筑波大学

博士（医学）学位論文

Caspase-independent cell death by CD300LF (MAIR-V), an inhibitory immunoglobulin-like receptor on myeloid cells

(抑制性免疫受容体 CD300LF（MAIR-V）による
カスパーゼ非依存の細胞死)

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Introduction

Activating and inhibitory cell surface receptors play important roles in regulation of immune responses (1, 2). Recent progress has demonstrated that many inhibitory receptors pair with activating, as well as inhibitory, isoforms (2, 3), both of whose genes are located in small clusters on a chromosome (4). The CMRF, recently assigned as CD300 (5), are a multigene family consisting of six genes on human chromosome 17 (6). The mouse counterparts of CD300 molecules, which were reported to be as myeloid-associated immunoglobulin (Ig)-like receptor (MAIR) (7-10) /CMRF-35-like molecule-1 (CLM) (11) /leukocyte mono-Ig-like receptor (LMIR) (12, 13) /DIgR (14, 15), were encoded by nine genes on chromosome 11, syntenic region of human chromosome 17. CD300a (MAIR-I) and MAIR-II (7), also named as CLM8/LMIR1 and CLM4/LMIR2/DIgR1, respectively (14, 11, 12), constitute paired activating and inhibitory receptors. CD300a is preferentially expressed on myeloid cells, including macrophages, neutrophils, dendritic cells (DCs) and bone marrow-derived cultured mast cells, and mediates an inhibitory signal through an immunoreceptor tyrosine-based inhibitory motif (ITIM) in the cytoplasmic portion (7, 8). On the other hand, MAIR-II,

expressed on macrophages, associates with either immunoreceptor tyrosine-based activating motif (ITAM)-containing adapters DAP12 or FcRγ chain and mediates an activating signal, resulting in inflammatory cytokine secretion from macrophages (7, 9).

We and others have recently reported that one of the genes in the mouse CD300 family, MAIR-IV, also named LMIR4 or CLM5 (16, 13), associates with the FcRγ chain and mediates an activating signal in neutrophils(10). The Ig-like domain of MAIR-IV in the extracellular portion shares 91% amino acid identity with that of MAIR-V (CD300LF), also named LMIR3 or CLM1 (16, 13, 10). CD300LF has a long cytoplasmic tail containing two consensus ITIMs, suggesting that MAIR-IV and CD300LF constitute paired activating and inhibitory receptors. In fact, CD300LF associates with SHP-1, and expression of CD300LF in a macrophage cell line, RAW264, effectively inhibits in vitro osteoclast formation (11). Recently, Shi et al reported dendritic cell (DC)-derived immunoglobulin receptor 2 (DIgR2), which lacks 7 amino acids (PAIQVPI) in the stalk region of CD300LF, suggesting that DIgR2 is a splicing variant of CD300LF (15). Although DIgR2 was reported to mediate negative

regulation of DC-induced antigen-specific T cell responses, whether CD300LF is also involved in the same function in DC remains undetermined.

Here, we investigated the role of CD300LF in immune responses and describe that CD300LF-mediated signals induce caspase-independent cell death in myeloid cells.

Materials and Methods

Mice and cell lines

C57BL/6J mice were purchased from Clea (Tokyo, Japan). All mice used in this study were 8-10 wk of age and were maintained in our animal facility under specific pathogen free conditions. All experiments were performed according to the guidelines of the animal ethics committee of the University of Tsukuba Animal Research Center. BW5147, RAW264, and P3U1 cells are murine T, macrophage and myeloma cell lines, respectively. All cell lines were purchased from American Type Tissue Culture Collection (Rockville, MD).

