferates, and the armed T-cells then migrate back to the skin to exert their actions. The CD4\(^+\) T-cell activates phagocytic cells (yellow) and further stimulates cytotoxic CD8\(^+\) T-cells. The CD8\(^+\) T-cells exert their effects on the KCs (light blue), inducing these to undergo apoptosis (blurred KC) through the Fas – FasL interaction. Meanwhile, Regulatory T-cells expressing CD4 and CD25 secrete inhibiting cytokines (IL-10 and TGF-β) to control the reaction. DC: Dendritic cell. PRR: Pattern recognition receptor. MHC: Major Histocompatibility Complex. KC: Keratinocyte. IL: Interleukin. TGF: Tumour growth factor. IFN: Interferon. CLA: Cutaneous Lymphocyte-associated antigen. FasL: Fas Ligand.
Examples of allergens and exposure

Depending on the type of allergen, different exposure routes are available. One of the most common allergens in western societies is nickel (Ni), and the symptoms from this type of allergy often occur after wearing Ni-containing jewellery (50). Some coins also contain Ni, which can be seen on the hands of Ni-allergic cashier workers. It is suggested that up to 20% of the general population suffer from Ni allergy (50).

Other common allergens include fragrances and preservatives (51). These can often be pinpointed as the causative agent, but the difficult thing is to discern which of the many ingredients in for example a topical lotion that is responsible for the rash. This is important to know, for future avoidance.

Another group of chemicals that often causes ACD in exposed workers are different plastics and rubbers used in gloves. Among nurses, 30% had patch tests positive to different rubber components (52). This trend can be seen in many different industries, where the exposed workers are more sensitized than the average population (52).

The focus of this thesis is ACD to gold. Approximately 10% of patch tested patients at dermatology clinics show a positive reaction to gold sodium thiosulphate (GSTS) (53, 54), the compound used to diagnose ACD to gold (53). While this might indicate sensitisation to gold, it is uncommon for these patients to experience any clinical symptoms from gold usage. There are however examples of patients with restorative gold in the oral cavity suffering from blistering related to the gold exposure, and ACD to gold is a common side effect in Rheumatoid Arthritis (RA) patients receiving gold salt treatment.

Oxidative states of gold

Gold exists in three different oxidative states (Au [0], Au [I] and Au [III]), which have very different effects in the body (55). Au [I] is most commonly used as jewellery, and the increasing popularity of piercing body parts with gold has been suggested as a
cause for ACD to gold (54). Metallic gold (Au [0]) is also used as restorative materials in the oral cavity (56, 57), and saliva has been shown to slowly dissolve gold and transport it via mucous membranes into the bloodstream (54). Dental gold can be associated with oral lesions and dermatitis, and there is a correlation between ACD and oral lesions in patients with oral gold materials (56-58).

Metallic gold has also been used to coat coronary stents in an effort to prevent restenosis after a coronary blockage (59, 60). There are reports stating a higher occurrence of restenosis with gold-plated stents than with titanium ones, and a recent thesis published in Lund, Sweden, states that ACD to gold is more common among patients with gold-plated stents than in the general population as well (61). This has resulted in a discontinuance of the use of gold-plated coronary stents.

Gold salts containing gold in the Au [I] oxidation state (55, 62), have been used for treatment of RA for the last 80 years (63, 64), and while it can have positive effects, a major drawback is adverse effects, including skin rashes, occurring in up to half of all treated patients (55). It has been shown that Au [I] is oxidized into Au [III] in lysosomes of macrophages (65), and Au [III] shows more prominent anti-inflammatory and toxic effects (66).

Mechanisms in gold allergy

In medicine, gold is mostly used for its immunosuppressive functions (63, 64). The mechanisms are not completely known, but one way might be through inhibition of the inflammatory mediator High mobility group box chromosomal protein 1 (HMGB1) (67). HMGB1 is translocated from the nucleus into the cytoplasm in monocytes in response to other inflammatory mediators, such as IFN-β and nitric oxide (67). Gold is also known to inhibit lymphocyte maturation, differentiation and function (68), as well as decrease the production of several cytokines in vitro (69-71), including IL-1β, TNF-α and IFN-γ. One way by which this is accomplished is through inhibition of transcription factors
such as Activator Protein (AP)-1 or Nuclear Factor (NF) κB, as well as inhibiting specific caspases needed for post-translational modification of proteins (69). Both of these transcription factors are heavily involved in the inflammation response, regulating the transcription of several cytokines and signal proteins.

**T-cells in ACD to metals**

Although it is well known that gold and other metal haptens can cause ACD, the details of the mechanism are still unknown (54, 72, 73). Gold bound to proteins is digested and presented to T-cells by APCs (34, 37-39), but hapten recognition has also been suggested to be APC independent (74). One suggestion is that gold binds directly to the MHC class II molecules, thereby changing their peptide-binding ability (39).

ACD is a type IV hypersensitivity reaction, and both CD3⁺CD8⁺ and CD3⁺CD4⁺ T-cells are reported to be participating in the reaction (34, 75). Different compartments of the body contain different percentages of metal-specific CD8⁺ T-cells in sensitized individuals; 33 % were found in the blood of sensitized individuals, but only 15 % in the skin (76). One theory states that both CD3⁺CD8⁺ T-cells and CD3⁺CD4⁺ Th1 cells, secreting cytokines such as IFN-γ, IL-13 and IL-2, are the effector cells in ACD (71, 77). Regulatory CD3⁺CD4⁺ T-cells and Th2 cells then exert negative feedback on the immune response by secreting inhibitory cytokines such as IL-10, IL-4 and IL-5 (36, 78, 79). Other publications identify only CD3⁺CD4⁺ cells of a Th1 type to be the major culprit in the ACD reaction (75, 80-82). Another theory states that the type of reaction (Th1 or Th2) is dependent on the microenvironment surrounding the naïve T-cells (76).

In non-allergic donors, Ni seems to activate both naïve (CD3⁺CD45RA⁺) and memory (CD3⁺CD45R0⁺) cells in vitro (83). However, in allergic subjects, Ni exclusively activates memory T-cells (CD3⁺CD45R0⁺), particularly CD3⁺CD4⁺CD45R0⁺ cells, leaving naïve T-cells (CD3⁺CD45RA⁺) unstimulated (80, 83). Ni-activated CD3⁺CD45R0⁺ cells also express the skin-homing receptor CLA and
the chemokine receptors CXCR3, CCR4 and CR10 (80). To our knowledge, no data regarding gold allergy in this respect have been published.

**Diagnosis of ACD**

Patch testing is considered the gold standard to identify ACD (84). The protocols for testing are well known, and extensive knowledge exists for many of the approximately 3000 allergens known to induce ACD (85). There are, however, some drawbacks with this technique: The procedure is time-consuming for both patient and physician, with one visit for the patch placement and at least one more visit to the clinic for test reading. The risk for a flare of allergic reactions in a previously sensitized individual is slight, but exists (86), and there is a risk of creating new sensitivity in the subject. The results may differ between different test occasions, and the reading of the test is subjective (86-88). Furthermore, the correlation between dermatological symptoms and patch test outcome relevance varies between contact allergens: In the majority of patch test positive cases, no clinical relevance can be found. Many patients show an irritant reaction (IR), which might be interpreted as a false positive result if the morphology is incorrectly read (85). The IR is caused by direct damage to the skin, mainly the keratinocytes (89), and is dependent on test substance concentration. At present, a dilution series of the test substance can be the only way to distinguish between an allergic and irritant reaction (IR), since decreasing concentrations of the allergen results in disappearing rashes if it is an IR. Repeated testing increases the cost for both patients and health care systems. Thus, interest in a blood sample based diagnosis has been, and still is, high.

One such blood-based method is the lymphocyte transformation test (LTT), which shows a relatively high correlation to patch test to gold, although the method has so far only proved useful at a group level (71, 90). The method relies on the reactivity of specific memory T-cells *in vitro* by specific allergen exposure (71, 91-93), and has been used as an aid in diagnosing allergy since the 1960’s.
Knowledge of the role of cytokines in ACD has increased markedly in the last decade. The use of cytokine fingerprinting has hitherto been used more frequently in the predictive setting rather than in a diagnostic situation, evaluating new chemical entities for their possible allergenic properties (94). With the development of DNA screening chips, protein profiles can be evaluated more easily, and one or several might surface as a candidate diagnostic marker.
**Aims**

The general aim of this thesis was to investigate mechanisms in gold allergy, by studying the activation of lymphocyte subsets.

Specific aims were:

1) To study mechanisms of gold stimulation and/or inhibition on lymphocytes from both allergic and non-allergic subjects.

