

CD4⁺ T cells producing interleukin (IL)-17, IL-22 and interferon- γ are major effector T cells in nickel allergy

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Summary

Background. It has been suggested that interleukin (IL)-17 and IL-22 play important roles in the elicitation of human allergic contact dermatitis; however, the frequencies of T cell subtypes producing IL-17 and IL-22 in human allergic contact dermatitis are unknown.

Objectives. To determine the frequencies of CD4⁺, CD8⁺ and $\gamma\delta$ T cells producing IL-17, IL-22 and interferon (IFN)- γ in the blood and skin from nickel-allergic patients.

Patients/materials/methods. Blood samples were collected from 14 patients and 17 controls, and analysed by flow cytometry. Biopsies were taken from 5 patients and 6 controls, and analysed by immunohistochemistry and flow cytometry of skin lymphocytes.

Results. We found an increased frequency of $\gamma\delta$ T cells in the blood, but no differences in the distribution of cytokine-producing CLA⁺ T cell subtypes in nickel-allergic patients as compared with controls. In nickel-allergic patients, there was massive cellular infiltration dominated by CD4⁺ T cells producing IL-17, IL-22 and IFN- γ in nickel-challenged skin but not in vehicle-challenged skin.

Conclusion. CD4⁺ T cells producing IL-17, IL-22 and IFN- γ are important effector cells in the eczematous reactions of nickel-induced allergic contact dermatitis in humans.

Key words: allergic contact dermatitis; CD4⁺ T cells; CD8⁺ T cells; IFN- γ ; IL-17; IL-22; nickel allergy; $\gamma\delta$ T cells.

Allergic contact dermatitis (ACD) is a T cell-mediated delayed-type hypersensitivity reaction. Both CD4⁺ and CD8⁺ T cells are involved in the elicitation phase of ACD.

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In mice, CD8⁺ T cells appear to be the main effector cells in the reaction, whereas CD4⁺ T cells seem to play both pro-inflammatory and anti-inflammatory roles (1, 2). In contrast, in humans, CD4⁺ T cells appear to be the major effector cells (3–5). Interferon (IFN)- γ is an important inflammatory cytokine produced by both CD4⁺ and CD8⁺ T cells during ACD in humans and mice (6, 7). However, during the last decade, it has become evident that other inflammatory cytokines, such as interleukin (IL)-17 and IL-22, may have an important role in the immune response to contact allergens (3, 8, 9). IL-17 is produced by T helper (Th)17 cells and other cell types, and is important in defence against extracellular bacteria.

Inappropriately regulated expression of IL-17 has been associated with inflammatory diseases (10).

The role of IL-17 has been highlighted in mouse models of contact allergy, as both IL-17-producing CD4⁺ and IL-17-producing CD8⁺ T cells have been identified (2, 11). IL-17-deficient mice have markedly reduced ear-swelling after exposure to either dinitrofluorobenzene or trinitrochlorobenzene as compared with wild-type mice (12). In humans, CD4⁺ and CD8⁺ T cell clones from nickel-allergic individuals have been shown to produce IL-17, and increased numbers of IL-17-producing cells have been found in skin sections from positive patch test biopsies from 2 patients (3, 13). Our group and others have found both nickel-specific Th1 cells and Th17 cells in nickel-allergic patients, but not in healthy controls (3, 8). Thus, IL-17 appears to be involved in human ACD, even though the contribution from CD4⁺ as opposed to CD8⁺ T cells requires further investigation.

Like IL-17, IL-22 was originally believed to be produced by Th17 cells; however, it has been shown recently that a specific skin-homing subtype of CD4⁺ T cells can produce IL-22 independently of IL-17 (14). CD4⁺ and CD8⁺ T cell clones that produce IL-22 have been found, derived from psoriatic lesional skin (15). We have found increased numbers of IL-22-producing cells and IL-22 receptor (IL-22R)-expressing cells in nickel-exposed skin as compared with vehicle-exposed skin in nickel-allergic individuals (8). Furthermore, increased serum levels of IL-22 have been found in nickel-allergic individuals (9). Thus, IL-22 appears to play a role in human ACD, even though the cells producing it during the response require further characterization.

IL-17, IL-22 and IFN- γ are produced not only by CD4⁺ and CD8⁺ T cells, but also by cells known to bridge the innate and adaptive immune systems, such as $\gamma\delta$ T cells and NKT cells (16, 17). In mice, $\gamma\delta$ T cells are important in immune surveillance, wound healing, and malignancy (18–20). It has been suggested that $\gamma\delta$ T cells play a role in wound healing in humans, but their functions in human skin remain elusive (21). Recently, it was shown that IL-17-producing $\gamma\delta$ T cells have an important role in the pathogenesis of psoriasis (22). In mice, $\gamma\delta$ T cells have been shown to have a role in induction of the allergic response (23–26). However, very little is known about the role of $\gamma\delta$ T cells in human ACD.

