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CD226 Expression Deficiency Causes High Sensitivity to Apoptosis in NK T Cells from Patients with Systemic Lupus Erythematosus

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Humans and mice with systemic lupus erythematosus (SLE) and related autoimmune diseases have reduced numbers of NK T cells. An association between NK T cell deficiency and autoimmune disease has been identified. However, the mechanisms for reduction of NK T cell number in patients with SLE are unknown. In the present study we report that NK T cells from active SLE patients are highly sensitive to anti-CD95-induced apoptosis compared with those from normal subjects and inactive SLE patients. CD226 expression is deficient on NK T cells from active SLE patients. The expression of one antiapoptotic member protein, survivin, is found to be selectively deficient in freshly isolated NK T cells from active SLE patients. CD226 preactivation significantly up-regulates survivin expression and activation, which can rescue active SLE NK T cells from anti-CD95-induced apoptosis. In transfected COS7 cells, we confirm that anti-CD95-mediated death signals are inhibited by activation of the CD226 pathway through stabilization of caspase-8 and caspase-3 and through activation of survivin. We therefore conclude that deficient expression of CD226 and survivin in NK T cells from active SLE is a molecular basis of high sensitivity of the cells to anti-CD95-induced apoptosis. These observations offer a potential explanation for high apoptotic sensitivity of NK T cells from active SLE, and provide a new insight into the mechanism of reduction of NK T cell number in SLE and understanding the association between NK T cell deficiency and autoimmune diseases.


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Abbreviations used in this paper: SLE, systemic lupus erythematosus; aSLE, active SLE; iSLE, inactive SLE; Pl, propidium iodide; SLEDAI, SLE disease activity index.
expression on platelets as well, and also known as DNA accessory molecule-1 (17–19), is a transmembrane glycoprotein characterized by two extracellular Ig-like domains and a cytoplasmic portion containing three tyrosine residues (17). CD226 belongs to an Ig superfamily (17–19) and, with ligands CD112 and CD155 (20), is highly conserved among humans, apes, and monkeys, suggesting very important biological functions (21, 22), such as mediating cytotoxicity and signaling transduction of T cell activation and differentiation (19–27). CD226 is expressed by virtually all human NK cells, T cells, monocytes, and a small subset of B cells (17–20). CD226 cooperates with other triggering NK receptors in the induction of NK-mediated cytotoxicity (20). CD226 mediates the intercellular binding between thrombin-activated platelets and HUVEC (25). CD226 and CD18/CD11a molecules are physically and functionally associated in normal cells and are involved in a costimulatory signal for naïve T cell differentiation and proliferation (26, 27). CD226/CD112 and CD155 interactions may play a role in the interaction between leukocytes and endothelial cells in the process of leukocyte recirculation (20). To date, there has been no report on the correlation between CD226 signals and regulation of cell apoptosis.

In the present study we report that NK T cells from active SLE patients are more sensitive to anti-CD95-induced apoptosis. CD226 is significantly down-regulated on NK T cells from active SLE patients. Survivin is selectively deficient in NK T cells from active SLE patients. In transfected COS-7 cells and human NK T cells, we confirm that anti-CD95-mediated death signals are inhibited by activation of the CD226 pathway through activation of survivin. A deficiency of CD226 and survivin in NK T cells from active SLE is the molecular basis of the high sensitivity of the cells to anti-CD95-induced apoptosis.

Materials and Methods

Patients, normal subjects, and cell purification

SLE patients who fulfilled at least four of the American Rheumatism Association 1982 revised criteria for SLE (26) were included. The 47 SLE patients (43 women and four men; median age, 32.7 years; range, 20–49 years) had a mean duration of disease of 4.2 years (0.5–11 years). Disease activity was assessed by a modified SLE disease activity index (SLEDAI) score (29). The mean SLEDAI for the active patients was 30 (range, 3–30), and that for inactive patients was 0 (29). All patients were informed consent according to institutional guidelines. A total of 20 healthy volunteers (18 women and two men; median age, 29.5 years; range, 18 – 46 years) had a mean duration of disease of 3–30 years, and that for inactive patients was 0 (29). All patients were informed consent according to institutional guidelines. The mean SLEDAI for the active patients was 14.8 (range, 5–27), and that for inactive patients was 0 (29). All patients were informed consent according to institutional guidelines. The mean SLEDAI for the active patients was 14.8 (range, 5–27), and that for inactive patients was 0 (29). All patients were informed consent according to institutional guidelines. The mean SLEDAI for the active patients was 14.8 (range, 5–27), and that for inactive patients was 0 (29).

Induction of apoptosis and CD226 activation or blockade of purified NK T cell

For induction of apoptosis of purified NK T cells using immobilized anti-CD95 mAb (ECOS9.1) or anti-CD3 mAb (UCHT1; from BD Pharmingen), the assays were conducted as described previously (32, 33). We generated a mouse anti-human mAb against CD226. For CD226 activation, anti-CD226 mAb was immobilized by adding it to the wells in culture plates (2 μg/ml) at 4°C overnight, followed by washing twice before use. A single cell suspension was added to the plate and cultured for 8 h before subsequent procedures. For CD226 blockade, signal cell suspension was added to soluble mAb against CD226 (5 μg/ml) and kept at room temperature for 4 h before subsequent procedures.

