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Skin Homing (Cutaneous Lymphocyte-Associated Antigen-Positive) CD8⁺ T Cells Respond to Superantigen and Contribute to Eosinophilia and IgE Production in Atopic Dermatitis¹

Mübeccel Akdis,^{2*} Hans-Uwe Simon,^{*} Lorenz Weigl,[†] Oliver Kreyden,[‡] Kurt Blaser,^{*} and Cezmi A. Akdis^{*}

In allergic inflammations of the skin, activation of CD4⁺ T cells was demonstrated to play an important role; however, a minor role for CD8⁺ T cells is implied. In the present study, we compared cutaneous lymphocyte-associated Ag (CLA)-expressing CD4⁺ and CD8⁺ subsets, which were isolated from peripheral blood and lesional skin biopsies in atopic dermatitis (AD) patients. We demonstrated that CD8⁺CLA⁺ T cells proliferate in response to superantigen and are as potent as CD4⁺CLA⁺ T cells in IgE induction and support of eosinophil survival. In atopic skin inflammation, the existence of high numbers of CD4⁺ and CD8⁺ T cells was demonstrated by immunohistochemistry and by culturing T cells from skin biopsies. In peripheral blood, both CD4⁺ and CD8⁺ subsets of CLA⁺CD45RO⁺ T cells were in an activated state in AD. The in vivo-activated CLA⁺ T cells of both subsets spontaneously released an IL-5- and IL-13-dominated Th2 type cytokine pattern. This was confirmed by intracytoplasmic cytokine staining immediately after isolation of the cells from peripheral blood. In consequence, both CD4⁺ and CD8⁺, CLA⁺ memory/effector T cells induced IgE production by B cells mainly by IL-13, and enhanced eosinophil survival in vitro by delaying eosinophil apoptosis, mainly by IL-5. These results indicate that in addition to the CD4⁺ subset, the CD8⁺CLA⁺ memory/effector T cells are capable of responding to superantigenic stimulation and play an important role in the pathogenesis of AD. *The Journal of Immunology*, 1999, 163: 466–475.

Activation of peripheral blood CD4⁺ T cells and their preferential secretion of Th2 cytokines in patients with asthma and atopic dermatitis (AD)³ has repeatedly been reported (1–6). These cells control IgE Ab synthesis by B lymphocytes and are capable of attracting, activating, and prolonging survival of nonspecific effector cells. Eosinophils are major effector cells in the pathogenesis of atopic inflammatory diseases such as AD and bronchial asthma (7, 8). Besides other mechanisms, it was shown that delayed apoptosis contribute to the accumulation of eosinophils in allergic tissue (9). Mainly the cytokines IL-5, IL-3, and GM-CSF were shown to enhance eosinophil survival and activation of eosinophils in vitro (1, 10–12) and in cultures of excised nasal polyps (9). In previous studies mostly using specific T cell clones, the allergen-specific responses were ascribed to CD4⁺ T cells (2–6). However, there is now broader evidence for heterogeneity of CD8⁺ T cell functions. CD8⁺ T cells may not act solely as potent effector cells concerning the elimination of viral

and other intracellular pathogens. They can secrete Th2 cytokines and help B cells for Ab production (13–17).

In AD, the pivotal role of CD45RO⁺ (memory/effector) T cells expressing the skin homing receptor, the cutaneous lymphocyte-associated Ag (CLA) was demonstrated (18–22). Almost all T cells in benign and malignant cell infiltrations of the skin express CLA on their surface (18, 22, 24). The CLA molecule was shown to be a posttranslational modification of the carbohydrate moiety of the P-selectin glycoprotein ligand, i.e., constitutively expressed on lymphocytes, by the activity of a fucosyltransferase (25). CLA interacts with its vascular counterreceptor E-selectin (CD62E), being expressed on inflamed superficial dermal postcapillary venules (19, 20). CLA⁺ T cells, isolated from peripheral blood of atopic individuals, were shown to contain and spontaneously release cytokines, IL-4 and IL-13 (21, 26). Furthermore, it was shown that in vitro staphylococcal enterotoxin B (SEB) treatment of atopic PBMC resulted in up-regulation of CLA expression on T cells and therefore may facilitate the homing of T cells in the AD skin (27). Moreover, evidence from several in vitro and in vivo studies suggests that bacterial superantigens possess the potency to trigger chronic T cell-mediated skin inflammation in AD (27–29).

In the present study, the functional properties of CD4⁺ and CD8⁺ subsets among CLA-expressing memory/effector T cells isolated from peripheral blood and lesional skin of AD patients were compared. We demonstrate that CLA⁺CD8⁺ T cells isolated from skin or peripheral blood of AD patients respond to superantigenic stimulation to the same extent as CD4⁺ T cells. They spontaneously proliferate ex vivo, secrete high levels of IL-5 and IL-13, and therefore are capable of preventing spontaneous eosinophil apoptosis and enhancing IgE similar to the CD4⁺ subset of CLA⁺ T cells.

*Swiss Institute of Allergy and Asthma Research (SIAF), Davos, Switzerland; [†]Clinic for Dermatology and Allergy, Davos, Switzerland; and [‡]Zürcher Höhenklinik Clavadel, Davos, Switzerland

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² Address correspondence and reprint requests to Dr. Mübeccel Akdis, Swiss Institute of Allergy and Asthma Research (SIAF), Obere Strasse 22, CH-7270 Davos, Switzerland. E-mail address: akdis@siaf.unizh.ch

³ Abbreviations used in this paper: AD, atopic dermatitis; CLA, cutaneous lymphocyte-associated Ag; SEB, staphylococcal enterotoxin B; ECD, PE-Texas red.

Materials and Methods

Study population

Nineteen patients, 11 female and 8 male, with chronic AD (mean age, 31 yr) who fulfilled the criteria of Hanifin and Rajka (30) and who required hospitalization were selected for the study. All were polyallergic and had positive cutaneous tests to at least three aeroallergens. None of these patients had asthma. Patients showed specific IgE Abs at radioallergosorbent test class ≥ 2 and total IgE of >400 U/ml (mean, 3219 ± 1862 IU/ml). None of the patients had systemic immunosuppressive treatment at least 1 mo before peripheral venous blood was taken. Skin biopsies were taken in five of the AD patients after receiving their informed consent. Total IgE of patients who underwent skin biopsy was 9132 ± 3265 IU/ml. Peripheral eosinophilia was $9.75 \pm 2.39\%$. They were all severely ill patients who required hospitalization. Five healthy individuals (mean age, 28 yr) with no history of atopy were included in the study as normal control group. Their mean IgE levels were 72 ± 18 IU/ml. The study was approved by the ethical committee of Davos, Switzerland.

Abs and reagents

The CLA-specific rat mAb HECA-452 was kindly provided by Dr. E. Butcher, Stanford University, Palo Alto, CA (18–20). All fluorescent- or biotin-labeled mAbs for cell purification or FACS analyses were purchased from Coulter (Hialeah, FL), Immunotech (Marseille, France), or PharMingen (San Diego, CA). Anti-CD4, anti-CD8, anti-CD14, anti-CD19, anti-mouse Ig, and streptavidin-conjugated magnetic microbeads for Magnet Activated Cell Sorting (MACS) cell separation were from Miltenyi Biotec (Marburg, Germany). Anti-CD2 (4B2 and 6G4) and anti-CD28 (15E8) mAbs were from the Red Cross Blood Transfusion Service, Amsterdam, The Netherlands. Anti-CD3 was produced by clone CRL 8001 obtained from American Type Culture Collection (Manassas, VA). Anti-CD4 mAb was obtained from Novocastra Laboratories (Newcastle, U.K.). Anti-CD8 and control mAbs were purchased from Dako (Zug, Switzerland). Neutralizing anti-IL-4 mAbs (8F12 and 3H4) and anti-IFN- γ mAb 45-15 were provided by Novartis (Basel, Switzerland). Anti-IL-13 mAbs (JES8-5A2 and JES10-2F9) and mutant IL-4 antagonist (Y124D) with IL-4- and IL-13-inhibitory activity were provided by DNAX Research Institute (Palo Alto, CA). Neutralizing anti-IL-5 and anti-GM-CSF were purchased from R&D Systems (Abingdon, U.K.) and PharMingen.

