

Skin-associated lymphocytes in the peripheral blood of patients with atopic dermatitis: Signs of subset expansion and stimulation

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Background: Skin-associated T cells are defined by the cutaneous lymphocyte-associated antigen (CLA). In atopic dermatitis (AD), CLA⁺ T cells harbor allergen-reactive memory cells, spontaneously secrete T_{H2} cytokines, and display signs of increased in vivo activation, thus relating the subset to the central disease pathomechanisms.

Objectives: It is not known whether the proportion of circulating CLA⁺ T cells might be expanded in AD. We were therefore prompted to compare the peripheral blood lymphocyte subpopulations of patients with AD with those of control subjects. **Methods:** We used 3-color flow cytometry to investigate age-matched peripheral blood samples of pediatric and young adult patients with mild (n = 21) or severe (n = 15) AD, patients with allergic/atopic diseases not involving the skin (n = 9), and healthy control subjects (n = 14).

Results: We found no differences among the study groups with respect to the general proportions of T cells, CD4⁺ T cells, CD8⁺ T cells, B cells, NK cells, CD103⁺ T cells, and CD25⁺ T cells among total circulating lymphocytes. However, there were slightly more CD4⁺ memory cells and clearly more HLA-DR⁺ T cells in patients with severe AD. Most remarkably, patients with severe AD had a significantly expanded proportion of CLA⁺ T cells ($P = .024$) and CLA⁺/CD4⁺ T cells ($P = .006$) but similar proportions of CLA⁺/CD8⁺ T cells compared with control subjects. Patients with severe AD also had distinctly more HLA-DR⁺/CLA⁺ T cells than control subjects ($P = .005$). Similar alterations were seen in patients with mild AD, but these were not statistically significant. After correction for age, all differences were significant only in probands less than 10 years of age.

Conclusions: Circulating skin-associated T cells (CLA⁺) show signs of subset expansion and enhanced activation in patients with AD. These alterations, compared with control values, affect CD4⁺ memory T cells in particular and are prominent only in children less than 10 years of age. (*J Allergy Clin Immunol* 1999;103:901-6.)

Key words: Atopic dermatitis, T cell, cutaneous lymphocyte-associated antigen, skin association, peripheral blood

The compartmentalization of T-cell responses by means of tissue-specific adhesion receptors represents one of the unraveled mysteries of immunology.¹ T cells with skin-selective recirculation capabilities, for example, are defined by a unique adhesion molecule, the cutaneous lymphocyte-associated antigen (CLA), which is the counter-receptor of the endothelial selection CD62E.^{2,3} Preferential vascular expression of CD62E at cutaneous sites and enhancement of transmigration of T cells through endothelial cell layers by means of CLA/CD62E interactions form the backbone mechanisms by which CLA⁺ T cells acquire their tissue-selective homing potentialities.^{4,5} Most T cells in normal skin, as well as about 16% of peripheral blood (PB) memory T cells (both CD4⁺ and CD8⁺ cells), express CLA.^{2,6} With respect to T cell-mediated cutaneous diseases, including atopic dermatitis (AD), it is not surprising that the infiltrating functional T cells also express CLA, as do allergen-specific T-cell clones from cutaneous challenge sites.^{2,7}

It has been a recurrent issue of investigation whether the lymphocytes in the PB of patients with AD show disease-related alterations with respect to their proportional composition, phenotype, or functional activity. Only recently, it was found that in AD, unlike in asthma, the allergen-specific T cells in PB exclusively reside in the CLA⁺ memory T-cell compartment and that these circulating CLA⁺ T cells also show signs of enhanced in vivo activation.^{8,9} In addition, it was demonstrated that the CLA⁺ T cells from the skin and PB of patients with AD spontaneously secrete T_{H2} cytokines,⁷⁻⁹ which is typical of the disease process in AD.¹⁰⁻¹⁴ These signs of linkage of the CLA⁺ T-cell subset to the central pathogenetic process of AD prompted us to ask whether the CLA⁺ T-cell compartment in the PB of patients with AD might also be expanded.

