

Increased activation-induced cell death of high IFN- γ -producing T_H1 cells as a mechanism of T_H2 predominance in atopic diseases

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Background: A dysregulated and T_H2-biased immune response appears to be a key pathogenetic factor in atopic diseases. Increased activation and massive infiltration of T cells in the dermis without any evidence for the expansion of their numbers in peripheral blood characterize atopic dermatitis.

Objective: To investigate differences and mechanisms of T_H1 and T_H2 cell activation-induced cell death (AICD) in atopic disease.

Methods: Naive (CD4⁺CD45⁺RA) and memory (CD4⁺CD45⁺RO) T cells were isolated from healthy and atopic individuals. T_H1 and T_H2 subsets were *in vitro* differentiated. High IFN- γ -producing T cells and CXCR3⁺ T cells were purified, and AICD of isolated cells was determined in addition to expression of apoptosis receptors and caspase activation. **Results:** T_H1 cells, particularly their high IFN- γ -producing fraction, and CXCR3⁺ T cells showed significantly increased apoptosis in atopic individuals. During their *in vitro* differentiation, both T_H1 and T_H2 cells of atopic individuals showed increased apoptosis compared with the healthy control group, with a significantly high apoptosis in T_H1 cells. Increased expression of Fas, Fas-ligand, tumor necrosis factor receptor-II, and caspase activation was detected on T_H1 cells that underwent apoptosis. Neutralization experiments demonstrated a dominant role of IFN- γ and Fas–Fas-ligand interaction-mediated suicide in T_H1 cell AICD.

Conclusion: Predominant T_H2 profile in atopic diseases might be a result of the increased tendency to activation and apoptosis of high IFN- γ -producing T_H1 cells. (J Allergy Clin Immunol 2008;121:652-8.)

Key words: T cells, activation-induced cell death, apoptosis, atopy, T_H1 cells, CXCR3

Abbreviations used

7AAD: 7-Amino-actinomycin D

AD: Atopic dermatitis

AICD: Activation-induced cell death

CLA: Cutaneous lymphocyte-associated antigen

Infection and damage induce the local production of distinct cytokines by tissue cells and antigen-presenting cells, initiating the differentiation of T cells reacting to the antigen into either type 1 or type 2 cells. IL-12 drives naive T-cell differentiation toward type 1 phenotype, and IL-4 drives toward type 2. CD4⁺ T_H1 cells are involved in cell-mediated inflammatory reactions. T_H2 cytokines support antibody production, particularly IgE response and eosinophil differentiation and function and associated allergic responses.^{1,2}

Atopic dermatitis (AD), asthma, and rhinitis are atopic diseases characterized by high IgE and particularly T-cell-mediated allergic inflammation.³ A polarized peripheral blood T_H2 cytokine pattern has been regarded as a specific feature reflecting immune dysregulation in the peripheral blood.⁴ In AD, a switch in cytokine pattern occurs toward T_H0/T_H1 after skin homing of T_H2 cells.⁵⁻⁷ T-cell turnover between skin and circulation is apparently associated with a switch of cytokine profiles such as T_H0/T_H1-like inside the skin and T_H0/T_H2 in the circulation and secondary lymphoid organs in atopic individuals.⁸ 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.^{9,10} Most of the T cells found in skin-draining lymphatics, which represent skin-dehoming memory/effector T cells, express increased levels of IFN- γ .¹¹ Certain chemokine receptors have been associated with T-cell homing to tissue-specific sites. T_H1 cells recruit to tissue through the cognate interaction with the T_H1-associated chemokine receptor CXCR3.¹²

To procure self-tolerance and downregulation of an immune response, elimination of T cells takes place in the periphery and involves induction of apoptosis.^{9,13} Cell death by apoptosis is a tightly regulated process that enables removal of unnecessary, aged, or damaged cells.¹⁴⁻¹⁶ It requires the interaction of specific receptors, termed *death receptors*, with their ligands. Especially Fas (CD95) and Fas-ligand (CD178) interactions are the major mechanism for activation-induced cell death (AICD) of CD4⁺ T cells.¹⁷⁻¹⁹ We have recently demonstrated that, in AD, circulating cutaneous lymphocyte-associated antigen (CLA⁺) CD45RO⁺ T cells with skin-specific homing property represent an activated memory/effector T-cell subset and undergo AICD.⁸ Freshly purified CLA⁺ CD45RO⁺ T cells of atopic individuals displayed distinct features of *in vivo* triggered apoptosis such as

