

ORIGINAL ARTICLE

Cytokine patterns *in vitro*, in particular IL-5/IL-8 ratio, to detect patients with nickel contact allergy

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Abstract

Background Patch test (PT) is the gold standard to reveal nickel (Ni) allergy. *In vitro* tests are under discussion. We aimed to establish a cytokine based *in vitro* assay to detect Ni sensitization.

Methods From 15 patients with positive (13f, 42–78 years) and 15 with negative PT to Ni (controls, 3f, 31–82 years) within a consecutive patient series, peripheral blood mononuclear cells (PBMC) were obtained. Six-day stimulation with three concentrations of NiSO₄ was performed. Proliferative response (uptake of tritiated thymidine) and production of the cytokines IL-1 β , IL-4, IL-5, IL-6, IL-8, IL-10, IL-17A, TNF- α , IFN- γ (cytometric bead array) were assessed.

Results Twelve of fifteen Ni PT-positive patients also had positive LTT, and all control patients were PT and LTT negative. The mean SI differed between Ni allergics and controls ($P < 0.01$). Upon Ni stimulation, PBMC of the allergic patients showed (i) enhanced IL-5 response ($P < 0.0001$) and (ii) reduced IL-8 production ($P < 0.01$). The IL-5/IL-8-ratio best distinguished allergics from non-allergics in all three Ni concentrations with a sensitivity and specificity of 93%.

Conclusion Assessment of the ratio of Ni-induced IL-5 and reduced IL-8 production *in vitro* is a helpful tool for a better characterization of Ni-allergic patients.

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Conflicts of interest

None declared.

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Introduction

In the general population, nickel (Ni) is the most prevalent contact allergen with a frequency of approximately 13% and with a predominance of female patients.^{1,2} The ‘gold’ standard method to confirm the clinical suspicion of Ni allergy is the patch test (PT). As its performance takes several days and might not always give 100% reproducible results,^{3–5} there is ongoing search for a suitable *in vitro* test. As allergic contact dermatitis is mediated by T lymphocytes, the lymphocyte transformation test (LTT) has been under development to establish a reliable method for verifying a diagnosis of Ni allergy. The LTT – under given culture conditions – can be a helpful tool for the diagnosis of drug hypersensitivity^{6,7} and has already been used for evaluation of Ni reactivity *in vitro*.^{8,9} However, the Ni stimulation of peripheral blood mononuclear cells (PBMC) may result in sometimes unspecific proliferative response as reflected in various publications by overlapping LTT reactivity in non-allergic and allergic individuals. Such nonspecific mitogenic effect might be explained by the binding of Ni to TLR4, as reported by Schmidt

*et al.*¹⁰ in 2010. In addition, for years the LTT has not yet been sufficiently optimized or validated as potential diagnostic tool for Ni allergy. We have recently shown in accordance with Pacheco *et al.*^{11,12} that optimized culture conditions can significantly increase the specificity of Ni LTT. To enhance the LTT sensitivity, many laboratories use the measurement of ³H-thymidine uptake in dividing, e.g. proliferating cells. With varying success, alternative methods for the detection of *in vitro* proliferative response to Ni have been described. Examples are the controversial ‘MELISA’¹³ using increased (monocyte reduced) PBMC numbers per well or the flow cytometric evaluation by CFSE dilution assay assessing the division depending ‘weakening’ of carboxyfluorescein succinimidyl ester (CFSE)-stained cells.^{14,15} Simultaneous assessment of released cytokines by ELISA or its ELISPOT variant might add to distinguish between allergic and non-allergic individuals.¹⁶ Accordingly, cytokine patterns such as IFN- γ , IL-2, IL-12 (TH1-related), IL-4, IL-5, IL-13 (TH2-related) and TH17-associated IL-17 have been investigated.^{17–22} A modification of this approach was the addition of ‘cytokine cocktails skewing lymphocytes towards type 1 or 2 phenotype’ while performing Ni stimulation *in vitro*.

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Accordingly, Spiewak *et al.*²¹ stated that the presence of type 2 skewing cytokines resulted in best correlation with contact allergy to Ni when using readout by IL-2 and IL-13 ELISPOT. In 2013, Martins and Reis reported on *in vitro* IL-13 production as potential marker to distinguish chromate-stimulated *in vitro* response of PBMC derived from allergic/non-allergic individuals.²³ In 2017, on the other hand, Akerman *et al.*²⁴ stated on their experiments with PBMC cultured with/without potassium dichromate that 'PBMCs from patients with ACD to chromium secreted significantly higher amounts of all cytokines tested, except IL-4'. Czarnobilska *et al.*²⁵ reported in 2009 that in their experiments regarding contact allergy to Ni, 'patch test score correlates with IL-5, but not with IFN-Gamma'. We felt that there was still an open question: The characterization of a particular pattern of cytokine response allows (i) to determine both unspecific and (ii) specific, e.g., allergy-linked *in vitro* reactions to nickel. For this purpose, we analysed the *in vitro* response of PBMC of Ni-allergic patients and controls after stimulation with Ni and evaluated the sensitivity and specificity of different potential marker cytokines.

