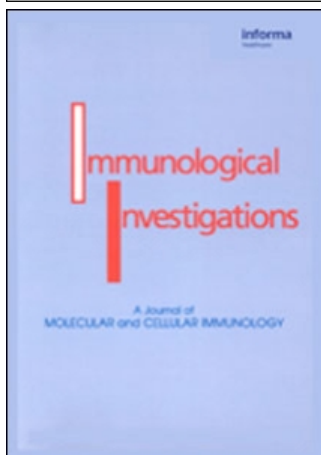


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ELISpot Displays a Better Detection over ELISA of T Helper (Th) 2-Type Cytokine-Production by *Ex Vivo*-Stimulated Antigen-Specific T Cells from Human Peripheral Blood

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Detection of cytokines secreted by *ex vivo* antigen-stimulated peripheral blood mononuclear cells (PBMC) by ELISA is hampered by low frequencies of specific T cells and cellular receptor consumption. We investigated if ELISpot, measuring cytokine production at the single cell level, facilitated a better detection of the Th2 cytokines IL-4 and IL-5. PBMC from nickel-allergic (n = 31) and non-allergic subjects (n = 10) were stimulated with nickel or tetanus toxoid (TT) and cytokine production assessed by ELISpot and ELISA. By IL-4 ELISpot, 74% of the allergic and 0% control subjects responded to nickel and 56% of all subjects to TT. ELISA detected IL-4 after nickel stimulation only in 13% of the allergic subjects. Also detection of TT-induced IL-5 was improved by ELISpot with 54% subjects responding versus 24% in ELISA. In contrast, detection of nickel-induced IL-5 was more comparable between methods, most likely due to the 7-fold higher IL-5 production per cell in response to nickel versus TT. The low IL-5 response to TT was associated with a higher induction of the down-regulatory cytokine IL-10 by TT as compared to nickel ($p < 0.001$). Overall, ELISpot displayed a better detection of IL-4 as well as low intensity IL-5 responses thus emphasizing the importance of selecting suitable methods for the measurement of cytokine production *ex vivo*.

Keywords ELISA, ELISpot, Interleukin-4, Interleukin-5, Interleukin-10.

INTRODUCTION

T-helper (Th) 2-type cytokines such as IL-4 and IL-5 are secreted by T cells in response to allergens, both in atopic and contact allergy (Jakobson et al., 2002; Parronchi et al., 1991; Romagnani, 1989), and helminthic antigens (Heinzel et al., 1989; Porto et al., 2001). Th2 cytokines down-regulate Th1-type immune responses (Mosmann and Coffman, 1989; Sasaki et al., 2000). More specifically, IL-4 promotes the development of IgE-mediated allergy (Steinke and Borish, 2001) by regulating switching in B cells to IgE and IgG₄ in humans (Del Prete et al., 1988; Punnonen et al., 1993; Wood et al., 2004). IL-5 plays a role in the differentiation and maturation of cells involved in IgE-mediated allergic reactions, such as mast cells and eosinophils (Fattah et al., 1990; Severinsson et al., 1989), inhibits certain macrophage functions (Rizzo, 1992) and acts as a growth factor for IgA-producing B cells (Sonoda et al., 1989).

Given the plethora of biological activities associated with IL-4 and IL-5, their measurement is often used in epidemiological studies (Leenstra et al., 2006; Romagnani et al., 1989), assessment of immunotherapeutic agents (Andersson et al., 2004; Hall et al., 2002) and studies on atopic or contact allergies (Minang et al., 2005; Parronchi et al., 1991). ELISA represents an established method for measuring secretion of soluble factors but can have a limited applicability for measurement of cytokine secretion by ex vivo/in vitro antigen-stimulated T cells. The number of specific cells and the amount of cytokine secreted per cell can be low, resulting in concentrations below the detection limit of the assay (Ekerfelt et al., 2002; Tanguay and Killion, 1994). Moreover, in the case of IL-4, the IL-4 receptor is up-regulated in a diverse population of cells upon antigenic stimulation (Carter and Swain, 1997; Renz et al., 1991) and consumption by cellular receptors can further decrease the net amount of cytokine accumulated in the cell culture supernatant (Ewen and Baca-Estrada, 2001).

