Clin Exp Immunol 1996; **106**:218–229

Preferential elimination of CD28$^+$ T cells in systemic lupus erythematosus (SLE) and the relation with activation-induced apoptosis

H. KANEKO*"", K. SAITO*"", H. HASHIMOTO"", H. YAGITA*, K. OKUMURA* & M. AZUMA* *Department of Immunology and ""Department of Internal Medicine, Division of Rheumatology, Juntendo University School of Medicine, Tokyo, Japan

(Accepted for publication 17 July 1996)

SUMMARY

CD28 on T cells provides a potent costimulatory signal for T cell activation. Down-regulation of CD28 on peripheral T cells has been reported in certain clinical conditions, but full studies on the mechanism and biological significance have not been performed. Our extensive phenotype analysis of peripheral blood lymphocytes (PBL) from SLE patients revealed that the absolute number of CD28$^+$ T cells of both CD4 and CD8 phenotypes was selectively decreased, while that of CD28$^-$ T cells was maintained. CD28$^+$ T cells from SLE patients exhibited mostly normal proliferative responses to both CD28-dependent and -independent stimulations. In contrast, CD28$^-$ T cells were hyporesponsive to anti-CD3 stimulation in both SLE and normal controls. These results implied that the selective decrease of CD28$^+$ T cells in SLE does not result from a hyporesponsiveness of CD28$^+$ T cells. To investigate the reason for the selective loss of CD28$^+$ T cells, we determined the appearance of apoptotic cells in culture with or without anti-CD3 stimulation. Apoptotic cells defined by merocyanine (MC)540 were gradually increased from 12 h to 24 h. Anti-CD3-induced apoptosis of CD28$^+$ T cells was significantly accelerated in SLE, whereas apoptosis of CD28$^-$ T cells was hardly detected in both SLE and normal controls. Comparative analysis between CD28$^+$ and CD28$^-$ T cells on CD95 (Fas) and Bcl-2 expression, which are related to activation-induced cell death (AICD), did not show a major difference, although CTLA4, which has been demonstrated to transmit an apoptosis-inducing signal, was expressed only on CD28$^+$ T cells. Our results suggest that CD28-mediated costimulation influences T cell susceptibility to AICD and may be involved in T cell lymphopenia in SLE.

Keywords CD28 apoptosis T cell activation systemic lupus erythematosus

INTRODUCTION

Recent studies have indicated that activation signals to T cells may lead to either proliferation, anergy, or cell death by apoptosis [1–3]. It appears that the activational state of cells and the pathway of an activation signal both play important roles in the decision process [4,5]. An optimal T cell activation requires two signals, the first signal through the ligation of TCR/CD3 complex and the second costimulatory signal delivered by direct interaction between T cells and antigen-presenting cells (APC). Triggering of TCR/CD3 alone, in the absence of a costimulatory signal, not only fails to induce proliferation but occasionally leads to a state of hyporesponsiveness or anergy [1,2,6]. One of the best known, a crucial costimulatory molecule, is CD28, which is expressed on most T cells and thymocytes. Ligation of CD28 by its ligands, CD80 (B7, B7-1) [7,8] or CD86 (B70, B7-2) [9,10] costimulates T cell proliferation, cytokine production and generation of cytotoxic T lymphocytes (CTL) [11,12].

Although essentially all TCR/CD3$^+$ thymocytes in man and mouse express CD28, a minor subset of human peripheral blood T cells lacks CD28 [13]. In healthy adult peripheral blood, CD28$^-$ T cells were preferentially found within the CD8 subset [14,15], while CD28$^-$4$^+$ T cells were observed in certain individuals [16]. CD28$^-$8$^+$ T cells were not observed in the thymus and were present only at low frequency in cord blood. In healthy adults, this subpopulation was gradually increased with age [17]. We previously demonstrated that CD28$^-$8$^+$ T cells were morphologically and phenotypically distinct from CD28$^+$8$^+$ T cells and exhibited unique functional characteristics, in that CD28$^-$8$^+$ T cells were

M.A. present address: Department of Allergy and Immunology, National Children's Medical Research Centre, 3-35-31, Taishido, Setagaya-ku, Tokyo 154, Japan.

Correspondence: Dr Hiroyuki Kaneko, Juntendo University School of Medicine, Department of Immunology, 2-1-1 Hongo, Bunkyo-ku, Tokyo 113, Japan.

cytolytic in response to anti-CD3 stimulation but unresponsive to anti-CD3-induced proliferation even in the presence of costimulation through CD80 or CD58 [15]. The CD28$^-$8$^+$ T cell population overlapped most with the CD57$^+$8$^+$ or CD11b$^+$8$^+$ T cell subset in normal adult individuals [18], and it has been demonstrated that CD57$^+$8$^+$ T cells from patients with AIDS and bone marrow transplantation (BMT) manifested non-specific suppressor function through the release of soluble factors [19,20]. All these data indicate that CD28$^-$ T cells may possess distinct functional roles from CD28$^+$ T cells in an immune response. The increase of this population has been reported in certain clinical situations, such as after BMT [21] or in the course of viral infections [22,23]. It seems that abnormal T cell activation by allo-antigens or viral antigens might be associated with such clinical situations *in vivo*.

SLE is a systemic autoimmune disease characterized by autoantibody production against selected intercellular antigens. In peripheral blood from SLE patients, abnormalities in T and B cell activation are generally observed and lymphopenia is seen in most cases. Although it is not well understood why lymphopenia occurs in SLE, one possible explanation is the existence of lymphocytotoxic antibodies [24], and another may be apoptosis resulting from an excessive lymphocyte activation. Activation through the TCR/CD3 results in the elimination of a proportion of the responding cells; this is called activation-induced cell death (AICD). Recent studies suggest that one of the important mechanisms for AICD within T cells is mediated by CD95 (Fas/Apo-1)- and the CD95 ligand-mediated pathway [25,26]. Many investigators have discussed abnormalities in apoptosis as the cause of autoimmunity [27]. In this study, we investigated the functional and phenotypic characteristics of CD28$^+$ and CD28$^-$ T cells from both SLE and normal subjects and compared the susceptibility to AICD between these two distinct T cell subsets. Based on our present results, we discuss the possibility that CD28-mediated costimulatory signals affect not only the choice of proliferative response or anergy but also the choice of apoptosis.

PATIENTS AND METHODS

Patients

Lymphocyte marker analysis was performed in 35 SLE patients and 20 healthy donors. Heparinized peripheral blood was obtained from 35 out-patients (34 women and one man) observed at the Rheumatology Clinic in our University. All patients met the American College of Rheumatology criteria (1982) for SLE [28]. The age of patients ranged from 16 to 72 years, with a mean ($\pm$ s.d.) of 36·8 $\pm$ 12·7 years. Thirty-one patients were receiving prednisolone treatment (all <30 mg/day). Normal controls (NC) were obtained from 20 healthy volunteers (14 women and six men, 28·8 $\pm$ 3·4 years old). At the same time as drawing blood for phenotyping, another blood sample was subjected to whole blood leucocyte count and haemogram on Micro Diff 18 cell counter (Coulter Corp., Hialeah, FL) in our central blood test laboratory. For the cell sorting study in NC, buffy coats were obtained from the Japanese Red Cross Central Blood Centre (Tokyo, Japan). Peripheral blood mononuclear cells (PBMC) were isolated by Ficoll–Hypaque gradient within 6 h after blood drawing.

Monoclonal antibodies

MoAbs against the following antigens were used: CD3 (OKT3, IgG2a; obtained from the American Type Culture Collection, Rockville, MD), CD3 (UCHT1, IgG1), CD4 (RPA-T4, IgG1), CD8 (RPA-T8, IgG1), CD5 (UCHT2, IgG1), CD45RO (UCHL1, IgG2a), CD45RA (HI100, IgG1), CD57 (NK-1, IgM), CD11b (44, IgG1), CD16 (3G8, IgG1), CD25 (M-A251, IgG1), CD69 (FN50, IgG1), HLA-DR (TÜ36, IgG2b), Bcl-2 (124, IgG1; Dako Japan Co. Ltd, Kyoto, Japan), CD28 (TN228, IgG1) and CD95 (DX2, IgG1; generated as previously described [12,29]), CTLA4 (11D4, IgG1; generously provided by Dr P. Linsley, Bristol-Myers Squibb Pharmaceutical Research Institute, Seattle, WA). All MoAbs were generously provided by PharMingen (San Diego, CA), unless otherwise stated.

