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Up-regulated expression of Fas antigen (CD95) by peripheral naive and memory T cell subsets in patients with systemic lupus erythematosus (SLE): a possible mechanism for lymphopenia

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SUMMARY

Fas antigen (CD95) is a membrane-associated molecule that mediates apoptotic cell death and may play a role in the induction and maintenance of T cell tolerance. To elucidate the involvement of Fas antigen in human autoimmune diseases, we analysed Fas antigen expression by peripheral T cells from patients with SLE and rheumatoid arthritis (RA), using three-colour flow cytometry. Both $CD4^+$ and $CD8^+$ T cells from SLE patients expressed Fas antigen in a higher density than did these cells from healthy donors and from RA patients. Enhancement of Fas antigen density was noted in $Fas^+CD45RO^+$ memory T cells from SLE patients. More remarkably, a significant expression of Fas antigen was observed in $CD45RO^-$ naive T cells from SLE patients. $CD4^+CD45RO^-$ T cells from SLE patients co-expressed Fas antigen and early to intermediate activation antigens such as CD25 and CD71, and late activation antigen HLA-DR in only $Fas^{hi}CD4^+$ naive T cells. Such up-regulation of Fas antigen expression in SLE patients seems to be clinically meaningful, because mean fluorescence intensity (MFI) of Fas antigen on $CD4^+$T cell subsets inversely correlates with the absolute size of $CD4^+$ T cell subsets in peripheral blood of SLE patients. These results suggest that T cells with increased Fas antigen expression may be highly susceptible to apoptotic cell death, *in vivo*. A putative mechanism for lymphopenia in SLE patients is discussed.

Keywords systemic lupus erythematosus Fas antigen T cell subsets lymphopenia

INTRODUCTION

Apoptotic cell death is thought to be involved in the negative selection of immature autoreactive T cells during differentiation and maturation in the thymus [1,2]. Yonehara *et al.* [3] and Trauth *et al.* [4] established mouse MoAbs, anti-Fas and anti-APO-1, respectively, both of which induce apoptosis in cells expressing an identical surface molecule, Fas/APO-1 antigen (CD95). Sequence analysis of human [5,6] and mouse [7] Fas antigen cDNA revealed this molecule to be a member of the tumour necrosis factor (TNF) receptor/nerve growth factor receptor family, as are CD40 [8], OX40 [9] and CD27 [10].

For both $CD4^+$ and $CD8^+$ T cells from human peripheral blood, Fas antigen is preferentially expressed by $CD45RO^+$ memory, but not by $CD45RO^-$ naive T cells [11]. Fas antigen is, however, inducible in naive T cells by *in vitro* stimulation with mitogen [11], and in IL-2-activated lymphocytes [12]. Of particular interest is that despite the expression of Fas antigen on the cell surface, memory T cells are resistant to anti-Fas-mediated apoptosis [11], whereas a murine cell line transfected with human Fas antigen cDNA [3] and a portion of IL-2-activated lymphocytes are sensitive to this apoptosis [12]. Thus, apoptosis appears to depend mainly on the periods of activation or stimulation, and seems to require particular *in vivo* conditions. Fas antigen expression might reflect previous or ongoing *in vivo* antigen-specific or non-specific activation. Whether Fas antigen on peripheral lymphocytes plays a crucial role in regulating immune responses to self or non-self antigens remains to be determined.

Mice bearing the *lpr* gene are characterized by massive lymphadenopathy and lupus-like autoimmunity [13]. Lymphadenopathy is a recessive trait and is due to the non-malignant expansion of unique T cell subsets. Watanabe-Fukunaga *et al.* [14] showed that *lpr* mice express little Fas antigen mRNA because of rearrangement of the Fas antigen gene, and they speculated that a defect in Fas antigen-mediated apoptosis may be responsible for massive lymphadenopathy and

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autoimmunity, resulting from the excessive release of self-reactive T cells escaping from apoptosis in the thymus. We and other groups demonstrated that the insertion of an early transposon into the intron of the Fas antigen gene suppresses the expression of intact Fas antigen mRNA [15–17] and leads to autoimmune disease [17]. The defect in Fas antigen-mediated apoptotic signalling thus appears to be closely associated with the induction or exacerbation of autoimmunity. In this study we present the results obtained by flow cytometric analysis of peripheral blood T cells from autoimmune diseases and by correlation analysis between Fas antigen mean fluorescence intensity (MFI) on T cell subsets and various clinical parameters.

