

# Expansion of Cytokine-producing CD4<sup>-</sup>CD8<sup>-</sup> T Cells Associated with Abnormal Fas Expression and Hypereosinophilia

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## Summary

The mechanisms of sustained overproduction of eosinophils in the idiopathic hypereosinophilic syndrome and in some human immunodeficiency virus (HIV)-1-infected individuals are largely unknown. We hypothesized that T cells may release soluble products that regulate eosinophilia in these patients, as has been previously shown in bronchial asthma. We identified one patient with idiopathic hypereosinophilic syndrome and one HIV-1-infected individual with associated hypereosinophilia who demonstrated high numbers of CD4<sup>-</sup>CD8<sup>-</sup> T cells in peripheral blood. CD4<sup>-</sup>CD8<sup>-</sup> T cells from both patients, although highly activated, did not express functional Fas receptors. In one case, the lack of functional Fas receptors was associated with failure of Fas mRNA and protein expression, and in another, expression of a soluble form of the Fas molecule that may have antagonized normal signaling of Fas ligand. In contrast to the recently described lymphoproliferative/autoimmune syndrome, which is characterized by accumulation of CD4<sup>-</sup>CD8<sup>-</sup> T cells and mutations within the Fas gene, this study suggests somatic variations in Fas expression and function quite late in life. Both genetic and somatic abnormalities in regulation of the Fas gene are therefore associated with failures to undergo T cell apoptosis. Furthermore, the expanded population of CD4<sup>-</sup>CD8<sup>-</sup> T cells from both patients elaborated cytokines with antiapoptotic properties for eosinophils, indicating a major role of these T cells in the development of eosinophilia. Thus, this study demonstrates a sequential dysregulation of apoptosis in different cell types.

Expansion of CD4<sup>-</sup>CD8<sup>-</sup> T cells has been observed in patients with a syndrome characterized by hypergammaglobulinemia, lymphocytosis, hepatosplenomegaly, lymphadenopathy, and autoimmunity (1). In two recent reports, mutations within the Fas gene were associated with this disorder (2, 3). Fas is a cell-surface protein that plays a major role in induction of apoptosis in lymphoid cells. Therefore, mutations within the Fas gene result in defects of Fas-induced apoptosis (2, 3). Moreover, the clinical, immunological, and molecular features of this human lymphoproliferative syndrome are reminiscent of those observed in *lpr/lpr* mice (1–3).

Eosinophilic granulocytes are proinflammatory cells that are primarily involved in immune defense against parasites. Furthermore, they play a major role in late-type inflammatory reactions, and are prominent in many chronic inflammatory diseases, particularly in allergic manifestations such as bronchial asthma and atopic dermatitis (4, 5). It has been

shown that these diseases can be associated with an increase of activated T cells that regulate eosinophilia by the release of cytokines such as IL-3, IL-5, and GM-CSF (6–8). Among T cells, the CD4<sup>+</sup> cells have been considered as major producers of eosinophil survival factors in human bronchial asthma (9) and in animal models (10). In contrast, the pathogenesis of eosinophilia in the idiopathic hypereosinophilic syndrome (11) and in HIV-infected individuals (12) is largely unknown.

In this article, we describe the accumulation of CD4<sup>-</sup>CD8<sup>-</sup> T cells due to abnormal Fas expression in two patients with hypereosinophilia, suggesting that these cells can not undergo apoptosis via the Fas pathway. In contrast to the recently published work where expansion of CD4<sup>-</sup>CD8<sup>-</sup> T cells was associated with mutations of the Fas gene (2, 3), the results of our study suggest that somatic variations in Fas expression and function may also lead to defects in Fas-induced apoptosis. The consequences of such

failure to regulate T cell apoptosis are the overproduction of regulatory cytokines for eosinophil growth and survival resulting in chronic inflammation.

## Materials and Methods

**Patients.** Patient 1 was a 64-yr-old man with a 20-yr history of idiopathic hypereosinophilic syndrome. The laboratory findings included marked eosinophilia ( $4,420/\text{mm}^3$ ) in the blood, liver, and bone marrow. Because of fluctuating symptoms and eosinophilia, ongoing treatment with steroids was required. After a 10-yr course of the disease, diarrhea became increasingly prominent. Gastrointestinal biopsies revealed a severe eosinophilic gastroenteritis. As a result of chronic gastroenteritis, the patient developed hypogammaglobulinemia. Absolute and relative B cell numbers were normal and autoantibody production was not observed. 20 yr after onset of initial symptoms, the patient died of a nonocclusive small bowel infarction with peritonitis. Studies were done using peripheral blood taken 2–4 mo before the patient's death. At this time, the patient had a peripheral blood eosinophilia of 11–14%, despite treatment with 50 mg of prednisone. Immunohistochemistry and genotypic studies were performed on both small intestine and colon specimens that were obtained after the patient's death.

Patient 2 was an HIV-1-infected, 37-yr-old man with recurrent skin lesions, oral candidiasis, and hypereosinophilia ( $3,690/\text{mm}^3$ ). The patient had a high serum IgE level (4.00 kU/liter), but normal levels for IgG, IgM, and IgA. Absolute and relative B cell numbers were normal, and autoantibodies were not detected. The patient did not suffer from allergic diseases or atopic dermatitis, nor did he show any documented inflammatory skin lesions before his seroconversion to HIV-1. He also had no detectable parasitic infection. Studies were done using blood taken before and after a 100-mg prednisone treatment that lasted 2 d. Despite this treatment, the patient maintained a peripheral blood eosinophilia of 41%.

**Collection of Blood and Bronchoalveolar Lavage.** Heparin anticoagulated blood (100 ml) was collected between 7:30 and 8:00 a.m. Bronchoalveolar lavage (BAL)<sup>1</sup> was performed according to the technical recommendation and guidelines of the Task Group on BAL of the European Society of Pneumology. Briefly,  $3 \times 50$  ml of 0.9% NaCl was instilled in the middle lobe, immediately aspirated into plastic tubes using a vacuum suction system, and kept at 4°C.

**Immunofluorescence Analysis.** To determine T lymphocyte subsets, cells were incubated in different combinations with FITC- or PE-conjugated mAbs against CD3 (clone UCHT1, this antibody reacts with human CD3 $\epsilon$ ; Dako, Zurich, Switzerland), CD4, CD8, CD16, and CD19. Purified CD4<sup>+</sup>CD8<sup>-</sup> T cells were analyzed with mAbs against TCR- $\alpha/\beta$  (Pan-TCR- $\alpha/\beta$ ) and TCR- $\gamma/\delta$  (TCR- $\delta$ 1) (both from T Cell Diagnostics, Cambridge, MA).

To analyze T cell activation, cells were incubated with FITC- or PE-conjugated mAbs against CD4, CD8, CD25 (IL-2R  $\alpha$  chain), and HLA-DR. The distribution of CD45 isoforms was assessed on T cells by incubation with PE-conjugated anti-CD45RA (naive T cells) and CD45RO (memory T cells, both from Dako).

The usage of different V $\beta$  elements of TCR was measured with the following FITC-conjugated mAbs: anti-V $\beta$ 2 (Immunotech, Marseille, France), anti-V $\beta$ 5(a), anti-V $\beta$ 5(b), anti-V $\beta$ 5(c),

anti-V $\beta$ 6, anti-V $\beta$ 8, anti-V $\beta$ 12 (all from T Cell Diagnostics), and anti-V $\beta$ 19 (Immunotech).

To determine protein expression of the Fas molecule, two anti-Fas mAbs (IgM from Biomol, Hamburg, Germany) and IgG3 (a kind gift from Dr. P.H. Krammer, German Cancer Research Center, Heidelberg, Germany) were used. For both mAbs, cells were incubated at 4°C for 1 h. Since these mAb were not conjugated, cells were additionally incubated with PE-conjugated goat anti-mouse Ab (Tago-Inotech, Dottikon, Switzerland) at 4°C for 30 min.

Unless stated otherwise, all other mAbs were from Coulter (Instrumentation Laboratory, Schlieren, Switzerland). All incubations were performed with saturating concentrations of mAb according to the manufacturer's instructions. Cells were analyzed using a cytofluorograph (Epics Profile or Epics XL; Coulter, Hialeah, FL). The number of immunofluorescence-positive cells was determined in 5,000 analyzed cells. Specific binding of mAb was controlled by subtraction of isotype-matched control mAbs.