Immunofluorescence and flow cytometry

Methods of immunofluorescent staining, flow cytometry, and data analysis have been described previously [30]. Cytoplasmic staining for Bcl-2 antigen was performed as described by Pollice *et al.* [18] with minor modifications. Briefly, cells were fixed with 0·25% paraformaldehyde in PBS for 15 min after cell surface staining with PE- and Cychrome (Cy)-conjugated MoAbs. Then the cells were incubated with 70% methanol at 4°C for 60 min for cell membrane permeabilization. After washing once, cells were incubated with FITC-conjugated anti-Bcl-2 MoAb or control mouse IgG1 MoAb for 30 min and were analysed after washing. Flow cytometry was performed using FACScan or FACStar^{plus} (Becton Dickinson Immunocytometry Systems, San Jose, CA) and data were analysed using Lysis II or Cell Quest software (Becton Dickinson).

Cell isolation

CD4$^+$ or CD8$^+$ cells were isolated from PBMC using anti-CD4- or anti-CD8-coated Dynabeads (M-450) and DetachaBeads (Dynal Inc., Oslo, Norway), according to the manufacturer's instructions. CD4$^+$ T cells were stained with PE-conjugated anti-CD28 (TN228) and then CD28$^+$ cells were collected by flow cytometric cell sorting and used as CD4$^+$28$^+$ cells. For isolation of CD8$^+$28$^+$ and CD8$^+$28$^-$ T cells, purified CD8$^+$ T cells were stained with FITC-conjugated anti-CD16 (3G8) MoAb and PE-conjugated anti-CD28 MoAb to distinguish contaminated CD8$^+$16$^+$28$^-$ natural killer (NK) cells and sorted for CD28$^+$16$^-$ or CD28$^-$16$^-$ fraction by flow cytometry. All isolated cells were reanalysed by flow cytometry and purity >95% was always assured.

T cell proliferation assays

Anti-CD3-coated plates were prepared as previously described [15]. In our preliminary studies, we determined that coating with 5 μg/ml of OKT3 (anti-CD3 MoAb) caused maximal proliferation of T cells and coating with 0·5 μg/ml of OKT3 was suboptimal. Freshly isolated PBMC (1×10^5 cells) or highly purified T cells (1×10^5 cells) were added at the indicated cell number to anti-CD3-coated or uncoated wells in 96-well flat-bottomed plates (Corning, New York, NY) in the presence or absence of 5 μg/ml anti-CD28 (TN228) MoAb and 50 U/ml recombinant IL-2 (Shionogi Pharmaceutical Inc., Osaka, Japan) and plates were cultured for the indicated period. Cultures were labelled for the final 18 h with 1 μCi/well ^{3}H-TdR (New England Nuclear, Boston, MA), were harvested using a Micro 96 Harvester (Skatron, Lier, Norway) and radioactivity was measured using a microplate β counter (Micro Beta Plus; Wallac, Turku, Finland).

Assessment of apoptotic cells

For identification of apoptotic cells in certain T cell subsets, we estimated the increased binding of a lipophilic fluorescent dye merocyanine (MC)540 (Sigma, St Louis, MO) and decreased forward scatter (FSC) by flow cytometry. Staining with MC540 was performed as described [31] with minor modifications. After cell surface staining with FITC-conjugated anti-CD28 and Cy-conjugated anti-CD3 MoAbs, cells were resuspended in 100 μl of 2% fetal calf serum (FCS)–PBS. MC540 (0·1 μg/ml) was added and the cells were incubated for 10 min at room temperature. After one washing, cells were analysed by flow cytometry on FACScan immediately.

RESULTS

Selective loss of CD28$^+$ T cells in SLE

Three-colour immunofluorescence analysis of peripheral blood lymphocytes (PBL) from 35 SLE patients and 20 healthy NC was performed to examine CD28 expression on T cell subsets. Representative results are presented in Fig. 1, and the mean percentage of each subset to total lymphocytes is shown in Fig. 2a. As shown in the left panel in Fig. 1, CD28 was exclusively expressed on CD3$^+$ T cells in both NC and SLE PBL. The percentages of CD3$^+$ T cells in NC and SLE were 72·8% and 60·3%, respectively (data not shown). Despite a minor decrease of total T cell percentage in SLE, the proportion of CD28$^+$ T (CD28$^+$3$^+$) cells was apparently decreased and, in contrast, that of CD28$^-$ T (CD28$^-$3$^+$) cells was significantly increased (Fig. 2a). Since we previously showed that discrimination of CD8$^+$ NK cells was difficult by two-colour staining with anti-CD28 and anti-CD8 MoAbs, we performed three-colour staining in the following experiments. An electronic 'gate' was placed on CD3$^+$ T cells stained with Cy-conjugated anti-CD3 MoAb to eliminate CD28$^-$ non-T cells and the percentages in total lymphocytes are presented (Fig. 2a). Within the CD4$^+$ T cell population, the percentage of CD28$^+$ cells in SLE (21·3%) was decreased to half of NC (42·9%). Consistent with previous observations, CD28$^-$ T cells were hardly detected in NC [16,18]. However, considerable percentages of CD28$^-$4$^+$ cells were observed in several individuals with SLE, as represented in Fig. 1. Within the CD8$^+$ T cell subset, no significant difference was seen in percentages of CD28$^+$ cells between NC and SLE, while a significant increase ($P < 0·05$) of CD28$^-$ cell percentage was observed in SLE. The percentages of CD28$^-$ T cells in the CD8 subset were remarkably variable in individual SLE patients (range 0·2–40·9%) in contrast with NC (range 1·0–14·7%). The proportions of CD28$^-$ cells in both CD4$^+$ and CD8$^+$ T cells were 11·8% and 45·4% in SLE, and 4·5% and 29·8% in NC, respectively (not shown). We examined the statistical correlation between the increased proportion of CD28$^-$ T cells and clinical parameters including duration of disease, total amount of received corticosteroids, and disease activity, but no correlation was observed (not shown). These results suggest that CD28$^-$ T cells might be expanded in SLE patients. However, lymphopenia is common in SLE, so the increase of percentages of certain cell subsets is not a direct indication of expansion.

We therefore calculated the absolute cell numbers (Fig. 2b). A remarkable reduction of CD28$^+$ T cells was observed, especially within the CD4 subset. This is consistent with a previous

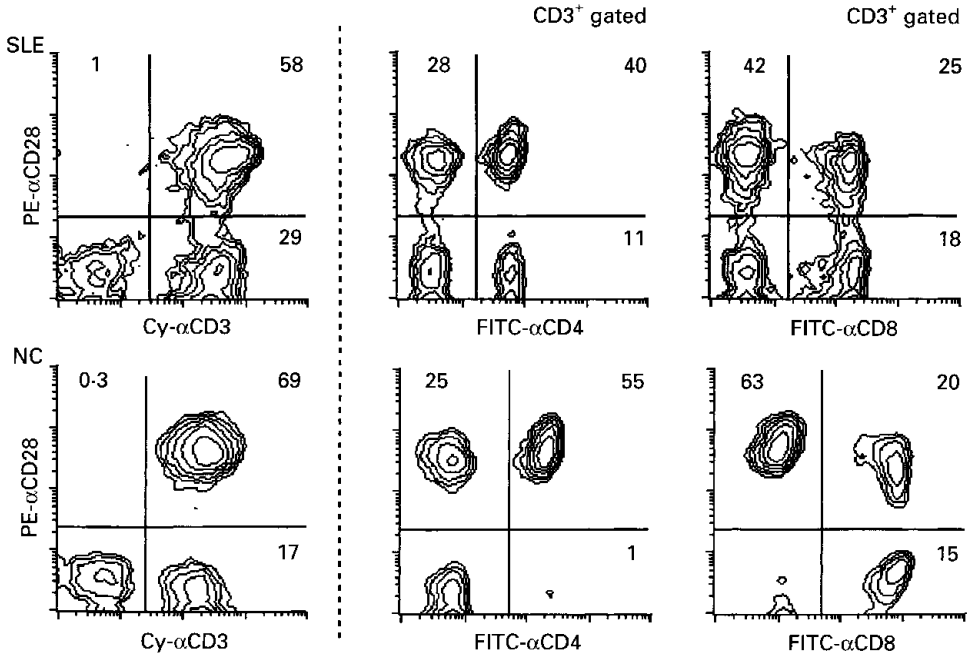


Fig. 1. Expression of CD28 on peripheral blood lymphocytes (PBL) from SLE patients (SLE) and normal controls (NC). Peripheral blood mononuclear cells (PBMC) from healthy adults and SLE patients were stained with FITC-anti-CD4 or anti-CD8, PE-anti-CD28 and Cy-conjugated anti-CD3 MoAbs, or with appropriate fluorochrome-control immunoglobulin (not shown). Samples were analysed by three-colour flow cytometry. Lymphocytes were identified by characteristic forward angle and side scatter profiles. Data are displayed as contour plots (4-decade log scale) and quadrant markers were positioned to include >98% of control immunoglobulin-stained cells in the lower left. In the middle and left panels, an electronic gate was set on CD3$^+$ T lymphocytes and expression of CD28 and CD4 or CD8 is displayed. Representative FACS profiles are presented.