METHODS

Patients and control subjects

A total of 59 children and young adults attending an outpatient allergy clinic for symptoms of potentially allergic disease were

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Abbreviations used

AD:	Atopic dermatitis
ADhi:	Group of patients with severe atopic dermatitis
ADlo:	Group of patients with mild atopic dermatitis
AL:	Group of patients with allergic/atopic diseases not involving the skin
CLA:	Cutaneous lymphocyte-associated antigen
CO:	Group of control subjects without atopy/allergy
PB:	Peripheral blood
PE:	Phycoerythrin

enrolled in the study after informed consent from the probands and/or their parents had been obtained. All probands were evaluated by their clinical and family history, by physical examination, by skin prick testing, and by measurement of specific and total IgE antibody levels to diagnose or exclude allergic/atopic disease. Skin prick testing was performed according to the technique described by Pepys.¹⁵ We used standardized allergen preparations (ALK Laboratories, Horsholm, Denmark) against a screening panel of inhalant and/or nutritive allergens, including pollen, mites, animal dander, molds, and food allergens, as well as 0.9% NaCl and histamine dihydrochloride as negative and positive controls, respectively. In infants, screening panels were adjusted to age and clinical symptoms. Circulating specific IgE antibody levels were measured for skin prick test-reactive allergens, as well as for a panel of inhalant allergens, by using the Pharmacia CAP RAST FEIA (Pharmacia Diagnostics, Uppsala, Sweden). Total IgE was measured with the Pharmacia CAP IgE FEIA (Pharmacia Diagnostics) according to the manufacturer's instructions. According to these cumulative data, probands were given a diagnosis of AD (as defined by the standard criteria of Hanifin and Rajka¹⁶), allergic/atopic disease not involving the skin (rhinoconjunctivitis and/or asthma bronchiale; $n = 9$, AL group), or no allergic disease ($n = 14$, CO group). Patients with AD were further subdivided by having either mild ($n = 21$, ADlo group) or severe ($n = 15$, ADhi group) disease according to the extent of skin involvement, which was estimated by using the rule of nines (percentage of total body surface area) and the total clinical severity grading according to Rajka.¹⁷ This clinical severity scoring considered 6 parameters, including erythema, edema/papulation/induration, pruritus, excoriations/erosions, scaling/dryness, and lichenification. Each was measured on a half-point incremental scale from 0 to 3: 1, mild; 2, moderate; and 3, severe. Dermatitis was classified as mild, with a total clinical score of 6 or less (median, 3.5) involving at the most 20% of the skin. Severe dermatitis was diagnosed with a total clinical score of at least 7 (median, 10) involving at least 20% of the body surface area. From all the probands, heparinized PB samples were collected for immunologic investigation. At the time of blood sampling, all patients with AD had active disease, and none had received oral steroid or other immunosuppressive therapy for at least 1 month.

Antibodies

The following FITC- or phycoerythrin (PE)-labeled or unconjugated murine mAbs were used: CD3 (UCHT1-FITC, -PE, pure), CD4 (MT310-FITC, -PE), CD8 (DK25-FITC, -PE), CD16 (DJ130c-FITC), CD19 (HD37-PE), CD45 (T29/33-FITC), CD45RO (UCHL1-FITC), and anti-MLA/CD103 (Ber-ACT8 pure) from Dako (Glostrup, Denmark); CD25 (2A3-PE) and anti-HLA-DR (L243-PE) from Becton Dickinson (Sunnyvale, Calif); and CD45RA (2H4-RD1 pure) from Coulter Immunology (Krefeld, Germany). The unlabeled rat mAb HECA-452 against the CLA antigen² was kindly provided by Dr Picker (University of Texas, Dallas).

