

Letter to the Editor

CD8⁺ T cells are recruited early to allergen exposure sites in atopy patch test reactions in human atopic dermatitis

To the Editor:

Atopic dermatitis (AD) is a T-cell-mediated chronic inflammatory skin disease associated with cutaneous hyper reactivity to environmental antigens that are innocuous to normal nonatopic individuals. The house dust mites (HDM) *Dermatophagoides pteronyssinus* (Der p) and *Dermatophagoides farinae* (Der f) are major allergens in AD pathophysiology. The eczematous skin reaction can be provoked in patients with AD by the epicutaneous application of aeroallergens, commonly referred to as the atopy patch test (APT).¹ Epicutaneous application of HDM by APT on uninvolved skin of patients with AD elicits eczema in 30% to 50% of these patients.^{1,2} The initial mechanisms that induce skin inflammation in patients with AD are unknown. CD4⁺ T_H cells are considered to be pivotal in the development of the eczema and skin eosinophilic inflammation by producing a mixed pattern of T_H1 and T_H2 cytokines including IL-4 and

IL-13, which are known to induce isotype switching to IgE synthesis, as well as IL-5, which plays an important role in eosinophil development and survival.¹ Recently, using a mouse model of Der f-induced AD, we have shown that CD8⁺ T cells were essential to the development of AD skin inflammation.³ CD8⁺ T cells were recruited in the Der f-exposed skin in the hours after Der f skin exposure, before the infiltration of other leukocyte subsets, suggesting that CD8⁺ T cells are involved in the initiation of the skin inflammation. However, although these results came from a representative mouse model, studies in human subjects were required to confirm the hypothesis that CD8⁺ T cells are important for the development of human AD skin lesions. Here, we analyzed the cellular infiltrate in APT to HDM using immunohistochemistry and molecular analysis. Nine patients with mild to moderate AD were recruited on the basis of the positivity of APT to HDM $\geq 2+$ (according to European Task Force on Atopic Dermatitis criteria) at the 72-hour reading in the 3 months preceding the study (summarized in Fig 1, A). Each participant provided informed consent. The study was approved by the ethics committee. Because the serum levels of chemokine (C-C motif) ligand 17 correlate with AD activity,⁴ we measured the level of this chemokine

