

# Increased expression of membrane TNF- $\alpha$ on activated peripheral CD8+ T cells in systemic lupus erythematosus

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**Abstract.** Membrane TNF- $\alpha$  is a precursor form of soluble TNF- $\alpha$  and exerts pro-inflammatory functions in a cell-to-cell contact manner. We showed that membrane TNF- $\alpha$  is induced upon activation on the cell surface of CD4+ T cells and CD8+ T cells. In patients with systemic lupus erythematosus (SLE), the percentage of membrane TNF- $\alpha$ -bearing CD8+ T cells (41.5 $\pm$ 12.3%) was significantly higher compared with those of healthy controls (26.7 $\pm$ 3.9%) (p=0.007) or patients with rheumatoid arthritis (29.8 $\pm$ 15.4%) (p=0.038). Membrane TNF- $\alpha$ -bearing CD8+ T cells from SLE patients displayed cytotoxic activity against L929 cells. It is possible that membrane TNF- $\alpha$  may be involved in the increased apoptosis and the generation of autoantigens in SLE.

## Introduction

Tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) is a pleiotropic cytokine that plays a critical role in health and diseases. The biological effects of TNF- $\alpha$  are mediated through two distinct cell surface receptors, TNF-RI (TNFRSF1A) and TNF-RII (TNFRSF1B) (1). Accumulating evidence suggests the involvement of TNF- $\alpha$  and its receptors in the pathogenesis and development of autoinflammatory disorders, such as rheumatoid arthritis (RA) and systemic lupus erythematosus (SLE) (2).

SLE is an inflammatory disorder affecting multiple organ systems and is considered to be the prototypic systemic autoimmune disease characterized by a variety of autoantibodies. Increased levels of serum TNF- $\alpha$  as well as soluble TNF receptors have been demonstrated in SLE (3,4), although it remains controversial whether soluble TNF receptor neutralizes

the TNF effect or stabilizes and prolongs the effect of TNF- $\alpha$ . A number of polymorphisms in the TNF- $\alpha$  gene and in the nearby TNF- $\beta$  gene have been shown to be associated with SLE (5-7). One of these polymorphisms, TNF- $\alpha$ -308A, located in the promoter region of the TNF- $\alpha$  gene, was shown to be associated with increased production of TNF- $\alpha$  (6). In a mouse model of SLE, TNF- $\alpha$  administration modulates its disease severity, depending on the dose and time of its administration (8,9).

Association of TNF receptors with SLE has also been demonstrated. Recent genome-wide linkage studies in multiplex SLE families have identified chromosome 1p36, where the gene for TNF-RII is located, as one of the candidate loci for SLE (10,11). A polymorphism at amino acid residue 196 (arginine at 196; 196R) of the TNF-RII gene was associated with the Japanese SLE patients (12,13). Increased production of IL-6 and cell death is observed in 196R TNF-RII-transfected HeLa cells compared with those transfected with wild-type TNF-RII (13). The molecular basis of a subset of the hereditary periodic fever syndromes has been demonstrated to reside in defects of the TNFRSF1A gene (*TNFRSF1A*) (14). Its clinical symptoms such as recurrent episodes of fever, skin rash, arthritis, and serositis, mimic those of SLE. We and others have reported that novel polymorphisms in the TNFRSF1A gene were associated with SLE (15,16). Moreover, a recent open-label study suggests that anti-TNF- $\alpha$  antibody, infliximab, may be effective to improve the inflammatory manifestations of SLE (17). These findings suggest that the TNF system may be involved in the pathogenesis of SLE.

TNF- $\alpha$  is generated from its precursor form, membrane TNF- $\alpha$ . Membrane TNF- $\alpha$  is expressed on the cell surface of resting natural killer (NK) cells as well as of activated T cells and macrophages. It has been demonstrated that membrane TNF- $\alpha$  exerts various pro-inflammatory functions in a cell-to-cell contact manner, which is distinct from the soluble form of TNF- $\alpha$  (18). In the present study, we report that membrane TNF- $\alpha$  is significantly induced on the cell surface of CD8+ T cells from SLE patients compared with those from healthy individuals and patients with RA. Membrane TNF- $\alpha$  on the CD8+ T cells from SLE patients exerted cytotoxic activity on L929 cells. These results suggest that membrane TNF- $\alpha$  might be involved in the pathogenesis of SLE.

