

# Characterization of the T cell response in allergic contact dermatitis caused by corticosteroids

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## Summary

**Background.** Delayed allergic hypersensitivity reactions have classically been described as type IV reactions, which are caused by T cells; however, the respective roles of CD4<sup>+</sup> and CD8<sup>+</sup> cells are yet to be defined. A central role for CD8<sup>+</sup> cytotoxic T cells as effector cells has been suggested.

**Objectives.** To determine the type of T cell involved in corticosteroid allergy.

**Methods.** We analysed the kinetics of T cell recruitment and the cytokine production profile in positive patch tests of 27 corticosteroid-sensitized patients, as compared with control sites and control subjects. Skin biopsies, collected at 8, 24 and 48 hr following drug application, were embedded in paraffin for histological and immunohistological staining, and, in some cases, also deep-frozen for gene expression analyses.

**Results.** CD3<sup>+</sup> T cells were rapidly recruited in concert with the positivity of the patch test sites. High levels of interleukin (IL)-4, IL-5 and, to a lesser extent, interferon- $\gamma$  suggested that both Th2 and Th1 cytokines were implicated. IL-4 was also produced by  $\gamma\delta$  T cell receptor (TCR) lymphocytes.

**Conclusions.** This study showed that, in allergic contact dermatitis caused by corticosteroids, the inflammatory infiltrate is composed of CD3<sup>+</sup> T cells with a predominant Th2 cytokine profile, among which IL-4 is also produced by  $\gamma\delta$  TCR lymphocytes.

**Key words:** allergic contact dermatitis; corticosteroid; CD3<sup>+</sup>; IL-4;  $\gamma\delta$  TCR lymphocytes.

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Corticosteroids (CSs), which constitute the anti-inflammatory treatment of choice in allergic disorders, may cause immediate and delayed hypersensitivity reactions themselves. Allergic contact dermatitis (ACD) is the most frequent presentation of the latter type; however, patients previously sensitized by topical use of a CS may also react upon systemic administration (1). CSs are thus potential contact allergens, as well as causes of systemic adverse drug reactions.

Delayed allergic reactions have classically been described as type IV reactions caused by T cells, but the respective roles of CD4<sup>+</sup> and CD8<sup>+</sup> cells are still being discussed (2–6). Differences in the clinical presentation and in the severity of delayed drug allergies have been related to the distinct natures of the effector T cell subsets involved. CD8<sup>+</sup> cytotoxic T cells mediate severe bullous diseases, that is, toxic epidermal necrolysis, in particular, whereas, non-bullous – and hence less severe – forms of allergic drug reaction are thought to be mediated by CD4<sup>+</sup> T helper (Th) cells (3). In this case, the current paradigm maintains that Th1 cells, and not Th2 cells, are the primary effector cells. However, recent studies by Rozières et al. (7–9) showed that CD8<sup>+</sup> T cells are present in the skin of allergic patients at a very early stage before the onset of clinical symptoms, and suggested their effector role in all drug allergies. In addition, CD4<sup>+</sup> T cells, which become the predominant T cell subset at the time of the development of clinical lesions, might play only a modulatory role. Similar findings have been observed in mice for ACD (10, 11) and atopic dermatitis (12, 13).

Despite the mounting evidence that the main effector cells of ACD are CD8<sup>+</sup> cytotoxic T cells, it is possible that the nature of the antigen and/or its access pathway may contribute to determining the cell type involved in the inflammatory response (5, 6). Besides the interferon (IFN)- $\gamma$ -producing CD8<sup>+</sup> T cells, importance has been attributed to Th1 T lymphocytes, and more recently also to Th17 cells, as a subpopulation implicated in the pathogenesis of various allergic disorders (14). A Th2 response seems to be only exceptionally induced (15–20).

With regard to delayed allergic hypersensitivity reactions to CSs, the influence of the intrinsic anti-inflammatory properties of these molecules on such reactions is unknown. Positive patch test sites, which are infiltrated by allergen/drug-specific T cells, represent an experimental model of a CS-induced immune reaction that allows study of the pathophysiology of delayed allergic hypersensitivity. In skin biopsies of positive patch tests for CSs, some authors (21) have observed a T cell infiltrate (CD3<sup>+</sup>), with CD4<sup>+</sup> cells predominating over CD8<sup>+</sup> T cells, which are present to a lesser extent.

More recently, the role of Th17 and Th1/Th2 cytokine gene expression was examined in positive patch test biopsies from 11 patients allergic to nine different allergens, 2 of whom were positive to tixocortol pivalate, and levels of interleukin (IL)-17, IFN- $\gamma$  and IL-4 were all found to be increased (22).

In the present study, we wanted to gain insights into the nature of the T cells involved in CS allergy. Therefore, we analysed the kinetics of T cell recruitment and the cytokine production profile in positive patch tests of 27

CS-sensitized patients as compared with patch tests with CSs to which patients reacted negatively, as well as normal skin.

## Materials and methods

### Patients

Twenty-seven patients with a history of a positive patch test reaction to at least one of 68 CS molecules tested (23), as well as 9 control subjects, were included in this study. The patients were otherwise healthy, and were only investigated when their dermatitis was clinically in remission. The study and data accumulation were conducted with the approval of the Institutional Ethical Committee, Commission d'Ethique Biomédicale Hospitalo-Facultaire de l'Université Catholique de Louvain (NCT 340320084407); informed consent for all of the diagnostic procedures was obtained from all study subjects.

The data from the all CS-sensitized and control subjects are summarized in Table 1. To include a sufficient number of subjects, the initial selection criterion was solely based on the existence of a previous positive patch test reaction to at least one CS, irrespective of the particular molecule. Wherever possible, tixocortol pivalate and budesonide, both of which are included in the European baseline series (24), diluted in petrolatum were chosen as the positive and/or negative control. For only 3 patients who were negative to these molecules were other CSs selected, according to their history.

To highlight the effects of each molecule, as budesonide and tixocortol pivalate have different anti-inflammatory properties, patients were initially classified by profile, and analyses were conducted in a distinct way.

All subjects were examined clinically and patch tested with CSs according to the profiles mentioned below:

- Type A: Patients (n = 8) who previously reacted to tixocortol pivalate and not to budesonide were patch tested with tixocortol pivalate as the positive molecule, and with budesonide as the control molecule (patients 1–7 and 25).
- Type B: Patients (n = 16) who previously reacted positively to budesonide but not to tixocortol pivalate were tested with budesonide as the positive molecule, and with tixocortol pivalate as the control molecule (patients 8–21 and 26, 27).
- Type 'other': Patients (n = 3) who previously reacted to a CS other than tixocortol pivalate and budesonide were tested with betamethasone 17-valerate (patient 22), hydrocortisone 17-butyrate (patient 23) or betamethasone dipropionate

