

Quantitative, absolute count-based T cell analysis of CD69 upregulation as a new methodology for *in vitro* diagnosis of delayed-type nickel hypersensitivity

Running title: New methodology of CD69 T cell analysis

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ABSTRACT

BACKGROUND: T cells play a major role in delayed-type hypersensitivity reactions. Their reactivity can be assessed by measuring the upregulation of the activation marker CD69, followed by proliferation and cytokine production. The aim of our study was to develop a novel, whole blood-based, quantitative, absolute count activation index (AI) analysis of CD69 upregulation on different subsets of T cells in nickel hypersensitive patients and compare it with the previously reported approaches.

METHODS: Ten patients with nickel allergy and nine healthy controls were included. CD69 expression of CD3+, CD3+CD4+ and CD3+CD8+ T cells in heparinized blood was determined with flow cytometry after incubation with nickel sulfate for 48 h. The absolute cell count of CD69+ cells was determined with microbeads. The production of the cytokines IL-2, IL-5, IL-13, and IFN- γ was determined after nickel sulfate stimulation of PBMCs for 48 h.

RESULTS: We showed that the most sensitive methodology is the absolute AI, which was calculated as the ratio between the absolute count of CD69-positive T cells stimulated with nickel and the absolute count of CD69-positive T cells in non-stimulated blood. This novel quantitative approach was more discriminative than the previously reported approaches in which T cell CD69 percentage AI and cytokine production are measured.

CONCLUSIONS: Our results demonstrated that measuring the absolute CD69 AI is an accurate new approach to quantify antigen-specific T cells in the blood of patients with hypersensitivity reactions to nickel. This approach may be useful for better *in vitro* assessment of patients with delayed-type hypersensitivity reactions.

Keywords: delayed-type hypersensitivity, nickel, whole blood, CD69.

RESUMEN

ANTECEDENTES: Los linfocitos T juegan un papel importante en las reacciones de hipersensibilidad de tipo retardado. Su actividad puede evaluarse midiendo la expresión del marcador de activación CD69, seguido de la proliferación y la producción de citocinas. El objetivo de nuestro estudio ha sido el desarrollar un novedoso análisis cuantitativo del índice de activación absoluto (AI) en sangre completa de la expresión de CD69, en diferentes subconjuntos de linfocitos T, en pacientes con hipersensibilidad al níquel, y compararlo con los métodos existentes.

MÉTODOS: Se estudiaron diez pacientes con alergia al níquel y nueve controles sanos. La expresión de CD69 de los linfocitos T CD3 +, CD3 + CD4 + y CD3 + CD8 + en sangre heparinizada se determinó con citometría de flujo, después de una incubación con sulfato de níquel durante 48 h. El recuento absoluto de células CD69 + se determinó con microesferas. La producción de las citocinas IL-2, IL-5, IL-13 e IFN- γ se cuantificó después de la estimulación de células mononucleares periféricas, durante 48 h, con sulfato de níquel.

RESULTADOS: Se demuestra que la determinación del índice AI absoluto es la metodología más sensible. Se calculó como la relación entre el recuento absoluto de linfocitos T CD69-positivos estimulados con níquel y el recuento absoluto de linfocitos T CD69-positivos en sangre no estimulada. Este nuevo enfoque cuantitativo fue más discriminativo que los enfoques publicados previamente en los que se midió el porcentaje de CD69 de linfocitos T y la producción de citocinas.

CONCLUSIONES: Nuestros resultados demostraron que la medición del AI absoluto de CD69 es un enfoque nuevo y preciso para cuantificar los linfocitos T específicos de antígeno en la sangre de pacientes con reacciones de hipersensibilidad al níquel. Este enfoque puede ser útil para una mejor evaluación *in vitro* de los pacientes con reacciones de hipersensibilidad de tipo retardado.

Palabras clave: hipersensibilidad de tipo retardado, níquel, Sangre completa, CD69.

