

Cytokine production, activation marker, and skin homing receptor in children with atopic dermatitis and bronchial asthma

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T cells are known to develop a critical role in the pathogenesis of atopic dermatitis (AD) and bronchial asthma. T cells involved in AD express the skin homing receptor CLA, but no lung homing receptor has been identified in bronchial asthma. We compared different cell markers and the cytokine production in T cells from children with AD or bronchial asthma. We studied the involvement of CLA⁺ and CLA⁻ T-cell subpopulations in these diseases. We studied 20 children with acute AD lesions, 15 with mild persistent asthma, and 15 non-atopic controls. All patients were sensitized to house dust mite (DP) and evaluated during the acute phase. Total and specific IgE were measured by immunoassay and the expression of different cell markers and the cytokine production was analyzed by flow cytometry in peripheral blood mononuclear cells. Total IgE was significantly higher in AD children and IgE to DP in the asthmatic children. There was a significant increase in CD25⁺CD4⁺ cells in asthmatic children and in HLA-DR⁺CD4⁺ and HLA-DR⁺CD8⁺ cells in AD. In the CD4⁺ subsets, there was an increase in IL-13, IL-5 and TNF- α in AD compared to controls, a decrease in IFN- γ in asthmatic children compared to controls, and an increase in IL-13, IL5, IL2, TNF- α , and IFN- γ in the AD compared to asthmatic children. Changes in cytokine production were mainly detected in CLA⁺ cells in AD and in CLA⁻ cells in asthma. Differences exist in total and specific IgE, activation markers, and cytokine patterns between AD children and children with asthma, with the former expressing a Th2 pattern whereas in asthmatic children we only detected a decrease in IFN- γ . Moreover, the subpopulations (CLA⁺ vs. CLA⁻) expressing these changes were different, indicating that the underlying mechanisms in the two diseases are not exactly the same.

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The concept of 'atopy' includes atopic dermatitis (AD), asthma, and allergic rhinitis, with AD usually being the first manifestation (1). The atopic march is the natural history of atopic manifestations, characterized by a progression of clinical signs of atopic disease, with some signs becoming more prominent while others subside (2).

Despite multiple studies of AD and asthma, their pathogenesis is still not completely understood, though T cells are known to play a critical role in both processes (3, 4). In immune responses, homing of lymphocytes from blood into organs is mediated by a sequential interaction between the lymphocytes and the vascular endothelium in specialized post-capillary venules

(5). In inflamed skin, T cells express the cutaneous lymphocyte associated antigen (CLA) and P-selectin ligand and the vascular epithelium expresses E-selectin and P-selectin (6); however, the adhesion molecules and chemokines for T-cell homing into airways, lungs and bronchi are not well-clarified (7).

Upon encountering antigen-carrying dendritic cells in secondary lymphoid organs, naive T-lymphocytes proliferate and differentiate into at least two distinct lineages, defined by their cytokine profiles: Th1 (mainly producing IFN- γ) and Th2 (secreting IL-4, IL-5, and IL-13) (8). There is evidence that abnormal Th1 and Th2 functions contribute not only to the production of IgE but also to the pathogenesis of allergic disorders, and this has been linked to excess production of Th2 cytokines (9).

It is known that AD patients with acute lesions have elevated Th2 and decreased Th1 expressing cells in peripheral blood (10). This Th2 pattern also exists in asthmatic patients (4, 11), though this is controversial (12–14). Atopic patients are known to have a reduced production of IFN- γ (15) and experiments in cord blood suggest that this deficit is a primary component of the atopic state, resulting in increased levels of Th2 cytokines, IgE levels, and mast-cell degranulation on exposure to antigen (16).

The aim of this study was to compare different markers and the cytokine production in peripheral blood lymphocytes, distinguishing between CLA⁺ and CLA⁻ subsets, in symptomatic asthmatic children compared to AD children with acute lesions and non-atopic control children, as well as to confirm, in children, our previous results found in adults with acute AD lesions. We found a clear Th2 pattern in children with AD, mainly produced by CLA⁺ cells. This pattern was not detected in asthmatic children, who expressed a reduced level of IFN- γ in CLA⁻ cells.

Materials and methods

Patients

Thirty-five children from the pediatric allergic unit of Carlos Haya Hospital were classified into two groups. The first group was formed by twenty children (8 girls and 12 boys, mean age: 10.5 yr, range: 7–13), diagnosed with AD according to Hanifin and Rajka (17). They had no other skin diseases or respiratory problems, and had received no systemic steroids, tacrolimus or psoralen-ultraviolet A for at least six months prior to the study. Lesions onset was less than

1 wk before the study and the mean severity score was 7.1 (18).