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procaspase degradation and active caspase-8 formation. The apoptosis of circulating memory/effector T cells was confined to atopic individuals, whereas nonatopic patients such as those with psoriasis, intrinsic-type asthma, contact dermatitis, intrinsic-type AD, and bee venom allergy, and healthy controls did not show any evidence for increased T-cell apoptosis. In the current study, we demonstrate that increased T-cell apoptosis in atopic individuals may play a role in T_H2 predominance, because particularly the high IFN- γ -producing T_H1 cells show increased AICD in atopic patients.

METHODS

Study population

Twelve patients with chronic AD (mean age, 36 years) who fulfilled the criteria of Hanifin and Rajka²⁰ are included in the study. All were polyallergic and showed positive cutaneous tests to at least 2 aeroallergens. Patients showed specific IgE against at least 2 aeroallergens at radioallergosorbent test class ≥ 2 and total IgE of >400 IU/mL (mean, 4763 ± 1785 IU/mL). None of the patients had any systemic immunosuppressive treatment for at least 1 month before testing. Fourteen healthy individuals (mean age, 35 years) with no history of atopy are included in the study as controls. Their mean serum IgE level was 69 ± 10 IU/mL. The study was approved by the Ethical Committee of Davos, Switzerland.

Purification of $CD45RA^+$, $CD45RO^+$, and $CXCR3^+$ T cells and *in vitro* differentiation of T_H1 -cell and T_H2 -cell subsets

Antibodies and reagents are listed in this article's Online Repository at www.jacionline.org. PBMCs were isolated by Ficoll (Biochrom KG, Berlin, Germany) density gradient centrifugation of peripheral venous blood as described.²¹ Pure T cells were isolated with human pan T-cell isolation kit (Miltenyi Biotec AG, Bergisch Gladbach, Germany). Briefly, anti-CD14, anti-CD16, anti-CD19, anti-CD56, anti-CD36, anti-CD123, and anti-CD235a were added to PBMCs for the depletion of B cells, natural killer cells, dendritic cells, monocytes, granulocytes, and erythroid cells. For the negative selection of $CD45RA^+$ naive T cells, anti-CD45RO microbeads were used in the second step. For the negative selection of $CD45RO^+$ memory T cells, anti-CD45RA microbeads were used in the second step. To purify $CXCR3^+$ T cells, PBMCs were initially labeled with anti- $CXCR3$ -phycoerythrin for 45 minutes at 4°C , and phycoerythrin-labeled cells were positively selected with anti-phycoerythrin magnetic beads. Control antibodies were phycoerythrin-conjugated or fluorescein isothiocyanate-conjugated mouse IgG₁ and mouse IgG₂ (PharMingen AG, San Diego, Calif). Fluorescence analysis was performed on an EPICS XL (Coulter Corp, Fullerton, Calif) with argon laser (488 nm). Purity of $CD45RA^+$ or $CD45RO^+$ T cells was more than 87% and $CXCR3^+$ T cells was more than 81.3%. The purification method of IFN- γ -secreting and IL-4-secreting T cells, T_H1 and T_H2 cell differentiation from $CD45RA^+$ T cells, and their cytokine profile and intracytoplasmic cytokine detection are described in the Online Repository.

Determination of cell viability, apoptosis, and receptors of apoptosis

PBMCs, $CD45RO^+$ and $CD45RA^+$ T cells, and T_H1 -derived and T_H2 -derived cells were cultured in 48-well tissue culture plates in complete RPMI 1640 medium. PBMCs and $CD45RO^+$ T cells were stimulated with plate-bound anti-CD3 mAb ($20 \mu\text{g/mL}$) for 48 hours. $CD45RA^+$ T cells and T_H1 and T_H2 cells were stimulated with anti-CD2/3/28 mAbs. Viability of T cells was assessed by uptake of $1 \mu\text{mol/L}$ ethidium bromide and flow-cytometric analysis.⁸ Membrane phosphatidylserine redistribution and cell viability were determined by phycoerythrin-conjugated annexin-V and 7-amino-actinomycin D (7AAD) staining and flow cytometry. Together with the analysis of forward and side scatter and cell size and preliminary experiments on time

kinetics, annexin-V and 7AAD-positive cells were referred to as *apoptotic cells* because of clear annexin-V staining in small cells. Controls were used for gating of live and apoptotic cells by overnight incubation with ethanol in different concentrations (0.1% to 10 %). Negative control was freshly purified naive T cells. The quadrants were set by gating 100% viable cells and 100% dead cells.

