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J Clin Invest. 1997;100(10):2622-2633. <https://doi.org/10.1172/JCI119806>.

Research Article

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Interleukin-10 Promotes Activation-induced Cell Death of SLE Lymphocytes Mediated by Fas Ligand

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Abstract

Immune function in SLE is paradoxically characterized by active T cell help for autoantibody production, along with impaired T cell proliferative and cytokine responses in vitro. To reconcile these observations, we investigated the possibility that the accelerated spontaneous cell death of SLE lymphocytes in vitro is caused by an activation-induced cell death process initiated in vivo. 27 SLE patients, three patients with systemic vasculitis, seven patients with arthritis, and 14 healthy subjects were studied. Patients with clinically active SLE or systemic vasculitis had accelerated spontaneous death of PBMC with features of apoptosis at day 5 of culture. A prominent role for IL-10 in the induction of apoptosis was observed, as neutralizing anti-IL-10 mAb markedly reduced cell death in the active SLE patients by 50%, from $22.3 \pm 5.2\%$ to $11.2 \pm 2.8\%$, and the addition of IL-10 decreased viability in the active SLE group, but not in the control group, by 38%. In addition, apoptosis was shown to be actively induced through the Fas pathway. The potential clinical relevance of T cell apoptosis in active SLE is supported by the correlation of increased apoptosis and IL-10 levels in vitro with low lymphocyte counts in vivo. We conclude that the spontaneous cell death observed in vitro in lymphocytes from patients with SLE and other systemic autoimmune disorders results from in vivo T cell activation, is actively induced by IL-10 and Fas ligand, and reflects pathophysiologically important events in vivo. Activation-induced cell death in vivo provides a pathogenic link between the aberrant T helper cell activation and impaired T cell function that are characteristic features of the immune system of patients with SLE. (*J. Clin. Invest.* 1997; 100: 2622–2633.) Key words: systemic lupus erythematosus • apoptosis • activation-induced cell death • interleukin-10 • Fas ligand

Introduction

SLE is the prototype systemic autoimmune disease, characterized by the generation of autoantibodies specific for components of intracellular nucleic acid–protein complexes (1, 2).

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Received for publication 7 April 1997 and accepted in revised form 17 September 1997.

J. Clin. Invest.

© The American Society for Clinical Investigation, Inc.
0021-9738/97/11/2622/12 \$2.00
Volume 100, Number 10, November 1997, 2622–2633
<http://www.jci.org>

The pathogenic lupus autoantibodies are mostly of the IgG isotype, and are thought to be antigen driven. While the character of these autoantibodies suggests a requirement for T helper cells, only recently have autoantigen-specific, CD4-positive T cells been demonstrated in these patients (2, 3). Many studies indicate chronic and ongoing triggering of SLE T cells in vivo, as evidenced by increased expression of activation markers such as Fas, CD45 isoforms, and adhesion molecules (4–7), as well as by increased secretion of the cytokines, IL-6 and IL-10 (8–13). We and others have also presented data consistent with augmented T helper cell activity in SLE. CD40 ligand (CD40L), a T cell transmembrane molecule responsible for cognate help to B cells and other CD40-positive target cells, is constitutively expressed on lymphocytes from some patients with active SLE and is prolonged in its expression after activation of lupus T cells in vitro (14, 15).

In contrast to these reports of active T helper cells in SLE, the majority of studies of T cell function in vitro describe impaired cellular immune activation (6, 7). It has been difficult to reconcile the evidence for active SLE T helper cell function with impaired proliferative and cytotoxic function in vitro, as well as impaired antigen-specific immune responses and increased susceptibility to infection in vivo (7, 16, 17).

Previous in vitro studies by Emlen et al. (18) identified an increase in spontaneous apoptosis of lymphocytes from patients with SLE. Mechanisms that might account for this spontaneous cell death include withdrawal of growth enhancing cytokines in in vitro culture systems (19), or an active form of cell death resulting from lymphocyte activation in vivo (20, 21). Most evidence to date indicates that the Fas and TNF α pathways are effectors of activation-induced cell death (AICD; 22, 23). We have investigated the mechanism of in vitro lymphocyte death in patients with SLE. Our results confirm an increase in spontaneous cell death reported previously (18), and show that IL-10, present at increased levels in active SLE, promotes T cell apoptosis through the Fas pathway.

Methods

Human subjects. Study subjects included patients with SLE ($n = 27$): 10 Hispanics, 9 Caucasians, 8 African Americans (age 38 ± 9.5 , range 19–61; 24 females and 3 males); systemic vasculitis ($n = 3$, 2 with Wegener's granulomatosis, and 1 with Takayasu's arteritis); disease control patients with arthritis (DC; $n = 7$; 4 with rheumatoid arthritis [RA], 1 with osteoarthritis, 1 with hepatitis C arthritis, and 1 with reactive arthritis secondary to chronic myelogenous leukemia); and healthy control subjects (NC; $n = 14$). All patients met the American

Abbreviations used in this paper: AICD, activation-induced cell death; APC, antigen presenting cell; DC, disease control; NC, healthy control subjects; PI, propidium iodide; RA, rheumatoid arthritis; SLEDAI, SLE disease activity index; TB, trypan blue; TCC, total cell counts; TCR, T cell receptor.

College of Rheumatology (ACR) diagnostic criteria for their diseases. SLE activity was assessed by a disease activity index (SLE-DAI) (24), and medical therapy, absolute lymphocyte, and neutrophil counts were recorded.

Cell separation. After obtaining informed consent, blood was drawn from patients and healthy control subjects by venipuncture, and PBMC were immediately isolated by Ficoll-Hypaque gradient centrifugation, washed three times with PBS, and resuspended in RPMI 1640 containing 2 mM glutamine, 100 U/ml penicillin, 0.1 mg/ml streptomycin (GIBCO BRL, Gaithersburg, MD), and 10% FCS (Gemini Bioproducts, Calabasas, CA).

Culture conditions. PBMC were incubated at a concentration of 1×10^6 cells/ml at 37°C, 5% CO₂ for 5 d. We chose to study unfractionated PBMC, rather than isolated mononuclear cell subpopulations, as we wanted to evaluate cell death that reflected interactions among populations and to detect cytokine effects that acted either directly or indirectly on the cells comprising PBMC preparations. Thus, the cultures were comprised of lymphocytes, monocytes, natural killer, and perhaps other mononuclear cell types. Culture conditions included: medium alone; rat anti-human IL-10 mAb (0.5–25 µg/ml), rat anti-human IL-4 mAb (0.5–25 µg/ml), or rat IgG1 isotype control antibody (all, PharMingen, San Diego, CA). As the optimal effects of anti-IL-10 mAb were observed at a concentration of ≥ 10 µg/ml, that dose was used for anti-IL-10, anti-IL-4, and isotype control antibodies in most experiments. Anti-FasL mAb (4H9, kindly provided by Dr. S. Nagata, Ogata Bioscience Institute, Osaka, Japan) (25) or anti-human TNFα mAb (Upstate Biotechnology, Inc., Lake Placid, NY) were used at concentrations of 5 and 20 µg/ml, respectively. Recombinant human IL-10 (Genzyme Corp., Cambridge, MA) was used at doses ranging from 0.2 to 20 ng/ml. As 20 ng/ml IL-10 produced the greatest effect on apoptosis in our cultures, that dose was chosen as standard for our cytokine experiments, and recombinant human IL-12 (Sigma Chemical Co., St. Louis, MO) was used at 20 ng/ml and recombinant IL-2 (Hemagen Diagnostic, Inc., Columbia, MD) was used at 20 U/ml. Allogeneic or autologous serum (10 or 20%), as a substitute for FCS, was used in some experiments. DNA synthesis was quantified by incubating 1×10^5 cells in 200 µl medium in a 96-well Falcon U-bottom plate. The cultures were performed in triplicate, for 5.5 d, and 1 mCi [³H]thymidine included for the last 18 h of culture. The cultures were harvested and counted in a liquid scintillation counter.

