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The Apoptotic Ligands TRAIL, TWEAK, and Fas Ligand Mediate Monocyte Death Induced by Autologous Lupus T Cells

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Individuals with systemic lupus erythematosus show evidence of a significant increase in monocyte apoptosis. This process is mediated, at least in part, by an autoreactive T cell subset that kills autologous monocytes in the absence of nominal Ag. We have investigated the apoptotic pathways involved in this T cell-mediated process. Expression of the apoptotic ligands TRAIL, TNF-like weak inducer of apoptosis (TWEAK), and Fas ligand on lupus T cells was determined, and the role of these molecules in the monocyte apoptotic response was examined. We report that these apoptotic ligands mediate the autologous monocyte death induced by lupus T cells and that this cytotoxicity is associated with increased expression of these molecules on activated T cells, rather than with an increased susceptibility of lupus monocytes to apoptosis induced by these ligands. These results define novel mechanisms that contribute to increased monocyte apoptosis characterizing patients with lupus. We propose that this mechanism could provide a source of potentially antigenic material for the autoimmune response and interfere with normal clearing mechanisms. The Journal of Immunology, 2002, 169: 6020–6029.

Abbreviations used in this paper: SLE, systemic lupus erythematosus; TWEAK, TNF-like weak inducer of apoptosis; FasL, Fas ligand; DR, death receptor; RA, rheumatoid arthritis; SLEDAI, SLE disease activity index; 6-MP, 6-mercaptopurine; PI, propidium iodide; MCF, mean channel fluorescence.

Materials and Methods

Abs and reagents

FN14: Fc (murine), a newly characterized receptor for the apoptotic ligand TWEAK (12), was a kind gift from Genentech (San Francisco, CA). Purified PHA was from Murex (Norcross, GA). The following reagents were all obtained from BD PharMingen (San Diego, CA) and, unless specified otherwise, the Abs were all mouse anti-human mAbs: FITC-annexin-V kit, FITC-anti-CD2, PE-anti-CD14, CYC-anti-CD3, purified anti-CD3, APC-and CYC-anti-CD4, purified anti-HLA-ABC, PE-anti-CD29, CYC- and FITC-anti-CD8, anti-human TRAIL/TNFSF10 mAb, biotin-anti-FasL, PE-anti-TRAIL, purified anti-TRAIL, APC-mouse IgG2a, PE mouse IgG1, FITC-goat anti-rabbit Ig-specific polyclonal Ab, FITC-goat anti-mouse Ig-specific polyclonal, streptavidin-CYC conjugate, affinity-purified polyclonal rabbit-human death receptor (DR) 4 (C-terminal) and affinity-purified polyclonal rabbit-human DR3 Abs, purified rat anti-human IL-4.
purified mouse anti-human IFN-γ, purified rat anti-human IL-10, and pu-
rified mouse anti-human IFN-α. PE mouse anti-human CD19 was obtained
from Coulter (Miami, FL). Recombinant human TRAIL R1/Fc chimera, re-
combinant human TRAIL and TRAIL inhibitor, and rabbit anti-human
TRAIL R2/DR5 were obtained from R&D Systems (Minneapolis, MN).

Purified mouse anti-human CD28 was from Southern Biotechnology As-
sociates (Birmingham, AL). FITC-conjugated and PE-conjugated affinity-
purified F(ab’)2 goat anti-mouse IgG (H + L) were obtained from Immu-
notech (Marseille, France). FITC-conjugated affinity-purified F(ab’)2
donkey anti-goat IgG (H + L), HRP-conjugated AffiniPure F(ab’)2 donkey
anti-goat IgG (H + L), purified goat anti-mouse-TWEAK, and recombi-
nant human TWEAK were obtained from R&D (Minneapolis, MN). FITC
and PE goat-F(ab’)2 anti-rabbit IgG (H + L) was from Caltag Laboratories
(Burlingame, CA). Recombinant human FasL, recombinant human TRAIL kit
(including TRAIL enhancer), and ApoSENSOR ADP/ATP Ratio Assay kit were
obtained from Alexis Biochemicals (San Diego, CA). Pan T cell isolation
kit, CD4 T cell isolation kit, monocyte isolation kit, and CD8 microbeads were
obtained from Miltenyi Biotec (Auburn, CA). The pan-
caspase inhibitor Ac-VAD-CHO was from Biosource International
(Camarillo, CA). Chloroquine, hydrocortisone, indomethacin, 6-mercaptopurine
(6-MP), PMSF, sodium orthovanadate, leupeptin, and aprotinin were ob-
tained from Sigma-Aldrich (St. Louis, MO).51 Cr was obtained from
Promega (Madison, WI). PerkinElmer (Boston, MA). Hoechst 33342 was from Molecular Probes
(Eugene, OR). The CellTracker 96 aqueous one solution cell proliferation
assay was from Promega (Madison, WI).