Immunofluorescence staining procedure

Mononuclear cells were isolated from the PB specimens by density gradient centrifugation and washed twice in RPMI-1640 medium containing 2% FCS (both from Gibco, Paisley, UK) before immunofluorescence labeling. Staining protocols used 3-color investigations in all experiments. All antibodies were used at concentrations titrated for optimal staining. In brief, 1 to 2×10^5 mononuclear cells were first incubated with the unconjugated mAbs and then with biotinylated rabbit F(ab')₂ anti-mouse immunoglobulins or (for HECA-452 detection) with biotinylated rabbit anti-rat immunoglobulins (both from Dako). To exclude nonspecific staining by means of unsaturated binding sites of the second step reagent, this was followed by incubation with unlabeled irrelevant murine IgG₁ (MOPC21; Sigma, St Louis, Mo). Without washing, the directly labeled mAbs were then added simultaneously and together with streptavidin-R670 (Gibco). Murine irrelevant isotype-matched fluorescent mAbs, as well as irrelevant rat immunoglobulins (all from Dako), were always included in the staining protocols as negative controls. All incubations were performed at 4°C in the dark over 30 minutes and were followed by washing steps with PBS solution (0.13 mol/L NaCl, 7 mmol/L Na₂HPO₄, and 3 mmol/L NaH₂PO₄) supplemented with 2% FCS. Before flow cytometry, cell suspensions were passed through a 30- μ m nylon mesh (Swiss Silk Bolting Manufacturing Co, Zurich, Switzerland).

Flow cytometry

Analyses were performed on a FACStar Plus (Becton Dickinson) equipped with an Argon laser tuned to 488 nm. Calibration beads (Calibrite, Becton Dickinson) were routinely used for monitoring and optimizing the instrument settings. Laser and photomultiplier parameters were kept constant for all experiments. Data were acquired with the LYSYS II or CELL Quest software (Becton Dickinson). Forward light scattering, orthogonal light scattering, and fluorescence signals (FL-1-FITC, FL-2-PE, FL-3-Red670) were stored in listmode data files. Each measurement contained 30,000 cells. The data were analyzed by using the PAINT-A-GATE (Becton Dickinson).

Statistical analysis

The Wilcoxon 2-sample test (2-sided) was generally used to investigate the conformity of the size of the various lymphoid subpopulations in patients and control subjects, as well as age and IgE distributions. In case of fewer than 10 samples within a proband group, exact P values were calculated.

RESULTS**Definition of study groups and correlation of age parameters**

The following 4 proband groups were investigated and compared with each other: 2 patient groups, with either mild (ADlo, $n = 21$) or severe (ADhi, $n = 15$) AD and 2 control groups, with either allergic diseases not involving the skin (AL group, $n = 9$) or without any allergic/atopic disease (CO group, $n = 14$). The median age of the ADlo group was 5.8 years (range, 2.5 to 31.4 years), that of the ADhi group was 10.7 years (range, 0.5 to 27 years), that of the AL group was 10.5 years (range, 3 to 16.25 years), and that of the CO group was 7.7 years (range, 2 to 29.5 years). In testing for the conformity of the age distributions among the 4 groups, we found no significant difference in any of the correlations.

TABLE I. Mean percentage values (SD) of various lymphoid subsets in the PB of probands of the 4 study groups

	CO (n = 14)	AL (n = 9)	ADlo (n = 21)	ADhi (n = 15)
T cells of lymphocytes (%)	62 (13)	61 (10)	57 (19)	54 (16)
B cells of lymphocytes (%)	28 (9)	30 (9)	35 (21)	35 (16)
NK cells of lymphocytes (%)	10 (8)	9 (5)	11 (8)	11 (7)
CD4 ⁺ T cells (%)	60 (11)	60 (11)	57 (10)	58 (12)
CD8 ⁺ T cells (%)	29 (6)	26 (6)	30 (9)	30 (10)
CD4 ⁺ /CD8 ⁺ ratio	2.3 (0.8)	2.4 (0.6)	2.1 (0.8)	2.3 (1.1)
CD45RO ⁺ CD4 ⁺ T cells (%)	33 (14)	45 (16)	41 (12)	46 (18)
CLA ⁺ T cells (%)	10 (5)	8 (2)	12 (6)	14 (4)
CLA ⁺ CD4 ⁺ T cells (%)	10 (5)	9 (3)	14 (8)	16 (7)
CLA ⁺ CD8 ⁺ T cells (%)	12 (5)	9 (3)	11 (7)	13 (7)
CD103 ⁺ T cells (%)	3 (1)	2 (1)	3 (2)	2 (1)
CD25 ⁺ T cells (%)	3 (1)	3 (2)	4 (2)	4 (2)
CD25 ⁺ CLA ⁺ T cells (%)	10 (3)	12 (6)	12 (4)	13 (5)
HLA-DR ⁺ T cells (%)	3 (1)	4 (4)	6 (5)	7 (4)
HLA-DR ⁺ CLA ⁺ T cells (%)	14 (7)	17 (9)	21 (11)	30 (16)
HLA-DR ⁺ CLA ⁻ T cells (%)	2 (1)	3 (4)	3 (2)	3 (2)