**Immunocytochemistry.** Cryopreserved postmortem colon sections from patient 1 were fixed in acetone for 5 min at -20°C, air-dried, and blocked for 20 min with PBS containing 10% goat serum. Adjacent sections were exposed with mAbs to anti-CD2, anti-CD3, anti-CD4, anti-CD5, anti-CD8 (all from Dako), anti-TCR- $\alpha/\beta$  ( $\beta$ F1), and anti-TCR- $\gamma/\delta$  (TCR $\delta$ 1; both from T Cell Diagnostics), for 30 min, washed extensively in PBS, and subsequently incubated with peroxidase-conjugated anti-mouse IgG diluted 1:80 in PBS containing 2% goat serum and 10% human serum. Diaminobenzidine was used as a substrate, and Mayer's hematoxylin was used as counterstain. Frozen tonsil sections served as positive controls.

**Cell Purifications.** T cells were isolated from blood as previously described (13). Briefly, PBMC were obtained by Ficoll-Hypaque (Seromed-Fakola AG, Basel, Switzerland) density gradient centrifugation. To purify the CD4<sup>+</sup>CD8<sup>-</sup> T subpopulation, cells were incubated with anti-CD14, anti-CD16, anti-CD19, anti-CD4, and anti-CD8 mAbs (Immunotech). Cells were washed twice with PBS/0.5% BSA, and were incubated with goat anti-mouse IgG magnetic microbeads (Miltenyi Biotec, Bergisch-Gladbach, Germany) for 15 min at 10°C. T cells were then negatively selected by an immunomagnetic procedure incorporating the MACS system (Miltenyi Biotec).

To obtain *in vivo*-activated T cells for functional Fas-mediated apoptosis assays, one patient with hypersensitivity pneumonitis was selected. A sample of BAL fluid of this patient was filtered through a 70- $\mu\text{m}$  nylon mesh (Falcon, Basel, Switzerland). Cells were centrifuged at 500 *g* for 10 min and resuspended in PBS. Cytologic examination of the BAL fluid was done after cytocentrifugation and staining with May-Gruenwald-Giemsa. The BAL fluid of this patient contained 80% lymphocytes. To further enrich the lymphocyte fraction, cells were centrifuged over a Percoll density gradient (Pharmacia Biotech, Dübendorf, Switzerland). The resulting cell pellet consisted of 96% pure CD3<sup>+</sup> T cells, as assessed by flow cytometry.

Eosinophil purification was performed as previously described (13–15).

**Cell Cultures and Cell Lines.** To prepare cell supernatants, PBMC, purified CD4<sup>+</sup> T cells, and purified CD4<sup>+</sup>CD8<sup>-</sup> T cells were cultured at  $2 \times 10^6$  cells/200  $\mu\text{l}$  in 96-well plates (Falcon). In some experiments, cells were stimulated with 10  $\mu\text{g}/\text{ml}$  PHA (Boehringer Mannheim, Rotkreuz, Switzerland). After incubation for 24 h at 37°C in 5% CO<sub>2</sub> in a fully humidified atmosphere, the supernatants were harvested and frozen at -80°C until analysis.

<sup>1</sup>Abbreviations used in this paper: BAL, bronchoalveolar lavage; nt, nucleotide.

To obtain *in vitro*-activated T cells, PBMC from control individuals were incubated with anti-CD3 mAb (0.5 µg/ml OKT 3; American Type Culture Collection, Rockville, MD) for 48 h and cultured as described above.

For comparisons with *in vivo*- and *in vitro*-activated non-transformed T cells, the human T cell line Jurkat was used. Jurkat cells were maintained by serial passage in RPMI 1640 containing 10% FCS (complete medium), and were used in their logarithmic growth phase.

**RNA Isolation and cDNA Synthesis.** Using an RNA purification kit (Stratagene, Heidelberg, Germany), RNA was isolated from  $5 \times 10^6$  lymphocytes (PBMC or purified T cell subsets) or from  $2 \times 10^7$  purified eosinophils derived from the patient with hypereosinophilic syndrome. First-strand cDNA synthesis was performed using total lymphocyte or eosinophil RNA, random hexadeoxynucleotides at 0.2 µg/µl as primers, and Moloney murine leukemia virus reverse transcriptase (Pharmacia) as previously described (15, 16).

**CD8α, CD8β, CD3γ, and Fas mRNA Expression.** mRNA expression was detected by a PCR technique. Primers for CD8α amplification were obtained from Clontech (LucernaChem, Lucerne, Switzerland). Primers for CD8β (5'-CTCCATGGCAACTCAGTCCTCC-3' and 5'-CCTGAGGTAACCGGCACACTCTCTTC-3'), CD3γ (5'-GGGGAAGGGCCTGGCTGTCC-3' and 5'-CCCAACAGCAAGGACGAAAATGC-3'), and for Fas (5'-GTGGGATCCCACCTCGGAGGATTGCTCAACAACC-3' and 5'-GTGCTGCAGTATGTTGGCTCTTCAGCGCTATA-3') amplifications were synthesized according to previously published sequences (17–19). After amplification, 8 µl of PCR product was run on 1% agarose gel and stained by ethidium bromide.

**DNA Sequencing.** PCR products after Fas amplification were subcloned into pCR™ II (Invitrogen, San Diego, CA). The plasmid DNA was sequenced directly by dideoxy chain termination using the Sequenase sequencing method (U.S. Biochemical Corp., Cleveland, OH).

**Analysis of Fas-mediated Cell Death.** To determine the cytolytic effect of anti-Fas mAb (Biomol), purified T cell subsets from the two patients with eosinophilia, resting and *in vitro*-activated non-transformed T cells from normal individuals, *in vivo*-activated BAL T cells from a patient with hypersensitivity pneumonitis, and Jurkat cells were cultured in the presence of 0.5 µg/ml anti-Fas mAb for 18 h. Cell viability was assessed by flow cytometry (14).

**Bioassay to Determine Soluble Fas Activity.**  $10^6$ /ml Jurkat cells were cultured in complete culture medium with 0.5 µg/ml anti-Fas mAb (Biomol) in the presence of supernatants (final dilution = 1:4) derived from PBMC and purified T cell subsets of both patients for 18 h. In addition, supernatants from normal human resting and PHA-activated PBMC were used as comparisons. Cell viability was assessed by flow cytometry (14).

**Analysis of TCR Gene Rearrangement Patterns.** Original protocols to analyze TCR-β gene rearrangements by Southern blotting were used (20). Nitrocellulose filters (Schleicher & Schuell, Dassel, Germany) were hybridized with a digoxigenin-labeled TCR-β1 cDNA probe (21). Color detection was done by using a digoxigenin nucleic acid detection kit (Boehringer Mannheim).

**Bioassay to Determine Eosinophil Survival Factors.** The determination of eosinophil viability was performed by flow cytometry as previously described (14).

**Cytokine Protein Expression.** The expression of cytokines that prolong eosinophil viability *in vitro*, IL-3, IL-5, GM-CSF, and IFN-γ, was measured in culture supernatants from PBMC and purified T cells by ELISA (22).

**Cytokine mRNA Expression.** To determine cytokine gene ex-

pression in PBMC, T cell subsets, and eosinophils, a PCR technique using oligonucleotides that recognize specific sequences within cytokine genes was used (15). In addition, β-actin amplification was performed to control the quality of cDNA samples. Primers for the following cytokines were used: IL-2, IL-3, IL-4, IL-5, IL-6, TNF-α, IFN-γ, and GM-CSF (all from Clontech Laboratories, Palo Alto, CA). All primers have been designed to span an intron within the cytokine genes. Amplification of any residual genomic DNA would therefore appear as an unexpected, larger PCR product. After amplification, 8 µl of each PCR product was run on 1% agarose gel and transferred to a nitrocellulose filter (Schleicher & Schuell). Radioactive probes were made using random priming (Oligonucleotide Labeling Kit; Pharmacia) from the appropriate cytokine cDNA, and the filters were hybridized with <sup>32</sup>P-labeled cytokine probes.