Patient selection

Patients with active SLE, inactive SLE, and rheumatoid arthritis (RA) were
recruited from the outpatient Rheumatology clinic and inpatient services at
the University of Michigan (Ann Arbor, MI), and from the Michigan Lupus
Cohort (Ann Arbor, MI). Healthy controls were obtained by advertising.
All SLE and RA patients fulfilled the American College of Rheumatology
criteria for these diseases (13, 14). SLE activity was assessed by the SLE
disease activity index (SLEDAI; Ref. 15), and those with SLEDAI >5
were considered to have active disease. RA activity was assessed by the
modified disease activity score (16). Patient and control T cells were paired
and studied in parallel. Information regarding the demographics, disease
activity, and use of medications by the subjects is provided in Table I.

PBMCs and T cell isolation

PBMCs were isolated by Ficoll-Hypaque (Amersham Pharmacia Biotech,
Uppsala, Sweden) gradient centrifugation as described (9). T cells were
isolated by negative selection using magnetic beads and instructions pro-
vided by the manufacturer (pan T cell isolation kit; Miltenyi Biotec), or by
rosetting with sheep erythrocytes (Colorado Serum, Denver, CO) as
described (9).

Drug treatment

Unstimulated and PHA-stimulated human T cells were cultured in 24-well
plates in RPMI/5% FBS (100,000 cells/ml) in the presence or absence of
graded concentrations (0.1–100 μM) of indomethacin, chloroquine, hydro-
cortisone, or 6-MP (17, 18). A stock solution of 6-MP was prepared in
100
mg/ml human-anti-CD28 in PBS. Cells were cultured at 37°C overnight,
harvested, and incubated with human TRAIL for 30 min at 4°C with
10–15 μM flurochrome-conjugated mAb following the manufacturer’s direc-
tions, washed three times again with standard buffer, then fixed in 1% paraformaldehyde
and analyzed in a FACScan flow cytometer (BD Biosciences, Mountain
View, CA) using previously described protocols (9). For identification of B
cells and monocytes, fluorochrome-conjugated anti-CD19 and anti-CD14
were used, respectively.

Where indicated, purified T cells from seven healthy controls were stim-
ulated using 96-well plates coated with 10 μg/ml human anti-CD3 and 1
μg/ml human-anti-CD28 in PBS. Cells were cultured at 37°C overnight,
harvested, and incubated with human FasL (Alexis Biochemicals), TRAIL
soluble (human) recombinant fusion protein, human recombinant
– TRAIL, or TWEAK, or FasL. Cells were then stained with 5 μg of annexin
V-FITC and 10 μg of PI. 0.05 μg/ml for 15 min at room temperature
in the dark. Cells were analyzed immediately using a FACScan flow
cytometer. In additional experiments, monocyte apoptosis was measured
in unfraccionated PBMCs by labeling cells with anti-CD14-PE, washing in
PBS, and then staining with annexin V/PI and PI as above. To measure
typical purity was ~93–96% by CD14 staining.

Measurement of apoptosis

Annexin V staining. Monocytes were isolated using negative selection with
magnetic beads. Approximately 50,000 cells were suspended in 100
μl of 1× annexin V binding buffer (0.1 M HEPES/NaOH (pH 7.4), 1.4 M
NaCl, 25 mM CaCl2). Cells were then stained with 5 μg of annexin V-FITC
and 10 μg of PI for 15 min at room temperature in the dark. Cells were analyzed immediately using a FACScan flow
cytometer. In additional experiments, monocyte apoptosis was measured
in unfraccionated PBMCs by labeling cells with anti-CD14-PE, washing in
PBS, and then staining with annexin V/PI and PI as above. To measure
monocyte apoptosis induced by lupus T cells, lupus and control monocytes
were isolated using negative selection with magnetic microbeads. Mono-
cytes were cultured with autologous T cells using a ratio of 25:1 T cell:
monocyte. Cells were cultured together for 18 h in the presence or absence of
increasing concentrations of blocking Abs or fusion proteins to
TRAIL, TWEAK, or FasL. Cells were then stained with anti-CD2PE and
anti-CD14 APC, washed, resuspended in 1× annexin V binding buffer, and
stained with annexin V/PI as described above. Cells were analyzed im-
ediately using a FACScan flow cytometer.

ADP:ATP ratio. The ApoSENSOR ADP/ATP Ratio Assay kit was used to
measure ADP:ATP ratios. This kit uses bioluminescent detection of the
ATP level via luciferase catalyzed reaction for a rapid screening of apo-
ptosis and cell viability in mammalian cells (21). A total of 50,000
monocytes were added to 96-well plates in RPMI/10% FBS in the presence of
human recombinant FasL (Alexis Biochemicals), TRAIL soluble (human) recombinant fusion protein or TWEAK soluble (human) recombinant
fusion protein (Research Diagnostics, Flanders, NJ), or isotype
control (BD Pharmingen). Where indicated, an affinity-purified rabbit anti-
mouse Ig (Alexis Biochemicals) was added to optimize cross-linking. The
cells were cultured for 18 h after which the medium was removed and cells
were treated with 100 μl nuclear releasing agent as per manufacturer’s
instructions for 5 min at room temperature with gentle shaking. ATP and
ADP levels were measured, and ADP:ATP ratios calculated according to
the manufacturer’s instructions.