The purpose of this study was to determine the role of CD4⁺, CD8⁺ and $\gamma\delta$ T cells in nickel allergy by investigating the distribution of skin-homing T cell subsets in blood-derived and skin-derived T cells and their production of IL-17, IL-22, and IFN- γ .

Materials and Methods

Blood and skin samples

Thirty-three individuals participated in the study. All of the skin biopsies and blood samples from patients and controls were collected after informed, written consent had been obtained, in accordance with the Declaration of Helsinki principles for research involving human subjects. The study was approved by the local ethical committees (H-4-2011-091) and the Danish Data Protection Agency.

Fourteen patients with patch tests positive for nickel were enrolled. The patients were tested with 5% NiSO₄ in petrolatum. The patch test was applied on the upper back under occlusion in Finn Chambers® on Scanpor® tape. The skin reactions were evaluated after 3 days according to the guidelines of the International Contact Dermatitis Research Group (27). Only patients with at least a 2+ reaction were included.

Elicitation reactions were provoked by applying nickel (5% NiSO₄) and vehicle (Petrolatum) on the lower back, with collection of punch biopsies and blood samples on D3. Three punch biopsies (4 mm) were collected from nickel-challenged and vehicle-challenged skin, two for culture of skin lymphocytes; one biopsy from positive reactions was preserved in formalin and paraffin-embedded for immunohistochemistry. Seventeen healthy individuals with no history of dermatitis were enrolled as controls. Punch biopsies were collected from the lower-back area, and a blood sample was collected at the same time point. Punch biopsies from nickel-challenged skin were collected from 2 healthy individuals on D3.

Immunohistochemistry

Immunohistochemical studies were performed on paraffin sections with anti-human $\gamma\delta$ TCR (cat. no. TCR1153; Thermo Fisher Scientific Pierce Biotechnology, Rockford, IL), anti-human CD4 (cat. no. NCL-L-CD4-368; Novocastra, Newcastle, UK), and anti-human CD8 (cat. no. M7103; Dako, Glostrup, Denmark). Negative controls were made by omission of the primary antibody.

Briefly, dewaxing and antigen retrieval were performed by immersing slides in EnVision™ FLEX Target Retrieval Solution, High pH (cat. no. K8004; Dako) and heating them in the PT-LINK module (Dako), according to the manufacturer's instructions. After pretreatment, the slides were incubated with the primary antibodies anti- $\gamma\delta$ TCR clone γ 3.20 (1:150), anti-CD4 clone 4B12 (1:100) and anti-CD8 clone C8/144B (1:400) for 30 min. Reactions were detected and visualized with a two-step horseradish peroxidase-conjugated polymer detection system (FLEX+, cat. no. K8002; Dako) together with

a standard chromogenic visualization system (DAB, cat. no. K8002; Dako), following the recommendations from the vendor. Finally, sections were counterstained with haematoxylin and mounted with Pertex.

Isolation of T cells from skin biopsies

Skin biopsies were kept in 24-well plates, one biopsy per well, with 1 ml of medium containing RPMI (R5886; Sigma Aldrich Denmark ApS, Broendby, Denmark), 500 mIU/l penicillin (LEO Pharmaceuticals, Ballerup, Denmark), 500 mg/ml streptomycin (Merck KGaA, Darmstadt, Germany), 60 U/ml recombinant IL-2 (Proleukin; Novartis Healthcare, Copenhagen, Denmark) and autologous serum for 11 days. Approximately 400 μ l of medium was replaced every other day. On D11, T cells that had emigrated from the biopsies were collected and placed in new wells with medium without IL-2. On D12, skin lymphocytes were purified by using CD3 beads (Dynabeads cat. no. 111.51D; Invitrogen, Carlsbad, CA) for positive selection, according to the manufacturer's instructions, and left in an incubator at 37°C for 7 hr until bead release. Beads were removed, and cells were incubated overnight, before they were restimulated with phorbol 12-myristate 13-acetate (PMA) and ionomycin for 4 hr in the presence of monensin and stained for flow cytometric analysis.