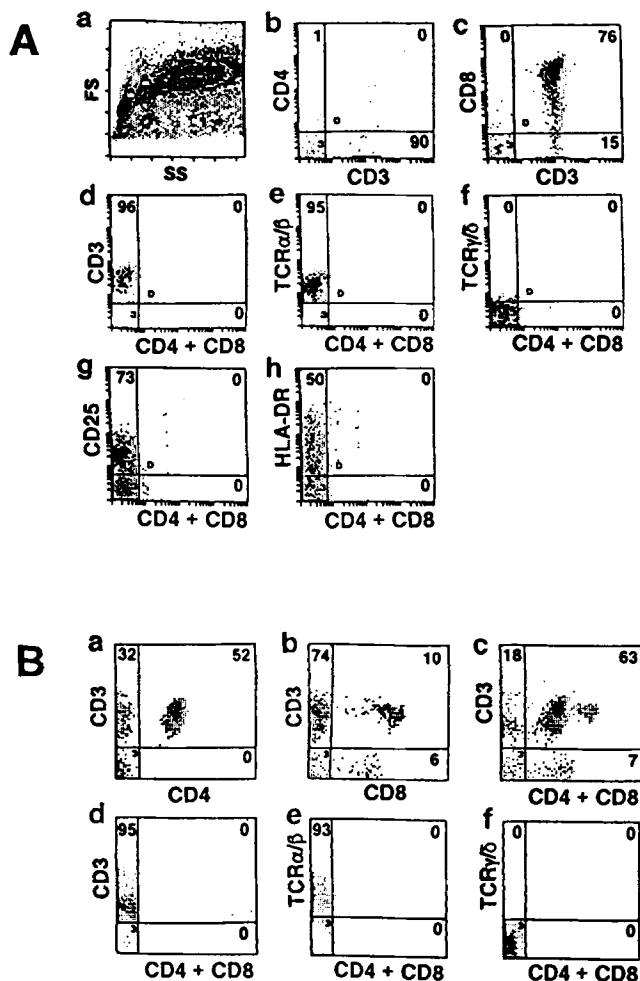
## Results and Discussion

**Identification of CD4<sup>+</sup>CD8<sup>-</sup> T Cells in Blood and Tissue.** Most mature T cells express either CD4 or CD8 molecules on their surface. In two patients with high blood eosinophilia, however, we observed high numbers of CD4<sup>+</sup>CD8<sup>-</sup> T cells (15 and 18% within the lymphocyte population) in blood (Fig. 1 A, b and c, and Bc). These T cells were TCR-α/β<sup>+</sup> and TCR-δ/γ<sup>-</sup> (Fig. 1 A, e and f, and B, e and f). In addition, microscopic evaluation of purified cells indicated a lymphocytic morphology (data not shown). Studies on normal blood cells from 50 donors revealed that the CD4<sup>+</sup>CD8<sup>-</sup> T subpopulation usually does not exceed 3% within the lymphocyte population (data not shown). In addition, using the postmortem tissue of one patient, we showed the presence of these cells in colon tissue. The tissue CD4<sup>+</sup>CD8<sup>-</sup> T cells were CD3<sup>+</sup> (Fig. 2 A) and TCRβ<sup>+</sup> (Fig. 2 E), but CD5<sup>-</sup> (Fig. 2 C) and TCRδ<sup>-</sup> (Fig. 2 F).

**In Vivo Clonality of CD4<sup>+</sup>CD8<sup>-</sup> T Cells.** We have attempted to identify the origin of the patient's CD4<sup>+</sup>CD8<sup>-</sup> T cell populations. Since purified CD4<sup>+</sup>CD8<sup>-</sup> T cells from both patients did not express CD16 (data not shown), it is unlikely that they represent so-called large granular lymphocytes (23, 24). In addition, since these cells were TCRβ<sup>+</sup> and TCRδ<sup>-</sup> (Figs. 1 and 2), it was concluded that they did not belong to the γ/δ<sup>+</sup> CD4<sup>+</sup>CD8<sup>-</sup> T cell subset (25–27).

It has been recently shown in mice that CD8<sup>+</sup> T cells develop into CD4<sup>+</sup>CD8<sup>-</sup> T cells in the presence of IL-4 (28). In this *in vitro* system, IL-4 downregulates the expression of CD8α, but not of CD8β (28). We therefore investigated the expression of CD8α and CD8β by purified CD4<sup>+</sup>CD8<sup>-</sup> T cells using PCR and specific primers for both genes. As shown in Fig. 3, CD4<sup>+</sup>CD8<sup>-</sup> T cells derived from patients 1 and 2 did not express CD8α or CD8β, suggesting that they are distinct from those observed in the murine *in vitro* model. Furthermore, CD4<sup>+</sup>CD8<sup>-</sup> cells from both patients expressed mRNA for CD3γ confirming that they indeed represented T cells (Fig. 3).

To investigate whether the increased numbers of CD4<sup>+</sup>CD8<sup>-</sup> T cells may be the result of a monoclonal T cell expansion, DNA was extracted from the highly T cell-infil-



**Figure 1.** Identification of CD4<sup>+</sup>CD8<sup>-</sup> T cells in peripheral blood. (A) HIV-1-infected individual (patient 2). (a) Dot plot of forward vs side light scatter of peripheral blood cells by flow cytometry showing "gated" lymphocytes that were later analyzed by two-color immunofluorescence. (b and c) Two-color immunofluorescence analysis of blood cells. No CD3<sup>+</sup>CD4<sup>+</sup> T cells were observed. 15% of the lymphocyte fraction contained CD3<sup>+</sup>CD4<sup>-</sup>CD8<sup>-</sup> T cells. (d-h) Two-color immunofluorescence analysis of purified CD4<sup>-</sup>CD8<sup>-</sup> T cells. Cells were purified by negative selection. CD4<sup>-</sup>CD8<sup>-</sup> T cells were TCRα/β<sup>+</sup> and TCRγ/δ<sup>-</sup>. Furthermore, they were highly activated as they expressed high levels of CD25 and HLA-DR. (B) Patient with idiopathic hypereosinophilic syndrome (patient 1). (a-c) Two-color immunofluorescence analysis of blood cells. 18% of the lymphocyte fraction contained CD3<sup>+</sup>CD4<sup>-</sup>CD8<sup>-</sup> T cells. (d-f) Two-color immunofluorescence analysis of purified CD4<sup>-</sup>CD8<sup>-</sup> T cells. Cells were purified by negative selection. CD4<sup>-</sup>CD8<sup>-</sup> T cells were TCRα/β<sup>+</sup> and TCRγ/δ<sup>-</sup>. Numbers at the upper right corner of each region indicate the percentage of positively stained cells.

trated colon of patient 1, and Southern blot analysis was performed using a probe of a constant region of the β chain of the TCR gene. Fig. 4 A demonstrates a band(s) in addition to the germline bands that indicates a pattern of clonal rearrangement. Thus, CD4<sup>-</sup>CD8<sup>-</sup> T cells from patient 1 were indeed monoclonal. In another case of idiopathic hypereosinophilic syndrome, a monoclonal expansion of CD4<sup>+</sup>CD3<sup>-</sup> lymphocytes has been observed (29). Thus, monoclonal proliferation of T cell subsets may frequently occur in this syndrome.