PATIENTS AND METHODS

Patients and clinical features

Studies were carried out on 25 Japanese patients with SLE (one man and 24 women). All patients satisfied the American Rheumatism Association criteria for SLE (1982) [18]. Their mean age at the time of examination was 35 ± 9 years. As to the Lupus Activity Criteria Count, designed by Urowitz *et al.* [19], six patients were categorized as active, and the others inactive. Most patients were taking oral corticosteroid in small doses up to 17 mg/day. To characterize disease activity, clinical signs and symptoms were estimated as well as haemoglobin, platelet, leucocyte and lymphocyte counts, serum C3, serum CH50, anti-DNA antibody (radioimmunoassay (RIA)), anti-RNP antibody (enzyme immunoassay (EIA)), anti-Sm antibody (EIA), ESR (Westergren), and serum IgG level. As controls, 18 healthy Japanese volunteers (three men and 15 women, mean age 32 ± 6 years) were examined, as well as 18 patients with rheumatoid arthritis (RA) (two men and 16 women, mean age 50 ± 14 years), who fulfilled criteria of the American College of Rheumatology [20].

Staining reagents

Mouse anti-human Fas MoAb (IgM) [3] was purchased from MBL (Nagoya, Japan). Biotin-labelled rat anti-mouse IgM MoAB (Zymed Labs Inc., South San Francisco, CA) and PE-labelled streptavidin (Biomeda Corp., Foster City, CA) were used for indirect staining following anti-Fas MoAb. Cells were phenotyped using peridinin chlorophyll protein (PerCP)-labelled anti-Leu-3a (CD4) and anti-Leu-2a (CD8) (Becton Dickinson Immunocytometry Systems, Mountain View, CA), and FITC-labelled UCHL-1 (CD45RO) (Dakopatts, Copenhagen, Denmark). FITC- or PE-labelled anti-Leu-M3 (CD14), anti-Leu-12 (CD19) and anti-Leu-16 (CD56) (Becton Dickinson) as well as FITC-labelled anti-Leu-4 (CD3), anti-Leu-3a (CD4) and anti-Leu-2a (CD8), were also used to determine the percentage of corresponding subpopulations. FITC-labelled mouse anti-human Fas MoAb (IgG) (MBL) was used to analyse sorted naive $CD4^+$ T cells, combined with PE-labelled anti-IL-2Rα (CD25), anti-HLA-DR (Becton Dickinson) and anti-transferrin receptor (CD71) (Leinco Technology Inc., St Louis, MO).

Flow cytometry and cell sorting

Heparinized venous blood samples were obtained from patients and healthy volunteers, after informed consent has been obtained. Peripheral blood mononuclear cells (PBMC) were isolated by Ficoll–Paque (Pharmacia LKB Biotechnology AB, Uppsala, Sweden) density gradient centrifugation and washed in PBS supplemented with 0·1% sodium azide (standard staining buffer). The cells were incubated sequentially with anti-Fas MoAb or irrelevant IgM MoAb, biotin-labelled rat anti-mouse IgM MoAb, and PE-streptavidin. Following incubation with 5% normal mouse serum as a blocking reagent, the cells were stained with FITC-labelled UCHL-1 and PerCp-labelled anti-Leu-3a or anti-Leu-2a MoAb. The appropriate concentrations of MoAb used were predetermined, and all incubations were run for 30 min at 4°C in the dark.