1. INTRODUCTION

Nickel is one of the most common causes of allergic contact dermatitis and delayed-type hypersensitivity reactions [1, 2]. A recent study showed that nickel sulfate affects approximately 15% of the population [3]. Allergic contact dermatitis (ACD) to nickel activation of nickel-specific T cells followed by the proliferation and induction of cytokine production [4, 5]. More specifically, in the sensitization phase nickel ions penetrate the skin which activates epithelial cells that produce various cytokines and chemokines. This is followed by complex immune responses resulting in activation of antigen presenting cell (APCs), such as Langerhans cells or dendritic cells. Activated APCs migrate to lymph nodes where they present the antigens to naïve T cells. In the elicitation phase subsequent re-exposure to the same allergen leads to the activation of antigen-specific T-cells, which enter the bloodstream where they activate, proliferate and produce various inflammatory cytokines and chemokines at the site of exposure that promote the allergic reaction and lead to development of characteristic skin lesions [6].

Nickel allergy hypersensitivity is mainly diagnosed by epicutaneous patch testing or an *in vitro* lymphocyte transformation test (LTT), which exploits the proliferative potential of antigen-specific T cells [7–11]. However, LTT imposes limitations in terms of using radioactive isotopes, and thus, there is a need for other methods that are easier to implement. Therefore, CD69 has been identified as the earliest T lymphocyte activation marker [12], and an alternative approach is the flow cytometric analysis of *in vitro* CD69 upregulation on T lymphocytes, also termed a lymphocyte activation test (LAT) [12–14]. LAT studies have applied analysis of the percentage activation index (AI), a ratio between the percentage of CD69-positive cells stimulated with nickel sulfate and the percentage of CD69-positive cells stimulated with culture medium only, and an AI above 2 has been considered the threshold for

positivity [13, 15]. However, the main limitation of this approach is that the difference between the percentage of CD69-positive cells stimulated with a test substance and the percentage of CD69-positive cells in non-stimulated blood is often small and less than 2-fold. This percentage-based AI also depends on the percentage ratio of other lymphocyte populations and frequently results in a grey zone interpretation (AI just beyond 2) without a clear distinction between positive and negative patients. These limitations could be overcome with a more precise and specific measurement of the absolute number (cells per μ l) of antigen-specific CD69-positive T cells following *in vitro* whole blood antigen stimulation.

The aim of our study was to develop a novel, whole blood-based, quantitative, absolute count activation index (AI) analysis of CD69 upregulation on different subsets of T cells in nickel hypersensitive patients and compare it with the previously reported method in which CD69 percentage AI and cytokine production are measured [16–20].

2. METHODS

Study population and blood collection

This study included 10 patients with a clear clinical history of contact allergy to nickel. The inclusion criterion was persuasive clinical anamnesis of nickel hypersensitivity. Eight out of 10 patients were confirmed with positive nickel patch test reactivity. Seven out of eight patients had +++ positive and one out of eight had ++ positive nickel sulfate patch result [21]. Nine healthy controls without previous evidence of nickel allergy were also included. Before recruiting patients/healthy controls, nine controls were tested for quantitative, absolute count analysis of CD69 upregulation after phytohemagglutinin (PHA) stimulation, which served as positive and methodological control of the novel test approach. Whole blood samples were

collected in heparinized blood tubes (BD Vacutainer, NJ, USA) and were further processed within 1 hour.

Measurements of CD69 activation

Then, 225 μ l of heparinized whole blood was incubated with 25 μ l of nickel sulfate hexahydrate ($\text{NiSO}_4 \cdot 6\text{H}_2\text{O}$) (Sigma Aldrich, Germany) at a final concentration of 0.5 or 5 $\mu\text{g/ml}$ or 25 μ l PBS as a negative stimulation control. For positive control, the same amount of heparinized blood was stimulated with PHA (Sigma Aldrich) in the final concentration of 20 $\mu\text{g/ml}$. All samples were incubated for 48 hours at 37°C and 5% CO_2 . Afterwards, CD69 expression of CD3+, CD3+CD4+ and CD3+CD8+ T cells was determined with flow cytometry from 50 μ l of stimulated/nonstimulated blood using anti-human CD4-FITC, CD3-PerCP, CD8-APC and CD69-PE (all BD Biosciences, NJ, USA). The absolute cell count was determined with SPHERO™ AccuCount Fluorescent Particles (Spherotech, IL, USA). Whole blood probes were lysed, washed, fixed and analysed within 2 hours on a FACSCalibur flow cytometer (BD Biosciences). Moreover, 15000 events were acquired in forward and side scatter lymphocyte gates in each tube analysed. **Figure 1** shows representative flow data and gating strategy in the absolute cell count assay. Percentage AI (Pct AI) was calculated as the ratio between the percentage of CD69-positive CD3+, CD3+CD4+ or CD3+CD8+ T cells stimulated with nickel sulfate and the percentage of the corresponding CD69-cells of non-stimulated blood (PBS alone). Absolute AI (Abs AI) was calculated as the ratio between the absolute number of CD69-positive CD3+, CD3+CD4+ or CD3+CD8+ T cells stimulated with nickel sulfate and the absolute number of the corresponding CD69-cells of non-stimulated blood.