Fifteen children (seven girls and eight boys, mean age: 9.5 yr, range: 8–14) with mild persistent atopic asthma according to GINA (19), with no skin disease and managed only with inhaled β_2 -agonists were evaluated during a symptomatic phase, formed the second group.

Fifteen non-atopic children (nine girls and six boys, mean age: 11 yr, range: 9–13) with no skin or respiratory diseases were, included as controls.

We selected patients allergic to the same allergen, house dust mite, to make comparisons in specific IgE and to evaluate exacerbations in the same period of time. Skin tests were done using ALK-Abello (Denmark) and quantification of total and specific IgE to *Dermatophagoides pteronyssinus* (DP) in serum (UniCAP, Pharmacia Diagnostic AB, Uppsala, Sweden) was done following the manufacturer's indications.

The study was approved by the institutional review board, and informed consent for all the diagnostic procedures was obtained from the parents of the patients and the controls.

Phenotypic immunofluorescence analysis

Peripheral blood mononuclear cells (PBMC) from each patient were isolated by density gradient centrifugation (Nycomed, Oslo, Norway) from 6 ml of heparinized venous blood for immunofluorescence staining, as described (20). Briefly, 5×10^5 cells were sequentially stained with different MoAbs at 4°C. Stained cells were fixed in 1% paraformaldehyde in PBS. Cell subsets were assessed with the following MoAbs: CD3-PerCP, CD4-APC, CD8-APC, CD45RO-PE and CD45RA-FITC, the skin homing receptor expression with CLA-FITC and the activation state by CD25-PE and HLA-DR-PE (all provided by Beckton–Dickinson, San Jose, CA). Intracellular cytokine production was determined in cells stimulated for 4 h at 37°C with phorbol 12-myristate 13-acetate (Sigma, St. Louis, MO) and ionomycin (Sigma) at a final concentration of 25 ng/ml and 1 μ g/ml cell suspension, respectively, in which the intracellular protein transport of cytokines was disrupted with monensin (Sigma) at 10 μ g/ml cell suspension. Following subset cell surface staining for 15 min, cells were fixed for 15 min using Fix and Per cell permeabilization kit (Caltag Laboratories, Burlingame, CA) and permeabilized with the same kit and stained intracellularly for 30 min at room temperature with different MoAbs to cytokines: IL-2-FITC, IFN- γ -PE, TNF- α -PE, IL-10-FITC, IL-4-PE, IL-5-PE, and IL-13-PE (all provided by

Becton–Dickinson). After washing, cells were analyzed by flow cytometry. Six-parameter analysis was undertaken on a FacsCalibur flow cytometer using Cell Quest software. Negative isotype controls were used to verify the staining specificity of the antibodies used. A disadvantage of phorbol 12-myristate 13-acetate activation is the down-regulation of certain surface proteins, including CD4. All CD4⁺ T cells are found within the CD3⁺/CD8⁻ population, and the majority of this population consists of CD3⁺/CD4⁺ T cells. Accordingly, in the analysis of the cytokine production we refer to the CD3⁺/CD8⁻ population as the CD4⁺ population.

Statistical study

Data are presented as box-plots displaying medians and interquartile ranges (IR). As the variables evaluated were not distributed normally, the mean comparisons were done by non-parametric analysis (Kruskal–Wallis and, if significant, Mann–Whitney tests). All reported p values represented two-tailed tests, with values ≤ 0.017 , after the Bonferroni correction, considered statistically significant. The statistical analysis was performed using the SPSS program version 11.5.

Results

Total and specific IgE to DP

Both total and specific IgE to DP were increased in AD and asthmatic patients compared to controls ($p < 0.017$). Comparisons between the two patient groups showed that total IgE was significantly higher in AD patients ($p = 0.014$)

and the specific IgE was higher in asthmatic patients ($p = 0.014$) (Fig. 1).

Cell surface expression in CD3 lymphocytes

The percentage of total CD4 and CD8 subpopulations in CD3⁺ cells and in CLA⁺ cells, and the percentage of the different naive/memory markers in CD4⁺ and CD8⁺ subpopulations is presented in Table 1. Differences existed in the percentage of CD8⁺ cells between the three groups ($p = 0.022$), with a decrease in AD ($p = 0.004$) and asthmatic patients ($p = 0.012$) compared to controls, indicating that both groups of atopic patients had a higher number of CD4⁺ than CD8⁺ cells. There were no significant differences in the percentage of CLA expression between the three groups, probably because in AD patients with acute lesions the CLA⁺ cells are in the skin but not circulating.