2) To discuss potential similarities and/or differences between the mechanisms of action for allergic contact dermatitis to gold and what is previously known about Ni allergy.

3) To evaluate the use of different blood markers as predictors and/or diagnostic tools for allergic contact dermatitis to gold.
Materials and methods

Patients

Paper I

The subjects were selected from patients on whom epicutaneous patch tests had been performed, at the outpatient clinics of the Departments of Dermatology at the University Hospitals of Linköping and Örebro, Sweden. The subjects were divided into three groups; one with subjects who had shown a morphologically positive patch test for gold (EPI+); one which had shown no positive test to the standard screening test panel patch test series (95, 96) (EPI-); and one which had shown an IR for gold (EPI-IR). In this way 77 subjects (63 women and 14 men) with an age range of 22-80 yrs (52.6 ± 14.8, mean ± SD), were recruited to the study.

The subjects were asked to answer a questionnaire about demographic data and exposure to gold, including medication with gold salts, gold jewellery usage and dental gold.

Paper II

Five allergic subjects were recruited at the outpatient clinic of the Department of Dermatology at Linköping University Hospital. These patch test positive subjects had been examined in our previous study (paper I:(71)), and were selected due to their high LTT SI values and high IFN-γ levels.

Non-allergic subjects were recruited from healthy laboratory workers with no clinical history of allergy to gold.
**Patch testing (paper I and II)**

The allergic subjects had undergone a patch test with a standard screening panel and shown a positive reaction to 0.5 % and/or 2.0 % gold sodium thiosulphate (Na₃[SO₃]₂) (GSTS), in petrolatum (Chemotechnique Diagnostics, Malmö, Sweden), applied on Finn Chambers (Epitest, Tuusula, Finland). The patches were left on for 48 hours, and tests were read on days 3 and 7, and graded as: (-), negative reaction; (+), weak, non-vesicular response; (++) , strong oedematus vesicular reaction, and; (+++), extremely strong reaction, according to the European Contact Dermatitis Research Group (ECDRG) guidelines (39). All reactions that achieved at least a (+) grade were considered to be positive reactions.

In paper I, thirty-three subjects (30 women and 3 men) showed a (+) – (+++) reaction to gold, 28 (21 women and 7 men) showed no reaction at all to gold, and 16 (12 women and 4 men) showed a morphologically separate IR. In paper II, the allergic subjects all showed a (+) reaction or stronger. The non-allergic subjects in paper II were not subjected to patch test.

Blood samples were taken at the dermatology department at Linköping University Hospital. All test subjects had given informed consent. The study was approved by the local ethics committee in Linköping.

**Cell preparation and purification**

**Paper I**

Venous blood was obtained in vacuum tubes containing Na-Heparin (Vacuette, Greiner Bio-One, Krems-Muenster, Austria). The cells were separated on Ficoll Paque Plus® (Amersham, Uppsala, Sweden) to obtain peripheral blood mononuclear cells (PBMC). The cells were then suspended in RPMI 1640 with 10 mM Hepes, 4 mM L-glutamine, gentamycin (8 mg/ml) and 10 % heat-inactivated AB+ serum (all from Gibco
BRL, UK), at a concentration of $1 \times 10^6$ cells/ml.

Six GSTS concentrations were used (1.56 - 50.0 µg/mL) (Chemotechnique Diagnostics) to stimulate the cells, as well as sterile Milli-Q water as solvent control. All samples were run in duplicate. The metal salt solution or sterile water was added with a total of $1 \times 10^6$ cells/well to flat-bottomed 24-well plates (Costar, Cambridge, Massachusetts, USA), and incubated for 5 days at 37 °C and 5 % CO₂.

**Paper II**

PBMC were obtained in the same manner as described for Paper I. Cells from the PBMC fraction were suspended in phosphate buffered saline (PBS) with 0.5 % human AB+ heat-inactivated serum (Invitrogen, Carlsbad, CA, USA) and 2 mM EDTA. The suspension was mixed with a magnetic bead kit for purifying T-cells (Pan T-cell kit, Miltenyi Biotech, Bergisch Gladbach, Germany) targeting CD14, CD16, CD19, CD36, CD56, CD123, and CD235a, leaving only CD3 cells unlabelled, and the cells were separated according to the manufacturer’s instructions. Briefly, the cell-antibody cocktail was incubated in room temperature (RT) for 15 minutes and then added to a magnetic separation column (Miltenyi Biotech).

Two different cell fractions were obtained: One unlabelled T-cell fraction (henceforth referred to as the T-cell fraction) and one labelled non-T cell fraction (henceforth referred to as the non-T fraction). The non-T fraction was stored on ice until used as a monocyte supply in the cultures.

The T-cell fraction was resuspended in PBS with 0.5 % human AB+ heat-inactivated serum and labelled with CD3-APC-Cy7, CD45R0-PE-Cy7 (e-bioscience, San Diego, CA, USA) and CD45RA-Pacific Blue (Becton Dickinson (BD), Stockholm, Sweden) for 15 minutes in RT. The cells were then washed with PBS and sorted on a FACS Aria II Cellsorter (BD), into two different fractions, one $CD3^+CD45RA^+$ enriched fraction, henceforth referred to as the CD45RA-EF and one $CD3^+CD45R0^+$ enriched fraction,
referred to as the CD45R0-EF. A summary of the separation procedure is shown in fig.4.

After separation, the different fractions obtained during the separation procedures (PBMC-fraction, T-cell fraction, non-T fraction, CD45RA-EF and CD45R0-EF) were analysed for purity, using the following antibodies: CD3-FITC, CD4-APC, CD45RA-PE, CD14-FITC, CD19-APC, CD56-PE (all from Miltenyi Biotech) and CD45R0-PE-Cy5 (BD). The staining procedure was the same as mentioned above, and the cells were analyzed on a FACS Aria (BD).

**Purity and enrichment**

The T-cell fraction consisted on an average of 92.2 % and 94.4 % CD3$^+$ cells in the allergic subjects and non-allergic subjects respectively, and they had been enriched 1.43 and 1.55 times compared with the PBMC fractions. Almost all monocytes (CD14$^+$), B-cells (CD19$^+$) and NK-cells (CD56$^+$) were depleted with the magnetic beads: 1.6 % of monocytes remained in the T-cell fraction obtained from allergic subjects after depletion.

The CD45RA-EF was very pure, with 99.4 % and 98.0 % CD45RA$^+$ cells in the allergic group and the non-allergic group, respectively. The CD45RA$^+$ cells had been enriched 2.15 times in the allergic group and 1.53 times in the non-allergic group compared with the T-cell fraction. For the CD45R0-EF, the CD45R0$^+$ cell fractions were 98.5 % in the allergic group and 95.7 % in the non-allergic group, and the CD45R0$^+$ cells had been enriched 3.08 times and 6.05 times, respectively, compared to the T-cell fraction.
For culturing of the CD45RA-EF and CD45R0-EF, 48-well flat-bottomed culture plates (BD) were coated with the non-T fraction suspended in RPMI 1640 with 10 mM HEPES, 4 mM L-Glutamin, Gentamycin (8 mg/ml) and 30 % human AB+ heat-inactivated serum (all from Invitrogen) at a concentration of 1 x 10^7 cells/ml for 4-6 hours, to obtain adherent monocytes in the wells. The plates were then washed with medium, and the CD45RA-EF and CD45R0-EF cells were added to 48-well flat-bottomed plates (BD) in RPMI 1640 with 10 mM HEPES, 4 mM L-Glutamin, Gentamycin (8 mg/ml) and 10 % human AB+ heat-inactivated serum at a concentration of 1 x 10^6 cells/ml. The PBMC cells were added directly to wells in the same concentration, since this fraction already contained monocytes.
The cells were cultured for 4 - 5 days in 37 °C and 5 % CO₂, with five different concentrations of GSTS (25.0– 200 µg/ml) in sterile Milli-Q water, which was also used as a solvent control.

**LTT**

**Paper I**

After 5 days of culturing as described above, LTT was performed according to Nordlind and Lidén (97) with some minor modification (90). 4 - 18 h before harvesting, 1 µCi ³H-thymidine, specific activity 12.7 GBq/mg (Amersham) in 10 µl of RPMI medium was added to each well. The cells were then harvested with an automatic cell harvester (Inotech, Minolab, Upplands Väsby, Sweden), and the radioactivity was measured using a 1450 Microbeta Plus (Wallac, Turkuu, Finland), and counts per minute (CPM) was recorded.