Cell viability and measurement of apoptosis. Total cell counts (TCC) and trypan blue (TB)-stained cells were quantified by light microscopy after vigorous suspension of cell cultures. Cell death (necrotic or apoptotic) was also quantified by propidium iodide (PI) uptake. In brief, PI was added to cells at a final concentration of 10 µg/ml in PBS, and the percentage of PI positive cells assessed by flow cytometry (FACScan; Becton Dickinson, San Jose, CA). Absolute cell viability was calculated from TCC and TB or PI positive cells. Apoptotic cells were detected by the method of Nicolletti et al. (26). The percentage of DNA in the subdiploid (M2) region reflects the proportion of apoptotic cells. Only single cells were analyzed; the doublets and cell clumps were excluded using a double discrimination FACS® module. In some experiments, cell death was also quantified by the Alamar blue assay, as described previously (27).

Cell surface marker analysis. 2×10^5 PBMC were washed and incubated for 30 min at 4°C with saturating concentrations of FITC-conjugated mouse anti-human CD3, CD14, or CD19 mAb (Immunotech, Inc., Westbrook, ME). An irrelevant IgG1 mAb was used as a control (Immunotech, Inc.). After three washes in cold buffer, PI (10 µg/ml) was added to cells, and analysis performed on the FACScan, gating on either the PI negative, viable cells, or the PI positive, dead or dying cells.

ELISA for IL-10. IL-10 in 5 d culture supernatants was determined by a sandwich ELISA (Biosource International, Camarillo, CA). The ELISA was performed according to the instructions provided by the manufacturer.

Statistical analysis. Patient and control groups were compared and analyzed for statistically significant differences using Student's

paired or unpaired *t* test, the Mann-Whitney test, or the Signed Ranked Wilcoxon, as appropriate.

Results

Increased apoptosis of SLE PBMC in vitro. To evaluate the viability of lymphocytes obtained from patients with SLE and other autoimmune and rheumatic diseases, PBMC were isolated from normal subjects and patients and cultured at 37°C for 1–5 d. Cell death was quantified immediately after isolation and on day 5 (and in some cases daily) by PI staining, as well as by TB exclusion, which showed a high degree of concordance. A small percentage of cells were dead immediately after isolation (*t*₀) in all groups tested (normal subjects [4.8±2.3%], arthritis controls [3.6±0.5%], SLE patients [4.9±1.3%], and vasculitis patients [9.1±2%]). At day 5, the percentage of dead PBMC from normal and arthritis controls increased slightly to 9.3±2.5% and 11.1±2.4%, respectively. Compared with these controls, increased cell death was observed in the SLE patients (19.1±5.9%) (*P* < 0.001 compared with normal and disease controls) and the three vasculitis patients (26±10.9%) (Fig. 1A). Clinically active SLE patients, as determined by a SLE-DAI count > 5, had a higher percentage of dead cells than did inactive patients (22.3±5.2% versus 14.3±4.6%; *P* < 0.001). As expected from the above results, the absolute TCC and the total number of viable cells at day 5 were markedly reduced in active SLE patients and vasculitis patients when compared with normal controls, inactive SLE patients, or arthritis control patients (Fig. 1, B and C; *P* < 0.0001). To confirm that the cells were dying by apoptosis, we performed PI staining followed by cell cycle analysis and quantified the M2 peak, comprising subdiploid, apoptotic cells. At day 5, the proportion of cells in the M2 peak was higher in the eight SLE patients studied, with a mean of 27.5±11.9% (*P* < 0.05), and in the three vasculitis patients (23.6±8.8%), when compared with six normal controls (mean of 11.4±3.9%) and three RA patients (15.2±1.7%). Moreover, in the SLE patients, an increase in apoptosis (27.9±8.4% of cells in the M2 peak) was observed in some patients as early as day 3 of culture. Representative histograms from a healthy subject, a patient with RA, and a patient with SLE, are demonstrated in Fig. 2. These results are consistent with those reported previously by Emlen et al. (18), in which cells from SLE patients, but not from RA patients or normal controls, showed accelerated spontaneous cell death in vitro.

Apoptosis of SLE PBMC in vitro is associated with high IL-10 production. To explore the idea that withdrawal from cytokines and growth factors induced the death of SLE T cells in vitro, we first cultured the SLE cells in human serum. Neither addition of 10 nor 20% autologous or allogeneic serum restored normal viability to the SLE T cells (data not shown), suggesting that growth factor withdrawal was unlikely to be the major mechanism for induction of cell death in vitro. We next considered whether an increase in cytokine levels could promote cell death. Increased levels of Th2 cytokines, including IL-6 and IL-10, have been reported in patients with SLE (8–13). IL-10 is of particular interest, as this cytokine has been shown to inhibit T cell proliferative function in vitro (28–33). When IL-10 concentrations in day 5 culture supernatants were measured by ELISA, IL-10 levels were significantly higher in the active SLE patients (500±580 ng/ml) and vasculitis patients (1202±1214 ng/ml) compared with the normal (36.7±32.3 ng/ml) or arthritis (41.1±26.8 ng/ml) controls, or the inac-