Flow cytometry

For detection of CD226, the cells were incubated with anti-CD226 mAb (5 μg/ml) at 4°C for 60 min, followed by an appropriately labeled secondary Ab. Analysis of Vα24+Vβ11+ NK T cells was performed on PBMC or purified NK T cell. FITC-labeled anti-human Vα24 mAb and PE-labeled anti-human Vβ11 mAb were used (Immunotech). For CD95 and CD95L detection, cells were first incubated with CD95 mAb (clone 13) or CD95L mAb (clone 33; BD Biosciences Europe) at 5 μg/ml or 5 μg/ml isotype-matched mAb (DakoCytomation) in staining buffer (34). After 20 min, the cells were washed twice with staining buffer and resuspended in 50 μl of CyChrome-conjugated secondary mAb (BD Pharmingen) for 20 min. For detection of apoptosis, cells were stained in staining medium (RPMI 1640, 2% FBS, and 0.1% sodium azide) with 1 μg/ml propidium iodide for 30 min at 4°C, then stained with FITC-conjugated annexin V with binding buffer (BD Pharmingen) as previously described (35). Coulter XL was used for analyses.

Real-time quantitative RT-PCR assay

For real-time quantitative RT-PCR, we used specific primers that were designed previously (36, 37). Briefly, RNA from purified NK T cells (1 × 10^6; purity >99%) was using Quick Prep total RNA extraction kit (Pharmacia Biotech) according to the manufacturer’s instructions. RNA was reverse transcribed using SuperScript II reverse transcriptase (Invitrogen Life Technologies). The real-time quantitative PCR was performed in special optical tubes in a 96-well microplate reader (Applied Biosystems) with an ABI PRISM 7700 Sequence Detector System. Using SYBR Green PCR Core Reagents Kit (Applied Biosystems; cat. No. 4304886), fluorescence signals were generated for each PCR cycle via the 5’ to 3’ endonuclease activity of AmpliTaq Gold to provide real-time quantitative PCR information. The sequences of the specific primers are listed below: CD226 sense, 5’-TCAATAAGCCA CATTTTGGGA-3’; CD226 antisense, 5’-AGGATATATGGC GTTTACAC-3’; and survivin sense, 5’-GTGTTTCCTCTGCTTCAAGGA AGGC-3’. CD226 cDNA probe, labeled by [α-32P]dCTP, was obtained by PCR amplification of the sequence mentioned above from total RNA of PBMCs (from PBMCs of patients and healthy subjects) and hybridized overnight with 1 × 10^5 cpm/μl [32P]-labeled probe, followed by intensively washing before being autoradiographed. For protein detection (Western blot analysis), the cells were lysed in lysis buffer (0.4% Nonidet P-40, 150 mM NaCl, 50 mM Tris-HCl, 1 mM sodium vanadate, 0.1 mM PMSF, and 2 μg/ml leupeptin and antipetase, pH 8.0) as previously described (21). Lysates were centrifuged at 10,000 g for 15 min at 4°C. Protein concentration was measured using a Bio-Rad protein assay. Protein (40 μg) was loaded onto 16% SDS-PAGE, transferred onto polyvinylidene difluoride membranes after electrophoresis, and incubated with the appropriate Abs (anti-Bcl-2, clone BCL-2/120; anti-caspase-3, clone CPP32–19 (BD Pharmingen); anti-caspase-8, clone H-134; anti-survivin, clone FL-142 (Santa Cruz Biotechnology); anti-β-actin (Sigma-Aldrich); all at 0.5 μg/ml). Analyses were conducted using ECL detection (Amer sham Biosciences).

Cell transient transfection

For mRNA detection (Northern blot), 2 μg of total RNA obtained from each sample was electrophoresed under denaturing conditions, followed by blotting onto Nitran membranes and cross-linking by UV irradiation as previously described (38). CD226 cDNA probe, labeled by [α-32P]dCTP, was obtained by PCR amplification of the sequence mentioned above from total RNA of PBMCs (from PBMCs of patients and healthy subjects) and hybridized overnight with 1 × 10^5 cpm/μl [32P]-labeled probe, followed by intensively washing before being autoradiographed. For protein detection (Western blot analysis), the cells were lysed in lysis buffer (0.4% Nonidet P-40, 150 mM NaCl, 50 mM Tris-HCl, 1 mM sodium vanadate, 0.1 mM PMSF, and 2 μg/ml leupeptin and antipetase, pH 8.0) as previously described (39). Lysates were centrifuged at 10,000 g for 15 min at 4°C. Protein concentration was measured using a Bio-Rad protein assay. Protein (~40 μg) was loaded onto 16% SDS-PAGE, transferred onto polyvinylidene difluoride membranes after electrophoresis, and incubated with the appropriate Abs (anti-Bcl-2, clone BCL-2/120; anti-caspase-3, clone CPP32–19 (BD Pharmingen); anti-caspase-8, clone H-134; anti-survivin, clone FL-142 (Santa Cruz Biotechnology); anti-β-actin (Sigma-Aldrich); all at 0.5 μg/ml). Analyses were conducted using ECL detection (Amer sham Biosciences).