From the LTT results, a stimulation index (SI) was calculated for each GSTS concentration and subject, using the following formula:

\[
SI = \frac{\text{mean CPM (GSTS stimulated)}}{\text{mean CPM (unstimulated)}}
\]

The GSTS concentration yielding the highest LTT SI values was considered optimal.

**Paper II**

After 4 days of culturing as described above, LTT was performed on the PBMC fraction from non-allergic subjects and the CD45RA-EF and CD45R0-EF from allergic and non-allergic subjects according to Nordlind and Lidén (97) with some modifications. 120 µl from each well was transferred to a 96-well, flat-bottomed plate (BD). To each well 1 µCi ³H-thymidine, specific activity 12.7 GBq/mg (Amersham) was
also added. After 5-6 hours of incubation, the cells were harvested using an automatic cell harvester (Inotech, Ninolab), and the radioactivity was measured using a 1450 Microbeta Plus (26) and CPM were recorded. The same formula for calculating SI values as described above was used.

**Cytokine assay**

**Paper I**

Eight cytokines were measured in the cell supernatants: IL-1β, IL-2, IL-4, IL-8, IL-10, IL-12, IL-13 and IFN-γ. The cells were cultured as described above for paper I, although only the GSTS concentrations 6.25 and 25.0 µg/ml were used. The supernatant was collected after 3, 4, and 5 days and frozen at -70 °C until further analysis. The multiple bead array Luminex (Linco Research Inc., Missouri, USA) was used to quantify the cytokines, according to the manufacturer’s instruction (Linco Research Inc., Missouri, USA) with RPMI medium as blank. A Luminex 100 IS instrument (Biosource, Nivelles, Belgium) with the Star Station acquisition program (v2 Applied Cytometry Systems, Sheffield, UK) was used to process the data. All samples were run in single wells, except the standard curve points, which were run in duplicate according to the manufacturer’s recommendations. “Net” concentrations were then calculated by subtracting values for unstimulated samples from the stimulated samples. Culture conditions (culture time and GSTS concentrations) yielding the highest mean net concentrations were considered optimal.

**Flow cytometry**

**Paper II**

Cells from the cultured PBMC fraction from non-allergic subjects were used for flow
cytometry analysis. Briefly, the cells were suspended in PBS with 0.5 % human AB+ heat-inactivated serum (Invitrogen) and labelled for 15 minutes in RT with the following antibodies: CD3-FITC, CD4-APC, CD45RA-PE, CD14-FITC (all from Miltenyi Biotech), CD8-PE-Cy5 and CD45R0-PE-Cy5 (BD). The cells were washed with PBS and analysed on a FACS Calibur (BD), using FACS CellquestPro software (BD).

The cultured cells from the PBMC fraction were also labelled with propidium iodide (PI) (BD) to determine viability. Briefly, the cells were stained with 2.5 % PI in PBS with 0.5 % human AB+ heat-inactivated serum (Invitrogen), then washed with PBS and analysed on a BD FACS Calibur (BD). The cells were plotted in a histogram, where the cells with positive PI-staining (considered as dead cells) were selected with a gate. These cells were then back-gated into a forward/side-scatter dot plot, to determine the location of the dead and living cells. Due to a shortage of cell material from the separated fractions, this procedure could not be performed on the CD45RA-EF and CD45R0-EF. However, since the staining and localization of the dead and living PBMC cells was highly reproducible, the above back-gating was also performed on the separated cell fractions, where the same localization of living and dead cells were clearly visible.

Cells from the cultured CD45RA-EF and CD45R0-EF were also analysed by flow cytometry. The staining procedure was the same as for the PBMC fraction described above, but included the additional antibodies CLA-PE, CD19-APC, CD56-PE (Miltenyi), CD25-APC, CCR4-PE, CXCR3-PE-Cy5 (BD) and CCR10-APC (R&D systems Europe Ltd., Abingdon, UK). The cells were analysed on a FACS Aria II Cellsorter (BD) using FACS Diva software (BD).
Statistics

Paper I

Specificity, sensitivity and accuracy were calculated using epicutaneous patch test as a reference, according to the following formulas:

\[
\text{Sensitivity} = \frac{\text{No of analysis positives (in the EPI+ group)}}{\text{Total No of EPI+}} \times 100
\]

\[
\text{Specificity (EPI-)} = \frac{\text{No of analysis negatives (in the EPI- group)}}{\text{Total No of EPI-}} \times 100
\]

\[
\text{Specificity (EPI-IR)} = \frac{\text{No of analysis negatives (in the EPI-IR group)}}{\text{Total No of EPI-IR}} \times 100
\]

\[
\text{Accuracy} = \frac{\text{(No of analysis positives in the EPI+ group + No of analysis negatives in the EPI- group + No of analysis negatives in the EPI-IR group)}}{\text{Total No of subjects}}
\]

To evaluate whether the methods used were able to identify the EPI-IR subjects as non-allergic, specificity was calculated separately for the EPI- and EPI-IR groups. All concentrations and SI values were used in the cut-off determinations. The cut off value (expressed as cytokine concentration and LTT SI) that yielded the highest accuracy was defined as the point which resulted in the highest simultaneous sensitivity and specificity. A prerequisite for these calculations was that both sensitivity and specificity each had to be > 50.0 %.

Fisher’s exact test was used to compare scores from the questionnaires. Correlations were calculated with Spearman’s non-parametric test. When correlating the cytokines to LTT, the GSTS concentration 25.0 µg/ml was used for both the cytokines and the LTT. When correlating the cytokines to each other, the same GSTS concentration and day was used for each calculation. Linear regression was calculated to evaluate the dose-response relationship for the LTT SI values and GSTS concentrations.

Results from the cytokine quantifications are presented for day 5 at GSTS concentrations of 6.25 or 25.0 µg/ml, depending on which concentrations caused the
highest net cytokine production. The non-parametric Kruskal-Wallis test and Bonferroni’s post hoc test were used for comparisons between the three subject groups. Friedman’s test was used to compare the SI response of the LTT at the different GSTS concentrations.

The analyses were performed using GraphPad Prism 3.0 (GraphPad Software, San Diego, California, USA) and MINITAB (Minitab Ltd., www.minitab.com).

Logistic regression

Logistic regression was calculated to evaluate the combined effect of detecting all the cytokines and LTT. The formula achieved looked as follows:

\[
k_1 + (k_2 \times \text{LTT}) + (k_3 \times \text{IFN-\gamma}) + (k_4 \times \text{IL-13}) + (k_5 \times \text{IL-2}) + (k_6 \times \text{IL-10}) = K;
\]

\[
e^K / 1 + (e^K) = K_p
\]

Where \(k_1 - 6\) represents constants given in the calculations, and \(K_p\) is the predictive value. The value attained is between 0 - 1, where 1 = truly allergic, and 0 = not allergic. Depending on the chosen cut-off, the accuracy varies. The analysis was performed on the EPI+ and the EPI-groups with patch test results as reference, and the predictive values were calculated for all subjects. The analysis was performed using MiniTab.

Paper II

To compare results between the subject groups, the non-parametric Kruskal-Wallis test was used, and unadjusted exact p values were calculated. To compare between the different concentrations of GSTS, Friedman’s test was used, and unadjusted p values were calculated. Special regard was taken to missing values. To help determine the effect of GSTS, linear regression was calculated for all the parameters as well. Statistical analyses were performed using GraphPad Prism 5.0, SPSS for Windows (SPSS Sweden AB, Kista, Sweden) and MiniTab.
Results

Subject data

Paper I

There were no differences between the three subject groups, EPI+, EPI- and EPI-IR, regarding jewellery usage and presence of dental gold. No patients had been treated with gold-containing medications.

Paper II

The allergic subjects were aged 49 ± 7.8 years, all were female, and 6 - 10 years had passed since patch testing. The non-allergic subjects were aged 27 ± 2.9 years (which was significantly different from the allergic subjects, p < 0.05), and all were female.

Viability

Paper II

The PBMC viability from non-allergic subjects showed an inverse dose-response relationship to the GSTS dose ($r^2 = 0.81$, $p < 0.001$) (fig. 5). There was a significant decrease in cell viability in the CD45RA-EF and CD45R0-EF from both allergic and non-allergic subjects after exposure to 50.0 $\mu$g/ml GSTS compared with unstimulated cells ($p < 0.01$ for all four groups) (fig. 5). The inverse dose-response relationship between viability and GSTS dose was significant for both the CD45RA-EF ($r^2 = 0.62$, $p < 0.01$) and the CD45R0-EF ($r^2 = 0.53$, $p < 0.05$) from the non-allergic subjects and for the CD45R0-EF ($r^2 = 0.7$, $p < 0.01$) from the allergic subjects.
Figure 5. Viability in the PBMC fraction from non-allergic subjects and in the CD45RA-EF and CD45R0-EF from both allergic and non-allergic subjects, from paper II. Briefly, cells were stained with propidium iodide and analyzed with a FACS Aria Flow Cytometer, to evaluate living and dead cell populations. GSTS: Gold sodium thiosulphate. **: p < 0.01. $: significant linearity, p < 0.05. $$: significant linearity, p < 0.01. $$$: significant linearity, p < 0.001. Significance levels were calculated using Friedman's test.