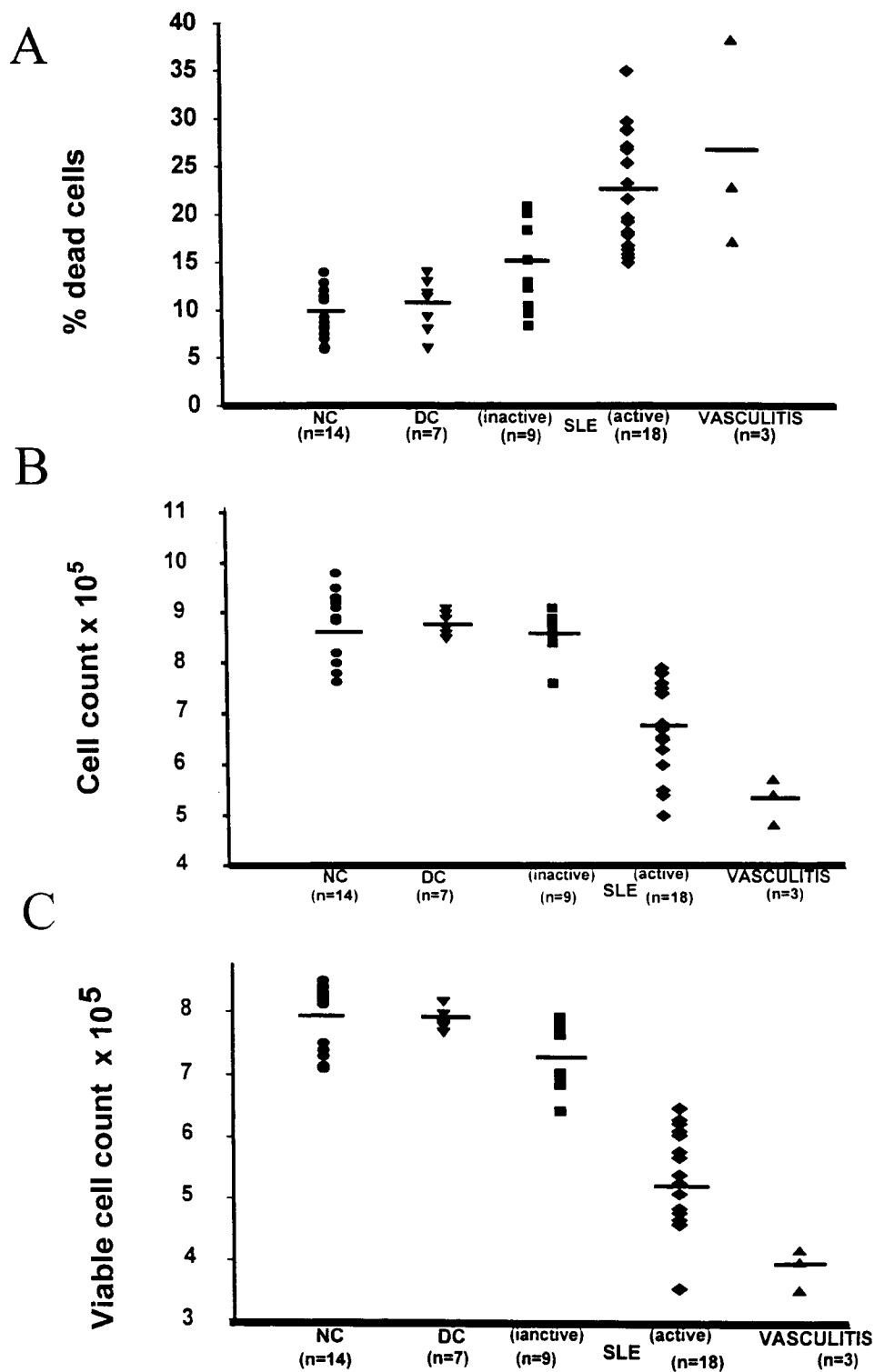


Figure 1. Impaired cellular viability in vitro in patients with active SLE or systemic vasculitis. Cell viability was quantified by PI incorporation (A, % dead cells), by absolute cell count (B; TCC), and by calculation of total viable cells (C; TCC $\times$ % PI-negative cells) on day 5 of culture of PBMC from normal donors (NC), disease controls (DC), inactive or active SLE patients, or from three patients with systemic vasculitis. The percent of dead cells was significantly higher ($P < 0.0001$), and TCC and total viable cell counts were significantly lower ($P < 0.0001$), in the active SLE and vasculitis patient groups when compared with normal and disease control groups.

tive SLE patients (85.3 ± 30.9 ng/ml) ($P < 0.0001$ for active SLE patients compared with inactive SLE and both control groups; Fig. 3). The IL-10 detected by ELISA was specifically adsorbed by anti-IL-10 mAb, confirming the identity of that cytokine in the SLE culture supernatants (data not shown).

Neutralization of IL-10 enhances survival of SLE PBMC in vitro. To directly examine whether IL-10 contributes to enhanced apoptosis in vitro, we cultured PBMC from SLE pa-

tients and controls with culture medium alone or with neutralizing antibodies to IL-10 or IL-4 or with isotype matched control rat antibodies. Cell viability was assessed at t_0 and day 5 of culture. The viable cell count and percentages of TB and PI positive cells were unchanged, or minimally changed, by culture of PBMC from normal controls ($n = 14$), inactive SLE patients ($n = 9$), and arthritis control patients ($n = 7$) with anti-IL-10, anti-IL-4, or isotype control mAb (Figs. 4 and 5).

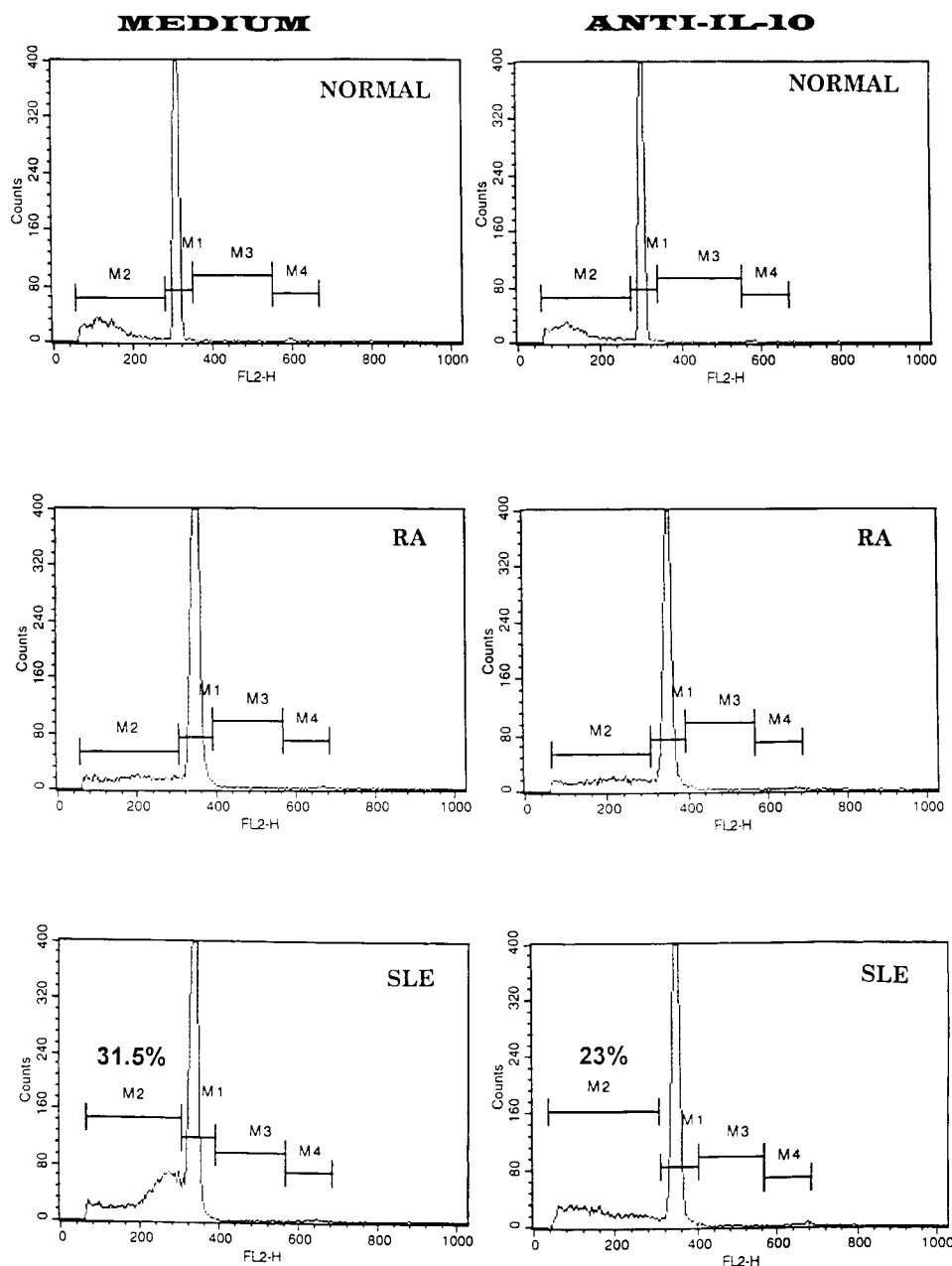


Figure 2. Increased SLE spontaneous cell death is apoptotic death, as measured by cell cycle analysis (DNA PI staining). PBMC from a normal subject (*top*), a patient with active RA (*middle*), and a patient with SLE (*bottom*) were assessed on day 5 of culture with medium alone (*left histograms*) or with anti-IL-10 mAb (*right histograms*) for the percentage of DNA in the M2 region, using the PI method, as described in Methods. The M1 region comprises cells in G0-G1; M2 indicates hypodiploid (apoptotic) cells; M3 indicates cells undergoing DNA synthesis; and M4 indicates cells in the G2-M cell cycle stage. Increased proportion of PI staining in the M2 region reflects an increased proportion of subdiploid cells, consistent with apoptotic cell death. Addition of anti-IL-10 to cultures (*right histograms*) decreased the proportion of cells in the M2 region in the SLE patient, but had no effect on PBMC from the NC or RA patient. Similar findings were observed in seven additional SLE patients.