Patients and methods

Patients

The study included 30 individuals within a consecutive patient series of our clinic who underwent allergological workup with at least the standard PT series and who either showed a positive or negative PT to Ni. All patients gave their written consent to blood donation for this investigation.²⁶ The here selected 15 patients with positive PT to Ni (13f, 42–78 years) and 15 individuals without PT reactivity to Ni ('controls', 3f, 31–82 years) were non-smokers, had no metallic orthopaedic-surgical implant and did not underwent immunosuppressive therapy. All patients were additionally asked for the presence of atopic diseases (e.g. allergic rhinoconjunctivitis, allergic asthma or atopic dermatitis). Eleven of the PT-positive patients and two of the control persons had at least one atopic disease. This investigation was approved by the local ethics committee. Characteristics of the 30 subjects are given in Table 1.

Table 1 Characteristics of the 30 patients

	Control patients	Patients with positive* Ni PT
Age	31–82 years, mean 61.47 years	42–78 years, mean 63.8 years
Sex		
Male	12	2
Female	3	13
History of suspected Ni allergy**	All negative	14/15 positive
Atopic disease***	2/15	10/15

Controls, Ni PT-negative patients; *, eight with '+' reaction, six with '++' reaction and one with '+++ reaction'; **, history of itching, erythema, eczema upon contact with jeans button, wrist watch, jewellery; ***, allergic rhinoconjunctivitis, allergic asthma and/or atopic eczema.

Patch testing

In every patch tested patient, at least the baseline standard series containing 29 allergens including Ni was placed on D0 on the patient's back for 48 h. Additional test series were used depending on the patients' personal history, e.g. suspected potential allergen exposure. On D2 and D3, reading was carried out according to the guidelines of the German Contact Dermatitis Research Group (DKG).²⁶ The cutaneous reactions were documented as negative = no reaction or positive graded as +, ++ and +++.

Lymphocyte transformation test

The LTT was performed according to Summer *et al.*^{17,27} with the optimizations reported by Ständer *et al.*¹² From all the 30 individuals, peripheral venous blood (60 mL) was obtained on the start date of PT to avoid booster by PT. In short, peripheral blood mononuclear cells (PBMC) were isolated from heparinized venous blood by density centrifugation. After isolation, PBMC were washed twice in 'Hanks balanced salt solution' (HBSS; Sigma, Darmstadt, Germany) and resuspended in RPMI1640 medium (1×10^6 cells/mL) completed with 10% autologous serum, non-essential amino acids and antibiotic/antimycotic solution (all reagents by Sigma). The cells were cultivated with the respective allergen and control substances for 6 days in quadruplicate. The following substances were used: T-cell mitogen phytohaemagglutinine (PHA; Biochrom, Berlin, Germany) 2.4 µg/mL, tetanus toxoid as control recall antigen (TT; Chiron Behring, Berlin, Germany) 5 µg/mL. The following Ni concentrations were used: 7.5×10^{-6} mol/L NiSO₄, 1×10^{-5} mol/L NiSO₄ and 2.5×10^{-5} mol/L NiSO₄. In addition, the 'irrelevant' metal antigen CoCl₂ was used as a second control (concentrations 5×10^{-5} mol/L, 2.5×10^{-5} mol/L, 1×10^{-5} mol/L). Further control were cells without stimuli, i.e. in medium alone. On day 5, ³H thymidine was added to the cells and proliferation was assessed by overnight incorporated radioactivity. The results of the proliferation test were given as stimulation index (SI), calculated by the ratio of incorporated radioactivity of stimulated vs. unstimulated cultures. SI ≥ 3 was regarded as positive result. In parallel with the 'radioactive' cultures, cell suspensions with the same cell and stimulus concentration were set up except for two modifications: first, to address the maximum (general unspecific) stimulatory response instead of PHA, a mixture of phorbol-myristate-acetate (PMA 15 ng/mL; Sigma-Aldrich, Munich, Germany) and ionomycin (1.5 µg/mL; Sigma-Aldrich) was used; and second, no ³H thymidine was added. All cultures were performed in quadruplicate.