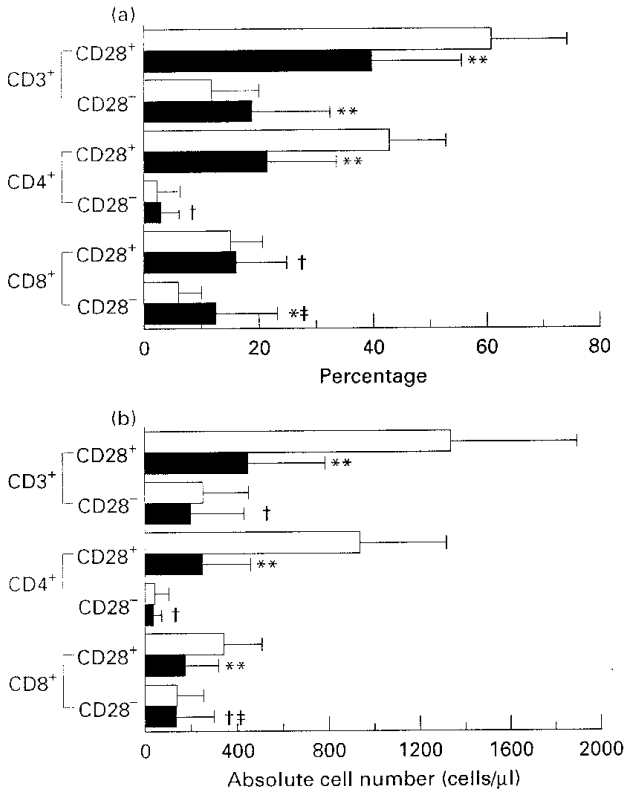


Fig. 2. Comparison of mean percentages (a) and absolute cell numbers (b) of CD28$^+$ and CD28$^-$ T cells between normal controls (NC) (□) and SLE (■). Peripheral blood mononuclear cells (PBMC) from 35 SLE patients and 20 NC were stained as described in Fig. 1. (a) The mean percentage of CD28$^+$ or CD28$^-$ cells co-expressing CD3 within total lymphocytes was determined. For CD4$^+$ or CD8$^+$ cells, an electronic gate was placed on CD3$^+$ T lymphocytes and then the percentage of CD28$^+$ or CD28$^-$ cells co-expressing CD4 or CD8 within total lymphocytes was presented. (b) The absolute cell number of the subsets was determined by multiplying the total lymphocyte count by the percentage of lymphocytes exhibiting the indicated phenotypes. Each value is presented as the mean ± s.d. of 35 (SLE) or 20 (NC) individuals. * $P < 0{\cdot}05$; ** $P < 0{\cdot}001$ compared with NC by Mann–Whitney *U*-test; †not significantly different from NC; ‡not significantly different from CD28$^+$ cells.

demonstration that lymphopenia in SLE was remarkable in the CD4 subset and the ratio of CD4 to CD8 was reduced [32]. We could not see any significant difference in absolute cell number of CD28$^-$ T cells in both CD4 and CD8 subsets between NC and SLE. This clearly indicates that the increase of CD28$^-$ T percentage in SLE resulted from the reduction of CD28$^+$ T cells rather than the expansion of CD28$^-$ T cells. Furthermore, it seems unlikely that CD28$^-$ T cells arose from CD28$^+$ T cells by down-regulation of CD28 expression during activation *in vivo*, because the mean fluorescence intensity of CD28 on CD28$^+$ T cells in each CD4 and CD8 subset from SLE and NC was similar, and we have not observed an obvious CD28^{dim} population in SLE. These observations revealed that CD28$^+$ T cells were selectively lost in PBL from SLE patients.

CD28$^+$ T cells are not generally hyporesponsive in SLE

It has been frequently reported that T cells from SLE patients have dysfunction in *in vitro* proliferation and IL-2 production [33]. This suggests that the decrease of CD28$^+$ T cells in SLE might result from a limited expansion of CD28$^+$ T cells *in vivo*. To see the proliferative ability of T cells from SLE and NC, we examined the proliferative response to CD28 costimulation. PBMC were cultured with optimal amounts of soluble anti-CD28 MoAb (5 μg/ml) or rIL-2 (50 U/ml) in the presence or absence of suboptimal (0·5 μg/ml) or optimal (5 μg/ml) anti-CD3 MoAb. As shown in Fig. 3a, PBMC from SLE showed only slightly lower proliferative responses than those from NC in both culture conditions. Since PBMC from SLE patients contained fewer CD4$^+$ and CD28$^+$ T cells, this minor decrease in proliferation might result from a decrease of such responder T cells. We previously demonstrated that CD28$^-$ T cells possess a limited proliferative capacity when stimulated with allo-antigens [16] or anti-CD3 MoAb [15]. To investigate whether the lower proliferative responses in SLE resulted from the hyporesponsiveness of CD28$^+$ or CD28$^-$ T cells, we compared the proliferative ability of purified CD28$^-$ or CD28$^+$ T cells from NC and SLE patients. In the case of the CD4$^+$ T subset, the isolation of CD28$^-$4$^+$ T cells from NC was difficult, so we compared proliferative responses of highly purified CD28$^+$4$^+$ cells (>97% pure) from NC and SLE. These cells were cultured with anti-CD28 and anti-CD3 MoAbs for 4 days. We observed no significant difference between NC and SLE, though the proliferative responses in SLE were slightly lower than NC (Fig. 3b). In our preliminary experiments, CD28$^-$4$^+$ T cells from SLE failed to proliferate (not shown). As shown in Fig. 3c, we compared the proliferative response of purified CD28$^+$8$^+$ and CD28$^-$8$^+$ T cells between NC and patients. CD28$^-$8$^+$ T cells responded poorly to stimulation with anti-CD3 MoAb and exogenous IL-2 in both NC and SLE. In contrast, CD28$^+$8$^+$ T cells from some SLE patients (as represented by S.O.) showed a lower response, but those from some patients (as represented by M.U.) showed a comparable response to NC. These results indicate that, although CD28$^-$ T cells are similarly hyporesponsive in both NC and SLE, CD28$^+$ T cells from SLE are not generally hyporesponsive to TCR/CD3-mediated stimulation. This suggests that the decrease of CD28$^+$ T cells in SLE does not result from a hyporesponsiveness of these cells *in vivo*.

Activation-induced apoptosis of CD28$^+$ T cells is accelerated in SLE

We next investigated an alternative possibility that CD28$^+$ T cells are more susceptible to elimination by AICD. TCR/CD3 cross-linking has been reported to induce apoptosis in previously activated T cells and T cell lines, but not in normal naive T cells [4,5]. However, there was a report that the spontaneous apoptosis of SLE-PBL was accelerated [34]. We therefore investigated whether activation-induced apoptosis is comparably induced in both CD28$^+$ and CD28$^-$ T subsets. We took advantage of the sensitive reactivity of a lipophilic fluorescent dye, MC540, to apoptotic cells and performed three-colour and five-parameter analysis by flow cytometry. Apoptotic cells were identified by their decreased FSC and increased binding of MC540, as shown in Fig. 4b. In our initial experiments, we did not observe a clear difference in percent apoptotic cells between the MC540 method and a common propidium iodide-staining for apoptotic cells [35] (not shown). Figure 4a shows the appearance of MC540^{high}FSC^{low} apoptotic T cells in cultures with or without anti-CD3 MoAb. In preliminary experiments, it was confirmed that the total cell