ELISpot is principally similar to ELISA but identifies single cytokine-secreting cells. This is achieved by incubating the cells in plates adsorbed with capture antibody followed by detection in a manner similar to ELISA but using a substrate that precipitates at the site of the cytokine-producing cell (Czerkinsky et al., 1983, 1988). The immediate capture of the cytokine upon secretion and the fact that the secreted cytokines accumulate locally, overcome several of the problems associated with cytokine analysis by ELISA.

For cell lines secreting various factors, ELISpot has been shown to display a better detection sensitivity than ELISA in terms of the number of cells needed for detection (Tanguay and Killion, 1994; Zuber et al., 2005). Based on its high detection sensitivity, ELISpot has become a standard assay for quantifying IFN- γ -secreting CD8⁺ T cells as an immune correlate of the cytotoxic capacity in the fields of vaccine development, tumour-induced immunity and

infectious diseases (Adams et al., 1991; Dheda et al., 2005; Schwartzentruber et al., 1991). Despite the potential benefits of the method, relatively few studies in the field of allergy, have utilized ELISpot for detection of Th2-type cytokines.

The aim of this study was to compare the capacity of ELISpot and ELISA to detect Th2-type cytokine responses to antigens by *ex vivo*-stimulated PBMC. The antigens used, nickel (Ni; a metal contact allergen) and tetanus toxoid (TT; a protein antigen), have previously been shown to elicit immune responses involving Th2-type cytokines, Ni by natural exposure and TT by vaccination against tetanus (elGhazali et al., 1993; Jakobson et al., 2002).

MATERIALS AND METHODS

Human Subjects and Isolation of Peripheral Blood

Mononuclear Cells

Blood samples were obtained by venepuncture and collected in sterile heparinised glass vials. PBMC were separated by centrifugation over Ficoll-Paque (Pharmacia, Uppsala, Sweden) and frozen as described (Minang et al., 2005). Prior to testing, PBMC were thawed at 37°C, washed with medium twice and resuspended in cell culture medium; RPMI-1640 supplemented with HEPES (10 mM), L-glutamine (2 mM), penicillin G sodium (100 U/ml), streptomycin sulphate (100 µg/ml) (all from Gibco BRL, Life Technologies Ltd, Paisley, UK) and 10% fetal bovine serum (FBS; HyClone Laboratories Inc., Logan, UT, USA). Cell viability was assessed by trypan blue exclusion (>90% viable cells).

The study included 31 females with contact allergy to Ni and a positive patch test to NiSO₄ according to the scoring system recommended by the International Contact Dermatitis Research Group (Brasch and Geier, 1997) and ten healthy volunteers (nine females and one male) with a negative patch test. The subjects were 27–70 years old and were either patients or staff of the Karolinska University Hospital, Stockholm, Sweden. The study was approved by the Ethics committee at the Karolinska University Hospital. All subjects gave their informed consent to participation.

Enumeration of Cytokine-Producing Cells by ELISpot

ELISpot was performed essentially as described (Minang et al., 2005) using polyvinyl difluoride plates pre-coated with capture monoclonal antibodies (mAbs) for IL-4 (mAb 82.4) or IL-5 (mAb TRFK5) (Mabtech, Nacka Strand, Sweden). PBMC suspensions of 2.5×10^5 cells with or without 50 µM NiCl₂ × 6H₂O (Merck, Darmstadt, Germany) or 10 µg/ml of tetanus toxoid (TT; SBL Vaccin, Stockholm, Sweden) in 100 µl cell culture medium

were added per well. As positive controls, 1×10^5 cells/well stimulated with phytohemagglutinin (PHA; 5 $\mu\text{g/ml}$; Orion Diagnostics, Trosa, Sweden) was used.