Sensitivity of SLE NK T cells to apoptosis

Sensitivity of SLE NK T cells to apoptosis was measured by a modified 5′-endonuclease activity of AmpliTaq Gold to provide real-time quantitative PCR information. The sequences of the specific primers are listed below: CD226 sense, 5′-TCAATAAGCCA CATTTTGGGA-3′; CD226 antisense, 5′-AGGATATATGGC GTTTACAC-3′; and survivin sense, 5′-GTGTTTCCTCTGCTTCAAGGA AGGC-3′. All unknown cDNAs were diluted to contain equal amounts of B-actin cDNA. The standard, no template controls, and unknown samples were added in a total volume of 50 μl of a reaction. The PCR conditions were 2 min at 50°C, 10 min at 95°C, and 40 cycles of 15 s at 95°C and 60 s at 60°C for each amplification. Potential PCR product contamination was digested to avoid any cross-contamination by dUTP. Lysates were electrophoresed and AmpliTaq Gold (PE Applied Biosystems) were applied according to the manufacturer’s instruction.
The gatings in the forward scatter and side scatter histograms were described in Materials and Methods. Cells were analyzed by flow cytometry for PI (x-axis) and FITC-conjugated annexin V (y-axis) as described in Materials and Methods. The cultures in the absence of stimuli showed the unstimulated basic lines (a, d, h, and l). The apoptotic responses in inactive SLE (iSLE) patients were marginally increased (e and j). As a positive control, cells were stimulated with camptothecin, which induces identical apoptosis in all three types of subjects (c, g, and k), whereas isotype Abs for anti-CD95 and anti-CD3 did not induce apoptosis in the cells (data not shown). The cell cultures for distinct subject groups in the absence of stimuli showed the unstimulated basic lines of cell apoptosis (d, h, and l). The summary of data (mean ± SD) for apoptotic cells (PL annexin V+) of six experiments performed in each subject group is shown in Fig. 1B, documenting the sensitivity to apoptosis of NK T cells from aSLE patients. As shown in Fig. 1C, the total fractions of dead cells (including apoptotic and necrotic) in NK T cells from normal subjects and iSLE patients were significantly lower than that in aSLE patients in anti-CD95- and anti-CD3-induced apoptotic assays, but identical levels were observed in camptothecin-induced apoptosis in all three types of subjects. These results could confirm that NK T cells from active SLE were highly sensitive to apoptotic induction by the CD95-CD95L pathway or the CD3-TCR pathway. To explore the reasons for the phenomena observed above, we examined the levels of CD95 and CD95L expression on NK T cells from various subjects. Flow cytometry revealed that CD95 was expressed in identical levels on NK T cells from different subjects (Fig. 2), and the expression of CD95L on NK T cells from aSLE patients could not be explained by the levels of CD95 and CD95L expression on NK T cells from various subjects; otherwise, there were no significant differences in sensitivity to anti-CD95-induced apoptosis in distinct types of cells among aSLE, iSLE, and normal subjects (data not shown).

FIGURE 1. Analysis of apoptotic and total dead (necrotic and apoptotic) NK T cells. Flow cytometric analysis of apoptotic (A and B) and total dead (C) NK T cells after stimulation of immobilized anti-CD95 mAb, anti-CD3 mAb, or camptothecin. NK T cells were first purified from aSLE patients, iSLE, and normal subjects (NML) as described in Materials and Methods, and followed culture with apoptotic inducers for 24 h. As a positive control, cells were cultured with camptothecin (5 μM) for 6 h (Comp.). The cultures in the absence of stimuli showed the basic lines (unstimulated). Cells were analyzed by flow cytometry for PI (x-axis) and FITC-conjugated annexin V (x-axis) as described in Materials and Methods. The gatings in the forward scatter and side scatter histograms were microliters of 3 × 10⁶ cell suspension mixed with 0.25 or 2.5 μg of cDNA was transferred to the provided cuvette and nucleofected with an Amaxa Nucleofector apparatus (Amaxa).