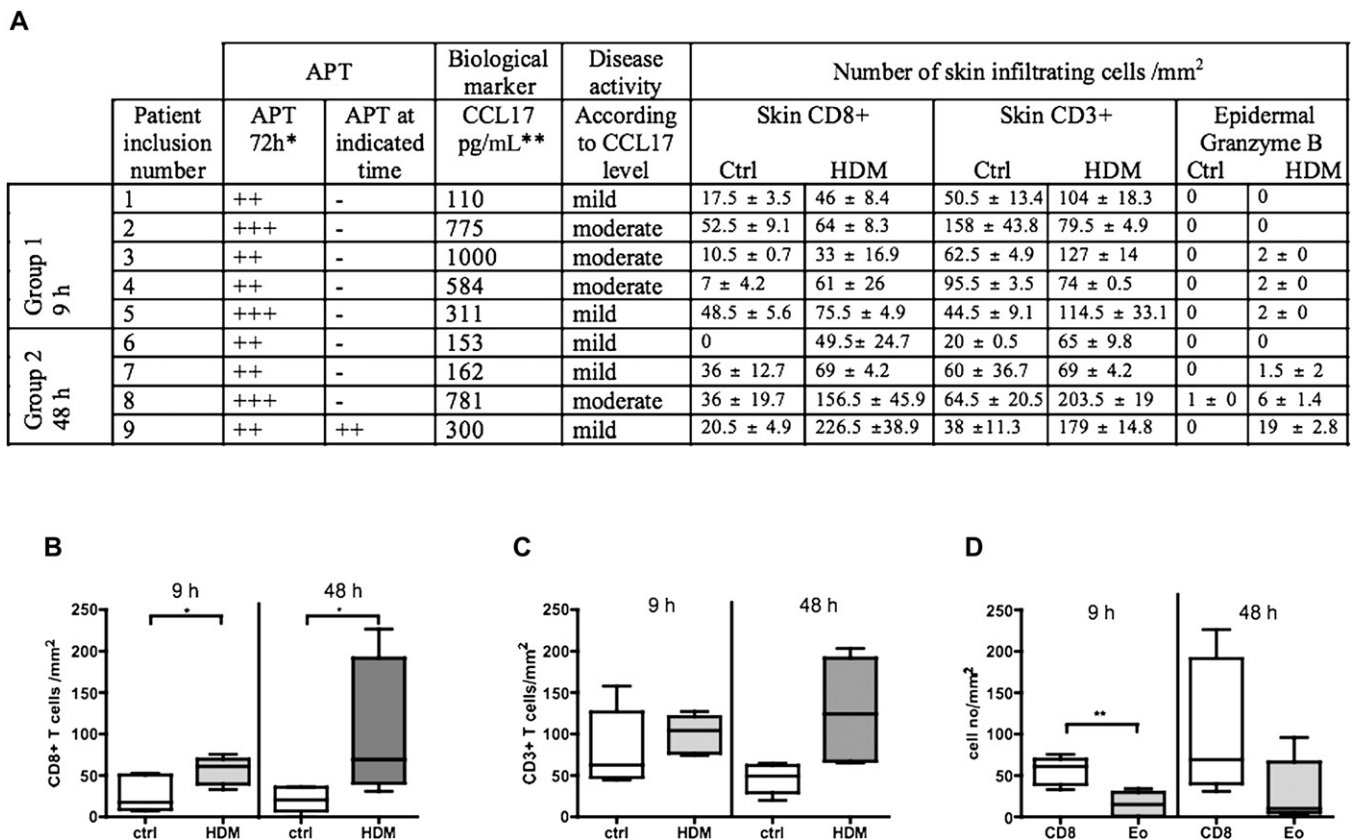


FIG 1. A, Summary of the 9 patients included in the study. *Positivity of the APT (according to European Task Force on Atopic Dermatitis consensus) at 9 and 48 hours as well as at 72 hours in the preselection of the study. The severity of the disease is defined as mild (<500 pg/mL) and moderate (>500 pg/mL) according to chemokine (C-C motif) ligand 17 (CCL17) serum level (**normal range, approximately 200 pg/mL). The numbers of CD8⁺ (**B**) and CD3⁺ (**C**) T cells/mm² in skin in control (ctrl) or HDM APT are represented at 9 and 48 hours. **D**, Comparison between the number of CD8⁺ T cells and eosinophils at 9 and 48 hours in HDM APT.