The cells were incubated at 37°C and 5% CO_2 in humidified air for 40 h. After incubation, the plates were washed in phosphate-buffered saline (PBS) and incubated with biotinylated detection mAbs (1 $\mu\text{g/ml}$ in PBS/0.5% FBS) for IL-4 (mAb 12.1) or IL-5 (mAb 5A10) (Mabtech) at room temperature (RT) for 2 h, followed by incubation with streptavidin-alkaline phosphatase conjugate and subsequently BCIP/NBT substrate (Mabtech). ELISpot assays were set up in triplicates. An automated ELISpot reader was used to enumerate the spots representing cytokine-producing cells.

Assessment of Cytokine Production by ELISA

In parallel with the ELISpot, cell suspensions with the same concentration of cells and stimuli were set up in polystyrene round-bottom tubes (Becton Dickinson Labware, San Jose, CA, USA) at 2 ml volumes. For the analysis of the impact of exogenous IL-10 on Ni-induced IL-5 levels, recombinant human (rh) IL-10 (R&D Systems, Minneapolis, MN, USA) was added to the cell cultures. Cells were incubated as above and the supernatants were collected and stored at -20°C prior to the ELISA, performed as described (Minang et al., 2005). The same capture and detection mAbs for IL-4 and IL-5 as in the ELISpot were used.

For the detection of IL-10, mAb 9D7 was used for capture and biotinylated mAb 12G8 for detection (Mabtech). Standard curves were obtained using recombinant human IL-4, IL-5 and IL-10 (Mabtech). Streptavidin-alkaline phosphatase conjugate (Mabtech) was used as a secondary detection reagent in combination with pNPP substrate (Sigma-Aldrich Corp, St. Louis, MO, USA). The mean concentration of the cytokine standard (from 10 independent experiments) that had an absorbance at 405 nm equivalent to 2x the background absorbance (0 pg/ml) + 2SD was used as the lowest detection limit (1.5, 2.1 and 5.0 pg/ml for IL-4, IL-5 and IL-10, respectively). ELISA values below the lowest detection limit were set to these values.

Statistical Analysis and Cut-off Values for Positive Responses

The Wilcoxon matched pair test was used to compare cytokine production in the absence (spontaneous) and presence of antigens (Ni and TT) and the Mann-Whitney U-test was used to compare the responses between Ni-allergic and non-allergic subjects. To evaluate the relationship between observed parameters, correlations were computed using the Spearman rank-order correlation coefficient r_s . For statistical analyses of antigen-induced responses and for definition of positive responses by cut off thresholds, the spontaneous production was subtracted.

Cut-off definitions for ELISA were set at an increase $>3\times$ the lowest detection limit value. For ELISpot, where no lowest detection limit can be applied, the cut off was based on the mean IL-4 response to Ni displayed by the control group + 3SD (2.6 spots/ 2.5×10^5 input cells). The same cut off was used for IL-5 ELISpot. A p -value <0.05 was considered to be statistically significant. All tests were performed using the software STATISTICA 5.1 (StatSoft, Tulsa, OK, USA).

RESULTS

ELISpot- and ELISA-Detection of IL-4 and IL-5 Induced by Nickel or Tetanus Toxoid

Production of IL-4 and IL-5, spontaneously or induced by Ni or TT, in PBMC from 31 Ni-allergic and 10 non-allergic subjects was assessed by ELISpot and ELISA. Whereas all donors potentially displayed reactivity to TT from previous vaccinations, a response to Ni was anticipated only in allergic subjects. The assays were set up as identical as possible. In ELISpot, PBMC at 2.5×10^6 cells/ml in 100 μ l/well, with or without antigen, were incubated for 40 h and supernatants for ELISA were generated in vials with the same PBMC and antigen concentration and with the same incubation time. Also, the capture and detection mAbs used for the assays were the same.

The spontaneous production of IL-4 and IL-5 was low and did not differ between allergic and non-allergic subjects. In the ELISpot, the average number of cytokine-producing cells (PC)/250,000 PBMC in all subjects was 2.2 ± 2.1 for IL-4 and 1.0 ± 1.1 for IL-5. In ELISA, the IL-4 levels were below the detection limit for all subjects whereas the IL-5 levels were 3.0 ± 3.1 pg/ml.

