

Th17/Tc17 infiltration and associated cytokine gene expression in elicitation phase of allergic contact dermatitis

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Summary

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Conflicts of interest

None declared.

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Background Allergic contact dermatitis (ACD) is a typical delayed-type hypersensitivity to sensitizing haptens mediated by T cells. Th1/Tc1 cells are currently considered to be the primary effectors in ACD. There is little information concerning the role played in ACD in humans by Th17/Tc17 cells, a recently defined subpopulation of effector T cells.

Objectives In the present report we attempted to characterize Th17/Tc17 cells in the infiltrates of the skin in the elicitation phase of ACD.

Methods Th17 as well as Th1/Th2 cytokine gene expression was examined by semiquantitative real-time polymerase chain reaction in paired samples of positive patch test biopsies and normal skin from 11 patients allergic to nine different allergens. The *in situ* characterization of interleukin (IL)-17-producing cells was carried out using anti-RORC and anti-T-cell subset antibodies by double immunofluorescence.

Results Compared with normal paired skin samples, gene expression of transcription factor for human Th17 cells, RORC, and Th17-related cytokines IL-17A, IL-17F and IL-23 was significantly increased in positive patch test biopsies. The mRNA for interferon- γ and IL-4 was also increased. In the dermal infiltrates, about 20% of the infiltrating cells were IL-17-producing cells as they expressed RORC, and such RORC-expressing cells were detected in both CD4+ (~30%) and CD8+ (~20%) subsets.

Conclusions This is the first demonstration of Th17/Tc17 cells in the elicitation phase of human ACD, showing that they are a regular participant in the immunopathology of this common allergic reaction regardless of the nature of the triggering allergen.

Allergic contact dermatitis (ACD) is a cutaneous delayed-type hypersensitivity reaction which is initiated when allergens, usually haptens, penetrate the skin and modify the host's structural proteins. Such altered self proteins are perceived by the host as foreign and are captured and conveyed by antigen-presenting cells (APCs) to the draining lymph nodes. There antigen-specific T lymphocytes, recognizing the antigen displayed in the major histocompatibility complex molecules on the surface of the APCs and in the presence of costimulation provided by APCs, undergo activation and clonal expansion.¹ The lymphocytes thus activated will then migrate to the peripheral tissue ready for subsequent encounter with the same antigen. In the effector or elicitation phase, the presence of even one antigen-specific T lymphocyte at the site of antigen

re-introduction in the skin triggers an antigen-specific immunological response to clear the antigen with inflammation and tissue destruction manifested by ACD symptoms such as erythema, oedema, and even blisters.¹ However, at the ACD elicitation sites, the frequency of antigen-specific T cells is low despite dense infiltration. Our knowledge concerning the nature of the infiltrating cells in ACD is incomplete, and both the specificity and the mechanisms of action of these cells require further investigation.

Both Th1 and Th2 T cells are involved in ACD,¹ but other cells such as NK T cells, NK cells and B1 cells not only contribute to the process but are essential for the efficient execution of the elicitation phase.^{2,3} Th17 cells are a recently defined lineage of effector T cells with the expression of

distinctive transcription factor ROR γ t (RAR-related orphan nuclear receptor γ t in mice; RORC, RAR-related orphan nuclear receptor C in humans), elaboration of a set of signature proinflammatory cytokines such as interleukin (IL)-17A, IL-17F, IL-21 and IL-22, and the expression of characteristic chemokine receptors such as CCR6.⁴ IL-23 is important for the development of the effector functions of Th17 cells.⁴ The function of Th17 cells is the defence against extracellular bacterial and fungal infections, but they are also the main inflammatory cells involved in tissue destruction in autoimmunity.^{4,5} Studies have shown that Th17 cells are involved in contact hypersensitivity (CHS) in murine models. IL-17, the signature cytokine of Th17 cells, has been detected in human T-cell clones specific for nickel,⁶ and immune response to contact allergens was impaired in IL-17-deficient mice.⁷ However, there are few studies that directly examine Th17 cells and their roles in ACD in humans.^{5,8}

In the present report we examine Th17 signature cytokine gene expression and the frequency of IL-17-producing cells in the infiltrate of positive patch test reactions to a variety of allergens, and find that gene expression of IL-17 and some related cytokines and RORC-positive CD4+ and CD8+ T cells are significantly increased in the lesions. These data indicate that Th17/Tc17 cells are regular participants in the immunopathology of this allergic reaction regardless of the nature of the triggering allergen.