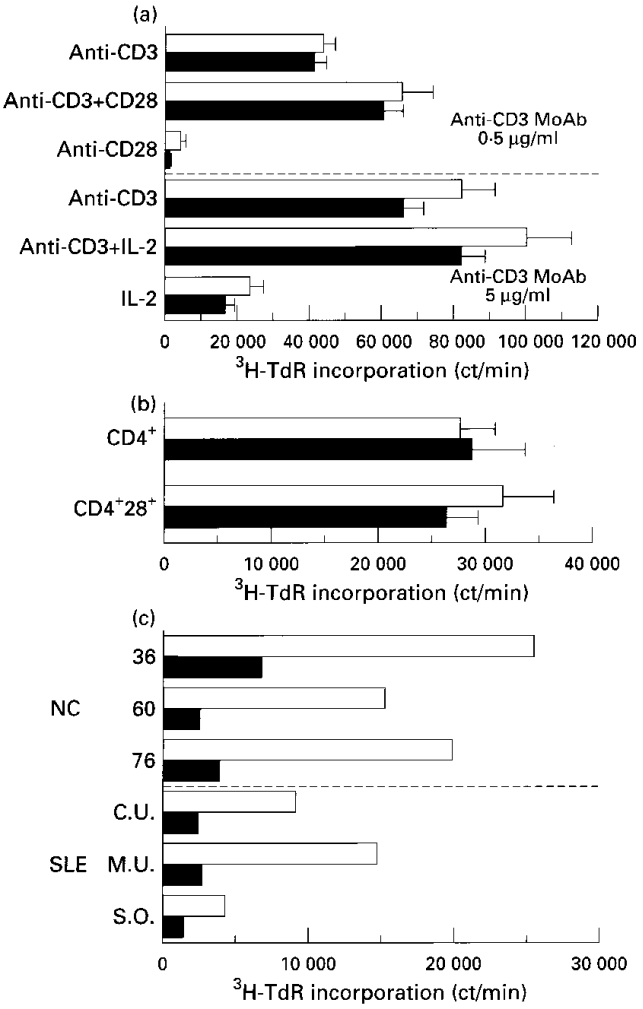


Fig. 3. CD28-dependent or CD28-independent proliferative responses of peripheral blood mononuclear cells (PBMC) or purified $CD4^+$ and $CD8^+$ T cells from SLE patients. (a) Freshly isolated PBMC (1×10^5 cells/well) were cultured with a suboptimal amount of immobilized anti-CD3 (OKT3, 0·5 μg/ml) and/or soluble anti-CD28 (TN228, 10 μg/ml), or with optimal amount of immobilized anti-CD3 (5 μg/ml) and/or 50 U/ml rIL-2, as indicated. Cultures were pulsed overnight with ^{3}H-TdR and harvested on day 4. Values represent the mean ± s.e.m. of five (NC; □) and nine (SLE; ■) individuals. (b) $CD4^+$ T cells were isolated by Dynabeads and DetachaBeads system and then $CD4^+28^+$ T cells were collected by flow cytometric cell sorting. Purified cells (1×10^5 cells/well) were cultured with immobilized anti-CD3 MoAb (0·5 μg/ml) and soluble anti-CD28 MoAb (5 μg/ml) for 4 days. Values represent the mean ± s.e.m. of four (NC; □) and eight (SLE; ■) individuals. (c) $CD8^+$ T cells were stained with FITC-anti-CD16 MoAb and PE-anti-CD28 MoAb and selected for $CD28^+16^-$ and $CD28^-16^-$ cells and used for the assay as $CD8^+28^+$ T cells (□) and $CD8^+28^-$ T cells (■), respectively. Sorted cells were stimulated with immobilized anti-CD3 MoAb (5 μg/ml) and 50 U/ml of rIL-2. Cultures were pulsed overnight with 1 μCi/well ^{3}H-TdR and harvested on day 7. Three individual results from normal controls (NC) and SLE were presented.

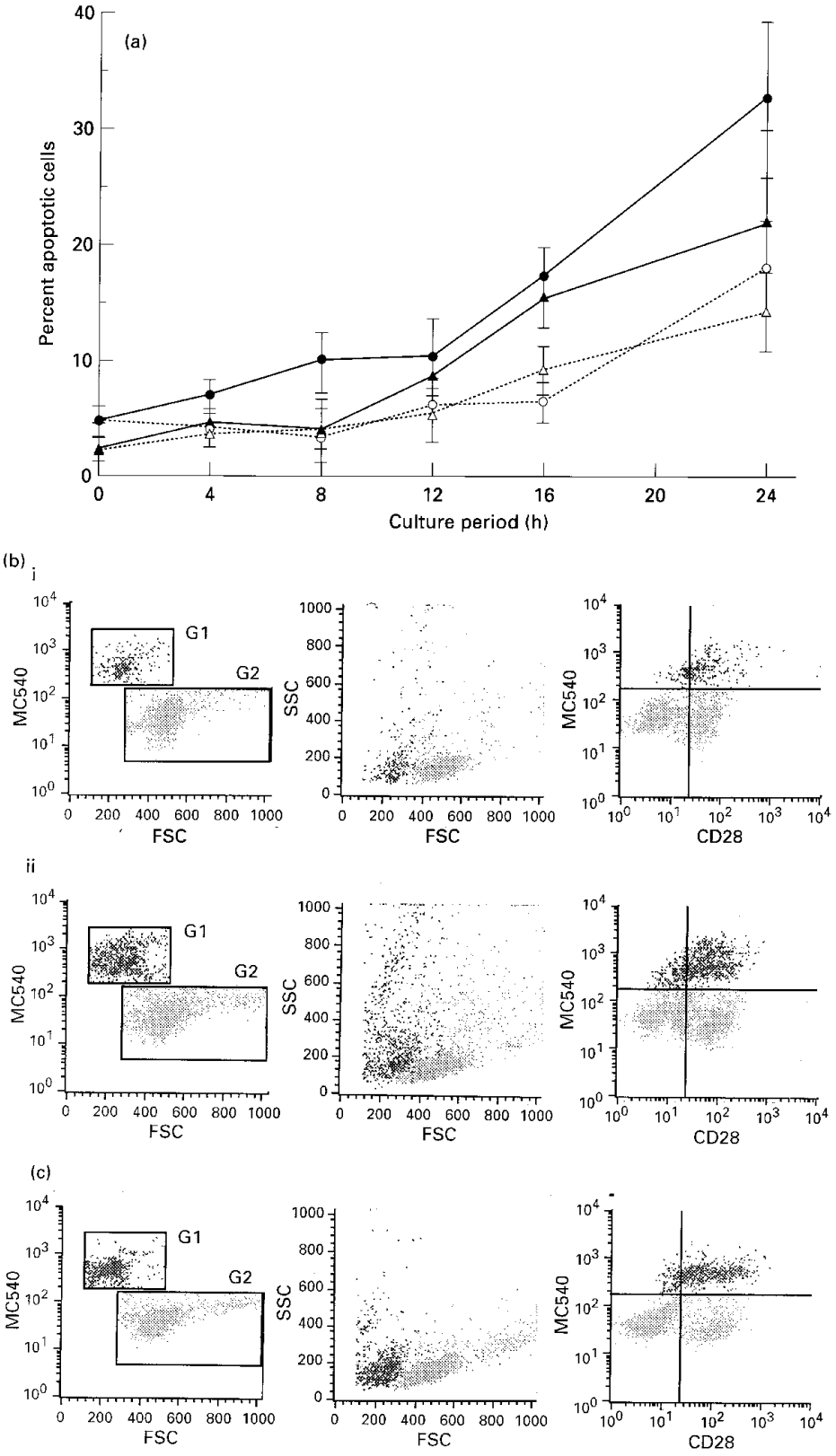
number determined by trypan blue exclusion did not change significantly within the culture up to 24 h. In freshly drawn PBL, 2·1 ± 0·8% and 4·7 ± 1·3% (mean ± s.e.m.) of $CD3^+$ T cells were apoptotic in NC and SLE, respectively. Spontaneous apoptosis in culture gradually occurred in both NC and SLE to a similar extent. Apoptosis of T cells in SLE tended to be augmented by anti-CD3 stimulation from 16 h to 24 h, but this was not statistically significant (Fig. 4a). Apoptotic cells defined by $MC540^{high}FSC^{low}$ were preferentially observed in the $CD28^+$ T cell population, and only a few $CD28^-$ T cells were apoptotic over 24 h in culture of SLE PBMC (Fig. 4b). Figure 5c represents the apoptosis of $CD8^+$ T cells from NC. As was the case in SLE PBMC, apoptotic cells arose preferentially from $CD28^+$ cells. We therefore compared the ratio of apoptotic cells within the $CD28^+$ T cell subset between NC and SLE. As shown in Fig. 4d, the percentage of apoptotic cells was significantly increased in SLE 24 h after anti-CD3 stimulation. These results suggest that $CD28^+$ T cells from SLE patients are more susceptible to activation-induced apoptosis, which may be responsible for the selective loss of $CD28^+$ T cells in SLE.

Expression of CD95 and Bcl-2 in $CD28^+$ and $CD28^-$ T cells

To investigate possible mechanisms for the susceptibility of $CD28^+$ T cells to AICD, we performed comparative analysis of apoptosis-related molecules between $CD28^-$ and $CD28^+$ T cells. CD95, which has been implicated in AICD of preactivated mature T cells, was predominantly expressed on $CD45RO^+$ 'memory' T cells, but not on $CD45RA^+$ 'naive' T cells in NC. Consistent with previous reports [36,37], a significant up-regulation of CD95 was observed in $CD45RA^+$ T cells from SLE. There was no apparent difference in expression patterns of CD95 between CD4 and CD8 T cell subsets (not shown). To compare the levels of CD95 expression between $CD28^+$ and $CD28^-$ T cells, we prepared overlaid histograms of an electronic gate placed on $CD28^+3^+$ and $CD28^-3^+$ cells (Fig. 5b). $CD28^-$ T cells showed a homogeneous and intermediate level of CD95 expression in both NC and SLE. In contrast, $CD28^+$ T cells consisted of two populations with high or low–negative CD95 expression. Some SLE patients, as represented by M.M., exhibited higher CD95 expression on $CD28^+$ T cells than NC, but this was not generally observed in other SLE patients tested. The product of proto-oncogene Bcl-2 has been shown to inhibit apoptosis and to be expressed on T and B cells [38]. Comparison of Bcl-2 protein level in $CD28^+$ and $CD28^-$ T cells showed no significant difference in both NC and SLE (Fig. 5c).