**Table 1.** Patient data and patch test results

| Patient number | Positive corticosteroid markers | Time between first corticosteroid sensitivity detection and study | Sex/age (years) | Atopy | Clinical presentation    | Clinical positivity at biopsy time | Positive reaction time (hr) | Immunochemistry | Quantitative PCR |
|----------------|---------------------------------|-------------------------------------------------------------------|-----------------|-------|--------------------------|------------------------------------|-----------------------------|-----------------|------------------|
| 1              | TP                              | 8 years                                                           | F/45            | +     | Facial eczema            | +                                  | 48                          | D               | ND               |
| 2              | TP                              | 13 years                                                          | F/68            | +     | Facial eczema            | +                                  | 48                          | D               | ND               |
| 3              | TP                              | 1 year                                                            | M/77            | —     | Generalized eczema       | —                                  | —                           | D               | ND               |
| 4              | TP                              | 1 year                                                            | F/61            | —     | Generalized eczema       | +                                  | 48                          | D               | D                |
| 5              | TP                              | 1 year                                                            | M/51            | —     | Hand eczema              | —                                  | 168                         | D               | ND               |
| 6              | TP                              | 6 months                                                          | F/60            | +     | Generalized eczema       | +                                  | 48                          | D               | D                |
| 7              | TP                              | 9 years                                                           | F/51            | +     | Leg eczema               | —                                  | —                           | D               | ND               |
| 8              | B                               | 2 years                                                           | M/37            | —     | Facial eczema            | +                                  | 48                          | D               | ND               |
| 9              | B                               | 9 years                                                           | F/52            | +     | Facial eczema            | +                                  | 48                          | D               | D                |
| 10             | B                               | 4 years                                                           | M/31            | +     | Facial eczema            | +                                  | 48                          | D               | D                |
| 11             | B                               | 11 years                                                          | M/72            | —     | Hand eczema              | —                                  | 96                          | D               | ND               |
| 12             | B                               | 5 years                                                           | F/56            | —     | Stomatitis               | —                                  | 168                         | D               | ND               |
| 13             | B                               | 8 years                                                           | F/54            | —     | Generalized eruption     | +                                  | 48                          | D               | ND               |
| 14             | B                               | 2 years                                                           | M/58            | —     | Generalized eruption     | +                                  | 24                          | D               | D                |
| 15             | B                               | 1 year                                                            | M/49            | —     | Generalized eruption     | —                                  | 168                         | D               | ND               |
| 16             | B                               | 13 years                                                          | M/64            | —     | Foot eczema              | —                                  | —                           | D               | ND               |
| 17             | B                               | 1 year                                                            | F/64            | +     | Generalized eruption     | —                                  | — (retest +)                | D               | ND               |
| 18             | B                               | 1 year                                                            | F/55            | +     | Generalized eruption     | +                                  | 24                          | D               | ND               |
| 19             | B                               | 7 years                                                           | F/57            | —     | Hand and foot eczema     | —                                  | —                           | D               | ND               |
| 20             | B                               | 8 years                                                           | M/75            | +     | Generalized eczema       | —                                  | —                           | D               | ND               |
| 21             | B                               | 8 years                                                           | F/43            | +     | Hand eczema              | +                                  | 24                          | D               | ND               |
| 22             | Betamethasone 17-valerate       | 4 months                                                          | M/47            | —     | Atopic dermatitis        | +                                  | 48                          | D               | D                |
| 23             | Hydrocortisone 17-butyrate      | 14 years                                                          | M/59            | —     | Atopic dermatitis        | —                                  | —                           | D               | ND               |
| 24             | Betamethasone dipropionate      | 2 years                                                           | M/32            | —     | Hand and foot eczema     | —                                  | —                           | D               | ND               |
| 25             | TP                              | 1 month                                                           | F/51            | +     | Hand and foot eczema     | +                                  | 48                          | ND              | D                |
| 26             | B                               | 1 month                                                           | F/54            | —     | Facial eczema            | +                                  | 48                          | ND              | D                |
| 27             | B                               | 1 month                                                           | M/63            | —     | Facial-periocular eczema | —                                  | — (retest +)                | ND              | ND               |
| 28             | Control                         | —                                                                 | F/54            | —     | —                        | —                                  | —                           | D               | ND               |
| 29             | Control                         | —                                                                 | M/66            | —     | —                        | —                                  | —                           | D               | ND               |
| 30             | Control                         | —                                                                 | F/56            | +     | —                        | —                                  | —                           | D               | D                |
| 31             | Control                         | —                                                                 | M/23            | +     | —                        | —                                  | —                           | D               | D                |
| 32             | Control                         | —                                                                 | M/21            | —     | —                        | —                                  | —                           | D               | D                |
| 33             | Control                         | —                                                                 | M/60            | —     | —                        | —                                  | —                           | ND              | D                |
| 34             | Control                         | —                                                                 | F/50            | —     | —                        | —                                  | —                           | ND              | D                |
| 35             | Control                         | —                                                                 | F/46            | +     | —                        | —                                  | —                           | ND              | D                |
| 36             | Control                         | —                                                                 | F/53            | —     | —                        | —                                  | —                           | ND              | D                |

Positive corticosteroid marker(s): B, budesonide; TP, tixocortol pivalate. Control: none. D, done; F, female; M, male; ND, not done; PCR, polymerase chain reaction.

(patient 24) as the positive molecule, and with budesonide (patients 22 and 23) or tixocortol pivalate (patient 24) as the control molecule.

The 9 control subjects (5 women and 4 men, with a mean age similar to that of the allergic patient group, except for 2 medical students) had never developed a hypersensitivity reaction in the course of topical and/or systemic CS administration, or had never been previously patch tested. All of them were patch tested with tixocortol pivalate and budesonide as a negative control.

### Patch tests

Besides tixocortol pivalate and budesonide being tested in pet. 0.1% and 0.01%, respectively, ethanol (Alcool 96%; Belgalco NV/SA, Gent, Belgium) was the vehicle used to test the three other molecules, in a 0.1% dilution (17). All CSs were compounded by the hospital pharmacy, except for the two screening agents officially added to the baseline series (24).

The patch test materials used were Haye's® chambers (Destaing, Grasse, France) covered on the upper back with Fixomull Stretch® (Smith and Nephew, Auckland, New Zealand).

The three series of patches were removed after 8, 24 and 48 hr, respectively, and the patch test reactions were evaluated at the same time points and at D4, and in many cases also at D7.

The patch test reactions were evaluated according to the International Contact Dermatitis Research Group criteria (25). All clearly positive tests were taken into account.

### Histology and immunohistochemistry

In order not to miss the preclinical stage and to take into account the anti-inflammatory properties of CSs, which are responsible for delayed positivity of patch tests as compared with other allergens, three different time points were chosen for the performance of skin biopsies. Three-millimetre punch biopsies from patch tests with the positively and negatively reacting CS molecules, whether positive or negative, were collected at 8, 24 and 48 hr following drug application. Before patch testing (0 hr), normal skin from 4 patients (numbers 4, 10, 25, and 26) and 4 control subjects (numbers 30, 31, 35, and 36) was also biopsied.

The biopsies were fixed in formaldehyde and embedded in paraffin for histological Haematoxylin-Eosin-Safran (HES) and immunohistological staining. Paraffin sections were cut (5 µm), deparaffinized, rehydrated, and then sequentially incubated with the primary monoclonal

antibodies anti-CD3 (lot 128; Dako, Glostrup, Denmark), anti-CD8 (clone C8/144B; Dako), and anti-CD4 (clone 4B12; Novocastra, Newcastle, UK).