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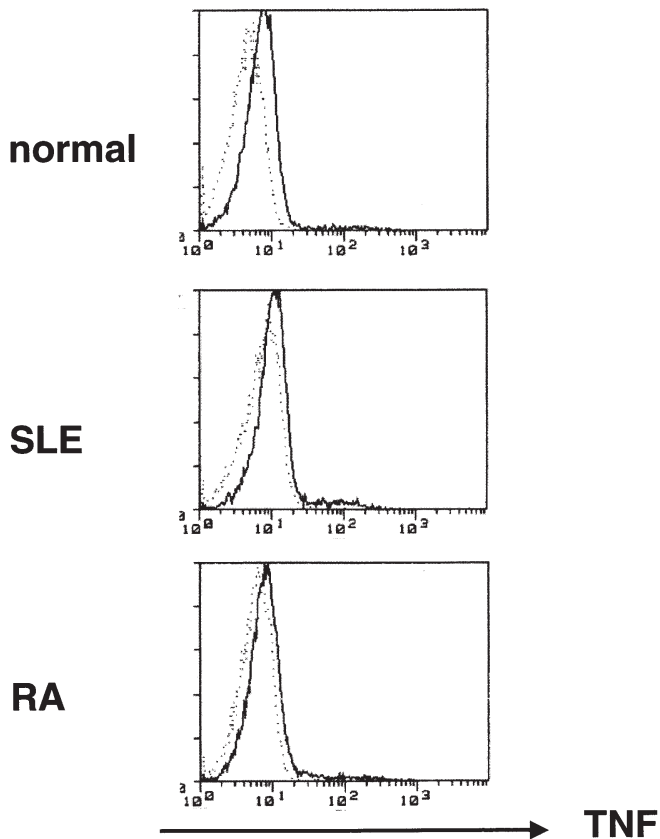


Figure 1. Membrane TNF- $\alpha$  expression on resting CD8+ T cells. CD8+ T cells were purified by using MACS CD8 microbeads from patients with SLE, RA and healthy individuals, followed by FACS analysis using mouse monoclonal anti-human TNF- $\alpha$  (solid line) or control mouse IgG1 antibody (dotted line).

## Materials and methods

**Subjects.** We studied 20 patients diagnosed as having SLE according to the 1982 revised criteria of the American College of Rheumatology (ACR) (19), and 10 patients with rheumatoid arthritis fulfilled the diagnostic criteria of 1987 revised criteria of the ACR (20). As a control, 10 healthy donors were enrolled. All of the patients and controls were Japanese, and the patients were followed at the rheumatology facility at Kyushu University Hospital. The disease activity of the SLE patients was stable and was administered for 5-10 mg of prednisolone.

**CD4+ and CD8+ T cell preparation.** Peripheral blood mononuclear cells (PBMCs) were prepared from heparinized blood of the patients with SLE, patients with RA, and the controls by centrifugation on lymphocyte separation medium (ICN Biochemicals, Aurora, OH, USA) gradients as described previously. T cells were separated by the Rosette purification method (Stem Cell Technologies, Vancouver, Canada), followed by Ficoll-Hypaque gradient centrifugation. CD4+ or CD8+ T cells were further separated from the T cells using MACS CD4 and CD8 microbeads, respectively, followed by MACS positive selection columns (Miltenyi Biotec, Auburn, CA) in accordance with the manufacturer's protocol. The purity of each isolated cell population routinely exceeded 95%.

**Immunofluorescence analysis of PBMCs.** Cells obtained from patients with SLE, RA or healthy individuals were treated with 10  $\mu$ g/ml of phytohemagglutinin (PHA) for 60 min. Then, the cells were washed 3 times in phosphate-buffered saline (PBS) supplemented with 2% bovine serum albumin (BSA), resuspended in the same buffer to a concentration of  $1 \times 10^5$  cells/25  $\mu$ l. Mouse monoclonal anti-human TNF- $\alpha$  antibody conjugated with fluorescein isothiocyanate (FITC) (R&D Systems, Minneapolis, MN) or anti-human CD8 antibody conjugated with phycoerythrin (PE) (Dako, Glostrup, Denmark) was added, and the cells were incubated for 30 min on ice and washed twice with PBS supplemented with 2% BSA. Mouse IgG1 (Dako) was used as a negative control. Flow cytometric analysis was performed with a FACScan (Becton Dickinson).