Activation-induced cell surface antigens on $CD28^+$ and $CD28^-$ T cells

To investigate whether the hypoproliferative capacity and resistance to AICD in $CD28^-$ T cells result from the defect in activation, we measured cell surface expression of CD25, CD69, and HLA-DR on both $CD28^+$ and $CD28^-$ T cells after CD3 stimulation (Fig. 6a). CD69 expression was induced during the first 4 h after CD3 stimulation on both $CD28^+$ and $CD28^-$ T cells, and reached a maximal level within 24 h. Although HLA-DR was expressed on freshly isolated T cells from SLE patients but not from NC, expression of CD25 and HLA-DR on $CD28^-$ T cells was comparable to that on $CD28^+$ T cells 24 h after activation. It has been recently reported that CTLA4, which is a structural homologue of CD28, mediated apoptosis of activated T cells *in vitro* [38]. Substantial expression of CTLA4 in normal T cells was detectable at 5 days after stimulation with anti-CD3 MoAb and IL-2. Figure 6b shows the FACS profiles of T cells from SLE stained with MoAbs against CD28 and CTLA4 at days 0, 5, and 7 after stimulation. CTLA4 was preferentially induced on $CD28^+$ T



(See next page for caption.)

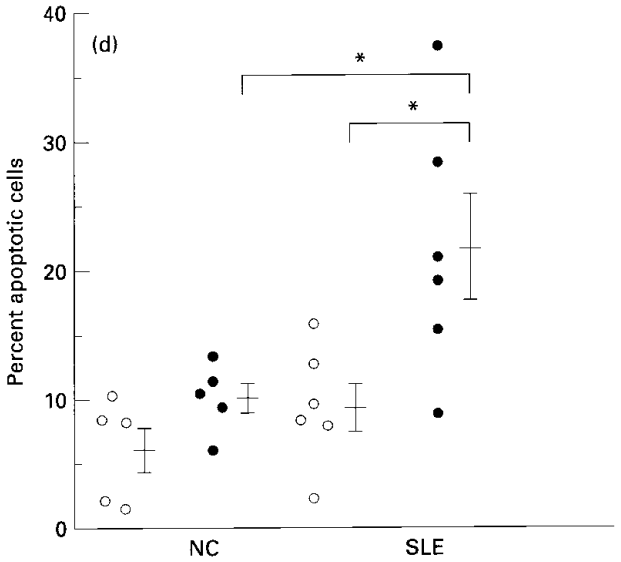


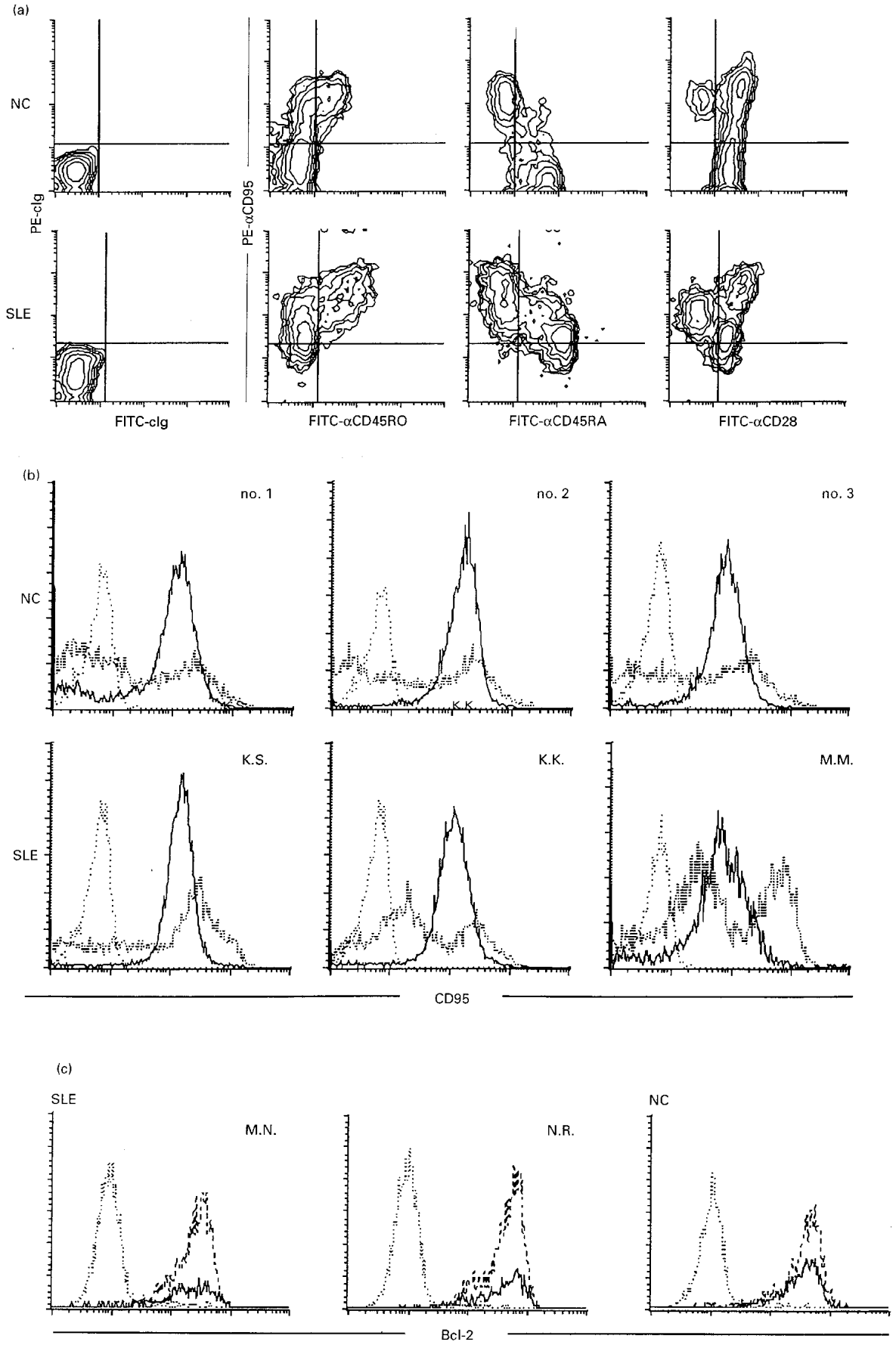
Fig. 4. (See previous page.) Appearance of apoptotic cells in culture. (a) Comparison between normal controls (NC) and SLE. Peripheral blood mononuclear cells (PBMC) from NC (triangles) and SLE (circles) were cultured for 0, 4, 8, 12, 16, and 24 h in anti-CD3 (5 μg/ml)-coated (closed symbols) or uncoated (open symbols) 48-well plates. Cells were recovered, stained with Cy-anti-CD3 MoAb, FITC-anti-CD28 MoAb, and MC540, and analysed by three-colour flow cytometry. An electronic gate was set on the live $CD3^+$ T cells by FSC and fluorescence intensity for CD3. The percentage of apoptotic cells was determined as $MC540^{high}FSC^{low}$ in total $CD3^+$ T cells. Values represent the mean percentage $\pm$ s.e.m. of four (NC) and seven (SLE) individuals. (b,c) CD28 expression on apoptotic cells. Freshly isolated (bi) or 24 h anti-CD3-stimulated (bii) PBMC from SLE and 24 h anti-CD3-stimulated $CD8^+$ cells from NC (c) were analysed. Stained cells were gated on the live $CD3^+$ T cells by FSC and fluorescence intensity and data are displayed as dot plots with the indicated parameters on abscissa and ordinate. In the left panels, rectangular markers were set on $FSC^{low}MC540^{high}$ cells for apoptotic cells (G1) and $FSC^{high}MC540^{low}$ cells for non-apoptotic cells (G2). Results are representative of at least five experiments. (d) The percentage apoptotic cells in $CD28^+$ T cells. PBMC from NC and SLE were cultured in the presence (●) or absence (○) of anti-CD3 MoAb and stained as described above. An electronic gate was set on $CD28^+$ cells and then the percentage of $MC540^{high}FSC^{low}$ cells was calculated. Values represent the mean percentage $\pm$ s.e.m. of five (NC) and six (SLE) individuals. *Statistically significantly different ($P < 0{\cdot}05$).

cells but not on $CD28^-$ T cells, though a significant difference in kinetics of CTLA4 induction between SLE and NC was not observed (not shown). This suggests that CTLA4 preferentially expressed on $CD28^+$ T cells might be responsible for the preferential apoptosis of these cells after activation.