Ni elicited increased IL-4 PC only in the allergic group ($p < 0.01$) whereas TT induced IL-4 PC in both groups (Figure 1). Antigen-induced IL-4 levels were low and did not differ between groups (Figure 1). The IL-5 ELISpot yielded results similar to the IL-4 ELISpot both for Ni and TT (Figure 1). Analysis of Ni-induced IL-5 by ELISA yielded results comparable to the ELISpot with higher IL-5 levels in allergic subjects (Figure 1; $p < 0.01$). Although TT elicited increased IL-5 PC in both groups, comparable to the numbers of Ni-induced IL-5 PC in allergic subjects, the IL-5 levels induced by TT in both groups were lower than the IL-5 levels induced by Ni in the allergic group (Figure 1); in the allergic group the mean IL-5 level induced by TT was 4.2 pg/ml *versus* 33 pg/ml for Ni ($p < 0.001$).

This latter finding suggests that Ni elicits higher IL-5 secretion per antigen-specific cell than TT. Subjects responding to Ni in both IL-5 ELISA and ELISpot ($n=12$) displayed mean responses of 79 pg/ml (i.e., per 2.5×10^6 PBMC) and 17 PC/250,000 PBMC yielding a ratio corresponding to a production of 0.46 pg per PC. In contrast, subjects responding to TT by both methods

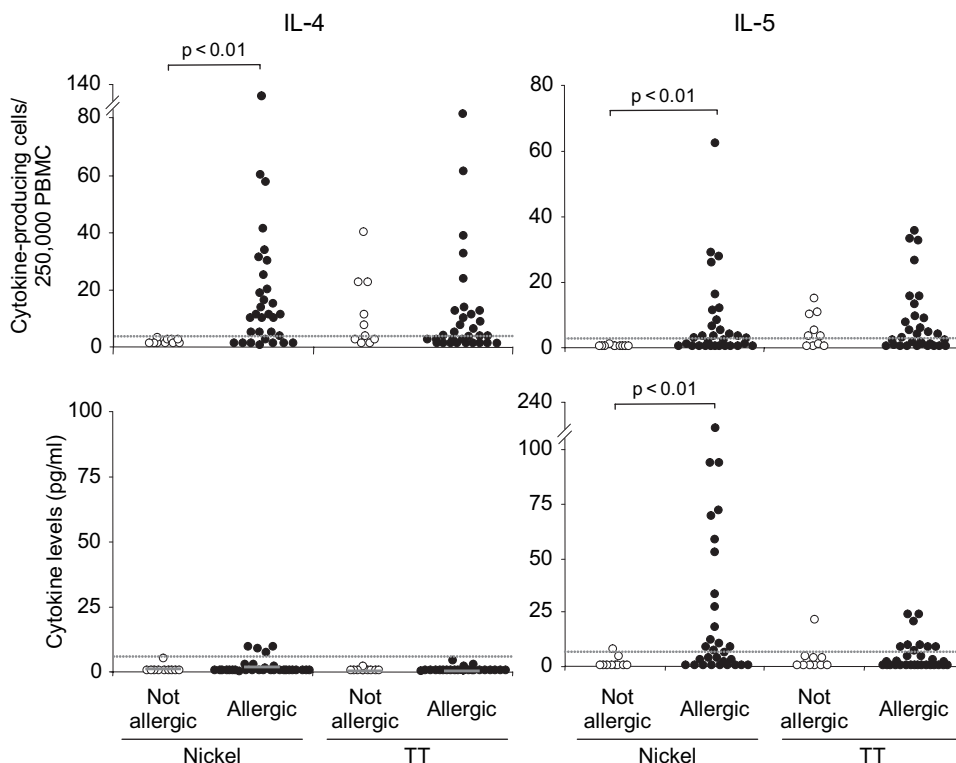


Figure 1: Individual IL-4 and IL-5 responses to Ni and TT as analysed by ELISpot and ELISA. PBMC from subjects with Ni allergy ($n = 31$) or non-allergic subjects ($n = 10$) were cultured for 40 h with or without antigens. The responses to the antigens are shown after subtraction of the spontaneous production (medium only). Dotted lines indicate cut off levels for positive responses to the respective antigens. Statistically significant differences between the responses of the two groups to the respective antigens are indicated.