In contrast, the spontaneous cell death of PBMC obtained from 18 active SLE patients and all three vasculitis patients was specifically abrogated by culture with the anti-IL-10 mAb ($P < 0.0001$). Correspondingly, the total number of viable cells increased from 5.3 ± 0.7 to $7.85 \pm 0.3 \times 10^5$ for SLE patients ($P < 0.0001$) and from 4.5 ± 0.5 to $6.9 \pm 0.3 \times 10^5$ for the vasculitis patients (Fig. 4). The rescue of SLE PBMC from cell death mediated by the anti-IL-10 mAb was associated with a reduction in the proportion of cells in the M2 (subdiploid) peak on PI cell cycle analysis in eight SLE patients studied (from a mean of $27.5 \pm 11.9\%$ to $19.8 \pm 8.9\%$; $P = 0.004$) (representative histograms in Fig. 2, *right panels*). To further confirm these results, proliferation of PBMC was measured by incorporation of [3 H]thymidine in the presence or absence of the neutralizing anti-IL-10, anti-IL-4, or control mAbs. In 13 of the 17 SLE subjects tested, anti-IL-10 mAb augmented base-

line [3 H]thymidine incorporation at day 5 of culture from 950 ± 911 to $2,418 \pm 1,955$ cpm ($P < 0.01$), while the control antibodies had little effect. Anti-IL-10 mAb did not alter proliferation of PBMC from control subjects (data not shown).

Two color flow cytometry, using PI to identify dead or dying cells and anti-CD3 mAb to identify T cells, revealed that the CD3-positive population was the dominant mononuclear cell population undergoing spontaneous cell death in vitro and rescued from apoptosis by anti-IL-10 mAb (Fig. 6). The absolute number of viable CD3-positive T cells at day 5 of culture in the active SLE patients was significantly lower than the number of viable T cells in the control groups ($P < 0.0001$ for active SLE compared with inactive SLE or normal controls, and $P < 0.001$ for active SLE compared with disease controls). Of the PI-negative viable cells at day 5, 33.9% were CD3-positive in the active SLE patients, while 64.2, 62.1, and 68.6% of

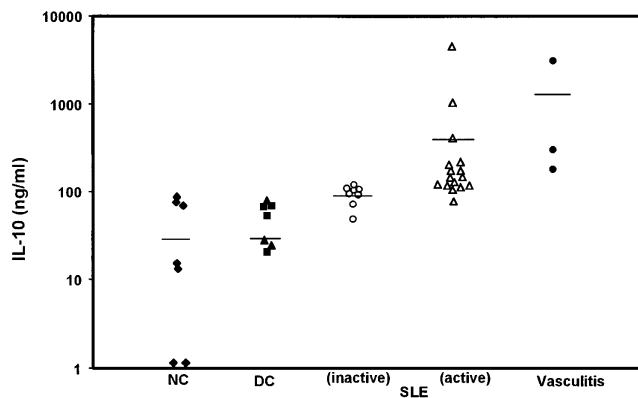


Figure 3. IL-10 is increased in 5 day culture supernatants from patients with active SLE. Culture supernatants were harvested at day 5 from PBMC cultures from NC, DC, inactive SLE patients, active SLE patients, or patients with systemic vasculitis, and then assayed for IL-10 by ELISA. IL-10 levels (ng/ml) in supernatants (log values on Y axis) are indicated for individuals in each patient group and means are indicated by a horizontal line. The levels of IL-10 in supernatants from the active SLE patients were significantly higher than those in the inactive SLE patients or controls ($P < 0.0001$).

the viable cells were CD3-positive in the normal controls, disease controls, and inactive SLE patients, respectively. These results were confirmed by directly detecting the percentage of CD3-positive cells among the PI-positive, dying, cell population, with the percent CD3-positive, PI-positive cells in the ac-

tive SLE patients significantly higher than in the disease and normal control groups ($P < 0.001$). Inclusion of anti-IL-10 mAb in the cultures increased the percentage of CD3-positive cells in the viable fraction to 53.1% in the active SLE patients ($P = 0.005$ for PBMC cultured with medium, compared with PBMC cultured with anti-IL-10 mAb). Taken together, these results support a role for IL-10 in promoting spontaneous apoptosis of T cells from patients with active SLE or systemic vasculitis.

IL-10 accelerates, and IL-12 and IL-2 retard, apoptosis of SLE PBMC in vitro. The studies described so far indicate that SLE PBMC produce high levels of IL-10 in vitro and that neutralization of this cytokine significantly reduces T cell apoptosis in vitro. To determine whether the direct addition of IL-10 would increase cell death, IL-10 (20 ng/ml) was added to normal and patient PBMC cultures and cell viability assessed over five days of culture. While IL-10 did not alter cell viability in nine normal controls and one RA patient tested, addition of IL-10 to PBMC from seven SLE and three vasculitis patients resulted in a decrease in TCC at day 5 from 5.9 ± 0.7 to $3.7 \pm 0.4 \times 10^5$ for the SLE patients ($P < 0.01$), and from 4.4 ± 0.5 to $3.1 \pm 0.5 \times 10^5$ for the vasculitis patients (Figs. 5 and 7). These results confirm a role for IL-10 in accelerating or augmenting apoptosis in vitro.

IL-10 has been shown to inhibit T cell proliferation by direct actions on T cells, such as inhibition of IL-2 production (29–31), and also indirectly, by inhibiting costimulatory molecule expression or IL-12 production by antigen presenting cells (APCs) (28, 32, 34). As IL-12 has been shown to protect some lymphocytes from apoptosis (35), we studied the effect of IL-12