Materials and methods

Patients

Patients were recruited from our cutaneous allergy clinic, and were being evaluated for ACD (see Table 1 for details). The intensity of reaction to various allergens was determined by the North American Contact Dermatitis Group scoring system.⁹ Paired biopsy samples of positive patch test sites and

normal control skin were collected and snap-frozen in liquid nitrogen before being stored at -80°C for later RNA extraction. Additionally, patch test-positive biopsies were obtained from additional patients and frozen in OCT compound for immunocytochemical studies. Two normal adult skin samples from surgical remnants and one tonsil were used as negative and positive controls for immunocytochemistry. The study was approved by the local institutional review committee and it followed the Declaration of Helsinki Principles for research involving human subjects.

Real-time polymerase chain reaction

Total RNA was isolated from the biopsies using an RNeasy kit (Qiagen, Valencia, CA, U.S.A.) after mechanical homogenization. cDNA was synthesized using a reverse transcription kit (Amersham, Piscataway, NJ, U.S.A.). Real-time polymerase chain reaction (PCR) was carried out in a LightCycler (Roche Applied Science, Indianapolis, IN, U.S.A.) using FastStart DNA Master SYBR Green kit (Roche Applied Science) according to the manufacturer's instructions and as described previously.¹⁰ The cytokine mRNA was normalized to the expression of 18S RNA (which showed comparable levels of expression in patch test lesions and normal skin samples in the present study; data not shown), and the fold changes were calculated by comparing mRNA levels of positive patch test skin with those from normal skin of the same patients. The primers for the cytokines and transcription factor are as follows: IL-4 sense: 5'-AGA AGA CTC TGT GCA CCG AGT TGA-3', antisense: 5'-CTC TCA TGA TCG TCT TTA GCC TTT-3'; interferon (IFN)- γ sense: 5'-GCA TCC AAA GAG TGT GGA G-3', antisense: 5'-GCT CTT CGA CCT CGA AAC-3'; IL-17A sense: 5'-ACC AAT CCC AAA AGG TCC TC-3', antisense: 5'-GGG GAC AGA GTT CAT GTG GT-3'; RORC sense: 5'-TTT TCC GAG GAT GAG ATT GC-3', antisense: 5'-CTT TCC ACA TGC TGG CTA CA-3'; IL-17F sense: 5'-TGA AGC TTG ACA TTG GCA TC-3', anti-

Table 1 Allergen and patient data

Age (years)/sex/race ^a	Allergen ^b	Score/duration	Study ^c
40/M/W	Balsam	1+/48 h	PCR
69/F/W	MMA	1+/72 h	PCR
49/M/A	Ni	1+/48 h	PCR
63/M/W	Ni	1+/48 h	PCR
57/M/W	Epoxy	2+/48 h	PCR
77/M/W	Bacitracin	2+/48 h	PCR
49/M/A	Ni	2+/96 h	PCR
43/F/AA	Tixocortol	2+/7 days	PCR
53/M/W	Epoxy	1+/48 h	PCR/ICC
49/F/AA	PPD	1+/48 h	PCR/ICC
36/M/W	Gold	2+/96 h	PCR/ICC
71/F/AA	Ni	2+/48 h	ICC
63/M/W	Thimerosal	2+/96 h	ICC
43/F/AA	Tixocortol	3+/96 h	ICC

^aRace: A, Asian; AA, African-American; W, white (caucasian). ^bAllergen: MMA, methyl-methacrylate; PPD, para-phenylenediamine. ^cStudy: PCR, real-time polymerase chain reaction; ICC, immunocytochemistry.

sense: 5'-TTC CTT GAG CAT TGA TGC AG-3'; IL-23p19 sense: 5'-CTG ATA GCC CTG TGG GCC AG-3', antisense: 5'-GCT GCC TTT AGG GAC TCA GG-3'; 18S sense: 5'-AAC CCG TTG AAC CCC ATT-3', antisense: 5'-CCA TCC AAT CGG TAG TAG CG-3'. The PCR was run under the following conditions: initial denaturation 10 min at 95 °C, and then denaturation at 94 °C for 5 s, annealing/detection at 56 °C for 20 s, and extension at 76 °C for 15 s for 40 cycles. The real-time PCR products were verified for the correct size by agarose gel electrophoresis and melting curve analysis.