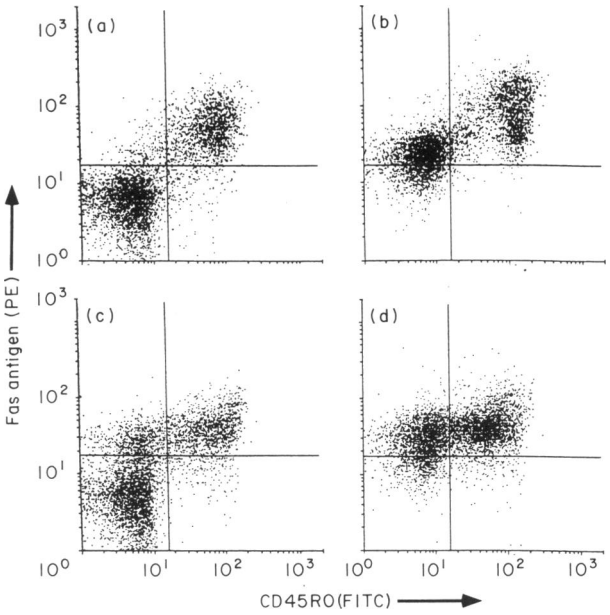

Fig. 1. Fas antigen expression by T cell subsets from SLE patients. Two-dimensional dot plots displaying Fas antigen (PE) and CD45RO (FITC) expression are shown for $CD4^+$ (a,b) and $CD8^+$ (c,d) T cells from healthy donors (a,c) and SLE patients (b,d). Staining protocols are as described in Patients and Methods.

Two- or three-colour analysis was performed on a FACScan flow cytometer (Becton Dickinson). A gate for lymphocyte population was defined by forward and side light scatter characteristics, and FL3 (PerCP)-strongly positive areas were further gated as $CD4^+$ or $CD8^+$ T cell populations for subsequent two-colour analysis. Cells in these gates were positive for CD3 and contained only a negligible percentage of monocytes (CD14), B cells (CD19) and natural killer (NK) cells (CD56) (data not shown). In other experiments, $CD4^+CD45RO^-$ T cells were sorted on FACS Vantage (Becton Dickinson), using PerCP-labelled anti-CD4 and FITC-labelled anti-CD45RO MoAb. Over 99% of sorted cells were positive for CD4 and negative for CD45RO. These populations were used for two-colour analysis on FACScan, after being stained with FITC-labelled anti-Fas MoAb and PE-labelled anti-CD25, anti-CD71 or anti-HLA-DR.

Statistical analysis

The Mann–Whitney U-test was used to determine the statistical significance of difference between MFI of Fas antigen on T