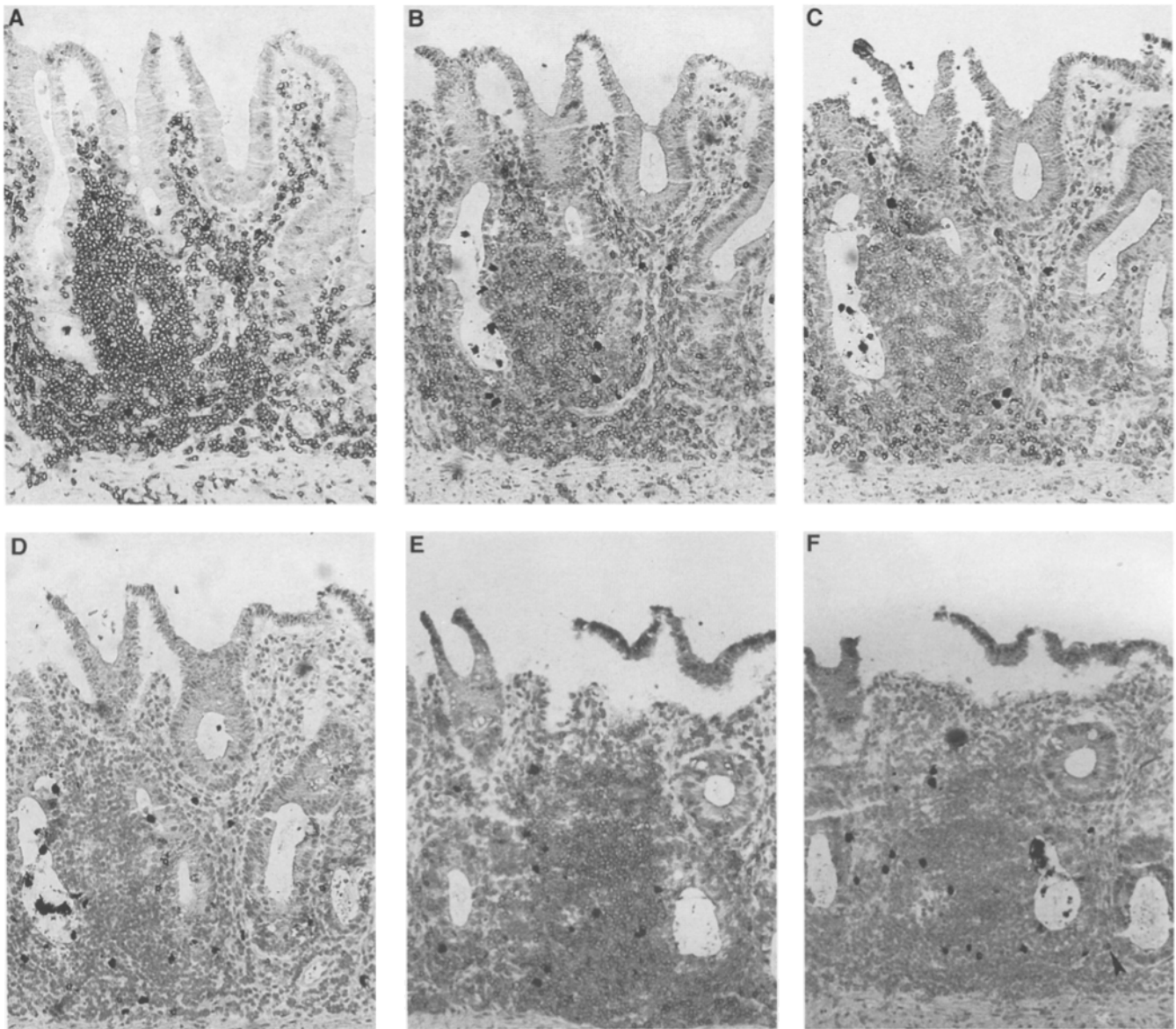
In the HIV-1-infected individual, TCR Vβ expression was determined in CD4<sup>-</sup>CD8<sup>-</sup> T cells and total lymphocytes using specific mAb and flow cytometry. As shown in Fig. 4 B, the Vβ repertoire of nonpurified T cells was heterogeneously diverse. In contrast, none of the mAbs used in this study stained purified CD4<sup>-</sup>CD8<sup>-</sup> T cells. These results suggest a limited number of TCR gene rearrangements compatible with the presence of an oligoclonal CD4<sup>-</sup>CD8<sup>-</sup> T cell subset in patient 2.

**Functional Fas Molecules Are Not Expressed by CD4<sup>-</sup>CD8<sup>-</sup> T Cells.** Next, we investigated the mechanism of CD4<sup>-</sup>CD8<sup>-</sup> T cell expansion. The Fas antigen, a 43-kD membrane protein and a member of the TNF receptor family, was originally defined by its capacity to induce apoptosis either by stimulation with agonistic mAb (30-32) or by its natural ligand (33). Mutations of the Fas gene are associated with a massive accumulation of CD4<sup>-</sup>CD8<sup>-</sup> T cells in association with autoimmune disease in humans (2, 3) and mice (34). We therefore considered Fas as a candidate responsible for increased numbers of CD4<sup>-</sup>CD8<sup>-</sup> T cells observed in patients 1 and 2.

Cell death of activated mature T cells can be triggered by treatment with anti-Fas mAb (32). Therefore, we first examined whether purified CD4<sup>-</sup>CD8<sup>-</sup> T cells express functional Fas receptors. While ~25% of in vitro- or in vivo-activated nontransformed or Jurkat T cells died after anti-Fas mAb exposure, anti-Fas mAb did not induce cell death in CD4<sup>-</sup>CD8<sup>-</sup> T cells from patients 1 and 2 (Fig. 5). The failure of CD4<sup>-</sup>CD8<sup>-</sup> T cells to die was not because of a lack of activation, since the surface expression of the activation markers CD25 and HLA-DR were similar compared to activated, control T cells (Fig. 1 A, g and h, and data not shown). Rather, these results suggested defective Fas expression by CD4<sup>-</sup>CD8<sup>-</sup> T cells from both patients.

Cell-surface analysis revealed that the Fas receptor was not expressed by CD4<sup>-</sup>CD8<sup>-</sup> T cells from patient 2 (Fig. 6 A). This is intriguing, since these T cells expressed the CD45RO<sup>+</sup> memory phenotype (data not shown) and should therefore demonstrate high surface staining by anti-Fas mAb (35). CD8<sup>+</sup> T cells expressed high Fas levels and served as a positive control in this experiment (Fig. 6 A). To confirm our data obtained from cell surface protein expression, we investigated Fas mRNA expression by PCR. The Fas mRNA transcript was amplified using oligonucleotide primers starting at nucleotide (nt) 170 (5' untranslated region) and ending at nt 1336 (3' untranslated region) of the published Fas cDNA sequence (19, 36). The amplified product had the expected size of 1,167 bp in PBMC from patient 2 (mostly CD8<sup>+</sup> T cells) which expressed Fas (Fig. 6 B). In contrast, the CD4<sup>-</sup>CD8<sup>-</sup> T cells did not express detectable amounts of Fas mRNA (Fig. 6 B), being consistent with the absence of Fas protein (Fig. 6 A).

A defect in Fas expression observed in *lpr* mutant mice causes lymphadenopathy (34). In addition, an association between mutations within the Fas gene and lymphadenopathy was recently observed in children (2, 3). This is probably caused by the inability of Fas-deficient lymphocytes to undergo apoptosis via the Fas pathway. Similarly, we ob-

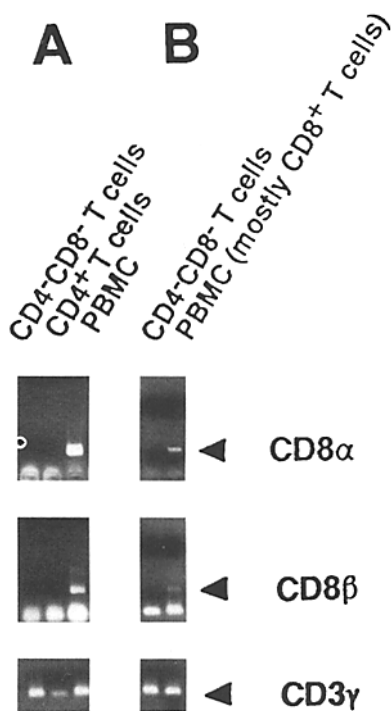


**Figure 2.** Identification of CD4<sup>-</sup>CD8<sup>-</sup> T cells in tissue. Cryopreserved postmortem colon sections of patient 1 were stained with the following mAbs: anti-CD3 (A), anti-CD4 (B), anti-CD5 (C), anti-CD8 (D), anti-TCRβ (E), and anti-TCRδ (F). Infiltrating T cells were primarily CD3<sup>+</sup>TCRβ<sup>+</sup>, but CD4<sup>-</sup>CD5<sup>-</sup>CD8<sup>-</sup>TCRδ<sup>-</sup>.

served an expansion of CD4<sup>-</sup>CD8<sup>-</sup> T cells that lacked Fas mRNA and protein expression in an adult HIV-1-infected individual. This patient, however, did not demonstrate hypergammaglobulinemia or any sign of autoimmunity, a finding that stands in contrast to these murine and human disease models. In addition, the numbers of CD4<sup>-</sup>CD8<sup>-</sup> T cells in peripheral blood was less compared to that of children with the lymphoproliferative syndrome (1–3). Moreover, PCR amplifications were performed using genomic DNA from PBMC of patient 2 and intron primers that amplify exons 3, 7, and 9 of the human Fas gene (3). Sequence analysis of the PCR products revealed no abnormalities within the Fas gene (data not shown). These data exclude the possibility of any known germline mutation within the

Fas gene (3), but suggest instead that Fas abnormalities in this HIV-infected individual could have arisen from somatic variations in gene regulation, since CD4<sup>-</sup>CD8<sup>-</sup> T cells expressed no Fas while CD8<sup>+</sup> T cells expressed normal levels of this protein.