Results

NK T cells from active SLE are more sensitive to apoptosis

Knowing the abnormality of apoptosis of some types of immune cells in patients with SLE and in mice with lupus (7–9), we examined the death-promoting effect of signals transmitted through CD95-CD95L and CD3-TCR on NK T cells from different subjects. Flow cytometric analysis (Fig. 1) revealed that the numbers of apoptotic and necrotic cells were significantly increased in cultures of NK T cells from active SLE patients (aSLE) in the presence of immobilized anti-CD95 or anti-CD3 mAb (Fig. 1, a and b; p < 0.001) compared with that in cell cultures from normal subjects (i and j). The apoptotic responses in inactive SLE (iSLE) patients were marginally increased (e and j). As a positive control, cells were stimulated with camptothecin, which induces identical apoptosis in all three types of subjects (c, g, and k), whereas isotype Abs for anti-CD95 and anti-CD3 did not induce apoptosis in the cells (data not shown). The cell cultures for distinct subject groups in the absence of stimuli showed the unstimulated basic lines of cell apoptosis (d, h, and l). The summary of data (mean ± SD) for apoptotic cells (PL annexin V+) of six experiments performed in each subject group is shown in Fig. 1B, documenting the sensitivity to apoptosis of NK T cells from aSLE patients. As shown in Fig. 1C, the total fractions of dead cells (including apoptotic and necrotic) in NK T cells from normal subjects and iSLE patients were significantly lower than that in aSLE patients in anti-CD95- and anti-CD3-induced apoptotic assays, but identical levels were observed in camptothecin-induced apoptosis in all three types of subjects. These results could confirm that NK T cells from active SLE were highly sensitive to apoptotic induction by the CD95-CD95L pathway or the CD3-TCR pathway. To explore the reasons for the phenomena observed above, we examined the levels of CD95 and CD95L expression on NK T cells from various subjects. Flow cytometry revealed that CD95 was expressed in identical levels on NK T cells from different subjects (Fig. 2), and the expression of CD95L on NK T cells from aSLE patients could not be explained by the levels of CD95 and CD95L expression on the cells. We also examined the sensitivity of total CD3+ T cells, B cells, and monocytes to anti-CD95-induced apoptosis. The results showed that aSLE CD3+ T cells were slightly, but significantly, more sensitive to anti-CD95-induced apoptosis than cells from iSLE and normal subjects; otherwise, there were no significant differences in sensitivity to anti-CD95-induced apoptosis in distinct types of cells among aSLE, iSLE, and normal subjects (data not shown).

CD226 expression is deficiently expressed on NK T cells from aSLE patients

We examined the expressions of some adhesion molecules (L-selectin, CD18/CD11a, CD15, CD28, CD29, CD31, CD34, CD44, adhesion to the lymphocyte region. The percentages of PI− annexin V− cells and PI+ annexin V+ cells are indicated. The data in A were from a single experiment, which was representative of six experiments performed. B, The data for apoptotic cells (PI− annexin V+) were the mean ± SD of six experiments performed. C, The data for total dead cells (PI− annexin V+ and PI+ annexin V+) were the mean ± SD of six experiments performed. Statistically significant differences compared with controls are indicated (*, p < 0.001).
CD49a-e, CD51, CD54/ICAM-1, CD62E, and VCAM-1/VLA-4) on NK T cells from three types of subjects, and either the results were in agreement with previous reports or no differences were found among the three types of subjects (data not shown). To search for the mechanism of the high apoptotic sensitivity of NK T cells from aSLE patients, we examined the expression of CD226 on NK T cells from aSLE patients, iSLE patients, and normal subjects. The results from flow cytometric analyses shown in Fig. 3 documented that there were different numbers of NK T cells in the three groups of subjects. There were only 1.5% of Vα24/Vβ11+ NK T cells of the total T cell population in aSLE patients and 21% of CD226-positive cells (Fig. 3A). There were 8% Vα24/Vβ11+ NK T cells of the total T cell population in normal subjects and 88% of CD226-positive cells (Fig. 3C), whereas there were 4% Vα24/Vβ11+ NK T cells of the total T cell population in inactive SLE patients and 47% of CD226-positive cells (Fig. 3B). To confirm the observation mentioned above, we performed a real-time quantitative RT-PCR assay to detect the different expressions of CD226 at the mRNA level. The results (Fig. 3D) showed that CD226 mRNA was expressed at high levels in freshly isolated NK T cells from normal subjects (1.3 × 10^4 copies). CD226 mRNA expressions was significantly decreased in NK T cells from aSLE patients (1.2 × 10^3 and 0.9 × 10^3 copies in two separate cases) compared with that in normal subjects, whereas there was an increase in NK T cells from the iSLE patients (6 × 10^3 copies). A linear relationship between cycle threshold and log starting quantity of standard DNA template or target cDNAs was detected (data not shown). In all experiments, the correlation coefficients were ~0.95–0.99. The same pattern of CD226 mRNA and protein expressions in NK T cells from aSLE

FIGURE 2. CD95 and CD95L expression on NK T cells. CD95 (A) and CD95L (B) expression on purified NK T cells was examined by flow cytometry. The cells were freshly isolated purified from aSLE patients, iSLE patients, and normal subjects (NML) as described in Materials and Methods, followed by Vα24/Vβ11+ staining for NK T cells and consequent CD95 or CD95L staining as described in Materials and Methods. Isotype Ab controls are expressed as dashed curves. The gatings in the forward scatter and side scatter histograms adhered to the lymphocyte region. The numbers represent the percentages of CD95+ and CD95L+ cells as indicated. The numbers in parentheses are the mean fluorescence intensities (MFIs). The data were from a single experiment, which was representative of five similar experiments performed.