Cytokine detection by cytometric beads assay

The accumulated cytokine production was determined by collecting supernatants at day 6. Analysis of samples from the control stimulation by TT and by the mixture of PMA and ionomycin should give information of 'predisposition' to

altered/dissimilar cytokine production in the two groups. Supernatants of the non-radioactive cultures were analysed by flow cytometry in a FACS Canto (BD Biosciences, Heidelberg, Germany) using a cytometric bead array (BD Biosciences, Heidelberg, Germany) for the presence of IL-1 β , IL-4, IL-5, IL-6, IL-8, IL-10, IL-17A, TNF- α and INF- γ . The respective detection limit was 0.5 pg/mL.

Statistical methods

Differences in the LTT reactivity (i.e. SI values) and in the cytokine production were assessed using the Mann–Whitney U-test. The here presented cytokine data were calculated as differences between the unstimulated and the Ni-exposed PBMC cultures. To evaluate the concordance of the differences in cytokine production with the Ni allergy status, the sensitivity and specificity of the LTT reactivity and cytokine reactions were calculated. In addition, the differences in the ratio between two different cytokines were assessed by the Student's *t*-test. The statistical software package SPSS for Windows (SPSS 23, IBM, Ehningen, Germany) was used for the calculations and evaluation.

Results

General patient characteristics and additional patch test reactivity

Eleven of fifteen patients with Ni allergy and 2/15 control had at least one atopic disease. Atopic diseases in the 15 patients with Ni allergy were allergic rhinoconjunctivitis in seven, allergic asthma in five and atopic eczema in four patients. History of cutaneous 'metal intolerance with suspicion of Ni allergy' was reported by 14 of the 15 PT-positive patients. In five of fifteen Ni PT-positive and in nine of fifteen Ni-negative patients, no other PT reaction was found (Table 2).

Within the Ni-allergic, i.e., Ni PT-positive patients, there were eight with '+', six with '++' and one with '+++ reaction.

In vitro lymphocyte proliferation

Twelve of the 15 Ni PT-positive patients also had positive LTT, i.e. SI ≥ 3 to at least one of the three stimulation concentrations. The three Ni allergics with negative LTT had a weak, i.e. '+' reaction in the PT to Ni. All of the 15 Ni PT-negative patients also had negative LTT (SI < 3). Accordingly, stimulation with NiSO₄, at 7.5×10^{-6} mol/L, 1×10^{-5} and 2.5×10^{-5} mol/L correctly gave significantly higher proliferative response (e.g. SI ≥ 3) in 80% of the consecutive Ni PT-positive patients. Compared with non-allergic subjects, the mean values were as follows: Ni 2.5×10^{-5} mol/L: 7.66 ± 1.82 vs. 1.72 ± 0.17 ; Ni 1×10^{-5} mol/L: 5.15 ± 1.41 vs. 1.25 ± 0.14 ; Ni 7.5×10^{-6} mol/L: 4.29 ± 1.24 vs. 1.03 ± 0.0 (Fig. 1). Interestingly, only the non-atopic blood donors with positive PT to Ni had a significantly increased proliferation rate in comparison with the non-atopic PT negative blood donors. The LTT test

Table 2 Positive patch test reactions and atopic disease in the single patients of the study (AR, allergic rhinitis; AA, allergic asthma; AE, atopic eczema)

	Atopy	PT positive to
Control 1	No	
Control 2	AR	
Control 3	No	
Control 4	No	
Control 5	No	Epoxy resin
Control 6	No	
Control 7	No	Thiuram Mix, Zinc
Control 8	No	Dibromodicyanobutane
Control 9	No	
Control 10	No	
Control 11	No	
Control 12	No	
Control 13	No	
Control 14	No	
Control 15	AR	
Ni allergic 1	No	Nickel
Ni allergic 2	AR, AA	Cobalt, Nickel
Ni allergic 3	AE	Balsam of Peru, Nickel, Bufexamac
Ni allergic 4	AR, AA, AE	Nickel
Ni allergic 5	No	Nickel
Ni allergic 6	No	Nickel
Ni allergic 7	No	Nickel
Ni allergic 8	AR, AA	Nickel
Ni allergic 9	AR, AA	Nickel
Ni allergic 10	AE	Nickel
Ni allergic 11	No	Nickel
Ni allergic 12	AR	Nickel
Ni allergic 13	AR	Cobalt, Balsam of Peru, Nickel
Ni allergic 14	AR, AE	Colophonium, Nickel
Ni allergic 15	AA	Nickel

demonstrated a perfect specificity with 100%; i.e., there were no false positive detected, and on the other hand, the sensitivity was low with 47–73% (Table 4).