PB lymphocyte subpopulations in patients and control subjects

There were no significant differences between the groups of patients with AD (ADlo and ADhi) and the control groups (CO and AL) with respect to the general proportions of T cells (CD3⁺), CD4⁺ T cells, CD8⁺ T cells, B cells (CD19⁺), and NK cells (CD16⁺CD3⁻) among total lymphocytes (CD3⁺ plus CD19⁺ plus CD16⁺) in the PB. The mean percent values and SDs for all these subpopulations are shown in Table I. The CD4⁺/CD8⁺ ratios were also similar in the patient and control groups (Table I). The memory cell compartment (CD45RO⁺) of CD4⁺ T cells, however, showed a trend to a slightly increased size in both AD cohorts, as well as in the AL group compared with the CO group, which reached marginal statistical significance only in the CO versus ADhi correlation ($P = .047$).

Circulating T cells expressing tissue-selective homing receptors in patients and control subjects

Investigating the proportions of circulating T lymphocytes that expressed the tissue-selective homing receptors CLA and CD103, we found that the range distributions of the 4 study groups clearly overlapped. However, CLA-expressing T lymphocytes were slightly more frequent in patients with AD (Table I, Fig 1). This difference was statistically significant only for the ADhi group correlations versus the CO group ($P = .024$) and versus the AL group ($P = .0005$). With respect to CD103 expression (Table I), which is the tissue-specific homing receptor of T cells at mucosal sites,^{18,19} there were no significant differences between patients with AD and control subjects. Because both CD4⁺ and CD8⁺ T lymphocytes may express CLA,² but CD4⁺ T lymphocytes predominate in the cellular dermal infiltrate in AD suggesting a major pathogenetic role of this T-cell subset in

particular,^{20,21} we also investigated T cell subset-specific CLA expression patterns. CD8⁺ T lymphocytes expressed CLA in similar proportions in patients and control subjects, but the subsets of circulating CD4⁺ T cells were expanded in patients with AD (Table I, Fig 1). Again, differences in the latter were more obvious for patients with severe AD when compared with the CO group ($P = .006$) and the AL group ($P = .007$), whereas differences were marginally nonsignificant for the ADlo group correlations. Reviewing these data in correlation to age (probands of groups ADhi and CO subdivided according to age less than or greater than 10 years), we found that these differences in CLA expression were particularly evident in children less than 10 years of age (T cells, $P = .008$ for ADhi vs CO; CD4⁺ T cells, $P = .012$ for ADhi vs CO), whereas they were nonsignificant for older probands (Table II). As shown in Table II, we found age-related increases in both the CO and the ADhi groups in general with respect to the proportions of CLA⁺ T cells (marginally nonsignificant) and of CLA⁺ CD4⁺ T cells ($P = .009$ for both groups).

Activation markers on circulating T cells in patients and control subjects

We found similar proportions of CD25 expression on circulating T cells in general and on CLA⁺ T cells in particular when comparing patients with AD and control subjects (Table I). HLA-DR, however, was more frequently expressed by T cells, and especially by CLA⁺ T cells, in patients with AD compared with control subjects, although the range distributions were overlapping to a certain extent (Table I, Fig 1). Differences were statistically significant for T cells (ADhi vs CO: $P = .002$; ADhi vs AL: $P = .037$; ADlo vs CO: $P = .036$) and for CLA⁺ T cells (ADhi vs CO: $P = .005$). After correction for age (Table II), these differences were still significant only in children less than 10 years of age (HLA-DR⁺ T cells: $P = .015$ for ADhi vs CO; HLA-DR⁺ CLA⁺ T cells: $P = .007$ for ADhi vs CO).