Flow cytometric analysis of peripheral blood mononuclear cells (PBMCs) and skin lymphocytes

PBMCs were separated by density gradient centrifugation over Lymphoprep (cat. no. 1114547; Nycomed-Pharmacia, Oslo, Norway) and collected at the interface. To separate the different T cell subsets and to determine the cytokine capability of PBMCs and skin lymphocytes by intracellular cytokine staining after restimulation, cells were incubated for 4 hr at 37°C in the presence of 1.25 μ g/ml ionomycin, 2.5 μ g/ml PMA, and 3 mmol/l monensin (all from Sigma Aldrich). After surface staining, the cells were fixed (Fixation/Permeabilization Concentrate, cat. no. 00-5123-43; eBioscience, San Diego, CA) and permeabilized (Permeabilization Buffer, cat. no. 00-8333-56; eBioscience), according to the manufacturer's instructions, before staining for intracellular proteins. Cytometric analysis was performed on a Becton Dickinson FACS Calibur flow cytometer, and the results were analysed with BD CELLQUEST PRO Software (BD Biosciences, Franklin Lakes, NJ).

The following monoclonal antibodies were used for flow cytometric studies of PBMCs: anti-cutaneous lymphocyte-associated antigen (CLA) biotin (559950; BD Biosciences), anti-streptavidin APC (554067; BD

Biosciences), anti-T cell receptor (TCR) $\gamma\delta$ fluorescein isothiocyanate (FITC) (347903; BD Biosciences), anti-CD4 FITC (555346; BD Biosciences), anti-CD8 FITC (555366; BD Biosciences), anti-IL-17A phycoerythrin (PE) (12-7178-42; eBioscience), anti-IL-22 PE (12-7229-42; eBioscience), and anti-IFN- γ (554552; BD Biosciences).

For flow cytometric studies of skin lymphocytes, the following monoclonal antibodies were used: anti-TCR $\gamma\delta$ PE (333141; BD Biosciences), anti-CD4 PE (555347; BD Biosciences), anti-CD8 FITC (555366; BD Biosciences), anti-IL-17A APC (17-7179-42; eBioscience), anti-IL-22 APC (17-7222-82; eBioscience), and anti-IFN- γ APC (17-7319-82; eBioscience). The gating procedure was the same for each sample.

Statistical analysis

The data are normally distributed and, according to Levene's test, variance homogeneity in the two groups could be assumed. Data were therefore analysed with Student's *t*-test.

Results

Increased frequencies of $\gamma\delta$ T cells in the blood from patients with nickel allergy

To compare the frequencies of CD4⁺, CD8⁺ and $\gamma\delta$ T cells in the blood from patients with nickel allergy with the frequencies in blood from healthy controls, blood samples were collected from 14 nickel-allergic patients and 17 healthy controls. We determined the percentages of T cell subtypes by flow cytometric analysis. For the percentages of CD4⁺ T cells and CD8⁺ T cells, no significant differences were found between nickel-allergic patients and healthy controls (mean $\pm$ standard deviation: CD4⁺ T cells in patients, 52.50% $\pm$ 10.15%; CD4⁺ T cells in controls, 49.20% $\pm$ 6.97%; CD8⁺ T cells in patients, 17.65% $\pm$ 7.41%; CD8⁺ T cells in controls, 19.92% $\pm$ 7.59%). In contrast, a significantly increased percentage of peripheral $\gamma\delta$ T cells was found in nickel-allergic patients (2.90% $\pm$ 1.50%; minimum 0.82%; maximum 5.80%) as compared with controls (1.85% $\pm$ 1.11%; minimum 0.45%; maximum 4.53%; *p* < 0.05) (data not shown).

Similar frequencies of skin-homing T cell subtypes in nickel-allergic patients and healthy controls

To determine the relative frequencies of different skin-homing T cell subsets known to be involved in skin inflammation, we analysed cells that coexpress the skin-homing receptor CLA. Th (CD3⁺ CD4⁺ CLA⁺) cells, Tc