Double immunofluorescence

Skin biopsies in OCT were cut into 5-µm sections and fixed in methanol. The slides were incubated overnight with a rabbit antihuman RORC polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.) at 1 : 100 dilution, followed by goat antirabbit IgG fluorescein isothiocyanate (FITC) conjugate. As negative control, normal rabbit serum was used in place of the first antibody. For double labelling of T cells and T-cell subsets, the slides were then incubated with phycoerythrin-labelled monoclonal anti-CD3, CD2, CD4 or CD8 antibodies. The slides were mounted in buffered glycerol containing 4',6-diamidino-2-phenylindole. The slides thus stained were examined by a fluorescence microscope and digital images recorded. The cells were counted on the digital pictures after superimposition of the RORC staining (green) and T-cell staining (red) images. On average nine fields from each double-stained section were counted with a mean of 130 infiltrating cells per field. RORC+ cells were recognized by the presence of FITC-positive stains over the nuclei of the cells that were also positive for a T-cell marker.

Data analysis

The Mann–Whitney U-test was used to analyse cytokine mRNA fold changes. $P < 0.05$ was regarded as statistically significant.

Results

Patient data

Table 1 summarizes the demographic information, the allergens tested, the reaction scores and duration as well as the types of studies performed. Fourteen biopsies from Asians ($n = 2$), African-Americans ($n = 4$) and caucasians ($n = 8$) were included in the study. These patients were allergic to nine allergens and the scores ranged from 1+ to 3+ (1+ in six cases, 2+ in seven cases and 3+ in one case).

Th1, Th2 and Th17 cytokine gene expression at allergic contact dermatitis elicitation sites

To characterize IL-17-producing Th17/Tc17 cells in human ACD, we examined 11 cases of positive patch test biopsies for

Th17 as well as Th1 and Th2 cytokine gene expression. Th1, Th2 and Th17 cytokine mRNAs were increased in the positive patch test sites compared with the paired normal skin samples from the same patients. Thus, IFN- γ mRNA was increased 6.9-fold (median; range 1.2–370, $P < 0.001$), IL-4 10.4-fold (median; range 1.6–38.4, $P < 0.005$), IL-17A 5.0-fold (median; range 1.3–186.1, $P < 0.005$), IL-17F 2.2-fold (median; range 0.6–5.1, $P < 0.05$) and IL-23p19 2.1-fold (median; range 1.1–13.5, $P < 0.005$) (Fig. 1a). The characteristic Th17 master transcription factor in humans, RORC, was also increased (median 3.2-fold, $P < 0.005$) (Fig. 1a). No correlation of IL-17 cytokine levels with that of either Th1 or Th2 cytokines were detected. However, in one case with nickel allergy, two reaction sites with different scores, 1+ at 48 h duration and 2+ at 96 h duration, were found to exhibit a decrease in IFN- γ but an increase in IL-17A mRNA expression as the reaction increased in severity (Fig. 1b). A similar correlation pattern between IL-17A and IL-4 gene expression was also observed in this case (data not shown). This seemed to indicate that more IL-17A but less IFN- γ or IL-4 was produced as the reactions aggravated. The above results in this case