Analysis of the expression of naive/memory markers showed a decrease in CD8⁺CD45RA⁺ cells and an increase in CD8⁺CD45RORA⁺ cells in AD patients compared to asthmatic children ($p < 0.001$) and controls ($p < 0.001$), with no differences between the asthmatic children and controls. The AD patients therefore presented a higher number of cells in an intermediate state with a lower number of naive cells.

CD25 and HLA-DR markers

The results of CD25 and HLA-DR markers in both CD4⁺ and CD8⁺ subpopulations are shown in Fig. 2. Significant differences were observed between the three groups in the

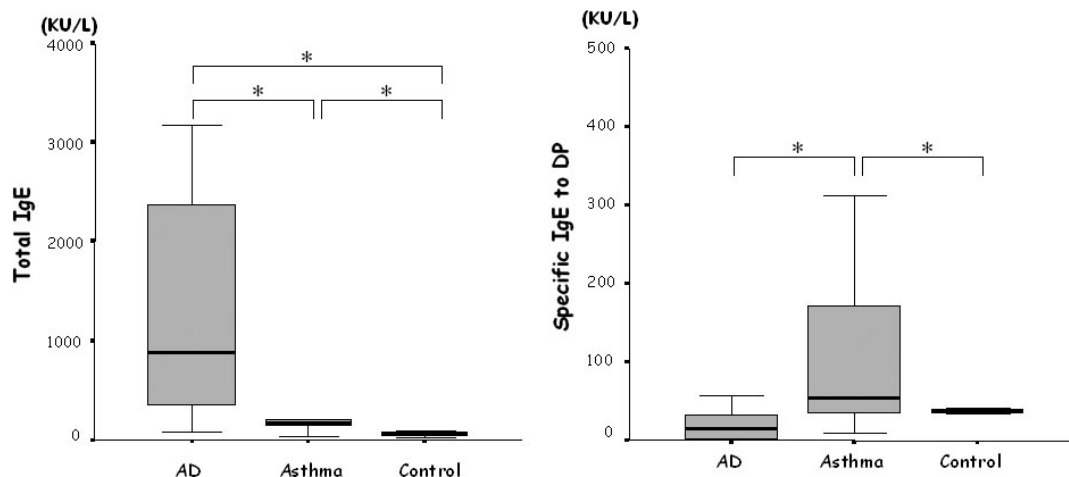


Fig. 1. Box plot of the total (left) and specific IgE to DP levels (right) measured by CAP and expressed in KU/L. Comparisons were made between the three groups of children studied: AD ($n = 20$), asthmatic ($n = 15$), and controls ($n = 15$). Statistical differences (*) were considered significant at $p < 0.017$ after Bonferroni correction.

Table 1. Median and IR of the different surface markers in the CD4⁺ and CD8⁺ subpopulations in AD, asthmatic and control children

	Atopic dermatitis		Asthma		Controls	
	Median	IR (75–25)	Median	IR (75–25)	Median	IR(75–25)
CD3 ⁺						
CD4 ⁺	54.42	60.88–50.62	63.09	63.48–51.94	48.37	61.40–34.77
CD8 ⁺	33.09	40.95–26.24	31.44	38.71–28.65	53.65	59.70–31.73
CLA ⁺						
CD4 ⁺	3.83	3.07–5.45	3.45	2.77–4.96	3.28	5.67–2.14
CD8 ⁺	1.25	0.94–1.69	1.35	0.63–1.56	1.30	2.22–0.49
CD4 ⁺						
CD45RO ⁺	32.76	35.99–27.78	30.76	35.63–23.43	33.13	47.65–18.56
CD45RA ⁺	50.06	56.36–46.29	47.32	58.69–29.05	53.47	63.24–33.45
CD45RO ⁺ RA ⁺	15.65	19.29–11.03	15.96	30.83–13.00	15.67	17.60–8.47
CD8 ⁺						
CD45RO ⁺	23.34	28.35–21.05	15.81	26.14–12.30	21.43	34.62–5.12
CD45RA ⁺	17.55	21.91–13.90	63.36	70.12–50.72	48.46	85.32–42.59
CD45RO ⁺ RA ⁺	56.76	61.72–50.20	19.83	29.48–13.20	20.33	28.15–9.23