**LTT results**

**Paper I**

The optimal GSTS concentration for LTT was 50.0 µg/ml. The EPI+ group had significantly higher SI values than subjects with a negative patch test did, at all GSTS concentrations (p < 0.05 or lower, data not shown), which was most significant at GSTS concentration 50.0 µg/ml (fig. 6A). There were no significant differences between the EPI+ group and the EPI-IR group at any GSTS concentration, with the single exception at GSTS concentration 3.13 µg/ml, where EPI+ subjects showed higher SI values (p < 0.05, data not shown). There were no significant differences between the EPI- group and
the EPI-IR group at any GSTS concentrations.

The SI values for all subjects showed a significant dose-response relationship to GSTS \( (r = 0.43, p < 0.001) \), with the strongest linearity in the EPI- group \( (r = 0.50, p < 0.001) \) (fig. 6A). The highest rise in SI in response to increased GSTS concentration was, however, found in the EPI+ group.

Maximal accuracy for diagnosing ACD with LTT, 75.3 %, was obtained using the SI cut-off 7.9 at GSTS concentration 50.0 \( \mu g/ml \). The sensitivity was 54.5 % and the specificities for the EPI- and the EPI-IR groups were 92.9 % and 87.5 %, respectively. (Table 2)

**Paper II**

While the cells from non-allergic subjects showed a decreased proliferation after GSTS exposure, the cells from allergic subjects showed an increased proliferation compared to the unstimulated cultures (fig. 6B). The CD45R0-EF and CD45RA-EF from non-allergic subjects showed a significant decrease in SI with increased doses of GSTS, with a significant inverse dose-response relationship \( (r^2 = 0.5, p = 0.0003) \) for the CD45RA-EF and \( r^2 = 0.39, p = 0.003 \) for the CD45R0-EF) (fig. 6B). The PBMC fraction from non-allergic subjects showed a slight increase in proliferation at 25.0 and 50.0 \( \mu g/ml \) GSTS, but this increase was not significant (fig. 6B). The CD45R0-EF from the allergic subjects showed a significant dose-response reaction \( (r^2 = 0.39, p = 0.002) \), with increasing SI following increasing GSTS exposure (fig. 6B).
Figure 6: LTT results. In A, the LTT results from paper I are shown, for the three subject groups (EPI+, EPI- and EPI-IR). In B, the results from paper II are shown, with all culture variants (PBMC from non-allergic subjects, CD45RA-EF and CD45RO-EF from both allergic and non-allergic subjects). The CD45RA-EF is plotted on the right axis due to the large variations in this group from both allergic and non-allergic subjects. PBMC: Peripheral blood mononuclear cells. LTT: Lymphocyte transformation test. GSTS: Gold sodium thiosulphate. PBMC: Peripheral blood mononuclear cells. LTT: Lymphocyte transformation test. SI: Stimulation index.
Table 2. Maximal accuracy, sensitivity and specificity for the different cytokines and LTT assays.

<table>
<thead>
<tr>
<th>Included variables</th>
<th>Accuracy (%)</th>
<th>Sensitivity (%)</th>
<th>Specificity (%)</th>
<th>Specitivity EPI-IR (%)</th>
<th>Cut-off</th>
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<td>72.7</td>
<td>89.3</td>
<td>81.3</td>
<td>0.55§</td>
</tr>
</tbody>
</table>

*Values obtained at a GSTS concentration of 50.0 µg/ml. †Values obtained at a GSTS concentration of 6.25 µg/ml. ‡Values obtained at a GSTS concentration of 25.0 µg/ml. §Values obtained at day 5. †§Values obtained at day 4. ‡§Values obtained at day 5. ¥Value shown is Stimulation index. ¥§Values shown are pg/ml. ¥Values shown from the logistic regression calculations have no unit. EPI-IR, Epicutaneous patch tested irritant reactive subject group. GSTS: Gold sodium thiosulfate. LTT, lymphocyte transformation test. SI, Stimulation index. IL, Interleukin. IFN, Interferon.

Cytokine assay results

Paper I

IFN-γ

Optimal culture conditions for the IFN-γ measurement were obtained at day 5, at GSTS concentration 6.25 µg/ml. The level of IFN-γ was significantly higher in the EPI+ group compared to the EPI- group and the EPI-IR group (fig. 7A), but no significant difference was found between the EPI- group and the EPI-IR group. A modest but significant correlation was found between IFN-γ for all days and LTT SI (Table 3).

The maximum accuracy for diagnosing ACD with IFN-γ assessment was 83.1 %, at the cut-off value 0.0 pg/ml, (GSTS concentration 6.25 µg/ml at day 5). The corresponding sensitivity was 81.8 % and the specificity 82.1 %. The specificity for the EPI-IR group was 87.5 % (Table 2).
**IL-13**

Optimal culture conditions for the IL-13 measurement were obtained at day 5, and GSTS concentration 25.0 µg/ml. The level of IL-13 was significantly higher in the EPI+ group at day five, compared to both the EPI- and the EPI-IR groups (fig. 7B). No differences in IL-13 secretion were found at any day between the EPI- group and the EPI-IR group. A modest but significant correlation was found at all days between IL-13 and LTT SI (Table 3).

When using IL-13 measurement to detect ACD, the maximum accuracy was 76.6 %, with a sensitivity of 78.8 %, and specificity of 71.4 % and 81.3 % for the EPI- and the EPI-IR groups, respectively (Table 2). The cut-off value was 0.0 pg/ml (obtained at GSTS concentration 25.0 µg/ml, at day 4).

**IL-2**

Optimal culture conditions for the IL-2 measurement were obtained at day 4, at GSTS concentration 6.25 µg/ml. The IL-2 level was significantly higher in the EPI+ group at all time points investigated, as compared with the EPI- group (fig. 7C). The same applied for the difference between the EPI+ group and the EPI-IR group after 4 and 5 days. There was no significant difference in IL-2 secretion between the EPI- group and the EPI-IR group. A relatively high, significant correlation existed between LTT SI and the IL-2 levels after 3, 4, and 5 days (Table 3).

The maximum accuracy when using IL-2 measurement to identify ACD was 74.0 % when using the cut-off value of 3.41 pg/ml. The corresponding sensitivity was 60.0 % and the specificity for the EPI- group and the EPI-IR group was 89.3 % and 75.0 %, respectively (Table 2). These values were obtained at GSTS concentration 6.25 µg/ml, at day 4.
Figure 7. Results from the Cytokine Assay. A shows the results for the IFN-γ assay, B shows the results from the IL-13 assay, C shows the results from the IL-2 assay, and D shows the results from the IL-10 assay. The significance levels were calculated using Kruskal-Wallis. *; p < 0.05. **; p < 0.01. ***; p < 0.001. IL: Interleukin. IFN: Interferon. EPI: Epicutaneous Patch test subject group (+: positive, -: negative, IR: irritant reaction).
**IL-10 levels**

Optimal culture conditions for the IL-10 measurement were obtained at day 4, and a GSTS concentration of 6.25 µg/ml. There were no significant differences in IL-10 secretion between any of the groups (Fig 7D). There was a weak correlation between IL-10 and LTT at day 5 (Table 3), but not when means or sums were calculated.

The maximum accuracy was lower for IL-10 than for the other cytokines, 64.9 %, with a sensitivity of 60.6 %, and a specificity of 71.4 % and 62.5 % for the EPI- and the EPI-IR groups, respectively (Table 2). These values were obtained using the cut-off value of 0.0 pg/ml, at GSTS concentration 25.0 µg/ml, at day 5.

**IL-1β, IL-4, IL-8 and IL-12**

For IL-1β, IL-4 and IL-12, almost all values were below detection limit (data not shown). For IL-8, more than half of the values were above detection limit. Due to lack of extra material, a second trial using diluted supernatants could not be performed, and these results are therefore not presented.

**Correlation between the different cytokines**

The most significant correlations were found using cytokine values at GSTS concentration 25.0 µg/ml at day 5, where all the cytokines correlated significantly to each other (p < 0.05, Table 3). The highest correlation was found between IL-13 and IFN-γ (r = 0.6744, p < 0.001).
Table 3. Correlation between the different cytokines and LTT.