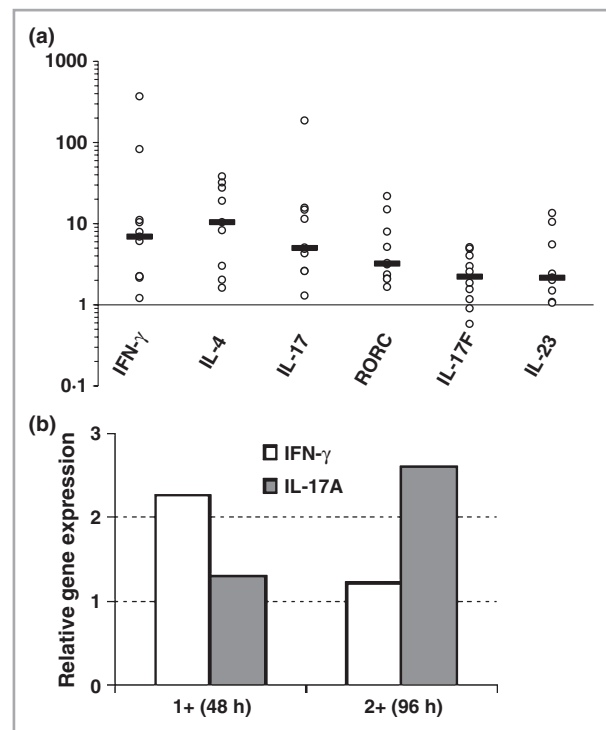


Fig 1. Increased Th1, Th2 and Th17 cytokine gene expression in allergic contact dermatitis positive patch test sites. (a) The relative increase of cytokine mRNAs (medians) is shown in log scales in comparison with their respective normal skin samples. By Mann–Whitney U-test, mRNA increased significantly in the patch test sites over paired controls for interferon (IFN)- γ ($P < 0.001$), interleukin (IL)-4 ($P < 0.005$), IL-17A ($P < 0.005$), RORC ($P < 0.005$), IL-17F ($P < 0.05$) and IL-23p19 ($P < 0.005$). (b) Relative fold change in cytokine mRNA expression as the patch test score increased over time in one patient with nickel allergy and a crescendo patch test reaction.

demonstrate that at the sites of ACD elicitation, there is an increased gene expression of IL-17A and related cytokines apart from increased gene expression of IFN- γ and IL-4, and a probable inverse relationship between IL-17 and Th1/Th2 cytokines. Whether this is generally true for all the cases and allergens remains to be investigated further.

RORC+ interleukin-17-producing T cells in allergic contact dermatitis infiltrates

Immunocytochemistry study was also carried out for Th17 transcription factor RORC and T-cell surface markers in positive patch test biopsies from six patients. RORC+ cells were mainly localized in the perivascular infiltrating patches of ACD elicitation sites (Fig. 2a). Of the total population of infiltrating CD3+ T cells, 18% were RORC+ (CD3+RORC+ IL-17-producing cells). Of the total population of infiltrating CD4+ T cells, 33% were RORC+ (CD4+RORC+ Th17 cells). Of the total population of infiltrating CD8+ T cells, 21% were RORC+ (CD8+RORC+ Tc17 cells) (Fig. 2b). Of all the RORC+ cells in the infiltrates, about 95% were CD3+ T cells,

58% CD4+ cells, and 26% CD8+ cells (Fig. 2c). There was a small number of CD3–RORC+ cells in the infiltrates. The RORC+ T cells were among the superficial dermal perivascular patches but no RORC+ T cells could be detected in the epidermis. No RORC-stained infiltrating cells were detectable in the healthy control skin samples examined, and there was a high percentage of RORC+ cells (~20–30%) in the tonsil specimen (in the parafollicular areas) (data not shown).

Discussion

Th17 cells, a recently defined lineage of T cells distinct from Th1 and Th2 cells, are the main effectors for defence against extracellular bacterial infections and for tissue destruction in inflammation and autoimmunity.⁴ Such Th17 cell function is achieved through the signature cytokines they produce, such as IL-17A, IL-17F, IL-21 and IL-22.¹¹ Th17 cells express the characteristic chemokine receptor CCR6 which determines their migration pattern into epithelial sites for defence and inflammation.^{12,13} In mice Th17 cells play a pathogenic role in autoimmune disease models such as experimental autoimmune