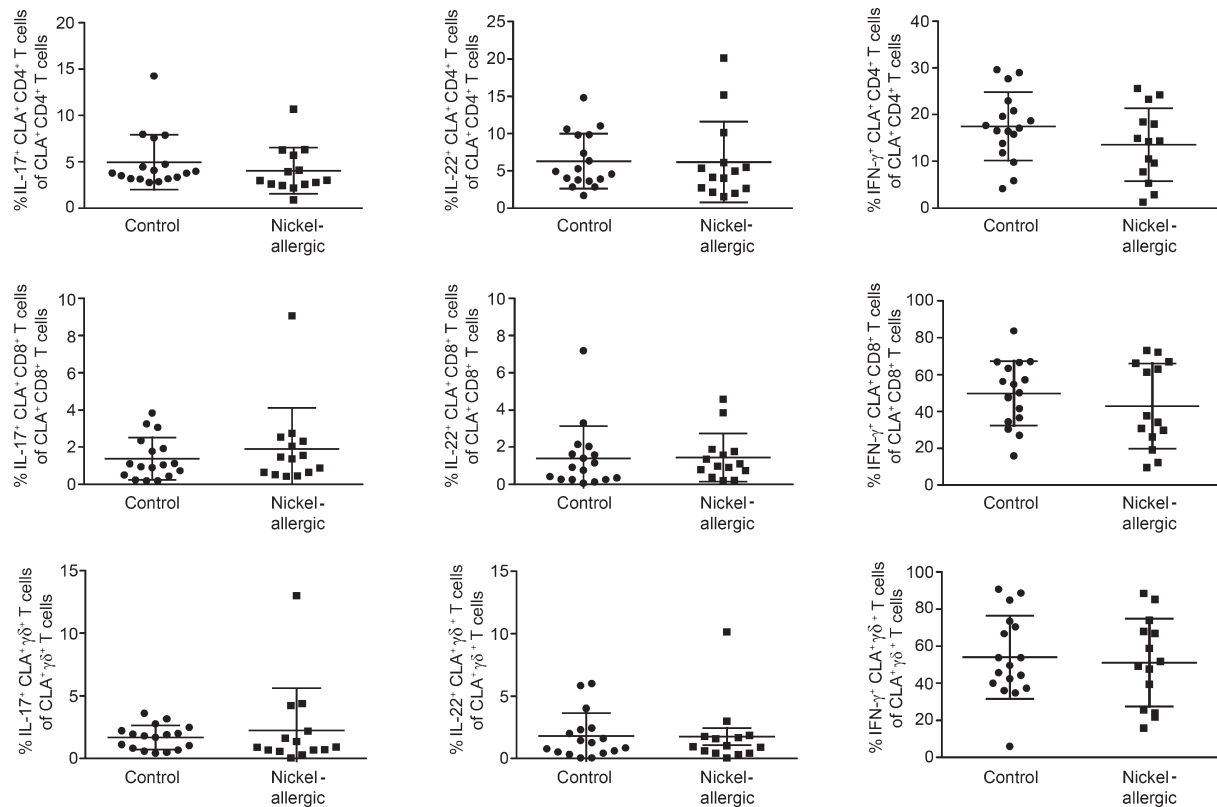


Fig. 1. Interleukin (IL)-17, IL-22 and interferon (IFN)- γ production in skin-homing T cells. The relative frequencies of skin-homing [cutaneous lymphocyte-associated antigen (CLA⁺)] T cell subsets in peripheral blood from nickel-allergic patients ($n = 14$) and healthy controls ($n = 17$). A fraction of the CLA⁺ T cell subsets analysed ($\gamma\delta$, CD4⁺, and CD8⁺) produced IL-17, IL-22, and IFN- γ . The percentages of IL-17⁺ and IL-22⁺ cells were highest in the CD4⁺ T cell subset. Expression of IFN- γ was prevalent in $\gamma\delta$ T cells and CD8⁺ T cells. No significant differences were found between the groups.

(CD3⁺ CD8⁺ CLA⁺) cells and $\gamma\delta$ T (CD3⁺ $\gamma\delta$ ⁺ CLA⁺) cells in the peripheral blood of nickel-allergic patients ($n = 14$) and healthy controls ($n = 17$) were analysed by flow cytometry after intracellular cytokine staining (Fig. 1). We found that the percentage of skin-homing Th cells producing IL-17 (patients, 4.15% $\pm$ 2.49%; controls, 5.07% $\pm$ 2.95%), IL-22 (patients, 6.07% $\pm$ 5.42%; controls, 6.19% $\pm$ 3.67%) or IFN- γ (patients, 13.38% $\pm$ 7.81%; controls, 17.29% $\pm$ 7.29%) were not significantly different between nickel-allergic patients and healthy controls. Similarly, the percentages of skin-homing Tc cells producing IL-17 (patients, 1.89% $\pm$ 2.21%; controls, 1.37% $\pm$ 1.14%), IL-22 (patients, 2.93% $\pm$ 2.58%; controls, 2.83% $\pm$ 3.47%) or IFN- γ (patients, 42.46% $\pm$ 23.10%; controls, 49.33% $\pm$ 17.43%) were not significantly different between patients and controls. In addition, the frequencies of skin-homing $\gamma\delta$ T cells producing IL-17 (patients, 2.22% $\pm$ 3.37%; controls, 1.65% $\pm$ 0.97%), IL-22 (patients, 1.71% $\pm$ 2.54%; controls, 1.74% $\pm$ 1.85%) or IFN- γ (patients,

57.57% $\pm$ 23.66%; controls, 54.45% $\pm$ 22.44%) were not significantly different between patients and controls (Fig. 1).