Hoechst 33342 staining. Monocytes from lupus patients and controls
were isolated using negative selection with magnetic beads. A total of
50,000 cells were then cultured in the presence or absence of 1–100 ng/ml
TRAIL soluble (human) recombinant fusion protein, human recombinant

| Table I. Characteristics of patients studied |
|-----------------|-----|-----|-----|
| **Variable**    | **Lupus** | **Control** | **RA** |
| Number studied  | 52  | 35  | 15  |
| Age (mean ± range) | 37.6 ± 11.4 | 28 ± 10.4 | 45 ± 10.3 |
| Females n (%)   | 47  (90.3) | 19  (54.2) | 9  (60) |
| Males n (%)     | 5   (9.7) | 16  (45.8) | 6  (40) |
| Disease Activity|     |     |     |
| SLEDAI ≥ 5 n (%) | 27  (52) | 25  (48) |
| SLEDAI > 5 n (%) |     |     |     |
| Medications (%) |     |     |     |
| Antimalarials   | 67.3 | 0   | 55  |
| Azathioprine    | 10  | 0   | 10  |
| Mycophenolate mofetil | 13.4 | 0   | 0   |
| Cyclophosphamide| 7.6 | 0   | 0   |
| Methotrexate    | 0   | 0   | 46  |
| Prednisone (<0.5 mg/kg/day) | 40 | 0   | 55  |
| Prednisone (0.5–1 mg/kg/day) | 21 | 0   | 25  |
| Prednisone (>1 mg/kg/day) | 3.8 | 0   | 2   |

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FasL, TWEAK soluble (human) recombinant fusion protein, or isotype control for 18 h in a total volume of 200 μl RPMI/3% FBS/penicillin/streptomycin. Cells were then washed, resuspended in 1 ml PBS, and incubated with 1 μl Hoechst 33342 stock solution and 10 μl PI. Cells were then incubated on ice for 20–30 min and analyzed immediately using a FACScan flow cytometer.

**Cytotoxicity assays**

For direct cytotoxicity assays, 50,000 monocytes/well were incubated with FasL, TRAIL, TWEAK, or a control mAb (mouse IgG1 or IgG2; BD PharMingen) in a total volume of 200 μl/well. Following manufacturer’s instructions, a specific enhancer Ab was added for cross-linking after incubation with TRAIL and FasL recombinant proteins. These enhancers are Abs that interact with TRAIL and FasL, recombinant proteins, increasing their activity. The enhancers were used at 2 μg/ml following the manufacturer’s instructions. As additional negative controls, cells were stimulated with enhancer without TRAIL or FasL. The cells were cultured for 18 h, then cell viability was determined by trypan blue exclusion. Quantitative analysis of apoptosis was done with annexin V/PI staining and/or ADP/ATP ratio calculation (see ADP/ATP ratio).

For cell-mediated cytotoxicity, 51Cr release assays were performed as previously described by our group (9). Briefly, 250,000 PBMC were adhered to round bottom microtiter wells in 100 μl RPMI/10% FBS for 1–2 h, after which the nonadherent cells were removed. The adherent cells were labeled with 2 μCi 51Cr/well for 3 h, and then washed. T cells were obtained by rosetting or negative selection using magnetic beads and added to the labeled monocytes at a ratio of 25:1. Cr release was determined 18 h later as described (9).

**T cell proliferation assay**

T cells from healthy controls or lupus patients were suspended in RPMI/5% FBS/penicillin/streptomycin media and treated with immobilized anti-CD3 (10 μg/ml) for 3 days in 96-well plates in the presence or absence of the following inhibitory Abs or fusion proteins: anti-human TRAIL, human Fn14:Fc (anti-TWEAK), anti-human FasL, and isotype control Ig at concentrations of 1–100 ng/ml. A total of 50,000 T cells/well were used, in a total volume of 200 μl RPMI/3% FBS/penicillin/streptomycin/well. At 72 h, T cell proliferation was measured using a colorimetric method (cellTiter 96 Aqueous one solution cell proliferation assay; Promega) following manufacturer’s instructions (22). In brief, 20 μl of the CellTiter 96 solution reagent was pipetted into each well and the plate was incubated for 3 h at 37°C in a humidified, 5% CO2 atmosphere. Absorbance was measured at 490 nm using a 96-well plate reader (Bio-Tek Instruments, Winooski, VT).

**Statistical analysis**

The difference between means was analyzed using Student’s t test or ANOVA, using Systat 10 software (Systat, Evanston, IL) and Stata 6.0 (Stata Corporation, College Station, TX). To determine whether current treatment with immunosuppressives was associated with markers of apoptosis, univariate linear regression was performed. Each marker of apoptosis was modeled separately as a dependent variable, with medications modeled as dichotomous independent predictors. Control subjects were also included from this portion of the analysis because a high degree of collinearity existed between case/control status and treatments due to the lack of immunosuppressive use in controls.