<table>
<thead>
<tr>
<th></th>
<th>LTT</th>
<th>IFN-γ</th>
<th>IL-13</th>
<th>IL-2</th>
<th>IL-10</th>
</tr>
</thead>
<tbody>
<tr>
<td>LTT</td>
<td>X</td>
<td>0.41***</td>
<td>0.44***</td>
<td>0.24*</td>
<td>0.56***</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>0.41***</td>
<td>X</td>
<td>0.67***</td>
<td>0.40***</td>
<td>0.58***</td>
</tr>
<tr>
<td>IL-13</td>
<td>0.44***</td>
<td>0.67***</td>
<td>X</td>
<td>0.32**</td>
<td>0.59***</td>
</tr>
<tr>
<td>IL-2</td>
<td>0.24*</td>
<td>0.40***</td>
<td>0.32**</td>
<td>X</td>
<td>0.42***</td>
</tr>
<tr>
<td>IL-10</td>
<td>0.56***</td>
<td>0.58***</td>
<td>0.59***</td>
<td>0.42***</td>
<td>X</td>
</tr>
</tbody>
</table>

Cytokine values were included at GSTS concentration 25 μg/ml and day 5, and LTT SI values were included at GSTS concentration 25 μg/ml. GSTS: Gold sodium thiosulphate. LTT: Lymphocyte transformation test. IFN: Interferon. IL: Interleukin*: p < 0.05. **: p < 0.01. ***: p < 0.001. Correlation was calculated using Spearman’s non-parametric test.

**Logistic regression**

This analysis was performed to evaluate whether the sensitivity and specificity could be improved by using the different cytokines simultaneously. Different combinations using cytokines and LTT were used to find the optimal combinations. The cytokines were included at the same GSTS concentration and culture day, and LTT was included at GSTS concentration 50.0 μg/ml. The conditions that yielded the highest accuracy at GSTS concentration 6.25 μg/ml, at day 5, yielded the following formula:

\[
e^{(-1.0258 + (0.15346 \times \text{LTT}) + (0.02545 \times \text{IFN-γ}))} / 1 + (e^{(-1.0258 + (0.15346 \times \text{LTT}) + (0.02545 \times \text{IFN-γ}))})
\]

The values obtained from this formula were 0 - 1. The highest accuracy, 80.5 %, was obtained when using 0.55 as a cut-off; the sensitivity was 66.7 %, the specificity was 92.9 %, and the sensitivity for the EPI-IR group was 87.5 % (Table 4). The conditions that yielded the highest accuracy when using the cytokines at GSTS concentration 25.0 μg/ml, at day 5, yielded the following formula:

\[
e^{(-1.0257 + (0.12575 \times \text{LTT}) + (0.01533 \times \text{IFN-γ}))} / 1 + (e^{(-1.0257 + (0.12575 \times \text{LTT}) + (0.01533 \times \text{IFN-γ}))})
\]

The highest accuracy, 80.5 %, was obtained when using 0.55 as a cut-off; the sensitivity was 72.7 %, the specificity was 89.3 %, and the sensitivity for the EPI-IR
group was 81.3 % (Table 2).

**Flow cytometry results**

**Paper II**

**CD3⁺ cell fraction**

In the PBMC fraction from non-allergic subjects, the CD3⁺ fraction of cells decreased at GSTS concentrations above 50.0 µg/ml (fig. 8), and was significantly reduced at 200 µg/ml GSTS (p < 0.05). The fraction of CD3⁺ cells in the CD45RA-EF and CD45R0-EF from both the allergic and non-allergic subjects (data not shown) was largely unchanged (mean range 73.4 %) within the GSTS dose range used (0.0 – 50.0 µg/ml).

**CD3⁺CD4⁺ and CD3⁺CD8⁺ cell fractions**

The fraction of CD3⁺CD4⁺ cells in the PBMC fraction from the non-allergic subjects decreased at a GSTS concentration of 200.0 µg/ml, while the fraction of CD3⁺CD8⁺ cells started to decrease at concentrations above 50.0 µg/ml (fig. 8). Thus the ratio between the CD3⁺CD4⁺ and CD3⁺CD8⁺ cells clearly increased at GSTS concentrations above 50.0 µg/ml (fig. 8).

In the CD45RA-EF from both allergic and non-allergic subjects, the fraction of CD3⁺CD4⁺ cells and CD3⁺CD8⁺ cells ranged from 49.5 % – 55.5 % and 33.6 % – 49.6 %, respectively, and did not change within the GSTS dose range 0.0 – 50.0 µg/ml (data not shown). In the CD45R0-EF from both allergic and non-allergic subjects (fig. 9), the fraction of CD3⁺CD4⁺ cells tended to increase after GSTS exposure, while the fraction of CD3⁺CD8⁺ cells tended to decrease, both in a dose-dependent manner. In the CD45R0-EF from the allergic subjects, this trend was statistically significant (p < 0.01). Thus the ratio between the mean fraction of CD3⁺CD4⁺ and CD3⁺CD8⁺ cells in the CD45R0-EF from both allergic and non-allergic subjects increased with the GSTS dose,
although the differences were not significant.

\[ \text{Figure 8. CD}^3\text{+, CD}^4\text{+ and CD}^8\text{+ cell fractions in PBMC from non-allergic subjects in paper II. GSTS: Gold sodium thiosulphate. *: p < 0.05. §§: Significant linearity, p < 0.01. Significances were calculated using Friedman’s test.} \]

**CD}^3\text{CD45RA}^+ \text{ and CD}^3\text{CD45R0}^+ \text{ cell fractions**}

In the PBMC fraction from non-allergic subjects, the CD3’CD45RA+ cell fraction showed a significant decrease at 200 µg/ml GSTS (significant from GSTS concentration 50.0 µg/ml, p < 0.05) while the fraction of CD3’CD45R0+ cells started to decrease at 50.0 µg/ml GSTS, and the inverse dose-response relationship was significant (\( r^2 = 0.28, \) p < 0.05) (fig. 10). Thus, the ratio between the CD3’CD45RA+ cells and the CD3’CD45R0+ cells increased with increasing GSTS concentrations (fig. 10).
Figure 9. CD4\(^+\) and CD8\(^+\) cell fractions in the CD45R0-EF from both allergic and non-allergic subjects in paper II. Plotted on the right axis is the ratio between CD4\(^+\) and CD8\(^+\), for both subject groups. GSTS: Gold sodium thiosulphate. **: p < 0.01. Significances were calculated using Spearman’s test.

In the CD45RA-EF from the allergic subjects, the minute CD3\(^+\)CD45R0\(^+\) cell fraction decreased significantly (p < 0.05) with GSTS exposure (0.9 % at 0.0 µg/ml - 0.1 % at 50 µg/ml GSTS) (data not shown). The fraction of CD3\(^+\)CD45R0\(^+\) cells remained unchanged regardless of GSTS dose (0.0 - 50.0 µg/ml) in the CD45R0-EF from allergic subjects (47.4 - 56.0 %) and the CD45RA-EF (0.2 - 4.5 %) and CD45R0-EF (74.8 - 78.9 %) from the non-allergic subjects (data not shown). The fraction of CD3\(^+\)CD45RA\(^+\) cells remained unchanged in response to GSTS in the CD45RA-EF (89.4 - 90.9 %) and CD45R0-EF (2.8 - 7.7 %) from the allergic subjects and in the CD45RA-EF (91.4 - 94.1 %) from the non-allergic subjects.
%) and CD45R0-EF (1.8 - 9.3 %) from the non-allergic subject group (data not shown).

Figure 10. CD45RA⁺, CD45R0⁺ and double positive CD45RA⁺CD45R0⁺ cell fractions in PBMC from non-allergic subjects in paper II. Plotted on the right axis is the ratio between CD45RA⁺ and CD45R0⁺ cell fractions. GSTS: Gold sodium thiosulphate. *: p < 0.05. $: Significant linearity, p < 0.05. Significances were calculated using Friedman’s test.

**CD3⁺CD45RA⁺CD45R0⁺ double positive cell fraction**

The CD45RA-EF from both subject groups had a small fraction of CD3⁺CD45RA⁺CD45R0⁺ double positive cells after culturing, which remained unchanged (4.1 - 10.4 %) after GSTS exposure. In the CD45R0-EF from both the allergic and non-allergic subjects, the fraction of CD45RA⁺CD45R0⁺ cells increased after GSTS exposure, which was significant for the CD3⁺CD4⁺CD45RA⁺CD45R0⁺ cells from the allergic subjects (p < 0.01 between GSTS concentrations 0.0 and 50.0 µg/ml) (Table 4).
Table 4. CD45RA⁺, CD45R0⁻ and CD45RA⁺CD45R0⁻ cells in the CD45R0-EF from allergic and non-allergic subjects.