Peroxidase-conjugated goat anti-mouse immunoglobulins or anti-rabbit IgG were used as secondary antibodies when appropriate, and revealed by incubation with 3-amino-9-ethylcarbazole. The sections were counterstained with haematoxylin. Negative controls included omission of primary antibody on the control tissue sections. Stained slides were digitized by automated whole-slide image capture, with a Mirax Midi scanner (Carl Zeiss MicroImaging, Zaventem, Belgium).

Surface sections of 2400 × 2000 µm (4.8 mm<sup>2</sup>) were delimited with AXIOVISION 40 V4 software (Carl Zeiss Imaging), and cell numbers from three different 'hot spots' in epidermal and dermal fields were twice counted manually, with a margin of error of <10%.

### RNA extraction and quantitative reverse transcription polymerase chain reaction (RT-PCR)

Skin biopsies from the reacting molecule and control sites (at the different time points) were also deep-frozen in liquid nitrogen for 8 patients (numbers 4, 6, 9, 10, 14, 22, 25, and 26) and 7 control subjects (numbers 30, 31, 32, 33, 34, 35, and 36) for the performance of gene expression analysis of secreted cytokines. Total RNA was isolated from the skin with TriPure isolation reagent (Roche, Mannheim, Germany). Reverse transcription was performed on 1 µg of total RNA with oligo(dT) primer (Roche) and murine Moloney leukaemia virus reverse transcriptase (Invitrogen, Carlsbad, CA, USA). Quantitative polymerase chain reaction (PCR) amplifications were performed from cDNA corresponding to 25 ng of total RNA with primer sets and Taqman probes corresponding to human EF-1, CD3, CD4, CD8, granzyme B, IFN-γ, tumour necrosis factor (TNF)-α, IL-17, IL-4, IL-5, and IL-9, with qPCR Mastermix Taqman (Eurogentec, Seraing, Belgium). The sequences of primers and probes used are listed in Table 2. Samples were first heated for 10 min at 95°C. cDNA was amplified as follows: 40 cycles of a two-step PCR program at 95°C for 10 seconds and 60°C for 1 min. A standard curve with various concentrations of a cloned cDNA fragment was obtained for each gene for accurate quantification.

### Flow cytometry on dermal cells of positive patch test skin biopsies

Flow cytometry on dermal cells from healthy skin and from positive patch test skin biopsies (at 48 hr following patch test application) of allergic patients was performed (numbers 4, 6, 25, 26, and 27). Skin biopsies from

**Table 2.** The sequences of primers and probes used for RNA extraction and quantitative reverse transcription polymerase chain reaction

|               | Primers                                                          | Probes                                   |
|---------------|------------------------------------------------------------------|------------------------------------------|
| hEF-1         | 5'-GCCGTGTGGCAATCCAAT-3'<br>5'-GCTTCACTGCTCAGGTGAT-3'            | 5'-AAATAAGCGCCGGCTATGCCCCTG-3'           |
| CD4           | 5'-GCTAGGCATCTTCTCTGTGTC-3'<br>5'-TTCCTGCCTGCGGACCAG-3'          | 5'-CAGATCCCCTGAGCCTCCC-3'                |
| CD8           | 5'-CAGCTGAGTGTGGTTGATTCC-3'<br>5'-ACCGGCACACTCTTCTTGA-3'         | 5'-CCCACCACTGCCCAGCCCAC-3'               |
| CD3           | 5'-TGAGGGCAAGAGTGTGTGAG-3'<br>5'-CTGTGACACGAGGAGCGG-3'           | 5'-CTGCATGGAGATGGATGTGATGTCGGTG-3'       |
| Granzyme B    | 5'-AGTGCGAATCTGACTTACGCCA-3'<br>5'-GCAGGCTCGTGGAGGCAT-3'         | 5'-CATTGTCTCCTATGGACGAA-3'               |
| IFN- $\gamma$ | 5'-CTAATTATTCGGTAACTGACTTGA-3'<br>5'-CGAAACAGCATCTGACTCTT-3'     | 5'-TCCAACGCAAAGCAATACGAATCATCC-3'        |
| TNF- $\alpha$ | 5'-CACGCTCTTCTGCTGCT-3'<br>5'-GCTTGCTACTCGGGGTTTC-3'             | 5'-CCAGGGACCTCTCTCTAATCAGCC-3'           |
| IL-17         | Kit AB Applied Biosystems                                        |                                          |
| IL-4          | 5'-AGAGCAGAAGACTCTGTGCA-3'<br>5'-AGCTGCTTGTGCTGTGGAA-3'          | 5'-TGCTGCCTCCAAGAACACAAGTGAAG-3'         |
| IL-5          | 5'-CTGATAGCCAATGAGACTTGA-3'<br>5'-GTGTTTATTACACCAAGAACTCTT-3'    | 5'-TCCTGTCTCTGTACATAAAAAATACCAACTGTGC-3' |
| IL-9          | 5'-TGTGACCAGTTGTCTCTGTTGG-3'<br>5'-GTGGTATTGGTCATCTGAGACAGTCT-3' | 5'-TTCCCTCTGACAAGTGCACAGACCA-3'          |

IFN, interferon; IL, interleukin; TNF, tumour necrosis factor.

control subjects were also analysed (numbers 30 and 31). Separation between dermis and epidermis was achieved by placing the biopsy in dispase II (1 U/ml Roche) at 4°C overnight. Dermis was further incubated in collagenase type III (200 U/ml; Whorlinton Biochemical Corp., Lakewood, NJ) for 2 hr at 37°C. The cell suspension was filtered (40- $\mu$ m filter) and stimulated with anti-CD3, anti-CD28 (Invitrogen) and Monensin (BD Biosciences, La Jolla, CA) for 4 hr at 37°C.

Extracellular staining was evaluated with anti- $\gamma\delta$  (clone B1; Biolegend, San Diego, CA) anti-CD3 (clone UCHT1), anti-CD4 (clone RPA-T4), and anti-CD8 (clone RPA-T8) (BD Pharmingen, La Jolla, CA) at 2  $\mu$ g/ml for 1 hr at 4°C. Samples were then (extensively) washed before cell permeabilization and fixation (Cytofix/Cytoperm kit; BD Biosciences) for 20 min at 4°C. Samples were again washed extensively, and intracellular labelling with anti-IL-4 (clone 8D4-8) and anti-IFN- $\gamma$  (clone B27) (Biolegend) for 1 hr at 4°C was performed. Stained cells were analysed with a FACS Fortessa instrument, and post-acquisition analysis was achieved with FLOWJO software (Tree Star, Ashland, OR).

### Statistical analysis

The data were analysed with generalized linear models, that is, Poisson regression with repeated measures (26) and logistic regression with repeated measures (27). Analyses were conducted with SAS<sup>TM</sup>. Significance levels of 95% and 99% were used. For quantitative PCR and

fluorescence-activated cell sorting analysis, statistical significance between groups was assessed by the use of a one-tailed unpaired Student's *t*-test or a one-way ANOVA with Tukey's or Bonferroni's multicomparison test, with INSTAT<sup>TM</sup> software.

## Results

### Clinical responses to CS patch testing

As we did not observe any significant difference between patients reacting to tixocortol pivalate and those reacting to budesonide, either in time points or in intensity, further analysis was conducted with all patients clustered in one unique group.