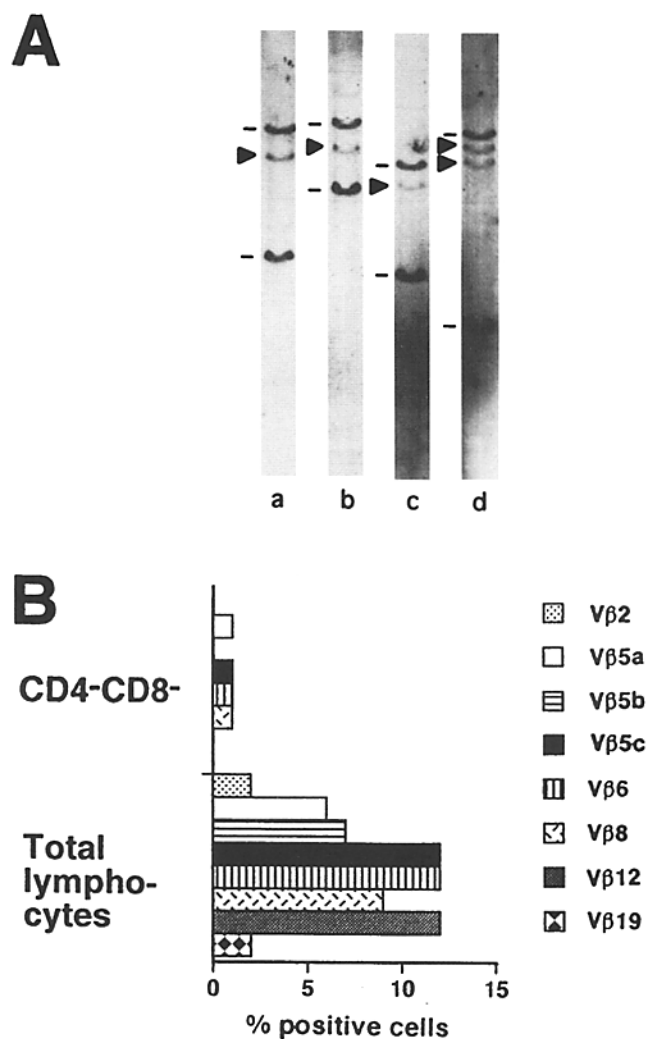
In contrast to the data observed in patient 2, the purified CD4<sup>-</sup>CD8<sup>-</sup> T cells of patient 1 showed high expression of the Fas protein (Fig. 7 A) and mRNA (Fig. 7 B). However, in addition to the expected 1,167-bp PCR product, a smaller product of ~1,100 bp was seen in CD4<sup>-</sup>CD8<sup>-</sup> T cells, but not in CD4<sup>+</sup> T cells of that patient or in PHA-activated PBMC from a normal control individual (Fig. 7 B). The structure of the Fas PCR products of CD4<sup>-</sup>CD8<sup>-</sup> T cells from patient 1 was analyzed by DNA sequencing,



**Figure 3.** CD8 mRNA expression by CD4<sup>-</sup>CD8<sup>-</sup> T cells assessed by PCR. Purified CD4<sup>-</sup>CD8<sup>-</sup> T cells from patients 1 and 2 did not express mRNA for CD8 $\alpha$  or CD8 $\beta$ . PBMC from both patients, as well as purified CD4<sup>+</sup> T cells from patient 1, served as controls. CD3 $\gamma$  expression confirmed that CD4<sup>-</sup>CD8<sup>-</sup> cells were T cells.

and revealed that the larger PCR product was indeed the anticipated Fas cDNA fragment. The smaller PCR product began with an identical nt sequence, but diverged at nt 700 (Fig. 7 C). Further sequence analysis showed that the smaller PCR product represented a Fas molecule with a 63-bp deletion resulting in a predicted protein that lacks 21 amino acid residues corresponding mostly to the transmembrane domain of Fas.

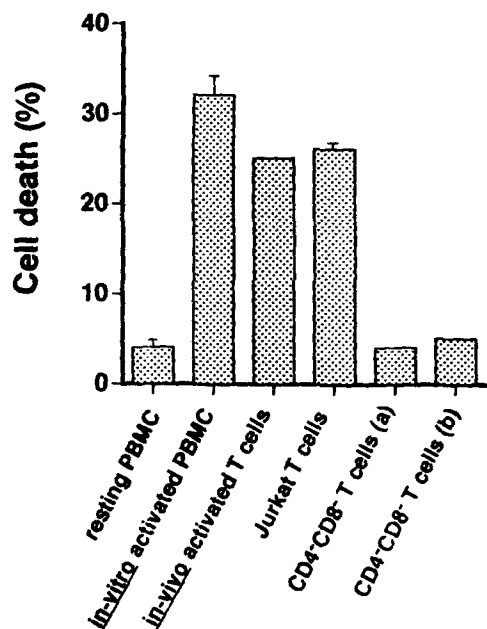
The existence of such a molecule was recently described and characterized as a soluble, secreted form of Fas resulting from alternative splicing (19, 37). Supernatants from cells transfected with this alternative splice form of Fas could block anti-Fas-induced apoptosis (19, 37). We therefore analyzed whether the supernatant of purified CD4<sup>-</sup>CD8<sup>-</sup> T cells from patient 1 contained soluble Fas activity. Jurkat cells were cultured with antibody to Fas in the presence of supernatants derived from PBMC and different T cell subsets of both patients. As shown in Fig. 7 D, supernatant of CD4<sup>-</sup>CD8<sup>-</sup>, but not of CD4<sup>+</sup> T cells from patient 1 or of CD4<sup>-</sup>CD8<sup>-</sup> T cells from patient 2, decreased by twofold the anti-Fas mAb-mediated death of the Jurkat T cells. In addition, other T cell supernatants, including those of PHA-activated PBMC from normal individuals, did not block anti-Fas-induced Jurkat cell death. These results suggest that CD4<sup>-</sup>CD8<sup>-</sup> T cells from patient 1 indeed produced soluble Fas protein, which neutralized the anti-Fas antibody. It has been reported that PHA-activated PBMC



**Figure 4.** Monoclonal expansion of CD4<sup>-</sup>CD8<sup>-</sup> T cells in vivo. (A) Southern blot analysis of genes coding for the  $\beta$  chain of the TCR. DNA of colon tissue from patient 1 was digested with EcoRI (a), EcoRV (b), HindIII (c), and XbaI (d) restriction endonucleases. A probe for the constant region of the  $\beta$  chain of the TCR gene was used. The arrows indicate the positions of the rearranged alleles. The results indicate clear evidence for monoclonal T cell expansion. (B) Analysis of TCR V $\beta$  gene expression by CD4<sup>-</sup>CD8<sup>-</sup> T cells assessed by mAbs and flow cytometry. None of the mAbs that were used stained purified CD4<sup>-</sup>CD8<sup>-</sup> T cells from patient 2. In contrast, total lymphocytes demonstrate a normal distribution of V $\beta$  gene usage.

from normal individuals may also express mRNA for the apoptosis-inhibiting form of Fas (37), but the amounts synthesized by these cells were not enough to have significant effects in the Jurkat system. Taken together, our data make it likely that soluble Fas prevented the CD4<sup>-</sup>CD8<sup>-</sup> T cells of patient 1 from undergoing Fas ligand-induced apoptosis.