DISCUSSION

In the present study, we demonstrate that $CD28^+$ T cells were selectively decreased in PBMC from patients with SLE. The $CD28^-$ T cell subset is mostly correspondent to the $CD11b^+$ or $CD57^+$ T cell subset in normal PBMC [14,15]. Increased $CD57^+$ or $CD11b^+$ T cell subset in PBMC has been reported after BMT [21] or in the course of infection by cytomegalovirus [22] and Epstein–Barr virus (EBV) [23]. Recently, an increased proportion of $CD28^-$ T cells was also reported in HIV infection [39–41]. In such clinical situations, lymphoproliferative and/or subsequent lymphoatrophic states are often observed in their clinical courses. The increased percentage of $CD28^-$ T cell subset could be caused by preferential expansion of $CD28^-$ T cells and/or preferential elimination of $CD28^+$ T cells in response to antigenic stimulation. We tested which is the case in SLE. Although the etiology of SLE is not clear, aberrant activation of both T and B cells in SLE patients has been reported [42,43]. It has been generally agreed that hyperactivation of B cells characterized by autoantibody production is driven by some autoantigen, T–B cognate interaction, and T cell-derived cytokines. Therefore, abnormal T cell activation by some autoantigen seems to be occurring in SLE *in vivo*. In this study, we demonstrate that $CD28^-$ T cells were not increased in SLE, and these cells from both SLE and NC were hyporesponsive to anti-CD3 stimulation even in the presence of exogenous IL-2. In contrast, $CD28^+$ T cells were selectively decreased in SLE. In our experiments using highly purified $CD4^+28^+$ T cells, we observed unimpaired proliferation of SLE T cells in response to anti-CD3 and -CD28 stimulation. Consistent with our data, Sfikakis *et al.* demonstrated that the anti-CD3-induced proliferative response costimulated with CD80-transfectant was not impaired in SLE patients [44]. It is well known that proliferative responses to phytohaemagglutinin (PHA) and recall antigens are lowered in SLE patients. Since these responses are dependent on CD28-mediated costimulation, as indicated by blocking with Fab fragments of anti-CD28 Fab or anti-CD80 and -CD86 MoAbs, hyporesponsiveness of SLE PBMC to these stimulations seems to result from the decrease of $CD28^+$ T cells. In addition, $CD28^-$ T cells have been proposed to have a suppressive effect against proliferative T cell responses [21,45]. Therefore, relatively increased $CD28^-$ T cells may also have a suppressive effect against proliferative $CD28^+$ T cells in SLE.

Lymphopenia is a common manifestation of human SLE and the murine lupus models. Although it is not well understood why lymphopenia occurs in SLE, several possible explanations have been proposed. First, antilymphocyte antibodies [24] may react



 (Caption on next page.)

specifically with a particular T cell subset. Second, apoptosis may result from aberrant lymphocyte activation. Third, lymphocytes may be sequestered at sites of inflammation or lymphoid tissues. In respect of T cell lymphopenia, certain T cell subsets may be affected selectively by these three factors. Consistent with the studies using two-colour analysis by Alvarado *et al.* [46], we demonstrated strictly that $CD28^+$ T cells are eliminated selectively in SLE. Sfikakis *et al.* [47] suggested that the decrease of $CD28^+8^+$ T cells in peripheral blood of rheumatoid arthritis (RA) patients might result from the migration of these cells to active sites of inflammation, from the comparative examination of both peripheral blood and synovial fluid T cells in RA patients. Although we cannot completely eliminate this possibility, it is unlikely that the migration of $CD28^+$ T cells is a common reason for T cell lymphopenia in SLE, since the homing of T cells to the specific organs is not typical except for certain cases. There were only few studies directly investigating abnormalities in lymphocyte apoptosis of lymphocytes in SLE. Emlen *et al.* reported the up-regulated spontaneous apoptosis of PBL from SLE patients *in vitro* [34]. We observed a significant increase of apoptotic T cells from SLE patients within the $CD28^+$ T cell subset, but not in whole T cell populations. Although this *in vitro* experiment did not reproduce the state of lymphocytes *in vivo*, it is possible that hyperactivated B cells or other APC expressing CD28 ligands augment T cell activation through CD28 costimulation. Therefore, we prefer the explanation that an excessive activation of $CD28^+$ T cells might result in acceleration of *in vivo* apoptosis in SLE patients. It has been demonstrated that CD95 and its ligand play a critical role in AICD of mature T cells [25,26]. On the other hand, Bcl-2 and its related molecules affected lymphocyte survival and resistance to CD95-mediated apoptosis [27,48,49]. Recent studies reveal that defects in the CD95 or CD95 ligand result in autoimmunity in lpr and gld mice, which develop a murine form of lupus [27,50,51]. Although the expression of CD95 and Bcl-2 in SLE was investigated by several groups, no report has focused on the relation to CD28 expression. In a comparative analysis between CD28 and CD95, we found relatively higher CD95 expression in the $CD28^+$ T cell subset. It remains to be determined whether these $CD95^{high}$ cells in the $CD28^+$ population are indeed susceptible to AICD. However, despite up-regulation of CD95 expression, increased apoptosis in SLE PBMC and B cells by the agonistic anti-CD95 MoAb in freshly isolated PBMC was not observed [37]. In addition, several reports indicate that expression of CD95 is not always associated with CD95-mediated apoptosis [52–54]. Recently, human CD95 ligand has been cloned [55] and the induction of CD95 ligand on T cells might be more critical to AICD. CD95 ligand was co-expressed with CD95 on T cells after activation [25,26]. However, at present, expression of CD95 ligand on various T cell subpopulations has not been clarified. We are now trying to detect surface expression of CD95 ligand on anti-CD3-activated T cells from NC and SLE using specific MoAbs against human CD95 ligand [56]. In the analysis for Bcl-2, a high level of Bcl-2 was constitutively expressed in peripheral blood T cells, and we found no significant difference in expression level between $CD28^+$ and $CD28^-$ T cells from SLE. This indicates that Bcl-2 is not critical for determining the susceptibility of $CD28^+$ T cells to AICD. Boise *et al.* reported that CD28 costimulation promoted T cell survival by enhancing the expression of Bcl-xL [57]. Therefore, Bcl-xL induction in $CD28^-$ T cells from SLE remains to be determined.

Recently, CD28 overlapped with CD95 on the sphingomyelin-ceramide signalling pathway. Signalling through both CD95 and CD28 caused sphingomyelin conversion to ceramide by an active form of sphingomyelinase [29,58,59]. The generated ceramide acts as a second messenger for activating a serine/threonine protein kinase [60] and Ras [61] and initiates induction of apoptosis. Therefore, the ceramide generated by the CD28-mediated signalling may act additively with that generated by the CD95-mediated signalling and, thus, enhance AICD of $CD28^+$ T cells. Although ceramide generation appears to be involved in both proliferation and apoptosis, further studies are required to know what factor decides between apoptosis and proliferation. Furthermore, Gribben *et al.* [62] recently suggested that CTLA4 triggers antigen-specific apoptosis. More recently, a negative regulatory role of CTLA4 has been demonstrated by *in vitro* studies [63,64] and by the generation of CTLA4-deficient mice [65,66]. It is possible that CTLA4 rather than CD28 could directly function to eliminate autoreactive T cells or antigen-specific T cells that were no longer needed. At present, it is difficult to discriminate the role of CTLA4 from CD28-mediated T cell activation, since the induction of CTLA4 expression seems to be dependent on CD28-mediated activation ([64] and Fig. 6b). It remains to be determined how CD28 and CTLA4 operate on T cell activation and apoptosis.

In this study, we found that $CD28^+$ T cells were selectively eliminated in PBMC from SLE patients, and surmised that this reduction of $CD28^+$ T cells might be partly due to AICD *in vivo*. In many clinical situations, such as cancer, viral infections, and autoimmune diseases, T cells are activated by certain antigens and then faced with a decision whether they should proliferate, be anergic, or die. To elucidate the exact regulatory mechanisms of the fate of activated T cells may provide an effective way of treatment of such human diseases.

ACKNOWLEDGMENTS

We thank Dr Peter Linsley (Bristol-Myers Squibb Pharmaceutical Research Institute, Seattle, WA) for providing anti-CTLA4 MoAb, and Mr Hironori Matsuda for expert assistance with flow cytometry. This work was supported by grants from the Ministry of Education, Culture, and Science and the Ministry of Health, Japan.