FIGURE 3. CD226 detection in Vα24/Vβ11+ NK T cells. Triple-color flow cytometric analysis of CD226 on Vα24/Vβ11+ NK T cells from aSLE patients (A), iSLE patients (B), and normal subjects (NML; C). The numbers are percentages of Vα24/Vβ11+ NK T cells or CD226-positive cells. The numbers in parentheses are the mean fluorescence intensities (MFIs). Dashed curves indicate isotype Ab controls. The data were from a representative of at least six similar experiments performed. E, Northern blot of CD226 mRNA in NK T cells. The hybridization signals for CD226 mRNA in NK T cells are shown in the upper panels, and 28S rRNA signals are shown in the lower panels. The data shown are from a representative experiment of six performed. F, CD226 protein examined by Western blot. The arrows were used to verify equivalent molecular weights of appropriate proteins in each lane. The illustrated data were from a representative experiment of four performed.
patients, iSLE patients, and normal subjects was confirmed by Northern and Western blots (Fig. 3, E and F). We also examined the CD226 expression levels in total CD3⁺ T cells, B cells, and monocytes. There were no significant differences in CD226 expression levels in total CD3⁺ T cells, B cells, and monocytes from aSLE, iSLE, and normal subjects, determined by real-time RT-PCR assay and confirmed by Northern and Western blot analyses (data not shown).

To further investigate the correlation between the sensitivity to apoptosis and the level of CD226 expression on the cells mentioned above, we conducted anti-CD95-induced apoptosis in NK T cells from normal, iSLE, and aSLE subjects by preactivating and blocking the function of CD226. We used immobilized or soluble anti-CD226 mAb to activate or block CD226 on the cells, respectively. As shown in Fig. 4A, preactivation of CD226 could rescue NK T cells from aSLE patients from anti-CD95-induced apoptosis, although CD226 expression was low in freshly isolated cells (Fig. 3). Compared with the results shown in Fig. 1Aa (55% of dead cells), there were only 14% of dead NK T cells from active SLE, in which the cells were preactivated by anti-CD226 (Fig. 4Aa). The blockade function of CD226 reduced the resistance of anti-CD95-induced apoptosis in NK T cells from normal subjects, although CD226 expression was high in freshly isolated cells. The percentage of total dead cells reached 58% (Fig. 4A). Isotype Ab had no such effects (Fig. 4A, c, f, and i). As shown in Fig. 4B, the total fractions of dead cells (including apoptotic and necrotic) in NK T cells from different subjects showed patterns similar to that in Fig. 4A, confirming that activation of CD226 could rescue NK T cells from anti-CD95-induced apoptosis, whereas blockade of CD226 function could significantly reduce the resistance of NK T cells from aSLE patients to anti-CD95-induced apoptosis. Of note, the explanation for the finding that CD226 activation still could rescue low expressed CD226 aSLE NK T cells from apoptosis could be that abundant binding sites of CD226 in experimental system (anti-CD226 mAb at 2 μg/ml was immobilized) were sufficient to activate aSLE NK T cells through limited CD226 receptors. We also observed that preactivation of CD226 could rescue NK T cells from active SLE patients from anti-CD95-induced apoptosis (data not shown), confirming the rescuing function of CD226 activation from apoptosis.

Mechanisms of CD226 involved in high sensitivity to anti-CD95-induced apoptosis in NK T cells from iSLE patients

To examine the mechanisms of CD226 expression and activation involved in high sensitivity to anti-CD95-induced apoptosis in NK T cells from active SLE patients, we first examined the expression levels of some important substrates of the apoptosis pathways in the cells. Western blots revealed that key apoptotic executioner molecules, procaspase-8 and procaspase-3, were expressed at identical levels in freshly isolated NK T cells from different subjects (Fig. 5A). Western blot showed that the protein levels of one antiapoptotic member, Bcl-2, in freshly isolated NK T cells from different subjects were also identical (Fig. 5B). Interestingly, one antiapoptotic member protein, survivin, was selectively expressed at very low levels or was absent in freshly isolated NK T cells from active SLE patients, whereas it was expressed at high levels in freshly isolated NK T cells from normal subjects and iSLE patients (Fig. 5B). To confirm deficient expression of survivin in NK T cells from aSLE patients, we examined the expression of survivin mRNA in NK T cells from aSLE patients, iSLE patients, and normal subjects. We conducted a real-time quantitative RT-PCR assay to detect the different expressions of survivin at the mRNA level. The results (Fig. 5C) showed that survivin mRNA was expressed at a very low level in freshly isolated NK T cells from active SLE patients (0.8 × 10³ copies). Survivin mRNA expression was significantly increased in NK T cells from the normal subjects (5.0 × 10⁵ copies) compared with that from aSLE patients, whereas there was also an increase in NK T cells from the iSLE patients (3.5 × 10⁵ copies). The same pattern of survivin mRNA expression in NK T cells from aSLE patients, iSLE patients, and normal subjects were confirmed by Northern blot (Fig. 5D). Thus, it might be important to maintain a certain level of expression of survivin to...
balance between proapoptotic and antiapoptotic pathways in cells. We also examined the survivin expression levels in total CD3⁺/H11001 T cells, B cells, and monocytes. The results showed that there were no significant differences in survivin expression levels in total CD3⁺/H11001 T cells, B cells, and monocytes from aSLE, iSLE, and normal subjects, determined by real-time RT-PCR assay and confirmed by Northern and Western blot analyses (data not shown).