Measurements of cytokine secretion

Peripheral blood mononuclear cells (PBMNCs) were isolated using a Ficoll-Paque density gradient, washed twice with PBS and resuspended in cell culture medium consisting of RPMI-1640 medium supplemented with 10% foetal bovine serum (FBS), 2 mM L-glutamine and 1% penicillin/streptomycin (all Sigma Aldrich, Germany). PBMNCs were seeded at a concentration of 2×10^5 cells per well and incubated with nickel sulfate hexahydrate ($\text{NiSO}_4 \cdot 6\text{H}_2\text{O}$) (Sigma Aldrich) at a final concentration of 0.5 or 5 $\mu\text{g/ml}$ or cell culture medium as negative stimulation control for 48 h at 37°C and 5% CO_2 . Four cytokines, namely, IL-2, IL-5, IL-13 and IFN- γ , were quantified in the supernatants with the Cytometric Bead Array (CBA; BD Biosciences) according to the manufacturer's protocol. The results were normalized to non-stimulated (cell culture medium) cytokine secretion. Samples were analysed within 2 hours on a FACSCalibur flow cytometer (BD Biosciences).

Statistical analysis

The Mann-Whitney U test was used to compare activation indexes and cytokine responses between patients and controls. Receiver operating characteristic (ROC) curve analysis was used for the flow cytometric assay of CD69 upregulation or cytokine production to predict clinical history. We used the nickel sulfate concentration of 5 $\mu\text{g/ml}$ for statistical analysis. A P value below 0.05 was considered significant. All statistical analyses were carried out using GraphPad Prism software (version 5, San Diego, CA, USA). All reported P -values are two-tailed.

3. RESULTS

Lymphocyte activation test

CD69 Pct AI (percentage-derived) in response to 5 µg/ml nickel sulfate treatment was significantly increased in patients compared to healthy controls in CD3+ cells (median 2.62 vs. 0.96; $P=0.002$), CD3+CD4+ cells (median 4.17 vs. 1.00; $P=0.006$) and CD3+CD8+ cells (median 1.73 vs. 0.95; $P=0.0002$). Median Pct AI increase was 2.73-fold for CD3+ cells, 4.17-fold for CD3+CD4+ cells and 1.82-fold for CD3+CD8+ cells.

CD69 Abs AI (absolute count-derived) in response to 5 µg/ml nickel sulfate treatment was significantly increased in patients compared to healthy controls in CD3+ cells (median 6.93 vs. 0.92; $P=0.0004$), CD3+CD4+ cells (median 14.59 vs. 0.93; $P<0.0001$) and CD3+CD8+ cells (median 3.14 vs. 1.03; $P<0.0001$). Median Abs AI increase was 7.53-fold for CD3+ cells, 13.66-fold for CD3+CD4+ cells and 3.04-fold for CD3+CD8+ cells. The exact flow cytometry Pct and Abs data are presented in **Table 1**.

Given a previously accepted threshold of AI>2 for positivity [15], 7/10 (70%) patients were CD3CD69-positive, 8/10 (80%) were CD4CD69-positive and 3/10 (30%) were CD8CD69-positive when Pct AI was applied. In comparison, 8/10 (80%) patients were CD3CD69-positive, 10/10 (100%) were CD4CD69-positive and 8/10 (80%) were CD8CD69-positive when Abs AI was applied (**Figure 2**).

ROC analysis demonstrated an AUC of 0.91 for CD3+ CD69 Pct AI and an AUC of 0.94 for CD3+ CD69 Abs AI. We found an AUC of 0.87 for CD3+CD4+ CD69 Pct AI, an AUC of

0.98 for CD3+CD4+ CD69 Abs AI, an AUC of 0.97 for CD3+CD8+ CD69 Pct AI and an AUC of 0.9 for CD3+CD8+ CD69 Abs AI (**Figure 3**).