**Cytotoxic assay.** CD8+ T cells prepared by MACS CD8 microbeads from SLE patients were stimulated with 10  $\mu$ g/ml of PHA for 24 h to express membrane TNF- $\alpha$  on the surface. Then, the cells were irradiated by 3,000 rad and were used as effector cells bearing membrane TNF- $\alpha$  on the cell surface. L929 cells are sensitive to TNF- $\alpha$  and are used as target cells. L929 cells were seeded in a 96-well flat-bottom plate ( $1.0 \times 10^4$  cells/100  $\mu$ l/well) and were incubated for 24 h. Irradiated effector cells (CD8+ T cells) were added on target cells (L929 cells) with variable E/T ratio and incubated for 30 or 64 h. The wells were washed and viable cells were measured by crystal violet staining at OD590.

**Statistical analysis.** Results are expressed as means  $\pm$  SD. Significance was determined by a two-tailed t-test and multi-variable statistical analysis.

## Results

**Membrane TNF- $\alpha$  expression on resting CD4+ and CD8+ T cells.** Purified CD4+ or CD8+ T cells were studied for membrane TNF- $\alpha$  expression on the cell surface. As shown in Fig. 1, resting CD8+ T cells from patients with SLE and RA as well as from healthy individuals did not express significant levels of membrane TNF- $\alpha$ . Only a trace amount of membrane TNF- $\alpha$  was detected in several SLE patients. Resting CD4+ T cells were also negative for membrane TNF- $\alpha$  (data not shown).

**Membrane TNF- $\alpha$  expression on activated CD4+ and CD8+ T cells.** CD4+ or CD8+ T cells prepared from patients with SLE or RA or from healthy individuals were activated with PHA, followed by FACS analysis for membrane TNF- $\alpha$  expression. Although there was no difference in the induction of membrane TNF- $\alpha$  on activated CD4+ T cells from SLE patients and healthy individuals, activated CD8+ T cells from SLE patients significantly induced membrane TNF- $\alpha$  compared with those from healthy individuals (Fig. 2). Fig. 3 shows the percentage of activated CD4+ or CD8+ T cells bearing membrane TNF- $\alpha$  in patients with SLE, RA or healthy individuals. Although the percentages of membrane TNF- $\alpha$ -expressing CD4+ T cells were not significantly different between SLE patients ( $33.2 \pm 6.3\%$ ) (n=5), RA patients ( $29.4 \pm 2.5\%$ ) (n=5) and healthy individuals ( $33.8 \pm 3.2\%$ )