To determine the mechanism of CD226 activation to rescue NK T cells from programmed death, we conducted a series of experiments to investigate the activities of a number of proapoptotic and antiapoptotic members during the process of anti-CD95-induced apoptosis in cells from aSLE patients under different conditions of CD226 activation, as indicated in Fig. 6. In the antiapoptotic pathway systems, Bcl-2 activity under different conditions of CD226 activation was seemingly identical in aSLE NK T cells. CD226 activation significantly up-regulated survivin activity, which rescued the cells from apoptosis (Fig. 6A). In the proapoptotic pathway systems, active forms of caspase-8 and caspase-3 were significantly reduced after activation of CD226, suggesting that anti-CD95-mediated death signals were inhibited by activation of the CD226 pathway (Fig. 6, B and C). Thus, abnormal expression of survivin and deficient expression of CD226 were the molecular bases of the high sensitivity of NK T cells from aSLE patients to anti-CD95-induced apoptosis.

CD226 activation protects CD95- and CD226-transfected COS-7 cells from apoptosis

To confirm the observation of a protective effect of CD226 from apoptosis of NK T cells from active SLE patients, we chose a cell line, COS-7, that was able to express CD95 and CD226 after transfection and in which apoptosis was inducible by culture in presence or the absence of CD95 mAb. CD226A, CD226 activation. CD226B, CD226 blockade. Isotype, isotype Ab control under immobilized condition. The cells were then lysed, and the total protein content was electrophoresed and blotted as described in Materials and Methods. The hybridization signals for survivin mRNA in NK T cells from different subjects are shown in the upper panels. The 28S rRNAs in the lower panels confirm the comparable amounts of loaded total RNA. The illustrated data are from a single representative experiment of three performed.
CD226 transfection rescues aSLE NK T cells from anti-CD95-induced apoptosis

To confirm the finding of a correlation between CD226 expression and resistance to anti-CD95-induced apoptosis in the COS-7 cell system, we applied Amaxa nucleofection technology to transfected cDNA encoding CD226 into human NK T cells from aSLE patients, iSLE patients, and normal subjects to investigate the effect of CD226 expression on resistance to anti-CD95-induced apoptosis. The data in Fig. 7, A and B, demonstrate that successful transfection of CD226 took place; particularly NK T cells from aSLE patients were well transfected with cDNA encoding CD226, as documented by Western blot (Fig. 7A) and flow cytometry (Fig. 7B). The results (Fig. 7, C and D) from flow cytometric analysis revealed that the number of apoptotic cells was significantly decreased in the culture of sufficient CD226-transfected (2.5 μg) NK T cell from active SLE patients in the presence of immobilized CD95 mAb for 24 h, followed by analysis by flow cytometry as described in Materials and Methods. The amount of transfected DNA was kept constant in each sample by adding control pcDNA3 vector. Untransfected cells, CD226 transfection in normal NK T cells had little effect on anti-CD95-induced apoptosis (Fig. 7C). A sufficient CD226 transfection (25 μg) could also rescue iSLE NK T cell from anti-CD95-induced apoptosis (Fig. 7D). Even the apoptotic responses in inactive SLE patients were marginal increased (Fig. 7C, i–l). CD226 transfection in normal NK T cells had little effect on anti-CD95-induced apoptosis (Fig. 7, C, i–l, and D), because normal NK T cells already expressed abundant CD226 (Fig. 3). However, experiments of CD226 transfection in normal NK T cells showed that the transfection procedure itself did not affect the sensitivity of cells to apoptosis (Fig. 7C, a, e, and i). Thus, the results confirmed the correlation between CD226 expression and resistance to anti-CD95-induced apoptosis in the human NK T cell system.