Cytokine release

Differences in cytokine production were found for four of the nine cytokines tested (IL-5, IL-8, TNF- α , INF- γ). As differences were most distinctly seen for IL-5 and IL-8, we focus on these results. Upon Ni stimulation, IL-8 production was lower in Ni allergics although the difference was not significant. The highest correlation to the proliferation response in LTT ($P < 0.0001$) was observed in Ni-induced IL-5 (Table 3). As shown in Fig. 2, Ni allergy-specific release of IL-5 as compared to the controls was the highest upon stimulation with NiSO₄, at 2.5×10^{-5} mol/L. A significant production of IL-5 upon Ni stimulation was found in atopic and non-atopic PT-positive patients. The mean levels of IL-5 (pg IL5/mL) were as follows:

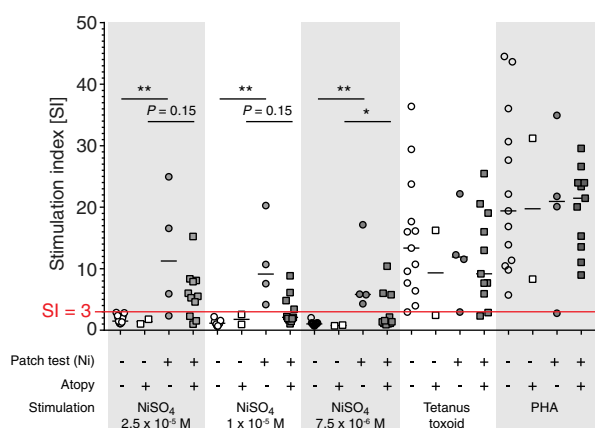


Figure 1 Proliferative response of PBMC of 15 controls (two atopic and 13 non-atopic, PT to Ni negative) and 15 Ni-allergic patients (11 atopic, four non-atopic) to the three Ni concentrations and tetanus toxoid (TT); * $P < 0.05$, ** $P < 0.01$; single values and median are given.

Table 3 Calculated sensitivity and specificity for lymphocyte transformation test (LTT), IL-5, IL-8 and the ratio of IL5/IL8

		Sensitivity (%)	Specificity (%)
LTT	Ni 2.5×10^{-5} mol/L	73	100
	Ni 1×10^{-5} mol/L	60	100
	Ni 7.5×10^{-6} mol/L	47	100
IL-5	Ni 2.5×10^{-5} mol/L	93	87
	Ni 1×10^{-5} mol/L	87	87
	Ni 7.5×10^{-6} mol/L	67	93
IL-8	Ni 2.5×10^{-5} mol/L	73	60
	Ni 1×10^{-5} mol/L	87	67
	Ni 7.5×10^{-6} mol/L	87	80
Ratio IL-5/IL-8	Ni 2.5×10^{-5} mol/L	93	93
	Ni 1×10^{-5} mol/L	87	93
	Ni 7.5×10^{-6} mol/L	73	93

249.36 ± 92.99 vs. 1.70 ± 0.61 (Ni 2.5×10^{-5} mol/L); 154.77 ± 64.19 vs. 0.33 ± 0.23 (Ni 1×10^{-5} mol/L); 68.83 ± 32.44 vs. 0.17 ± 0.17 (Ni 7.5×10^{-6} mol/L). A baseline, spontaneous IL-5 production was found only in two blood donors in the 'medium control'. Moreover, the Ni-induced cytokine production also showed inverse correlation with allergy status in the case of IL-8 (Fig. 3). Its production was significantly higher in the non-allergic individuals: 3911 ± 943.4 vs. 8119 ± 1969 (Ni 2.5×10^{-5} mol/L); 2388 ± 661.8 vs. 9851 ± 3559 (Ni 1×10^{-5} mol/L); 2367 ± 693.5 vs. 8239 ± 1815 (Ni 7.5×10^{-6} mol/L). When assessing the relation between proliferative response (SI) in general with cytokine release upon Ni stimulation, best positive correlation was with IL-5 and negative correlation with IL-8. An overview is given in Table 2.

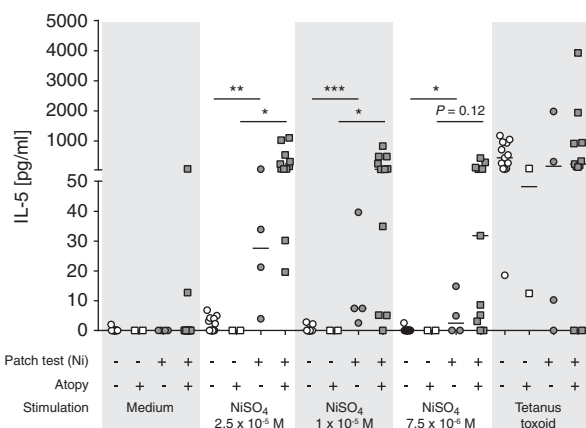


Figure 2 IL-5 response of PBMC of 15 controls (two atopic and 13 non-atopic, PT to Ni negative) and 15 Ni-allergic patients (11 atopic, four non-atopic) to the three Ni concentrations, tetanus toxoid (TT) and medium alone; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$; single values and median are given.

