

T helper (Th) 2 predominance in atopic diseases is due to preferential apoptosis of circulating memory/effector Th1 cells

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ABSTRACT T cells constitute a large population of cellular infiltrate in atopic/allergic inflammation and a dysregulated, Th2-biased peripheral immune response appears to be an important pathogenetic factor. In atopic dermatitis, circulating cutaneous lymphocyte-associated antigen-bearing (CLA⁺) CD45RO⁺ T cells with skin-specific homing property represent an activated memory/effector T cell subset. They express high levels of Fas and Fas ligand and undergo activation-induced apoptosis. The freshly purified CLA⁺ CD45RO⁺ T cells of atopic individuals display distinct features of in vivo-triggered apoptosis such as procaspase degradation and active caspase-8 formation. In particular, the Th1 compartment of activated memory/effector T cells selectively undergoes activation-induced cell death, skewing the immune response toward surviving Th2 cells in atopic dermatitis patients. The apoptosis of circulating memory/effector T cells was confined to atopic individuals whereas non-atopic patients such as psoriasis, intrinsic-type asthma, contact dermatitis, intrinsic type of atopic dermatitis, bee venom allergic patients, and healthy controls showed no evidence for enhanced T cell apoptosis in vivo. These results define a novel mechanism for peripheral Th2 response in atopic diseases.—Akdis, M., Trautmann, A., Klunker, S., Daigle, I., Küçüksezer, U. C., Deglmann, W., Disch, R., Blaser, K., Akdis, C. A. T helper (Th) 2 predominance in atopic dermatitis is due to preferential apoptosis of circulating memory/effector Th1 cells. *FASEB J.* 17, 1026–1035 (2003)

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To ASSURE self-tolerance and down-regulation of an immune response, elimination of T cells takes place in the periphery and involves induction of apoptosis (1). Under certain conditions, some of the activated T cells die by activation-induced cell death (AICD) (2). AICD plays an important role in maintaining homeostasis of the immune response and prevention of excessive immune reactivity. Peripheral T cell death requires the

action of death receptor/ligand systems, particularly Fas (CD95) and Fas ligand interaction (3–5). The death signaling function is ensured by the presence of a cytoplasmic protein–protein interaction motif termed death domain (6). Upon triggering of CD95, the adaptor molecule, Fas-associated death domain-containing protein and pro-caspase-8 are recruited to the receptor, forming the death-inducing signaling complex. Binding of pro-caspase-8 leads to its autoproteolytic activation, upon which the active enzyme is released into the cytosol (7, 8). Once activated, caspase-8, also known as FLICE, can initiate apoptosis of cells (9).

In allergic inflammations of the skin, the pivotal role of activated CD45RO⁺ (memory/effector) T cells expressing the cutaneous lymphocyte-associated antigen (CLA) was demonstrated (10). Purification of CLA⁺ T cells from peripheral blood enables to study features of these cells in atopic dermatitis (AD), whereas a peripheral blood T cell marker has not been identified in allergic asthma, allergic rhinitis, or other organ-specific autoimmune and inflammatory diseases so far. A number of triggering factors, including aeroallergens, food allergens, and superantigens are highlighted as being involved in T cell activation in AD (11–13). Accordingly, peripheral blood CLA⁺ memory/effector cells demonstrate typical features of activated T cells in AD (14–16). Both CD4⁺ and CD8⁺ subsets of freshly isolated CLA⁺ T cells express significantly higher levels of CD25, CD40 ligand, and HLA-DR. Additional evidence for in vivo activation of CD4⁺ and CD8⁺ subsets of CLA⁺ T cells in AD emerges from spontaneous proliferation, IgE induction by B cells, and augmentation of eosinophil survival without further activation. In contrast, the peripheral blood CLA[−] CD45RO⁺ T cell population represents a resting memory T cell fraction (14–16).

T cell turnover between skin and circulation apparently is associated with a switch of cytokine profiles such

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as Th0/Th1-like inside the skin, Th0/Th2-like in the circulation, and secondary lymphoid organs in atopic individuals. Because apoptosis of mature T cells is a powerful mechanism for deleting T cells, it raises the interesting possibility that unequal apoptosis of Th1 and Th2 effector cells may lead to preferential deletion of one subset over another. The present study demonstrates that immediately after purification from peripheral blood, a fraction of CLA⁺ CD45RO⁺ T cells show direct evidence for in vivo-initiated AICD. Apoptosis of circulating memory/effector T cells was mostly confined to the Th1 cells, which may explain the Th2-skewed, allergen-specific immune response in polyallergic individuals with AD.

MATERIALS AND METHODS

Study population

Forty-three patients with chronic AD (mean age, 29 years) who fulfilled the criteria of Hanifin and Rajka were studied (17). All were polyallergic and had positive cutaneous tests to at least three aeroallergens. Patients showed specific IgE Abs against at least three allergens at radioallergosorbent test class ≥ 2 and total IgE of >400 U/mL (mean, 3219 ± 1669 IU/mL). None of the patients had systemic immunosuppressive treatment at least 1 month before peripheral venous blood was taken. Nine healthy individuals (mean age, 30 years) with no history of atopy were included in the study as non-atopic healthy control group. Their mean serum IgE level was 71 ± 18 IU/mL. In addition, four psoriasis patients, three non-atopic asthma (intrinsic-type) patients, five non-atopic bee venom allergic patients, three intrinsic type of AD (non-atopic dermatitis) patients, and four contact dermatitis patients were studied as non-atopic patients for comparison. None of the patients in the non-atopic group (n , 19) had serum IgE levels over 83 IU/mL (mean 54 ± 13 IU/mL). They did not show skin test reactivity or specific IgE against environmental allergens. Only bee venom allergic patients who were monoallergic to honey bee stings had specific IgE to bee venom (*Apis mellifera*). 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 skin pieces were fixed with OCT compound (Tissue-Tek, Diatex, Nurnberg, Germany) and further processed for staining. The study was approved by the Ethical Committee of Davos, Switzerland.