Antibodies and reagents

Control rat and mouse IgGs, anti-mouse CD11b (Mac1), CD11c, Ly6G (Gr-1), CD3, NK1.1 mAbs and PE-conjugated annexin V were purchased from BD Biosciences (San Diego, CA); anti-Flag mAb from Sigma-Aldrich (St. Louis, MO); and PR-conjugated rabbit anti-rat IgG from Rockland (Gilbertsville, PA). Anti-CD300LF (TX70 and TX73) and anti-Flag (TX74) mAbs were generated in our laboratory. In brief, 1 x 10^7 BW5147 transfectants expressing Flag tagged-CD300LF suspended in 500 μl PBS was

emulsified with the same volume of CFA. A rat was s.c. injected in each footpad with 500 μl of the suspension of BW5147 transfectants on day 1 and 8. On day 11, popliteal lymph node cells were harvested and fused with the Sp2/0 myeloma cell line by a standard method. BW5147 transfectants expressing Flag-tagged CD300LF, CD300a, MAIR-II, or MAIR-IV were used for screening of specificity to CD300LF or Flag protein by flow cytometry. Wortmannin, LY294002, cytochalasin D, dithiothreitol (DTT), 3-Methyladenine (3-MA), N-Acetyl-L-cystein (NAC), staurosphorine, etoposide, LPS, Earle's balanced salts solution (EBSS) and monodansylcadaverine (MDC) were purchased from Sigma-Aldrich. N-benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone (z-VAD-fmk) was purchased from Calbiochem (San Diego, CA). Zymozan and CpG DNA (ODN1826) were purchased from Molecular Probes (Eugene, OR) and Invivogen (San Diego, CA), respectively.

Establishment of transfectants expressing CD300LF

The *CD300LF* cDNA tagged with the Flag cDNA at the NH_2 terminus was subcloned

into the pMX retroviral vector (17). To generate site-directed CD300LF mutants at residues Y^{241}, Y^{289} and Y^{325}, we designed PCR primers containing a codon for F (TTT) instead of Y (TAT). BW5147, P3U1, and RAW264 cells stably expressing Flag-tagged CD300LF at the N-terminus were established as described previously (18).

Induction and measurement of cell death

One million transfectants expressing CD300LF or thyoglycollate-elicited peritoneal macrophages (more than 80% purity, as determined by flow cytometry) were incubated in 1 ml of RPMI medium for 30 min in the presence of 2.5 μg of premixed rat anti-CD300LF, anti-MAIR-I, anti-MAIR-IV, or anti-Flag mAbs with 4 μg of rabbit anti-rat IgG. Cells were then washed with cold PBS twice and stained with annexin-V and propidium iodide (PI), as described (19). Briefly, cells were resuspended in a binding solution (10 mM HEPES/NaOH, pH 7.4, 140 mM NaCl, 2.5 mM $CaCl_2$) containing PE-conjugated annexin-V, at the dilutions recommended by the manufacturer (BD Biosciences). After 15 minutes, cells were resuspended in the binding solution containing PI and analyzed by flow cytometry. For inhibition of caspase activation,

cells (10^6/mL) were pretreated with 50 μM pan-caspase inhibitor z-VAD-fmk for 24 hours at 37°C, and then incubated as described above. For autophagy induction by amino acid starvation, cells were washed with PBS three times and incubated with 1ml EBSS at 37°C for 4 hours. For inhibition of autophagy, cells (10^6/mL) were pretreated with 10 μM of NAC or 10 mM of 3-MA for 12 hours at 37°C, and then incubated as described above. Intracellular autophagic vacuoles were labeled with monodansylcadaverine (MDC), as described previously (20). In brief, following induction of autophagy by amino acid starvation or stimulation by anti-CD300LF, the cells were incubated with 0.05 mM MDC in PBS at 37°C for 10 minutes (21). After incubation, cells were washed four times with PBS and collected in 10 mM Tris-HCl, pH 8 containing 0.1% Triton X-100. Intracellular MDC was measured by fluorescence photometry (excitation wavelength 380 nm, emission filter 525 nm) in a Varioskan of ThermoFisher Scientific (Waltham, MA). To normalize the measurements to the number of cells present in each well, a solution of ethidium bromide was added to a final concentration of 0.2 μM and the DNA fluorescence was measured (excitation wavelength 530 nm, emission filter 590 nm). The MDC incorporated was expressed as

specific activity (arbitrary units).

DNA staining

Cells were incubated in a hypotonic buffer (0.1% sodium citrate, 0.1% Triton X-100) containing 50 μg/ml propidium iodide (PI) overnight at 4°C and analyzed by flow cytometry.