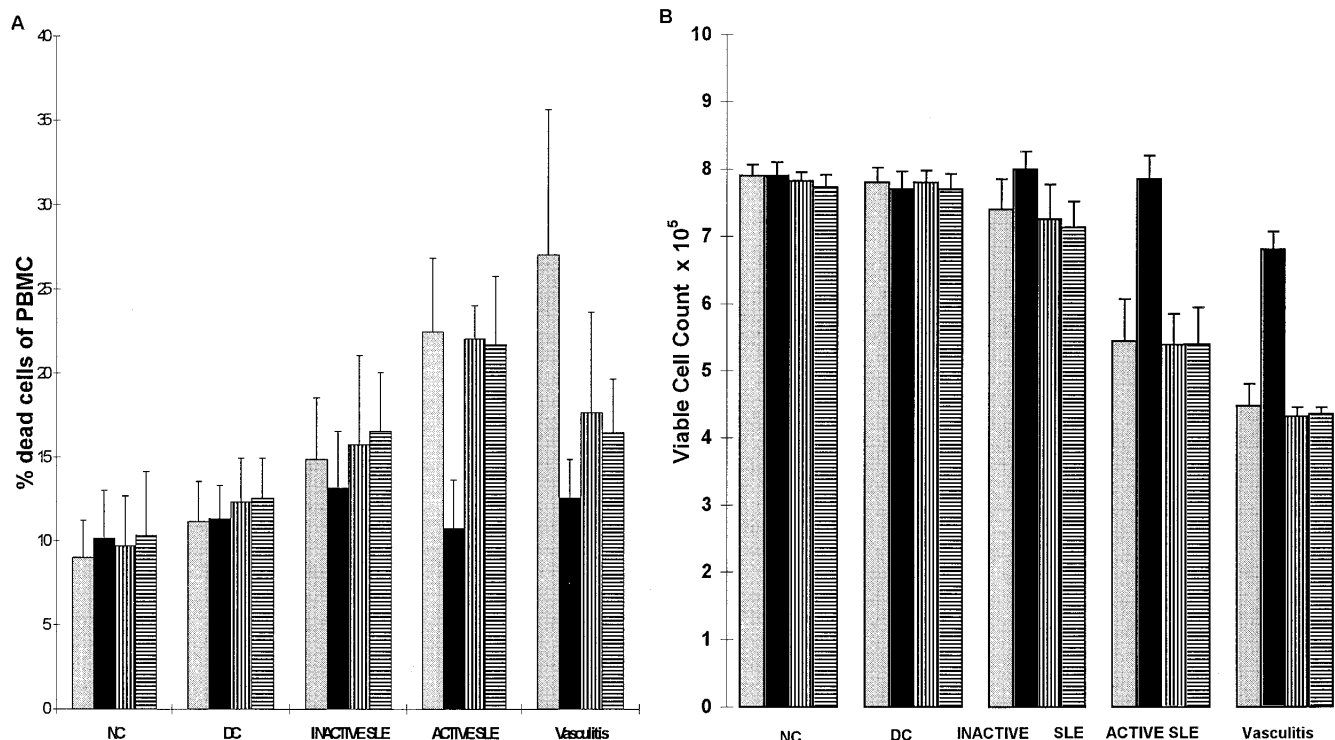


Figure 4. Anti-IL-10 mAb promotes cell viability in patients with active SLE or systemic vasculitis. NC ($n = 14$), disease control patients ($n = 7$), inactive SLE patients ($n = 9$), active SLE patients ($n = 18$), and patients with systemic vasculitis ($n = 3$) were studied for cell viability after 5 days of culture with medium alone (shaded bars), anti-IL-10 mAb (black bars), anti-IL-4 mAb (vertically-lined bars), or IgG1 control antibody (horizontally-lined bars). (A) Percentage of dead cells as determined by PI incorporation. (B) Total number of viable cells.

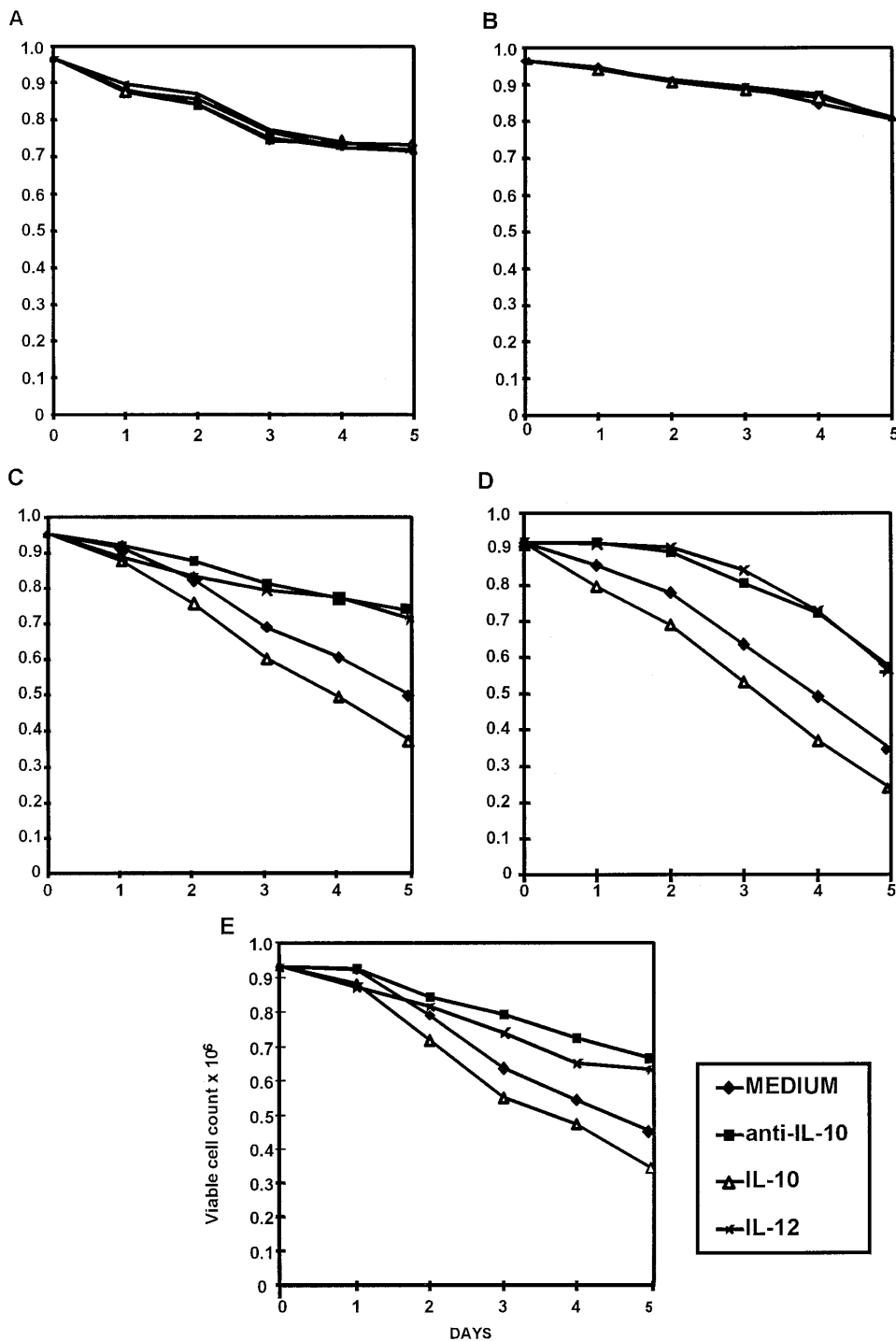


Figure 5. IL-10 decreases, and IL-12 augments, cell viability in PBMC from active SLE and systemic vasculitis patients. Total number of viable cells was determined daily over 5 days of culture in a NC subject (A), an RA patient (B), an active SLE patient (C), and two patients with systemic vasculitis (D and E). 1×10^6 PBMC were cultured with medium alone (diamonds), IL-10 (triangles), anti-IL-10 mAb (squares), or IL-12 (x) for the indicated number of days. The absolute number of cells multiplied by the percentage of PI-negative cells is expressed as total number of viable cells on the Y axis.

on spontaneous apoptosis of SLE PBMC. Addition of IL-12 (20 ng/ml) to the cultures increased the number of viable SLE cells from 5.9 ± 0.7 to $7.7 \pm 0.2 \times 10^5$ ($n = 5$; $P = 0.001$) and of all three vasculitis patients from 4.4 ± 0.5 to $6.9 \pm 0.4 \times 10^5$, but had no effect on cells from five normal controls (Figs. 5 and 7). To examine the effect of IL-2, 20 U/ml IL-2 was added to the cultures and the TCC assayed at day 5 in three SLE patients and three normal controls. As in the case of IL-12, IL-2 increased SLE cell viability at day 5 from 4.4 ± 0.4 to $8.1 \pm 0.4 \times 10^5$ ($P = 0.002$; Fig. 7). These results raise the possibility that the

capacity of IL-10 to promote T cell apoptosis is mediated, in least in part, by its known inhibitory effects on production of IL-12 and IL-2 (29, 33, 34, 36).