Sensitivity of IL-5 production and IL-8 inhibition was better as the LTT reactivity (67–93% for IL-5, 73–87% for IL-8), and on the other hand, the specificity for these marker cytokines was below the LTT (Table 4).

IL-8/IL-5 ratio to distinguish between allergic and non-allergic individuals

We observed that PT-positive individuals responded to Ni most prominently with IL-5 production but also with an inhibition of IL-8 production. On the other hand, there was high IL-8 release by PBMC of both PT- and LTT-negative individuals. Thus, we tested the hypothesis that (i) IL-5 production in response to Ni correlated positively with the extent of allergy status while at the same time was negatively correlated with the corresponding IL-8 levels and (ii) this could be a special discrimination mark. In fact, when calculating the respective ratio, a clear-cut distinction between both patient groups was possible at all three nickel concentrations ($P < 0.0001$). Stimulation at Ni 2.5×10^{-5} mol/L was the best 'differentiator' (Fig. 4). This ratio also showed the best compromise for sensitivity and specificity (93% sensitivity and 93% specificity (Table 3).

Discussion

The established gold standard of confirming the clinical suspicion of Ni allergy is the PT. In our study, 14 of the 15 patients with positive PT gave a self reported history of suspected Ni allergy. Such high specificity is in accordance with previous publications.^{4,5,28} In contrast, a substantial part of subjects with 'putative history of Ni allergy' may not show positive PT reactions to Ni^{29,30} being true- or false-negative cases. Thus, several authors point to a potential supportive role of LTT as diagnostic tool.^{8,9,11,12,17,18,27} Our LTT proliferation data are in line with

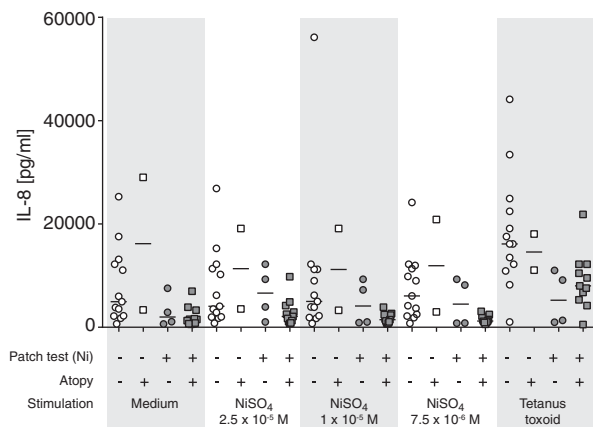


Figure 3 IL-8 inhibition of PBMC of 15 controls (two atopic and 13 non-atopic, PT to Ni negative) and 15 Ni-allergic patients (11 atopic, four non-atopic) to the three Ni concentrations, tetanus toxoid (TT) and medium alone; single values and median are given.

Table 4 Correlation between stimulation index (SI) and cytokine production (correlation to Spearman test)

	Correlation SI/IL-8 (Spearman)	Correlation SI/IL-5 (Spearman)
NiSO ₄ 2.5 × 10 ⁻⁵ mol/L	-0.286	0.640**
NiSO ₄ 1 × 10 ⁻⁵ mol/L	-0.409*	0.709**
NiSO ₄ 7.5 × 10 ⁻⁶ mol/L	-0.311	0.691**
Tetanus toxoid	0.043	0.061

*Correlation is significant on the 0.05 level.

**Correlation is significant on the 0.01 level.

these observations although the sensitivity was quite low. Furthermore, an established opinion regarding cellular mechanisms in Ni allergy was for long time that they are characterized by Th1-cytokines like IFN- γ .^{31,32} In analogy to Minang *et al.*,^{19,22} the role of concomitant TH2-(IL-4, IL-5) cytokine production is now being increasingly discussed. Finally, a nonspecific mitogenic effect by the binding of Ni to TLR4, as reported by Schmidt *et al.*¹⁰ in 2010, might be an argument against LTT. Thus, we aimed at the refinement of *in vitro* LTT – apart from assessing proliferative response – as an alternative for the detection of Ni-allergic individuals. Based on PT- and LTT-negative patients, we could show that the enhanced release of IL-5 and the inversely related, i.e., diminished release of IL-8 in response to Ni were characteristic for the Ni-allergic individuals. In fact, detection of IL-5 release *in vitro* has been described both in individuals with suspected drug allergy or Ni allergy,^{20,25,33} is rather ascribed to being produced by T cells within the PBMC and seemed to be more closely related to Ni allergy than IFN- γ production.^{22,25} A substantial part of our 15 Ni-allergic individuals