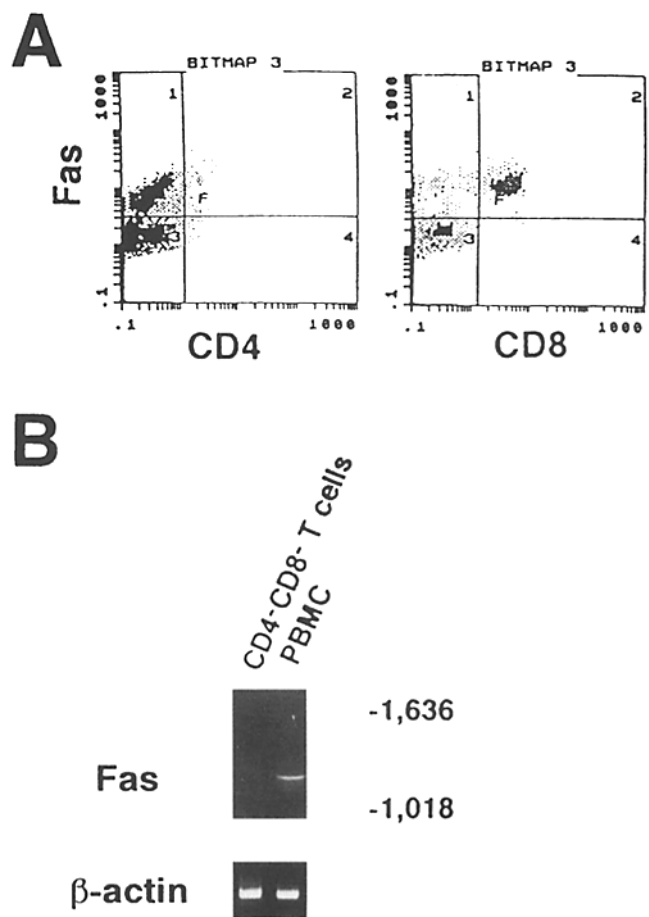
The programmed cell death of TCR-activated mature T cells probably serves to terminate an immune response. Recent work provides evidence that autocrine stimulation of Fas is essential for TCR-induced death of transformed T cells and activated PBMC in vitro (38-41). Our data suggest that activated T cells expressing abnormal Fas avoid



**Figure 5.** Inhibition of Fas-induced cell death in CD4<sup>-</sup>CD8<sup>-</sup> T cells in vitro. Anti-Fas mAb induced significant cell death in vivo- and in vitro-activated T cells, as well as in Jurkat T cells after an 18-h cell culture. In contrast, anti-Fas mAb failed to induce cell death in highly activated ex vivo CD4<sup>-</sup>CD8<sup>-</sup> T cells derived from patient 1 (a) or patient 2 (b). Cell viability before and after 18-h culture without anti-Fas mAb ranged between 95 and 98%. Data on PBMC and Jurkat represent mean values and standard deviations of three independent experiments. Data on in vivo-activated and -purified CD4<sup>-</sup>CD8<sup>-</sup> T cells are from one experiment that was performed in duplicate, which revealed identical results.

cell death in vivo, and therefore strongly support the hypothesis for a critical role of Fas-Fas ligand molecular interactions to preserve peripheral T cell homeostasis (38–41). Thus, the unusual CD4<sup>-</sup>CD8<sup>-</sup> T cells accumulating in the two patients may represent previously activated peripheral T cells that failed to undergo apoptosis at the end of an immune response. Moreover, our data show that in two different human diseases, Fas expression is critical for the normal function of the immune system.

**Production of Eosinophil Survival Factors by CD4<sup>-</sup>CD8<sup>-</sup> T Cells.** Since both patients of this study demonstrated increased numbers of CD4<sup>-</sup>CD8<sup>-</sup> T cells and eosinophils, we investigated whether these T cells may contribute to the eosinophilia observed in vivo. As shown in Fig. 8, PHA-activated, but not resting PBMC from control individuals, secrete eosinophil survival factors. In contrast, the resting PBMC from both patients produced soluble products that prolonged eosinophil survival in vitro. Addition of PHA to these cells did not significantly enhance the secretion of eosinophil survival factors. To determine whether the double negative T cell subset may produce eosinophil survival factors as observed previously in CD4<sup>+</sup> and CD8<sup>+</sup> T cell subsets derived from patients with bronchial asthma (6), CD4<sup>-</sup>CD8<sup>-</sup> T cells were purified by negative selection (Fig. 1, *Ad* and *Bd*). Purified CD4<sup>-</sup>CD8<sup>-</sup> T cells from both patients constitutively secreted factors that extended

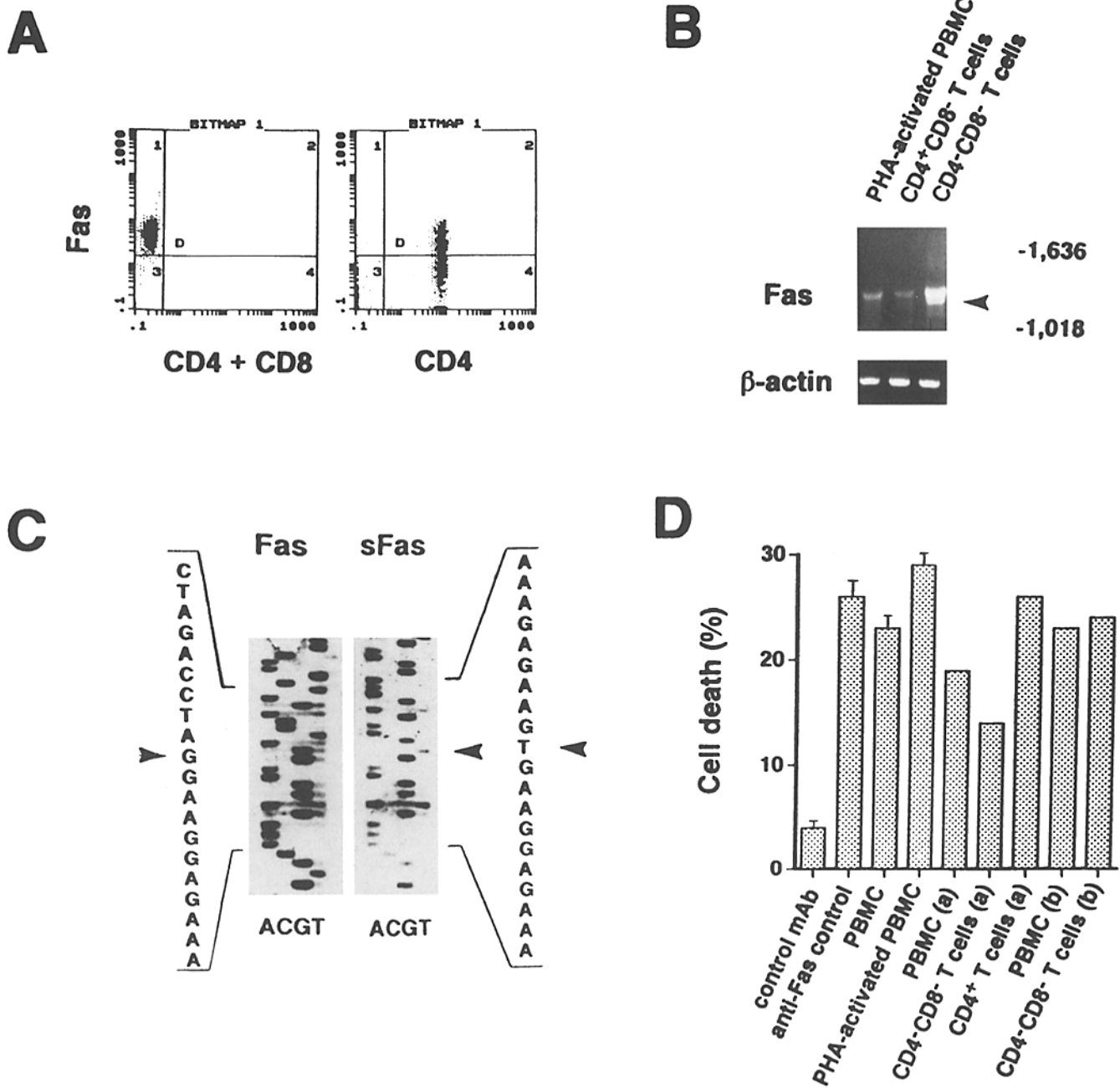


**Figure 6.** Fas gene expression by CD4<sup>-</sup>CD8<sup>-</sup> T cells from patient 2. (A) Two-color immunofluorescence analysis to determine Fas protein expression in lymphocytes. CD8<sup>+</sup>, but not CD4<sup>-</sup>CD8<sup>-</sup> T cells, expressed Fas surface protein. (B) mRNA expression assessed by PCR. Purified CD4<sup>-</sup>CD8<sup>-</sup> T cells did not express detectable Fas mRNA. In contrast, PBMC of this patient (primarily CD8<sup>+</sup> T cells, see Fig. 1 *A*, *b* and *c*) expressed Fas mRNA. Units are in basepairs. As a control, amplification of the β-actin gene was performed from each template.

the survival of eosinophils in vitro (Fig. 8). CD4<sup>-</sup>CD8<sup>-</sup> T cells derived from patient 1 generated even more eosinophil survival factors than purified CD4<sup>+</sup> T cells cultured under the same conditions. In addition, the supernatant of PBMC (primarily CD8<sup>+</sup> T cells, Fig. 1 *A*, *b* and *c*) from patient 2 also contained eosinophil survival factors, supporting previously published work that CD8<sup>+</sup> T cells have an active role in the regulation of the immune response (42).