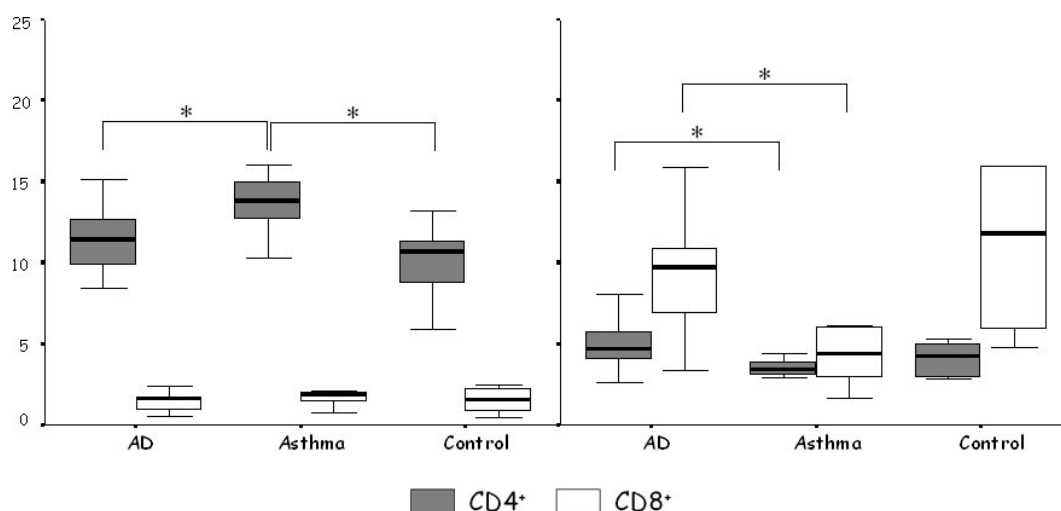


Fig. 2. Box plot of the percentage of CD25⁺ (left) and HLA-DR⁺ (right) cells in both CD4⁺ and CD8⁺ subpopulations. Comparisons were made between the three groups of children studied: AD (n = 20), asthmatic (n = 15), and controls (n = 15). Statistical differences (*) were considered significant at p < 0.017 after Bonferroni correction.

CD25⁺CD4⁺ cells (p = 0.029). Two by two comparisons showed an increase in the CD25⁺CD4⁺ cells in asthmatic patients (median: 13.83, IR: 15.52–12.48) compared to controls (median: 10.70, IR: 11.74–8.00; p = 0.01), and an increased tendency (non-significant after Bonferroni adjustment) in asthmatic patients (median: 13.83, IR: 15.52–12.48) compared to AD children (median: 11.45, IR: 12.63–9.63; p = 0.035). The population of CD8 cells expressing the CD25 marker was similar in all groups. This indicates that regulatory or activated T cells are increased in asthmatic children. Significant differences were also observed between the three groups in HLA-DR⁺CD4⁺ (p = 0.015) and HLA-DR⁺CD8⁺ (p = 0.043) T lymphocytes. The HLA-DR⁺CD4⁺ and HLA-DR⁺CD8⁺ T

lymphocytes were increased in AD patients (HLA-DR⁺CD4⁺; median: 4.67, IR: 5.85–3.95; HLA-DR⁺CD8⁺, median: 9.71, IR: 11.03–6.87) compared to asthmatic children (HLA-DR⁺CD4⁺; median: 3.40, IR: 3.93–2.81; p = 0.004; HLA-DR⁺CD8⁺; median: 4.37, IR: 6.08–2.09; p = 0.025), with no significant differences compared to controls.

Intracellular cytokine production in CD4⁺ and CD8⁺ subpopulations

The median and IR of the percentage of CD4⁺ and CD8⁺ subpopulations expressing the different cytokines (IL-4, IL-13, IL-5, TNF-α, IFN-γ, IL-2, IL-10) are shown in Table 2 and Fig. 3. Comparisons of cytokine production in the

Table 2. Median and IR of the CD4⁺ and CD8⁺ subpopulations producing IL-4, IL-13, IL-5, TNF- α , IFN- γ , IL-2, and IL-10 in AD, asthmatic and control children