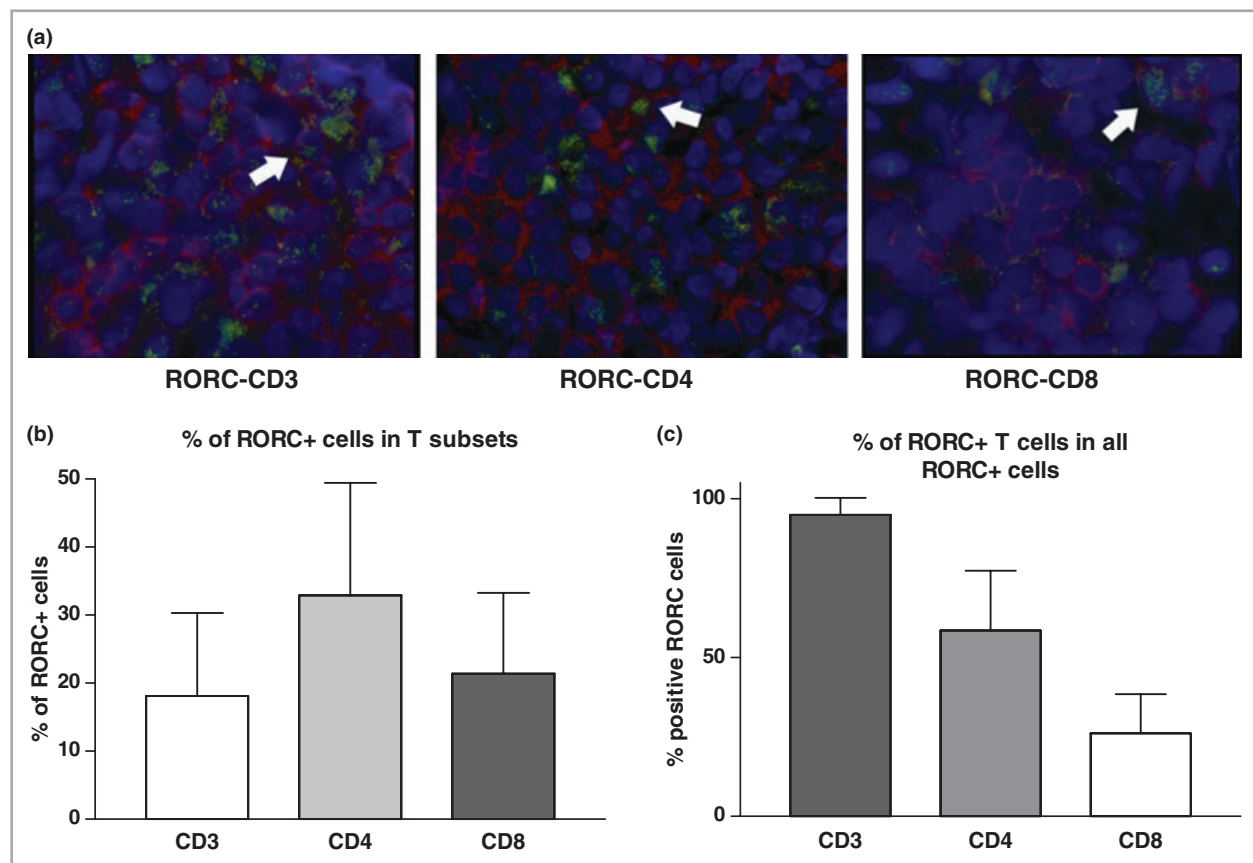


Fig 2. RORC+ cells in allergic contact dermatitis. (a) The skin sections are stained with RORC antibody (green) and phycoerythrin-labelled anti-T-cell monoclonals (anti-CD3 or CD4 or CD8) (red). The nuclei are counterstained by 4',6-diamidino-2-phenylindole (blue). (b) Percentage RORC+ cells in T-cell subsets. Among the six cases of positive patch test biopsies studied by immunofluorescence, $18.1 \pm 12.2\%$ (mean $\pm$ SD) of RORC+ cells were CD3+ T cells, $32.9 \pm 16.5\%$ RORC+ cells were CD4+, and $21.4 \pm 11.9\%$ were CD8+. (c) Of all RORC+ cells, $94.9 \pm 5.4\%$ were CD3+, $58.5 \pm 18.9\%$ were CD4+, and $26.2 \pm 12.3\%$ were CD8+.