CD4⁺ T cells are the main cells infiltrating nickel-exposed skin

To investigate the distribution of T cell subsets in the skin, we stained skin sections from nickel-allergic patients ($n = 5$) for CD4⁺, CD8⁺, and $\gamma\delta$ TCR (Fig. 2). The skin biopsies were taken on D3 from skin exposed to nickel for 48 hr. As a control, a biopsy was collected from each allergic patient at the same time, from a vehicle-treated area (Fig. 2). All patients had to have at least a 2+ positive nickel patch test reaction on the upper back before inclusion. In some patients, the clinical manifestation of the locally elicited allergic contact dermatitis was, as expected, milder on the lower back than on the upper back (Table 1). The infiltration of CD4⁺ and CD8⁺ T cells was assessed blind and scored from 0 to 6 for each staining

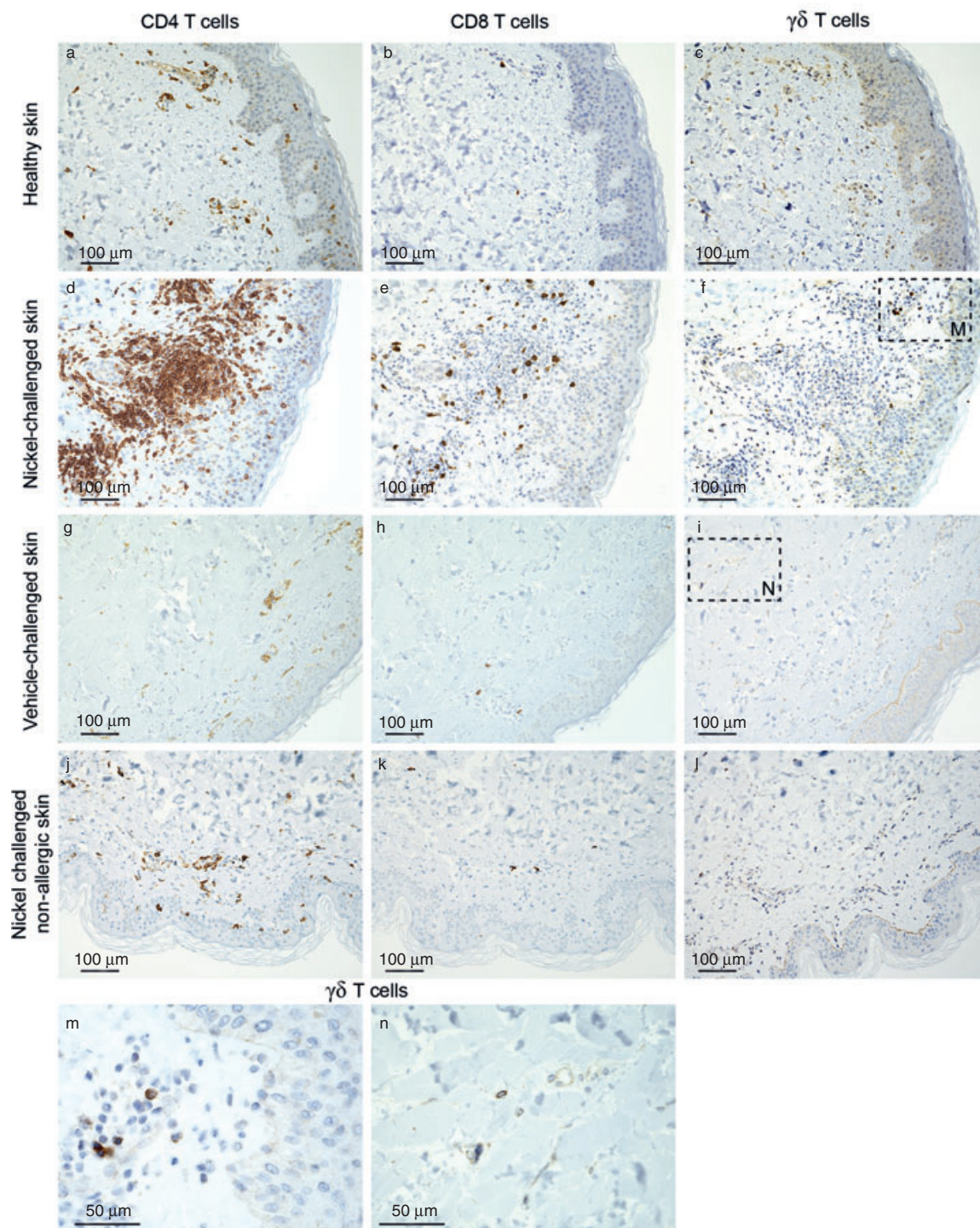


Fig. 2. Nickel exposure induces massive CD4⁺ T cell infiltration. To investigate the distribution of T cell subsets in the skin, we stained skin sections from healthy controls vehicle-challenged skin (a–c) and nickel-challenged skin (j–l) and from nickel-allergic patients nickel-challenged skin (d–f) and vehicle-challenged skin (g–i) with antibodies against CD4 (a, d, g, j), CD8 (b, e, h, k), or $\gamma\delta$ TCR (c, f, i, l). (d) In nickel-challenged skin, CD4⁺ T cells accounted for most of the T cells found in infiltrates in the dermis. (e–f) The numbers of CD8⁺ T cells (e) and $\gamma\delta$ T cells (f) were increased in nickel-challenged skin as compared with vehicle-challenged skin and healthy skin. Magnification: $\times 200$. (m, n) Higher magnification of $\gamma\delta$ TCR⁺ cells in nickel-challenged skin and vehicle-challenged skin from a nickel-allergic patient.

(Table 1). The number of infiltrating cells correlated with the clinical score. Most of the cells infiltrating the dermis were CD4⁺ cells (Fig. 2; Table 1). Skin sections were stained with the macrophage marker CD68, as macrophages can also express CD4. We found that only a small proportion of the infiltrating cells were CD68⁺ (data not shown), indicating that CD4⁺ T cells account for most of the CD4⁺ cells. CD8⁺ T cells were also found to infiltrate the allergen-exposed skin, but to a lesser extent than the CD4⁺ T cells (Fig. 2; Table 1).

The $\gamma\delta$ TCR staining was assessed by counting the number of positive cells, owing to the low prevalence of these cells (Table 1). Interestingly, the number of $\gamma\delta$ T cells – which are low in number as compared with CD4⁺ and CD8⁺ T cells – showed a marked increase in the most intense skin reactions. The control stains performed on nickel-challenged skin of non-allergic individuals showed no infiltration of the T cell subtypes investigated (Fig. 2J–L).