This study confirmed the large variation in the reaction times and the importance of repeated and late readings for detecting CS sensitization: few patients were positive before 48 hr, and, in some cases, late readings, that is, after 4 or even 7 days, were necessary (25). Indeed, clinical responses were not observed at 8 hr following patch test application. Three patients tested positively at 24 hr, 11 at 48 hr, 1 at 96 hr, and 3 at a later reading (7 days).

A number of patients who had previously reacted positively to CSs did not react during this study. Several possibilities could account for this discrepancy: possible loss of sensitization, hypo-reactivity or anergy, false-positive results during previous patch tests (vasodilatation), false-negative results during the study (insufficient quantity of allergen in the patch test chamber, or inadequate

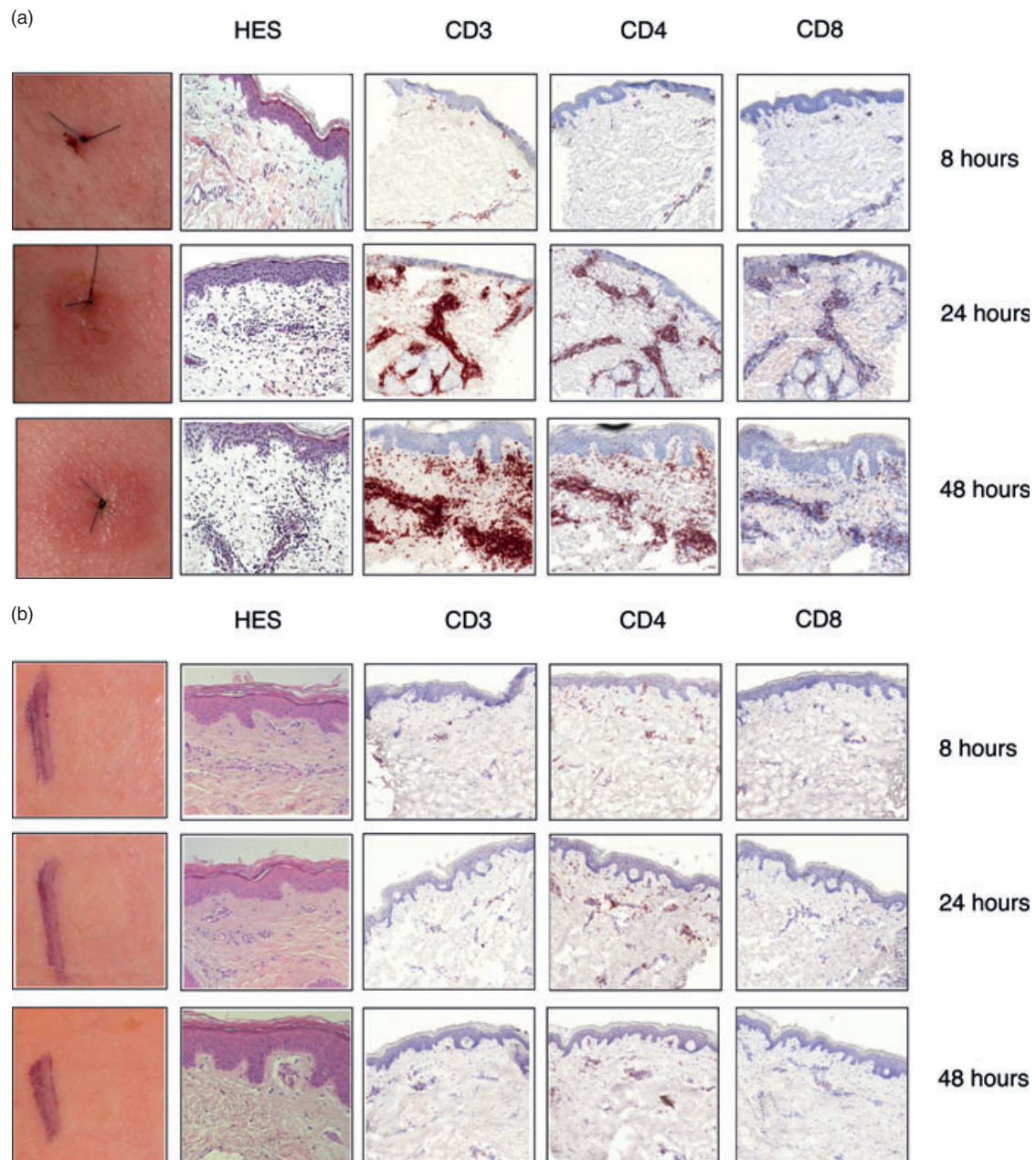


**Fig. 1.** Clinical aspects, HES epidermal and dermal infiltrate and CD3<sup>+</sup>, CD4<sup>+</sup> and CD8<sup>+</sup> immunostaining at 8, 24 and 48 hr after patch testing of a corticosteroid-allergic patient (number 14) (a) and a control subject (b).

occlusion), and concomitant systemic treatment, such as leflunomide (patient 7) or methylprednisolone (patient 23). Altogether, we confirmed a reproducibility of patch testing of 70%, which is in agreement with the literature (28, 29).

As already shown in a previous study (23), the prevalence of an atopic background is higher in the group of CS-allergic patients.

These results are summarized in Table 1.

#### Tissue infiltration by CD3<sup>+</sup> T cells

In order to characterize the cell infiltrate associated with the allergic response to CSs, we collected skin biopsies at 8, 24 and 48 hr following drug application, for histological and immunohistological staining. Only patients with

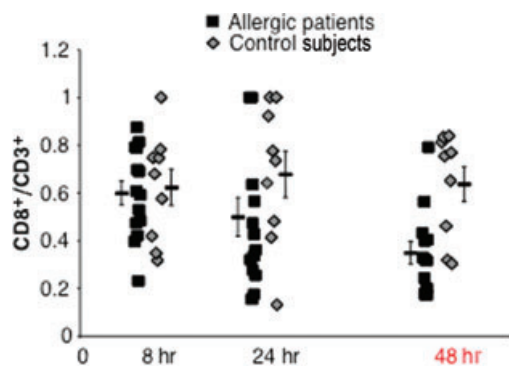


**Fig. 2.** Graphic comparing the CD8<sup>+</sup>/CD3<sup>+</sup> T cell ratio over time in patch test biopsies of corticosteroid-sensitized patients and control subjects.

clearly positive reactions at at least one biopsy time were taken into consideration (patients 1, 2, 4, 6, 8, 9, 10, 13, 14, 18, 21 and 22 for immunochemistry, and patients 4, 6, 9, 10, 14, 22, 25 and 26 for quantitative RT-PCR).

At 8 hr, contrasting with the absence of clinical response, histological analysis showed, in most cases, small changes, with a discrete mononuclear cell infiltrate, mainly in the dermis and often in a peri-annexial distribution (Fig. 1). Immunostaining showed that a high proportion of infiltrating dermal and epidermal cells were CD3<sup>+</sup> T cells, of which CD8<sup>+</sup> T cells constituted the majority (60%). However, the numbers of CD3<sup>+</sup> and

CD8<sup>+</sup> T cells were not statistically different from those in controls.

At 24 hr, the clinical aspect of the patch test sites was still normal for the majority of the patients (3/14); however, histological analysis showed a dermal infiltrate for all of them. Immunohistochemical analysis confirmed marked infiltration of CD3<sup>+</sup> T cells (Fig. 1).