encephalomyelitis and collagen-induced arthritis.^{4,14} IL-17-producing cells are now considered to be the main conveyors of autoimmunity previously presumed to be mediated by Th1 cells.^{4,14} ROR γ t, RAR-related orphan nuclear receptor γ t, or its human equivalent RORC, is the master transcription factor dictating T-cell differentiation down the Th17 rather than the Th1 or Th2 pathways.^{4,14,15} Transforming growth factor- β , together with inflammatory cytokines such as IL-6, IL-1 β or IL-21, has been shown to be critical for the development of Th17 cells, while Th1 and Th2 cytokines, IFN- γ and IL-4, could inhibit Th17 cell development.^{4,11} Such mode of actions between these subsets of T cells satisfies the 'self-promoting' yet 'mutually regulating' characteristics in defining T-lymphocyte lineages, establishing Th17 cells' distinct identity. Recent data showed that ROR γ t could directly bind to the specific ROR responsive element in the IL-17 gene promoter region, leading to increased IL-17 expression.¹⁶ Indeed, the forced expression of ROR γ t or RORC in naïve T cells could induce Th17 cell differentiation in mice or in humans.^{15,17} These results indicate that ROR γ t is an appropriate marker for IL-17-producing T cells. ROR γ and ROR γ t differ only at the N-terminal amino acid (AA) residues in humans as well as in mice. ROR γ t does not have the first 24 AA residues of ROR γ but, instead, it has an additional three AA residues (Met-Arg-Thr) at the very N-terminal end. This makes it difficult to raise antibodies with sufficient specificity to distinguish the two ROR γ isoforms. The antibody we used in the study recognizes the middle portion of the protein and, thus, both of the isoforms. However, as we only identified the cells positive for both ROR γ and a T-cell marker, we can be reasonably confident that they were the T cell-specific isoform of the transcription factor.

The current paradigm maintains that Th1 cells are the primary effectors while Th2 cells are minor effectors in ACD/CHS.¹ Most chemical haptens, such as dinitrofluorobenzene (DNFB), trinitrochlorobenzene (TNCB) and oxazolone, induce a Th1 response while only some, such as FITC and trimellitic anhydride, induce a Th2-dominant CHS response. However, there is also experimental evidence demonstrating that in both IFN- γ - and IFN- γ receptor 1-deficient mice, CHS developed normally to TNCB and DNFB. In contrast, CHS to TNCB and DNFB was reduced in mice deficient in either IL-4 or STAT-6, a Th2 cell-specific transcription factor. These studies indicate that Th2 as well as Th1 cytokines are important in CHS response.⁸ Characterization of the new Th17 lineage and the study of its role in human ACD and murine CHS have helped to redefine the primary effectors in this disease. Thus, nickel-specific CD4+ clones isolated from humans can produce IL-17 which can enhance local inflammation in ACD by recruiting other cells and stimulating keratinocytes to elaborate proinflammatory cytokines.⁶ In IL-17-deficient mice, experimentally induced CHS response is impaired with reduced ear swelling and cellular infiltration as well as reduced chemokine production and intercellular adhesion molecule (ICAM)-1 expression.⁷ However, T cells from IL-17-deficient mice can still sustain a weakened CHS response,⁷ indicating that either

other Th17 cytokines such as IL-21 and IL-22 are still functional in this situation, or that Th17 is just one of the participants in CHS. In our study, the presence of significant quantities of RORC+ IL-17-producing T cells (about 20%) of both CD4 and CD8 subsets within the perivascular cellular infiltrates in superficial dermis, the site of ACD onset, indicates that they might be (one of) the effectors in ACD. This increased presence of IL-17-producing cells in the dermis represents a significant enrichment of such cells as IL-17-producing cells comprised only 1.5% of T cells in the regional lymph nodes from allergen-primed mice.¹⁸ IL-17 exerts its effects through IL-17 receptors which are widely expressed by both immune and nonimmune cells.¹¹ The major functions of IL-17 include the recruitment of inflammatory cells, induction of chemokines (such as CXCL1, CXCL2, CXCL5 and CXCL8) and proinflammatory cytokines (such as IL-1, IL-6 and tumour necrosis factor- α) by endothelial and epithelial cells, and the induction of adhesion molecule expression (such as ICAM-1 and vascular cell adhesion molecule-1).¹¹ The inflammation in ACD is thus likely to be instigated and sustained by the proinflammatory effects of IL-17A/IL-17F and other cytokines produced by allergen-specific Th17/Tc17 cells in the lesions.