XBP-1 splicing

XBP-1 splicing was assessed using RT-PCR with primers that spanned the splice site in the XBP-1 mRNA: forward, ACGAGAGAAAACTCATGGC; reverse, ACAGGGTCCAACTTGTCCAG. PCR products were sequenced to confirm their identities.

Statistics

Statistical analyses were performed using the unpaired Student's *t*-test.

Results

Generation of monoclonal antibodies against CD300LF

To determine the functional characteristics of CD300LF, we generated two monoclonal antibodies (mAbs) (TX70 and TX73) against CD300LF and a mAb against Flag protein by immunizing a rat with a mouse T cell line, BW5147, transfected with Flag-tagged *CD300LF*. To confirm the specificity of these mAbs to CD300LF, we also established BW5147 transfectants stably expressing CD300LF, CD300a, or MAIR-IV tagged at the N-terminus with Flag. TX70 and TX73 mAbs specifically stained the transfectants expressing CD300LF, but not those expressing CD300a or MAIR-IV, indicating that these mAbs are specific to CD300LF (**Fig 1A**).

Analyses by flow cytometry using TX70 mAb showed that CD300LF was expressed on macrophages, but not on B cells, T cells or granulocytes, derived from the spleen and peritoneal cavity (**Fig. 1B**). Thyoglycollate-elicited peritoneal macrophages expressed an amount of CD300LF comparable to peritoneal macrophages from naïve mice. In vitro stimulation of peritoneal macrophages with the ligands for toll-like receptors, such as LPS (1~100 μg/ml), zymozan (1~100 μg/ml), or CpG DNA (100 nM~1 μM), did not

affect the expression of CD300LF on peritoneal macrophages (data not shown). We confirmed similar results by using TX73 anti-CD300LF mAb (data not shown).

CD300LF mediates cell death

We found that CD300LF contains a consensus immunoreceptor tyrosine-based switching motif (ITSM) as well as two consensus ITIMs in the cytoplasmic tail. To examine whether CD300LF-mediated signals affect cell activation or proliferation, BW5147 transfectant expressing CD300LF was stimulated with anti-CD300LF (TX70) in the presence of the secondary anti-rat IgG antibody for 30 min. As demonstrated in **Fig 2A**, cross-linking CD300LF with the antibody significantly induced cell death, as analyzed by flow cytometry for binding with annexin-V and/or PI, compared with the transfectant stimulated with the IC of a control rat IgG with the secondary anti-rat IgG antibody. In contrast, cross-linking MAIR-I or MAIR-IV on BW5147 transfectants with anti-MAIR-I or anti-MAIR-IV mAbs, respectively, did not induce cell death (**Fig 2B**). Since the BW5147 cell is a T cell line and did not express the receptors for IgG (FcγRs) (data not shown), FcγRs were excluded to be involved in the effect of anti-

CD300LF and the secondary antibody. Cross-linking CD300LF with anti-CD300LF mAb also induced cell death in *CD300LF*-transfected myeloma cell line P3U1 and macrophage cell line RAW264 (**Fig 2C**). Moreover, cell death was also induced by TX73 anti-CD300LF and anti-Flag mAbs (**Fig 2D**). Taken together, these results suggested that the cell death was specifically mediated by CD300LF. Finally, we examined whether CD300LF mediated cell death also occurred in primary cells. As demonstrated in **Fig 2E,** cross-linking CD300LF with TX70 anti-CD300LF mAb significantly induced cell death in peritoneal macrophages, suggesting that CD300LF plays an important role in survival of peritoneal macrophages.