At 48 hr, all patients presented positive patch test reactions, associated with a significant dermal infiltrate. Discrete epidermal signs, such as spongiosis and exocytosis, were present in only 3 cases. The numbers of CD3<sup>+</sup> T cells clearly increased at this time point, in comparison with the controls. Although the absolute numbers of CD8<sup>+</sup> T cells also increased, their proportion among CD3<sup>+</sup> T cells actually decreased at 48 hr as compared with the results obtained at 8 hr ( $p < 0.005$ ) (Fig. 2). This increased infiltration by T cells in the dermis of allergic patients was also significant ( $p < 0.05$ ) by flow cytometry performed on positive patch test skin biopsies (Fig. 3), with a major contribution of CD4<sup>+</sup> cells relative to CD8<sup>+</sup> cells.

Gene expression analysis confirmed the increased number of CD3<sup>+</sup> T cells, which was correlated with the higher numbers of CD4<sup>+</sup> and CD8<sup>+</sup> cells (Fig. 4). We also analysed the expression of granzyme B, which should reflect the presence of cytolytic T cells and natural killer cells. After 48 hr, we observed increased expression of granzyme B mRNA (Fig. 4).

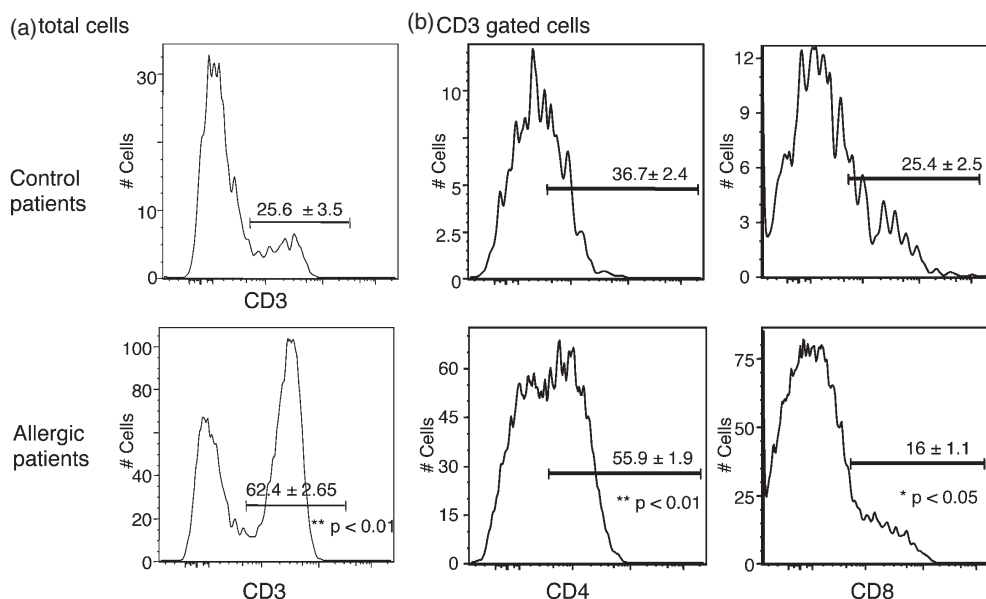


**Fig. 3.** Flow cytometry on dermal cells from healthy skin and positive patch test biopsies (at 48 hr) of allergic patients. (a) CD3<sup>+</sup> staining on total cells. (b) CD8<sup>+</sup> and CD4<sup>+</sup> labelling on gated CD3<sup>+</sup> T cells (mean  $\pm$  standard error of the mean for 4 patients) (\* $p < 0.05$  and \*\* $p < 0.01$  for allergic patients versus control subjects).

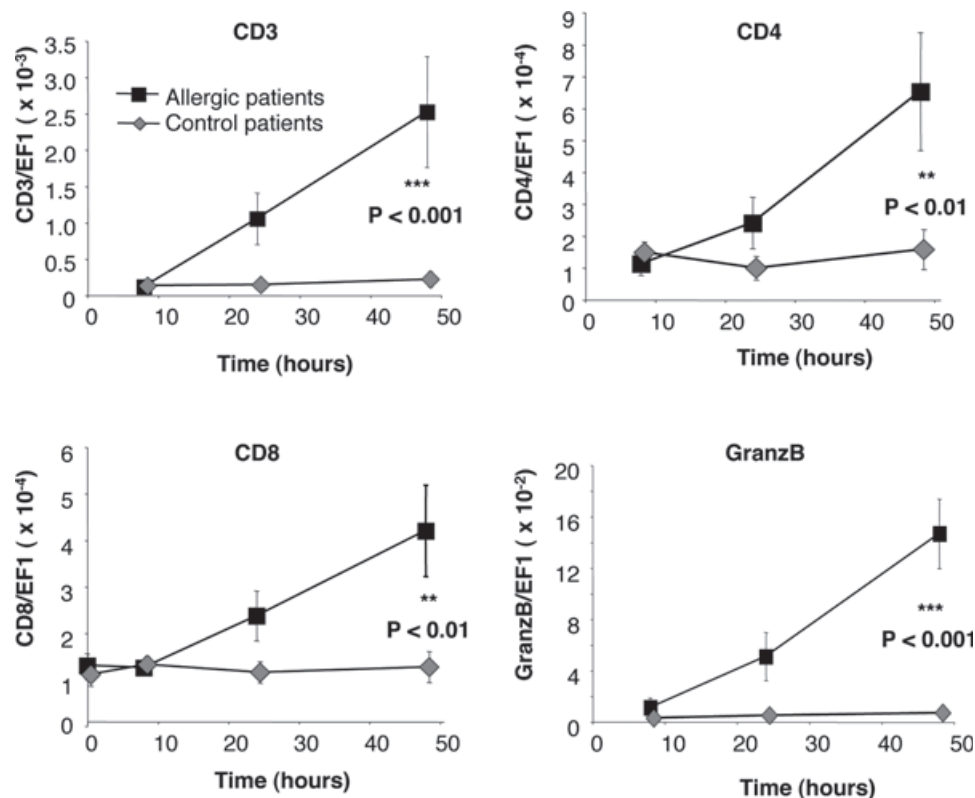


**Fig. 4.** Quantitative reverse transcription polymerase chain reaction for CD8, CD4, CD3, granzyme B and EF-1 mRNA expression. RNA was isolated from skin biopsies performed at 0, 8, 24 and 48 hr in corticosteroid-sensitized patients and control subjects (\* $p < 0.05$ , \*\* $p < 0.01$  and \*\*\* $p < 0.001$  for allergic patients versus control subjects).

### Cytokine production profile

In order to better characterize the T cells that infiltrate the dermis of positive patients, we analysed the skin expression of cytokine mRNA corresponding to the different subpopulations of Th cells. The onset of the skin lesion development was correlated with strong induction of the Th2 cytokine IL-4 and, to a lesser extent, of IL-5, IFN- $\gamma$ , and TNF- $\alpha$ , suggesting that Th2 and Th1 cytokines play a role in this mechanism. IL-9, another Th2 cytokine, was not significantly induced after 48 hr. Finally, IL-17 was slightly increased in three cases (numbers 6, 14, and 26) (Fig. 5), but this induction was not significant, suggesting that Th17 cells are not essential in the allergic response to CSs.