High IL-10 production in vitro is correlated with lymphopenia in vivo. To determine whether IL-10-facilitated AICD of SLE lymphocytes observed in vitro was clinically relevant, we quantified the absolute neutrophil and lymphocyte counts of the SLE patients studied. The absolute neutrophil count was not associated with disease activity, increased spontaneous apoptosis in vitro, or IL-10 production in vitro (data not shown). In

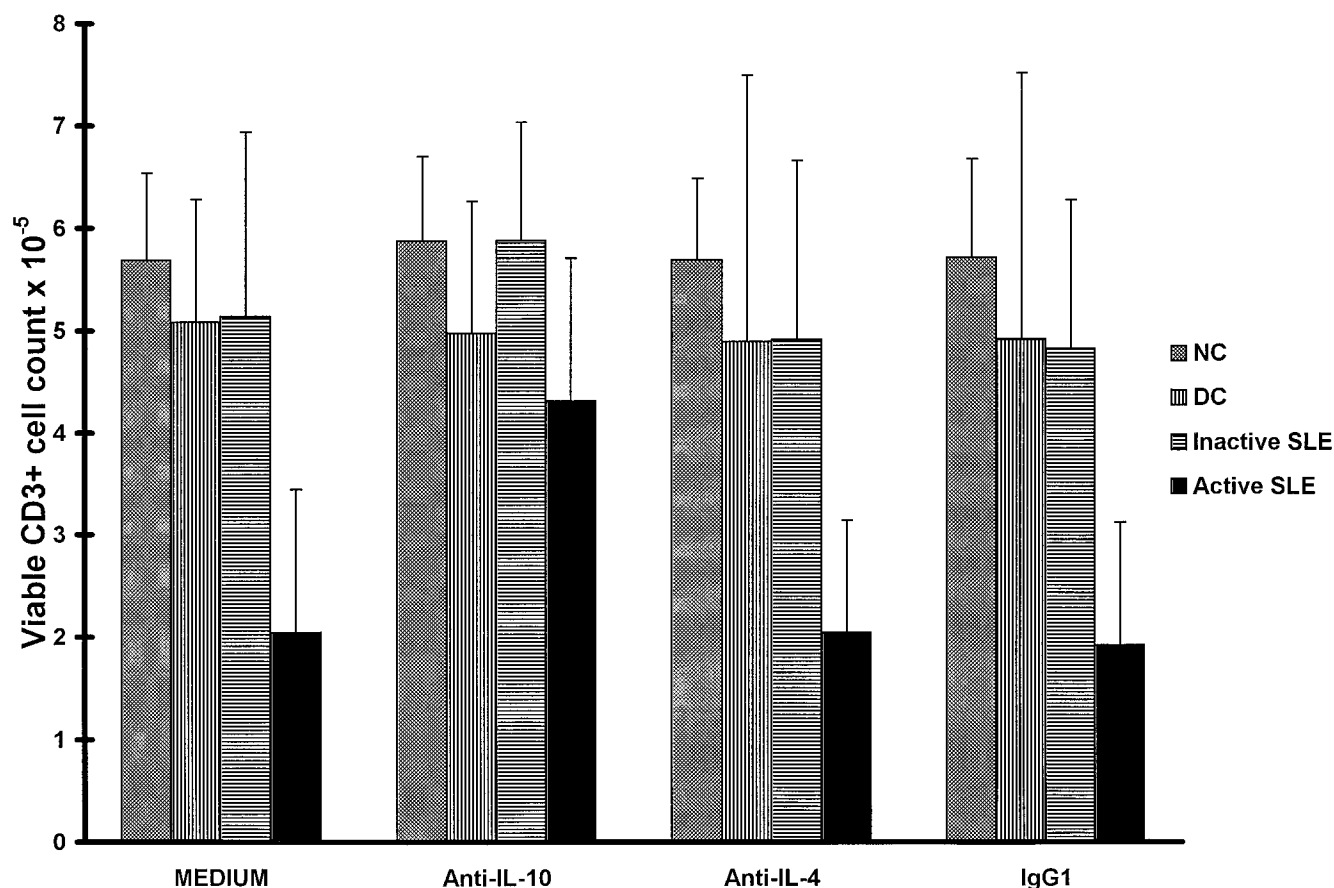


Figure 6. CD3⁺ PBMC are increased by culture with anti-IL-10 neutralizing mAb. PBMC from NC ($n = 9$), DC ($n = 7$), inactive SLE ($n = 10$), or active SLE ($n = 15$) patients were cultured for 5 days with medium alone, anti-IL-10 mAb, anti-IL-4 mAb, or IgG1 control antibody. Two parameter FACS[®] analysis was performed, using PI to identify dead or dying cells and anti-CD3 mAb to identify CD3-positive T cells. The figure indicates the absolute number of CD3-positive cells among the PI-negative (viable) cells. The mean absolute number of viable CD3-positive cells in the active SLE patients was significantly greater in the presence than the absence of neutralizing anti-IL-10 mAb ($P < 0.001$). When data are expressed as the percentage of PI-negative (viable) cells that are CD3-positive, active SLE cell viability was also greater in the presence than the absence of anti-IL-10 mAb ($P = 0.005$, as indicated in the text).

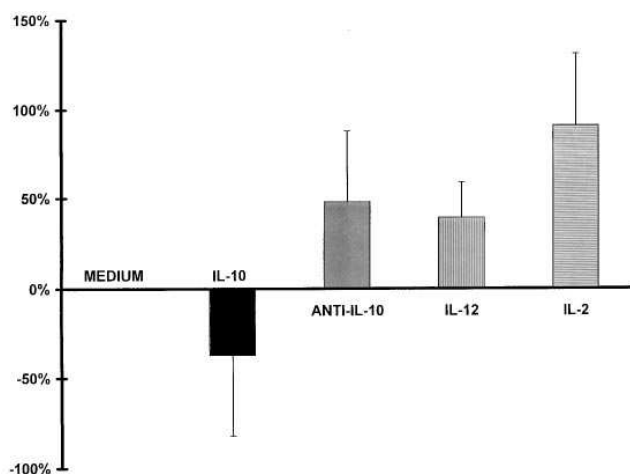


Figure 7. Effect of cytokines on cell viability of PBMC from patients with active SLE. Total viable cell count was determined in PBMC from patients with active SLE after 5 days culture in medium alone ($n = 27$), or with IL-10 ($n = 7$), anti-IL-10 mAb ($n = 27$), IL-12 ($n = 5$), or IL-2 ($n = 3$), as described in Methods. Viability of each

subject's PBMC for each cell culture condition was compared with the viability of those cells in medium alone, and results are expressed as a percentage of baseline viability (PBMC cultured in medium alone). IL-10 decreased cell viability (negative values; $P < 0.01$ compared with baseline viability), while anti-IL-10 antibodies, IL-12 and IL-2 promoted cell viability (positive values).