	Atopic dermatitis		Asthma		Controls	
	Median	IR (75–25)	Median	IR (75–25)	Median	IR (75–25)
IL-4						
CD4	1.65	2.71–1.13	1.47	2.27–1.10	2.04	2.09–1.39
CD8	1.03	1.74–0.74	0.72	0.96–0.50	1.07	1.19–0.77
IL-13						
CD4	1.15	1.93–0.72	0.33	0.43–0.21	0.35	0.48–0.12
CD8	0.87	1.03–0.65	0.59	0.94–0.40	0.61	0.89–0.45
IL-5						
CD4	1.08	1.81–0.78	0.44	0.53–0.30	0.80	1.11–0.41
CD8	0.64	0.85–0.36	0.31	0.34–0.16	0.49	0.58–0.11
TNF- α						
CD4	12.43	15.18–7.24	1.88	2.17–1.17	3.65	7.51–2.28
CD8	5.27	8.98–3.41	3.61	4.05–2.59	6.79	9.15–5.12
IFN- γ						
CD4	8.04	11.51–4.91	3.62	4.96–2.62	8.43	9.29–7.18
CD8	5.46	11.59–3.58	2.51	3.65–1.35	5.73	11.22–1.27
IL-2						
CD4	3.26	4.53–1.28	0.94	1.27–0.76	1.85	2.87–0.79
CD8	1.04	1.57–0.58	0.39	0.91–0.21	0.53	0.94–0.16
IL-10						
CD4	1.77	2.74–1.39	1.84	3.73–1.23	2.21	2.76–1.40
CD8	0.86	1.45–0.71	0.85	1.64–0.62	1.48	1.91–0.99

CD4⁺ subpopulation showed a significant increase in IL-13 and TNF- α in AD patients compared to controls ($p < 0.001$ and 0.003 , respectively). IL-2 and IFN- γ were also increased

in AD compared to the asthmatic children ($p = 0.009$ and 0.003 , respectively), but as they were not increased compared to controls, we believe that this is due to their low expression in

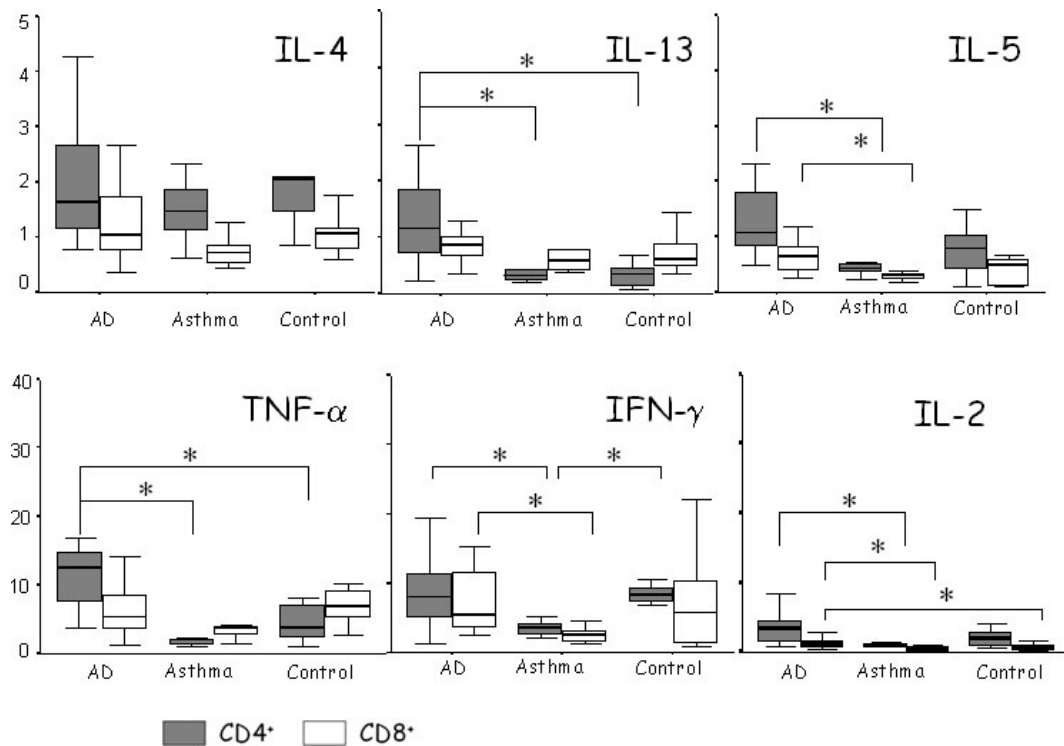


Fig. 3. Box plot of the percentage of CD4⁺ and CD8⁺ subpopulations producing IL-4, IL-13, IL-5, IL-2, TNF- α , and IFN- γ . Comparisons were made between the three groups of children studied: AD ($n = 20$), asthmatic ($n = 15$), and controls ($n = 15$). Statistical differences (*) were considered significant at $p < 0.017$ after Bonferroni correction.

asthmatic patients. Indeed, a significant decrease in IFN- γ was observed when asthmatic patients were compared with controls ($p = 0.017$). Thus, a clear Th2 pattern was observed in the AD children but not in children with asthma.