Culture of T cells from skin biopsies

Biopsies from AD skin lesions that were not treated by any topical treatment were incised at 8 mm diameters with 1% Xylocaine as local anesthetic. The biopsy specimens were minced with two scalpels; no proteolytic enzymes were used. Skin fractions from biopsy specimens were cultured in 25 U/ml IL-2 containing RPMI 1640 supplemented with 1 mM sodium pyruvate, 1% MEM nonessential amino acids and vitamins, 2 mM L-glutamine, 100 U/ml penicillin, 100 μ g/ml streptomycin, 50 μ M 2-ME (all from Life Technologies, Basel, Switzerland), and 10% heat-inactivated FCS. The biopsy specimens were stimulated with the combination of mAbs to T cell surface molecules anti-CD2 (two mAbs, 4B2 and 6G4, each 0.5 μ g/ml), anti-CD3 (5 μ g/ml), and anti-CD28 (1 μ g/ml). The combination of these mAbs provided an effective T cell growth from skin biopsies. The CD4:CD8 ratio and CLA expression were measured between days 7 and 10 of skin biopsy cultures as soon as enough T cells were obtained for FACS analysis. Stimulation of the skin biopsies to grow T cells may also induce CLA. The possibility that the CD4:CD8 ratio alters among T cells during culturing of skin biopsies for 7–10 days for FACS analysis was excluded by PBMC or purified T cells stimulations with IL-2 and mAbs to CD2, CD3, and CD28 (data not shown). This type of polyclonal T cell stimulation expands both CD4 $^{+}$ and CD8 $^{+}$ T cell subsets to the same degree. CD4 $^{+}$ and CD8 $^{+}$ T cell subsets from skin biopsies were positively selected by MACS at a purity of 99.8%.

Demonstration of the CD4 $^{+}$ and CD8 $^{+}$ cell infiltrate in the skin by immunohistochemistry

CD4 $^{+}$ and CD8 $^{+}$ cells were identified from skin biopsies by using specific mAb and immunohistochemistry (9). Briefly, skin tissues were fixed in 4% paraformaldehyde solution. Paraffin sections were mounted on poly-L-lysine-coated slides, stored at room temperature, deparaffinized in xylene, and rehydrated through graded concentrations of ethanol. Ag retrieval was achieved by the microwave oven heating procedure. CD4 and CD8 stainings were performed with the Dako catalyzed signal amplification system according to the manufacturer's instructions.

Isolation of CLA $^{+}$ and CLA $^{-}$ CD45RO $^{+}$ T cells of CD4 $^{+}$ and CD8 $^{+}$ subsets from peripheral blood

PBMC were isolated by Ficoll (Biochrom, Berlin, Germany) density gradient centrifugation of peripheral venous blood (31). Cells were washed three times and resuspended in DMEM (Life Technologies), supplemented with 10 mM HEPES (Life Technologies), 5 mM EDTA (Fluka Chemie, Buchs, Switzerland), 2% heat-inactivated FCS (SerA-Lab, Sussex, U.K.), 100 U/ml penicillin, and 100 μ g/ml streptomycin (both from Life Technologies). CLA $^{+}$ and CLA $^{-}$ cells were isolated with the MACS magnet-activated cell separation system (Miltenyi Biotec) as described in detail (21, 23, 26). In brief, anti-CD14- and anti-CD19-depleted cells were incubated with MACS microbead-conjugated anti-CD45RA and anti-CD16 mAbs and anti-CD4 or anti-CD8. The negatively selected CD4 $^{+}$ CD45RO $^{+}$ and CD8 $^{+}$ CD45RO $^{+}$ T cells were sequentially incubated with anti-CLA mAb HECA-452, biotin-conjugated goat anti-rat IgM, and streptavidin-conjugated microbeads. The magnetic (CD4 $^{+}$ CLA $^{+}$ CD45RO $^{+}$ or CD8 $^{+}$ CLA $^{+}$ CD45RO $^{+}$) and nonmagnetic (CD4 $^{+}$ CLA $^{-}$ CD45RO $^{+}$ or CD8 $^{+}$ CLA $^{-}$ CD45RO $^{+}$) T cell fractions were recovered by sequential elution from the MACS column. This procedure yielded 5×10^5 – 1.5×10^6 CLA $^{+}$ CD4 $^{+}$ cells, 9×10^5 – 2.4×10^6 CLA $^{-}$ CD4 $^{+}$ cells, 3×10^5 – 9×10^5 CLA $^{+}$ CD8 $^{+}$ cells, and 6×10^5 – 1.7×10^6 CLA $^{-}$ CD8 $^{+}$ T cells from 10^8 PBMC. The CD4 $^{+}$ T cell contamination in the CD8 $^{+}$ subset was $<0.1\%$, and CD8 $^{+}$ T cell contamination in CD4 $^{+}$ T cells was $<0.2\%$ as assessed by FACS analysis. Although CLA $^{+}$ CD45RO $^{+}$ T cells were positively selected by MACS purification, it was clearly demonstrated in the previous studies that ligation of CLA with HECA-452 mAb induces neither proliferation nor cytokine production (23, 26).

Flow cytometric analysis

After purification, 5×10^4 cells were sequentially stained with FITC-conjugated anti-CLA mAb HECA-452, together with anti-CD4-ECD or anti-CD8-ECD and either anti-CD45RO-PE. Stained cells were fixed in 2% paraformaldehyde. The controls were FITC-conjugated rat IgM and FITC-, PE-, or ECD-conjugated mouse IgG1. The purity of the CLA $^{+}$, CD4 $^{+}$, and CD8 $^{+}$ populations ranged from 93 to 99%.

Intracellular staining of cytokines in CLA $^{+}$ and CLA $^{-}$ T cells was performed by fixing and permeabilizing the cells immediately after purification from PBMC with a paraformaldehyde and saponin solution (ORTHOPERMEAFIX, Ortho Diagnostic Systems, Raritan, NJ) (26, 32). After washing with PBS containing 5% FCS, 1.5% BSA (Sigma, St. Louis, MO), and 0.0055% EDTA (Fluka Chemie), cells were stained with 0.5 μ g/ml R-PE-labeled anti-IL-4, anti-IL-5, anti-IL-13, or anti-IFN- γ mAbs and R-PE-labeled rat IgG1 and rat IgG2a control Ab (all from PharMingen) for 30 min at 4°C. The flow cytometric analysis was performed by an Epics Profile II (Coulter).

T cell cultures

Freshly isolated CLA $^{+}$ and CLA $^{-}$ T cells were resuspended in the above supplemented RPMI 1640 medium. Spontaneous cytokine secretion was determined in supernatants of 10^5 freshly isolated, unstimulated cells, cultured in 200 μ l medium for 24 h. Cultures were performed in 96-well flat-bottom plates (Costar, Cambridge, U.K.). Time points for harvesting the supernatants were chosen according to previous kinetic studies (26). The spontaneous proliferation of the cell subsets was analyzed by a [^{3} H]TdR incorporation of 10^5 cells in 200 μ l medium in 96-well flat-bottom tissue culture plates that were pulsed with 1 μ Ci/well [^{3} H]TdR for 24 h immediately after purification. The CLA $^{+}$ CD4 $^{+}$ or CLA $^{+}$ CD8 $^{+}$ T cells isolated from peripheral blood or skin of the patients were stimulated with different doses of SEB in 96-well flat-bottom plates (5×10^4 /200 μ l/well) in triplicates by using 3000-rad-irradiated autologous PBMC (5×10^4) as APC. [^{3} H]TdR incorporation was measured after 3 days after an 8-h incubation with 1 μ Ci/well [^{3} H]TdR. The plates were harvested on glass fiber plates, and radioactivity was measured in a β Plate Reader (Pharmacia-Wallac, Turku, Finland). Cytokine production by SEB stimulation was determined in parallel cultures from supernatants taken after 3 days.