CD300LF-mediated cell death was independent of ITIM or ITSM

To investigate the molecular mechanism of CD300LF-mediated cell death, we first examined whether cross-linking CD300LF induces cell death at 4° C. We observed that culture of the BW5147 transfectant at 4° C abrogated cell death mediated by stimulation with anti-CD300LF mAb (**Fig 3A**), suggesting that a CD300LF-mediated signal is involved in cell death. To examine whether ITIM or ITSM in the cytoplasmic

region of CD300LF is involved in the cell death, we established BW5147 transfectants stably expressing mutant CD300LF, in which the cytoplasmic region was deleted (Δ cyto) or the tyrosines (Y) at residue 241, 289, and 325 were replaced with phenylalanines (F) ($Y\text{-}F^{241, 289, 325}$) (**Fig. 3B**). These transfectants expressed an amount of CD300LF comparable to that of the transfectant expressing WT CD300LF (**Fig 3C**). We found that cross-linking the mutant CD300LF lacking the cytoplasmic region (Δ cyto) with TX70 mAb did not induce cell death (**Fig 3D**), indicating that the cytoplasmic region of CD300LF is responsible for the cell death. However, cross-linking $Y\text{-}F^{241, 289, 325}$ mutant CD300LF still induced cell death comparably to wild type CD300LF (**Fig 3D**). These results indicated that the cytoplasmic region other than ITIMs or ITSM played an essential role in induction of cell death.

CD300LF has a phosphoinositide-3 kinase (PI3K) binding motif (Y^{276}xxM) in the cytoplasmic region. To investigate whether the cell death is mediated by PI3K, we pretreated the BW5147 transfectant with the PI3K inhibitors, wortmannin (1 μ M) or LY294002 (1 μ M), and stimulated the transfectant with TX70 anti-CD300LF mAb in

the presence of the PI3K inhibitors. However, we did not observe any effect of the PI3 kinase inhibitors on the cell death (data not shown), suggesting that CD300LF-mediated cell death was also independent of PI3K.

CD300LF-mediated cell death was independent of caspases.

To examine whether caspase activation is implicated in CD300LF-mediated cell death, BW5147 transfectant expressing CD300LF was stimulated with TX70 anti-CD300LF in the presence or absence of a broad-spectrum caspase inhibitor, z-VAD-fmk. While z-VAD-fmk effectively suppressed caspases-dependent cell death induced by etoposide (40 μM) (**Fig 4A**), CD300LF-mediated cell death was not affected by z-VAD-fmk, suggesting that it is independent of caspases. Caspase activation induces cell death with nuclear features, such as chromatin condensation and DNA fragmentation. Flow cytometric analysis by staining of nucleic acids with PI showed no significant DNA fragmentation in the BW5147 transfectant stimulated with TX70 anti-CD300LF (**Fig 4B**). Moreover, we failed to demonstrate internucleosomal DNA laddering (data not shown). Taken together, these results indicated that CD300LF-mediated cell death was

independent of caspases.

CD300LF-mediated cell death was independent of ER stress.

Endoplasmic reticulum (ER) stress caused by misfolded proteins or cytotoxic drugs induces programmed cell death (22), which is not prevented by caspase inhibitors. Activation of unfolded protein response (UPR) causes IRE1-dependent splicing of a transcription factor X-box binding protein-1 (XBP-1) mRNA (23). To test whether ER stress is involved in CD300LF-mediated cell death, we examined XBP-1 splicing in BW5147 transfectant expressing CD300LF. While the splicing isoform of *XBP-1 (XBP-1s)* was detected in the transfectant after treatment with DTT, an ER stress-inducer, it was not detected in the transfectant stimulated with TX70 anti-CD300LF mAb **(Fig 5)**. These results indicated that CD300LF-mediated cell death was independent of ER stress.

CD300LF-mediated cell death was independent of autophagy

Autophagic programmed cell death associated with autophagosomes is another type of

cell death (24, 25). To examine whether CD300LF mediates autophagic programmed cell death, BW5147 transfectant expressing WT CD300LF was pretreated with autophagy inhibitors 3-MA or NAC (26, 27) and then stimulated with TX70 anti-CD300LF mAb. While these inhibitors effectively suppressed the autophagic programmed cell death induced by staurosporine, they did not affect CD300LF-mediated cell death **(Fig 6A).** Moreover, while BW5147 transfectant expressing WT CD300LF was effectively stained with MDC, a specific autophagolysosome marker (20), after autophagy induction by amino acid starvation, MDC did not stain the transfectant stimulated with TX70 anti-CD300LF mAb **(Fig 6B).** Together, these results indicate that CD300LF-mediated cell death was independent of autophagy.