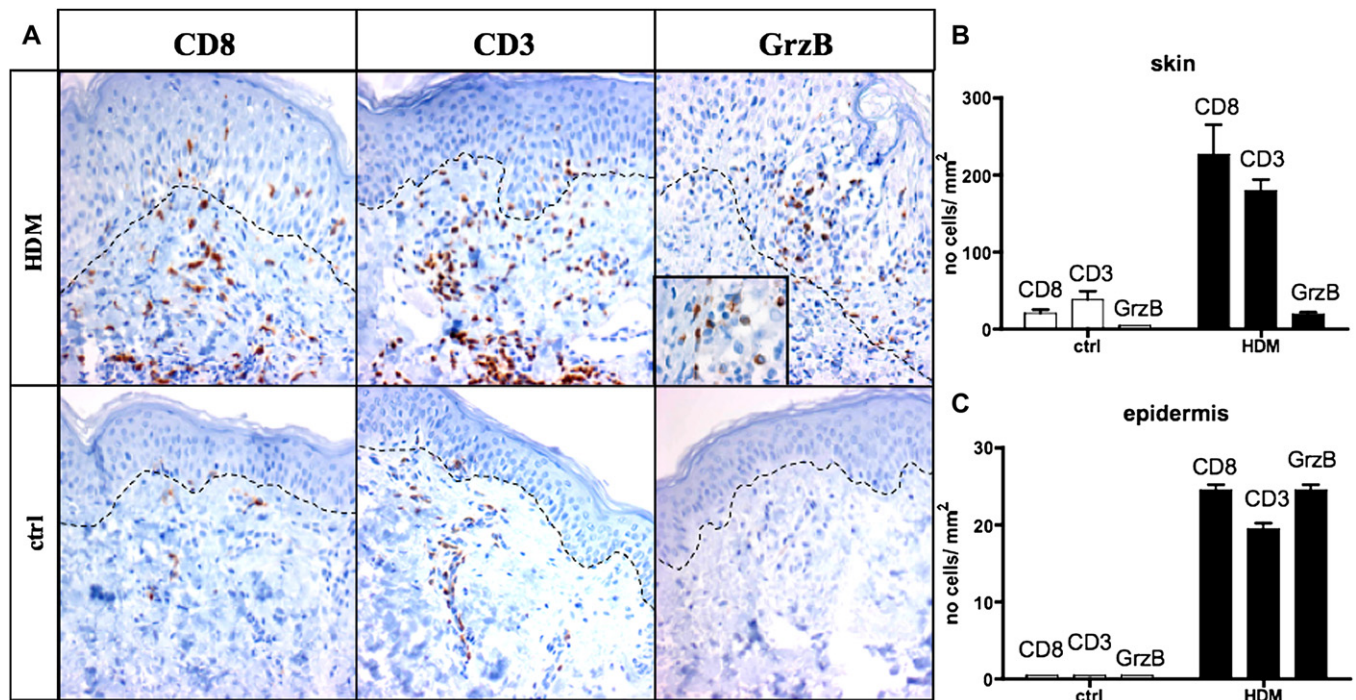


FIG 2. A, Immunostaining of HDM APT or control APT in patient 9 with anti-CD8, anti-CD3, and anti-granzyme B (GrzB). Original magnification is $\times 200$. Inset is magnified view ($\times 400$). Quantification of the number of CD8⁺, CD3⁺, and granzyme B⁺ cells/mm² in HDM compared with control (ctrl) in whole skin (B) and in epidermis (C).

by ELISA, and we classified the 9 patients as mild and moderate (Fig 1, A). At inclusion in the study, the patients were patch-tested again with APT to HDM and petrolatum as control. The patients were randomized into 2 groups according to the occlusion time: 9 hours (group 1, patients 1-5) and 48 hours (group 2, patients 6-9) after application of the test. None of the APT and control tests was positive in any patient the at time of the skin biopsy (9 and 48 hours) except for patient 6, who had a positive APT 2+ to HDM, but not to petrolatum, at 48 hours. This is in keeping with clinical experience showing that APTs are usually positive at 72 hours.

Skin sections from paraffin-embedded biopsies were stained by anti-CD8, anti-CD3, anti-granzyme B, and an isotype control mAb (all from BD Biosciences, San Jose, Calif) following previously described methods,³ and the number of positive cells was recorded. We have also counted the number of eosinophils by using hematoxylin-phloxine-safran staining. The results are given in Fig 1. At 9 hours, CD8⁺ T cells were already observed in increased numbers in the skin of the HDM APT versus the control test in all 5 patients of group 1 (Fig 1, B; $P = .0452$). For patients 2 and 4, CD3⁺ T cells were almost exclusively composed of CD8⁺ T cells (Fig 1, A). In APT to HDM, CD8⁺ T-cell numbers increased more importantly at 48 hours ($P = .049$). At that time, although most CD8⁺ T cells were found in the dermis, some epidermal CD8⁺ T cells were observed. No major change in the number of CD8⁺ T cells was observed in control APT over time (Fig 1, B). Granzyme B is a cytotoxic molecule produced by activated CD8⁺ T cells and is expressed in lesional skin.⁵ Granzyme B⁺ cells were found almost exclusively in the epidermis of 48-hour HDM APT (Fig 1, A). We estimated the number of CD3⁺ T cells found in each skin sample. Fig 1, C, shows that the mean number of CD3⁺ T cells recruited in the control and