Induction of IgE and IgG4 production

B cells were purified by MACS microbead-conjugated anti-CD19 after depletion of monocytes with microbead-conjugated anti-CD14 (26). Either CD4 $^{+}$ or CD8 $^{+}$ subsets of CLA $^{+}$ or CLA $^{-}$ T cells (10^5 /200 μ l/well, 96-well plate) were cocultured with the same number of purified B cells at 37°C in a 5% CO $_2$ atmosphere. IgE and IgG4 were determined in supernatants taken after 12 days. Inhibition of Ig production was attempted by

addition of neutralizing mAbs to IL-4 or IL-13, each at 10 μ g/ml. The neutralization capacity of the anticytokine mAbs was previously established (26, 33). Mutant IL-4 and IL-13 antagonist Y124D is used at 100 ng/ml (34). Mouse IgG1 (10 μ g/ml) (Immunotek) was used as a control. All experiments were performed in triplicate.

Quantification of cytokines and Ig isotypes

Solid phase sandwich ELISAs for IFN- γ , IL-3, IL-4, IL-5, IL-13, and GM-CSF are described (26, 31, 35, 36). Briefly, microtiter plates (Maxisorb, Nunc, Roskilde, Denmark) were coated with mAb 43-11 to human IFN- γ and developed with biotinylated mAb 45-15. The sensitivity of the IFN- γ ELISA was ≤ 10 pg/ml (mAbs and IFN- γ standard were gifts from Dr. S. S. Alkan, Novartis, Basel, Switzerland).

IL-4 was measured by using mAb 3H4 for coating and biotinylated mAb 8F12 for detection (mAbs and IL-4 standard were provided by Dr. C. H. Heusser, Novartis). The sensitivity of IL-4 ELISA was ≤ 30 pg/ml.

IL-5 was determined by using mAb TRFK5 for coating and biotinylated mAb JES15-A10 for detection (mAbs and IL-5 standard were from Pharmingen). The detection limit of the IL-5 ELISA was 50 pg/ml. IFN- γ , IL-4, and IL-5 ELISAs were developed by peroxidase-labeled ExtrAvidine (Sigma), and o-phenylenediamine HCl in citrate buffer, pH 5.5, was used as substrate. Optical density was measured at 490 nm in an ELISA reader (Molecular Devices, Menlo Park, CA) after stopping the reaction with 0.5 N H₂SO₄.

For the detection of IL-13, the mAb JES10-2F9 (kindly provided by DNAX Research Institute) was used for coating. Recombinant IL-13 from PeproTech (Rocky Hill, NJ) was used as a standard. Rabbit anti-IL-13 (PeproTech) and alkaline phosphatase-labeled goat anti-rabbit Ab (Zymed Labs, San Francisco, CA) were used for detection. The detection limit was 300 pg/ml IL-13. GM-CSF and IL-3 were detected by commercial ELISA kits (R&D), with a sensitivity of ≤ 5 pg/ml. In IL-3, IL-13, and GM-CSF ELISAs, the chromogenic substrate was 4-nitrophenyl phosphate-disodium hexahydrate (E. Merck, Darmstadt, Germany) in diethanolamine buffer, pH 9.8. Optical density was measured at 405 nm. The IL-2 activity was measured by [³H]TdR uptake of the IL-2-dependent cytotoxic T lymphocyte line cells as described (37, 38).

IgE and IgG4 in supernatants were measured in duplicates by sandwich ELISA as described (31, 35, 36). The assays reached a sensitivity of 0.2 ng/ml human IgE standard (Behringwerke, Marburg, Germany) and 0.6 ng/ml IgG4 World Health Organization 67-97 reference standard.

Determination of eosinophil death and apoptosis

Eosinophils were purified by negative MACS separation as previously described (39). Briefly, PBMC were separated from peripheral blood of patients with moderate eosinophilia (5–8%) by Ficoll-Hypaque centrifugation. The remaining cell population, mainly granulocytes and erythrocytes, were treated with erythrocyte lysis solution (155 mM NH₄Cl, 10 mM KHCO₃, and 0.1 mM EDTA, pH 7.3). The resulting granulocyte population was incubated with anti-CD16 mAb microbeads. CD16⁺ granulocytes were depleted by passing the through a MACS column. The resulting cell population contained 99% eosinophils as controlled by staining cell smears with Diff-Quick (Baxter, Düringen, Switzerland) and light microscopy.

Eosinophils (10⁵ cells/well) were cultured in 96-well tissue culture plates in RPMI 1640, either with spontaneously secreted 25% supernatants of CLA⁺ or CLA[−] T cell or with recombinant cytokines at different concentrations for indicated time points. Cell death of eosinophils was assessed by uptake of 1 μ M ethidium bromide and flow cytometric analysis as described (40).

Oligonucleosomal DNA fragmentation, a characteristic feature of cells undergoing apoptosis, was assessed as previously described (41). Briefly, MACS-purified eosinophils were cultured in the presence or absence of CLA⁺ or CLA[−] T cell supernatants for the indicated conditions and indicated times. After culture, the cells were resuspended in 0.3 ml hypotonic fluorochrome solution (50 μ g/ml propidium iodide, 0.1% sodium citrate, 0.1% Triton X-100) and incubated for 6 h at 4°C in the dark. The relative amounts of apoptotic eosinophils were determined by flow cytometric discrimination of DNA fragmentation in hypodiploid and diploid cells.

Membrane phosphatidylserine redistribution from the inner to the outer membrane leaflet takes place in apoptotic cells. Annexin V is a phosphatidylserine-binding protein and is used to detect apoptotic cells (42). Briefly, 2 $\times$ 10⁵ eosinophils were washed twice with cold PBS and resuspended in 1 ml binding buffer (HEPES, 0.25 mM CaCl₂). FITC-conjugated annexin V (100 ng/ml) and propidium iodide (500 ng/ml) were added to the cells. After gentle vortexing and incubating for 15 min in the dark, cells were immediately analyzed for annexin V binding and propidium iodide uptake by flow cytometry.

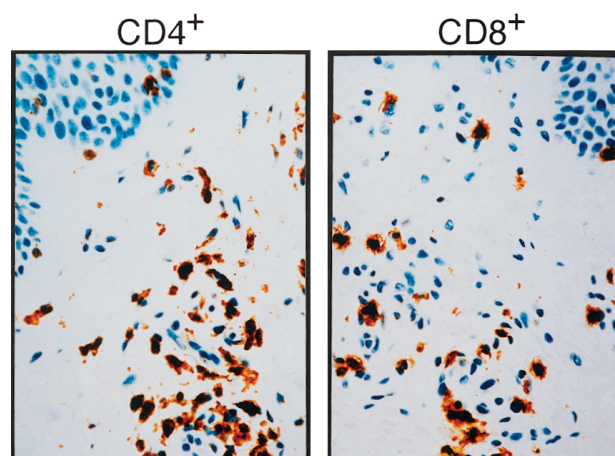


FIGURE 1. CD4⁺ and CD8⁺ cells in lesional skin of AD patients. The majority of the cells in the inflammatory cellular infiltrate in dermis represented CD4⁺ and CD8⁺ T cells. Two CD4⁺ T cells and one CD8⁺ T cell are stained in the basal cell layer of epidermis. Results shown are representative of five skin biopsies from five AD patients. Cumulative results of enumeration of cells are shown in Table I.