CD4⁺ T cells producing IFN- γ and IL-17 are the predominant cells in nickel-exposed skin

To further investigate the different T cell subtypes in the skin during the elicitation reaction, we isolated T cells from skin biopsies. As expected, there was a marked difference between the numbers of T cells obtained from biopsies taken from nickel-treated and vehicle-treated skin from nickel-allergic patients and the numbers obtained from skin from healthy controls. For both CD4⁺, CD8⁺ and $\gamma\delta$

T cells, we found an increased number of cells derived from nickel-exposed skin relative to those derived from vehicle-treated skin and skin from healthy donors (Fig. 3). In agreement with the histological data, we found that CD4⁺ T cells were the predominant cells in the skin following nickel exposure. However, we found a large variation in the number of cells recovered. This variation correlated with the histological scores of infiltration found for the patients, and this correlation was found for all the T cell subsets analysed.

To investigate the immune response further, skin-derived T cells were restimulated with PMA and ionomycin, and the numbers of IL-17⁺, IL-22⁺ and IFN- γ ⁺ cells were determined by flow cytometry. We found that skin-derived CD4⁺, CD8⁺ and $\gamma\delta$ T cells were able to produce IL-17, IL-22 and IFN- γ in the nickel-challenged skin after polyclonal stimulation. However, for CD8⁺ and $\gamma\delta$ T cells, only low numbers of cytokine-producing cells were found (data not shown). In contrast, a clear increase in the number of CD4⁺ T cells producing IL-17, IL-22 and IFN- γ was found in the most infiltrated skin biopsies (Fig. 3). Interestingly, the percentage of IL-17⁺ CD4⁺ T cells was, on average, twice as high in cells derived from the skin than in the skin-homing cells in the periphery from the same individuals (skin, 7.78% $\pm$ 3.86%; blood, 3.07% $\pm$ 0.58%), which highlights the important role of IL-17 in the skin.

Discussion

In this study, we have shown that CD4⁺ T cells producing IL-17, IL-22 and IFN- γ are important effector cells in the eczematous reactions of nickel-induced ACD in humans. In the blood, we found an increased frequency of the total number of $\gamma\delta$ T cells in nickel-allergic patients as compared with healthy controls (data not shown). However, the fractions of skin-homing CD4, CD8 and $\gamma\delta$ T cells producing IL-17, IL-22 or IFN- γ in the blood did not differ between nickel-allergic patients and healthy controls.