Antibodies and reagents

The CLA-specific rat mAb HECA-452 was kindly provided by Dr. E. Butcher, (Stanford University, Palo Alto, CA, USA) (10). All fluorescent- or biotin-labeled mAbs for cell purification or flow cytometry analyses were purchased from Immunotech (Marseilles, France) or PharMingen (San Diego, CA, USA). Anti-CD14, anti-CD16, anti-CD19, anti-CD45RO, anti-CD45RA, anti-mouse Ig, and streptavidin-conjugated magnetic microbeads were used for magnet-activated cell sorting (MACS, Miltenyi Biotec, Marburg, Germany). Human IL-2 and IL-4 were provided by Novartis (Basel, Switzerland). Human IL-15, IL-16, IL-18 were purchased from PeproTech (Rocky Hill, NJ, USA). Ethidium bromide was from Sigma Chemical Co. (St. Louis, MO, USA). Apoptosis-inducing anti-Fas mAb (CH-11) was purchased from Immunotech. Cell permeable caspase-3 (CPP32) inhibitor (DEVD.CHO) was

from Biomol (Hamburg, Germany). The recombinant human Fas-Fc, soluble Fas ligand, tumor necrosis factor α (TNF- α), TNF-related weak inducer of apoptosis (TWEAK), and TNF-related apoptosis-inducing ligand (sTRAIL) were from Alexis Corp. (San Diego, CA, USA). Anti-caspase-8 mAb was kindly provided by Dr. M. Peter (German, Cancer Research Center, Heidelberg, Germany). Isotype matching control Abs were rat IgM, mouse IgG1 and mouse IgG2a (Immunotech). Fibronectin, collagen VI, laminin, tenascin, transferrin, and bovine serum albumin were purchased from Sigma.

Isolation of CLA⁺, CLA⁻ CD45RO⁺, and CD45RA⁺ T cells from peripheral blood

PBMC were isolated by Ficoll (Biochrom KG, Berlin, Germany) density gradient centrifugation of peripheral venous blood. Cells were washed three times and resuspended in DMEM. CLA⁺ and CLA⁻ cells were isolated using the MACS as described (16). CD14⁻ and CD19-depleted cells were incubated with anti-CD45RA and anti-CD16 mAbs followed by goat anti-mouse Ig-conjugated microbeads. The negatively selected CD45RO⁺ T cells were sequentially incubated with anti-CLA HECA-452 mAb, biotin-conjugated goat anti-rat Ig, and streptavidin-conjugated microbeads. The magnetic (CLA⁺ CD45RO⁺) and nonmagnetic (CLA⁻ CD45RO⁺) T cell fractions were recovered by sequential elution from the MACS column. The CD45RA⁺ T cells were negatively selected by depletion of CD14⁺, CD19⁺, CD16⁺, and CD45RO⁺ positive T cells by using MACS. The whole cell purification procedure was performed on ice. The cell yield and purity of CLA⁺ and CLA⁻ CD45RO⁺ T cells obtained from PBMC with the same purification method were discussed previously (14–16).

After purification, 10^5 cells were stained with FITC-labeled anti-CLA mAb and PtdEtn-labeled anti-CD45RO, PtdEtn-labeled anti-CD45RA and anti-CD95-FITC, or anti-CD95L-FITC and ECD-labeled anti-CD3. Stained cells were fixed in 2% paraformaldehyde. Control Abs were rat IgM and FITC-conjugated goat anti-rat IgM and FITC-, PtdEtn-, or ECD-conjugated mouse IgG1. Fluorescence analysis was performed on an EPICS XL (Coulter Corp.) with an argon laser (488 nm). The purity of the CLA-enriched population ranged from 88 to 99% (mean: 94%). CLA⁺ T cell contamination in CLA⁻ fraction was $<2\%$.

T cell cloning

T cell clones were generated from freshly purified CLA⁺ CD45RO⁺ T cells of two polyallergic atopic patients and two healthy controls. Briefly, 100 CLA⁺ CD45RO⁺ T cells from each donor was plated as 0.3 cell/well in 96-well round bottom plates with 3000 rad irradiated allogeneic PBMC (10^5 /well, feeder cells) in the presence of 20 ng/mL IL-2 and 2 μ g/mL phytohemagglutinin (Sigma) in four different conditions. The first condition was cloning immediately after purification. In other three conditions, cells were incubated in 96-well round bottom plates for 24 h in the presence of fibronectin, collagen VI, laminin, tenascin (5 μ g/mL each, soluble), in the presence of 500 ng/mL sFas-Fc and 25 μ M caspase-3 inhibitor DEVD.CHO, or without any ECM protein or apoptosis inhibitors. These three groups of CLA⁺ CD45RO⁺ T cells were cloned with IL-2, PHA, and irradiated feeder cells as above in the presence of the same doses of ECM proteins and apoptosis inhibitors. After 15 days, growing clones were selected, washed, and restimulated (5×10^3 cell/well 96-well flat bottom plates) with 2 μ g/mL PHA for 72 h for cytokine detection from supernatants. Th1 clones

were characterized by <100 pg/mL IL-4 and IL-5, and >1200 pg/mL IFN- γ production. Th2 clones were characterized by <100 pg/mL IFN- γ and >550 pg/mL IL-4, IL-5, and IL-13. T cell clones that did not fit to Th1 or Th2 criteria were characterized as Th0.

Detection of cytokines

For intracytoplasmic cytokine detection, cells were stimulated with 50 ng/mL phorbol ester (PMA) and 500 ng/mL Ca^{2+} ionophore (ionomycin) for 4 h. Monensin (Sigma) was added at final concentration of 1 μM . The cells were fixed and permeabilized with paraformaldehyde/saponin solution (Ortho Permeafix, Ortho Diagnostic Systems Inc., Raritan, NJ, USA), stained with RPE- or FITC-conjugated isotype control Abs (rat IgG1 and rat IgG2a), anti-IL-4, anti-IL-5, anti-IL13, anti-IFN- γ , mAbs (all from PharMingen) for 30 min at 4°C, and analyzed by flow cytometry.

The solid phase sandwich ELISAs for IFN- γ , IL-4, IL-5, and IL-13 were performed as described previously (16). The sensitivity of the IFN- γ ELISA was 10 pg/mL. The sensitivity of IL-4 ELISA was 20 pg/mL (mAbs and IL-4 and IFN- γ standard were provided by Dr. C.H. Heusser, Novartis, Basel). The detection limit of IL-5 and IL-13 ELISA was 50 pg/mL (PharMingen).

Determination of T cell viability and apoptosis

$\text{CLA}^+ \text{CD45RO}^+$ or $\text{CLA}^- \text{CD45RO}^+$ T cell subsets or CD45RO^+ whole memory T cell fraction were cultured in 96-well tissue culture plates in RPMI 1640 medium supplemented as described (16) in the absence or presence of 10% fetal calf serum (FCS; SeraLab, Sussex, UK). Titrated doses of cytokines, ECM proteins, apoptosis-inducing anti-Fas mAb, soluble (s)Fas-Fc, sFas ligand, sTRAIL, TNF- α , TWEAK, and caspase-3 inhibitor were used. High density cultures were performed with $10^7/\text{mL}$ cells in 100 μL in 96-well round bottom plates; low density cultures were performed in $10^4/\text{mL}$ cells in 24-well plates. Viability of T cells was assessed by uptake of 1 μM ethidium bromide and flow cytometric analysis (16).