### IL-4 production by $\gamma\delta$ T cells

To further investigate IL-4-producing cells in skin reactions, we analysed the production of this cytokine by flow cytometry. As shown in Fig. 6, in contrast to IFN- $\gamma$ -producing cells, IL-4-producing cells were clearly induced in allergic patients, and consisted of 13% CD8<sup>+</sup>

and 26% CD4<sup>+</sup> cells (Fig. 6a). As most of the CD3<sup>+</sup> T cells were negative for both CD4 and CD8, we considered that these cells could be  $\gamma\delta$  T cell receptor lymphocytes ( $\gamma\delta$  T cells). By using an anti- $\gamma\delta$  T, we showed that two-thirds of these IL-4-producing cells were  $\gamma\delta$  T cells (Fig. 6a). Thus, these T cells rather than Th2 cells constitute the major source of IL-4 after CS exposure. Skin infiltration by  $\gamma\delta$  T cells was monitored by quantitative RT-PCR for the mRNA encoding the  $\gamma$ -chain of T cell receptor (TCR), and significant recruitment of  $\gamma\delta$  T cells was observed at 24 hr following drug application in allergic patients (Fig. 6b). The increase in the number of  $\gamma\delta$  T cells was confirmed by flow cytometry (Fig. 6c).

### Discussion and Conclusion

This study has shown that, in delayed allergic hypersensitivity to CSs, CD3<sup>+</sup> T cells with a predominant Th2 cytokine profile are rapidly recruited, along with the clinical response, in contrast to certain studies that have highlighted the role of CD8<sup>+</sup> T cells as effector cells in both drug and contact allergies. Moreover, we observed,

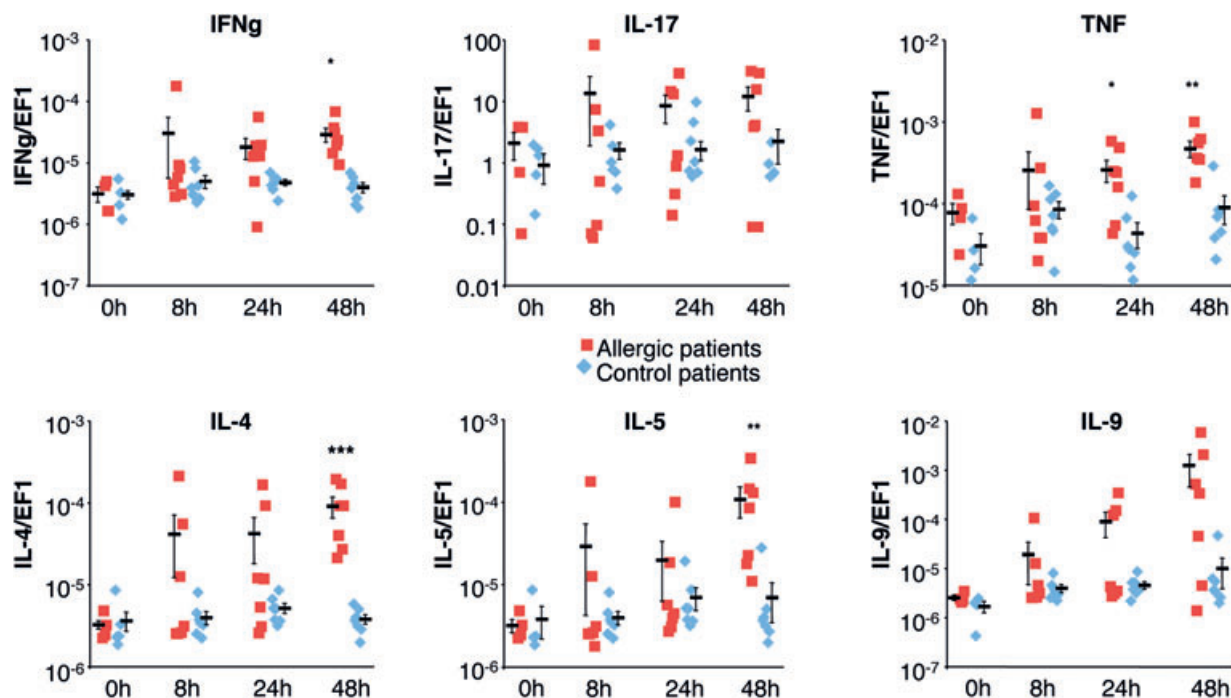


**Fig. 5.** Quantitative reverse transcription polymerase chain reaction for interferon (IFN)- $\gamma$ , interleukin (IL)-17, tumour necrosis factor (TNF)- $\alpha$ , IL-4, IL-5, IL-9 and EF-1 mRNA expression. RNA was isolated from skin biopsies performed at 0, 8, 24 and 48 hr in corticosteroid-sensitized patients and control subjects (\* $p < 0.05$ , \*\* $p < 0.01$  and \*\*\* $p < 0.001$  for allergic patients versus control subjects).

for the first time in patients with allergic hypersensitivity to CSs, an increase in the number of IL-4-producing  $\gamma\delta$  TCR<sup>+</sup> lymphocytes.

We showed that increasing numbers of CD3<sup>+</sup>, CD4<sup>+</sup> and, to a lesser extent, CD8<sup>+</sup> T cells correlate with patch test positivity. On the basis of immunostaining and quantitative RT-PCR findings, we suggest that dermal T cell infiltration does not really precede the onset of skin lesions, but that CD3<sup>+</sup> T cell recruitment rapidly increases with the positivity of the patch test sites. The predominance of CD8<sup>+</sup> T cells as effector cells was absent in CS allergy. In fact, the CD8<sup>+</sup>/CD3<sup>+</sup> T cell ratio of allergic patients was not statistically different from that of the controls. Moreover, the T cell infiltrate was not really different between control sites with and without CS exposure. Finally, the percentages of CD8<sup>+</sup> T cells were similar in patch test biopsies of tixocortol pivalate and of budesonide, despite the stronger anti-inflammatory effect of the latter, suggesting that the anti-inflammatory effect does not limit the infiltration of CD8<sup>+</sup> T cells induced by the allergic response.

The CD8<sup>+</sup>/CD3<sup>+</sup> T cell ratio rapidly decreased with time and with patch test positivity, probably because of the increased numbers of CD4<sup>+</sup> and  $\gamma\delta$  T lymphocytes. The presence of CD4<sup>+</sup> T cells seems to be associated with the onset of clinical inflammation, as has been suggested

by Padial et al. (21). These cells might play a modulatory role in the development of skin inflammation induced by the allergic response.

In addition, the  $\gamma\delta$  T cells could also play a regulatory role in the allergic response, because their numbers are increased with the positivity of the patch test. The conditions that lead to responses of  $\gamma\delta$  T cells are not fully understood, and current concepts of  $\gamma\delta$  T cells as 'the first line of defence', 'regulatory cells' or 'the bridge between innate and adaptive responses' address only some facets of their complex behaviour (30, 31).

The role of these cells as regulatory cells has already been highlighted in a mouse model of contact hypersensitivity (CHS) (32) to *p*-phenylenediamine (PPD) (33). In fact, it was shown that depletion of  $\gamma\delta$  T cells reduced the response elicited 12–24 hr after PPD challenge. In contrast, it was also shown that adoptive transfer of  $\gamma\delta$  T cells reconstituted the ability of  $\alpha\beta$  T cells to induce an allergic response, and that these  $\gamma\delta$  T cells were neither antigen-specific nor major histocompatibility complex-restricted.