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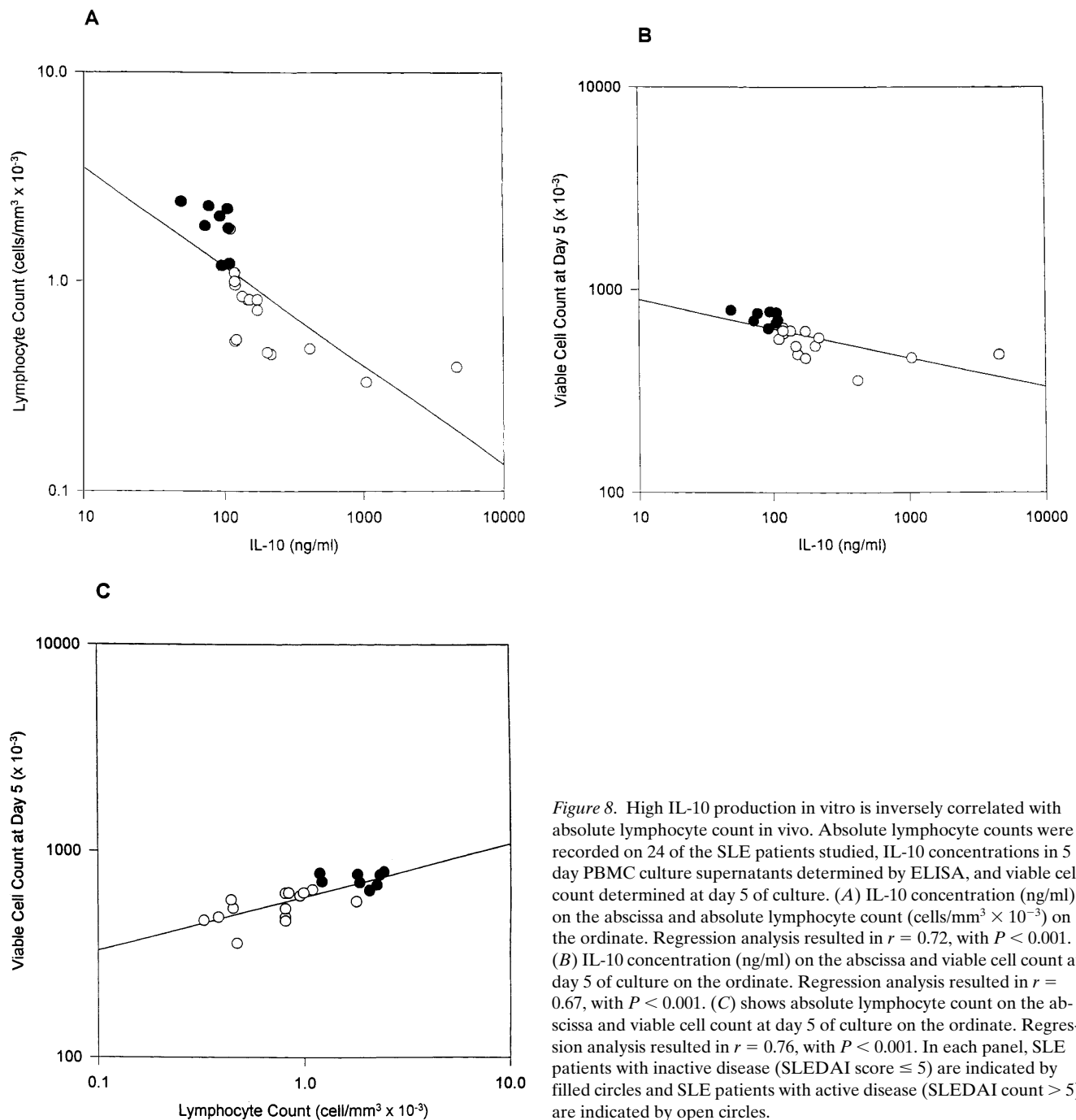


Figure 8. High IL-10 production in vitro is inversely correlated with absolute lymphocyte count in vivo. Absolute lymphocyte counts were recorded on 24 of the SLE patients studied, IL-10 concentrations in 5 day PBMC culture supernatants determined by ELISA, and viable cell count determined at day 5 of culture. (A) IL-10 concentration (ng/ml) on the abscissa and absolute lymphocyte count (cells/mm³ × 10⁻³) on the ordinate. Regression analysis resulted in $r = 0.72$, with $P < 0.001$. (B) IL-10 concentration (ng/ml) on the abscissa and viable cell count at day 5 of culture on the ordinate. Regression analysis resulted in $r = 0.67$, with $P < 0.001$. (C) shows absolute lymphocyte count on the abscissa and viable cell count at day 5 of culture on the ordinate. Regression analysis resulted in $r = 0.76$, with $P < 0.001$. In each panel, SLE patients with inactive disease (SLEDAI score ≤ 5) are indicated by filled circles and SLE patients with active disease (SLEDAI count > 5) are indicated by open circles.

portant role for IL-10 in augmenting AICD in vivo in patients with SLE.

Cell death is mediated through the FasL/FasR pathway. To examine whether FasL (38) or TNF α (23) was responsible for inducing cell death, we established cultures of PBMC from four active SLE patients and four normal subjects in medium alone or in the presence of neutralizing anti-FasL or anti-TNF α mAb. The addition of 5 μ g/ml anti-FasL mAb to SLE PBMC at t_0 resulted in increased cell survival at day 5 of culture (from $1,313 \pm 232$ to $2,359 \pm 366$ counts; $P < 0.05$), which was of similar magnitude to the improved survival in the presence of anti-IL-10 ($2,225 \pm 47$ counts). Addition of anti-FasL

mAb and IL-10 together to cell cultures resulted in a partial improvement in cell viability. In contrast, 20 μ g/ml anti-TNF α mAb had no effect on cell survival (Fig. 9). These results identify FasL as the major effector mediating the spontaneous apoptosis of SLE T cells in vitro.

Discussion

Recent reports have described lymphocyte apoptosis in vitro in the setting of active viral infection, such as HIV or hepatitis B, in patients with systemic autoimmunity, and in patients who have received extensive burn trauma, a trigger of generalized

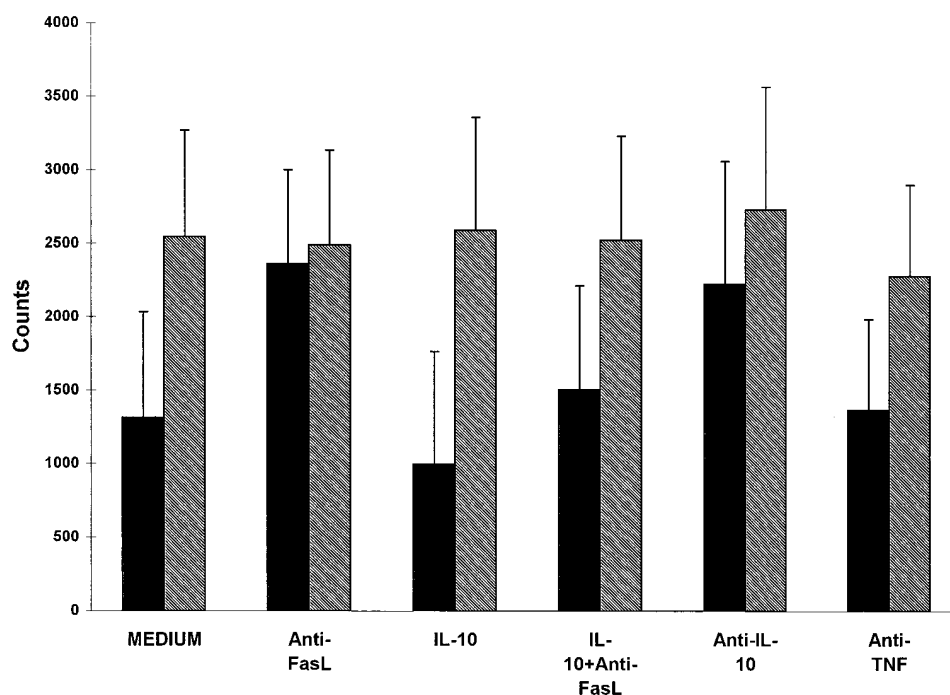


Figure 9. FasL mediates spontaneous in vitro apoptosis in active SLE. The alamar blue method was used to assess relative cell viability, as described in Methods. PBMC from four normal subjects (hatched bars) and from four patients with active SLE (black bars) were cultured for 5 days with medium alone, or with the indicated antibodies or cytokines, and cellular metabolism measured by spectroscopy (shown as counts on the Y axis). Mean values are shown for each subject group for each culture condition. Cell viability was significantly greater in the presence than in the absence of anti-FasL mAb ($P < 0.05$), and in the presence of anti-IL-10 mAb, compared with medium control ($P < 0.05$) in the SLE patients studied.

immune system activity (18, 35, 39–44). In this study, we report that the increased apoptosis of SLE lymphocytes in vitro (18) is, in part, due to the effect of IL-10 and is mediated by FasL. This phenomenon was not, however, specific for SLE, as high proportions of apoptotic cells were observed in patients with systemic vasculitis, and IL-10 also promoted apoptosis in PBMC obtained from those patients. It appears, then, that increased apoptosis in vitro is a common pathway for T cells that are activated in vivo in a number of pathologic conditions.