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 (18). Briefly, 2×10^5 CLA^+ or $\text{CLA}^- \text{CD45RO}^+$ T cells were washed twice with cold PBS and resuspended in 1 mL binding buffer (HEPES, 0.25 mM CaCl_2). FITC-conjugated annexin V (100 ng/mL) and propidium iodide (500 ng/mL) were added to the cells (both from R&D Systems, London, UK). 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.

FITC-labeled caspase binding peptide (inhibitor) Val-Ala-DL-Asp-fluoromethylketone (VAD-FMK) was used to stain the global activation status of caspase pathway (19). FITC-VAD-FMK (Promega Corp.) was added to T cells at 5 μM final concentration. After 20 min incubation, the cells were washed, fixed with 2% paraformaldehyde in PBS, and analyzed by flow cytometry.

Immunohistology

Tissue samples were placed in OCT compound (Tissue-Tek, Diatec, Nurnberg, Germany) and stored at -80°C. Cryostat sections (4 μm) were prepared on gelatin-coated slides (Merck, Darmstadt, Germany). After air-drying sections were fixed in acetone (10 min, 4°C). A three-step streptavidin-

biotin complex peroxidase method (strept-ABCComplex-peroxidase, Dako AG, Wiesentheid, Germany) was used. Sections were incubated with the primary mAb (Fas ligand, Alexis Corp., Fas, Ancell Corp.) at 4°C overnight, followed by incubation with biotin-conjugated rabbit anti-mouse IgG and preformed streptABCComplex-peroxidase (Dako AG) at RT for 1 h. Finally, visualization was obtained by incubation with the peroxidase-specific substrate 3-amino-9-ethylcarbazole (AEC, Sigma). Hematoxylin was used as a counterstain. For control purposes the primary mAb was replaced by an irrelevant isotype-matched mAb, which consistently yielded negative results.

Apoptotic cells were demonstrated in situ by histochemical techniques staining double-strand DNA breaks. Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) was used, as described (20). TUNEL reaction mixture (Boehringer, Mannheim, Germany) was added to the samples which were incubated for 60 min at 37°C. Incorporated fluorescein was detected by anti-fluorescein Ab Fab fragments from sheep, conjugated with alkaline phosphatase. HOECHST staining was performed as described (21), in lesional skin sections. After fixation with 4% paraformaldehyde and 200 mM dihydrogen phosphate (pH 7.0) overnight at 4°C, staining was performed with HOECHST 33342 dye (1 $\mu\text{g}/\text{mL}$, Sigma) for 5 min. Stained sections were evaluated with a UV microscope (Axiovert 405M, Carl Zeiss AG, Feldbach, Switzerland).

Immunoblotting analysis

Freshly purified $\text{CLA}^+ \text{CD45RO}^+$, $\text{CLA}^- \text{CD45RO}^+$, and CD45RA^+ T cells were lysed as described previously (22). Immunoblots were performed on nitrocellulose membranes (Amersham Life Science, Buckinghamshire, UK) with an anti-caspase-8 mAb (kindly provided by Dr. Markus Peter, Heidelberg, Germany) and visualized by chemiluminescence detection system (ECL, Amersham Pharmacia Biotech Limited, Buckinghamshire, UK). This mAb recognizes the 55 kDa pro-caspase-8 and the 18 kDa active caspase-8 as well as the 43 and 37 kDa cleavage intermediates (7). β -Actin was stained as control.

Statistical interpretation

Results are shown as mean $\pm$ SD. Student's *t* test for paired samples, Student's *t* test for unpaired samples, and Chi square test were used for statistical analysis. Nonparametric statistical comparison between groups of fewer than seven samples was performed by Mann Whitney U test. Because of less cell yield ($<1.5 \times 10^6$ $\text{CLA}^+ \text{CD45RO}^+$ T cells per donor), different experiments had to be performed with cells from different individuals. The number of donors indicated in figure legends includes all patients analyzed in the relevant experiment.

RESULTS

Increased death of peripheral blood $\text{CLA}^+ \text{CD45RO}^+$ T cells in atopic dermatitis

There is massive CLA^+ T cell infiltration in skin lesions (10, 23) and very active traffic from the skin via lymph to circulation in AD (24). Several studies have shown in vitro expansion of $\text{CLA}^+ \text{CD45RO}^+$ T cells by different means of T cell activation (12, 25); however, there is no

evidence for the expansion of CLA⁺ CD45RO⁺ T cells in the circulation in AD (23). Accordingly, we investigated the mechanisms that control CLA⁺ T cell numbers in peripheral blood. Life span of freshly purified CLA⁺ and CLA⁻ T cells of AD patients, various non-atopic patients, and healthy individuals was determined without any stimulation (Fig. 1A). The viability of CLA⁺ T cells in AD was significantly lower than viability of CLA⁺ T cells purified from healthy individuals as well as psoriasis, contact dermatitis, intrinsic form of asthma, intrinsic form of AD, and bee venom allergy patients. In contrast, CLA⁻ T cells did not show increased death and there was no difference between patients and controls. AD patients did not show any difference related to coexpression of atopic asthma or allergic rhinitis. CLA is expressed on about ~one-third of CD45RO⁺ T cells, and it has been demonstrated that the ligation of CLA with HECA-452 mAb induces neither proliferation nor cytokine production (14–16). Freshly purified CD45RO⁺ T cells also showed increased death in AD due to CLA⁺ T cell content. We investigated whether ligation of CLA affects the life span of T cells. Here, we demonstrate that ligation of CLA by anti-CLA mAb and cross-linking of the CLA epitope does not influence the viability of CD45RO⁺ T cells (Fig. 1B).