the HDM test was roughly similar, especially at the 9-hour time point. Although CD8⁺ T cells made up a high proportion of skin-infiltrating CD3⁺ T cells, other cells type including CD4⁺ T cells and natural killer T cells have been described to infiltrate the APT.⁶ This observation suggests that the inflammation reaction induced by the occlusive test is a dynamic process that consists of migration of cells to and from the skin at the same time. Because normal skin is infiltrated by low numbers of CD4⁺ T cells, we interpret this observation as a consequence of the emigration of the resident CD4⁺ T cells on inflammatory signals delivered by the patch test. In a mouse model of eczema, it has been previously shown that CD4⁺ T cells that play a regulatory role leave the skin at the early time points.⁷ Eosinophils are important in the skin inflammation in AD. We show here that very few eosinophils were observed at any time points, and their numbers never exceeded those of CD8⁺ T cells, suggesting that CD8⁺ T cells are recruited in the first place (Fig 1, D). Therefore, these data, obtained from noninflammatory/normal looking skin of patients with AD, show that development of positive APT to HDM is associated with a early skin infiltration by CD8⁺ T cells.

Patient 9 is the only patient of group 2 who had developed a positive APT at 48 hours, the time of the skin biopsy. Analysis of the skin sections found a prominent infiltration by CD3⁺ T cells, almost exclusively composed of CD8⁺ T cells (Fig 2, A and B). Expression of granzyme B was restricted to epidermal infiltrating T cells (Fig 2, A and C), suggesting that epidermal but not dermal CD8⁺ T cells are activated and display a cytotoxic phenotype. In contrast, the negative control test showed very mild skin changes with a moderate CD3⁺/CD8⁺ perivascular staining. No granzyme B⁺ cells were observed.