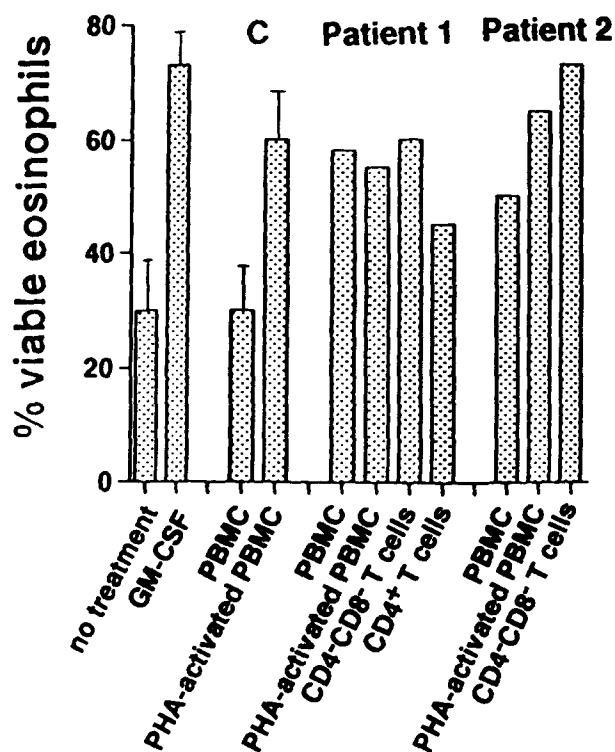
To determine which cytokines prolong the survival of eosinophils in culture, we tested multiple recombinant cytokines. IL-3, IL-5, GM-CSF, and IFN-γ are cytokines that inhibited eosinophil death in vitro. Other cytokines such as IL-1, IL-2, IL-4, IL-6, IL-8, IL-10, IL-13, TNF-α, TNF-β, and TGF-β had no effect on eosinophil viability (data not shown).

Since IL-3, IL-5, GM-CSF, and IFN-γ are able to prolong eosinophil viability in vitro, we measured the concentration of these cytokines in supernatants of purified CD4<sup>-</sup>CD8<sup>-</sup> T cells by ELISA and compared them with other T cell



**Figure 7.** Fas gene expression by CD4<sup>-</sup>CD8<sup>-</sup> T cells from patient 1. (A) Two-color immunofluorescence analysis to determine Fas protein expression in purified CD4<sup>-</sup>CD8<sup>-</sup> and CD4<sup>+</sup> T cells. All CD4<sup>-</sup>CD8<sup>-</sup>, but not all CD4<sup>+</sup> T cells express Fas surface protein. (B) mRNA expression assessed by PCR. Purified CD4<sup>-</sup>CD8<sup>-</sup> T cells express high levels of Fas mRNA. In addition, a distinct smaller PCR product of ~1,100 bp was observed in CD4<sup>-</sup>CD8<sup>-</sup> T cells, which was not expressed in PHA-activated PBMC from normal donors nor in the CD4<sup>+</sup> T cell subset. As a control, amplification of the  $\beta$ -actin gene was performed from each template. (C) Sequence analysis of Fas PCR products. The smaller PCR product observed in CD4<sup>-</sup>CD8<sup>-</sup> T cells represents a soluble form of Fas that lacks the hydrophobic transmembrane domain. This PCR product started with an identical nt sequence, but began to differ at nt 700 (arrow). (D) Soluble Fas activity in supernatants from purified T cells and PBMC of patient 1 (a) and patient 2 (b) compared to supernatants from resting and PHA-activated PBMC of three normal individuals. Jurkat cells were cultured in the presence of supernatant and anti-Fas mAb. Supernatant from CD4<sup>-</sup>CD8<sup>-</sup> T cells of patient 1 inhibited ~50% of the Jurkat cell death induced by anti-Fas mAb. In contrast, supernatant from CD4<sup>+</sup> T cells of the same patient did not contain detectable soluble Fas activity. In addition, supernatants from CD4<sup>-</sup>CD8<sup>-</sup> T cells and PBMC of patient 2 or resting and PHA-activated PBMC of normal individuals did not contain detectable soluble Fas activity. Data on normal PBMC and control experiments (without supernatants) represent mean values and standard deviations of three independent experiments. Data on PBMC and purified T cells from both patients are from one out of two experiments that showed identical results.





**Figure 8.** Effects of nonstimulated or stimulated T cell supernatants on eosinophil survival. PHA-activated, but not resting PBMC from control individuals, produce eosinophil survival factors ( $n = 6$ ). In contrast, supernatants from resting PBMC and purified CD4<sup>-</sup>CD8<sup>-</sup> T cells derived from both hyper-eosinophilic patients prolong eosinophil viability. Data on controls (no treatment, GM-CSF, supernatants from resting and PHA-activated PBMC) represent mean values and standard deviations of six independent experiments. Data on supernatants from PBMC and purified T cells of both patients are from one experiment that was performed in duplicate, which revealed identical results.

populations from the same patient, as well as from control individuals. As shown in Table 1, activated, but not resting PBMC from normal control individuals secrete significant amounts of IL-3, IL-5, GM-CSF, and IFN- $\gamma$ . In contrast,

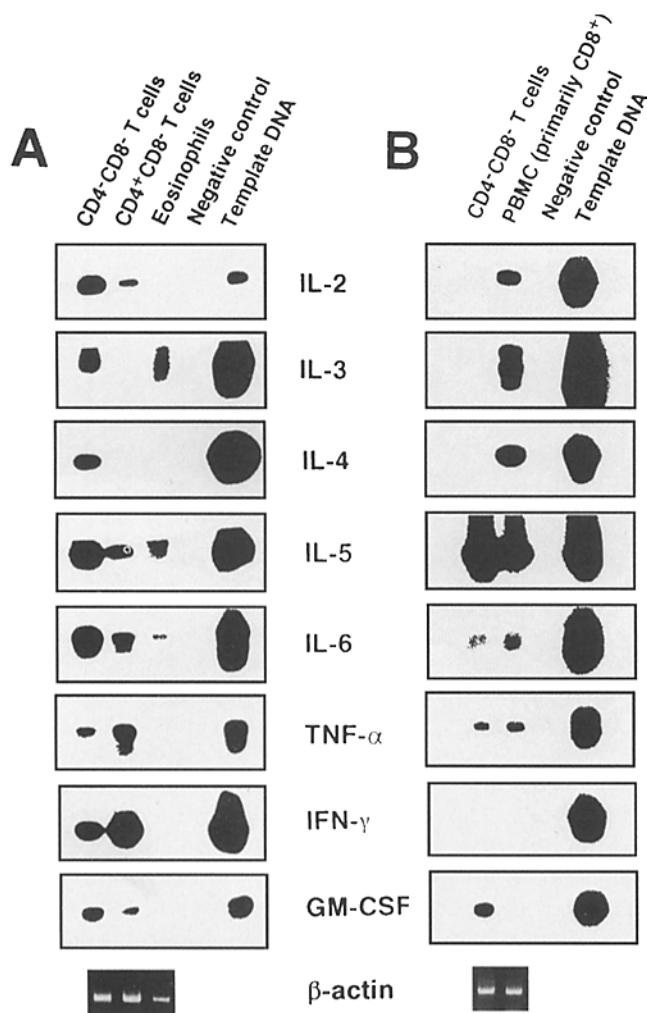
the resting PBMC from both patients released significant amounts of IL-3 and IL-5, but not IFN- $\gamma$  or GM-CSF (Table 1). This suggests that these PBMC were previously activated in vivo. Patient 2 released much higher amounts of IL-5 compared to patient 1, consistent with the higher degree of peripheral blood eosinophilia at the time of the study. In addition, the capacity of PHA-activated PBMC to secrete IL-5 seemed to be much higher in patient 1 compared to normal control individuals. The purified CD4<sup>-</sup>CD8<sup>-</sup> T cells from patient 1, in the absence of any stimulating agent, released significant amounts of IL-3 and IL-5, but no detectable amounts of IFN- $\gamma$  or GM-CSF (Table 1). In contrast, purified CD4<sup>+</sup> T cells from the same patient produced high concentrations of IFN- $\gamma$ , but not IL-3, IL-5, or GM-CSF (Table 1). These results suggest that both CD4<sup>-</sup>CD8<sup>-</sup> and CD4<sup>+</sup> T cell subsets of patient 1 contributed to the eosinophilia observed in vivo, however, by producing different cytokines. Furthermore, PBMC (primarily CD8<sup>+</sup> T cells, see Fig. 1 A, b and c) and the purified CD4<sup>-</sup>CD8<sup>-</sup> T cells from patient 2 spontaneously produced high amounts of IL-5, whereas levels of IL-3, GM-CSF, and IFN- $\gamma$  were low or undetectable (Table 1).