In this study, we also analysed the cytokine expression of positive patch test biopsies in order to address the question of the activation pattern of drug-induced T cells. In contrast to the classically described primary effector function of Th1 cells and the minor effector function of

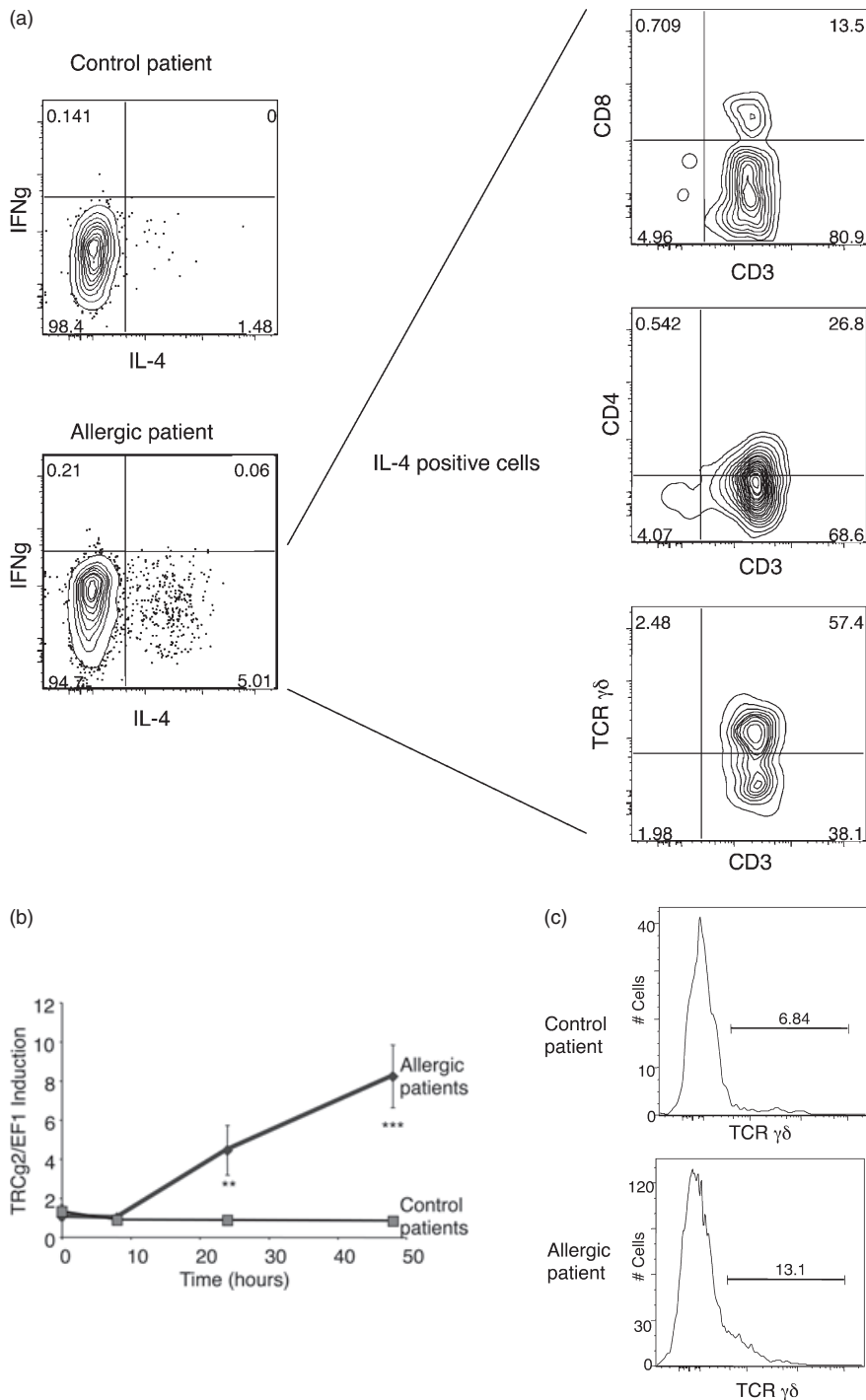


**Fig. 6.** (a) A dermal cell suspension was stimulated with anti-CD3<sup>+</sup>, anti-CD28<sup>+</sup> and Monensin for 4 hr at 37°C. Then, flow cytometry was performed on these stimulated cells from healthy skin and positive patch test biopsies (at 48 hr) of allergic patients, with interleukin (IL)-4 and interferon (IFN)- $\gamma$  cytokine staining, as well as CD3<sup>+</sup>, CD8<sup>+</sup>, CD4<sup>+</sup> and  $\gamma\delta$ <sup>+</sup> labelling on IL-4-producing cells. This staining was performed for 3 allergic patients and 3 control subjects. (b) Quantitative reverse transcription polymerase chain reaction for T cell receptor (TCR) $\gamma$ 2 and EF-1 mRNA expression. RNA was isolated from skin biopsies performed at 0, 8, 24 and 48 hr in corticosteroid-sensitized patients and control subjects. (c) Flow cytometry on dermal cells from healthy skin and positive patch test biopsies (at 48 hr) of allergic patients with  $\gamma\delta$ <sup>+</sup> labelling. This staining was performed for 3 allergic patients and 3 control subjects.

Th2 cells in ACD (12), we observed that the cytokines produced were mainly of the Th2 subtype, even though we observed an increase in Th1 cytokine expression, albeit to a lesser extent. Indeed, we showed that the onset of the skin lesions was correlated with strong induction of IL-4 expression, even at early time points, as well as significant

IL-5 production. A role for Th2 cytokines has already been shown in the mouse model of ACD caused by other allergens, such as PPD, nickel, and other metals (16–20). For instance, in PPD allergy, it has been shown that ear swelling induced by topical application of PPD is reduced in mice that are deficient in Th2 cytokines, showing that

Th2 cytokines play an essential role in the induction of CHS as compare with wild-type mice (17). However, in CS allergy, we showed that IL-4 was predominantly produced by  $\gamma\delta$  TCR lymphocytes.

IL-17, a Th17 cytokine, was slightly increased with the positivity of the patch test sites in 3 patients; hence, we were not able to confirm the recent observations suggesting an important role of Th17 T cells in the pathogenesis of various allergic diseases, including allergic contact hypersensitivity (34).

With respect to the kinetics and type of T cell responses, an atopic background does not seem to influence the infiltration of patch test reaction sites, as we did not observe differences between atopic and non-atopic subjects and all atopic patients whose dermatitis was actually in remission.

In conclusion, we have shown that, in ACD caused by CSs, CD3<sup>+</sup> T cells are rapidly recruited in concert with the positivity of the patch test, and cells produced predominantly Th2 cytokines. Among the CD3<sup>+</sup> T cell population,  $\gamma\delta$  TCR<sup>+</sup> lymphocytes constitute the major source of IL-4.