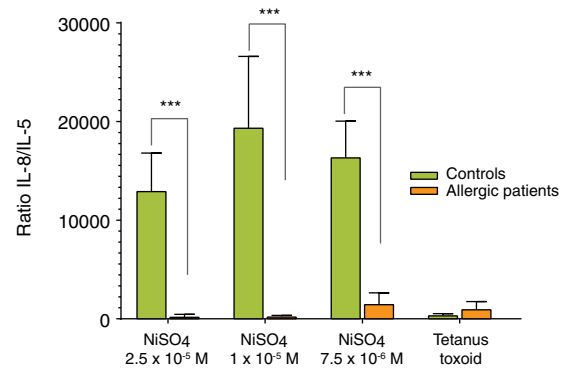


Figure 4 IL-5/IL-8 ratio of PBMC of 15 controls (C, PT to Ni negative) and 15 Ni-allergic patients (P) to the three Ni concentrations and tetanus toxoid (TT); *** $P < 0.001$.

also reported atopy. We however do not feel that this promotes IL-5 response to Ni, first, because in parallel TT-related cytokine response was dominated by IFN- γ , and second, because coexistence of atopy and allergic contact dermatitis has been described, however with no strict relationship.³⁴ Buchvald *et al.*³⁵ found an impaired response of PBMC to nickel in patients with nickel-allergic contact dermatitis and concomitant atopic dermatitis. In comparison with his results, we found also an elevated IL-5 production in PT-positive patients with atopic disease. In contrast to Rustemeyer *et al.* and Spiewak *et al.*,^{8,21} we have not studied PBMC response to Ni under the influence of added cytokines, e.g. IL-7/IL-4 to favour Th2-type response. In addition, we tried to avoid parallel usage of different cytokine measurement techniques like ELISA and ELISPOT, but rather used the simultaneous technique of cytometric bead assay. Thus using freshly isolated, Ni-exposed PBMC in our culture system, we intended to also detect potential release of ‘unspecific, innate’ cytokine pattern by PBMC of non-allergic individuals. We did not focus on the release of immunoregulatory cytokines such as TGF- β or IL-10^{8,36} that might reveal patients tolerized to Ni, i.e. without PT reactivity. However, to not overlook such phenomenon we have included the assessment of IL-10 production, but did not observe significant production in our control patients. We analysed not only T-cell cytokines as also the other cells in a PBMC cell culture participate in the immunologic answer. Among the unspecific, ‘innate’ cytokine pattern we found that individuals with negative patch test and negative LTT proliferative response produced markedly higher amounts of IL-8 as ‘inflammatory’ response to Ni. The potential underlying mechanism of such nickel-induced IL-8 production could be that described by Lin and coworkers in 2016. They postulated an ‘IL-8 gene expression via the L-type Ca(2+) channel, Toll-like receptor-4 (TLR-4) and nuclear factor NF- κ B signal transduction pathways’.³⁷ This would be in accordance with results published by Tsou *et al.*³⁸

Thus, IL-8 production seems to be among the consequences of 'innate immune signal' by nickel or cobalt.^{10,39} The role of IL-9 in allergic skin inflammation is increasingly discussed.⁴⁰ Accordingly, *in vitro* stimulation of PBMC with Ni is reported to induce IL-9 production in Ni allergics – and elevated IL-9 values were detected in blister fluids of respective PT reactions.^{41,42} Its proinflammatory but also potential regulatory role is currently investigated. In our experiments, we did not address this topic.

The inverse relation between IL-8 and IL-5 offers a new approach to distinguish between allergic and non-allergic individuals. Our results would give the rationale for a consecutive, blinded confirmatory study. Release of multifunctional IL-8 has been described in response to multiple agents. However, the present results indicate that nickel-allergic individuals can be best characterized, according to sensitivity and specificity by bringing together the substantial IL-5 production and diminished IL-8 release. Best discriminatory stimulation condition was provided by NiSO₄ at 2.5×10^{-5} mol/L. In line with this, as compared to Ni-allergic patients, evaluation of such cytokine profile in addition to 'standard' proliferation assay may help to identify 'true' PT-negative non-allergic individuals. In conclusion, assessing the IL-8/IL-5 ratio may offer a new additional tool to characterize Ni-allergic individuals.