Elevation of $\gamma\delta$ T cells in the peripheral blood has been associated with activation of the immune system, as seen in patients with autoimmune diseases (28, 29). However, whether this is a general phenotype of patients with ACD requires further investigation. We found no difference between nickel-allergic patients and controls regarding the distribution of the CLA⁺ subset of peripheral blood cells and their associated cytokine production. We have previously found nickel-specific Th1 and Th17 cells in peripheral blood from nickel-allergic patients but not in healthy controls (8). In this study, we examined the cytokine profile after polyclonal restimulation rather than after nickel-specific stimulation, as we wanted to

Table 1. Punch biopsies were collected from nickel challenged skin (from the lower back) from five nickel allergic patients with a minimum 2+ reaction on the upper back

	Clinical score		CD4 infiltration	CD8 infiltration	$\gamma\delta$ T cell count per section
	Upper back	Lower back			
43	Control	–	1+	0	0
44	Control	–	3+	0	0
45	Control	–	2+	1+	0
46	Control	–	2–3+	0–1+	0
47	Control	–	3+	0–1+	0
48	Control	–	2+	0–1+	0
49	++	+	3+	0	1
50	++	+	2+	0	5
51	+++	+++	6+	4+	31
53	+++	++	6+	4+	36
55	++	+	3+	1+	6

As a control, healthy skin from 6 donors was collected. Skin sections were stained with antibodies against $\gamma\delta$ T cell receptor, CD4, and CD8, and the infiltration of the T cell subtypes in the skin was evaluated in a blinded manner on a scale from 0 to 6. $\gamma\delta$ T cells were counted, owing to their low prevalence on the sections.

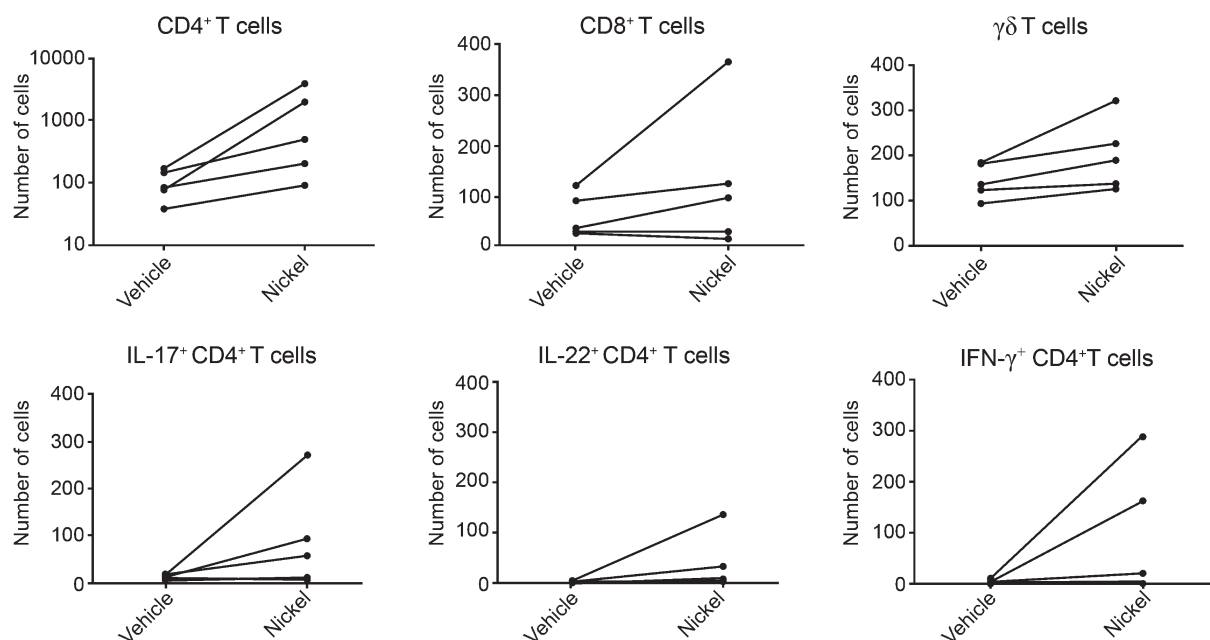


Fig. 3. T cell subtype composition and cytokine production in skin-derived T cells. We compared the number of T cells isolated from biopsies of skin, either nickel-challenged or vehicle-challenged, from nickel-allergic patients. There was a marked increase in the number of CD4⁺ T cells (log scale), but also an increase in the numbers of γδ T cells and CD8⁺ T cells, in nickel-challenged skin. We also compared the number of cytokine-producing CD4⁺ T cells in nickel-challenged skin and vehicle-challenged skin from paired biopsies. In the most infiltrated skin biopsies (by histological evaluation), we found the highest number of cytokine-producing cells and a marked increase in the number of interleukin (IL)-17⁺, IL-22⁺ and interferon (IFN)-γ⁺ CD4⁺ T cells. In the less infiltrated skin biopsies, there was no detectable increase.

examine the response directly without having the cells in culture. Thus, specific stimulation appears to be required for detection of differences in skin-homing T cells in the peripheral blood of allergic individuals and healthy controls. Furthermore, as the ligand for γδ T cells still needs to be characterized, it is unknown whether γδ T cells can be activated by nickel in the same way as αβ T cells (30). It has recently been shown in mice that γδ T cells can be activated in a TCR-independent and cytokine-dependent way, underlining that αβ and γδ T cells are probably activated differently by nickel (31).