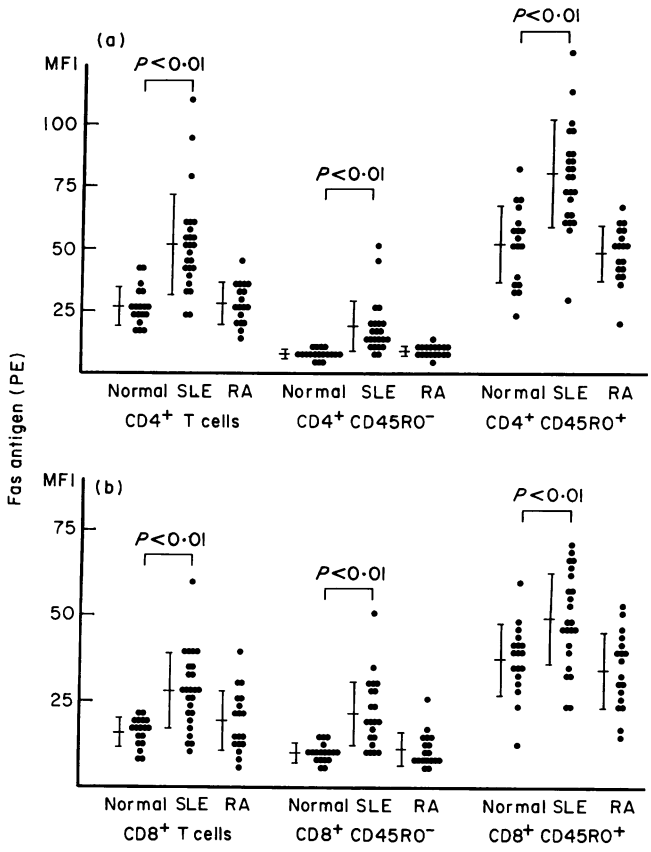

Fig. 2. A significant increase in mean fluorescence intensity (MFI) of Fas antigen expressed by T cell subsets from SLE and rheumatoid arthritis (RA) patients. MFI of Fas antigen on $CD4^+$ (a) and $CD8^+$ (b) T cell subsets from healthy donors ($n = 18$), SLE patients ($n = 25$) and RA patients ($n = 18$) is indicated on the ordinate. The horizontal lines and vertical bars represent mean MFI ± s.d. of each group. The difference in MFI of Fas antigen between normal and SLE in each T cell subset is statistically significant ($P < 0{\cdot}01$, Mann–Whitney U-test).

cell subsets from patients and from normal healthy donors. Correlations between the Fas antigen MFI and disease activity parameters of SLE were calculated by Spearman's rank correlation procedures.

RESULTS

Fas antigen expression on T cell subsets from SLE and RA patients

We examined Fas antigen expression by peripheral blood T cells obtained from SLE and RA patients. To investigate differences in Fas antigen expression among T cell subpopulations and their maturational stages identified by CD45RO expression, we performed a three-colour flow cytometric analysis using FACScan, and obtained two-colour dot plots of Fas antigen and CD45RO on gated $CD4^+$ or $CD8^+$ T cells. As described previously [11], Fas antigen was constitutively expressed by CD45 RO^+ memory T cells from SLE and RA patients as well as from healthy donors. In our analysis of normal T cells, however, a very small population of $CD4^+CD45RO^-$ and a significant percentage of $CD8^+CD45RO^-$ T cells also expressed Fas antigen, albeit at a lower density (Fig. 1a,c). Of particular interest was that all $CD4^+CD45RO^-$ or $CD8^+CD45RO^-$ T cells from SLE patients expressed Fas antigen, yet the expression remained weaker than seen with $CD45RO^+$ T cells (Fig. 1b,d). Since the Fas antigen-positive fraction in each subset could not be demarcated by its continuous expression in most patients, we used MFI to evaluate the degree of surface Fas antigen expression. As shown in Fig. 2, the results of individual MFI clearly indicate that Fas antigen density was significantly enhanced in all fractions of T cells from SLE patients. There was no significant increase in Fas antigen expression in T cell subsets from patients with RA (Fig. 2).