**Results**

**Monocytes from lupus patients undergo accelerated apoptosis**

Monocytes from 16 lupus patients with active lupus (SLEDAI >5) and 8 controls were isolated by negative selection with magnetic beads, incubated with annexin V/FITC and PI, then analyzed by flow cytometry. Fig. 1A shows a representative histogram. Patients with lupus had significantly greater percentages of apoptotic monocytes (annexin V+, PI−) relative to controls (Fig. 1B). To exclude the possibility that the isolation procedure induced apoptosis in lupus monocytes, these findings were confirmed in un fractionated PBMCs staining with anti-CD14+ and annexin V/PI. Apoptotic monocytes in six controls represented 4 ± 2.5% of CD14+ cells. In six patients with SLE, 45 ± 15.2% of CD14+ cells were apoptotic, p < 0.01. We also confirmed that lupus T cells kill autologous monocytes and that this phenomenon correlates with disease activity. In addition, we found that this phenomenon appears to be disease-specific. Fig. 2 shows that T cells from patients with lupus, but not from RA patients or controls, spontaneously kill autologous monocytes (p < 0.0001, SLE vs RA and controls). In addition, T cells from patients with active SLE kill monocytes better than those from patients with inactive disease (p < 0.001, active SLE vs inactive SLE).

**Lupus T cells show increased expression of apoptotic ligands**

We then proceeded to investigate the pathways involved in T cell-mediated monocyte death in SLE. Because we had previously reported that the apoptotic ligands TRAIL, TWEAK, and FasL mediate T cell-induced cytotoxicity of Ag-specific macrophages in murine systems (10), we compared expression of these apoptotic ligands on T cells from 35 healthy controls, 52 patients with SLE, and 15 individuals with RA. Using flow cytometry, we found that SLE patients had significantly greater expression of TRAIL, TWEAK, and FasL on CD4+ and CD8+ T cells when compared with controls. Representative histograms for CD4+ T cells are shown in Fig. 3. Fig. 4A shows that the percentage of CD4+ and CD8+ T cells expressing TRAIL, TWEAK, and FasL is significantly increased. When mean channel fluorescence (MCF) was analyzed, FasL was significantly increased on lupus CD4+ T cells, and TRAIL was significantly increased on lupus CD4+ and CD8+ T cells when compared with controls (Fig. 4B). RA patients showed increased levels of TRAIL on CD4+ and CD8+ T cells when compared with controls (control CD4 81 ± 20 vs RA CD4 475.6 ± 48, and control CD8 89 ± 12 vs RA CD8 362 ± 54 by MCF; mean ± SE; p < 0.01), but not of TWEAK or FasL. However, there were no differences in the percentage of cells expressing these ligands when compared with controls.
The findings were specific for T cells. There was no difference in expression of these apoptotic ligands on B cells from SLE patients when compared with controls (data not shown). Previous studies have found no consistent defect in the expression or function of the Fas receptor in SLE (3). Similarly, we observed no significant differences in the expression of DR3 (also known as TRAMP, LARD, WSL-1, or Apo-3), DR4 (also known as TRAIL-R1 or Apo-2), or DR5 (also known as TRAIL-R2 or Trick2) on monocytes from these same SLE patients when compared with controls (data not shown).

Association of ligand expression with disease activity and T cell activation

When percentage of expression was analyzed according to disease activity, patients with active lupus (SLEDAI >5) had greater expression of TRAIL and FasL on CD4⁺ and CD8⁺ T cells compared with individuals with inactive disease (SLEDAI ≤ 5; Fig. 4C). When expression of apoptotic ligands was analyzed by MCF according to disease activity, lupus patients with more active disease had a higher expression of TRAIL and FasL, but not TWEAK, on CD4⁺ T cells than patients with inactive lupus (Fig. 4D). To determine whether these molecules are overexpressed on activated T cells, two-color flow cytometry was performed using CD25 and CD29 as activation markers. CD29 (β1 integrin) is the β-chain associated with the integrin α subunits 1–6 known as very late Ags, and is expressed very late after T cell stimulation (23).

FIGURE 2. Lupus T cells kill autologous monocytes. PBMC from 5 controls, 16 lupus patients (8 with SLEDAI ≤5 and 8 with SLEDAI >5), and 4 RA patients were isolated by Ficoll gradient centrifugation. Monocytes were isolated by adherence and/or negative selection using magnetic microbeads and radiolabeled with 2 µCi/well Cr⁺⁺ using 96-well plates for 3 h. Cells were washed twice and resuspended in RPMI/10% FBS medium. In parallel, autologous T cells were obtained by either rosetting or using negative selection through magnetic microbeads. T cells were then added to the monocyte culture at an E:T (T cell:monocyte) ratio of ~25:1, in the absence of Ag and cultured for 18 h. Supernatants from each well were transferred to scintillation vials and cell lysates were then obtained by adding 100 µl/well PBS/0.1% Triton X and then transferring the lysate to scintillation vials. Cr release assay was then performed using a scintillation counter. *, p < 0.001. Results represent percentage of monocyte death, calculated as

\[
\text{supernatant CPI} = \frac{\text{supernatant CPI} - \text{cell pellet CPI}}{\text{background monocyte }^{51}\text{Cr release}}.
\]

Background monocyte $^{51}\text{Cr}$ release was $35 \pm 15\%$ for lupus patients, $20 \pm 11\%$ for controls, and $22 \pm 10.5\%$ for RA patients (mean percent ± SEM).