Statistical interpretation

Data are expressed as means $\pm$ SEM. Statistical analysis for paired comparisons was conducted by Student's *t* test and for percentages by the *z* test.

Results

Comparison of CLA-expressing CD4⁺ and CD8⁺ T cells in the lesional skin and PBMC of AD patients

The CD4⁺ and CD8⁺ T cell subsets in lesional skin of AD patients were identified in punch biopsies by immunohistochemistry. The CD4 and CD8 T cells in the eczema lesions in AD are shown in Fig. 1. The majority of the cells in the inflammatory cellular infiltrate represent CD4⁺ and CD8⁺ T cells. This suggests a role for both T cell subsets in AD. The percentages of CD4⁺ and CD8⁺ T cells in PBMC and skin biopsies are shown in Table I. The ratio of CD4:CD8 T cells in blood and skin appeared to be similar (1.74 and 1.62), indicating that both CD4⁺ and CD8⁺ T cell subpopulations are equally recruited into the inflammatory skin of AD patients.

As shown in Table II, the ratio of CLA-bearing CD4:CD8 T cells in PBMC was 1.97 $\pm$ 0.33 and was significantly lower in T cells cultured from skin biopsies (0.85 $\pm$ 0.73) (*p* < 0.001). CLA-expressing CD4⁺ (70%) and CD8⁺ (88%) T cells in skin biopsies were significantly higher than peripheral blood (30%: CD4⁺ and 27%: CD8⁺, *p* < 0.001). High CLA expression on skin T cells

Table I. Distribution of CD4⁺ and CD8⁺ T cells in PBMC and skin biopsies of AD patients^a

	Control (PBMC)	Atopic Dermatitis	
		PBMC	Skin
% CD4	49.20 $\pm$ 7.25	43.14 $\pm$ 3.43	502 $\pm$ 78
% CD8	33.05 $\pm$ 6.42	24.82 $\pm$ 4.29	297 $\pm$ 56
CD4:CD8	1.49 $\pm$ 0.31	1.74 $\pm$ 0.24	1.62 $\pm$ 0.20

^a PBMC from five AD patients and five healthy controls were stained immediately after purification. CD3⁺ cells were gated, and the percentages of CD4⁺ or CD8⁺ T cells was measured by FACS. The skin-infiltrating CD4⁺ and CD8⁺ T cells were determined by immunohistochemistry per square millimeter of skin section in five AD patients. Results are shown as mean $\pm$ SE.

Table II. CLA expression in CD4⁺ and CD8⁺ T cells in PBMC and skin biopsies of AD patients^a

	Control (PBMC)	Atopic Dermatitis	
		PBMC	Skin
% CD4 ⁺ CLA ⁺	9.75 ± 0.75*	13.06 ± 1.69	31.26 ± 8.86**
% CD4 ⁺ CLA ⁻	39.45 ± 7.75	30.08 ± 2.63	12.97 ± 3.07
% CD8 ⁺ CLA ⁺	5.45 ± 1.95	6.62 ± 1.38	36.94 ± 5.35**
% CD8 ⁺ CLA ⁻	27.60 ± 5.60	18.20 ± 5.18	5.24 ± 2.25
CLA ⁺ CD4:CD8	1.78 ± 0.71	1.97 ± 0.33	0.85 ± 0.73**

^a PBMC from five AD patients and five healthy controls were stained immediately after purification, and skin T cells were stained after 7–10 days of culture with IL-2 and anti-CD2, anti-CD3, anti-CD28 mAb mixture. The percentages of CD4⁺ or CD8⁺ T cell subsets with and without CLA were measured by FACS. Results are shown as mean ± SE. *, Significant difference between control group and AD PBMC (*p* < 0.001). **, Significant difference between AD PBMC and skin (*p* < 0.001).

assures that the experiments are done with real skin T cells but not the T cells inside the vessels of the skin biopsies.

Peripheral blood CLA⁺ T cells from AD patients represent an *in vivo*-activated memory T cell subset

The CLA⁺ memory/effector T cells demonstrated typical features of *in vivo* activation in AD. CLA⁺CD45RO⁺ T cells highly expressed CD25 and HLA-DR in contrast to the CLA⁻ T cell population of AD patients and both CLA⁺ and CLA⁻ subsets of healthy individuals (21, 26). Additional evidence for *in vivo* activation appeared from the fact that both CD4⁺ and CD8⁺CLA⁺ T cells showed spontaneous proliferation *in vitro* without further activation. This was demonstrated by [³H]TdR incorporation in cells that were pulsed immediately after purification from PBMC with [³H]TdR for 24 h. As shown in Fig. 2, freshly isolated CD4⁺ and CD8⁺CLA⁺ T cells spontaneously proliferated, whereas the CLA⁻ T cell subsets from AD patients and control subjects remained resting (*p* < 0.001). CD4⁺CLA⁺ T cells of AD patients showed higher spontaneous proliferation than CD4⁺CLA⁺ T cells of the control group (*p* < 0.05).

CD8⁺ T cells respond to superantigenic stimulation to the same extent as CD4⁺ T cells and secrete a Th2 cytokine profile in AD

One explanation for the existence and *in vivo* activation of CD8⁺ T cells in skin lesions in amounts similar to those of CD4⁺ T cells could be that their responsiveness to superantigenic stimuli released by *Staphylococcus aureus* colonizing the skin. This was

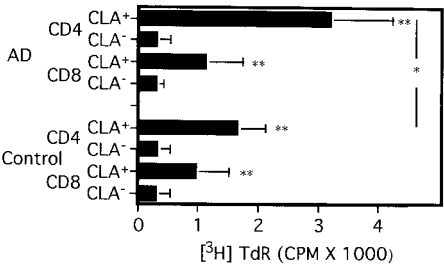


FIGURE 2. CLA⁺ T cells are *in vivo* activated. Peripheral blood CLA⁺ T cells show spontaneous proliferation. In five AD patients and five healthy controls, freshly isolated CD45RO⁺, CD4⁺, or CD8⁺, T cells with and without CLA were pulsed with [³H]TdR for 24 h immediately after isolation. Both CD4⁺ and CD8⁺ subsets of CLA⁺ T cells spontaneously proliferated, whereas the CLA⁻ T cells from AD patients and controls remained resting (**, *p* < 0.001; *, *p* < 0.05). Values are means ± SD of triplicate cultures.

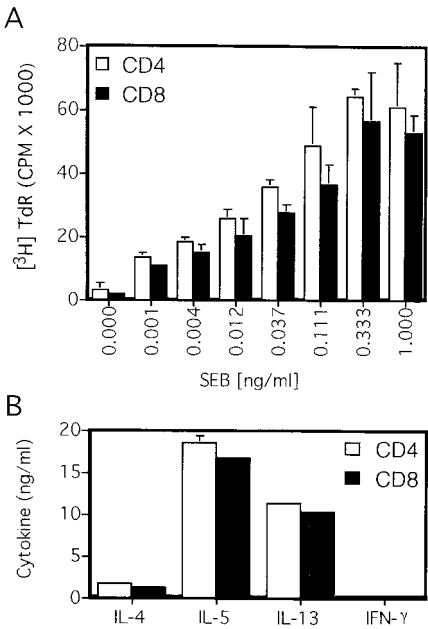


FIGURE 3. CLA⁺CD4⁺ and CD8⁺ T cells isolated from peripheral blood or skin proliferate by superantigenic stimulation. A, CD4⁺ or CD8⁺ T cells cultured and purified from AD lesional skin biopsies were stimulated by SEB in the presence of autologous irradiated PBMC as APC. [³H]TdR incorporation was measured at day 3. SEB dose-dependent proliferation was observed in both CD4⁺ and CD8⁺ subsets. B, Cytokines produced in parallel cultures from 1 ng/ml SEB stimulation were determined from supernatants harvested after 3 days. Cytokine production in unstimulated cultures was <7% of the SEB stimulated cultures and subtracted. Values are means ± SD of triplicate cultures. There is no significant difference between CD4⁺CLA⁺ and CD8⁺CLA⁺ T cells in cytokine production. Same results were observed in CLA⁺ subsets isolated from two PBMC and the CD4⁺ and CD8⁺ subsets isolated from two skin biopsy samples.

analyzed by stimulating the CD8⁺ and CD4⁺ subsets of CLA⁺ T cells from peripheral blood and skin biopsies of AD patients with SEB, by using irradiated autologous PBMC as APC. As shown in Fig. 3A, both CD4⁺ T cells and CD8⁺ T cells of AD patients responded to SEB in a dose-dependent manner. The cytokines released from CD4⁺ and CD8⁺ T cells isolated from skin showed a similar pattern by SEB stimulation (Fig. 3B). Both CD4⁺ or CD8⁺ T cells released high amounts of IL-5 and IL-13 and relatively lower amounts of IL-4 and very little IFN-γ.