Fas, TNFR-I, TNFR-II, and Fas-ligand expression on T_H1 and T_H2 cells of patients with AD and healthy subjects was analyzed by flow cytometry. Briefly, T_H1 and T_H2 cells derived from naive T cells were stained with fluorescein isothiocyanate-labeled anti-Fas-ligand and antihuman TNFR-I, phycoerythrin-labeled antihuman CD95, and antihuman TNFR-II.

Statistical interpretation

Results are shown as means $\pm$ SDs. Nonparametric statistical comparison between groups was performed by Mann-Whitney *U* test.

RESULTS

Increased AICD in T cells in AD

To analyze differences in cell viability caused by activation of peripheral blood T cells, we stimulated PBMCs of patients with AD and healthy donors with titrated doses of plate-bound anti-CD3 mAb and cultured for 48 hours (Fig 1, A). AICD of PBMCs correlated with the dose of anti-CD3 and was significantly higher in patients with AD compared with healthy controls in high doses ($20 \mu\text{g/mL}$) of anti-CD3 stimulation ($7.28 \pm 2.2\%$ and $14.02 \pm 5.0\%$, respectively; $P < .002$; Fig 1, B). $CD45RO^+$ memory T cells represent *in vivo* preactivated circulating T cells in AD, and approximately 10% to 25% of these cells express skin-homing-related CLA antigen on their surface, together with *in vivo* activation and spontaneous proliferation.²² To analyze further whether the viability of these preactivated $CD45RO^+$ T cells showed any difference in atopic patients, $CD45RO^+$ T cells were purified and cultured for 48 hours without any stimulation. Subsequently, a significantly decreased viability of $CD45RO^+$ T cells was observed in patients with AD compared with nonatopic controls ($83.4 \pm 5.6\%$; $91.1 \pm 3.3\%$; $P < .04$; Fig 1, C).

Increased apoptosis of T_H1 cells in atopic disease

After the demonstration of increased AICD in atopic individuals, we hypothesized whether increased apoptosis of one subset over the other might play a role in the predominance of T_H2 cells in atopy. Accordingly, we investigated whether AICD of peripheral blood naive T cells *in vitro* differentiated into T_H1 and T_H2 cells showed any difference between healthy and atopic individuals. Both T_H1 and T_H2 cells of atopic individuals showed significantly increased apoptosis with a preferential predominance in T_H1 cells compared with the healthy control group. Increased early and late stages of apoptosis of T_H1 cells were observed both in patients and in healthy controls compared with T_H2 cells ($P < .05$; Fig 2).

Increased expression of Fas, Fas-ligand, and TNFR-II on T_H1 cells of patients with AD

To investigate further the mechanism underlying increased apoptosis in T_H1 cells of atopic individuals, expression of apoptosis-related receptors and ligands is determined on the surface of both *in vitro* differentiated T_H1 and T_H2 subsets (Fig 3). Although Fas and Fas-ligand expression is higher in T_H1 cells of both healthy and atopic individuals, T_H1 cells of patients with AD