The best diagnostic cutoff values were obtained for CD3+CD4+CD69+ Abs AI at 2.24 with 100% sensitivity and 89% specificity and for CD3+CD8+CD69+ Abs AI at 1.76 with 90% sensitivity and 100% specificity (**Table 2**).

In addition, all nine controls demonstrated strong CD69 positivity in all subsets of T cells after PHA stimulation of whole blood for 48h. Median CD69 Abs AI in response to 20 µg/ml PHA treatment was 2650.0 (range 236.3-9441.0) in CD3+ cells, 10031.0 (204.6-31629.0) in CD3+CD4+ cells and 1126.0 (47.3-2037.0) in CD3+CD8+ cells. Median CD69 Pct AI was 52.3 (17.4-95.7) in CD3+ cells, 108.4 (14.7-182.7) in CD3+CD4+ cells and 33.3 (7.7-46.8) in CD3+CD8+ cells, respectively (**Supplemental Figure 1**).

Cytokine secretion measurements

Four different cytokines were measured: IL-2 and IFN- γ , which are specific for the Th1 immune response, and IL-5 and IL-13, which are specific for the Th2 immune response. We found significantly increased secretion of IL-2 (median 182 vs. 3 ng/ml; $P=0.0004$), IL-5 (median 9 vs. 0 ng/ml; $P=0.0025$), and IL-13 (median 36 vs. 0.5 ng/ml; $P=0.0004$) in response to 5 µg/ml nickel sulfate in patients compared to healthy controls. Moreover, cytokine secretion in patients was concentration dependent. In contrast, no difference was found for IFN- γ (median 2 vs. 0 ng/ml; $P=0.34$) (**Figure 4**).

4. DISCUSSION

In this study, we present a novel, whole blood, quantitative, absolute count-based T cell analysis of CD69 upregulation as a new methodology for *in vitro* diagnosis of delayed-type hypersensitivity to nickel, which has better diagnostic value than previously reported analyses based on Pct AI. Importantly, our Abs AI analysis showed much larger differences between stimulated and non-stimulated samples than percentage AI analysis for all subsets of T cells (CD3+, CD3+CD4+ and CD3+CD8+). We also compared our results of CD69 activation to cytokine secretion (IL-2, IL-5, IL-13 and IFN- γ) and found increased IL-2, IL-5 and IL-13 secretion. These observations suggest that absolute counting of antigen specific, CD69-positive T cells could be a novel approach in the diagnosis of delayed-type hypersensitivity reactions.

CD69 is the earliest activation marker of T cells *in vivo* and *in vitro* and is detectable within hours of TCR ligation [22]. Our study of CD69 measurements suggests that between 0.6% and 10.0% of CD3+ T cells reacted to nickel stimulation, which is in agreement with the results of Beeler et al., who reported between 0.5% and 3% drug reacting CD3+CD4+ T cells [15]. In contrast to other studies of CD69 upregulation on T cells [12, 13, 15], our approach utilizes whole blood and therefore does not involve PBMNC isolation, which is time consuming and does not allow for exact cellular quantification and absolute counting of drug activated T-cells. Importantly, 48 hour incubation of nonstimulated heparinized whole blood resulted only in minimal activation of T cells, approximately 1% for CD3+, 0.3% for CD3+CD4+ and 1.5% for CD3+CD8+ T cell subsets.

To normalize for background activation, the CD69 results are analysed as AI, which is calculated as activation in the presence of the tested substance (stimulated) divided by

activation in the absence of the tested substance (nonstimulated). Previous CD69 T cell studies of delayed-type hypersensitivity used Pct AI values [13, 15]. The novelty of our study is the use of Abs AI values, which showed higher AUC and diagnostic utility than Pct AI for all analysed T cell subsets. The main advantage of the Abs AI approach is the much larger differences between stimulated and nonstimulated samples in hypersensitive patients, with the comparable AI values observed in control subjects. Consequently, there is greater discrimination between patients and control subjects. Accordingly, we showed a median 7.5-fold increase in CD3+ in patients compared to control subjects when Abs AI was applied and only 2.9-fold when Pct AI was applied. Similar differences were also observed for CD3+CD4+ and CD3+CD8+ T cell subsets.