In the CD8⁺ subpopulation, we found an increase in the expression of IL-2 in AD compared to controls ($p = 0.009$), with no changes in asthmatic children, and an increase in IL-5, IL-2, and IFN- γ in AD compared to asthmatic patients ($p = 0.001$, 0.005 and 0.001, respectively).

Differences in the CLA⁺ and CLA⁻ subpopulations

The increase in the percentage of CD25 in asthmatic children versus AD children and controls was further analyzed by classifying the lymphocyte subsets into CLA⁺ and CLA⁻ cells. Although the CLA⁺ cells expressed a greater percentage of CD25, there were no significant differences between the three groups. The differences in the CD4⁺ and CD8⁺ T lymphocytes were only significant in the CLA⁻ cells (CD4⁺: AD; median: 17.27, IR: 20.16–14.20; asthmatic; median: 21.10, IR: 24.72–19.20; controls; median: 16.46, IR: 21.81–15.15; $p < 0.05$; CD8⁺: AD; median: 2.02, IR: 2.86–1.40; asthmatic; median: 4.17, IR: 4.91–3.10; controls; median: 2.98, IR: 4.30–1.40; $p < 0.05$) (Fig. 4). Comparisons by Mann–Whitney test showed that these differences were due to an increase in both CD4⁺ and CD8⁺ subpopulations in asthma compared to AD ($p = 0.011$ and 0.004, respectively) (Fig. 4).

As the changes in cytokine production were mainly observed in the CD4⁺ subpopulation, we further analyzed these data according to their CLA⁺ and CLA⁻ expression. We found that the

increase in IL-13 (AD; median: 9.88, IR: 14.90–7.83; controls; median: 3.29, IR: 6.24–2.65; $p = 0.001$), IL-5 (AD; median: 6.24, IR: 9.53–3.73; controls; median: 1.66, IR: 3.30–0.40; $p = 0.001$), and TNF- α (AD; median: 33.07, IR: 47.20–26.07; controls; median: 29.05, IR: 33.07–16.28; $p = 0.012$) observed in AD compared to controls was mainly due to the CLA⁺ cells, whereas the decreased production of IFN- γ in asthmatic patients was confined to the CLA⁻ subpopulation (asthmatic; median: 5.60, IR: 8.59–4.40; controls; median: 10.26, IR: 21.81–4.87; $p = 0.014$) (Fig. 5).

Discussion

Atopic dermatitis and atopic asthma are thought to share similar underlying mechanisms, although they differ in their target organs. We compared both disorders by analyzing several lymphocyte markers and the cytokine production in PBMC from two groups of atopic patients sensitized to house dust mite, one group with AD and one with bronchial asthma, as well as from a control group of non-atopic children.

IgE plays a central role in the mechanism of both diseases. As has been reported elsewhere (21, 22), this study showed that total IgE levels were higher in AD than asthmatic children, but the asthmatic children had higher levels of specific IgE to DP. This may be explained by different genes controlling total and specific IgE, as has been described in genetic linkage studies (23, 24). Moreover, in clinical practice AD patients showed multiple specificities whereas asthmatic patients presented a unique response.

Despite the fact that AD affects the skin whereas asthma does not, we found no differences between groups in the expression of T-cell