CD4⁺ CLA⁺ and CD8⁺ CLA⁺ memory/effector T cells in AD display an IL-5- and IL-13-dominated Th2 cytokine profile

To analyze *in vivo*-activated cytokine patterns of CLA⁺ and CLA⁻ CD45RO⁺ T cells among CD4⁺ and CD8⁺ subsets, we visualized their IL-4, IL-5, IL-13, and IFN-γ cytokine content in the cytoplasm immediately after isolation from PBMC. In Fig. 4, the intracellular IL-4, IL-5, IL-13, and IFN-γ content of CLA⁺ and CLA⁻ T cells from an AD patient and a healthy nonatopic control are shown. In AD patients, the number of IL-5- and IL-13-positive cells was significantly higher in CD8⁺CLA⁺ cells (IL-5, 71 ± 9%; IL-13, 77 ± 8%) compared with CD4⁺CLA⁺ cells (IL-5, 52 ± 11%; IL-13, 55 ± 7%) (*p* < 0.01). Neither CD4⁺ nor CD8⁺ CLA⁺ T cells contained significant amounts of intracytoplasmic IL-4 and IFN-γ. In healthy individuals, only 2–17% of the CD4⁺ or CD8⁺CLA⁺ T cells contained intracellular IL-5 and IL-13 but no IL-4 and IFN-γ.

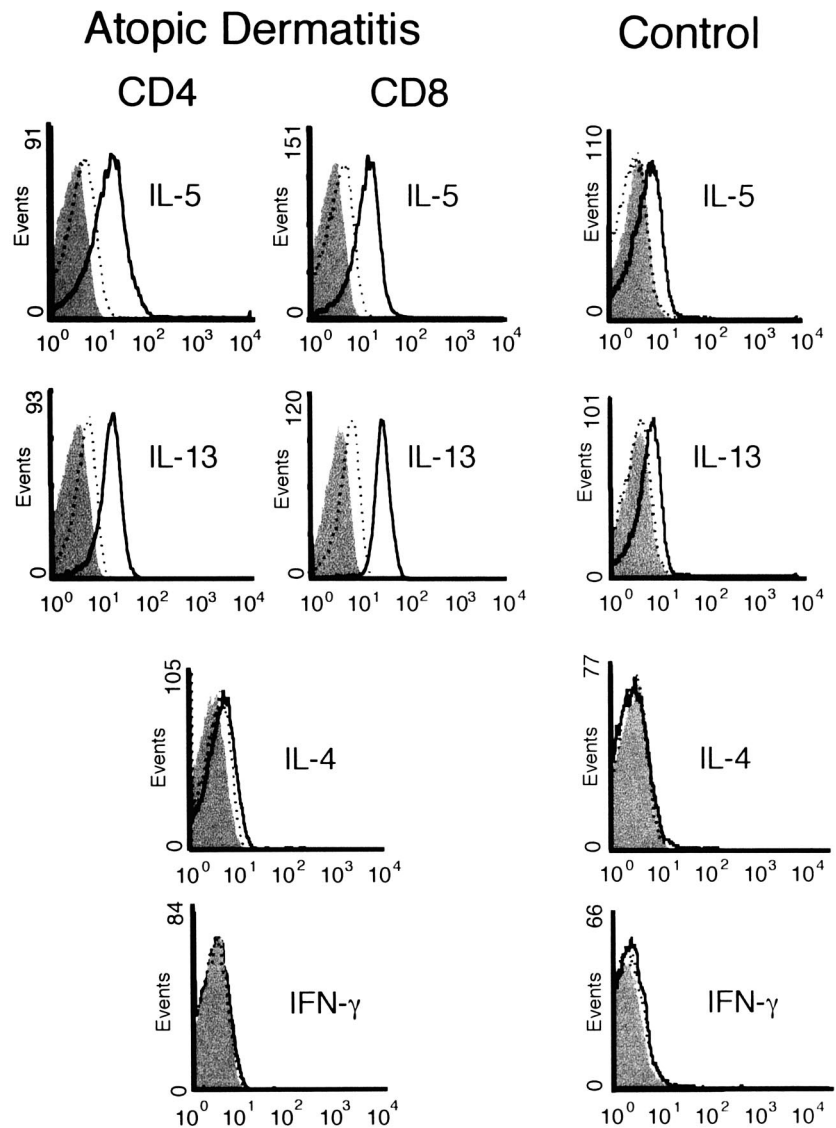


FIGURE 4. Intracytoplasmic cytokine content of peripheral blood CLA⁺ and CLA⁻ CD45RO⁺ T cells immediately after purification. CLA⁺ and CLA⁻ T cells were fixed and permeabilized immediately after purification. PE-labeled anti-IL-4, anti-IL-5, anti-IL-13, and anti-IFN- γ were used for staining of cytokines. Filled curve, control Ab; thick line, CLA⁺ T cells; dotted line, CLA⁻ cells. IL-5 and IL-13 showing significant shift in CLA⁺ cells were studied in both CD4⁺ and CD8⁺ subsets of CLA⁺CD45RO⁺ cells. One patient and one control are shown from similar results of five AD patients and three nonatopic healthy individuals.

The spontaneously secreted cytokine profile was determined in freshly isolated peripheral CLA⁺CD45RO⁺ T cells with no further *in vitro* stimulation. As shown in Fig. 5, the CLA⁺CD45RO⁺ T cells spontaneously released large amounts of IL-5. The amount of IL-5 released from CD4⁺ cells was 4.22 ± 0.59 ng/ml, and the amount from CD8⁺ cells was 8.43 ± 0.71 ng/ml. The amount of IL-13 released from CD4⁺ cells was 0.74 ± 0.19 ng/ml, and that from CD8⁺ cells was 2.73 ± 0.34 ng/ml. Only low amounts of IL-4 (CD4⁺, 0.18 ± 0.02 ; CD8⁺, 0.32 ± 0.03 ng/ml) and IFN- γ (CD4⁺, 0.09 ± 0.02 ; CD8⁺, 0.19 ± 0.03 ng/ml) was secreted in both CLA⁺ subsets. Significantly high amounts of Th2 cytokines were released by CLA⁺ T cells from AD patients in comparison with healthy subjects ($p < 0.01$). Similar to intracellular cytokine staining, the amounts of IL-4, IL-5, and IL-13 secreted by CD8⁺CLA⁺ T cells were significantly higher than those from the CD4⁺CLA⁺ subset ($p < 0.01$). Although the spontaneous cytokine release by the CLA⁺ T cell subsets reached higher levels in cells from AD patients than from the controls, it was in both groups significantly higher among the CLA⁺ populations than among the CLA⁻ T cells ($p < 0.001$). On the other hand, there was no significant difference for IFN- γ between patients and controls. Neither the CLA⁺ nor the CLA⁻ cell subsets secreted detectable amounts of IL-2, IL-3, and GM-CSF.