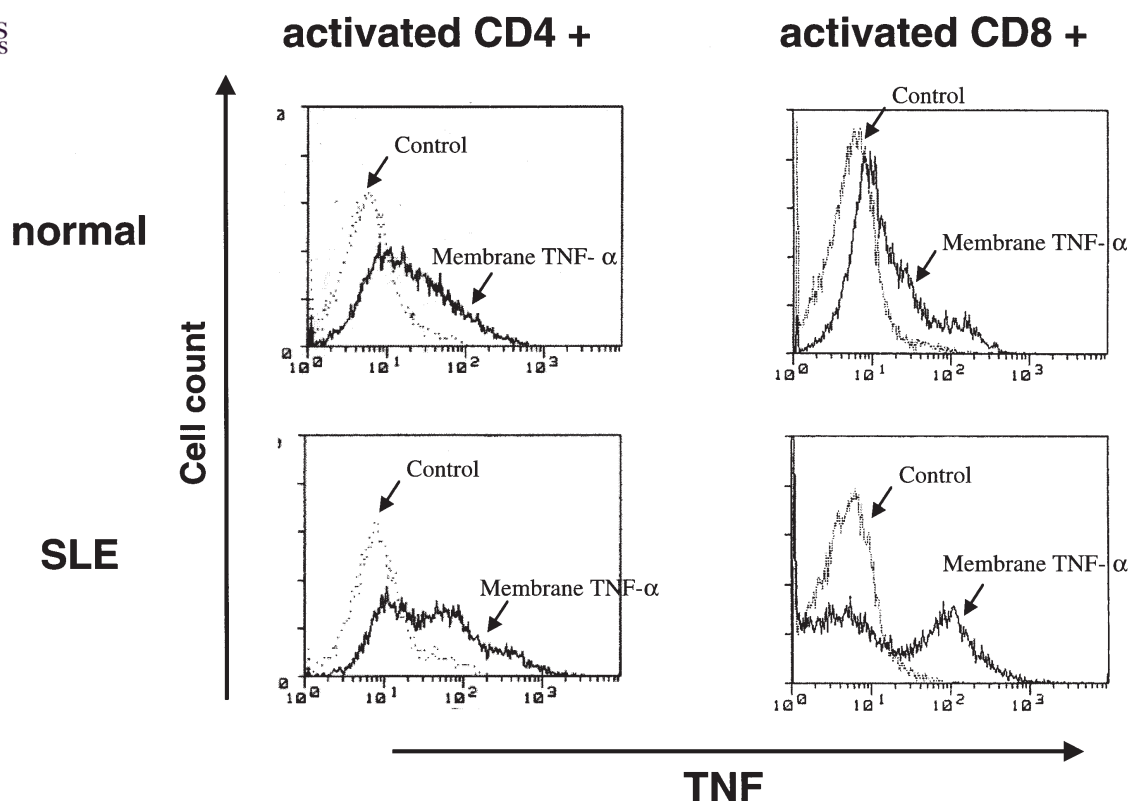


Figure 2. Expression of membrane TNF- $\alpha$  on activated CD4+ and CD8+ T cells. CD4+ or CD8+ T cells purified from SLE patients or from healthy individuals were stimulated with 10  $\mu$ g/ml of PHA for 24 h. Expression of membrane TNF- $\alpha$  was detected by FITC-conjugated mouse anti-TNF- $\alpha$  monoclonal antibody using FACS analysis (solid line). Mouse IgG1 was used as a negative control (dotted line).

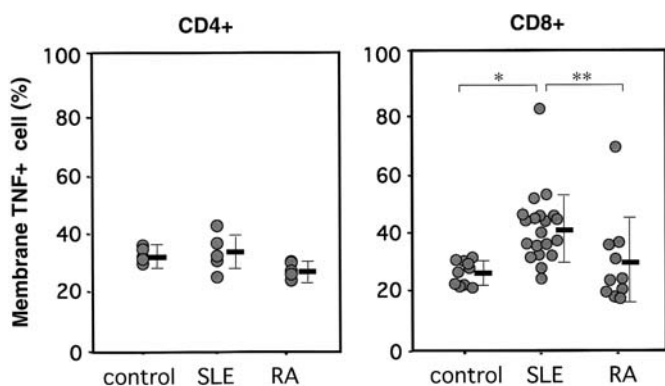


Figure 3. Increased expression of membrane TNF- $\alpha$  on activated CD8+ T cells in patients with SLE. The bars represent mean and SD of each group. Statistical evaluation was conducted by paired Student's t-test. \* $p=0.007$ , \*\* $p=0.038$ .

( $n=5$ ), the percentage of membrane TNF- $\alpha$  positive CD8+ T cells in SLE patients ( $41.5 \pm 12.3\%$ ) ( $n=20$ ) was significantly increased compared to those of healthy individuals ( $26.7 \pm 3.9\%$ ) ( $n=10$ ) ( $p=0.007$ ). There was no significant increase of membrane TNF- $\alpha$  in RA patients ( $29.8 \pm 15.4\%$ ) ( $n=10$ ), which was significantly low compared to that of SLE ( $p=0.038$ ).

**Cytotoxic activity of membrane TNF- $\alpha$  on CD8+ T cells.** Functional activity of the membrane TNF- $\alpha$  on CD8+ T cells of SLE patients was determined by using TNF- $\alpha$ -sensitive L929 cells. In order to exclude the possibility of the involvement of soluble factors, PHA-activated CD8+ T cells were irradiated