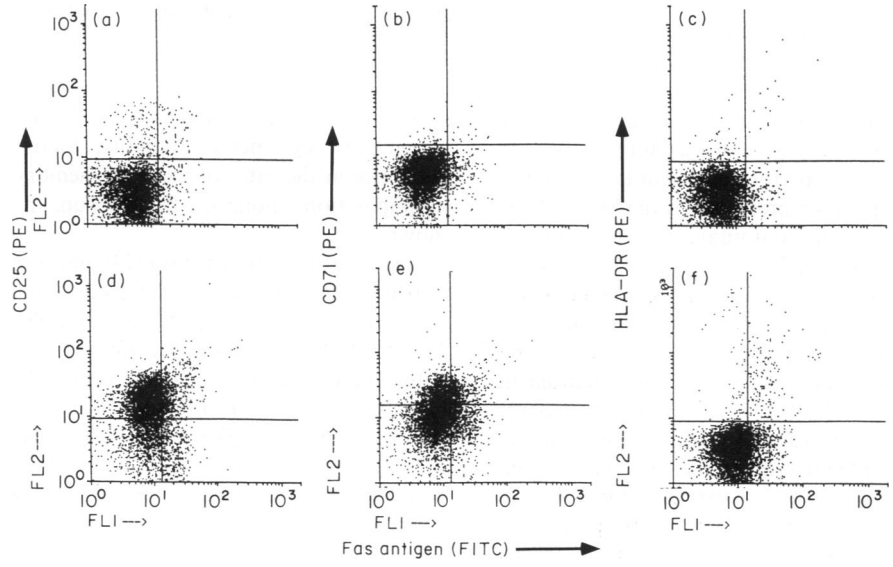

Fig. 3. Representative profiles of Fas antigen and other activation antigens co-expressed on naive $CD4^+$ T cell subset from a healthy adult (a,b,c) and a patient with SLE (d,e,f). Naive $CD4^+$ T cells (peridinin chlorophyll protein (PerCP)-$CD4^+$ FITC-$CD45RO^-$) were sorted, and further stained for Fas antigen (FITC) *versus* CD25 (a,d), CD71 (b,e), or HLA-DR (c,f) (PE).

Co-expression of other activation antigens on naive T cells from SLE patients

To elucidate the profiles of $CD4^+CD45RO^-$ T cells characteristic of SLE patients, we further examined the expression of other surface activation antigens by this T cell subset. Cells strongly positive for CD4 (PerCP) and negative for CD45RO (FITC) were sorted and subjected to two-colour analysis using anti-Fas MoAb (FITC) and MoAb to activation antigens (PE), CD25, CD71 or HLA-DR (Fig. 3). In SLE patients, naive $CD4^+$ T cells co-expressed Fas antigen and activation antigens, CD25 and CD71. This subset from SLE patients also expressed HLA-DR, but interestingly, the expression was limited to the subpopulation of Fas antigen^{hi} naive $CD4^+$ T cells.

Correlation between Fas antigen density and size of T cell subsets

We next analysed the correlation between the up-regulation of Fas antigen and the disease activity of lupus. Fas antigen intensity exhibited no significant correlation with any but one of the blood test parameters of lupus activity, including platelet count, serum complement level, anti-DNA antibody titre, the dose of corticosteroid at the time of examination, and so on (data not shown). There was, however, a statistically significant inverse correlation between the absolute size of $CD4^+$ T cell subsets in peripheral blood and Fas antigen MFI on the same subsets. As can be seen in Fig. 4, as they expressed Fas antigen at a higher density, circulating $CD4^+$ T cells, of both naive and memory subsets, were reduced in number, and this was not the case in $CD8^+$ T cell subsets.

DISCUSSION

In the present study, we obtained evidence that the surface expression of Fas antigen is up-regulated on both naive and memory subsets in $CD4^+$ and $CD8^+$ T cell subpopulations from SLE patients. Recently, Mysler *et al.* [21] reported an increase in the percentage of Fas/APO-1 antigen positive T cells in both $CD4^+$ and $CD8^+$ T cell compartments from SLE and RA patients. Since Fas antigen is constitutively expressed by $CD45RO^+$ memory T cells [11], interpretation should be made with care, as SLE patients have a changed ratio of naive to memory T cells. To avoid any ambiguity, we adopted three-colour flow cytometric analysis with a combination of MoAb to Fas antigen, CD45RO, and CD4 or CD8. Therefore, our results should reflect direct expression of Fas antigen by each T cell subset. Furthermore, we performed quantitative studies on relative Fas antigen densities represented by the MFI values, for the following reasons: (i) the continuous expression of Fas antigen by naive T cells cannot delineate negative and positive fractions; and (ii) all memory T cells from any donor constitutively express Fas antigen at a higher density. Thus, our data clearly indicate that Fas antigen MFI of both naive and memory subsets in $CD4^+$ and $CD8^+$ T cells is enhanced in SLE patients compared with healthy donors and RA patients (Fig. 2). However, our data regarding Fas antigen expression in RA patients are inconsistent with data of Mysler *et al.* [21], who found a greater increase in Fas antigen expression in RA than in SLE patients. This may be partly explained by the relative increase in memory T cells in RA patients, who are generally older than SLE patients, since there is an age-related shift from a naive to a memory phenotype in the $CD4^+$ T cell compartment [22]. In this respect, it seems advantageous to analyse each