An AI of 2 was previously accepted as the cutoff value to discriminate between nonreactive and reactive T cells in CD69 LAT testing [15]. According to our ROC analysis, a very similar cutoff was also demonstrated for Abs AI, indicating that a cutoff of AI>2 might also be suitable for Abs analysis. It seems that CD3+CD8+CD69+ Abs AI and CD3+CD4+CD69+ Abs AI have the highest diagnostic accuracy. This observation also suggests that both helper and cytotoxic T cells contribute to the hypersensitivity reaction to nickel. We acknowledge that a previous report failed to detect a change in CD69 upregulation on CD4+ CLA+ T cells of nickel hypersensitive patients [14]. However, that study used a less sensitive method to detect CD69 positive, antigen specific T cells than the approach employed in our study.

We also demonstrated increased cytokine secretion of IL-2, IL-5 and IL-13 in nickel hypersensitivity patients. This result is comparable with the result of Minang et al., who also showed IL-13 secretion, and suggests that nickel-induced PBMNC cytokine production could be used as a marker of nickel hypersensitivity [23]. These observations indicate that both Th1 and Th2 cytokines may contribute to nickel delayed-type hypersensitivity reactions. In

contrast to our data and those of Minang et al. [23], some previous studies suggested that nickel-induced allergic contact dermatitis is mainly mediated by Th1-type cytokines [24, 25], whereas Th2-type cytokines or IL-10 could have downregulatory effects [4, 26].

There are some important weaknesses of our study, which should be considered when interpreting the results. The major limitation applies to the small number of persons/samples involved in this study. Therefore, larger studies to confirm the methodological and clinical utility of this approach are needed. In addition, the specificity and sensitivity of this novel method should be also elaborated in patients with equivocal patch test results. Another thing which could influence baseline counts of CD69 activation is time since last nickel exposure: this was a prospective study and all patients had recent history (median 1 year) of nickel allergy and were tested for CD69 activation shortly (median 6 months) after initial clinical diagnosis and patch testing. Accordingly, baseline counts of CD69 were comparable between patients.

In conclusion, our results point to the potential of quantitative, microbead-based T cell analysis of CD69 upregulation to be an accurate new approach to identify antigen-reactive T cells in the peripheral blood of patients with nickel hypersensitivity reactions. Abs AI seems to be more discriminative than previously reported Pct AI and complements analysis of cytokine secretion. These findings suggest the sufficient diagnostic utility and technical reproducibility of CD69 Abs AI analysis to warrant further validation and replication studies, especially in clinically complex patients with hypersensitivity reactions to drugs.

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Table 1. Flow cytometry Pct and Abs data for CD69 measurements after nickel sulfate stimulation (0.5, 5 µg/ml) or no stimulation (PBS) of whole blood for 48 hours.

	PBS (median, IQ range)	0.5 µg/ml nickel sulfate (median, IQ range)	5 µg/ml nickel sulfate (median, IQ range)
<i>Percentage of CD69-positive cells</i>			
CD3+	0.975 (0.600-1.418)	1.020 (0.698-1.590)	2.695 (1.193-5.640)
CD3+CD4+	0.350 (0.258-0.448)	0.435 (0.205-1.043)	1.850 (0.673-5.135)
CD3+CD8+	1.645 (1.298-2.218)	1.805 (1.370-2.203)	2.855 (2.433-5.948)
<i>Absolute count (cells/µl) of CD69-positive cells</i>			
CD3+	0.141 (0.044-0.252)	0.112 (0.079-0.308)	1.322 (0.050-5.013)
CD3+CD4+	0.018 (0.008-0.076)	0.044 (0.009-0.131)	0.488 (0.171-3.080)
CD3+CD8+	0.158 (0.044-0.317)	0.141 (0.072-0.273)	1.200 (0.232-4.663)

Table 2. Optimal cutoffs for the parameters of CD69 upregulation after nickel sulfate stimulation (5 µg/ml) of whole blood for 48 h.