Both CD4⁺ and CD8⁺ subsets of CLA⁺ T cells induce IgE production primarily by IL-13

We then determined the capacity of both T cell subsets to induce and regulate IgE synthesis *in vitro*. In the first set of experiments, the two memory T cell populations and autologous B cells, purified from PBMC of AD patients, were cocultured without further stimulation. Fig. 6 demonstrates that either CD4⁺ or CD8⁺ subsets of CLA⁺ T cells were capable of inducing IgE in B cells. In contrast the enhancement of IgE by CLA⁻ T cells was much less pronounced. IL-4 and IL-13 possess similar isotype-regulatory properties on IgE production (43–45). To analyze whether IL-4 or IL-13 is responsible for IgE production in AD, either CD4⁺ or CD8⁺ subsets of CLA⁺ memory/effector T cells were cultured with autologous B cells in the presence of neutralizing anti-IL-4, anti-IL-13, or mutant IL-4 antagonist Y124D. Neutralization of IL-4 in cultures had a small effect (11.1–18.4%) on suppression of the IgE production, whereas IL-13 neutralization and Y124D mutant IL-4, which also inhibits IL-13 (34), decreased IgE levels by 84.2–91.7%. These results point out the pivotal role of IL-13 but not IL-4 in induction of IgE in AD, and CD4⁺ and CD8⁺ T cells were similarly potent in IgE induction by B cells.

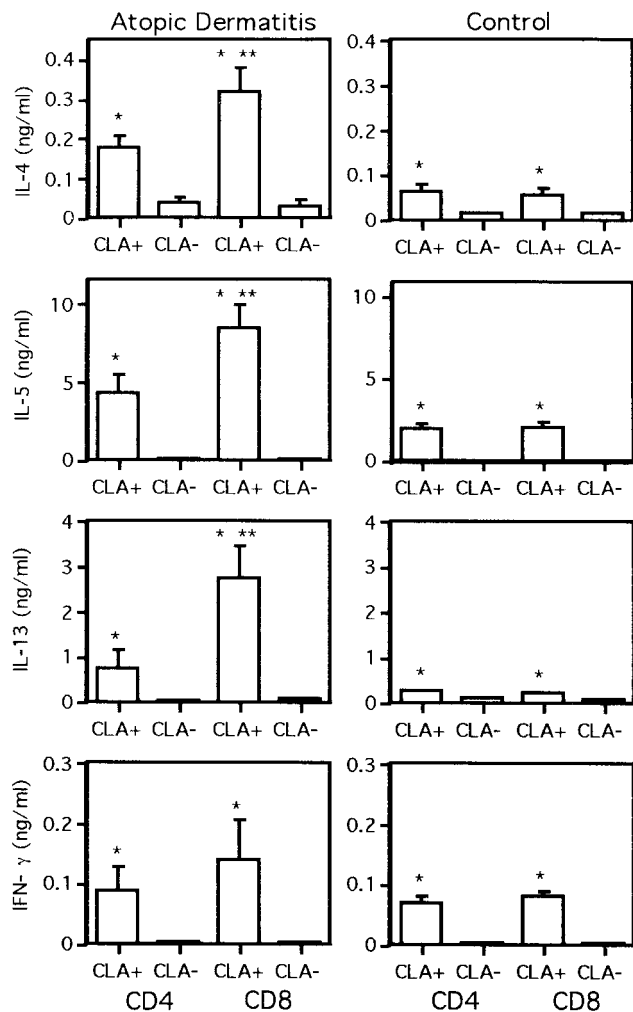


FIGURE 5. Peripheral blood CD4⁺ and CD8⁺ subsets of CLA⁺CD45RO⁺ T cells spontaneously release a Th2 pattern of cytokines in AD. Purified CD4⁺ and CD8⁺ subsets of CLA⁺ and CLA⁻ T cells of nine AD patients and five controls were cultured without stimulation. IL-4, IL-5, IL-13, and IFN-γ were measured from the supernatants harvested after 24 h. The results are shown as mean $\pm$ SD. Significantly higher amounts of IL-4, IL-5, and IL-13 were released by either CD4⁺ or CD8⁺, CLA⁺ T cells of AD patients than by control subjects ($p < 0.01$). The amounts of IL-4, IL-5, and IL-13 secreted by CD8⁺CLA⁺ T cells were significantly higher than those from the CD4⁺CLA⁺ subset in AD patients (**, $p < 0.01$). In both groups, the spontaneous cytokine release was significantly higher among the CLA⁺ populations than among the CLA⁻ T cells (*, $p < 0.001$).

In vivo-activated CLA⁺CD45RO⁺ T cells of both CD4⁺ or CD8⁺ subsets prevent eosinophil apoptosis

The spontaneously secreted cytokine pattern of CLA-bearing CD4⁺ and CD8⁺ memory/effector T cells suggested the investigation of the role of CLA⁺ T cells on eosinophil survival and apoptosis of AD patients. Therefore, the number of viable eosinophils, measured by ethidium bromide exclusion, was quantified after cocultivation with supernatants from freshly isolated, non-stimulated CD4⁺ or CD8⁺, CLA⁺ or CLA⁻, CD45RO⁺ T cells. As shown in Fig. 7A, supernatants from CLA⁺ T cells of both CD4⁺ and CD8⁺ subsets extended the life span of freshly purified eosinophils in vitro. In contrast, supernatants of CLA⁻ T cells exhibited no effect on eosinophil survival ($p < 0.001$). Also supernatants of CLA⁺ T cells from healthy controls generated some prolongation of eosinophil survival; however, much less pro-

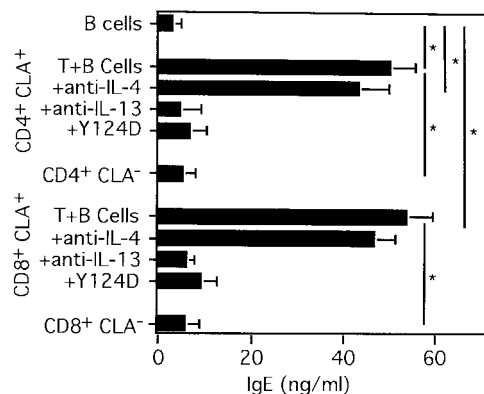


FIGURE 6. Both CD4⁺ and CD8⁺ subsets of peripheral blood CLA⁺ T cells induced IgE by IL-13. Purified B cells were cocultured with autologous purified CD4⁺ or CD8⁺ subsets of CLA⁺ or CLA⁻ T cells. The cells did not receive additional stimulation. IgE and IgG4 were measured from supernatants harvested after 12 days. IL-4 and IL-13 were neutralized by mAbs to these cytokines and by mutant IL-4 antagonist Y124D added from the start of culture. A mouse IgG1 was used as a control mAb. Results represent mean of triplicate cultures $\pm$ SD from three different experiments (*, $p < 0.001$).

nounced than the supernatants of cells from AD patients. The difference between CLA⁺ T cell supernatants of AD patients and control group on eosinophil survival was significant after 48, 72, and 96 h of incubation ($p < 0.01$). The supernatants of CD8⁺CLA⁺ T cells were significantly more effective than those from CD4⁺CLA⁺ T cells after 48, 72, and 96 h ($p < 0.05$).

During numerous attempts to find out the spontaneously secreted cytokine from CLA⁺ T cells that may play a role in prolonged eosinophil survival, we neutralized IL-4, IL-5, IL-13, IFN-γ, and GM-CSF and inhibited IL-4 and IL-13 activity by a mutant IL-4 antagonist Y124D. Only neutralization of IL-5 from the spontaneously secreted CD4⁺ or CD8⁺ supernatants decreased the life span of eosinophils (Fig. 7B). Repeated attempts to neutralize cytokines responsible for eosinophil survival with different mixtures of mAbs did not decrease eosinophil viability to background levels. In addition, combination of IL-5- and GM-CSF-neutralizing Abs did not inhibit eosinophil survival more than IL-5 alone.