The superficial perivascular dermis is the site of ACD onset during the elicitation phase. The lymphocytes as well as other innate and acquired immune cells respond to the challenged antigen in the vicinity of superficial vessels as the immune cells are coming out of the blood into the tissue in an attempt to contain and eliminate the antigen. The epidermal involvement is minimal initially, and severe inflammatory damage in the epidermis happens only as the reaction intensifies. That most RORC+ IL-17-producing cells were found within dermal infiltrates in our cases seems to be concordant with such an immunopathological scenario. In our cases the prominent infiltration was located in the superficial dermis with less pronounced epidermal changes as the scores of our patch test biopsies were mostly 1+ to 2+. One of the effects of IL-17 is the recruitment of neutrophils to the inflammatory sites. In contrast to mouse CHS, neutrophil infiltration is not a feature in ACD of humans due to species differences.¹⁹ The effector role of IL-17 in ACD, therefore, must be through its other proinflammatory effects. In addition to IL-17A, IL-17F and IL-23p19 were also increased in ACD in our cases. These cytokines could also play a role in ACD, and indeed, IL-23-deficient mice did show impaired hypersensitivity reaction.²⁰ The role played by other Th17 cytokines in ACD, such as IL-21 and IL-22, remains to be studied.

CD8 T cells are the primary effectors in ACD while CD4 T cells play a regulatory role.¹⁸ In mice deficient in CD8 T cells, CHS response was impaired, but in CD4 T cell-deficient mice CHS was either detected as normal or enhanced. IL-17 can be produced by both CD4 and CD8 T-cell subsets although disparity exists about the role of these two IL-17-producing subsets in murine CHS. Thus, in IL-17-deficient mice, CD4 but not CD8 cells from the regional lymph nodes had defective proliferation and IFN- γ production,⁷ yet in another study, in mice depleted of CD4 cells, IL-17 blockade, but not IFN- γ

blockade, led to reduced tissue swelling after hapten challenge.¹⁸ The situation in humans is less clear. Our results seem to indicate that both CD4 and CD8 subsets probably contribute to the ACD immune response as they both have the capacity to produce IL-17 and are present in significant numbers in the infiltrate. If IL-17 rather than IFN- γ is the primary cytokine in driving the inflammatory reactions in ACD in humans as suggested by many recent studies on the function of IL-17-producing cells,^{4,5,8,11,14} then both CD4 and CD8 subsets seem to be equally capable of sustaining the response as both can produce IL-17 in view of the master transcription factor they express. Thus, the primary effectors of ACD are probably Th17/Tc17 cells rather than Th1/Tc1 cells as stipulated by the current paradigm. ACD is generally construed as being caused by harmless hapten chemicals. However, most of these chemicals have the capacity to modify AA residues of cutaneous structural proteins¹ which may indeed have acquired altered properties if permitted to persist. Thus, such modification produces in the skin neoantigens which are recognized by the immune system as foreign. These neoantigens are, therefore, cleared by IL-17-producing cells in a similar fashion to extracellular bacteria. Previous studies have shown that IL-17 could be produced by nonlymphocytes such as monocytes and macrophages,²¹ albeit only weakly in a small percentage of them. Our staining of the infiltrates showed some RORC+CD3[−] cells which, though not further characterized in our study, might represent such cells.

In conclusion, our results indicate that IL-17-producing (Th17/Tc17) cells are regular participants in the cellular infiltrates and thus might play an important role in the elicitation phase of ACD in humans as we identified transcripts for Th17 cytokines and RORC⁺ lymphocytes in allergic skin reactions to nine different allergens. The study of these cells and the cytokines they produce may shed light on a new aspect of the immunopathology of the disease, and the present Th1/Th2 paradigm for ACD should thus be broadened to incorporate Th17/Tc17 lymphocytes.

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