Parameters	AUC ROC	Cutoff	Sensitivity (%)	Specificity (%)
CD3+CD69+ Abs AI	0.94	1.33	90.00 (55.50-99.75)	88.89 (51.75-99.72)
CD3+CD4+CD69+ Abs AI	0.98	2.24	100.00 (69.15-100.0)	88.89 (51.75-99.72)
CD3+CD8+CD69+ Abs AI	0.99	1.76	90.00 (55.50-99.75)	100.00 (66.37-100.0)

Figure 1. Representative flow cytometric analysis in absolute count-based and percentage-based T cell analysis of CD69 upregulation in CD3+CD8+ cells for one patient after 48h of stimulation of whole blood with (A) PBS or (B) 5 μ g/ml nickel sulfate. The absolute cell count was determined with SPHERO™ AccuCount Fluorescent Particles, which were added to antibody-labelled blood samples prior to flow cytometric analysis.

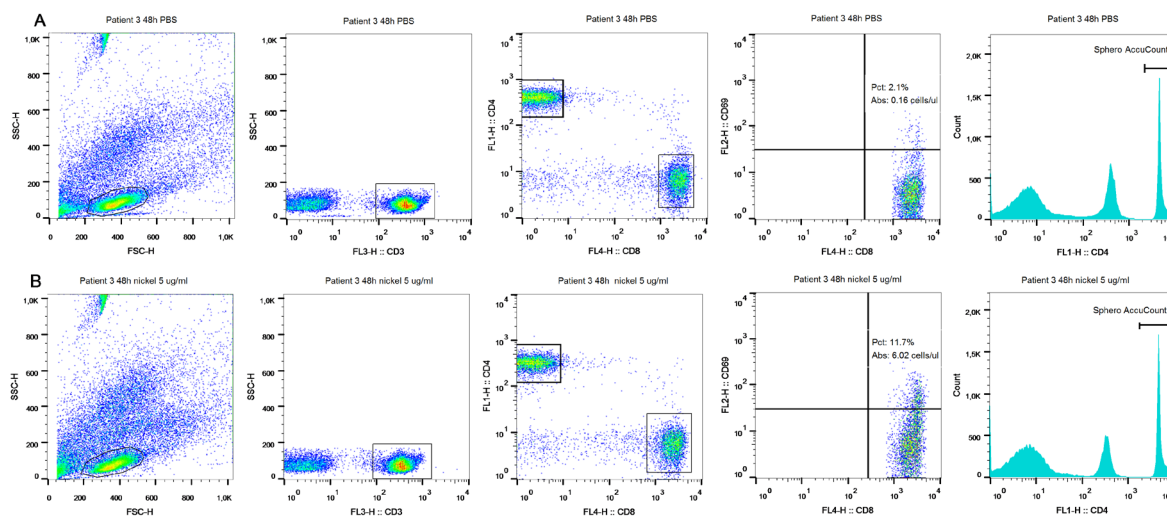


Figure 2. CD69 activation in CD3+, CD3+CD4+ and CD3+CD8+ T cells after nickel sulfate stimulation (0.5 and 5 $\mu\text{g/ml}$) of whole blood for 48 hours. Activation index is expressed as either a percentage (red) or absolute count (blue) value. AI: activation index; Pct: percentage; Abs: absolute.