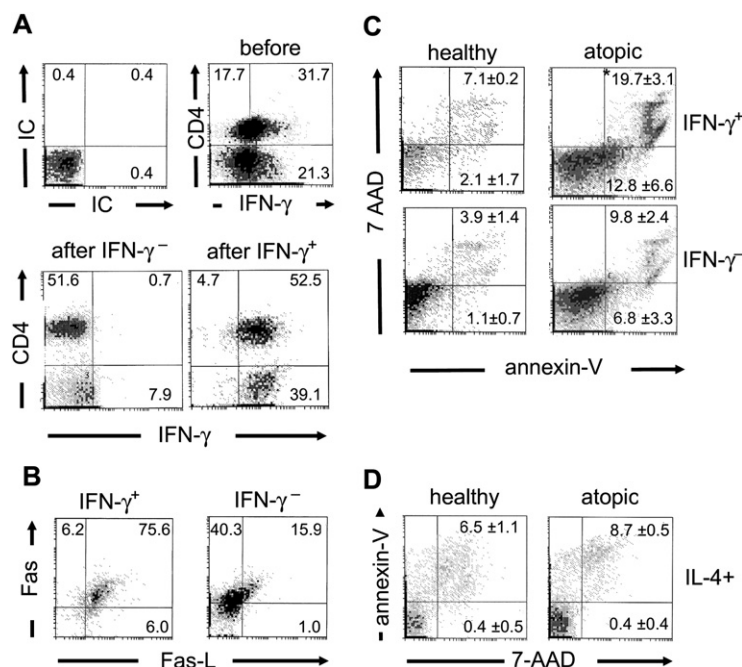


FIG 4. High IFN- γ -expressing T cells undergo AICD in atopy. **A**, Purification of IFN- γ -secreting and IFN- γ -nonsecreting CD4 $^{+}$ T cells. **B**, Increased expression of Fas and Fas-ligand on purified IFN- γ -secreting CD4 $^{+}$ T cells in patients with AD. **C**, IFN- γ -secreting CD4 $^{+}$ T cells of atopic patients showed increased apoptosis compared with healthy subjects and IFN- γ -nonsecreting CD4 $^{+}$ T cells (* P < .01). **D**, Purified CD4 $^{+}$ IL-4-secreting T cells. IC, Isotype control; Fas-L, Fas-ligand.

Increased apoptosis of CXCR3 $^{+}$ T cells in atopic disease

To support further the findings of increased T_H1 apoptosis in atopic individuals, we analyzed the apoptotic properties of gated CXCR3 $^{+}$ T cells. This provides a suitable model to investigate apoptosis of circulating T cells directly without any culture. Immediately after isolation of PBMCs, annexin-V-positive but 7AAD-negative T cells, which represent early apoptotic cells, were significantly high in atopic individuals compared with healthy subjects (P < .05). After 3 days of cultures, late apoptotic T cells with annexin-V and 7AAD positivity were significantly high in AD compared with healthy subjects (P < .05; Fig 6, A). In addition, late apoptotic cells in purified CXCR3 $^{+}$ T cells were significantly high in atopic individuals compared with healthy subjects (P < .05; Fig 6, B).

DISCUSSION

Although massive T-cell activation and skin or lung infiltration is one of the characteristic features of AD and asthma, it is of note that there is no accompanying lymphocytosis.⁵ Apoptosis is a powerful mechanism for deleting highly activated T cells, and it raises the interesting possibility that unequal apoptosis of T_H1 and T_H2 effector cells may lead to preferential deletion of one subset over the other. Because a polarized peripheral blood T_H2 cytokine pattern has been a specific feature reflecting immune dysregulation in atopy, the fate of activated T cells is investigated. The current study demonstrates that T_H1 cell apoptosis, particularly in a high IFN- γ -producing fraction of T cells, is much more prominent in atopic patients compared with healthy controls as a mechanism of T_H2 predominance. In addition, the study demonstrates that IFN- γ acts as the main cytokine for the increased

AICD of T_H1 cells in AD. Supporting these findings, high IFN- γ -secreting T_H1 cells that do not efficiently develop into long-term memory T_H1 cells in mice were shown to be short-lived.^{23,24} By using *in vitro* generated T_H1 and T_H2 cells, it was suggested that unequal susceptibility to AICD may be related to increased expression of a Fas-associated phosphatase-1 in T_H2 cells.²⁵ In addition, AICD susceptibility of the T_H1 subset in HIV infection was found to be related to variable levels of Bcl-2 expression,²⁶ whereas the T_H2 subset represents cells rather resistant to AICD. Although several different mechanisms may be involved, upregulation of phosphatidylinositol 3'-kinase, which inhibits caspase-8 cleavage at the death-inducing complex, has been reported as a mechanism for T_H2 resistance to Fas-mediated apoptosis.²⁷ To support the data in increased apoptosis on high IFN- γ -expressing cells, we investigated the apoptotic properties of circulating CXCR3 $^{+}$ T cells. Similarly, as a representative of circulating T_H1-like cells,¹² CXCR3 $^{+}$ T cells showed a very significant apoptosis indirectly analyzed by gating fresh PBMCs and purified cells. It has been demonstrated that TNFR-II may also induce apoptosis in mature T cells.²⁸ In the current study, T_H1 cells in atopic individuals expressed high TNFR-II, but neutralization of TNF- α did not show any change in T-cell apoptosis.