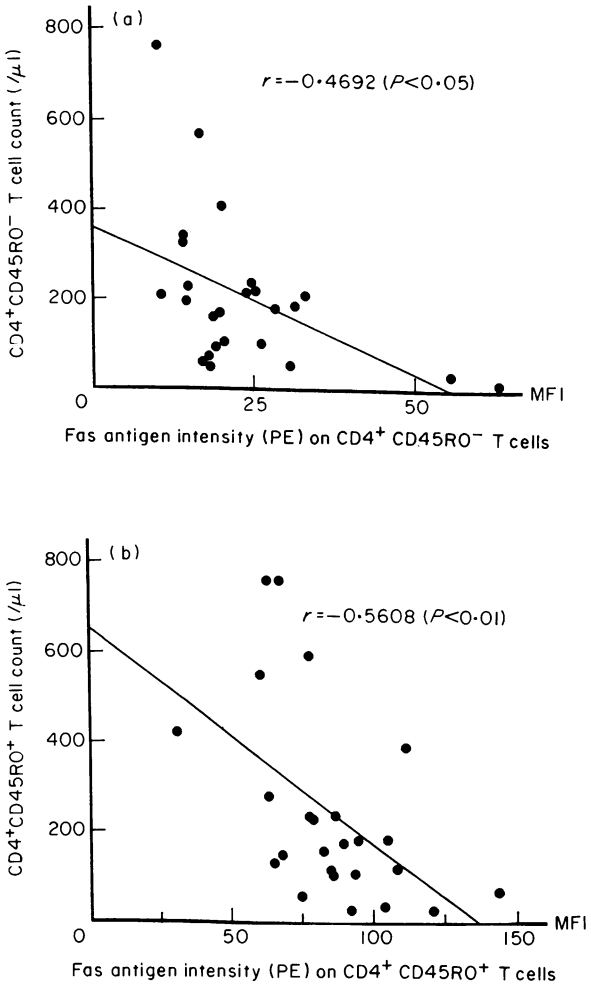

Fig. 4. Inverse correlations between mean fluorescence intensity (MFI) of Fas antigen on $CD4^+$ T cell subsets and their absolute numbers in peripheral blood of SLE patients. Cell numbers of $CD4^+CD45RO^-$ (a) and $CD4^+CD45RO^+$ (b) T cell subset were calculated from its percentage and total lymphocyte counts when examined. Each *r* and *P* value is indicated (Spearman's rank correlation test).

subset independently, and not T cells as a whole, by multi-colour flow cytometry. Actually, there was no significant difference in the ratio of naive to memory cells in each T cell subpopulation among the three groups in our study (data not shown).

Deficient IL-2 production [23] and increased expression of activation antigens such as CD25 [24] and MHC class II [25,26] were noted with peripheral blood T cells from SLE patients. Taken together with the inducibility of Fas antigen after *in vitro* mitogenic stimulation of naive T cells [11], the up-regulation of Fas antigen observed in this study might reflect *in vivo* T cell activation processes. We wish to stress that $CD45RO^-$ naive T cells express Fas antigen as a characteristic of SLE patients, because they have been reported to be negative for Fas antigen in healthy adults [11]. We were, therefore, interested to investigate the co-expression of Fas antigen and other activation antigens by naive T cells from SLE patients. To do so, we restained sorted $CD4^+CD45RO^-$ T cells with anti-Fas MoAb and MoAb to CD25, CD71, or HLA-DR. As expected, $CD4^+$