T cell death may occur in a suicidal way in which they

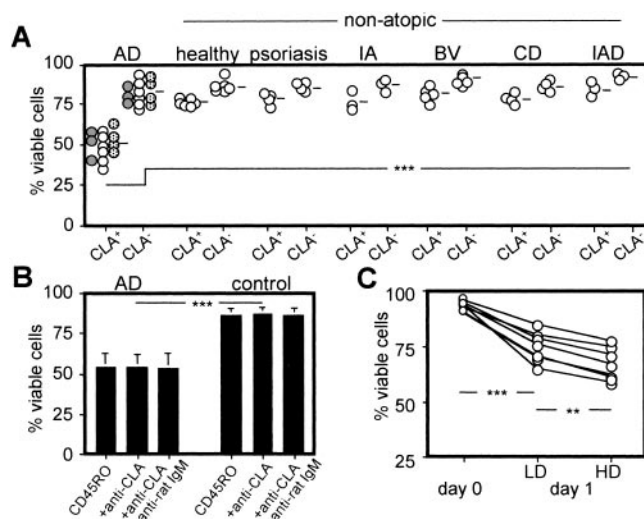


Figure 1. Increased death in CLA⁺ CD45RO⁺ T cells in atopic dermatitis. **A)** After purification, CLA⁺ and CLA⁻ CD45RO⁺ T cells of 13 AD patients (3 with asthma and rhinitis, filled circles; 4 with rhinitis, dotted circles), 6 healthy controls, 4 psoriasis, 3 intrinsic-type asthma (IA), 5 bee venom allergic (BV), 4 contact dermatitis (CD), and 3 intrinsic type of AD (IAD) were cultured for 48 h. **B)** Purified CD45RO⁺ T cells from AD patients and healthy controls were cultured, in the presence of anti-CLA mAb or anti-CLA mAb cross-linking anti-Rat IgM antibody. Results represent mean $\pm$ SE of 3 experiments from different donors. **C)** CLA⁺ CD45RO⁺ T cells from 7 AD patients were cultured in high (HD, 10⁷/mL) and low (LD, 10⁴/mL) densities without any stimulation in 10% FCS containing RPMI medium. Cell viability was measured by ethidium bromide staining and flow cytometry. ****P* < 0.001, ***P* < 0.01.

kill only themselves or in a fratricidal way, where they kill other bystander cells (3–5). The culture of CLA⁺ T cells at high and low densities demonstrated that they die by both suicide and murder (Fig. 1C). CLA⁺ CD45RO⁺ T cells from AD patients showed increased death at low cell densities after 1 day in culture, which was further enhanced at high cell density cultures.

Circulating CLA⁺ CD45RO⁺ T cells undergo Fas-mediated activation-induced cell death in AD

In AD, circulating CLA⁺ CD45RO⁺ T cells showed high Fas and detectable Fas ligand expression (Fig. 2A). Fas expression on CLA⁺ CD45RO⁺ T cells in AD (79 $\pm$ 12%) was significantly higher than bee venom allergic patients (14 $\pm$ 3%, *n*, 4), psoriasis patients (18 $\pm$ 4%, *n*, 3), contact dermatitis patients (15 $\pm$ 5%, *n*, 4), and healthy controls (12 $\pm$ 4%, *n*, 5) (*P* < 0.001). In contrast, CLA⁻ CD45RO⁺ T cells expressed neither Fas nor Fas ligand. Therefore, we analyzed Fas-mediated apoptosis in CLA⁺ and CLA⁻ subsets of CD45RO⁺ T cells from AD patients. As demonstrated by surface phosphatidylserine expression, there was significantly increased apoptosis in CLA⁺ T cells without any stimulation. This was further enhanced by triggering the Fas (Fig. 2B). In contrast, CLA⁻ cells showed significantly less cell death and were rather resistant to Fas-mediated apoptosis.

To investigate whether apoptotic pathways other than Fas play a role in death of activated skin-homing T cells; CLA⁺ CD45RO⁺ T cells were cultured with TRAIL, TNF- α , TWEAK, and sFas ligand. About 38% of the cells showed spontaneous death. Only sFas ligand further triggered the death of CLA⁺ CD45RO⁺ T cells whereas soluble TRAIL, TNF- α , and TWEAK had no effect (Fig. 2C).

To test that highly expressed Fas on CLA⁺ CD45RO⁺ T cells is triggered by Fas ligand from activated T cells, the human Fas-Fc protein was used as a competitive inhibitor of Fas ligand–Fas interaction (26). The cells showed spontaneous death and only about half (47.2 $\pm$ 7.2%) were alive after 24 h. Fas-Fc inhibited apoptosis of CLA⁺ T cells and the viability increased up to 64.3 $\pm$ 8.3% (Fig. 2D). In addition, spontaneous apoptosis of CLA⁺ CD45RO⁺ T cells was dose-dependently inhibited by the caspase-3 inhibitor DEVD.CHO (27) (Fig. 2E). In repeated experiments the death of ~one-third of CLA⁺ CD45RO⁺ T cells could not be inhibited by these approaches, suggesting that a segment of the cells is in vivo primed for AICD.

Apoptotic program of circulating CLA⁺ CD45RO⁺ T cells is initiated in vivo

Whether the apoptotic pathway in a subset of CLA⁺ CD45RO⁺ T cells has already been initiated in vivo in AD was further elucidated. Freshly purified T cells were lysed and immunoblots were performed with an anti-caspase-8 mAb (Fig. 3A). A fraction of freshly purified CD45RO⁺ T cells expressed caspase-8 degradation