<table>
<thead>
<tr>
<th></th>
<th>Allergic CD45R0-EF</th>
<th>Non-allergic CD45R0-EF</th>
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<tbody>
<tr>
<td></td>
<td>0.0 µg/mlᵃ</td>
<td>25.0 µg/mlᵃ</td>
</tr>
<tr>
<td>CD3⁺CD4⁺CD45RA⁺CD45R0⁻</td>
<td>22.4 ± 23.2</td>
<td>26.2 ± 20.8</td>
</tr>
<tr>
<td>CD3⁺CD4⁺CD45RA⁺⁺</td>
<td>2.2 ± 1.1</td>
<td>5.7 ± 3.6</td>
</tr>
<tr>
<td>CD3⁺CD4⁺CD45R0⁺⁺</td>
<td>68.6 ± 28.7</td>
<td>60.8 ± 22.6</td>
</tr>
</tbody>
</table>

**: p < 0.01 from CD3⁺CD4⁺CD45RA⁺CD45R0⁻ from allergic subjects at GSTS concentration 0.0 µg/ml, calculated using Friedmans repeated measurement ANOVA test. ⁺, GSTS concentration. GSTS: Gold sodium thiosulphate.

The fraction of CD3⁺CD45RA⁺CD45R0⁻ cells was larger in the CD45R0-EF (14.7 - 23.2 %) than in the CD45RA-EF (4.1 - 5.7 %) from the non-allergic subjects (the difference was significant at GSTS concentration 50.0 µg/ml, p < 0.05). The same relationship between the CD45R0-EF (38.5 - 49.3 %) and CD45RA-EF (8.2 - 10.4 %) was evident for the allergic subjects, but here the differences were not significant. The CD3⁺CD8⁺CD45RA⁺CD45R0⁻ cell fraction remained unchanged in the CD45RA-EF and CD45R0-EF from both the allergic and non-allergic subject groups (data not shown).

In the PBMC fraction from non-allergic subjects, the CD3⁺CD45RA⁺CD45R0⁻ cell fraction remained unchanged (3.5 - 7.1 %) regardless of GSTS dose (0.0 – 200.0 µg/ml, fig. 10).

**CLA expression**

CLA was not expressed on unstimulated CD3⁺CD45RA⁺ cells (mean fluorescence
intensity, MFI, 0 – 301), which did not change with GSTS exposure (fig. 11). The expression of CLA was significantly higher in the CD45R0-EF than in the CD45RA-EF from both allergic and non-allergic subjects in the GSTS dose range 0.0 - 50.0 µg/ml (p < 0.05, data not shown). There was a dose-dependent down-regulation of CLA expression in the CD45R0-EF from non-allergic subjects in response to GSTS exposure, which was significant for the CD3⁺CLA⁺ (r² = 0.46, p < 0.05) and CD3⁺CD4⁺CLA⁺ (r² = 0.6, p < 0.05) (fig. 11). The same trend could be seen for CD3⁺CD8⁺CLA⁺, but was not significant (data not shown).

![Figure 11](image)

Figure 11. CLA expression on CD45RA-EF and CD45R0-EF cells from both allergic and non-allergic subjects in paper II. GSTS: Gold sodium thiosulphate. CLA: Cutaneous Lymphocyte-associated Antigen. MFI: Mean fluorescence intensity. **: p < 0.01. Significances were calculated using Spearman’s test.

The CD45R0-EF from allergic subjects behaved somewhat differently regarding CLA expression. The CLA expression in the CD45R0-EF from allergic subjects remained unchanged at GSTS concentration 25.0 µg/ml compared to unexposed cells, but was
significantly decreased at GSTS concentration 50.0 µg/ml (p < 0.01) (fig. 11).

Chemokine receptor-expressing cells

Of the CD3⁺ cells, 1.6 – 46.6 % in the CD45RA-EF and 36.3 – 58.4 % in the CD45R0-EF from both allergic and non-allergic subjects expressed the CXCR3 receptor, but no significant differences were found between the CD45RA-EF and CD45R0-EF from the allergic or the non-allergic subject groups, or between the subject groups, for CXCR3. The expression was also not affected by GSTS exposure.

For CCR10, there was a weak trend of increasing CD3⁺CCR10⁺ cells after GSTS exposure in the CD45RA-EF and CD45R0-EF from both the allergic and non-allergic subjects (fig. 12A). A minimum of 3 % of the cells showed CCR10 without GSTS, to a maximum of 25 % of the CD3⁺ cells expressing this receptor at 50.0 µg/ml GSTS in both subject groups. This trend did not, however, reach statistical significance.

The mean fraction of CD3⁺CCR4⁺ cells was visibly higher in the CD45R0-EF than in the CD45RA-EF in both allergic and non-allergic subjects (fig. 12B), but the difference was only significant in the allergic subjects (p < 0.05). The fraction of CD3⁺CCR4⁺ cells in the CD45R0-EF from both subject groups was unaffected by the GSTS exposure. In contrast, there was a trend towards increased CD3⁺CCR4⁺ cell fractions after GSTS stimulation in the CD45RA-EF from both allergic and non-allergic subjects, but the dose-response relationship was only significant for the non-allergic subjects (r² = 0.48, p < 0.05).
Figure 12. Chemokine receptor-expressing cells in the CD45RA-EF and CD45R0-EF from both allergic and non-allergic subjects in paper II. A shows CCR10, and B shows CCR4. GSTS: Gold sodium thiosulphate. *: p < 0.05. Significances were calculated using Kruskal-Wallis test.
Discussion

Many studies have investigated the toxicology of different gold salts \textit{in vivo}, both in animals and in humans (98-101), but few studies have reported the \textit{in vitro} effects of gold salts. Our findings show that GSTS has a cytotoxic effect on lymphocytes in concentrations higher than 50.0 µg/ml, (102 µM). Approximately 40 % (40.8 µM) of the salt consists of Au [I]. RA patients receiving either gold sodium thiomalate (GSTM) or auranofin, the oral gold salt treatment, obtain blood levels ranging between 0.1 – 0.4 µg/ml when in steady state (57, 59, 102), which for GSTM equals 0.13 - 0.51 µM active Au [I], and for Auranofin equal 0.045 - 0.18 µM Au [I]. These concentrations are more than a 100-fold less than the concentrations found to be toxic in the \textit{in vitro} study in paper II. To compare with mercuric chloride HgCl$_2$, a highly toxic metal salt, toxic \textit{in vitro} levels range from 1-20 µM (0.74 - 14.8 µM pure Hg) (reviewed in (103)), while NiCl$_2$ levels of up to 1 mM (0.45 µM pure Ni) has been proven non-toxic \textit{in vitro} (104). Auranofin has proven to be toxic in the 0.3 - 3 µM (active Au [II]) range \textit{in vitro}, but many other gold salts have been regarded as virtually non-toxic \textit{in vitro} (105). The differences in toxicity might be explained by different properties of the molecule carrying the gold ion, i.e. that different structures release the gold at different rates, leaving different concentrations of available gold in the culture medium.

Furthermore, the \textit{in vitro} environment is limited in simulating the \textit{in vivo} conditions, where individual differences in carrier-protein levels (such as albumin and globulins for gold) can contribute. Individual properties of monocyte lysosomal enzymes influence the oxidation rate of the Au [I] salt into the more reactive oxidizer Au [III], which also affects the concentration at which different gold salts cause toxic effects.

We have shown that on a group level, LTT can be used to distinguish between subjects allergic (EPI+) and non-allergic (EPI-) to gold. In paper I we used SI cut-off 7.9
to gain maximal sensitivity and specificity of 54.5 % and 92.9 %, respectively. These results are not completely in agreement with an older study showing a sensitivity of 70 % and a specificity of 58 %, but there another cut-off (2.0), and different GSTS concentrations were used (90). Many publications have used cut-off values of 2-3 when calculating sensitivity and specificity for LTT (90, 106-108), but since this is just an arbitrary value, there is no golden standard for this.

Using the lower GSTS concentration in paper I (6.25 µg/ml), yielded an accuracy of 67.5 %, a sensitivity of 54.5 % and a specificity of 78.5 % (cut-off 1.97). It has previously been shown with NiSO₄ that higher concentrations in test culture results in an unspecific activation of T-cells, not unlike mitogens (109). To our knowledge, no similar results have been reported for gold. Our results show the largest difference in SI between allergic and non-allergic subjects at a high GSTS concentration. Therefore, we suggest that the GSTS used in paper I did not evoke any unspecific proliferation.