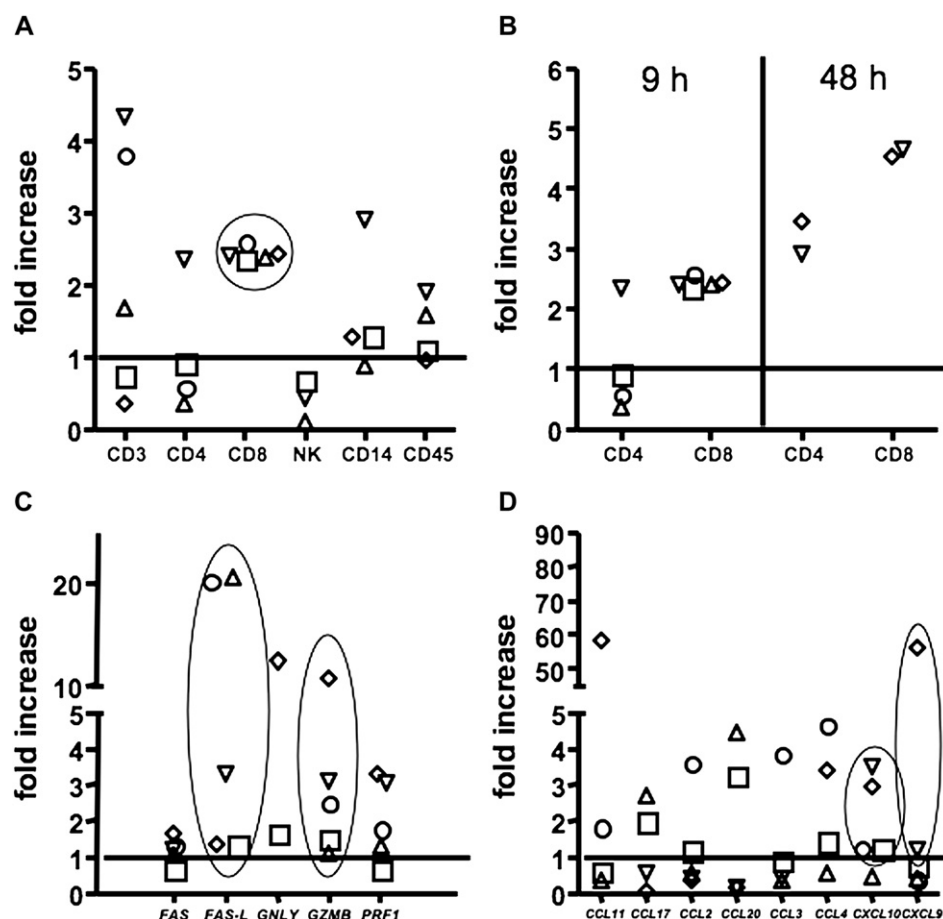


FIG 3. Quantification of the mRNA by the TaqMan low-density array technique for several molecules indicated in the figure. Total mRNA was extracted from APT to HDM and control. The mRNA was normalized to 3 housekeeping genes (hypoxanthine phosphoribosyltransferase [HPRT], ubiquitin, and actin). Results are expressed in fold increase in HDM APT compared with control (petrolatum). **A, C, D,** Fold increase of indicated molecules at 9 hours. **B,** Fold increase of CD8 and CD4 mRNA at 9 and 48 hours. *CCL*, Chemokine (C-C motif) ligand; *GNLY*, granulysin; *GZMB*, granzyme B; *NK*, natural killer; *PRF1*, perforin.

In addition, 5 patients were recruited for molecular studies, all with positive APT to HDM at 72 hours. All patients were biopsied early (9 hours), and 2 of them were biopsied in addition at 48 hours. mRNA levels of several chemokines and T-cell markers were quantified by TaqMan low-density array real-time PCR (Applied Biosystems, Carlsbad, Calif). All 5 patients showed an early increase of CD8 mRNA at 9 hours. In contrast, only 1 patient showed an increased CD4 mRNA at 9 hours, but at 48 hours, both CD4 and CD8 mRNA were upregulated. Furthermore, all 5 patients expressed upregulated Fas ligand (Fas-L) and granzyme B, as well as the chemokines (C-x-C motif) ligand 10 (CXCL10) and/or CXCL9 involved in CD8⁺ T cell recruitment⁸ (Fig 3, *C* and *D*). These molecular studies confirm and extend the immunohistochemical data and show that development of APT to HDM is associated with early infiltration of the skin by CD8⁺ T cells.

Previous work concerning the nature of cellular infiltrate in APT has shown few data concerning CD8⁺ T cells in the AD. Thepen et al⁹ showed that T-cell numbers increased rapidly until they reached a maximum at 24 hours for CD8⁺ T cells and 48 hours for CD4⁺ T cells, results that sustain our data of early recruitment of CD8⁺ T cells. Holm et al¹⁰ also showed in a kinetic study from 6 to 72 hours that a small but significant

number of CD8⁺ T cells were rapidly recruited to the epidermis (6 hours). In contrast, the number of CD4⁺ T cells in the epidermis did not change at different time points (6, 24, and 48 hours).¹⁰

In our opinion, the role of CD8⁺ T cells in the pathophysiology of AD has been underestimated essentially because of the early and transient presence of a small number of CD8⁺ T cells before clinical lesions occurred. We have previously shown in a mouse AD model that very few CD8⁺ T cells infiltrating the skin (about 5% of CD45⁺ cells) are sufficient to initiate the HDM-induced AD lesions.³ However, their presence was mandatory because depletion with anti-CD8 mAbs totally abrogated the inflammatory response.³ Here we show that very few CD8⁺ granzyme B⁺ T cells infiltrating the epidermis are associated with the development of positive APT. We hypothesize that these cytotoxic CD8⁺ are responsible for the apoptosis of keratinocytes and for the epidermal spongiosis, which are both pathological hallmarks of AD.^{11,12}

In summary, our data suggest an early and critical role for CD8⁺ T cells in the development of human AD lesions. Further experiments should identify which signals are required for the recruitment and activation *in situ* of the CD8⁺ T cells.

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