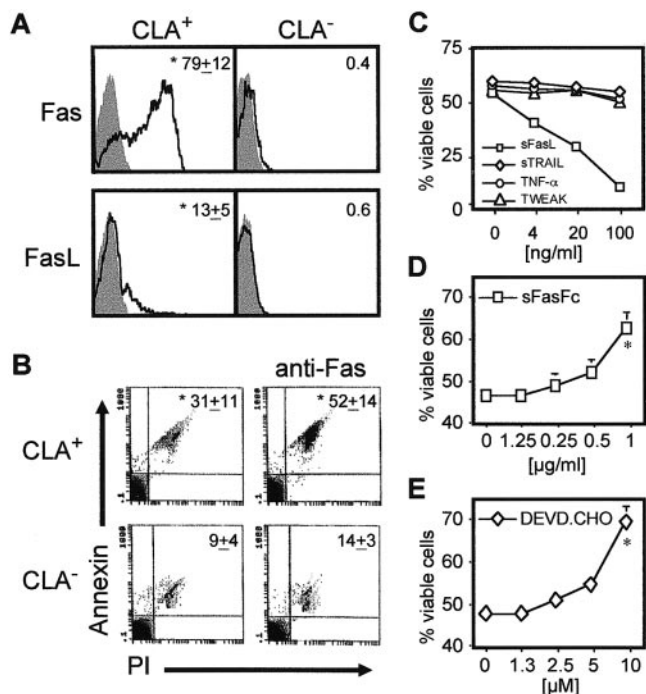


Figure 2. Fas-mediated AICD of peripheral blood CLA⁺ CD45RO⁺ T cells in AD. **A)** Fas and Fas ligand (FasL) were stained on the surface of freshly purified CLA⁺ and CLA⁻ subsets of CD45RO⁺ T cells. Mean $\pm$ SD of 5 individuals is shown on the upper right corner of each histogram. $*P < 0.001$. **B)** Spontaneous and Fas-mediated apoptosis of CLA⁺ and CLA⁻ subsets of CD45RO⁺ T cells was determined by membrane phosphatidylserine staining with FITC-labeled annexin V and propidium iodide (PtdIns) after 24 h. Mean $\pm$ SD of apoptotic cells in 7 individuals is shown on the upper right corner of each histogram. $*P < 0.001$. **C)** CLA⁺ CD45RO⁺ T cells were stimulated with sFas ligand, sTRAIL, TNF- α , and TWEAK. Cell viability was measured by ethidium bromide staining after 24 h. Results shown are representative of 3 experiments. **D, E)** CLA⁺ CD45RO⁺ T cells were cultured with sFas-Fc and caspase-3 inhibitor (DEVD.CHO). Cell viability was measured by ethidium bromide staining after 24 h. Results shown are representative of 4 experiments. $*P < 0.01$.

products as well as the 18 kDa active caspase-8 in AD. These findings in CD45RO⁺ T cells were confined to the CLA⁺ subset. In contrast, the CLA⁺CD45RO⁺ subset showed intact pro-caspase-8 and did not show any active caspase-8. The procaspase-8 band was repeatedly weaker in CLA⁺ T cells of AD patients immediately after purification, suggesting increased in vivo degradation. In comparison, CD45RO⁺ T cells of healthy individuals showed no active caspase-8.

Additional evidence for in vivo-initiated apoptosis of CLA⁺ CD45RO⁺ T cells was obtained using an FITC-labeled cell permeable caspase binding peptide (Fig. 3B). Again, immediately after purification the CLA⁺ CD45RO⁺ T cell subset showed markedly increased caspase degradation. High numbers of CLA⁺ CD45RO⁺ T cells showed low fluorescent (38 $\pm$ 11%) and high fluorescent (8 $\pm$ 4%) caspase degradation in AD. In contrast, the CLA⁻ CD45RO⁺ T cells showed only 7 $\pm$ 2% of low fluorescence caspase degradation. With this method, the high fluorescent cells have been

identified as late apoptotic cells and the low fluorescence cell population included early apoptotic cells and intact cells (28). CLA⁺ CD45RO⁺ T cells of healthy individuals did not show >4% caspase degradation. Similarly, no significant caspase degradation was observed in CD45RO⁺ T cells of contact dermatitis and bee venom allergic patients. These data demonstrate that circulating CLA⁺ CD45RO⁺ T cells show in vivo-initiated apoptosis in AD.

T cells express Fas and Fas ligand without showing apoptosis in the lesional skin in atopic dermatitis

The histological appearance of acute lesional AD skin is characterized with dermal mononuclear cell infiltration consisting mostly of CD4⁺ T cells and lower numbers of CD8⁺ T cells (16, 23). Apoptotic features in AD skin was investigated, by Fas and Fas ligand expression as well as two different methods for apoptosis (Fig.

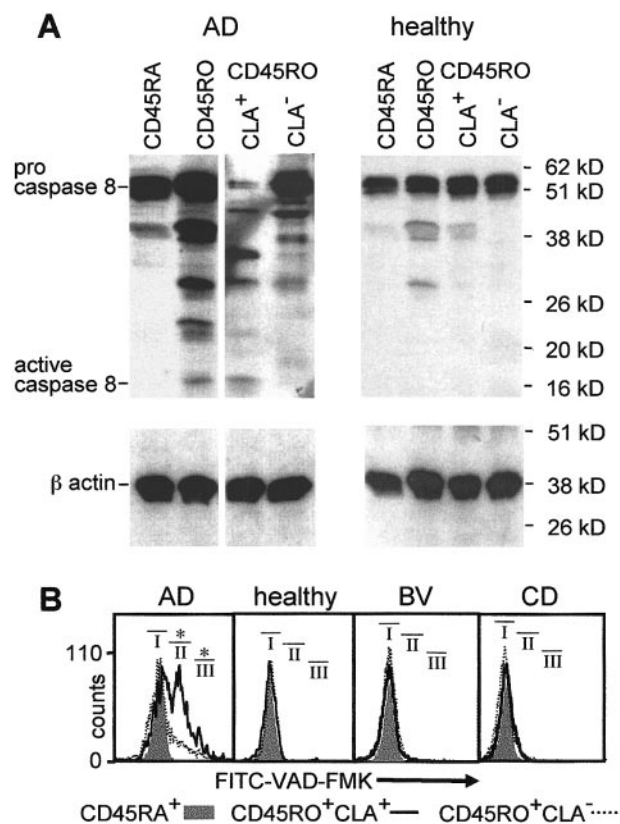


Figure 3. In vivo-triggered apoptosis of CLA⁺ CD45RO⁺ T cells in AD. **A)** Freshly purified CLA⁺ and CLA⁻ subsets of CD45RO⁺ T cells and CD45RA⁺ T cells were lysed and immunoblots were stained with anti-caspase-8 mAb. β -Actin was stained for control. Equivalent results were obtained in 3 AD patients and 3 healthy individuals. **B)** Freshly purified CLA⁺ and CLA⁻ subsets of CD45RO⁺ T cells and CD45RA⁺ T cells were stained with FITC-VAD-FMK and analyzed by flow cytometry. I: intact caspase in CD45RA⁺ cells; II: low fluorescence caspase degradation; III: apoptotic cell fraction showing high fluorescence caspase degradation. One of 3 experiments each with cells from AD, healthy controls, bee venom allergic individuals (BV) and contact dermatitis (CD) is shown. $*P < 0.001$.