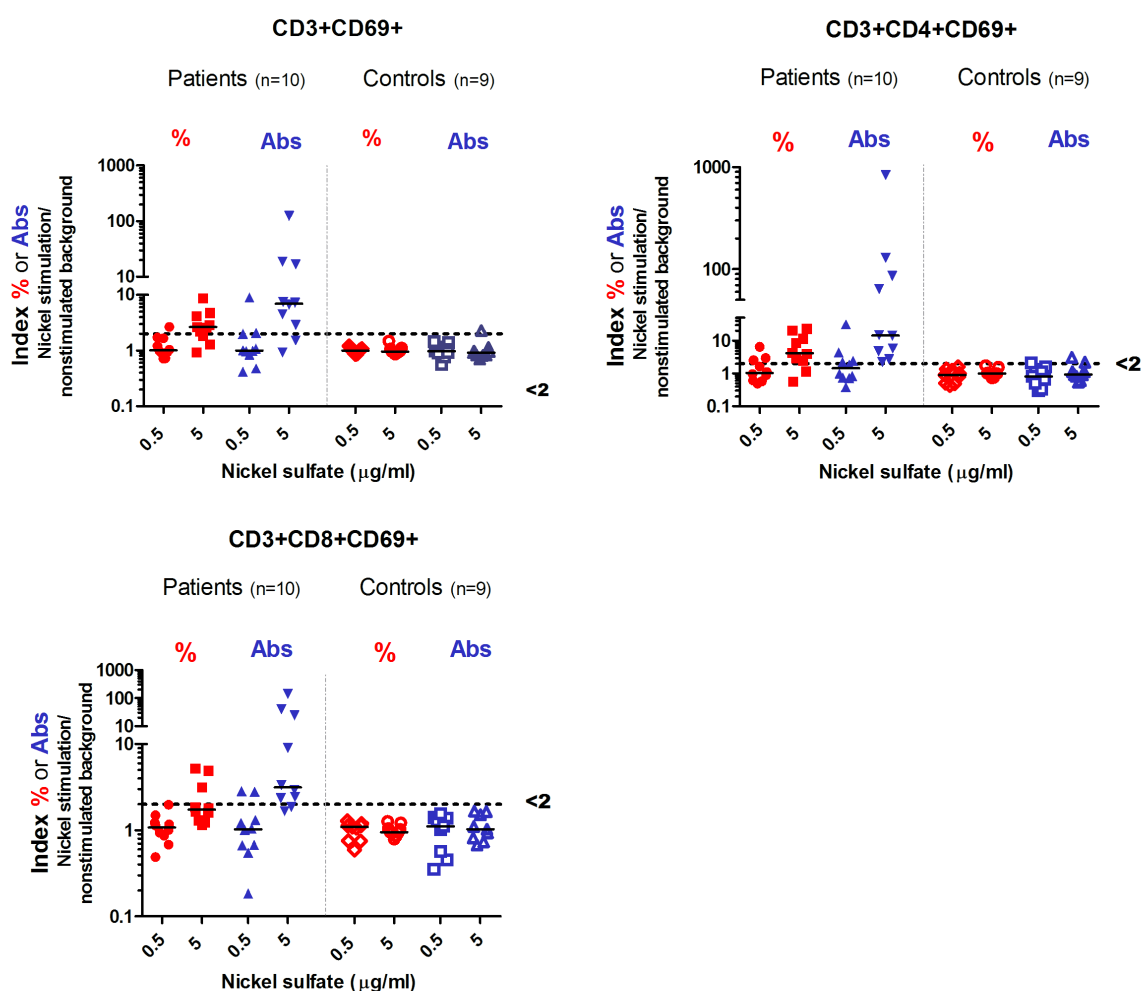


Figure 3. ROC analysis of CD69 activation index of CD3+, CD3+CD4+ and CD3+CD8+ T cells after 5 µg/ml nickel sulfate stimulation for 48 hours. Activation index is expressed either as **A**, percentage (red) or **B**, absolute (blue) count values. AUC: area under the ROC curve; AI: activation index; Pct: percentage; Abs: absolute.