Despite the fact that we only included patients with at least a 2+ patch test reaction to nickel on the upper back, we saw milder reactions in some of the patients following patch testing on the lower back, which is where the biopsies were collected. There was a clear correlation between the clinical evaluation, the histological assessment, and the number of T cells and their distribution in skin cultures. The control staining performed on skin sections from healthy, non-allergic individuals challenged with nickel showed no noteworthy T cell infiltration. This allows us to conclude, as previous studies have indicated, that the histological results showing infiltration in nickel-challenged skin

from allergic individuals are not attributable to the irritant properties of nickel sulfate (4). In accordance with previous studies, we found that CD4⁺ T cells represent the majority of infiltrating cells (3–5). Studies of skin-derived T cell clones from nickel-challenged skin of allergic individuals have emphasized the importance of CD4⁺ T cells, with a minority of T cells being CD8⁺ T cells (32).

The marked increase in the number of CD4⁺ T cells producing IL-17, IFN-γ and IL-22 in nickel-challenged skin that we found is well in line with previous findings of increased gene expression of the transcription factor RORC and IL-17A, IL-17F and IFN-γ in positive patch test biopsies from allergic patients with different contact allergies (33). Both CD8⁺ cells and γδ T cells appear to be involved in the elicitation phase of ACD, although to a lesser degree than CD4⁺ T cells, as these cells are recruited to and produce effector cytokines in the skin (Figs. 2 and 3, data not shown). It has been shown previously that T cells can co-produce IL-17/IFN-γ, IL-17/IL-22, and IL-22/IFN. In the present study, we did not investigate the presence of cytokine-co-producing T cells. It has recently been shown that only a minor fraction of skin-infiltrating T cells are antigen-specific, and it was suggested that non-specific T cells are important players

in the pathogenesis of ACD(3). In our study, the specific response of skin-infiltrating T cells was not tested. However, the cytokine-producing skin-infiltrating T cells found in our study most likely comprise both nickel-specific and non-specific cells, and it must be assumed that both groups are involved in the immune response to nickel.

The presence of $\gamma\delta$ T cells in human skin, and especially in ACD skin, has been known for some time, whereas the biological role and repertoire have not been completely elucidated (34–36). In this study, we found $\gamma\delta$ T cells producing IL-17, IL-22 and IFN γ in the skin, although only a small proportion of skin-derived $\gamma\delta$ T cells produced cytokines. This may be because the skin-derived $\gamma\delta$ T cells have functions other than cytokine production, or because they produce cytokines other than the ones examined. $\gamma\delta$ T cells have been suggested to have an anti-inflammatory role in mouse models of ACD, and therefore the human skin-derived $\gamma\delta$ T cells might be IL-10-producing (37–39). However, this needs further investigation. Furthermore, dermal $\gamma\delta$ T cells have been shown to produce IL-17 following stimulation with IL-23 and IL-1 β independently of TCR stimulation, and whether this can be detected after the polyclonal reactivation used in the present study is unknown (40).

The increased numbers of IL-22-producing CD4⁺ T cells in the patients with the highest clinical scores suggest that IL-22 has a role in ACD. Recent studies have shown that IL-22 is important for the maintenance of epithelial homeostasis, with keratinocytes being well-characterized

IL-22 target cells, and in the blood Th22 cells are abundant in the skin-homing memory T cell subset (8, 14). Upregulation of IL-22R on keratinocytes is found in inflammatory skin conditions such as ACD and psoriasis (8, 41). *In vitro* studies and murine studies have shown that IL-17 and IL-22 stimulate keratinocytes to release IL-1 β , a cytokine that has also been linked to ACD (42). In this way, IL-1 β provides positive feedback for more IL-17 and IL-22 production (43). Thus, our results support the idea that the same mechanism is used in human ACD, even though further investigations are required.

In conclusion, our study shows that CD4⁺ T cells producing IL-17, IL-22 and IFN- γ are important effector cells in the eczematous reactions of ACD caused by nickel. In view of the pro-inflammatory effects of these cytokines, the CD4⁺ T cells producing IL-17, IL-22 and IFN- γ most probably contribute to the immunopathology of nickel-induced contact dermatitis. Further research is required to understand the underlying processes and their interactions in the pathogenesis and temporal development of ACD.

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