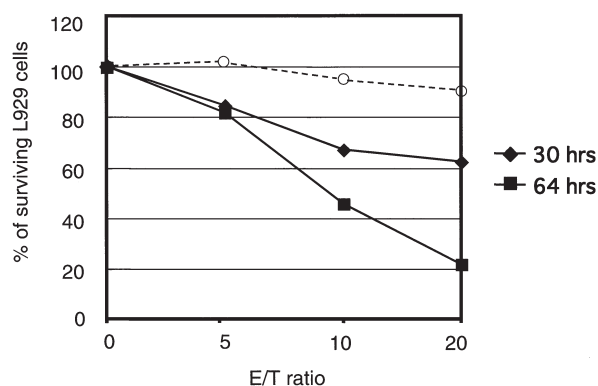


Figure 4. Cytotoxic activity of membrane TNF- $\alpha$  on activated CD8+ T cells. CD8+ T cells were stimulated with 10  $\mu$ g/ml of PHA followed by irradiation by 3,000 rad. Irradiated effector cells (CD8+ T cells) were added on target cells (L929 cells) with variable E/T ratio and incubated for 30 h (diamonds) or for 64 h (squares). The irradiated nonactivated CD8+ T cells incubated with L929 cells for 30 h are shown as a control (circles). The percentages of surviving L929 cells is detected by crystal violet. Representative results of 3 independent experiments are shown.

before incubation with L929 cells. As shown in Fig. 4, cell surface membrane TNF- $\alpha$  dose-dependently showed cytotoxic activity from effector/target cell ratio (E/T ratio) of 5-20. After 30 h of incubation, 62% of the L929 cells were alive at the E/T ratio of 20, while only 21% of the L929 cells were surviving at the E/T ratio of 20 after 64 h of incubation. Resting irradiated CD8+ T cells that are negative for membrane TNF- $\alpha$  on their surface did not show any cytotoxic activity against L929 cells.

## Discussion

We demonstrated that membrane TNF- $\alpha$  expression is significantly enhanced on activated CD8+ T cells from SLE patients compared with those from RA patients or healthy controls. The membrane TNF- $\alpha$  on the CD8+ T cells displayed cytotoxic activity against mouse L929 cells.

The etiologic importance of T cells in SLE has been widely recognized. Recent studies have shown the important role of cytotoxic CD8+ T cells in the pathogenesis of SLE. Increase in perforin- and/or granzyme B-positive, activated CD8+ T cells correlated with disease activity in SLE patients (21). These CD8+ T cells generated high amounts of soluble nucleosomes as well as granzyme B-dependent unique autoantigen fragments from their target K562 cells, which would imply the positive role of CD8+ T cells in the production of excess amount of autoantigens. We here showed the possibility that CD8+ T cells in SLE patients express a larger amount of membrane TNF- $\alpha$  upon activation, and are more prone to exert cytotoxic activity. It is possible that membrane TNF- $\alpha$  is an effector molecule in addition to perforin and granzyme B in CD8+ T cells. Increased soluble and T cell-associated TNF-related apoptosis-inducing ligand (TRAIL), a member of the TNF ligand family proteins like TNF- $\alpha$ , have been reported in active SLE patients (22). Considering its pro-apoptotic activity, TRAIL may amplify the abnormal apoptotic process in SLE.

Membrane TNF- $\alpha$ , a precursor form of soluble TNF- $\alpha$ , is expressed constitutively on NK cells as well as on activated macrophages, T cells and B cells. The biological activities of membrane TNF- $\alpha$  are mediated through cell-to-cell interaction, which is a manner distinct from that of soluble TNF- $\alpha$ . Membrane TNF- $\alpha$  acts both as a ligand and a receptor; therefore, it is considered to be a bipolar molecule that modulates local inflammation (23-25). A number of biological functions have been reported as a ligand, such as cytotoxic activity and antibody production (26,27). The biological functions of membrane TNF- $\alpha$  as a receptor is generated by signals directed from outside to inside, recently identified as a novel mechanism of membrane TNF- $\alpha$ . This reverse signal generates a number of biological functions, such as E-selectin expression, cell cycle arrest and apoptosis of the membrane TNF- $\alpha$ -expressing Jurkat T cells (28). In addition, the involvement of membrane TNF- $\alpha$  expressed on CD8+ T cells is also implicated for the pathogenesis of mouse autoimmune diabetes (29).

In conclusion, we have demonstrated increased membrane TNF- $\alpha$  expression on activated CD8+ T cells from SLE patients. Membrane TNF- $\alpha$  on activated CD8+ T cells from SLE patients displayed cytotoxic activity to L929 cells. *In vivo* function of membrane TNF- $\alpha$  is complex; however, the cytotoxic effect of membrane TNF- $\alpha$  on activated CD8+ T cells may be one of the factors contributing to the excess apoptosis and autoantigen presentation in SLE.

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