Activation-induced cell death of peripheral T cells requires the expression of functional Fas and Fas ligand by the T-cell population. Because qualitatively similar signals from the T-cell receptor (TCR) are responsible for both T-cell expansion in inflammation and T-cell elimination by AICD, regulating the balance between these opposing functions plays a crucial role in successful responses to pathogens and tumors while minimizing autoimmunity.^{17,19} Several studies have demonstrated major pathogenic events related to dysregulated apoptosis and survival of T cells,⁸ eosinophils,²⁹ and epithelial cells³⁰ in allergic

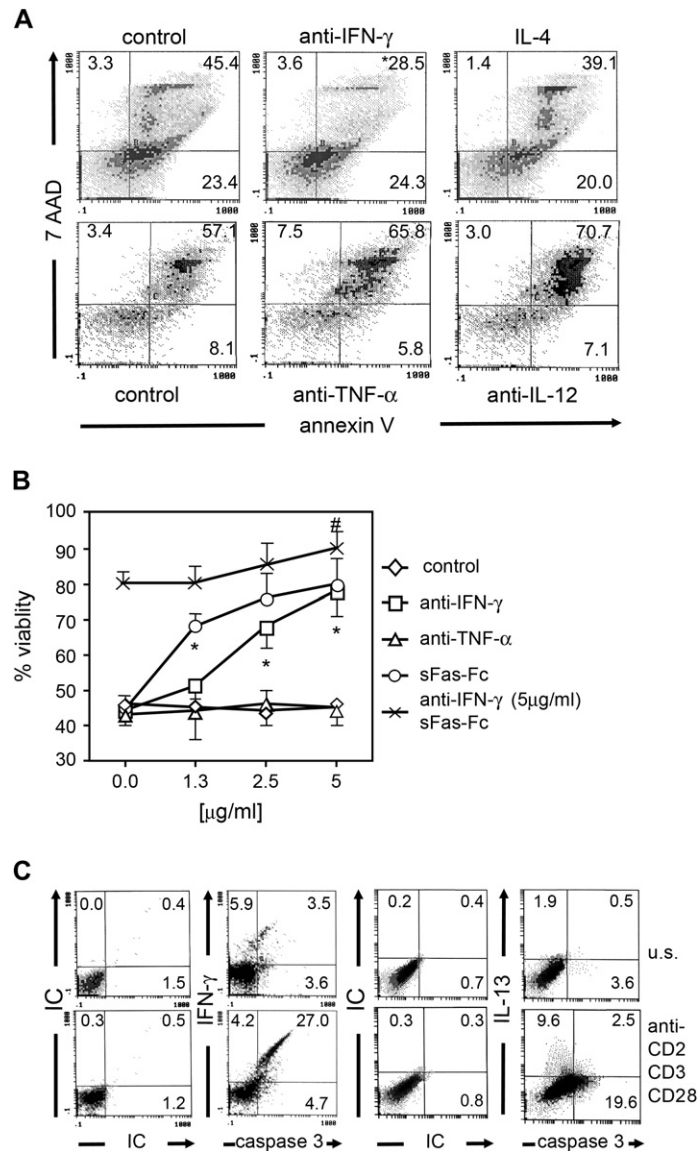


FIG 5. IFN- γ plays an essential role in TH1 cell AICD in atopic diseases. **A**, TH1 cells from atopic donors were cultured with anti-IFN- γ , anti-TNF- α , anti-IL-12, or IL-4 and stimulated with mAb against CD2, CD3, and CD28 for 4 days. **B**, TH1 cells from atopic donors were stimulated with anti-CD2/CD3/CD28 mAbs in the presence of different doses of anti-IFN- γ , anti-TNF- α , soluble Fas-Fc (sFas-Fc), the combination of 5 μ g/mL anti-IFN- γ and titrated amounts of sFas-Fc, and isotype control mAb (* P < .001 for the sum of early and late apoptotic cells compared with nonneutralizing conditions; # P < .05 compared to without sFas-Fc). **C**, IFN- γ -expressing but not IL-13-expressing T cells show active caspase-3 expression. Data represent 1 of 3 independent experiments. IC, Isotype control; u.s., unstimulated.