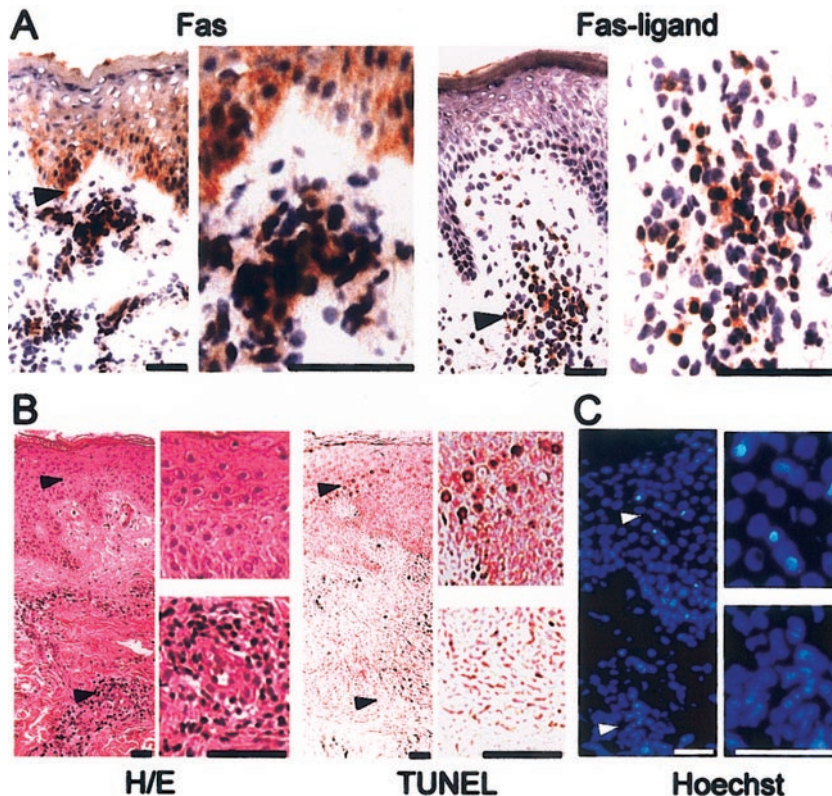


Figure 4. T cells do not undergo apoptosis in AD lesions. *A*) Skin-infiltrating T cells express Fas and Fas ligand in AD. Immunohistological staining with AEC substrate and counterstaining with hematoxylin. $\times 400$. *B*) Sequential sections from lesional AD skin. $\times 200$. Hematoxylin/eosin (H/E) staining and TUNEL staining. Inflammatory cells in the dermis show no sign of apoptosis. Red condensed and partly fragmented nuclei indicate positive staining for keratinocyte apoptosis in epidermis. *C*) Hoechst staining of lesional AD skin. $\times 400$. Results shown are representative of 7 different samples of lesional AD skin (biopsies were performed 2–5 days after flare-up of lesions in chronic AD patients, scale bar: 50 μ m at the lower right corner). Higher magnification of the area indicated by arrows is shown on the right-hand side of each figure.

4). T cell infiltrate highly expressed Fas in acute AD lesions. In addition, the basal and suprabasal compartment of the epidermis was strongly reactive for Fas. T cells in the dermis also showed Fas ligand immunoreactivity whereas keratinocytes showed no Fas ligand expression (Fig. 4A). Therefore, Fas ligand on T cells was presumed to trigger Fas on the same cell or bystander T cells as well as keratinocytes. Accordingly, sequential sections of the same skin biopsies from AD were analyzed using the TUNEL technique and HOECHST staining (Fig. 4B). The T cell infiltrate did not show any TUNEL staining in several biopsy samples taken from 3- to 7-day-old lesions, whereas TUNEL-stained keratinocytes were visible in epidermis in all of these samples. Similarly, condensed and fragmented nuclei of apoptotic keratinocytes were HOECHST stain positive in epidermis. Again, the T cell infiltrate showed no sign of apoptosis (Fig. 4C). These results demonstrate that skin-infiltrating T cells are resistant to apoptosis although they express both Fas and Fas ligand.

Selective AICD in Th1 cells define peripheral Th2 response in atopy

To investigate the factors responsible for prolonged T cell survival in the skin, freshly purified CLA⁺ CD45RO⁺ T cells were cultured with different cytokines in serum-free medium. The T cell growth factors IL-2, IL-4, and IL-15 significantly prevented CLA⁺ T cell apoptosis whereas IL-16 and IL-18 had no effect. We tested whether ECM proteins influence skin-homing T cell life span. The ECM proteins, fibronectin,

collagen IV, laminin, and tenascin tested significantly inhibited CLA⁺ T cell apoptosis. Transferrin, a serum and tissue factor, enhanced the life span of CLA⁺ T cells. (Fig. 5A)

After demonstrating the in vivo-initiated apoptosis and factors that prevent AICD of CLA⁺ CD45RO⁺ T cells, we investigated whether there is a change in T cell cytokine profile related to AICD. CD45RO⁺ T cells, which also show increased AICD because of their $\sim$ one-third CLA⁺ T cell content, were cultured alone or in the presence of fibronectin, collagen, tenascin, and laminin. After 24 h, the CD45RO⁺ T cells that were cultured without ECM proteins showed significantly less IFN- γ production in AD patients, but not in healthy controls ($P < 0.01$) (Fig. 5B). The same findings were observed by intracytoplasmic cytokine determinations in AD patients (Fig. 5C).

The data presented above suggest that increased survival in T cells affects cytokine profile and that IFN- γ secretion increases with enhanced T cell survival by ECM proteins. We further investigated the influence of memory/effector T cell AICD to cytokine profiles at the single cell level. With this approach, the possibility that ECM may booster IFN- γ production was ruled out. CLA⁺ CD45RO⁺ T cells from two AD patients and two healthy individuals were randomly cloned under four different conditions. In the first condition, CLA⁺ CD45RO⁺ T cells were cloned with IL-2 and PHA immediately after purification. In the second and third conditions, T cells were incubated with survival factors such as ECM proteins or apoptosis inhibitors such as sFas-Fc and DEVD.CHO for 24 h and cloned in the