Fig. 5. Expression of apoptosis-associated antigens in $CD28^-$ and $CD28^+$ T cells. (a) Correlation between CD95 and CD45R or CD28 expression. (b) CD95 expression. (c) Bcl-2 expression. Fresh peripheral blood mononuclear cells (PBMC) from normal controls (NC) and SLE were stained with Cy-anti-CD3, FITC-anti-CD28, CD45RO, and CD45RA, and PE-anti-CD95 MoAbs or with an appropriate fluorochrome-control immunoglobulin. An electronic gate was set on $CD3^+$ T cells and correlated expression of CD95 *versus* other antigens is displayed as contour plots (4-decade log scale) (a). In (b,c), an electronic gate was set on $CD3^+28^-$ (solid line) and $CD3^+28^+$ (broken line) cells and expression of CD95 (b) and Bcl-2 (c) are presented as histograms (4-decade log scale) with the immunoglobulin control histograms (dotted line) in each panel. Representative FACS profiles of at least five donors each from NC and SLE are presented.

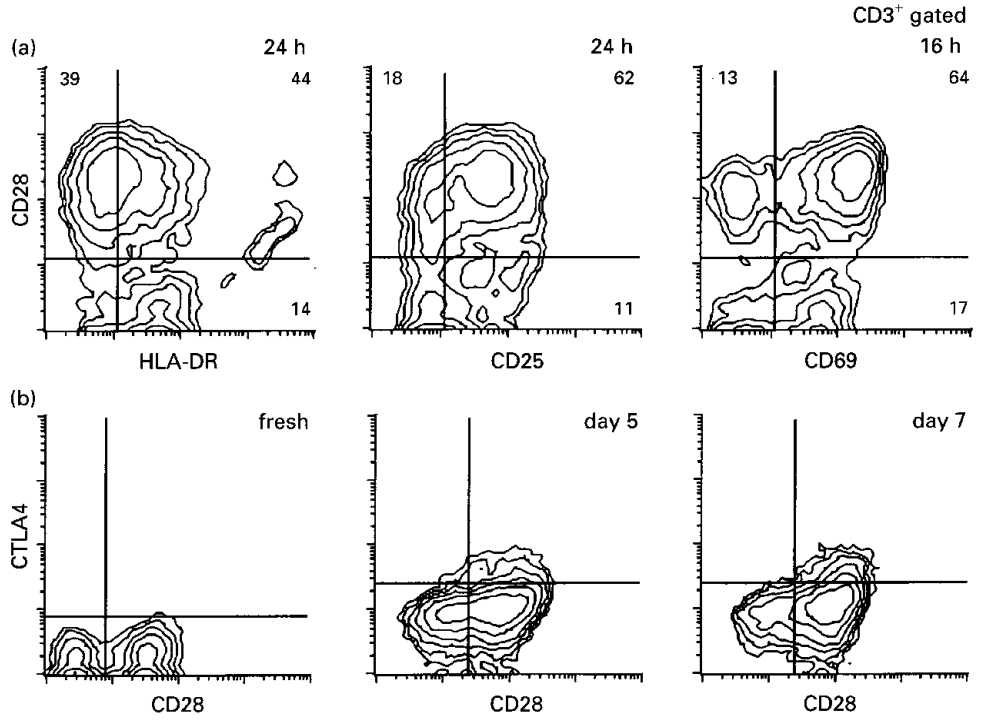


Fig. 6. Activation-induced cell surface antigens on CD28$^+$ and CD28$^-$ T cells. (a) Peripheral blood mononuclear cells (PBMC) from SLE patients were cultured with immobilized anti-CD3 MoAb (5 μg/ml) for 16 or 24 h. Cells were stained with FITC-anti-CD69, anti-CD25, or anti-HLA-DR and PE-conjugated anti-CD28, and Cy-anti-CD3, or with an appropriate fluorochrome-control immunoglobulin (not shown). (b) PBMC were stimulated with immobilized anti-CD3 MoAb for 3 days, then further cultured with rIL-2 (50 U/ml) and harvested for CTLA4 and CD28 expression at the indicated days. Cells were stained with biotinylated anti-CTLA4 (11D4), FITC-anti-CD28, and Cy-anti-CD3 MoAbs, followed by PE-streptavidin or with appropriate fluorochrome controls (not shown). Data are displayed as contour plots (4-decade log scale) and quadrant markers are positioned to include >98% of control immunoglobulin-stained cells in the lower left. Representative data from three patients are shown.

REFERENCES

1 Schwartz RH. A cell culture model for T lymphocyte clonal anergy. Science 1990; **48**:1349–56.

2 Harding FA, McArthur JG, Gross JA *et al.* CD28 mediated signaling costimulates murine T cells and prevents induction of anergy in T cell clones. Nature 1992; **356**:607–9.

3 Russell JH, White CL, Loh DY *et al.* Receptor-stimulated death pathway is opened by antigen in mature T cells. Proc Natl Acad Sci USA 1991; **88**:2151–5.

4 Radvanyi LG, Mills GB, Miller RG. Religation of the T cell receptor after primary activation of mature T cells inhibits proliferation and induces apoptotic cell death. J Immunol 1993; **150**: 5704–15.

5 Wesselborg S, Janssen O, Kabelitz D. Induction of activation-driven death (apoptosis) in activated but not resting peripheral blood T cells. J Immunol 1993; **150**:4338–45.

6 Mueller DL, Jenkins MK, Schwartz RH. An accessory cell-derived costimulatory signal acts independently of protein kinase C activation to allow T cell proliferation and prevent the induction of unresponsiveness. J Immunol 1989; **142**:2617–28.

7 Freeman GJ, Freedman AS, Segil JM *et al.* B7, a new member of the Ig superfamily with unique expression on activated and neoplastic B cells. J Immunol 1989; **143**:2714–22.

8 Freedman AS, Freeman G, Horowitz JC *et al.* B7, a B cell-restricted antigen that identifies preactivated B cells. J Immunol 1987; **139**: 3260–7.

9 Azuma M, Ito D, Yagita H *et al.* B70 antigen is a second ligand for CTLA-4 and CD28. Nature 1993; **366**:76–79.

10 Freeman GJ, Gribben JG, Boussiotis VA *et al.* Cloning of B7-2: a CTLA-4 counter-receptor that costimulates human T cell proliferation. Science 1993; **262**:909–11.

11 Azuma M, Cayabyab M, Buck D *et al.* CD28 interaction with B7 costimulates primary allogeneic proliferative responses and cytotoxicity mediated by small, resting T lymphocytes. J Exp Med 1992; **175**:353–60.

12 Lanier LL, O'Fallon S, Somoza C *et al.* CD80 (B7) and CD86 (B70) provide similar costimulatory signals for T cell proliferation, cytokine production and generation of cytotoxic T lymphocytes. J Immunol 1995; **154**:97–105.

13 Hansen JA, Martin PJ, Nowinski RC. Monoclonal antibodies identifying a novel T-cell antigen and Ia antigens of human lymphocytes. Immunogenetics 1980; **10**:247–60.

14 Yamada H, Martin PJ, Braun MP *et al.* Monoclonal antibody 9·3 and anti-CD11 antibodies define reciprocal subsets of lymphocytes. Eur J Immunol 1985; **15**:1164–8.

15 Azuma M, Phillips JH, Lanier LL. CD28$^-$ T lymphocytes: antigenic and functional properties. J Immunol 1993; **150**:1147–59.

16 Morishita Y, Sao H, Hansen JA *et al.* A distinct subset of human CD4$^+$ cells with a limited alloreactive T cell receptor repertoire. J Immunol 1989; **143**:2783–9.

17 Hoshino T, Yamada A, Honda J *et al.* Tissue-specific distribution and age-dependent increase of human CD11b$^+$ T cells. J Immunol 1993; **151**:2237–46.

18 Pollice AA, McCoy JPJ, Shackney SE *et al.* Sequential paraformaldehyde and methanol fixation for simultaneous flow cytometric analysis of DNA, cell surface proteins, and intracellular proteins. Cytometry 1992; **13**:432–44.