inflammation. T-cell AICD characterizes the death of a fraction of antigen-activated T lymphocytes to avoid accumulation of harmful cytokine-releasing cells. Similarly, keratinocyte apoptosis is a major tissue injury mechanism in AD, which leads to spongiosis in the epidermis and has been demonstrated to be related to AICD of keratinocytes. By IFN- γ stimulation, MHC class I and II molecules; cytokines such as IL-1, TNF, and GM-CSF^{31,32}; and chemokines such as IFN- γ inducible protein-10, monokine induced by IFN- γ , and IFN- γ inducible α chemoattractant are strongly up-regulated in keratinocytes.³³ However, as in activated T cells, these keratinocytes upregulate Fas expression, which is induced by IFN- γ , and become susceptible to apoptosis.³⁰ These studies

suggest it is not only T cells but also epithelial cells that become more prone to AICD in atopic diseases.

Interaction of Fas-ligand with Fas leads to the activation of cysteine proteases of the caspase family. This oligomerization triggers the activation of caspase-8 and caspase-3.⁹ It has been demonstrated that caspase-3 expression levels are selectively up-regulated in effector T cells, a population susceptible to AICD, which is dependent on early signaling events mediated by the TCR.³⁴ In agreement with these findings, we showed that high IFN- γ -expressing T cells also upregulate active caspase-3, in contrast with IL-13-expressing cells. IFN- γ can upregulate caspase-1, caspase-3, caspase-4, caspase-7, caspase-8, and

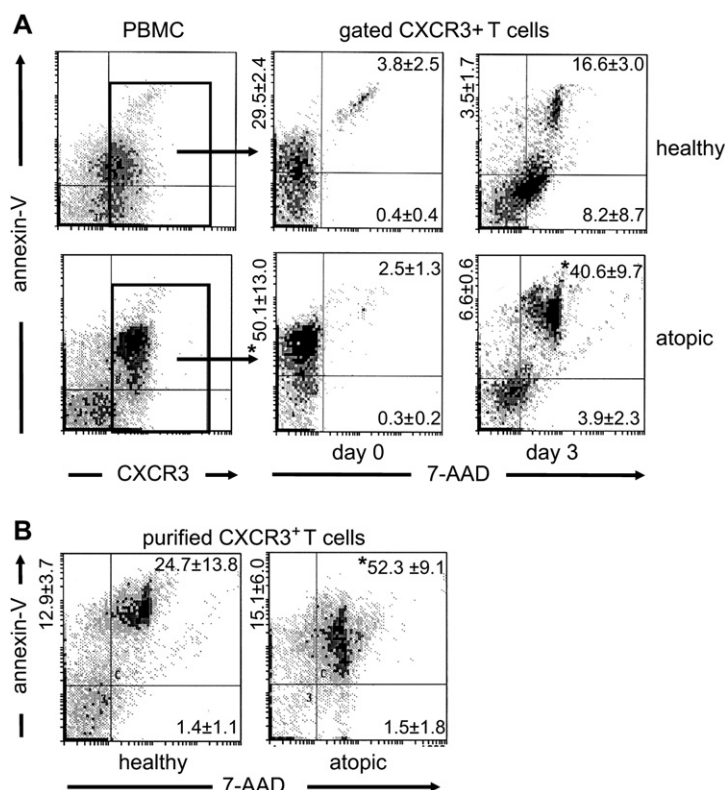


FIG 6. Increased apoptosis of CXCR3⁺ T cells in atopic dermatitis. **A**, PBMCs of patients with AD and healthy donors are labeled with antihuman CXCR3-phycoerythrin mAb. Apoptotic cells are measured immediately after purification and on day 3 (**P* < .05). **B**, CXCR3⁺ T cells are purified with antiphycoerythrin microbeads after phycoerythrin labeling of CXCR3⁺ T cells from PBMCs. Unstimulated cultures are analyzed on day 3 (**P* < .05).