Corticosteroid medication affects CD226 and survivin expression as well as sensitivity to apoptosis in NK T cells from aSLE patients

To examine the effects of corticosteroid medication on CD226 and survivin expression as well as sensitivity to apoptosis in NK T cells from aSLE patients, as shown in Table I, six patients with active SLE were recruited and were administrated prednisone as a major therapy. At the time of treatment, blood samples were taken. Could rescue COS-7 cells from anti-CD95-induced apoptosis (data not shown). As expected, the blockade of CD226 restored the sensitivity of double-transfected COS-7 cells to anti-CD95-induced apoptosis (data not shown). Isotype Ab for anti-CD226 had no such effect (data not shown). To confirm the mechanisms of the rescuing function of CD226 activation in COS-7 cells from anti-CD95-induced apoptosis, we first examined the expression levels of some important substrates of apoptosis pathways in the cells during the apoptotic process (data not shown). Western blot revealed that key apoptotic executioner molecules, procaspase-8 and procaspase-3, were stabilized by preactivation of CD226, where COS-7 cells were rescued from anti-CD95-induced apoptosis (data not shown). Western blot also showed that the protein levels of one antiapoptotic member, Bcl-2, in COS-7 cells under different conditions were identical (data not shown). Interestingly, the antiapoptotic protein, survivin, was selectively activated under the condition of CD226 preactivation, where COS-7 cells were rescued from anti-CD95-induced apoptosis (data not shown). Thus, we confirmed that in the transfected COS-7 cell system, anti-CD95-mediated death signals were inhibited by activation of the CD226 pathway through stabilization of caspase-8 and caspase-3 and through activation of survivin.

FIGURE 7. Analysis of apoptosis of CD226-transfected human NK T cells. Human NK T cells from aSLE patients, iSLE patients, and normal subjects (NML) were transfected with vectors of increasing concentrations of encoding CD226 at 0.25 μg (CD226 (l)) or 25 μg (CD226(h)) as described in Materials and Methods. The amount of transfected DNA was kept constant in each sample by adding control pcDNA3 vector. Untransfected cells. CD226 transfection in the various cells was examined using Western blot analyses (A) and flow cytometry (B) as described in Fig. 3. B. Data for apoptotic cells (PI+/annexin V+) are the mean ± SD of six experiments performed. Statistically significant differences compared with controls are indicated (*, p < 0.001).
The data showed that CD226 expression in NK T cells at the mRNA and protein levels was significantly increased after successful treatment with prednison. It began at wk 4 of treatment. Survivin expression in NK T cells was also significantly increased. It began at wk 6 of treatment. The anti-CD95-induced apoptosis in NK T cells also appeared to significantly decrease at wk 6 of treatment as the SLEDAI score decreased.

Together these data show that NK T cells from active SLE patients were highly sensitive to anti-CD95-induced apoptosis compared with that in normal subjects and iSLE patients. CD226 expression was deficiently expressed on NK T cells from iSLE patients. Survivin was found to be selectively expressed at a very low level in freshly isolated NK T cells from active SLE patients. CD226 preactivation significantly up-regulated survivin activation, which could rescue NK T cells from iSLE patients from anti-CD95-induced apoptosis. The observations were confirmed in transfected COS-7 cells and human NK T cells in terms of the correlation between activation of CD226 and stabilization of death signals.

Discussion

Development of SLE is associated with the emergence of autoreactive Th cells (43) and is accompanied by a reduction in regulatory T cells (44, 45). The nature and specificity of regulatory T cells that inhibit autoantibody production and development of lupus remain largely undefined. Some regulatory T cells are cells that inhibit autoantibody production and development of lupus (43) and is accompanied by a reduction in regulatory T cell network to inhibit the induction of SLE. To understand the mechanism of reduction of NK T cell number in SLE, we focused our study on the sensitivity of NK T cells to induction of apoptosis. The observations were confirmed in transfected COS-7 cells and human NK T cells in terms of the correlation between activation of CD226 and stabilization of death signals.

Table I. CD226 and survivin expression, sensitivity to apoptosis of NK T cells from aSLE patients during corticosteroid treatment

<table>
<thead>
<tr>
<th>Duration (wk)</th>
<th>Prednison (mg/day)</th>
<th>SLEDAI</th>
<th>CD226 (%)</th>
<th>CD226 (×10³)</th>
<th>Survivin (×10³)</th>
<th>Apoptosis (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>32 ± 14</td>
<td>19 ± 10</td>
<td>0.9 ± 0.6</td>
<td>0.9 ± 0.6</td>
<td>52 ± 15</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>30 ± 5</td>
<td>30 ± 17</td>
<td>22 ± 12</td>
<td>1.1 ± 0.7</td>
<td>0.8 ± 0.3</td>
<td>56 ± 14</td>
</tr>
<tr>
<td>2</td>
<td>30 ± 5</td>
<td>27 ± 12</td>
<td>20 ± 15</td>
<td>1.4 ± 0.3</td>
<td>1.8 ± 0.9</td>
<td>50 ± 18</td>
</tr>
<tr>
<td>4</td>
<td>30 ± 5</td>
<td>25 ± 16</td>
<td>31 ± 9</td>
<td>2.2 ± 0.5</td>
<td>1.4 ± 0.7</td>
<td>46 ± 16</td>
</tr>
<tr>
<td>6</td>
<td>25 ± 5</td>
<td>16 ± 18</td>
<td>35 ± 17</td>
<td>2.8 ± 0.4</td>
<td>1.9 ± 0.8</td>
<td>30 ± 10</td>
</tr>
<tr>
<td>8</td>
<td>15 ± 5</td>
<td>4 ± 2</td>
<td>40 ± 14</td>
<td>3.1 ± 0.3</td>
<td>2.7 ± 0.4</td>
<td>22 ± 12</td>
</tr>
<tr>
<td>10</td>
<td>10 ± 5</td>
<td>0</td>
<td>47 ± 18</td>
<td>5.3 ± 0.2</td>
<td>3.9 ± 0.2</td>
<td>20 ± 16</td>
</tr>
</tbody>
</table>