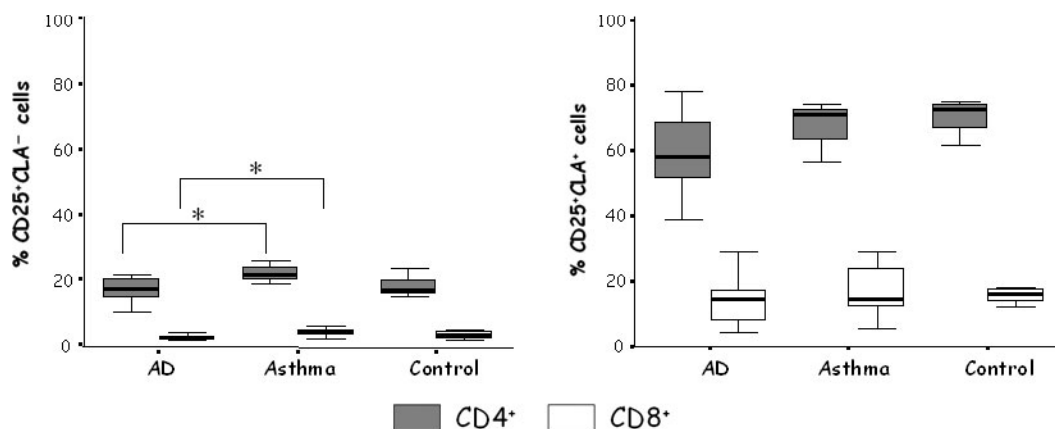


Fig. 4. Box plot of the percentage of CD25⁺CLA⁻ cells (left) and CD25⁺CLA⁺ cells (right) in both CD4⁺ and CD8⁺ subpopulations. Comparisons were made between the three groups of children studied: AD ($n = 20$), asthmatic ($n = 15$), and controls ($n = 15$). Statistical differences (*) were considered significant at $p < 0.017$ after Bonferroni correction.

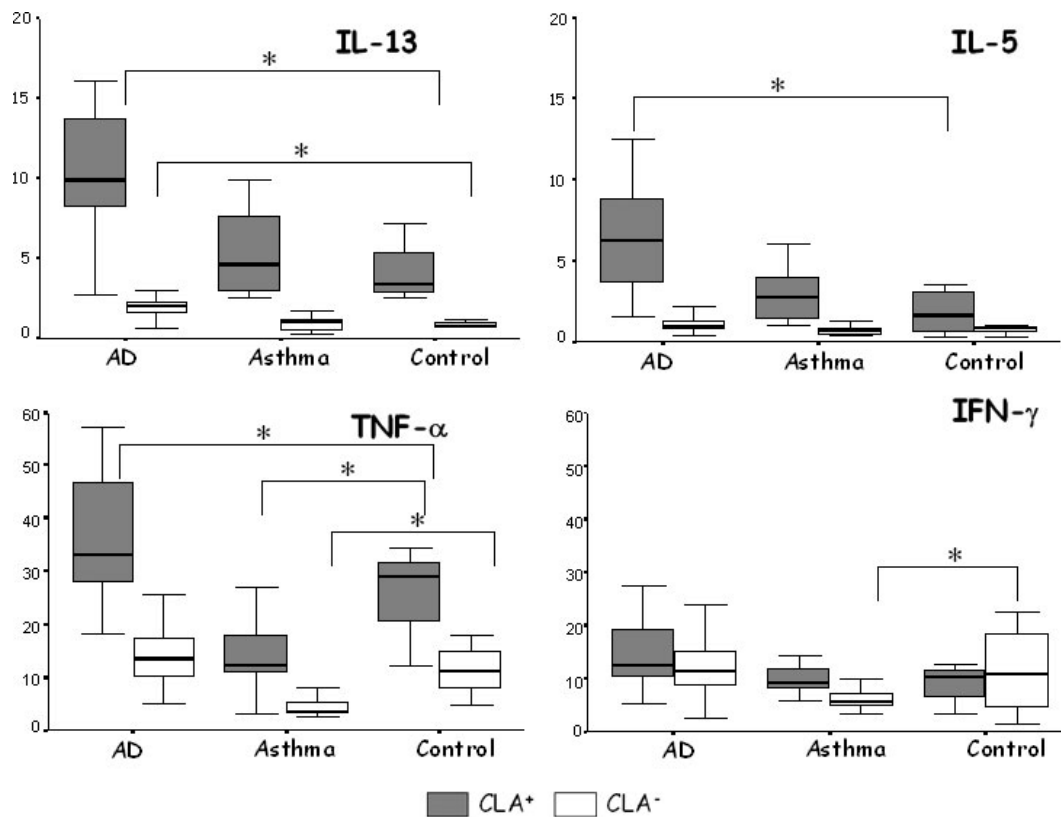


Fig. 5. Box plot of the percentage of CLA⁺CD4⁺ and CLA⁻CD4⁺ subpopulations producing IL-13, IL-5, TNF- α , and IFN- γ . Comparisons were made between the three groups of children studied: AD (n = 20), asthmatic (n = 15), and controls (n = 15). Statistical differences (*) were considered significant at $p < 0.017$ after Bonferroni correction.

skin homing receptors. These findings are in agreement with previous results, which have shown that circulating CLA⁺ cells are only increased in AD patients with chronic lesions (10) but not in AD patients with acute lesions. One reason may be that these CLA⁺ T cells are presumably located in the skin, and the increase is not therefore reflected in peripheral blood, as has been shown for some allergic reactions to drugs (25).