FIGURE 3. Lupus patients show increased expression of apoptotic ligands in CD4⁺ T cells when compared with controls. T, CD4⁺, and CD8⁺ cells were isolated from 15 controls and 20 SLE patients with Ficoll-Hypaque gradient and negative or positive selection using magnetic microbeads. Cells were washed, resuspended in standard buffer (PBS/3% horse serum, 0.01% sodium azide), and incubated with combinations of either mouse anti-human TRAIL PE, goat anti-human TWEAK-FITC, or mouse anti-human FasL-CYC for 30 min. Cells were then washed three times, resuspended in 10% formaldehyde, and analyzed using a FACScaner. Results are shown as representative histograms from one healthy control (control) and one lupus patient (SLE) and represent expression of FasL (A), TWEAK (B), and TRAIL (C) or isotype control on CD4⁺ T cells.
The lupus patients had higher numbers of activated T cells than controls (for CD29, 3.8% vs 2.1% for CD29, 7.9 vs 2.1 for CD25, control vs lupus, mean SEM, p 0.01; for CD25, 1.2 vs 10.8 for CD25) were increased on a subset of activated T cells, as measured by CD29 T cells and CD25 T cells, when compared with controls. Fig. 5 shows the average increase of the proapoptotic ligands on the activated cells. Our findings for FasL confirm previous observations by Kovacs et al. (24).

To determine whether T cell activation could contribute to the increased expression of these apoptotic ligands on lupus T cells, T cells from seven healthy control subjects were stimulated with immobilized anti-CD3 and anti-CD28 overnight. The percentage of T cells expressing TRAIL, TWEAK, and FasL expression increased after stimulation (for TRAIL, 6.6% vs 25.2% in stimulated T cells vs 25.2% in unstimulated T cells; for TWEAK, 5.9% vs 13.8 in unstimulated T cells vs 27.0% in stimulated T cells; for FasL, 1.4% vs 16% in stimulated T cells vs 16% in unstimulated T cells; mean SEM, p < 0.05 for all variables). Representative histograms are shown in Fig. 6. These results suggested that the increase in expression of apoptotic ligands seen in activated lupus T cells is similar to what is seen in T cells from healthy controls after CD3/CD28 stimulation.

Effect of medications

To exclude the possibility that medications altered the expression of these apoptotic ligands, unstimulated and stimulated human T cells were cultured in the presence or absence of graded concentrations (0.1–100 μM) of medications that are commonly used in lupus treatment: indomethacin (for nonsteroidal anti-inflammatory drugs), chloroquine (for antimalarials), hydrocortisone (for steroids), and 6-MP (for azathioprine), or combinations, as previously reported (17–19). After culture for 1, 6, 12, and 24 h, TRAIL, TWEAK, and FasL expression was determined by flow cytometry and compared with untreated cells. None of these medications caused significant changes in the expression of these ligands, suggesting that medications do not account for the differences in expression (data not shown). To determine whether current treatment with immunosuppressives was associated with markers of apoptosis, univariate linear regression was performed. Each marker of apoptosis was modeled separately as a dependent variable, with medications modeled as dichotomous independent predictors. Control subjects were excluded from this portion of the analysis because a high degree of collinearity existed between case/control status and treatments due to the lack of immunosuppressive use in controls. There was no correlation of specific medications with
proapoptotic molecule expression in these patients. In addition, there was no correlation between medications and SLEDAI, which also correlates with the ability of the T cells to kill monocytes (9).

**Lupus monocytes have equal susceptibility to cell death induced by apoptotic ligands when compared with controls**

To evaluate whether these apoptotic pathways are functional in monocytes from patients with SLE, and whether they differ from normal controls, we treated monocytes from five lupus patients and five controls with graded concentrations of TRAIL, TWEAK, or FasL recombinant proteins for 18 h. Using $^{51}$Cr release assays, we found a significant increase in cell death induced by these ligands (Fig. 7A, $p < 0.05$ for the three apoptotic ligands at a concentration of 100 ng/ml, relative to control Ig). There was no statistically significant difference between lupus and control patient monocytes in their susceptibility to apoptosis secondary to recombinant protein stimulation. In addition, TRAIL cytotoxicity was inhibited by adding a specific TRAIL inhibitor (human recombinant TRAIL-R1:Fc), confirming specificity (25 ± 1.2% cytotoxicity for TRAIL agonist vs 1.8 ± 0.9% for TRAIL antagonist + agonist, mean ± SEM). The change in ADP:ATP ratios has been used to differentiate different modes of cell death and viability. Decreased levels of ATP and increased levels of ADP are recognized markers of apoptotic cells (25). Using ADP:ATP ratio assays, we confirmed that monocyte cell death after ligand stimulation demonstrates an apoptotic pattern (Fig. 7B). This was also confirmed using Hoechst 33342:PI staining (data not shown).