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## References

- Baeck M, Marot L, Nicolas J F, Pilette C, Tennstedt D, Goossens A. Allergic hypersensitivity to topical and systemic corticosteroids: a review. *Allergy* 2009; **64**: 978–994.
- Coombs R R A, Geel P G H. Classification of allergic reactions for clinical hypersensitivity and disease. In: *Clinical Aspects of Immunology*, 3rd edition, Gell P G H, Coombs R R A, Lachman R (eds): London, Oxford, Blackwell Scientific, 1975: pp. 761–781.
- Pichler W J. Delayed drug hypersensitivity reactions. *Ann Intern Med* 2003; **139**: 683–693.
- Posadas S J, Pichler W J. Delayed drug hypersensitivity reactions – new concepts. *Clin Exp Allergy* 2007; **37**: 989–999.
- Peiser M, Tralau T, Heidler J et al. Allergic contact dermatitis: epidemiology, molecular mechanisms, in vitro methods and regulatory aspects. *Cell Mol Life Sci* **212**: 763–781.
- Martins L E, Reis V M. Immunopathology of allergic contact dermatitis. *An Bras Dermatol* 2011; **86**: 419–433.
- Rozières A, Vocanson M, Rodet K et al. CD8<sup>+</sup> T cells mediate skin allergy to amoxicillin in a mouse model. *Allergy* 2010; **65**: 995–1003.
- Rozières A, Hennino A, Rodet K et al. Detection and quantification of drug-specific T cells in penicillin allergy. *Allergy* 2009; **64**: 534–542.
- Rozières A, Vocanson M, Said B B, Nosbaum A, Nicolas J F. Role of T cells in nonimmediate allergic drug reactions. *Curr Opin Allergy Clin Immunol* 2009; **9**: 305–310.
- Vocanson M, Hennino A, Rozières A et al. Skin exposure to weak and moderate contact allergen induces IFN gamma production by lymph node cells of CD4<sup>+</sup> T-cell-depleted mice. *J Invest Dermatol* 2009; **129**: 1185–1191.
- Vocanson M, Hennino A, Cluzel-Tailhardat M et al. CD8<sup>+</sup> T cells are effector cells of contact dermatitis to common skin allergens in mice. *J Invest Dermatol* 2006; **126**: 815–820.
- Hennino A, Vocanson M, Toussaint Y et al. Skin-infiltrating CD8<sup>+</sup> T cells initiate atopic dermatitis lesions. *J Immunol* 2007; **178**: 5571–5577.
- Hennino A, Jean-Decoster C, Giordano-Labadie F, Debeer S, Vanbervliet B, Rozières A. CD8<sup>+</sup> T cells are recruited early to allergen exposure sites in atopy patch test reactions in human atopic dermatitis. *J Allergy Clin Immunol* 2011; **127**: 1064–1067.
- Van Beelen A J, Teunissen M B, Kapsenberg M L, de Jong E C. Interleukin-17 in inflammatory skin disorders. *Curr Opin Allergy Clin Immunol* 2007; **7**: 374–381.
- Ogawa A, Yoshizaki A, Yanaba K et al. The differential role of L-selectin and ICAM-1 in Th1-type and Th2-type contact hypersensitivity. *J Invest Dermatol* 2010; **130**: 1558–1570.
- Sinigaglia F, Scheidegger D, Garotta G. Isolation and characterisation of Ni-specific T cell clones from patients with Ni-contact dermatitis. *J Immunol* 1985; **135**: 3929–3932.
- Yokozeki H, Wu M H, Sumi K et al. Th2 cytokines, IgE and mast cells play a crucial role in the induction of para-phenylenediamine-induced contact hypersensitivity in mice. *Clin Exp Immunol* 2003; **132**: 385–392.
- Coulter E M, Jenkinson C, Farrell J et al. Measurement of CD4<sup>+</sup> and CD8<sup>+</sup> T-lymphocyte cytokine secretion and gene expression changes in p-phenylenediamine allergic patients and tolerant individuals. *J Invest Dermatol* 2010; **130**: 161–174.
- Czarnobilska E, Jenner B, Kaszuba-Zwoinska J, Kapusta M, Obtułowicz K, Thor P, Spiewak R. 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.
- Minang J T, Areström I, Trove-Blomberg M, Lundeborg L, Ahlberg 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.
- 21 Padial A, Posadas S, Alvarez J et al. Nonimmediate reactions to corticosteroids suggest an immunological mechanism. *Allergy* 2005; **60**: 665–670.
  - 22 Zhao Y, Balato A, Fischelevich R, Chapoval A, Mann D L, Gaspari A A. Th17/Tc17 infiltration and associated cytokine gene expression in elicitation phase of allergic contact dermatitis. *Br J Dermatol* 2009; **161**: 1301–1306.
  - 23 Baeck M, Chemelle J A, Terreux R, Drieghe J, Goossens A. Delayed hypersensitivity to corticosteroids in a series of 315 patients: clinical data and patch test results. *Contact Dermatitis* 2009; **61**: 163–175.
  - 24 Isaksson M, Brandao F M, Bruze M, Goossens A. Recommendation to include budesonide and tixocortol pivalate in the European standard series. *Contact Dermatitis* 2000; **43**: 41–63.
  - 25 Isaksson M. Corticosteroid contact allergy – the importance of late readings and testing with corticosteroids used by the patients. *Contact Dermatitis* 2007; **56**: 56–57.
  - 26 Frome E L, Checkoway H. Use of Poisson regression models in estimating incidence rates and ratios. *Am J Epidemiol* 1985; **121**: 309–323.
  - 27 Peduzzi P, Concato J, Kemper E, Holford T R, Feinstein A R. A simulation study of the number of events per variable in logistic regression analysis. *J Clin Epidemiol* 1996; **49**: 1373–1379.
  - 28 Mimesh S, Pratt M. Allergic contact dermatitis from corticosteroids: reproducibility of patch testing and correlation with intradermal testing. *Dermatitis* 2006; **17**: 137–142.
  - 29 Bircher A J, Thurlimann W, Hunziker T et al. Contact hypersensitivity to corticosteroids in routine patch test patients. A multi-centre study of the Swiss Contact Dermatitis Research Group. *Dermatology* 1995; **191**: 109–114.
  - 30 Holtmeier W, Kabelitz D. Gammadelta T cells link innate and adaptative immune responses. *Immunol Allergy* 2005; **86**: 151–183.
  - 31 Laggner U, Meglio P, Perera G K et al. Identification of a novel proinflammatory skin-homing V(gamma)9V(delta)2 T cell subset with a potential rôle in psoriasis. *J Immunol* 2011; **187**: 2783–2793.
  - 32 Ptak W, Askenase P W. Gamma delta T cells assist alpha beta T cells in adoptive transfer of contact sensitivity. *J Immunol* 1992; **149**: 3503–3508.
  - 33 Yokozeki H, Watanabe K, Igawa K, Miyazaki Y, Katayama I, Nishioka K. Gammadelta T cells assist alphabeta T cells in the adoptive transfer of contact hypersensitivity to para-phenylenediamine. *Clin Exp Immunol* 2001; **125**: 351–359.
  - 34 Vocanson M, Rozières A, Hennino A et al. Inducible costimulator (ICOS) is a marker for highly suppressive antigen-specific T cells sharing features of Th17/Th1 and regulatory T cells. *J Allergy Clin Immunol* 2010; **126**: 280–289.