19 Sadat-Sowti B, Debre P, Idziorek T *et al.* A lectin-binding soluble factor released by $CD8^+/CD57^+$ lymphocytes from AIDS patients inhibits T cell cytotoxicity. Eur J Immunol 1991; **21**:737–41.
20 Autran B, Leblond V, Sadat-Soeti B *et al.* A soluble factor released by $CD8^+CD57^+$ lymphocytes from bone marrow transplantated patients inhibits cell mediated cytolysis. Blood 1991; **77**: 2237–41.
21 Leroy E, Calvo CF, Divine M *et al.* Persistence of $T8^+/HNK1^+$ suppressor lymphocytes in the blood of long-term surviving patients after allogeneic bone marrow transplantation. J Immunol 1986; **137**: 2180–9.
22 Maher PA, Singer SJ. Anomalous interaction of the acetylcholine receptor protein with the nonionic detergent Triton X-114. Proc Natl Acad Sci USA 1985; **82**:958–62.
23 Nakata M, Kawasaki A, Azuma M *et al.* Expression of perforin and cytolytic potential of human peripheral blood lymphocyte subpopulations. Int Immunol 1992; **4**:1049–54.
24 Osman C, Swaak AJG. Lymphocytotoxic antibodies in SLE: a review of the literature. Clin Rheumatol 1994; **13**:21–27.
25 Alderson MR, Tough TW, Davis-Smith T *et al.* Fas ligand mediates activation-induced cell death in human T lymphocytes. J Exp Med 1995; **181**:71–77.
26 Ju ST, Panka DJ, Cui H *et al.* Fas (CD95)/FasL interactions required for programmed cell death after T-cell activation. Nature 1995; **373**:444–8.
27 Tan EM. Autoimmunity and apoptosis. J Exp Med 1994; **179**:1083–6.
28 Tan EM, Cohen A, Fries JF *et al.* The 1982 revised criteria for the classification of systemic lupus erythematosus. Arthritis Rheum 1982; **25**:1271–7.
29 Cifone MG, de Manial R, Roncaioli P *et al.* Apoptotic signaling through CD95 (Fas/Apo-1) activates an acidic sphingomyelinase. J Exp Med 1994; **177**:1547–52.
30 Lanier LL, Recktenwald DJ. Multicolor immunofluorescence and flow cytometry. In: Methods: a companion to methods in enzymology 1991; **2**:192–9.
31 Schlegel RA, Stevens M, Lumley-Sapanski K *et al.* Altered lipid packing identifies apoptotic thymocytes. Immunol Letters 1993; **36**:283–8.
32 Bakke AC, Kirkland PA, Kitridou RC *et al.* T lymphocyte subsets in systemic lupus erythematosus. Arthritis Rheum 1983; **26**: 745–50.
33 Horwitz DA, Stohl W. T lymphocyte, cytokine, and immune regulation in systemic lupus erythematosus. In: Wallace DJ, Hahn BH, eds. Dubois' lupus erythematosus, 4th edn. London: Lea & Febiger, 1993:83–96.
34 Emlen W, Niebur J, Kadera R. Accelerated *in vitro* apoptosis of lymphocytes from patients with systemic lupus erythematosus. JImmunol 1994; **152**:3685–92.
35 Nicoletti I, Migliorati G, Paglicci MC *et al.* A rapid and simple method for measuring thymocyte apoptosis by propidium iodide staining and flow cytometry. J Immunol Methods 1991; **139**:271–9.
36 Amasaki Y, Kobayashi S, Takeda T *et al.* Up-regulated expression of Fas antigen (CD95) by peripheral naive and memory T cell subsets in patients with systemic lupus erythematosus (SLE): a possible mechamism for lymphopenia. Clin Exp Immunol 1995; **99**:245–50.
37 Mysler E, Bini P, Drappa J *et al.* The apoptosis-1/Fas protein in human systemic lupus erythematosus. J Clin Invest 1994; **93**:1029–34.
38 Boise LH, Ding L, Lindsten T *et al.* bcl-x, a bcl-2-related gene that functions as a dominant regular of apoptotic cell death. Cell 1993; **74**:597–608.
39 Lewis DE, Ng Tang DS, Adu-Oppong A *et al.* Anergy and apoptosis in $CD8^+$ T cells from HIV-infected persons. J Immunol 1994; **153**:412–20.
40 Borthwick NJ, Bofill M, Gombert WM *et al.* Lymphocyte activation in HIV-1 infection. II. Functional defects of $CD28^-$ T cells. AIDS 1994; **8**:431–41.
41 Choremi-Papadopoulou H, Viglis V, Gargalianos P *et al.* Downregulation of CD28 surface antigen on $CD4^+$ and $CD8^+$ T lymphocytes during HIV-1 infection. J AIDS 1994; **7**:245–53.
42 Klinman DM. B cell abnormalities characteristic of systemic lupus erythematosus. In: Wallace DJ, Hahn BH, eds. Dubois' lupus erythematosus, 4th edn. London: Lea & Febiger, 1993:77–82.
43 Via CS, Handwerger BS. B-cell and T-cell function in systemic lupus erythematosus. Curr Opin Rheumatol 1993; **5**:570–4.
44 Sfikakis PP, Oglesby R, Sfikakis P *et al.* B7/BB1 provides an important costimulatory signal for CD3-mediated T lymphocyte proliferation in patients with systemic lupus eythematosus (SLE). Clin J Immunol 1994; **96**:8–14.
45 Koide J, Engleman EG. Differences in surface phenotype and mechanism of action between alloantigen-specific $CD8^+$ cytotoxic and suppressor T cell clones. J Immunol 1990; **144**:32–40.
46 Alvarado C, Alcocer-Varela J, Llorente L *et al.* Effect of CD28 antibody on T cells from patients with systemic lupus erythematosus. J Autoimmun 1994; **7**:763–73.
47 Sfikakis PP, Zografou A, Viglis V *et al.* CD28 expression on T cell subsets *in vivo* and CD28-mediated T cell response *in vitro* in patients with rheumatoid arthritis. Arthritis Rheum 1995; **38**:649–54.
48 Oltvai ZN, Milliman CL, Korsmeyer SJ. Bcl-2 heterodimerizes *in vivo* with a conserved homolog, bax, that accelerates programed cell death. Cell 1993; **74**:609–19.
49 Itoh N, Tsujimoto Y, Nagata S. Effect of bcl-2 on Fas antigen-mediated cell death. J Immunol 1993; **151**:621–7.
50 Watanabe-Fukunaga R, Brannan CI, Copeland NG *et al.* Lymphocyte proliferation disorder in mice explained by defects in Fas antigen that mediates apoptosis. Nature 1992; **356**:314–7.
51 Cohen PL, Eisenberg RA. The lpr and gld genes in systemic autoimmunity: life and death in the Fas lane. Immunol Today 1992; **13**:427–8.
52 Miyawaki T, Uehara T, Nibu R *et al.* Differential expression of apoptosis-related Fas antigen on lymphocyte subpopulations in human peripheral blood. J Immunol 1992; **149**:3753–8.
53 Owen-Schaub LB, Yonehara S, Crump III WL *et al.* DNA fragmentation and cell death is selectively triggered in activated human lymphocytes by Fas antigen engagement. Cell Immunol 1992; **140**: 197–205.
54 Klas C, Debatin K-M, Jonker RR *et al.* Activation interferes with the APO-1 pathway in mature human T cells. Int Immunol 1993; **5**:625–30.
55 Takahashi T, Tanaka M, Inazawa J *et al.* Human Fas ligand: gene structure, chromosomal location and species specificity. Int Immunol 1994; **6**:1567–74.
56 Kayagaki N, Kawasaki A, Ebata T *et al.* Metalloproteinase-mediated release of human Fas ligand. J Exp Med 1995; **182**:1777–82.
57 Boise LH, Minn AJ, Noel PJ *et al.* CD28 costimulation can promote T cell survival by enhancing the expression of bcl-xL. Immunity 1995; **3**:87–98.
58 Boucher L-M, Wiegmann K, Fütterer A *et al.* CD28 signals through acidic sphingomyelinase. J Exp Med 1995; **181**:2059–68.
59 Chan G, Ochi A. Sphingomyelin-ceramid turnover in CD28 costimulatory signaling. Eur J Immunol 1995; **25**:1999–2004.
60 Haimovitz-Friedman A, Kan C-C, Ehleiter D *et al.* Ionizing radiation acts on cellular membranes to generate ceramide and initiate apoptosis. J Exp Med 1994; **180**:525–35.
61 Gulbins E, Bissonnette R, Mahboubi A *et al.* Fas-induced apoptosis is mediated via a ceramid-initiated RAS signaling pathway. Immunity 1995; **2**:341–51.
62 Gribben JG, Freeman GJ, Boussiotis VA *et al.* CTLA4 mediates antigen-specific apoptosis of human T cells. Proc Natl Acad Sci USA 1995; **92**:811–5.
63 Klummel MF, Allison JP. CD28 and CTLA-4 have opposing effects on the response of T cells to stimulation. J Exp Med 1995; **182**:459–65.
64 Walunus TL, Lenshow DJ, Bakker CY *et al.* CTLA-4 can function as a negative regulator of T cell activation. Immunity 1994; **1**:405–13.

65 Waterhouse P, Penninger JM, Timms E *et al.* Lymphoproliferative disorders with early lethality in mice deficient in Ctla-4. Science 1995; **270**:985–8.

66 Tivol EA, Borriello F, Schweitzer N *et al.* Loss of CTLA-4 leads to massive lymphoproliferation and fatal multiorgan tissue destruction, revealing a critical negative regulatory role of CTLA-4. Immunity 1995; **3**:541–7.