caspase-10 expression, suggesting a role for this cytokine in the induction of caspase gene expression.³⁵ Particularly the early and high IFN- γ -expressing T cells showed significantly upregulated Fas and Fas-ligand expression, and neutralization experiments revealed the role of both IFN- γ and Fas-ligand in T_H1 cell apoptosis in atopy. IFN- γ itself does not induce apoptosis because its receptor does not contain death domains. Apparently, it sensitizes the T_H1 cells to Fas-Fas-ligand-mediated suicide in an autocrine fashion. IFN- γ induces an increase in Fas and Fas-ligand expression by allergen-stimulated CD4⁺ T cells in patients with asthma and inhibits allergen-specific CD4⁺ T-cell proliferation by inducing apoptosis, whereas no change was observed in nonatopic control subjects.³⁶ It was also hypothesized that T_H2 bias and decreased IFN- γ production could be a result of dissociation between the levels of apoptosis within the T_H1 and T_H2 cells in the bronchial tissue. IFN- γ ⁺ but not IL-4⁺ T cells in the asthmatic biopsies showed significantly higher proportions of apoptotic cells compared with the control group.³⁷ These 2 studies support our hypothesis that the T_H2 imbalance in atopy results from apoptosis of allergen-activated IFN- γ -producing cells. In addition, we have previously demonstrated that circulating CLA-bearing CD45RO⁺ T cells with skin-specific homing property represent an activated memory/effector T-cell subset.³⁸ 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. The apoptosis of circulating memory/effector T cells was confined to atopic individuals, whereas patients with nonatopic conditions such as

psoriasis, intrinsic-type asthma, contact dermatitis, intrinsic type AD, and bee venom allergy, and healthy controls did not show any evidence for enhanced T-cell apoptosis *in vivo*.⁸

Although a general immunodeficiency to infections is not characteristic for AD, there is increased propensity toward disseminated infections with herpes simplex or vaccinia virus. Susceptibility to severe viral infections such as eczema herpeticum or eczema vaccinatum may be linked to severity of atopy.³⁹ In addition, smallpox vaccination is contraindicated in patients with AD unless there is imminent danger of exposure to smallpox.⁴⁰ One of the intriguing questions remaining to be addressed is why severe immunodeficiency does not develop as a result of preferential apoptosis of T_H1 cells in AD. Prevention of T-cell apoptosis in the tissues maybe one of the reasons because, after migration to skin, T cells are protected from apoptosis mainly by survival signals from extracellular matrix proteins and cytokines such as IL-2, IL-4, and IL-15.⁸

It must be noted here that allergic inflammation is characterized by a chronic ongoing T and B memory cell response, which is aggravated by allergen exposure. At the time of diagnosis, the sensitization phase has already been completed, and patients have mounted a predominant T_H2 and IgE response against relevant allergens. Accordingly, models that envisage responsible nuclear factors for naive T-cell differentiation may not fully explain the continuous T_H2 response in atopy. This study suggests a novel mechanism in T_H2-cell predominance by demonstrating an increased AICD of particularly high IFN- γ -producing T_H1 cells in atopic individuals.

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Clinical implications: Our findings offer an insight into a novel mechanism for T_H2-cell predominance in atopic disease.

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ANTIBODIES AND REAGENTS

Human IL-2, IL-4, and anti-IL-4 were provided by Novartis (Basel, Switzerland). IL-12 and anti-IL-12 were from Pharmingen (San Diego, Calif). Neutralizing anti-TNF- α mAb was from R&D Systems Inc (Abingdon, United Kingdom). Anti-IFN- γ was from Novartis (Basel, Switzerland). Soluble (s) Fas-Fc was from Alexis Corp (San Diego, Calif). Ethidium bromide was from Sigma Chemical Co (St Louis, Mo). Isotype matching control antibodies were mouse IgG₁ and mouse IgG_{2a} (Immunotech AG, Marseilles, France). For detection of apoptosis in flow cytometry, phycoerythrin-conjugated annexin-V and 7AAD from Pharmingen were used. Anti-CD2, anti-CD3, and anti-CD28 mAbs were purchased from CLB (Amsterdam, The Netherlands). Fluorescein isothiocyanate-labeled anti-Fas (CD95) was from Immunotech AG. Phycoerythrin-labeled anti-Fas-ligand (CD178) was from Ancell (Byport, Minn). Fluorescein isothiocyanate-labeled mouse antihuman TNFR-I and phycoerythrin-labeled TNFR-II were from Pharmingen. Antihuman CXCR3-phycoerythrin mAb was from R&D Systems Inc. Antiphycoerythrin magnetic microbeads for MACS were purchased from Miltenyi Biotec (Bergisch Gladbach, Germany).