($n = 7$) displayed mean values of 15 pg/ml and 21 PC/250,000 PBMC corresponding to 0.068 pg per PC, a value 7-fold lower than for Ni. The corresponding values for calculations based on all subjects ($n = 41$) were similar, 0.46 pg/PC and 0.059 pg/PC for responses to Ni and TT, respectively.

The differences observed between the methods were also evident when defining positively responding subjects based on cut-off definitions (Figure 1). By IL-4 ELISpot, 23/31 allergic and 0/10 non-allergic subjects responded to Ni whereas only 4 allergic subjects had IL-4 responses above cut-off in IL-4 ELISA. Reactivity to TT were displayed by 17/31 allergic and 6/10 non-allergic subjects in the IL-4 ELISpot, whereas no subject responded according to IL-4 ELISA. IL-5 ELISpot identified 16/31 allergic and 0/10 non-allergic subjects responding to Ni, whereas IL-5 ELISA identified 15/31 allergic and 1/10 non-allergic subjects. A response to TT was found in 16/31 allergic and 6/10 non-allergic subjects by IL-5 ELISpot. In line with the relatively low levels of IL-5 induced by TT, the corresponding numbers for ELISA were 9/31 and 1/10.

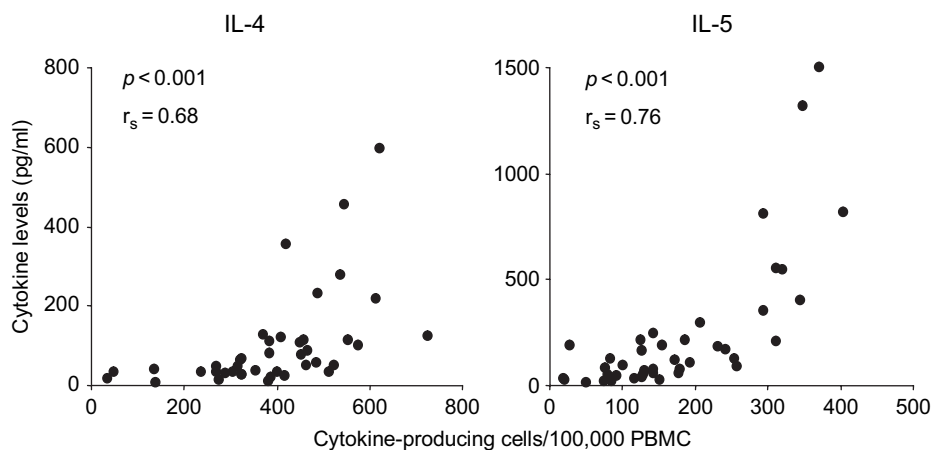


Figure 2: Positive correlation between ELISpot and ELISA analysis of mitogen (PHA)-induced IL-4 and IL-5 production. PBMC from subjects with Ni allergy ($n = 31$) or non-allergic subjects ($n = 10$) were stimulated for 40 h with PHA. The graphs display values for all subjects after subtraction of the spontaneous production (medium only).

In accordance with the poor detection of IL-4 by ELISA, the correlation between IL-4 ELISA and ELISpot measurement of antigen-induced responses was low as was the correlation between the methods for TT-induced IL-5 ($p < 0.01$; $r_s < 0.46$ for all analyses). The IL-5 response induced by Ni, yielded a better correlation between methods ($p < 0.001$; $r = 0.70$). A higher correlation was also found between ELISpot and ELISA analysis of IL-4 and IL-5 production induced by the mitogen PHA (Figure 2; $p < 0.001$; $r_s = 0.76$ and 0.68 , respectively). PHA was included in the test as a cell viability control and yielded variable but detectable responses in all PBMC.