**Lupus T cells kill autologous monocytes through apoptotic pathways involving the ligands TRAIL, TWEAK, and FasL**

We next asked if lupus T cells use these proapoptotic molecules to kill autologous monocytes. Monocytes from lupus patients and controls were radiolabeled with $^{51}$Cr and cultured with autologous T cells at a ratio of 25:1 T cells:monocytes in the presence or absence of graded concentrations of blocking Abs or fusion proteins to TRAIL, TWEAK, FasL, or a control Ig. A pancaspase inhibitor (Ac-VAD-CHO) was used as a positive control. Inhibiting these molecules partially blocked T cell-mediated macrophage killing (Fig. 8A), with anti-FasL being the most potent ($p < 0.005$). Anti-TRAIL and anti-TWEAK also gave significant inhibition ($p < 0.05$ at 100 ng/ml). The combination of anti-FasL, TRAIL, and TWEAK was not more inhibitory than any of the Abs alone.

T cells from lupus patients were then fractionated into CD4$^{+}$ and CD8$^{+}$ subsets using magnetic beads and negative selection to prevent activation or inhibition of the cells through interactions with the Abs. Interestingly, cytotoxicity levels were greater using unfractionated CD4$^{+}$ and CD8$^{+}$ T cells than with either subset alone (58 ± 5% for unfractionated T cells vs 20.7 ± 5.5 for CD4$^{+}$ T cells).
T cells and 12.5 ± 3.5% for CD8⁺ T cells, p < 0.05, n = 16 for unfractionated T cells, and 8 for CD4⁺ and CD8⁺ T cells), suggesting a requirement for both subsets for optimal killing.

Because we had previously reported that inhibiting T cell activation with mAb to molecules such as LFA-1 or class II MHC also prevents APC killing (9), it was important to exclude the possibility that blocking these apoptotic ligands could inhibit T cell proliferation/activation, we stimulated lupus T cells with anti-CD3 for 3 days, in the presence or absence of graded concentrations of anti-CD3. The results showed that blocking anti-CD3 with a monoclonal antibody (mAb) decreased T cell proliferation, suggesting a requirement for both subsets for optimal killing.

**Figure 7.** TRAIL, TWEAK, and FasL induce cytotoxicity of lupus and control monocytes. A, PBMCs from six SLE patients and five controls were isolated with Ficoll-Hypaque gradient, monocytes were isolated by negative selection kit with magnetic beads or by adherence to round-bottom microtiter wells in 100 μl RPMI/10% FBS for 2 h. Monocytes were radiolabeled with 2 μCi/well Cr⁵¹ for 3 h, washed and cultured in the presence or absence of graded concentrations of recombinant human TRAIL, TWEAK, FasL, or a control Ig for 18 h. When necessary, a secondary Ab was used for cross-linking. After 18 h, the cells were lysed and percentage of cytotoxicity calculated using a scintillation counter. Results represent the mean ± SEM of five independent experiments done in quadruplicate determinations. The percentages have been calculated after subtracting background monocyte apoptosis. B, Monocyte cytotoxicity induced by recombinant DR ligands is secondary to apoptosis. PBMCs from three SLE patients and two controls were isolated with Ficoll-Hypaque gradient, monocytes were isolated by negative selection kit with magnetic beads or by adherence to round-bottom microtiter wells in 100 μl RPMI/10% FBS for 2 h. Mononuclear cells were removed. Monocytes were cultured in the presence or absence of human recombinant FasL, TRAIL, soluble (human) recombinant fusion protein or TWEAK soluble (human) recombinant fusion protein, or control Ig. When indicated, an affinity-purified rabbit anti-mouse Ig was added to optimize cross-linking as described. The cells were cultured for 18 h after which the medium was removed and cells were treated with 100 μl nuclear releasing agent for 5 min. ATP and ADP levels were measured and ADP/ATP ratios calculated according to the manufacturer’s instructions. The percentages have been calculated after subtracting background monocyte apoptosis. Results represent the mean ± SEM of five independent experiments done in quadruplicate determinations. * p < 0.05 when compared with control Ig.