Taken together, these results demonstrate that the CD4<sup>-</sup>CD8<sup>-</sup> T cells derived from the patient with hyper-eosinophilic syndrome and from the HIV-1-infected individual secreted a cytokine profile that is characteristic for Th2 cells (43). Moreover, they are in agreement with recent findings describing the establishment and cytokine profile of CD4<sup>-</sup>CD8<sup>-</sup> T cell clones derived from HIV-1-infected individuals (44). Our results also support the notion that IL-5 plays a role in the pathogenesis of eosinophilia in human disease (9, 45, 46) and in animal models (47).

**Cytokine mRNA Expression by CD4<sup>-</sup>CD8<sup>-</sup> T Cells and Eosinophils.** Since purified T cell subsets in both patients secreted, at least partially, differing cytokine profiles, we next examined the expression of these cytokines at the RNA level using the reverse transcriptase PCR technique. As shown in Fig. 9 A, CD4<sup>-</sup>CD8<sup>-</sup> T cells from patient 1

**Table 1.** Cytokine Production by CD4<sup>-</sup>CD8<sup>-</sup> T Cells Compared to Other T Cell Subsets

|   | IL-3 | IL-5  | GM-CSF | IFN- $\gamma$ |
|---|------|-------|--------|---------------|
|   |      | pg/ml |        |               |
| <b>Patient 1</b>                          |      |       |        |               |
| PBMC                                      | 11   | 15    | 8      | <100          |
| PBMC + PHA                                | 29   | 669   | 299    | 260           |
| CD4 <sup>-</sup> CD8 <sup>-</sup> T cells | 34   | 26    | <5     | <100          |
| CD4 <sup>+</sup> CD8 <sup>-</sup> T cells | <5   | <5    | <5     | 318           |
| <b>Patient 2</b>                          |      |       |        |               |
| PBMC (primarily CD8 <sup>+</sup> )        | 25   | 145   | <5     | <5            |
| CD4 <sup>-</sup> CD8 <sup>-</sup> T cells | <5   | 156   | <5     | <5            |
| <b>Control individuals (n = 6)</b>        |      |       |        |               |
| PBMC                                      | <5   | <5    | 10     | <100          |
| PBMC + PHA                                | 47   | 77    | 283    | 459           |



**Figure 9.** Cytokine mRNA expression by CD4<sup>-</sup>CD8<sup>-</sup> T cells assessed by PCR. (A) Patient with idiopathic hyper eosinophilic syndrome (patient 1). Purified CD4<sup>-</sup>CD8<sup>-</sup> T cells expressed high levels of mRNA for the Th2 cytokines IL-4, IL-5, and IL-6. However, they also expressed all other investigated cytokine genes. The CD4<sup>+</sup> T cell subset demonstrated high level mRNA expression for the Th 1 cytokines IFN- $\gamma$  and TNF- $\alpha$ . (B) HIV-1-infected individual (patient 2). Purified CD4<sup>-</sup>CD8<sup>-</sup> T cells showed a prominent IL-5 mRNA expression. The CD8<sup>+</sup> T cell subset produced mRNA for Th 1 and Th 2 cytokines. Only CD8<sup>+</sup>, but not CD4<sup>-</sup>CD8<sup>-</sup> T cells, expressed IL-4 and may therefore account for high IgE levels. Neither CD4<sup>-</sup>CD8<sup>-</sup> nor CD8<sup>+</sup> T cells expressed mRNA for IFN- $\gamma$ .

expressed more IL-3 and IL-5 mRNA than CD4<sup>+</sup> T cells from the same patient. In contrast, CD4<sup>+</sup> T cells may express more IFN- $\gamma$  mRNA. Furthermore, CD4<sup>-</sup>CD8<sup>-</sup> T cells derived from patient 1 expressed IL-2 mRNA. Thus, at this level of sensitivity, we demonstrated that CD4<sup>-</sup>CD8<sup>-</sup> T cells can express, besides Th2 (IL-4, IL-5, and IL-6), also some Th1 (IL-2, IFN- $\gamma$ , and TNF- $\alpha$ ) cytokines. These data

are in agreement with the view that categorizing for only two types of T cell responses may be an oversimplification (48, 49).

The CD4<sup>-</sup>CD8<sup>-</sup> T cells from patient 2 showed a more restricted spectrum of cytokine gene expression (Fig. 9 B). The most prominent cytokine mRNA was IL-5. In addition, the PBMC population of this patient (primarily CD8<sup>+</sup> T cells) also demonstrated high levels of IL-5 mRNA in addition to IL-3 and IL-4, and low levels of IL-2, IL-6 and TNF- $\alpha$  mRNA (Fig. 9 B). Thus, both CD4<sup>-</sup>CD8<sup>-</sup> and CD8<sup>+</sup> T cells from patient 2 are likely to cause eosinophilia. In contrast, only CD8<sup>+</sup>, and not CD4<sup>-</sup>CD8<sup>-</sup>, T cells are a source of IL-4 mRNA that may stimulate IgE production in this HIV-infected patient. CD8<sup>+</sup> T cells were recently associated with high IgE production in patients with progression toward AIDS (44, 50). Interestingly, the highly activated T cells of this HIV-1-infected individual did not express mRNA for IFN- $\gamma$ . These data are in agreement with the hypothesis that progression to AIDS is characterized by loss of IFN- $\gamma$  and IL-2 production concomitant with increases in IL-4 (12).

Taken together, the results on mRNA expression of cytokines with eosinophil antiapoptotic properties are consistent with the ELISA results (Table 1). Moreover, at the level of PCR sensitivity, we demonstrated that CD4<sup>-</sup>CD8<sup>-</sup> T cells from both patients expressed some mRNA for GM-CSF, even though the protein was not detectable using the ELISA technique (Table 1).

We also examined cytokine gene expression by eosinophils derived from patient 1. It is known that eosinophils have the capacity to produce and release certain cytokines (15). This suggests that the eosinophil is not only an effector cell at the end of an immunological cascade, but can also actively participate in a cytokine network regulating the immune response. As shown in Fig. 9 A, eosinophils from patient 1 expressed mRNA for IL-3, IL-5, and IL-6, but not for IL-2, IL-4, TNF- $\alpha$ , IFN- $\gamma$ , or GM-CSF. The expression of IL-3, IL-5, and IL-6 by eosinophils is in agreement with previously published work (51–53).

In summary, we have demonstrated that blood and tissue eosinophilia can be associated with increased numbers of CD4<sup>-</sup>CD8<sup>-</sup> T cells. The expansion of CD4<sup>-</sup>CD8<sup>-</sup> T cells may occur in vivo as a result of somatic variations in Fas expression and function occurring quite late in life. Failure to express Fas or expression of a soluble form of Fas were associated with inhibition of apoptosis in these T cells. Moreover, activated T cells that do not undergo apoptosis are potentially dangerous. For example, CD4<sup>-</sup>CD8<sup>-</sup> T cells expressed cytokines promoting eosinophil survival and may therefore have inhibited eosinophil apoptosis in vivo (8). Thus, our findings further point to the importance of dysregulation of programmed cell death in pathogenic processes (54).

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