References

- Schafer T, Bohler E, Ruhdorfer S *et al.* Epidemiology of contact allergy in adults. *Allergy* 2001; **56**: 1192–1196.
- Thyssen JP, Linneberg A, Menne T, Johansen JD. The epidemiology of contact allergy in the general population—prevalence and main findings. *Contact Dermatitis* 2007; **57**: 287–299.
- Gollhausen R, Przybilla B, Ring J. Reproducibility of patch tests. *J Am Acad Dermatol* 1989; **21**: 1196–1202.
- Ale SI, Maibach HI. Reproducibility of patch test results: a concurrent right-versus-left study using TRUE Test. *Contact Dermatitis* 2004; **50**: 304–312.
- Schaeffer AC, Andersen KE, Bindslev-Jensen C, Mortz CG. The reproducibility of nickel, cobalt and chromate sensitization in patients tested at least twice in the period 1992–2014 with TRUE Test(R). *Contact Dermatitis* 2016; **75**: 111–113.
- Nyfeler B, Pichler WJ. The lymphocyte transformation test for the diagnosis of drug allergy: sensitivity and specificity. *Clin Exp Allergy* 1997; **27**: 175–181.
- Pichler WJ, Tilch J. The lymphocyte transformation test in the diagnosis of drug hypersensitivity. *Allergy* 2004; **59**: 809–820.
- Rustemeyer T, von Blomberg BM, van Hoogstraten IM, Bruynzeel DP, Scheper RJ. Analysis of effector and regulatory immune reactivity to nickel. *Clin Exp Allergy* 2004; **34**: 1458–1466.
- Werfel T, Hentschel M, Renz H, Kapp A. Analysis of the phenotype and cytokine pattern of blood- and skin-derived nickel specific T cells in allergic contact dermatitis. *Int Arch Allergy Immunol* 1997; **113**: 384–386.
- Schmidt M, Raghavan B, Muller V *et al.* Crucial role for human Toll-like receptor 4 in the development of contact allergy to nickel. *Nat Immunol* 2010; **11**: 814–819.
- Pacheco K, Barker L, Maier L, Erb S, Sills M, Knight V. Development of a validated blood test for nickel sensitization. *J Allergy Clin Immunol* 2013; **132**: 767–769.
- Stander S, Oppel E, Thomas P, Summer B. Evaluation of lymphocyte transformation tests as compared with patch tests in nickel allergy diagnosis. *Contact Dermatitis* 2017; **76**: 228–234.
- Koene RA. The 'memory lymphocyte immunostimulation assay' (MELISA) is useless for the detection of metal allergy. *Ned Tijdschr Geneesk* 2005; **149**: 2090–2092.
- Tsuge I, Okumura A, Kondo Y *et al.* Allergen-specific T-cell response in patients with phenytoin hypersensitivity; simultaneous analysis of proliferation and cytokine production by carboxyfluorescein succinimidyl ester (CFSE) dilution assay. *Allergol Int* 2007; **56**: 149–155.
- Milovanova TN. Comparative analysis between CFSE flow cytometric and tritiated thymidine incorporation tests for beryllium sensitivity. *Cytometry B Clin Cytom* 2007; **72**: 265–275.
- Jakobson E, Masjedi K, Ahlborg N, Lundeberg L, Karlberg AT, Scheynius A. Cytokine production in nickel-sensitized individuals analysed with enzyme-linked immunospot assay: possible implication for diagnosis. *Br J Dermatol* 2002; **147**: 442–449.
- Summer B, Paul C, Mazooghian F *et al.* Nickel (Ni) allergic patients with complications to Ni containing joint replacement show preferential IL-17 type reactivity to Ni. *Contact Dermatitis* 2010; **63**: 15–22.
- Cederbrant K, Anderson C, Andersson T, Marcusson-Stahl M, Hultman P. Cytokine production, lymphocyte proliferation and T-cell receptor Vbeta expression in primary peripheral blood mononuclear cell cultures from nickel-allergic individuals. *Int Arch Allergy Immunol* 2003; **132**: 373–379.
- Minang JT, Arestrom I, Troye-Blomberg M, Lundeberg L, Ahlborg N. Nickel, cobalt, chromium, palladium and gold induce a mixed Th1- and Th2-type cytokine response *in vitro* in subjects with contact allergy to the respective metals. *Clin Exp Immunol* 2006; **146**: 417–426.
- Merk HF. Diagnosis of drug hypersensitivity: lymphocyte transformation test and cytokines. *Toxicology* 2005; **209**: 217–220.
- Spiewak R, Moed H, von Blomberg BM *et al.* Allergic contact dermatitis to nickel: modified *in vitro* test protocols for better detection of allergen-specific response. *Contact Dermatitis* 2007; **56**: 63–69.
- Minang JT, Troye-Blomberg M, Lundeberg L, Ahlborg N. Nickel elicits concomitant and correlated *in vitro* production of Th1-, Th2-type and regulatory cytokines in subjects with contact allergy to nickel. *Scand J Immunol* 2005; **62**: 289–296.
- Martins LE, Reis VM. IL-13: a marker of chromium contact allergy. *J Eur Acad Dermatol Venereol* 2013; **27**: e390–e393.
- Akerman L, Klein T, Israeli M *et al.* Luminex liquichip system for the evaluation of cytokine levels in patients with contact dermatitis to potassium dichromate. *Dermatology* 2016; **232**: 748–751.
- Czarnobilska E, Jenner B, Kaszuba-Zwoinska J *et al.* Contact allergy to nickel: patch test score correlates with IL-5, but not with IFN-gamma nickel-specific secretion by peripheral blood lymphocytes. *Ann Agric Environ Med* 2009; **16**: 37–41.
- Schnuch A, Aberer W, Agathos M *et al.* Performing patch testing with contact allergens. *J Dtsch Dermatol Ges* 2008; **6**: 770–775.
- Summer B, Sander CA, Przybilla B, Thomas P. Molecular analysis of T-cell clonality with concomitant specific T-cell proliferation *in vitro* in nickel-allergic individuals. *Allergy* 2001; **56**: 767–770.
- Brasch J, Henseler T, Aberer W *et al.* Reproducibility of patch tests. A multicenter study of synchronous left-versus right-sided patch tests by the German Contact Dermatitis Research Group. *J Am Acad Dermatol* 1994; **31**: 584–591.
- Josefson A, Farm G, Meding B. Validity of self-reported nickel allergy. *Contact Dermatitis* 2010; **62**: 289–293.
- Thomas B, Kulichova D, Wolf R, Summer B, Mahler V, Thomas P. High frequency of contact allergy to implant and bone cement components, in particular gentamicin, in cemented arthroplasty with complications: usefulness of late patch test reading. *Contact Dermatitis* 2015; **73**: 343–349.
- Kapsenberg ML, Wierenga EA, Stiekema FE, Tiggeleman AM, Bos JD. Th1 lymphokine production profiles of nickel-specific CD4⁺ T-lymphocyte clones from nickel contact allergic and non-allergic individuals. *J Invest Dermatol* 1992; **98**: 59–63.

