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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 Dermatitis

Mübeccel Akdis,* 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.

A ctivation of peripheral blood CD4⁺ T cells and their preferential secretion of Th2 cytokines in patients with asthma and atopic dermatitis (AD) 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.

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

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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 therefore 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 ± 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 IgG, and streptavidin-conjugated magnetic microbeads for Mag- net Activated Cell Sorting (MACS) cell separation were from Miltenyi Biotech (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 mAbs 45-15 were provided by Novartis (Basel, Switzerland). Anti-IL-13 mAbs (JES8-5A2 and JES10-2F9) and mutant IL-4 antagonist (Y124D) with neutralizing anti-IL-5 and anti-GM-CSF were purified 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 minced with two scalpels; no proteolytic enzymes were used. Skin fragments 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 μg/mL streptomycin, 50 μM 2-ME (all from Life Technologies, Basel, Switzerland), and 10% heat-inactivated FCS (SerA-Lab, Sussex, U.K.), 2% heat-inactivated by immunohistochemistry 1 saline-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 from CD4+ and CD8+ subsets from peripheral blood

PBMC were isolated by Ficoll (Biochrom, Berlin, Germany) density gra- dient 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 μg/mL streptomycin (both from Life Tech- nologies). CLA+ and CLA− cells were isolated with the MACS magnet- activated cell separation system (Miltenyi Biotech) as described in detail (21, 23, 26). In brief, anti-CD4+ and anti-CD19-depleted cells were incubated with MACS micro bead-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-CD45RA-goat anti-mouse IgG, anti-CD45RO-PE, and anti-CD45RO-PE-labeled magnetic 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 × 104–1.5 × 105 CLA+CD4+ cells, 9 × 104–2.4 × 104 CLA−CD4+ cells, 3 × 104–9 × 103 CLA+CD8+ cells, and 6 × 103–1.7 × 104 CLA−CD8+ cells from 105 PBMC. The CD4+ T cell culture supernatants taken after 12 days of culture were incubated with 1% Xylocaine as local anes-

Flow cytometric analysis

After purification, 5 × 104 cells were sequentially stained with FITC-conjugated anti-CLA mAb HECA-452, together with anti-CD4-EC or anti-CD8-EC and either anti-CD45RO-PE, anti-CD14-PE, or anti-CD19-PE conjugated anti-CLA mAb HECA-452, together with anti-CD4-ECD or anti-CD28-ECD for 30 min at 4°C. The flow cytometric analysis was performed by an Epics Profile II (Coulter).
addition of neutralizing mAbs to IL-4 or IL-13, each at 10 μg/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 μg/ml) (Immunotech) 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 using mAb 3H4 for coating and biotinylated mAb 1F12 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 ExtrAvidin (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 [3 H]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 Ficol-Hypaque centrifugation. The remaining cell population, mainly granulocytes and erythrocytes, were depleted with erythrocyte lysis solution (155 mM NaCl, 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, Düdingen, 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 μM 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 μg/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 × 105 cells were washed twice with cold PBS and resuspended in 1 ml binding buffer (HEPES, 0.25 mM CaCl2). FITC-conjugated annexin V (Oligonucleotide Research Inc., Ann Arbor, MI) and propidium iodide (500 ng/ml) were added to the cells. After gentle vortexing and incubating for 15 min in the dark, the cells were immediately analyzed for annexin V binding and propidium iodide uptake by flow cytometry.

Statistical interpretation

Data are expressed as means ± 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 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.

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

<table>
<thead>
<tr>
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<th>Control (PBMC)</th>
<th>Atopic Dermatitis (PBMC)</th>
<th>Skin</th>
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<tbody>
<tr>
<td>% CD4</td>
<td>49.20 ± 7.25</td>
<td>43.14 ± 3.43</td>
<td>502 ± 78</td>
</tr>
<tr>
<td>CD4</td>
<td>33.05 ± 6.42</td>
<td>24.82 ± 4.29</td>
<td>297 ± 50</td>
</tr>
<tr>
<td>CD4/CD8</td>
<td>1.49 ± 0.31</td>
<td>1.74 ± 0.24</td>
<td>1.62 ± 0.20</td>
</tr>
</tbody>
</table>

*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 ± SE.
showed higher spontaneous proliferation than CD4
1
1
CD8
tion. Both CD4
and anti-CD2, anti-CD3, anti-CD28 mAb mixture. The percentages of CD4
1
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
1
1
1
1

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 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
 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
 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 repressed CD25 and HLA-DR in contrast to the CLA
 subset.

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.

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. [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.

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 ± 6%) 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 3. CLA
 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 lesions showed spontaneous proliferation in vitro without further addition of SEB, by using irradiated autologous 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.

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

<table>
<thead>
<tr>
<th></th>
<th>Control (PBMC)</th>
<th>Atopic Dermatitis (PBMC)</th>
<th>Skin</th>
</tr>
</thead>
</table>
| % 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 ± 1.28              | 36.94 ± 5.35** |
| CLA
 CD4
CD8 | 27.60 ± 5.60   | 18.20 ± 5.18             | 5.24 ± 2.25    |

* 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 < 0.003).
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.

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.
IL-5, and IL-13 secreted by CD81 cells were cocultured without additional 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 ± 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 pronounced 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 ± 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). 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 μg/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 ± 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 were 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 Vβ 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
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 overexpressing 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$\beta$ 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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**References**


**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.