IL-10 Production Induced by Nickel and Tetanus Toxoid

To assess if the lower IL-5 levels found after TT stimulation were associated with a differential capacity of the antigens to simultaneously induce factors that potentially could down-regulate the IL-5 secretion, the levels of the down-regulatory cytokine IL-10 were measured. Whereas TT elicited IL-10 production in both groups, Ni elicited significant IL-10 production only in the allergic group. In contrast to the IL-5 levels, the IL-10 levels induced by TT elicited were higher (in both groups) than those induced by Ni in allergic subjects (Figure 3); in the allergic group, Ni induced mean IL-10 levels of 25 pg/ml whereas mean TT-induced levels were 89 pg/ml ($p < 0.001$).

To investigate if the lower levels of endogenous IL-10 induced by Ni could have a favorable effect on the Ni-induced IL-5 secretion, Ni-induced IL-5 was

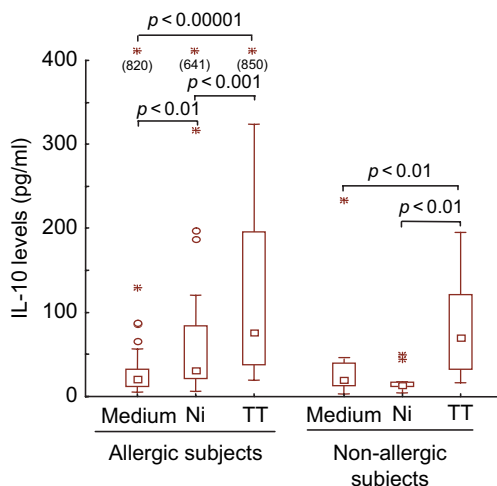


Figure 3: IL-10 levels induced by Ni and TT. PBMC from subjects with Ni allergy ($n = 31$) or non-allergic subjects ($n = 10$) were cultured for 40 h with or without antigens and IL-10 levels were measured by ELISA. The box plots show the median ($\circ$), min ($\pm$), max ($\mp$), 25th % and 75th % ($\square$) for PBMC cultured without stimuli (medium) or with Ni or TT. Statistically significant differences between antigen-induced responses and spontaneous production (medium only) within each group are indicated.

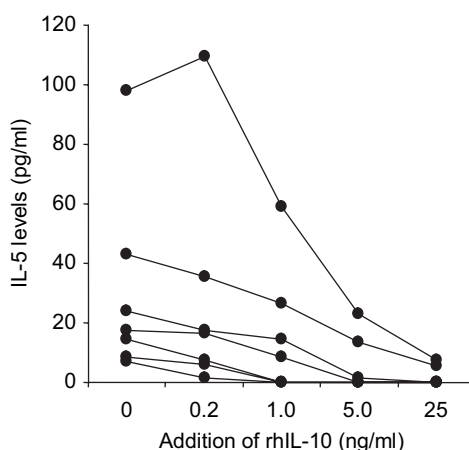


Figure 4: Impact of exogenous recombinant human (rh) IL-10 on Ni-induced IL-5 production. PBMC from seven subjects with Ni allergy were incubated for 40 h in the presence of Ni ($50 \mu\text{M}$) with or without the addition of rhIL-10 at concentrations ranging from 0.2 to 25 ng/ml. The levels of IL-5 accumulated in supernatants were measured by ELISA and are shown after subtraction of the spontaneous production (medium only).

analysed after addition of exogenous rhIL-10 to PBMC from 7 allergic subjects. RhIL-10 reduced the Ni-induced IL-5 secretion in a dose-dependent manner with a decrease in IL-5 production in 6/7 subjects at 0.2 ng/ml and a 50% reduction on average at 1 ng/ml (Figure 4).

DISCUSSION

Although ELISpot and ELISA measure different aspects of cytokine production, one method enumerating the cytokine-secreting cells and the other quantifying the cytokine levels accumulated in the supernatant, either method can confirm the production of a cytokine and define the magnitude of a response. However, the results herein demonstrate that the ELISA-based detection of antigen-induced production of IL-4 as well as IL-5, depending on the type of stimuli, after short-term stimulation of T cells (i.e., *ex vivo*-stimulated PBMC rather than T-cell lines or clones) is more likely to fail.