a Six active SLE patients who fulfilled at least four of the American College of Rheumatology 1982 revised criteria for SLE (28) were included in this study.

b All subjects included were inpatients. At the time intervals indicated during hospitalization, blood samples were taken. NK T cells were isolated as described in Materials and Methods.

c The numbers shown are percentages of apoptotic cells in NK T cells determined by flow cytometry.

d The numbers shown are survivin mRNA copies in 25 μg of cDNA from NK T cells by the real-time quantitative RT-PCR assay.

e The numbers shown are percentages of CD226-positive cells in NK T cells determined by flow cytometry.

The sensitivity of SLE NK T cells to apoptosis is associated with the emergence of autoreactive Th cells (43) and is accompanied by a reduction in regulatory T cell network to inhibit the induction of SLE. To understand the mechanism of reduction of NK T cell number in SLE, we focused our study on the sensitivity of NK T cells to induction of apoptosis. The observations were confirmed in transfected COS-7 cells and human NK T cells in terms of the correlation between activation of CD226 and stabilization of death signals.

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cell apoptosis inhibitor system, resulting in resistance of cells to apoptotic signals.

To explore the molecular basis of CD262 rescuing functions on NK T cells from active SLE patients, we examined the activity of a number of adaptor proteins during the process. Two major cell death pathways, termed the extrinsic and intrinsic pathways of apoptosis, exist in mammalian cells (50). The extrinsic pathway is initiated by engagement and aggregation of TNF family death receptors (such as CD95) through a series of death domain-containing adaptor molecules that recruit and directly activate cytosolic caspase 8, which, in turn, converts procaspase 3 to caspase 3, the central executioner of the apoptotic process (51, 52). The intrinsic pathway is intimately tied to the integrity of mitochondria and other intracellular organelles. The normal and healthy cells are maintained by a carefully regulated balance of proapoptotic and antiapoptotic members of the Bcl-2 family of proteins. In mitochondria of normal and healthy cells, resident antiapoptotic proteins, such as Bcl-2 and Bcl-xL, effectively bind up and neutralize proapoptotic proteins (53) and prevent perturbation of the outer mitochondrial membrane. A series of endogenous caspase inhibitors, known as inhibitors of apoptosis, normally interact with and inhibit the enzymatic activity of the initiator caspase 9 and the effector caspases 3 and 7 (54). Both the location and the activation state of proapoptotic and antiapoptotic components of the cell death pathway are crucial for controlling cellular decisions about life and death. Survivin, a member of the inhibitors of apoptosis family, is potentially involved in both the inhibition of apoptosis and the control of cell division (55–57). Being essential for embryonic development (55), survivin expression in adult human tissues is usually reported in the context of its relationship to cancer. Survivin is selectively expressed at very low levels in NK T cells from aSLE patients, compared with the level in freshly isolated NK T cells from normal subjects (Fig. 6). Furthermore, CD262 activation up-regulate significantly the expression and activation of survivin, which may be one of the reasons to rescue the cells (Fig. 7).

What is the essential correlation between the defective expression of CD262 and the signaling abnormalities identified to date in aSLE NK T cells? There is a mounting body of evidence indicating increased intracellular calcium in T cells from SLE patients due to defective expression of the TCR-ζ chain (61–65). Several previous studies of CD262 (20, 21, 26, 27) indicate that this molecule is a signaling element that triggers cytoxicity by engagement of extracellular ligand or cellular counter-receptor (CD155 and CD112) and postulate that CD262 is induced upon T cell activation, and the expression persists unless cells are exposed to signal that elevates intracellular free calcium (21). Hence, the increased intracellular free calcium concentration in SLE T cells may lead to down-regulation of CD262 expression and, thus, increased apoptosis of NK T cells.

Generally, deficient expression of survivin and CD262 on aSLE NK T cells establishes a molecular basis of sensitivity of the cells to anti-CD95-induced apoptosis. These observations offer a potential explanation for the high apoptotic sensitivity of aSLE NK T cells and provide a new insight into the mechanism of reduction of NK T cell number in SLE and understanding the association between NK T cell deficiency and autoimmune diseases.

References


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CD226 (DNAM-1) is involved in lymphocyte function-associated antigen 1 co-stimulatory signal for naive T cell differentiation and proliferation. J. Exp. Med. 198:1829.


Letter of Retraction


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