In the asthmatic children, we found increased levels of CD25 cells in the CD4⁺ subpopulation, limited to the CLA⁻ cells. In recent years, a specific subset of regulatory T cells bearing a CD4⁺CD25⁺ T-cell phenotype has been the focus of extensive investigation (26, 27). These T cells, endowed with distinct immunomodulatory properties, are important components of the homeostasis of the immune system, as impaired CD4⁺CD25⁺ T-cell activity can cause autoimmune diseases and allergy (28). However, our study was unable to distinguish whether they were merely activated CD4⁺ or regulatory cells, which might play a critical role in controlling the development of allergic disease and asthma.

In the AD children, we found an increase in IL-5, IL-13, and TNF- α in the CD4⁺ cells, though

only significant for IL-13 and TNF- α , with no change in IL-4. This may be explained by the transient expression of IL-4, shown by other authors (29), while IL-13 is produced in rapid response to activation of peripheral blood T cells and maintained over a longer period of time. These increases in the AD group support a Th2 response, which agrees with other studies (30, 31), as well as with our earlier results (10). The fact that Th1 cells have a greater susceptibility to activation-induced cell death in AD patients may contribute to the polarized Th2 response (32). As has been reported in AD, the increased production of TNF- α may also contribute to the up-regulation of the expression of chemokines, such as CCL17 (33) and CXCL10 (34), produced by keratinocytes, and adhesion molecules (E-Selectin, VCAM-1 and ICAM-1), produced by endothelial cells. These are all implicated in the development of atopic dermatitis, facilitating the endothelial extravasation of CLA⁺CCR4⁺ T cells into lesional skin (35). The overproduction of TNF- α plays a part in the pathophysiological mechanism of atopic dermatitis.

The most striking finding in the asthmatic group was a decrease in IFN- γ as compared with the AD patients and the control group. As these

results were accompanied by non-significant differences in the other cytokines compared to controls, the Th2 pattern found can be attributed to the reduction in IFN- γ . These results have been observed by others, independently of whether IL-4 was increased (36, 37) or not (15), although they used T cells stimulated *in vitro* with DP. Furthermore, in severe ongoing asthma, a significant decrease in the production of allergen-induced IFN- γ has also been found (38). These data suggest that defective IFN- γ release is a generalized feature of atopic asthma.

In this study, we attempted to determine the pattern of production of these cytokines depending on the expression of skin homing receptors. We found increased levels of IL-13, IL-5, and TNF- α in the CD4⁺CLA⁺ cells, while the decrease detected in IFN- γ production in asthmatic children was only found in the CD4⁺CLA⁻ subsets. This agrees with previous studies (6), which showed a preferential proliferation of circulating memory T cells to DP, of CLA⁺ memory cells in AD patients and of CLA⁻ in asthmatic patients and in patients with both diseases. Taken together, these results confirm the involvement of CLA⁺ cells in AD (3, 6, 10) and suggest the existence of a CLA⁻ subpopulation that participates in asthma, although we are unable to clarify whether these cells express a specific homing marker to the lung. Whether this can be changed to influence the atopic march is at present unknown.

Nowadays, broader evidence exists for the functional heterogeneity of CD8⁺ T cell; they can secrete Th2 cytokines and help B cells with antibody production (39) and they appear to participate in the induction of high IgE levels, although the exact role is still controversial (40). A similar pattern of cytokine production in CD4⁺ as in CD8⁺ cells has been found in asthmatic patients (41) and in AD patients (39). In our study, although changes in the CD8 subpopulation were found, these were minor compared with those found in the CD4 subpopulation.

We conclude that although AD and asthma are both atopic diseases, important immunological differences can be detected in peripheral blood. There are changes in total and specific IgE levels, activation markers, and cytokine production patterns, with AD patients expressing a Th2 pattern while asthmatic patients just show a decrease in IFN- γ . Moreover, the T-cell subpopulation involved is different, since the cytokine changes appear mainly in CLA⁺ T cells in AD but in CLA⁻ T cells in asthma. These data will contribute to the understanding of these two atopic manifestations.

Acknowledgments

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