In principle, ELISpot may detect cytokine-secreting cells in PBMC with a low frequency of antigen-specific T cells since the assay, in contrast to ELISA, is not affected by dilution of the cytokine in the culture medium. In line with this, we found a generally better ability of the ELISpot assay to detect low frequency events whereas detection of mitogen-induced IL-4 and IL-5 production was more comparable between methods. In addition to the dilution effect, consumption or degradation of cytokines can influence their detection. The poor IL-4 detection in ELISA, in spite of demonstrable numbers of IL-4-producing cells in ELISpot, may thus be due to cellular consumption rather than low production levels (Ekerfelt et al., 2002; Ewen and Baca-Estrada, 2001). Receptor consumption may affect the detection also of other cytokines but has not been reported. The dilution effect, however, will be an issue for the ELISA analysis of any cytokine secreted at lower levels.

Additional factors may affect the detection of low frequency responses. TT induced lower levels of IL-5 than Ni, despite similar numbers of responding cells measured by ELISpot, suggesting a 7-fold lower IL-5 production by TT-reactive cells. This may render the total levels of IL-5 induced by TT below the detection limit for ELISA but still sufficient for the detection of single secreting cells by ELISpot.

Notably, the lower IL-5 levels induced by TT coincided with a higher induction of IL-10. Considering that IL-10 may down-regulate both Th1 and Th2 cytokines (Akdis et al., 1998; Minang et al., 2006), the lower IL-10 response to Ni may explain the higher levels of IL-5. In line with this, we found that rhIL-10 inhibited the Ni-induced IL-5 secretion although relatively high levels of rhIL-10 were required to decrease the levels to the low IL-5 levels induced by TT. However, the dose of rhIL-10 required to inhibit the Ni-induced IL-5 production is difficult to compare directly to the potential inhibition of IL-5 production by TT-induced endogenous IL-10 since TT may induce other down-regulatory cytokines acting in synergy.

The main source of IL-10 in response to antigens is regulatory T cells that produce additional down-regulatory cytokines such as TGF- β (Taylor et al., 2006). It is also possible that the TT preparation used triggers IL-10 production by monocytes in a non-specific manner. The TT was produced for vaccination of humans but could nevertheless contain other *Clostridium tetani*-derived

molecules. Whether such contaminations, or even TT itself, has the capacity to trigger IL-10 production by non-T cells will have to be investigated.

Tassignon et al. (2005) recently showed low levels of TT-induced IFN- γ in supernatants from human PBMC, despite considerably elevated numbers of TT-specific IFN- γ -producing cells by ELISpot. The proposed explanation was that long term memory cells in the absence of recent antigenic boost may show a low cytokine production upon antigen encounter in vitro; similar to the present study, most of the subjects included in that study were 40 years or older and would have been vaccinated more than 2 decades ago. In contrast, PBMC from subjects sensitized to Ni are likely to be continuously exposed to Ni due to the wide-spread distribution and usage of Ni. Thus, in addition to the differential induction of IL-10, differences in the antigen exposure pattern may contribute to the differences between IL-5 secretion induced by TT and Ni.

Neither TT nor Ni induced a detectable Th2 response in all potentially reactive subjects. Although both antigens have been shown to elicit mixed Th1- and Th2-type responses, some subjects may display responses below the detection capacity of the cytokine assays used. In the case of Ni, it is also possible that certain subjects have been incorrectly diagnosed due to false positive reactions in the skin provocation test (patch test) used to diagnose contact allergy (Minang et al., 2005).

Our data show a considerably higher detection sensitivity of ELISpot over ELISA for antigen-specific Th2-type (notably IL-4) cytokine production in ex vivo-stimulated PBMC. Also the detection of IL-5 production differed between methods with ELISpot being better at detecting low-grade IL-5 production. In addition, the data from the study suggests that co-induction of regulatory cytokines may have an impact on the capacity of assays to detect a given cytokine.

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