We considered several possible mechanisms for enhanced in vitro apoptosis of T cells in SLE and systemic vasculitis. The cell death observed could be attributable to growth factor withdrawal, as has been shown for certain cell lines upon withdrawal of IL-2 (19). However, supplementation of the cultures with 20% autologous or allogeneic serum did not rescue the cells from accelerated death. While we cannot rule out the possibility that supplementation with greater quantities of serum might restore normal viability in the SLE cells, our experiments do not support cytokine withdrawal as the sole or major mechanism of accelerated in vitro apoptosis in these patients. Cytokines have been shown to profoundly affect cell viability in vitro in a number of other diseases (35, 39, 40, 45, 46). IL-10 has been reported to either augment or protect immune system cells from apoptosis, depending on the cell type being studied and its activation status (45–47). Based on neutralization, addition and correlative associations, we observed that IL-10, but not IL-4, was in part responsible for the accelerated apoptosis of SLE and systemic vasculitis patients' T cells in vitro. The observation that IL-10 did not alter viability of control PBMC suggests that IL-10 selectively impairs the viability of activated T cells.

The capacity of IL-10 to augment apoptosis of T cells from patients with systemic autoimmunity may reflect actions that directly alter target T cell function or those that act indirectly,

through effects on macrophages or other APCs. Direct mechanisms of modulation of T cell apoptosis by IL-10 could include inhibition of T cell production of IL-2, a cytokine known to partially overcome T cell anergy or apoptosis (29, 30). Most intriguing is the possibility that T cell receptor for antigen (TCR), IL-10-, and FasR-stimulated second messenger pathways interact in the T cell to shift the pattern of activated transcription factors away from those that mediate full T cell activation and toward a profile that favors cell death. In this regard, the recent report from Groux et al. (48) describes the capacity of IL-10 to directly induce long-lasting antigen-specific anergy in human CD4-positive T cells. In those experiments, T cells triggered in an allogeneic mixed lymphocyte reaction or with anti-CD3 mAb, in the presence of IL-10, failed to proliferate, produce cytokines, or upregulate expression of the IL-2 receptor (CD25) when retriggered with the initial stimulus. The cells successfully received TCR-mediated signals, as they demonstrated normal Ca^{2+} fluxes. The SLE T cells that we have studied, which are likely to have been repeatedly triggered in vivo and have been shown to be relatively anergic in vitro, are also shown, in this report, to undergo programmed cell death. Many studies suggest that anergy and apoptosis represent a continuum of responses to TCR-mediated activation (21, 48).

Among the indirect actions of IL-10 that could alter cell viability is its inhibition of expression of the costimulatory molecules, CD80 and CD86, on APCs, an effect that would promote TCR-mediated T cell activation in the absence of CD28 signals (28, 32, 49). The consequences of this partial T cell activation might be an anergic phenotype, manifest clinically as impaired delayed-type hypersensitivity and poor immune responses against some external pathogens, while perhaps allowing sufficient TCR signal transduction for the generation of the early T cell activation gene products, CD40L and FasL. This

partial activation profile could also prime the CD4 T cell for programmed cell death (50). IL-10 also inhibits monocyte production of IL-12, a cytokine that has been reported to protect T cells from AICD (34, 35). In our studies, addition of IL-12, or IL-2, partially inhibited apoptosis of SLE T cells, suggesting that some of the effect of IL-10 may be indirect. Similar observations have been reported in other studies, including those that have demonstrated the capacity of IL-12 or IL-2 to prevent in vitro apoptosis in patients infected with HIV (35, 36, 39). Whatever the mechanism of the IL-10 effect observed, our results suggest that high levels of this cytokine may be clinically important. Our data document a highly significant correlation between IL-10 production in vitro and lymphopenia in vivo. Thus, the patients with clinically active disease, and increased spontaneous apoptosis and high IL-10 production in vitro, are the patients who are markedly lymphopenic in vivo.

Recent reports have emphasized the importance of FasL and TNF α in the induction of apoptosis in AICD (22, 23, 43). Analysis of FasR expression and function in human SLE has demonstrated that this death pathway is intact (4, 5). In fact, cell surface FasR is higher on SLE T and B lymphocyte populations compared with controls, consistent with other measures of in vivo lymphocyte activation (4–7). To determine whether these death effectors were involved in the enhanced apoptosis observed in SLE and vasculitis, neutralizing antibodies to either FasL or TNF α were included in the PBMC cultures. Anti-FasL antibody significantly reversed the spontaneous apoptosis of SLE PBMC, while anti-TNF α mAb had no effect, indicating that FasL was the dominant death effector in this system. These findings are most compatible with the hypothesis that the enhanced cell death observed in vitro reflects AICD initiated in vivo.

The process of apoptosis is important, not only for deletion of unwanted lymphocyte specificities, but also for downregulation of an appropriately generated immune response. Thus, AICD is likely to occur in the normal course of events, following antigen-induced T cell activation (20–23). In systemic autoimmune diseases, however, AICD may be excessive, either by virtue of the extensive spectrum of T cell specificities triggered, or by alterations in regulatory mechanisms, such as increased production of IL-10 (8–12). Augmented programmed cell death may have deleterious clinical consequences, such as lymphopenia and impaired cellular immune function, common features of patients with SLE that may contribute to impaired handling of micro-organisms (17), as occurs in AIDS patients (35, 39–41, 51, 52). Abrogation of T cell function has also been observed in other clinical conditions in which we and others have observed augmented lymphocyte apoptosis. Impaired autologous mixed lymphocyte reactions, impaired proliferative responses to mitogens, or impaired T suppressor function, commonly cited abnormalities in SLE, have been reported in patients with polymyositis/dermatomyositis (53, 54), polyarteritis nodosa (55), hemophiliacs who are hepatitis C infected but HIV negative (56), patients with chronic hepatitis B infection (57, 58), and in patients who have undergone severe burn trauma (42, 59). These studies, together with the data presented in this study, support the suggestion that AICD and impaired T cell function, in vivo and in vitro, may be pathogenetically linked.

AICD in vivo may have other consequences for disease morbidity and pathogenesis in SLE. The necrotizing lymphadenitis that is sometimes observed in SLE, and is pathologically

indistinguishable from Kikuchi-Fujimoto disease, could reflect excessive in vivo apoptosis (60). As has been suggested by a number of investigators, exposure of the immune system to the contents of apoptotic cells, highly enriched in particles containing lupus autoantigens, may serve to perpetuate systemic autoimmunity and autoantibody production (18, 61–64).

In summary, our data confirm the observation of increased spontaneous apoptosis of SLE T cells in vitro. This process is not specific to SLE, but is a feature of lymphocytes from patients with a range of immune system disorders characterized by polyclonal and chronic T cell activation, whether stimulated by viral antigens, autoantigens, or undefined triggers. In SLE patients with immunologically active disease, the high levels of IL-10, in addition to promoting B cell differentiation and hyperactivity (11, 65–67), may augment T cell death in vivo, contributing to defective cellular immune function and generating a source of the self antigens relevant to autoantibody production in SLE. Additional studies will be necessary to understand how the second messenger molecules activated by IL-10, reportedly STAT1 α , STAT3, tyk2 and Jak1 (68), interact with the intracellular factors activated through the TCR and Fas to promote apoptosis, rather than positive effector functions, in T cells.

Acknowledgments

The authors thank Dr. Shigekazu Nagata for providing the anti-Fas ligand monoclonal antibody.

This work was supported by NIH P50 AR42588(SCOR; M.K. Crow, K.B. Elkon); NIH R01 AR38915 (K.B. Elkon), The S.L.E. Foundation, Inc. (M.K. Crow), and The Arthritis Foundation, New York Chapter (M.K. Crow).

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