naive T cells from SLE patients expressed CD25 strongly and CD71 intermediately as well as Fas antigen, compared with those from healthy donors (Fig. 3). It appeared that CD71 rather than CD25 expression was more in parallel with Fas antigen expression. In marked contrast, HLA-DR was expressed only by cells bearing Fas antigen in a higher density, thereby indicating that $CD4^+$ naive T cells from SLE patients can be divided into two subpopulations, $Fas^{low}DR^-$ and $Fas^{hi}DR^+$. As these activation antigens are expressed in their own kinetics after *in vitro* stimulation of T cells [27], it may be possible to distinguish the activation stage of T cells as early (CD25), intermediate (CD71) and late (HLA-DR) markers. Such differential expression of activation antigens suggests that most $CD4^+$ naive T cells in SLE are predominantly in an early to intermediate stage of activation after recent antigenic stimulation *in vivo*, while a minor subpopulation of naive T cells expressing $Fas^{hi}DR^+$ is in a later stage of activation. Since it has been shown that *in vitro* activation of naive T cells from adults results in phenotypic and functional conversion of $CD45RA^+$ to $CD45RO^+$ cells [28,29], it is of importance to define functionally two subpopulations of $CD4^+$ naive T cells from SLE patients with regard to transition into memory cells.

Although the up-regulation of Fas antigen was accompanied by an increased expression of early activation molecules by $CD4^+$ naive T cells in SLE, we found no correlation between Fas antigen MFI on T cells and various serological parameters reflecting SLE activity, i.e. the up-regulation of Fas antigen was observed equally in active and inactive SLE patients. The one exception was a significant inverse correlation between Fas antigen intensity and absolute number of each $CD4^+$ T cell subset in the peripheral blood samples (Fig. 4). This finding suggests a putative role for the enhanced Fas antigen in lymphopenia, a common manifestation in SLE patients. Although anti-lymphocyte autoantibodies [30] and T cell adhesion abnormalities [31] have been noted in such cases, whether or to what extent they play a role in inducing lymphopenia is unknown. The apoptosis-susceptibility of lymphocytes from lupus patients has recently been reported [32]. In general, mature peripheral T cells are known to be resistant to the induction of apoptosis by anti-Fas MoAb [11] or anti-CD3 MoAb [33], while anti-Fas-mediated apoptosis has been shown to be inducible in peripheral T cells subjected to extended stimulation with phytohaemagglutinin (PHA) and IL-2 [12]. We confirmed that freshly isolated PBMC from SLE patients also exhibited resistance to anti-Fas-mediated apoptosis in cell viability and DNA fragmentation assays, and found no significant change in percentage of T cell subpopulations and subsets during *in vitro* 2-day culture with anti-Fas MoAb (data not shown). Additional *in vivo* activation or conditions that are not easily reproduced *in vitro* seem to be required for activated T cells from SLE patients to be triggered to undergo apoptosis by Fas antigen–ligand interaction. Although human Fas ligand has not yet been identified, it is of interest to examine whether Fas ligand as well as Fas antigen is dysregulated in SLE. Alternatively, the CD4 molecule itself may be involved in the apoptotic mechanism for CD4-depleting lymphopenia in SLE. Cumulative evidence has shown that ligation or cross-linking of the CD4 molecule by anti-CD4 MoAb [34] or HIV envelope molecule gp120 [35] induces apoptotic T cell death. Intriguingly, anti-CD4-mediated T cell apoptosis is not induced in Fas antigen-defective MRL/Mp-*lpr*/*lpr* mice, indicating that this type of T cell depletion is Fas antigen-dependent [36]. Furthermore, Fas antigen expression is significantly increased in T cells from $HIV\text{-}1^+$ children [37]. All these results lead to the notion that CD4 engagement may up-regulate Fas antigen expression directly or indirectly, and render $CD4^+$ T cells susceptible to apoptotic cell death. The natural ligand for CD4 is MHC class II. In SLE, enhanced MHC class II [25,26] may act like anti-CD4 antibodies or HIV gp120, and may be a potent inducer of CD4-mediated T cell depletion. We are currently examining whether or not CD4 ligation facilities anti-Fas-mediated apoptosis of $CD4^+$T cell subsets from SLE patients.

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