In Fig. 8, the effect of different memory/effector T cell supernatants on eosinophil apoptosis is demonstrated. DNA fragmentation, a characteristic feature of apoptotic cells, was assessed by staining DNA with propidium iodide in Triton X-100 permeabilized cells and flow cytometric analysis. Exposure of purified eosinophils to supernatants of CLA⁺ T cells of either the CD4⁺ or the CD8⁺ subset from AD patients prevented DNA fragmentation as observed significantly after 24 h (Fig. 8A). Furthermore, flow cytometric analysis of surface phosphatidylserine expression, representing an early marker of apoptotic cell death, demonstrated that the eosinophil apoptosis-associated phosphatidylserine translocation was inhibited by supernatants of CLA⁺ T cells in both CD4⁺ and CD8⁺ subsets (Fig. 8B). Eosinophils cultured for 48 h with in vivo-activated CLA⁺ T cell supernatants were 30.7% for CD4⁺ and 29.2% for CD8⁺ T cells propidium iodide and annexin V stained. In comparison, eosinophils cultured with CLA⁻ T cell supernatants showed significantly high apoptosis. Propidium iodide and annexin V staining of eosinophils was 67.1% for CD4⁺ CLA⁻ and 65.4% for CD8⁺ CLA⁻ T cell supernatants.

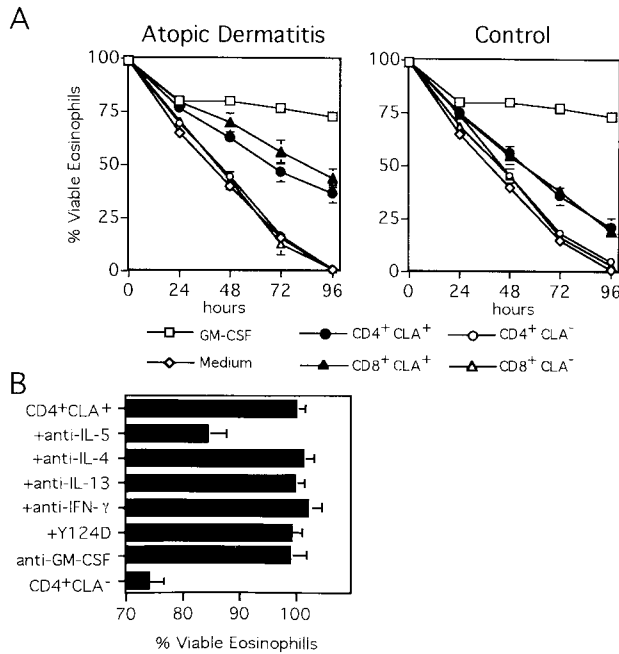


FIGURE 7. Spontaneously secreted supernatants from CD4⁺ and CD8⁺ subsets of CLA⁺CD45RO⁺ T cells enhance eosinophil survival. **A**, Freshly isolated human eosinophils were exposed to spontaneously secreted supernatants of CD4⁺ or CD8⁺ subsets of CLA⁺ or CLA⁻ T cells. GM-CSF, 50 ng/ml, was used as positive control. Cell death was assessed by uptake of ethidium bromide and flow cytometry. Results represent mean $\pm$ SD of five AD patients and five control individuals. Supernatants from CLA⁺ T cells of both CD4⁺ and CD8⁺ subsets extended the life span of freshly purified eosinophils in AD patients and healthy controls ($p < 0.001$). The increase in eosinophil survival by CLA⁺ T cell supernatants of AD patients was significantly higher than the control group after 48, 72, and 96 h of incubation ($p < 0.01$). The supernatants of CD8⁺CLA⁺ T cells were significantly more effective than those from CD4⁺CLA⁺ T cells after 48, 72, and 96 h ($p < 0.05$). **B**, Inhibition of cytokines from supernatants. The supernatants containing spontaneously secreted cytokines were preincubated with neutralizing Ab against IL-4, IL-5, IL-13, GM-CSF, IFN- γ (each 10 μ g/ml), and mutant IL-4 Y124D for 2 h at 4°C. Viability in eosinophils added to neutralized supernatants was measured after 48 h by ethidium bromide exclusion. The viability of eosinophils in CD4⁺CLA⁺ T cell supernatants was accepted as 100%, and the rest is calculated. Results are means $\pm$ SD of duplicate cultures representing three experiments.

Discussion

Activation of peripheral blood T cells and preferential secretion of Th2 cytokines are associated with atopic syndromes such as asthma and AD (2–6). In previous studies investigating the mechanisms of allergy, CD4⁺ T cells were inscribed as pivotal cells and the main producers of Th2 type proinflammatory cytokines and inducers of allergic inflammation (2–6). In contrast, there was limited evidence for involvement of CD8⁺ T cells in human allergic responses (46, 47). Culturing of T cells from AD skin lesions allowed the comparison of CD4⁺ and CD8⁺ T cell subsets in the present study. Previously, it was demonstrated that in blisters of AD skin almost the entire T cell population was of the CLA⁺CD45RO⁺ phenotype (18, 20). Accordingly, CLA⁺CD45RO⁺ T cells of CD4⁺ and CD8⁺ subsets were purified from peripheral blood for comparison.

The present study dissects CD45RO⁺ T cells into two distinct subsets by their skin-homing ligand expression. The CLA⁺CD45RO⁺ T cells of both CD4⁺ and CD8⁺ subsets express immunological features and functional properties of in vivo-activated memory/

effector cells, whereas CLA⁻CD45RO⁺ T cells represent resting memory T cells in AD. Most of the previous studies to demonstrate intracytoplasmic cytokines have been conducted by a full T cell stimulation with a phorbol ester and ionomycin combination (32, 47). To demonstrate in vivo-generated intracytoplasmic IL-5 and IL-13, the cells were not further stimulated but only fixed and stained immediately after purification, because CLA⁺ T cells were in vivo activated in AD patients. CD8⁺CLA⁺ T cells appeared to be higher producers of IL-5 and IL-13 and more potent in inhibition of apoptotic eosinophil death. Both subsets induced IgE to the same extent. In certain diseases including *Schistosoma mansoni* infection, leishmaniasis, lepromatous leprosy, and AIDS, a CD8⁺ T cell subset has been identified with a cytokine profile usually attributed to CD4⁺ Th2 type cells (13–15, 48). In allergic diseases, CD8⁺ T cells seem to exhibit diverse effects. In a study comparing IgE induction by IL-4 and IL-13, IL-13 was shown to be the major IgE-inducing cytokine secreted from CD8⁺ T cell clones (33). However, in mouse and rat systems of allergic sensitization, IgE production and allergic inflammation was dependent on CD4⁺ T cells, whereas CD8⁺ T cells were shown to be negative regulatory T cells (49, 50). Apparently, these animal models of allergic sensitization and CD4⁺ T cell accumulation in aeroallergen-induced cutaneous late phase responses do not adequately represent the immune responses in AD (49–53).

A number of pathogenetic mechanisms leading to T cell activation in AD including aeroallergens, food allergens, and superantigens have been emphasized. The role of aeroallergens in T cell activation in AD has been extensively studied (2–6, 51, 52). Aeroallergens can induce both immediate type and delayed type responses in the skin (51, 52). The frequency of aeroallergen-specific T cells was investigated in AD lesions, and they were found to be <1% in nonchallenged AD lesions (54). Besides, such allergen-specific T cells can be detected in the skin of atopic patients after allergen administration, without signs of AD lesions (53). The contribution of food allergens in the exacerbation of AD by T cell activation has also been studied. Food allergen-specific T cells have been cloned from lesional skin of patients with clinically relevant hypersensitivity responses to foods; however, this type of allergy plays a role in a minority of adult AD (55, 56). It is obvious that allergen-specific T cell responses in food and aeroallergen allergy are confined to CD4⁺ T cells; this does not explain the activation and recruitment of CD8⁺ T cells in AD skin lesions.