**Figure 8.** Lupus T cells induce autologous monocyte apoptosis through multiple apoptotic ligands. A, PBMCs were isolated from 16 SLE patients, PBMCs were adhered to round-bottom microtiter wells in 100 μl RPMI/10% FBS for 1–2 h, after which the nonadherent cells were removed. Monocytes were also obtained by negative selection using magnetic beads. The adherent cells were labeled with 2 μCi ⁵¹Cr/well for 3 h, and then washed. T cells were obtained by rosetting or negative selection with microbeads. T cells were added to the labeled monocytes at a ratio 25:1 E:T, in the presence or absence of graded concentrations of either mouse anti-human TRAIL, FN14:Fc (anti-TWEAK), anti-FasL, Ab, isotype control, or pancaspase inhibitor (Ac-VAD-CHO), and cultured for 18 h. Cr release assay was then measured using a scintillation counter. Results represent the mean ± SEM of 16 independent experiments each performed in quadruplicate. p < 0.05 for all reagents at 100 ng/ml. B, Blocking apoptotic ligands has no effect on proliferation/activation of lupus T cells. T cells were obtained from lupus patients by negative selection using magnetic beads resuspended in RPMI/5% FBS/penicillin/streptomycin media and treated with immobilized anti-CD3 (10 μg/ml) for 3 days in 96-well plates, in the presence or absence of similar of identical concentrations of anti-human TRAIL, human Fn14:Fc (anti-TWEAK), anti-human FasL, and isotype control Ig as the ones used in A. T cell proliferation was then measured using a colorimetric method following manufacturer’s instructions. Absorbance was measured at 490 nm using a 96-well plate reader. Results represent the percentage of proliferation relative to control ± SEM of two independent experiments, each performed in quadruplicate. p > 0.05 when comparing CD3 responses with responses after adding blocking Abs.
blocking Abs or fusion protein to TRAIL, TWEAK, FasL, or a control Ig. We then measured T cell proliferation using a colorimetric method. As seen in Fig. 8B, these molecules had no effect in inhibiting T cell proliferation, suggesting that an inhibition in T cell activation did not account for the inhibition of monocyte apoptosis by these blocking Abs.

Finally, the cytokines IL-10, IL-4, IFN-α, and IFN-γ have been reported to be involved in monocyte cytotoxicity (20, 27–30). To exclude a role for soluble cytokines in inducing/blocking monocyte apoptosis in this model, we tested whether mAb to IL-4, IL-10, IFN-α, and IFN-γ could inhibit lupus T cell-mediated monocyte killing. No significant inhibition was observed using concentrations up to 100 ng/ml, as previously reported by our group for Ag-specific monocyte killing by human T cells (31) (maximum inhibition = 4.5 ± 2.2% for anti-IL-10, 5.5 ± 3.1% for anti-IL-4, 6.6% ± 3.4 for anti-IFN-γ, and 6% ± 4.2 for anti-IFN-α, mean ± SEM, p > 0.05 for all values when compared with isotype control). This suggests that the T cell-mediated cytotoxicity occurs largely through the proapoptotic ligands on the lupus T cells.

Discussion

The present study describes the apoptotic ligands that mediate monocyte apoptosis induced by autologous lupus T cells. Our results indicate that patients with lupus show an increase in expression of TRAIL, TWEAK, and FasL in CD4⁺ and CD8⁺ T cells. Because our previously published studies show that anti-class II Abs can completely abrogate T cell-mediated autologous monocyte cytotoxicity and anti-class I was less effective (9), we propose that CD4⁺ T cells are required for the killing, although CD8⁺ T cells may play an additional role, since CD4⁺ T cells alone kill less efficiently than unfractionated T cells. The importance of a finely tuned cellular collaboration between CD4⁺ and CD8⁺ T cells for cytotoxicity has been described by other groups (32–34). We had previously shown that this phenomenon is specific for autologous monocytes, since SLE T cells do not kill allogeneic monocytes from normal or other lupus patients, and the killing was inhibited with mAbs to class II MHC determinants (9). The SLE patients had increased numbers of activated T cells relative to normal controls, and there was increased expression of these apoptotic ligands on the activated cells. We used CD29 as an activation marker since previous studies have reported that this marker is increased in lupus T cells (35), and it has been used by other groups to study expression of apoptosis markers on activated lupus PBMCs (3). Disease activity, measured by SLEDAI, also correlated with TRAIL, TWEAK, and FasL expression on CD4⁺ T cells but not with other T cell subset markers. These observations suggest that the increased expression of these molecules may be due to in vivo T cell activation. This conclusion is supported by our observation that expression of these apoptotic ligands increased following stimulation of T cells from healthy controls, as reported by others for TRAIL and FasL in human and murine T cells (10, 21, 36), and for TWEAK in murine T cells (10). Another group reported that TWEAK was not expressed on human T cells (20). The discrepancy may be related to differences in Abs used for stimulation or to variability in the degree of T cell stimulation achieved.

These studies also demonstrate that lupus T cells mediate autologous monocyte cytotoxicity using the same apoptotic ligands and receptors as those used by normal murine T cells to kill Ag-presenting macrophages. These results also indicate that TRAIL can induce cytotoxicity in a percentage of normal human monocytes, further supporting other reports that this molecule, although primarily toxic on transformed lines, has also an effect on non-transformed cells (10, 37–40). Although a slight increase in cytotoxicity inhibition was observed when the blocking Abs were used in combination (data not shown), no clear synergism was found when multiple pathways were blocked. This suggests that the signals given by one of these ligands might be sufficient to inhibit apoptotic signals given by other members of the TNF family. Although FasL and TRAIL have been proposed to act in a cooperative way in inducing cytotoxicity (41), a lack of true synergism for these molecules has previously been reported in cancer lines (42). Our results confirm these findings. The apparent redundancy in apoptotic pathways involved in T cell-mediated monocyte cytotoxicity in normal states, as well as in lupus, may indicate that each of these molecules could have a different role in APC apoptosis that still remains to be characterized. It has been shown that other cell types, such as keratinocytes, can be target of multiple CTL responses that may have distinct roles in tissue injury (43). The residual monocyte death after blocking TRAIL, TWEAK, and FasL could be secondary to another apoptotic ligand or pathway as yet unidentified. Our group has previously shown that TNF-α was not involved in monocyte/macrophage cytotoxicity induced by Ag-specific CD4⁺ T cells (10, 31). Investigations on the role of other molecules including perforin and granzymes are currently underway, and could potentially explain the residual monocyte death induced by lupus T cells that was observed in this study.