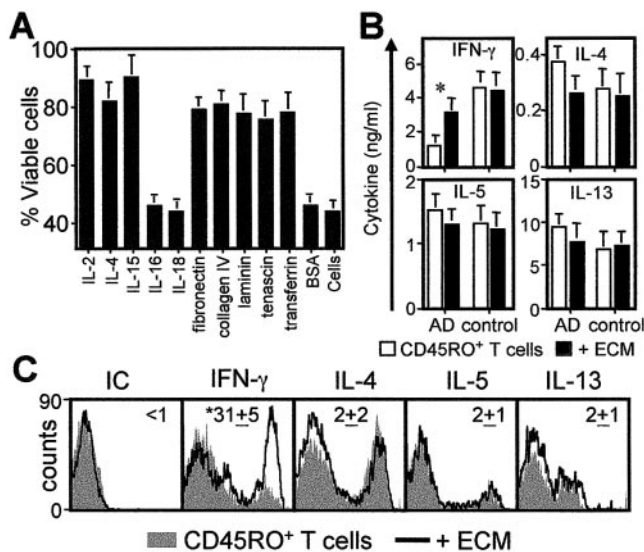


Figure 5. ECM proteins prevent AICD predominantly in IFN- γ -secreting T cells in AD. A) CLA⁺ CD45RO⁺ T cells were cultured in serum-free medium with different cytokines, ECM proteins, and transferrin. IL-2 was used at 20 ng/mL, IL-4, IL-15, IL-16 and IL-18 were used at 100 ng/mL, transferrin was used at 50 μ g/mL and ECM proteins were used at 10 μ g/mL. Bovine serum albumin (BSA) was used at 50 μ g/mL as control. Cell viability was determined by ethidium bromide staining and flow cytometry after 48 h. B) Peripheral blood CD45RO⁺ T cells from 3 AD patients and 3 healthy controls were cultured 24 h in the presence or absence of 10 μ g/mL fibronectin, collagen IV, laminin, and tenascin. Cells were stimulated with anti-CD2, anti-CD3, and anti-CD28 mAbs for 24 h and cytokines were detected by ELISA. C) Cells were stimulated with PMA/ionomycin for 4 h and cytokines were detected by intracytoplasmic staining. Percent difference in cytokine-expressing cells with and without ECM is shown in the upper part of histograms (* $P < 0.001$).

presence of the same ECM proteins or apoptosis inhibitors. In the forth condition, CLA⁺ CD45RO⁺ T cells were allowed to undergo apoptosis for 24 h and cloned without any ECM proteins or apoptosis inhibitors (Fig. 6). After 24 h, $43 \pm 4\%$ of the CLA⁺ CD45RO⁺ T cells from AD patients and $11 \pm 8\%$ from healthy controls died. Significantly less clonability was observed in cells that were left for 24 h without any apoptosis inhibitors in AD. Cytokine profile of CLA⁺ T cell clones of AD patient demonstrated a striking difference by cloning in the absence of any survival factors or apoptosis inhibitors. In these clones, IFN- γ decreased whereas IL-4 and IL-13 significantly increased compared with healthy control and conditions that prevent the AICD. In addition, cloning of T cells in the absence of apoptosis inhibitors did not lead to the generation of any Th1 clone in AD (Fig. 6D). In contrast, cloning in the presence of ECM proteins or Fas pathway inhibitors resulted in the generation of both Th1 and Th2 clones. In our previous attempts to increase the efficiency of T cell cloning in bee venom allergic patients, the use of sFas-Fc or ECM proteins did not influence cytokine profiles similar to healthy individuals (data not shown). Together, these data demonstrate that T cells, which

undergo AICD in AD, are the main Th1 cells, suggesting a mechanism for the Th2 predominance in atopy.

DISCUSSION

The balance between production and death is important in the control of cell numbers within physiological ranges. Inflammatory disorders are characterized by an expansion of hematopoietic effector cells. Activated T cells mediate most features of allergic inflammation in close interaction with mast cells, eosinophils, and epithelial cells (16, 23, 29–31). Such cell accumulation in

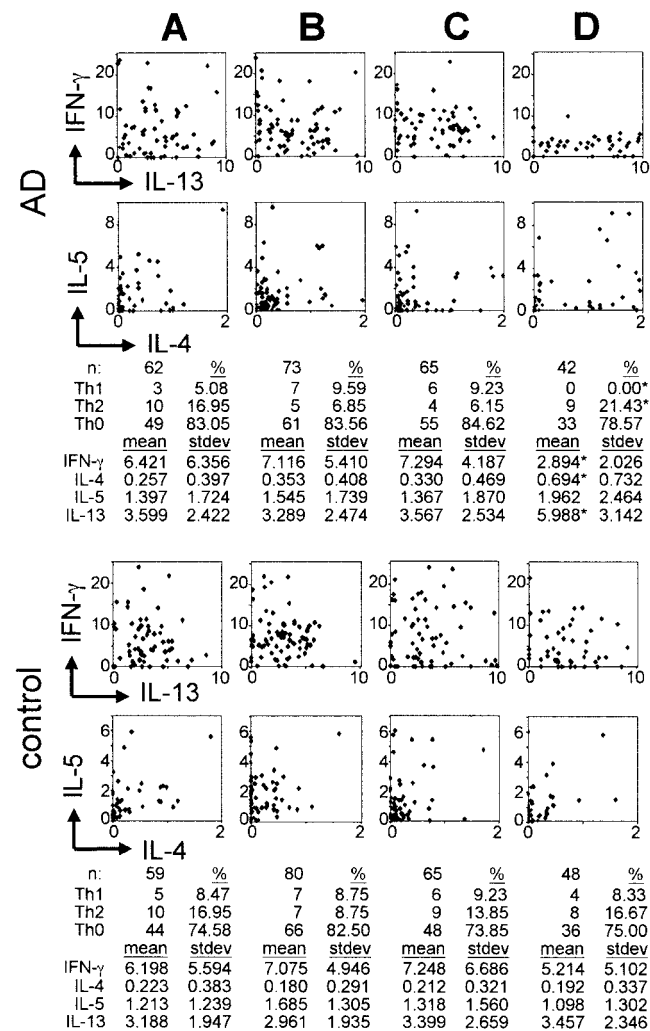


Figure 6. Predominant apoptosis in Th1 cells in AD. Peripheral blood CLA⁺ CD45RO⁺ T cells from 2 AD patients and 2 healthy controls were A) cloned immediately after purification; B) 24 h cultured in the presence of fibronectin, collagen, tenascin, and laminin and cloned in the presence of the same ECM proteins; C) 24 h cultured in the presence of caspase inhibitor DEVD.CHO and soluble Fas-Fc and cloned in the presence of the same apoptosis inhibitors; D) 24 h cultured and cloned without any ECM proteins and apoptosis inhibitors. T cell clones were stimulated with PHA and cytokine profiles of single clones were determined by ELISA (ng/mL) (* $P < 0.01$). Cutoff values for the definition of Th1 and Th2 clones are described in Materials and Methods.

the tissues may be a consequence of either increased cell production or decreased cell death. The present study demonstrates that highly activated peripheral blood CLA⁺ CD45RO⁺ T cells undergo AICD. Direct evidence is demonstrated that the apoptotic program is initiated *in vivo*. Immediately after purification, a fraction of these cells show active caspase-8 and increased caspase degradation, in contrast to their CLA⁻ counterpart, CLA⁺ T cells of healthy individuals, and several non-atopic disorders. AICD of CLA⁺ T cells could be inhibited by caspase cascade inhibition and by blocking Fas/Fas ligand interaction. This essential finding represents a novel mechanism in peripheral Th2 response in atopic individuals.