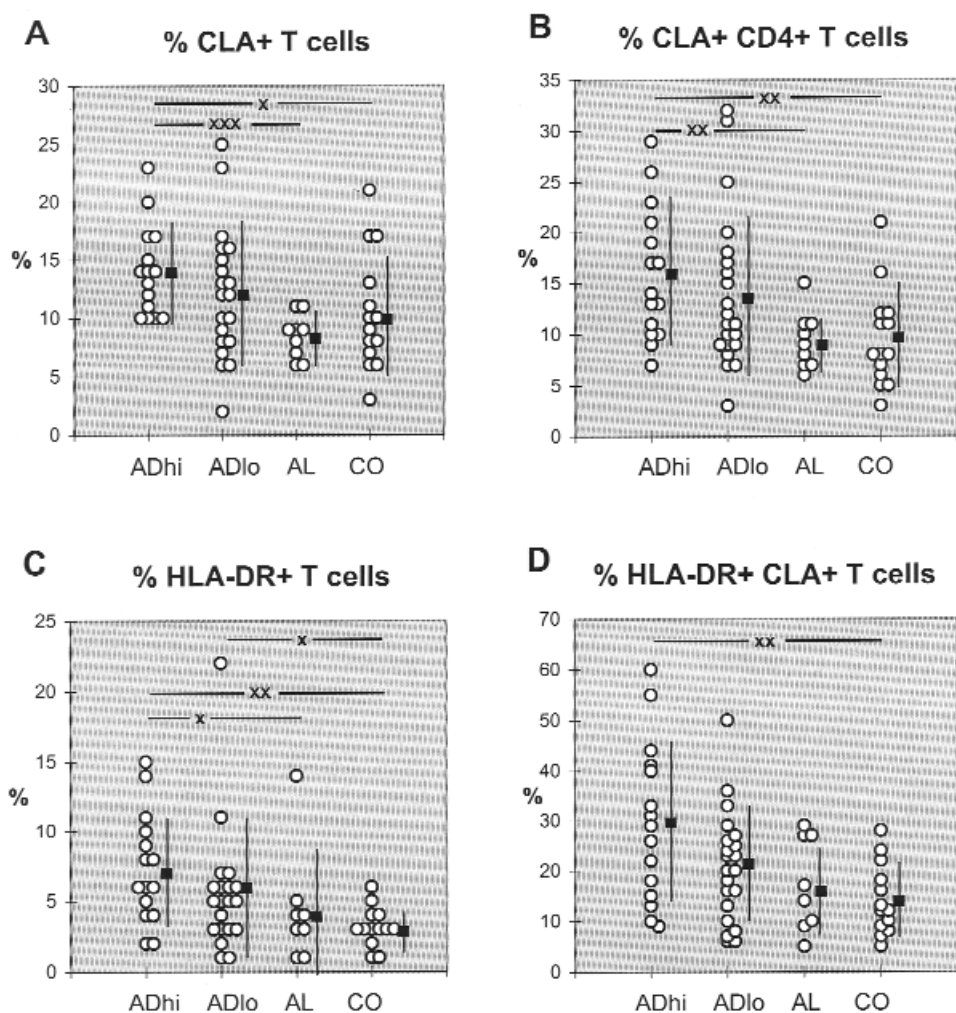


FIG 1. Comparison of the proportions of CLA⁺ T cells (**A**), CLA⁺ CD4⁺ T cells (**B**), HLA-DR⁺ T cells (**C**), and CLA⁺ T cells expressing HLA-DR (**D**) in the PB of probands from the 4 age-matched study groups. Each data point represents the proportion of respective T cells of an individual proband. The mean values of each group of probands are depicted by filled squares (SDs are indicated by the attached lines). Note that atopic patients (mild and severe) have trends to higher proportions than control groups in all T-cell subsets shown, although range distributions are considerably overlapping. Statistically significant correlations are indicated as follows: * $P < .05$, ** $P < .01$, *** $P < .001$.

TABLE II. Mean percentage values (SD) of lymphoid subsets in the PB of probands with respect to age groups (less than vs greater than 10 years of age)

	CO		ADhi	
	<10	>10	<10	>10
CLA ⁺ T cells (%)	8 (3)	14 (6)	12 (2)	16 (4)
CLA ⁺ CD4 ⁺ T cells (%)	7 (2)	13 (5)	11 (3)	20 (6)
HLA-DR ⁺ T cells (%)	3 (2)	4 (1)	8 (5)	6 (3)
HLA-DR ⁺ CLA ⁺ T cells (%)	15 (7)	13 (8)	37 (16)	24 (14)

CO: n = 7 less than 10 years of age and 6 older than 10 years of age; ADhi: n = 7 less than 10 years of age and 8 older than 10 years of age.