DETECTION OF CYTOKINES

For intracytoplasmic cytokine detection, T cells were stimulated with 50 ng/mL phorbol 12-myristate 13-acetate and 500 ng/mL Ca₂⁺ ionophore (ionomycin) for 4 hours in the presence of 10 μ g/mL brefeldin (Sigma Chemical Co). The cells were permeabilized with cytofix/cytoperm solution (PharMingen AG); fixed with paraformaldehyde 2%; stained with fluorescein isothiocyanate-labeled IFN- γ , phycoerythrin-labeled active caspase-3, and PE- or fluorescein isothiocyanate-conjugated isotype control antibodies; and analyzed by flow cytometry.

PURIFICATION OF IFN- γ -SECRETING AND IL-4-SECRETING T CELLS

PBMCs were isolated by Ficoll (Biochrom) density gradient centrifugation of peripheral venous blood, and cells were washed 3 times and resuspended in supplemental RPMI 1640 medium. Cells 2.5×10^7 were stimulated with anti-CD2/3/28 mAbs in

5 mL medium in 6-well plates in duplicates. After 16 hours of stimulation in humidified 5% CO₂, cells were harvested and labeled with 50 μ g/mL either anti-IFN- γ Ab or anti-IL-4 Ab (Miltenyi Biotec)²² for 10 minutes at a concentration of 10^8 cells/mL in ice-cold RPMI 1640 medium. The cells were diluted with 37°C warm medium to a final concentration of 10^6 cells/mL and allowed to secrete and capture the respective cytokines for 45 minutes at 37°C. After capturing the secreted cytokines on their surface, cells were centrifuged at 300g for 5 minutes at 4°C and resuspended at a concentration of 10^8 cells/mL in ice-cold buffer containing 0.5% BSA and 5 mmol/L EDTA in PBS. The cells were stained with either 5 μ g/mL phycoerythrin-conjugated anti-IFN- γ or anti-IL-4 for 10 minutes at 4°C. The cells were washed and resuspended in 400 μ L BSA-EDTA PBS and magnetically labeled for 15 minutes at 4°C and resuspended with 100 μ L antiphycoerythrin microbeads. After washing, labeled cells were purified by immunomagnetic separation (AutoMacs; Miltenyi Biotec). IFN- γ -secreting or IL-4-secreting and nonsecreting CD4⁺ T cells were analyzed by flow cytometry before enrichment and after enrichment. The purity of IFN- γ ⁺ T cells and IL-4⁺ T cells was more than 82% in all experiments.

T_H1 AND T_H2 CELL DIFFERENTIATION FROM CD45RA⁺ T CELLS

T_H1 cells were differentiated from naive CD45RA⁺ T cells (1×10^5) in 48-well flat bottom plate in 500 μ L complete RPMI medium supplemented with IL-2 (20 ng/mL), IL-12 (15 ng/mL), and a combination of anti-CD2 (clones: 4B2 and 6G4, each 0.5 μ g/mL), anti-CD3 (clone: CRL 8001, 0.5 μ g/mL), and anti-CD28 (clone: 15E8, 0.5 μ g/mL) mAbs and anti-IL-4 mAb (10 μ g/mL). T_H2 cells were differentiated in the presence of IL-2 (20 ng/mL), IL-4 (25 ng/mL), anti-CD2, anti-CD3, and anti-CD28 mAb and anti-IL-12 (2 μ g/mL). Cytokine production by T_H1 and T_H2 cells revealed distinct profiles. T_H1 cells 10^6 /mL secreted 14.2 ng/mL IFN- γ , <0.1 ng/mL IL-4, <0.1 ng/mL IL-5, and <0.3 ng/mL IL-13. T_H2 cells secreted 3.7 ng/mL IL-4, 9.62 ng/mL IL-5, 12.2 ng/mL IL-13, and <0.1 ng/mL IFN- γ by 10 μ g/mL plate-bound anti-CD3 mAb stimulation in 48 hours (mean of 2 cell purifications).