- 32 Lindemann M, Bohmer J, Zabel M, Grosse-Wilde H. ELISpot: a new tool for the detection of nickel sensitization. *Clin Exp Allergy* 2003; **33**: 992–998.
- 33 Sachs B, Erdmann S, Malte Baron J, Neis M, al Masaoudi T, Merk HF. Determination of interleukin-5 secretion from drug-specific activated ex vivo peripheral blood mononuclear cells as a test system for the in vitro detection of drug sensitization. *Clin Exp Allergy* 2002; **32**: 736–744.
- 34 Spiewak R. Contact dermatitis in atopic individuals. *Curr Opin Allergy Clin Immunol* 2012; **12**: 491–497.
- 35 Buchvald D, Lundeberg L. Impaired responses of peripheral blood mononuclear cells to nickel in patients with nickel-allergic contact dermatitis and concomitant atopic dermatitis. *Br J Dermatol* 2004; **150**: 484–492.
- 36 Thomas P, Iglhaut G, Wollenberg A, Cadosch D, Summer B. Allergy or tolerance: reduced inflammatory cytokine response and concomitant IL-10 production of lymphocytes and monocytes in symptom-free titanium dental implant patients. *Biomed Res Int* 2013; **2013**: 539834.
- 37 Lin CH, Chung CA, Wong JH *et al.* Involvement of L-type Ca(2)(+) channel and toll-like receptor-4 in nickel-induced interleukin-8 gene expression. *Environ Toxicol* 2016; **31**: 5–12.
- 38 Tsou TC, Liou SH, Yeh SC, Tsai FY, Chao HR. Crucial role of Toll-like receptors in the zinc/nickel-induced inflammatory response in vascular endothelial cells. *Toxicol Appl Pharmacol* 2013; **273**: 492–499.
- 39 Potnis PA, Dutta DK, Wood SC. Toll-like receptor 4 signaling pathway mediates proinflammatory immune response to cobalt-alloy particles. *Cell Immunol* 2013; **282**: 53–65.
- 40 Clark RA, Schlapbach C. TH9 cells in skin disorders. *Semin Immunopathol* 2017; **39**: 47–54.
- 41 Liu J, Harberts E, Tammaro A *et al.* IL-9 regulates allergen-specific Th1 responses in allergic contact dermatitis. *J Invest Dermatol* 2014; **134**: 1903–1911.
- 42 Gutin L, Tammaro A, Fischelevich R, Gaspari AA. Elevation of IL-9 in extreme patch test reactions suggests it is an inflammatory mediator in allergic contact dermatitis. *Dermatitis* 2016; **27**: 35–36.