DISCUSSION

Central to the major T cell-related pathogenetic process in AD, which is mediated by CD4⁺ memory T cells with a T_H2 cytokine profile,^{10,12,14,20-24} the skin-

homing T-cell subset, defined by the adhesion receptor CLA, has gained attention. It was found that these CLA⁺ T cells, derived from the skin and from the PB of patients with AD, harbor allergen-reactive memory cells, display

signs of in vivo activation, and spontaneously secrete T_H2 cytokines.⁷⁻⁹ In this study we corroborate and extend these findings. We show that the most significant proportional alteration with respect to lymphoid subpopulations in the PB of patients with AD affects the CLA^+ T-cell subset. In addition, we demonstrate that, apart from signs of increased activation (HLA-DR expression), the circulating CLA^+ T-cell subsets are proportionally expanded in patients with AD compared with control subjects (healthy probands and patients with allergic/atopic diseases not involving the skin). Interestingly, these differences were statistically significant only for patients with severe cutaneous manifestations of AD. Suggestively, this may imply a relationship of the phenomenon to the severity of actual disease; however, further investigations, including follow-up experiments, are needed for final proof. Our results indicate also that it is the $CD4^+$ subset within the CLA^+ T-cell compartment that preferentially contributes to the expansion of the whole subset. This has not been shown by the former studies but fits well into the concept, anticipated from studies of the cellular infiltrate in inflamed atopic skin, that $CD4^+$ memory T cells play the major pathogenetic role.^{20,21} Related to our observations, 2 other studies have described an expansion of the circulating CLA^+ T-cell pool, namely in patients with drug-induced cutaneous hypersensitivity reactions and in children with milk-induced eczema.^{25,26} The latter study compared 7 children with milk allergy and cutaneous atopic manifestations with several control groups, including children with milk-induced or allergic enterocolitis and healthy adults. Compared with this study, which deals with only 1 out of an array of possible allergic mechanisms leading to atopic skin manifestations, our investigation, which encompasses considerably more patients, proves the observed expansion of CLA^+ T cells for AD in general. Using age-matched patient and control groups, we could also show that this phenomenon is clearly related to age, finding that the cohort of children less than 10 years of age in particular contributed to these differences. Apart from this, we found that the proportion of CLA^+ cells among circulating T cells in general increases with age; in addition to the above data, we found that only 5% of T cells are CLA^+ in infants at 6 months of age (unpublished observation) compared with 16% in adults.² Such an increase with age is well-established for the global circulating memory T-cell pool.²⁷ Accordingly, older probands have larger numbers of circulating memory cells with different specificities, which may make an expansion of a certain subset less obvious and which may provide an explanation for the lack of differences between the proband groups older than 10 years of age.

The expansion of the CLA^+ T-cell pool in the PB of children with severe AD is interesting also with respect to the possible mechanisms leading to the generation of this specific T-cell type. Data in the literature seem to prove that CLA^+ T cells in atopic patients and normal subjects do not differ fundamentally regarding their functional capacity (eg, cytokine profile) but are decisively

more active and are generated more abundantly on allergen stimulation in AD.^{9,26} Apart from the question of why certain allergens (including nutritive and inhalant) induce T cells to mature toward this skin-associated T-cell subset with a basic propensity for T_H2 cytokines, the mechanisms leading to the increase in circulating CLA^+ T cells in AD need to be clarified. CLA expression is induced during antigen-dependent virgin to memory transition of T cells and is triggered and upregulated preferentially in skin-associated environments.²⁸ However, it remains elusive whether antigenic stimuli may generate CLA^+ T cells also at extracutaneous sites, thus focusing their recirculation potential by means of the PB to the skin, or whether these cells are first generated in skin-associated tissues, proliferate, and then recirculate to the PB in extended quantities, reflecting an enlarged CLA^+ T-cell pool in general. More detailed insights into this question may considerably contribute to a better understanding of the mechanisms that precipitate allergic/atopic conditions.

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