From a number of studies, it could be concluded that bacterial superantigens contribute to the pathogenesis and exacerbation of AD. Staphylococcal superantigens were isolated from AD skin (28, 29). The superantigen patch test elicits skin inflammation in AD patients (57) and in the human SCID mouse model (58). In addition, superantigens up-regulate the CLA molecule (27). The present study shows that CD8⁺ T cells cultured from skin or CLA⁺CD8⁺ T cells freshly isolated from peripheral blood efficiently proliferate by superantigenic stimulation. Furthermore, purified CD4⁺ or CD8⁺ T cells cultured from skin biopsies secrete a Th2-like cytokine profile with high IL-5 and IL-13 by SEB stimulation. Bacterial superantigens can interact with certain V β elements of the TCR, leading to activation, expansion, anergy, or deletion of T cells. It is evident from mouse studies that superantigen response of T cells is not restricted to CD4⁺ or CD8⁺ subsets (59, 60) and even CD4⁻CD8⁻ T cells can respond to superantigenic stimuli (61).

The functional Ig isotype-regulatory capacity of peripheral CLA⁺ and CLA⁻ memory/effector T cells from AD patients was investigated by coculturing isolated T cell subpopulations with purified B cells or T cell-depleted PBMC. The IgE production by B cells mainly depended on secreted IL-13. Previous reports and our

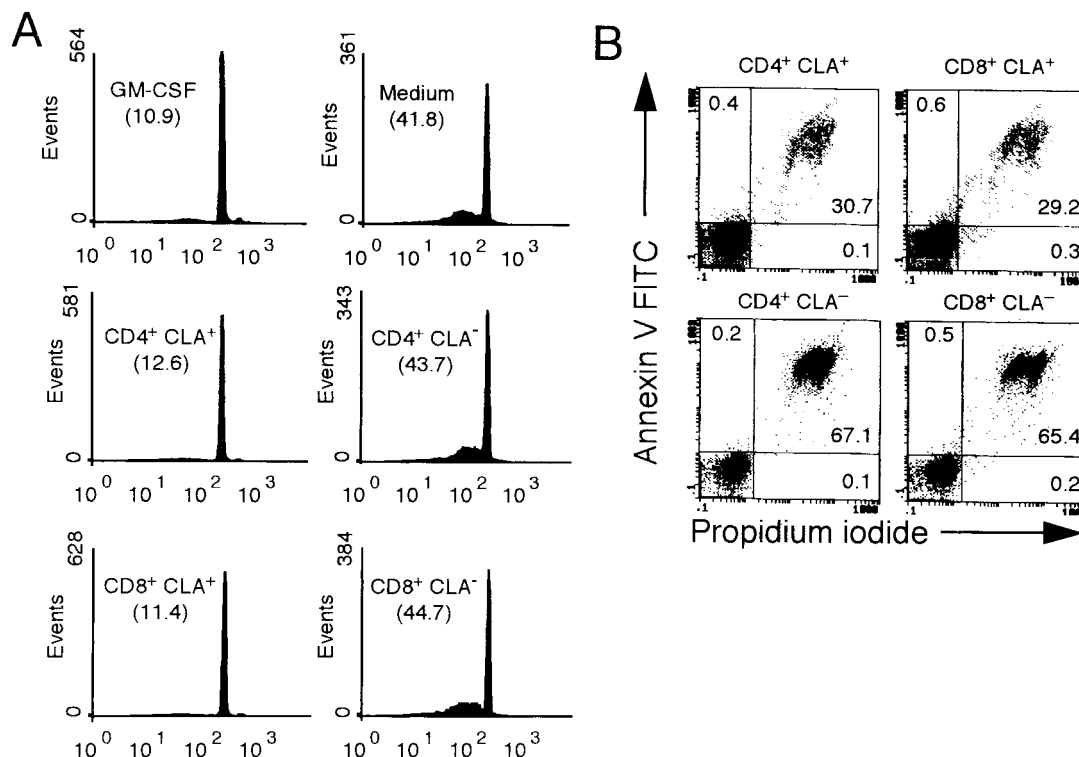


FIGURE 8. Effect of different memory/effector T cell supernatants on eosinophil apoptosis. **A**, DNA fragmentation was determined by DNA staining with propidium iodide in Triton X-permeabilized cells and flow cytometric analysis after 24 h incubation with 25% supernatants of peripheral blood CD4⁺ and CD8⁺ subsets of CLA⁺ or CLA⁻ T cells. GM-CSF, 50 ng/ml, was used as positive control. Numbers in parentheses are the percentage of DNA fragmented cells. The eosinophil apoptosis-associated with DNA fragmentation was inhibited by supernatants of CLA⁺ T cells in both CD4⁺ and CD8⁺ subsets. **B**, Surface phosphatidylserine expression in freshly purified eosinophils was assessed by flow cytometric analysis after 48 h incubation with 25% supernatants of peripheral blood CD4⁺ and CD8⁺ subsets of either CLA⁺ or CLA⁻ T cells. The eosinophil apoptosis-associated phosphatidylserine translocation was inhibited by supernatants of CLA⁺ T cells in both CD4⁺ and CD8⁺ subsets. Results are representative of four independent experiments.

present findings indicate that IL-4 inhibition is not sufficient to suppress IgE in atopic diseases. An IgE-inducing activity, which could be attributed to IL-13, has been found in PBMC cultures of atopic but not of normal individuals (62, 63). Accordingly, IL-13 produced by the skin-selective homing T cells in high amounts may have an important role in the pathogenesis of chronic AD. In contrast to IL-4, there is no feedback or priming mechanism exerted by IL-13 on T cells, because human T cells do not display specific receptors for IL-13 (34). Therefore, IL-4 may be decisive at the initial phase of allergic responses and in the priming and development of Th2 cells (64, 65), whereas IL-13 becomes more prominent in IgE induction in atopy.

Increased numbers of blood and tissue eosinophils are regularly observed in subjects suffering from AD and/or bronchial asthma (7, 8). Inhibition of apoptosis by survival factors, such as IL-3, IL-5, and GM-CSF, was demonstrated to substantially contribute to the prolonged survival of eosinophils (1, 11, 12, 40). In the present study, the functional eosinophil antiapoptotic properties were demonstrated in two ways by DNA fragmentation and phosphatidylserine translocation. Although IL-5 appeared to be the major cytokine, it may not be the only cytokine released by CLA⁺ T cells that is responsible for the prolonged eosinophil survival. The isolated CLA⁺ T cells did not release any detectable IL-3 and GM-CSF; therefore, other cytokines released from CLA⁺ T cells may also contribute to eosinophil life span. Similar to the present study, IL-5 was repeatedly reported as an eosinophil-specific differentiation and survival factor (11, 66). In vivo, as observed in nasal polyps, IL-5 was determined as the major cytokine inhibiting eosinophil apoptosis (9). Moreover, transgenic mice overexpress-

ing the IL-5 gene associate eosinophilia with IL-5 (67). In this context, it is now more reasonable that both CD4⁺ and CD8⁺ T cell populations expressing the appropriate TCR V β can be activated by superantigen in the presence of HLA class II-expressing cells. This suggests an explanation for the existence and activation of CD8⁺ T cells in the eczema lesions contributing to IgE production and eosinophil survival similar to CD4⁺ T cells and development, chronicity, and exacerbation of AD.

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