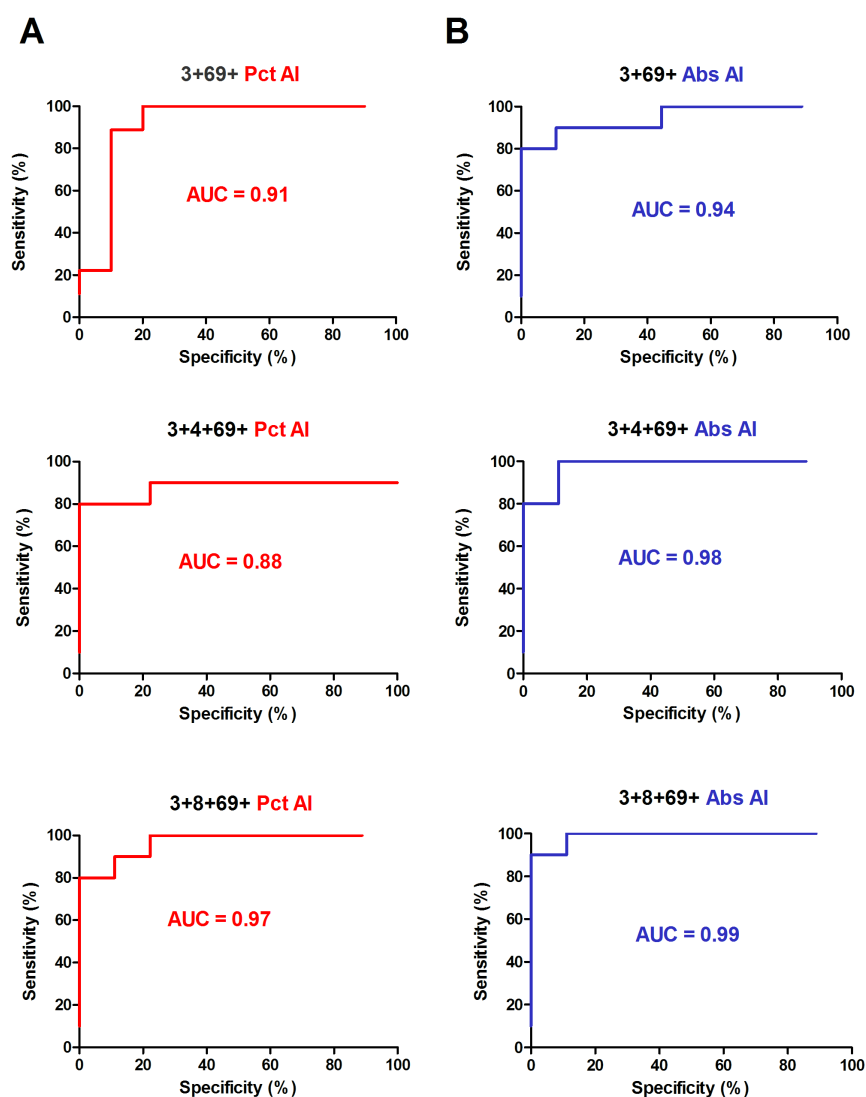
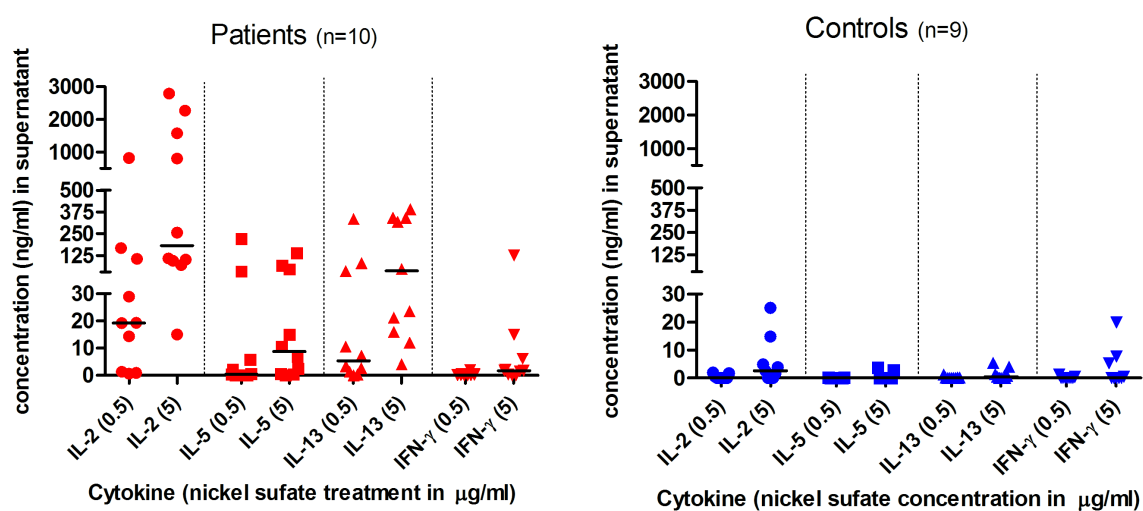


Figure 4. IL-2, IL-5, IL-13 and INF- γ cytokine expression after nickel sulfate stimulation (0.5 and 5 $\mu\text{g/ml}$) of PBMNCs for 48 hours.



Supplemental figure 1: CD69 activation in CD3+, CD3+CD4+ and CD3+CD8+ T cells after PHA stimulation (20 μ g/ml) of whole blood for 48 hours in 9 healthy donors. Activation index is expressed as either a percentage (red) or absolute count (blue) value. AI: activation index; Pct: percentage; Abs: absolute.