Importantly, both control and lupus monocytes are equally susceptible to apoptosis mediated by these ligands. We found no significant difference in the susceptibility of these cells to apoptosis after treatment with TRAIL, TWEAK, or FasL recombinant proteins, suggesting that the increased susceptibility of lupus monocytes to undergo apoptosis is not associated with a decreased threshold for apoptotic signals.

Other mechanisms of monocyte death, such as complement toxicity, could contribute to increased monocyte apoptosis in lupus, as lupus sera are known to induce apoptosis of normal monocytes, possibly through complement-mediated damage (44). However, this phenomenon was observed only by a minority of lupus patients’ sera, suggesting that serum factors do not play a dominant role in the monocyte apoptosis seen in SLE patients. We considered the possibility that medications could contribute to the increase in the T cell apoptotic ligands. No effect on their expression was seen in short-term cultures. Although it is possible that this approach does not reflect chronic in vivo exposure, we also could not find any association between specific medications the lupus patients were taking and the expression of apoptotic ligands on their T cells. The monocyte apoptosis induced by autologous T cells seems to be specific for SLE, since this phenomenon was not observed in PBMC from patients with RA. This raises the possibility that the killing is mediated by a uniquely autoreactive subset present in lupus but not RA patients. Although RA T cells had higher levels of TRAIL than controls, there were no significant increases in the other apoptotic ligands, suggesting that the increase in this molecule is not sufficient to induce cytotoxicity. Multiple groups including our own have reported that T cells from patients with active lupus overexpress LFA-1 (9). We have previously reported that the T cells overexpressing LFA-1 mediate the monocyte killing in lupus (9). In other studies, we have shown that LFA-1 overexpression induces T cell autoreactivity and identical monocyte killing in vitro, and a lupus-like disease in vivo (11). The autoreactivity caused by LFA-1 overexpression is due in part to overstabilization of TCR-MHC interactions, permitting T cell activation by class II MHC molecules presenting inappropriate Ags (45). It is possible that LFA-1 overexpression in lupus T cells similarly overstabilizes TCR-MHC interactions in lupus patients, resulting in T cell activation by the monocytes with subsequent...
killing. An alternative interpretation is that the T cells are responding to as yet unidentified Ags presented by the lupus monocytes. However, the lack of a similar response in patients with RA, another autoimmune disease with T cell participation, argues for a different mechanism.

Our data demonstrate that monocyte apoptosis induced by lupus T cells is mediated by an increased expression of apoptotic ligands on autoreactive T cells, rather than to an increased sensitivity of SLE monocytes to these ligands. It is certainly possible that their overexpression could be due to T cell activation. Augmentation of monocyte apoptosis could account for an increase in the source of autoantigens and potentiate the immune response seen in SLE. An increase in the apoptosis of cells involved in phagocytosis, such as monocytes and macrophages, could also induce an increase in apoptotic cells by both augmenting apoptotic load and interfering with apoptotic clearance. In the absence of phagocytes, apoptotic cells eventually undergo necrosis (46, 47). Such necrosis may promote the maturation of dendritic cells that could then present the apoptotic material to T cells (48). It is known that dendritic cells, but not macrophages, efficiently cross present Ag derived from apoptotic cells to autologous cytotoxic T cells and induce class I-restricted CTLs after acquiring Ag from apoptotic cells (49). Interestingly, dendritic cells are known to engulf apoptotic monocytes (49). It has also been proposed that failure of efficient phagocytic clearance of cells dying by apoptosis results in tissue injury, and tissue protective mechanisms operating in interstitial fluid can be inactivated during apoptosis. The available evidence supports the contention that phagocyte clearance of intact cells dying by apoptosis protects tissues from inflammatory and coagulative injury initiated by cellular constituents (50). Finally, the combination of apoptotic monocyctic lines cells and lupus IgG is known to be a potent IFN-α inducer (51), a molecule that has recently been shown to promote monocyte differentiation into dendritic cells in SLE (52), and to induce increased expression of TRAIL on T cells (53). These suggest additional mechanisms potentially contributing to the autoimmune response.

In conclusion, we have identified a novel mechanism by which lupus monocytes undergo accelerated apoptosis. This phenomenon could be involved in inducing an increased apoptotic load in lupus, which could provide an increased source of autoantigens. In addition, increased apoptosis of the monocyctic/macrophage lineage could potentially interfere with clearance of other apoptotic material, in which turn would further potentiate an autoimmune response. Having identified some of the molecules involved in this phenomenon indicates that potential therapeutic interventions could be designed.

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