When looking at the LTT results in paper II, we saw that both CD3⁺CD45RA⁺ and CD3⁺CD45R0⁺ cells from the allergic subjects proliferated in response to GSTS, with maximal mean SI values of 8.0 for the CD45RA-EF and 3.3 for the CD45R0-EF. In this study only one SI value in the separated cell fractions reached above the 7.9 cut-off limit, but it is not surprising that the separated cells might proliferate at a different rate than a complete lymphocyte population.

The SI values for the unseparated PBMC cell fractions from the non-allergic subjects proliferated at a rate clearly below the 7.9 limit. All GSTS exposed, separated cells from the non-allergic subjects had SI below 1.0, indicating a suppressive effect of GSTS. As to why both the CD45RA-EF and CD45R0-EF from the allergic subjects proliferated, we can only speculate. It is however plausible to hypothesise that the CD45R0 cells included most of the regulatory T-cells as well (22), leaving the CD45RA-EF without a suppressing cell cohort.

Even though the LTT method could predict the EPI-IR group as non allergic with
rather high accuracy (87.5%), there was no significant difference between this subject group and the group with positive patch test in paper I. The p-value was, however, very close to significant (p = 0.059, data not shown). Taken together with the non-significant p-value between the EPI- and the EPI-IR groups (p = 1, data not shown), it can be postulated that the EPI-IR group is more similar to the EPI- group than to the EPI+ group. To our knowledge, this has not been tested before. The observation opens the possibility for LTT to be used as a complement to patch testing where the morphological outcome has been uncertain. Confirmation of negativity could be equally as valuable as confirmation of positivity.

To evaluate which cell types had proliferated, we examined the cell cultures in paper II with flow cytometry. We saw that neither the CD45RA-EF nor CD45R0-EF cultures from the allergic subjects changed their CD3+ cell fractions in response to the GSTS exposure. The discrepancy between the LTT and flow cytometry might result from the two methods measuring two different aspects of cell proliferation. LTT provides a view of the actual proliferation during the 4 - 5 hours of labelled thymidin incorporation after 4 - 5 days of culturing, while flow cytometry shows the accumulated proliferation after 4 - 5 days of culture, including the net effect of cell proliferation and death. The reason that the CD3+ cell fractions did not change in the flow cytometry analysis might thus be due to a balance between cell death and cell proliferation.

In the CD45R0-EF from both allergic and non-allergic subjects, the ratio between the CD3+CD4+ and CD3+CD8+ cells increased in a GSTS-dose-dependent manner. This might be due to either an increase in CD3+CD4+ cell number, or a decrease in CD3+CD8+ cell number, or a combination of both. Some studies indicate CD3+CD4+ cells as the main culprit involved in ACD to various haptens (77, 80, 106), while others state that both CD3+CD4+ and CD3+CD8+ cells work as effector cells in ACD to Ni, but that non-allergic patients also express Ni-specific CD3+CD4+ T-cell clones (75, 79, 110,
111), but not CD3+CD8+ cells. We saw that the CD3+CD8+ cells did not react at all when exposed to GSTS, suggesting that CD3+CD8+ proliferation might not be a general phenomenon in ACD, but occurring specifically in Ni allergy, or that gold has additional immunomodulatory mechanisms.

The cells that responded to GSTS were almost exclusively of a CD3+CD4+CD45R0+ phenotype originating from the allergic subjects, responding by changing their expression to include both CD45RA and CD45R0. This can plausibly be interpreted as being in a transition state, since it has been shown that the expression of different CD45R isoforms on T-cells in vitro is cyclic rather than unidirectional (25-28), and that reactivated CD45R0+ T-cells express CD45RA as well (112, 113).

CLA was almost exclusively expressed on CD3+CD45R0+ cells, equally distributed among the CD3+CD4+ and the CD3+CD8+ cells. Both of the latter cell types from the allergic subjects retained their expression of CLA at the lowest GSTS concentration used, while the cells from the non-allergic subjects down-regulated their expression even at the lowest GSTS dose. This is in keeping with what has been found regarding Ni allergy, where several studies have shown that CLA+ cells are necessary for Ni-specific proliferation (11, 80, 81, 83, 114-116). CD3+CD4+CLA+ and CD3+CD8+CLA+ levels are increased after metal hapten stimulation in vitro, including AuCl₄⁻ (114), but this study did not investigate memory status of the cells analysed. Since we saw no proliferation of CD3+CD8+ cells in response to GSTS, we conclude that the CD3+CD4+CD45R0+CLA+ cells that are retained by GSTS exposure are the cells specific for gold allergy.

The chemokine receptor CCR4 seemed to be more expressed on CD3+CD45R0+ cells than CD3+CD45RA+ cells, from both the allergic and non-allergic subjects, which did not change with GSTS exposure. Ni-responsive CD3+CD4+CD45R0+CLA+ cells express
the chemokine receptors CCR4, CXCR3 and CCR10 (80, 117). CCR4 is normally co-expressed with CLA, and also directs cells to the skin (118, 119). Unfortunately, due to technical circumstances, this receptor could not be co-analysed with CLA in our study (paper II), but since a fairly large fraction of CD3^+CD45R0^+ cells expressed CCR4 (up to 50 %, fig 12), it seems reasonable to hypothesize that a substantial fraction of these cells expressed CLA as well.

CXCR3^+ cells have been shown to be infiltrating skin patch tested with allergens, but not irritants (118). In paper II, 40 – 60 % of all T-cells expressed this receptor, but we found no differences between the allergic and non-allergic subjects. However, since we were unable to co-analyse this receptor with CD4 and CD8 (also due to technical circumstances), we can not be certain that this is not due to a balance between CD3^+CD4^+ cell proliferation and CD3^+CD8^+ cell death, similar to the CD3^+ cell results.

We saw a trend towards an increase of CD3^+CCR10^+ cells after GSTS exposure in all separated cell fractions from both subject groups in paper I, but the increase was not significant. The trend supports what has previously been reported for Ni (80, 117), namely that CCR10 is mostly associated with skin homing (119), and CCR10-expressing cells have been associated with patch tested skin even a long time after Ni exposure (117, 119).

Previous studies using PBMC from gold-treated arthritic patients reported a decrease in mitogen-induced IFN-γ secretion (120) after gold salt exposure. However, another study using blood samples from gold-allergic subjects indicated an increased secretion of IFN-γ by gold salt-exposed lymphocytes (121). In the allergic subjects in paper I, IFN-γ levels were found to be elevated, which tallies with previous suggestions that ACD is mainly a Th1 response (37, 122, 123). IFN-γ detection discriminates the allergic subject group from both the negative and the irritant group, with a sensitivity and
specificity of 83.8 % and 81.8 %, respectively. Vannes et al (121) showed maximum sensitivity 89 % and specificity 73 % of IFN-γ as assessment method after 4 days of cell culturing, but it is possible that cell culturing for 5 days could have increased the sensitivity and specificity in that study. Assessment of IFN-γ in paper I was also able to correctly identify 87.5 % of the EPI-IR subjects as non-allergic, which is the highest discriminatory value obtained in this study for EPI-IR.

In paper I, a significant increase was found in the IL-13 secretion in the EPI+ subjects compared to the other two subject groups. IL-13 has been shown to be increased in blood cultures from Ni-allergic patients when exposed to Ni^{2+} (124). One theory is that the elicitation phase of ACD is mainly maintained by a Th1 response, while the containment and inhibition of the reaction is attained by Th2 or Treg cytokines such as IL-4, IL-5 or IL-10 (36, 75, 79). This might explain the increased secretion in the allergic subjects in the present study. The IL-13 assay was quite good at diagnosing ACD to gold, with a sensitivity and specificity of 78.8 % and 71.4 % respectively, but this was not quite as effective as the IFN-γ assay.

IL-2 is a cytokine primarily associated with Th1 responses, causing antigen-specific proliferation in memory T-cells, and is a key driver in T-cell proliferation (125). The findings in paper I of elevated IL-2 levels in allergic subjects were in concordance with the concept that gold exposure on memory lymphocytes causes specific proliferation, which is also reflected in the correlation with the LTT SI values. The IL-2 levels were significantly higher in the EPI+ group than in both the EPI- and EPI-IR groups, which points to a specific reactivity towards gold. Previous studies have shown that different gold salts decrease secretion of IL-2 in both blood samples from arthritic patients and in normal blood cell lines (126, 127). However, none of these studies included patch tested patients or patients with adverse effects to treatment, so it is possible that none of these patients were allergic.