Characterization of cytokine profile of T cells that resist or undergo AICD revealed unequal death in Th1 and Th2 cells. In the absence of survival factors and Fas pathway inhibitors, a Th2-skewed cytokine profile was observed in CD45RO⁺ T cells and CLA⁺ CD45RO⁺ T cell clones. Increased death of IFN- γ -secreting CD4⁺ T cells have been previously demonstrated (32, 33). Supporting these findings, high IFN- γ -secreting Th1 cells that do not efficiently develop into long term memory Th1 cells in mice were shown to be short-lived (34). By using with *in vitro* generated Th1 and Th2 cells, it was suggested that unequal susceptibility to AICD may be related to increased expression of a Fas-associated phosphatase, FAP-1, in Th2 cells (35). In addition, AICD susceptibility of Th1 subsets in HIV infection was found related to variable levels of Bcl-2 expression (36). Up-regulation of phosphatidylinositol 3'-kinase, which inhibits caspase-8 cleavage at the death-inducing complex, has been reported as a mechanism for Th2 resistance to Fas-mediated apoptosis (37).

A polarized peripheral blood Th2 cytokine pattern was regarded as a specific feature reflecting immune dysregulation in atopy. A switch in cytokine profile occurs toward Th0/Th1 after skin homing of T cells in AD. IFN- γ predominates over IL-4 in chronic skin lesions and older patch test reactions, whereas, IL-5 and IL-13 remain at high levels (23, 38, 39). IL-12 and IL-18 produced by cells in the microenvironment are likely predominant mediators for the induction of IFN- γ in T cells after homing to skin (40, 41). Studies of the induction of CLA on Th2 cells have also shown that CLA is induced mainly by IL-12 stimulation of Th2 cells (12, 25). Most of the T cells found in skin-draining lymphatics, which represent skin dehomings memory/effector T cells, express CLA and increased levels of IFN- γ (24, 42). In contrast, most studies to date have not detected abundant expression of IFN- γ in the lungs of asthmatics; growing evidence suggests that the nature of antigen presentation and T cell education in the lung is inherently biased toward a Th2-like response (43, 44).

From the cumulative data of the present study, peripheral blood CLA⁺ CD45RO⁺ T cells appeared to contain three fractions according to their apoptotic properties. In one fraction, apoptosis had already begun *in vivo*. A second fraction is predisposed to apo-

ptosis and shows spontaneous apoptosis, which is inhibitable by caspase inhibitors and soluble Fas-Fc. A third fraction is almost resistant to apoptosis, like the CLA⁻ CD45RO⁺ T cells. Our efforts to analyze the specificity of the findings to AD involved several T cell-mediated non-atopic diseases related to skin or allergy. Intrinsic-type asthma and non-atopic dermatitis are common diseases in which T cells play a role without showing Th2 bias and related hyper IgE or type 1 skin test reactivity to allergens (23, 45). Psoriasis and contact dermatitis are T cell-mediated skin diseases, also not related to atopy. In addition, monoallergic patients to bee venom, who do not show any atopy were studied (46). In all of the non-atopic T cell-mediated diseases, significantly less CLA⁺ CD45RO⁺ T cell apoptosis was observed, demonstrating that increased peripheral blood activation-induced T cell apoptosis is confined to AD.

Loss of attachment to matrix causes apoptosis in many cell types, including T cells (47–50). This phenomenon, referred to as anoikis (homelessness), was assumed to prevent cells that had lost contact with their original surroundings from establishing themselves at inappropriate locations. Inflammatory tissues and active lymphoid organs particularly provide this microenvironment. Recently, characterization of the cells in the afferent skin-derived lymph demonstrated the dominance of CLA-bearing and IFN- γ -producing T cells as direct evidence for active lymphatic dehomings of Th0/Th1 effector cells from the skin via afferent lymph (24, 42). During dehomings of T cells from skin via lymph or reverse transmigration, active deprivation of survival signals provided from tissue cytokines and ECM proteins occurs.

T cells infiltrating the AD skin are protected from apoptosis by ECM proteins and cytokines, although they express both Fas and Fas ligand. Inflammatory cells reside in a protein network in the tissues, the ECM, which exerts profound control over them. The effects of ECM are primarily mediated by integrins that attach cells to the matrix and mediate mechanical and chemical signals. Integrins can recognize several ECM proteins; conversely, a single ECM protein can bind to several integrins (51). During inflammation, leukocytes migrate into tissues and interact with ECM proteins. Cell adhesion to the ECM has been implicated in protection from apoptosis in anchorage-dependent cell types (52). Apparently, integrin signaling by ECM represents an important survival signal to T cells, although they do not require anchorage in the tissues.

IL-2, IL-4, and IL-15 prevented AICD in skin-homing T cells. The common γ chain shared by IL-2, IL-4, and IL-15 receptors as well as all other known T cell growth factor receptors is an essential signaling component. IL-15 shares many biological activities with IL-2 and signals through the IL-2 receptor β and γ chains. However, IL-15 and IL-2 differ in their control of expression and secretion, their range of target cells, and their functional activities. IL-2 induces or inhibits T cell apoptosis *in vitro* depending on T cell activation,

whereas IL-15 inhibits cytokine deprivation-induced apoptosis in activated T cells (6). Furthermore, blocking the γ chain in mice inhibits T cell proliferation and induces T cell apoptosis that leads to stable allograft survival (53). The role of cytokines that prolongs T cell survival was not explored in the present study because of their potent effects on T cell cytokine profiles.

T cell turnover between skin and circulation is associated with a switch of cytokine profiles that is Th0/Th1-like inside the skin, Th0/Th2-like in the circulation, and secondary lymphoid organs in atopic individuals. Most probably T cell-mediated effector functions such as eczema/spongiosis formation depend on a Th1-like profile in the skin (29, 54). In contrast, hyper-IgE production depends on a Th2-like profile throughout circulation and secondary lymphoid organs (55). In conclusion, the present study elucidates why peripheral blood lymphocytosis is not observed in atopic skin inflammation, although enormous numbers of T cells are actively homing to skin to make eczematous lesions and dehoming via lymphatic vessels. The unequal susceptibility to AICD between Th1 and Th2 cells that controls the T cell fate may eventually cause an imbalance in Th cell subsets leading to peripheral blood Th2 response in polyallergic individuals with AD. Based on the data we have presented, we propose a novel mechanism for understanding how Th2 responses are dominant in atopic individuals with dermatitis. **[F]**

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