The sensitivity and specificity for IL-2 measurement in diagnosing gold allergy were
both quite high, but not as high as for IFN-γ or IL-13. They are similar to the values of LTT alone, and there was a significant correlation to the LTT SI values.

IL-10 is a cytokine that has mainly immunosuppressant properties, and functions by downregulating expression of cytokines and co-stimulatory molecules (128). Some studies show that IL-10 secretion is increased upon epicutaneous allergen stimulation, which in turn downregulates Th1 cytokines, thereby limiting the immune reaction (59). One study showed that in Ni allergy, IL-10 production was increased in the allergic patients’ PBMC’s in vitro (106). Interestingly, in we found no significant differences between any of the subject groups, suggesting that Ni induces IL-10 secretion in a manner that gold does not.

One interesting find is that the cut-offs for all three cytokines are low, for IFN-γ and IL-13 it was 0.0 pg/ml, and for IL-2 3.41 pg/ml. Also, many of the net values are negative, mostly in the EPI- and the EPI-IR groups. However, since gold is known to have suppressive effects on T-cells, then it is to be expected that without gold-reactive T-cells, which should be the case in the non-allergic subjects, there will be an inhibition of cytokine production after gold exposure. Since the cells can be disturbed, and thereby slightly activated, by the purification procedure, as well as from being in an in vitro environment, the cultures without GSTS will secrete a base level of cytokines; thus the GSTS-exposed cultures from the non-allergic subjects turn out with a negative net value. In the cultures from the allergic subjects, the specific activated T-cells secrete enough cytokines to compensate for the inhibitory effect on the non-specific T-cells; therefore the net values from the allergic subjects come close to 0. High accuracy in testing can thus be achieved due to the pharmacologically inhibitory effect of gold on T-cells in non-allergic subjects.
Th1 and Th2 cytokines are known to inhibit one another (129), which might explain why the maximum sensitivities and specificities are achieved at different GSTS concentrations and different days for the different cytokines. To evaluate whether Th1 or Th2 cytokines were most prominent in ACD to gold, correlations between the different cytokines were calculated. There was a high degree of correlations between all cytokines, with the highest found between IL-13 and IFN-\(\gamma\). Possibly, the early T-cell response is to secrete Th1 cytokines such as IFN-\(\gamma\) and IL-2, which also correlates well with IL-13. To contain and stop the reaction, other T-cells not specific for gold allergy then respond by producing IL-13 to inhibit the immunological response. If the cytokine assay had been performed at closer time intervals and/or for a longer time, an increase in IL-13 might have been detected, with a subsequent decline in IFN-\(\gamma\) and IL-2 production.

To evaluate whether any or all of the in vitro methods could be combined to increase the accuracy of diagnosis, logistic regression was performed. First, correlations between the different cytokines and LTT were calculated, with the hypothesis that if the cytokines correlated well to LTT, they might not provide any improvement for diagnostic accuracy. The cytokines correlating the least to LTT (which turned out to be IL-10) would then yield a higher accuracy when included in the logistic regression calculation. The approach that yielded the highest accuracy did not include IL-10, however, but rather consisted of LTT, IFN-\(\gamma\) and IL-13. This yielded an accuracy of 80.5 %, which was very close to the one achieved with only IFN-\(\gamma\), 83.1 %.

Another aspect to consider is that of cost-benefit. One might consider the use of IFN-\(\gamma\) detection as a single diagnostic tool, using a simple ELISA method instead of the rather costly Luminex assay. Removing LTT from the procedure would also remove the
need for radioactive materials, and reduce number of culture plates. The IFN-γ assessment could then be performed by any routine diagnostic laboratory. This method could preferably be used to verify irritant epicutaneous patch test reaction in conjunction with, or as an alternative to, repeated patch tests with serial dilution of antigens.

**Conclusions**

When we compared our findings to what has been published on Ni, we found that the main similarity to gold allergy was that the T-cells from allergic individuals, when proliferating in response to relevant metal salt, are of the CD3⁺CD4⁺CD45R0⁺CLA⁺ phenotype. In contrast to Ni allergy, however, the chemokine receptors CCR4, CXCR3 and CCR10 were not triggered in gold allergic subjects in response to GSTS *in vitro*. But since the clinical outcomes of these two allergies differ significantly, different mechanisms of action may perhaps be expected.

It is also very possible that we would be able to discern differences if we collected cells at different time points in the development of ACD, and followed them over an extended time period. When culturing cells it is always difficult to accurately pinpoint the timing of protein expression and secretion.

Our findings in paper I indicate that both Th1 and Th2 cells are involved in the mechanisms of ACD to gold. This is in keeping with some publications on Ni (82), while others state that it is mostly Th1 cells that are involved in the Ni reaction (82, 130). Thus, this might be another reason why the clinical outcome for Ni allergy and gold allergy are so different.

We also suggest that a blood test followed by IFN-γ assessment with a simple ELISA assay could be used as a complement to patch testing, especially to rule out an IR. This could make the diagnostic process easier for both patient and physician.

In summary, contact allergy to gold seems to involve mainly memory CD4⁺CLA⁺ T-
cells, of both Th1 and Th2 type. Further studies are warranted, to examine whether different cell subsets are in effect at different time points, and whether other allergens compare to Ni or to gold. The differences between Ni and gold allergy, particularly regarding the receptors guiding T-cells towards the skin, could help explain the different clinical outcomes of the different allergens. Possibly this could also be extended to other allergens in the future, helping to explain the different allergenicity of different haptens.
Acknowledgements

I would like to thank my supervisor, professor Per Hultman, for giving me the opportunity to make this project. Since I began my university studies, I have always wanted to work in the immunology field, so this was the perfect chance for me. Thank you so much for inspiring me and always being there to answer questions, sometimes even in the middle of the night. Thanks also to my co-supervisors Karin Cederbrant and Chris Anderson. You’ve both helped me a lot, and you’ve really stepped up to the plate during the last difficult months. Thank you also to Said Havarinasab, Christer Bergman and Marie-Louise Eskilsson, who always help out with everything you can, whether it be putting up shelves or helping me with technical stuff.

I would also like to thank dr Klara Martinsson, my office-buddy and shoe-friend. You make it fun to come to the office, and thank you for introducing me to “pralin-kvällar” and pyssel! Thank you also to Jenny Mjösberg, who is the greatest discussion partner on lab-related and non-lab-related things, and also the best hairdresser in the world! If your research should unexpectedly turn sour, you know you can always fall back on that. Thank you also to Marie Rubér, who introduced me to my favourite hobby: Knitting! You also helped me so much when I first began at AIR with my very first Luminex assay! Thanks also to Jimmy Ekstrand, for always being so positive and willing to discuss anything both research and everything else. We sure miss you at the office when you’re away in Umeå!

Thanks also to all present and past, co-workers at AIR, none mentioned and none forgotten! I have felt welcome from the beginning, and it’s always easy to find someone to ask if I wonder something. It seems we have experts on almost everything in our department.

Thanks also to my co-writers at Örebro University, Gunilla Färm and Ruth Eid-Forest. You helped a great deal with patient recruiting and data interpretation. Also thanks to Inga-Lill Erikssohn for helping with interpreting patient journals.

I also want to thank Kristina Karlsson, who was the one who started the project and made significant laboratory work for the first paper. And thanks to Barbro
Gunnarsson, who has gathered all patients in Linköping and organized all patient data.

Thanks also to the statistician at Linköping University, Olle Eriksson. You helped me so much, and I am so grateful that you even made some of the analyses when you were on paternity leave.

Thank you also to all my friends who don’t work with me: Christine, Martin & the boys, Jonas, Anders, Fredrik, Martin & Gunilla, Marcus, Micke, Mackan & Micke and Elna. Thank you for taking my mind off research for a while, and always being supportive and helpful. You’ve all really helped me through this long and winding process and helped me find the light at the end of the tunnel when I sometimes felt like I had lost the way. I love you all!

Thanks also to my family, who although they don’t have a clue of what I’m working on, always support me and seem proud of me. Thank you so much, mom, dad, Sanna, mormor & Micael, and morfar. I couldn’t have done it without you.

Also, thanks to my biggest stress-reliever, Hobbe. When you curl up in my lap, everything feels a little better. I just wish your brother was still around to keep us company.

Finally, thank you Thomas, my wonderful husband. Thank you for always being there and picking up the slack when I have intensive periods where I don’t have time to clean and do laundry. Thank you for loving me and taking care of me and Hobbe. I love you so much.
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