Morphology of CD300LF-mediated cell death

To further characterize the CD300LF-mediated cell death, morphological analyses were performed by electron microscope (EM). As demonstrated in **Fig 7**, scanning EM (SEM) demonstrated that BW5147 transfectant expressing WT CD300LF that had been

stimulated with TX70 anti-CD300LF mAb showed cell shrinkage and aggregation. Notably, we observed lack of fine blebs on the cell surface of dead BW5147 transfectant expressing WT, but not mutant (Δ cyto), CD300LF. The similar morphological feature was also observed in apoptotic BW5147 transfectant induced by UV, but not in necrotic BW5147 transfectant induced by heat shock at 56 $^{\circ}$ C. However, transmitted EM (TEM) showed no obvious morphological difference in the cytoplasm and nuclei between BW5147 transfectants expressing WT and mutant (Δ cyto) CD300LF (data not shown).

Discussion

This is the first report to demonstrate that cross-linking CD300LF induced cell death in primary peritoneal macrophages as well as in several transfectants expressing CD300LF. We showed that CD300LF-mediated cell death was dependent on the cytoplasmic region. However, the tyrosines in the ITIM or ITSM were not required for the cell death. CD300LF has another tyrosine at residue 276 in the cytoplasmic region, which may be involved in PI3 kinase binding. We showed that this tyrosine may also not be responsible for the cell death, because PI3 kinase inhibitors, wortmannin or LY294002, did not affect cell death mediated by CD300LF. At present, which domain in the cytoplasmic region of CD300LF is responsible for cell death remained undetermined.

Although the morphological feature of the dead cells mediated by CD300LF revealed by SEM was similar to that of apoptotic cells, we showed that the cell death was independent of caspases activation, a major signaling event in programmed cell death in many developmental and physiological settings (28, 29). Recent evidence has demonstrated that programmed cell death can occur in the absence of caspases (30). Several cell surface receptors have been reported to mediate caspase-independent

programmed cell death in immune cells. For example, sialic acid-binding immunoglobulin-like lectin (Siglec)-9, which is expressed on neutrophils and monocytes, mediates in part caspase-independent cell death in neutrophils (26). Morphological analyses of Siglec-9-mediated cell death showed autophagosomes in the cytoplasm (26), suggesting that cross-linking Siglec-9 induced autophagic programmed cell death. In fact, an autophagy inhibitor NAC partially inhibited Siglec-9-mediated cell death (26). CD47 also mediates caspase-independent cell death in chronic lymphocytic leukemia cells (31, 32), T cells (33), monocytes and DC (34). Although the molecular mechanisms of the caspase-independent cell death in these cell types are still undetermined, CD47-mediated cell death was suppressed by cytochalasin D, an inhibitor of actin polymerization, suggesting that cytoskeleton reorganization may be involved in CD47-mediated, caspase-independent cell death (32). In contrast, CD300LF-mediated cell death was not inhibited in the presence of NAC, 3-MA, or cytochalasin D (data not shown), indicating that the mechanism of CD300LF-mediated, caspase-independent cell death is different from that induced by Siglec-9 or CD47.

Since ER stress also induces caspase-independent cell death (22), we examined the splicing variant of XBP-1 produced by ER stress in dead cells mediated by CD300LF. However, we did not detect any splicing variant of XBP-1, indicating that CD300LF-mediated cell death was not caused by ER stress. Future studies are required to clarify the molecular mechanism of CD300LF-mediated cell death.

Among the nine molecules in the mouse CD300 (MAIR) family, CD300a (MAIR-I) and CD300LF have ITIMs in the cytoplasmic region that mediates inhibitory signals. We previously demonstrated that coligation of CD300a (MAIR-I) and FcεRI inhibited IgE-mediated degranulation from bone marrow-derived cultured mast cells and a rat basophil cell line, RBL-2H3, transfected with *CD300LF* (7, 8). In contrast, the inhibitory function of CD300LF in immune responses remains unclear. In this study, we demonstrated that CD300LF mediated cell death was induced in peritoneal macrophages as well as transfectants expressing CD300LF. Regulation of survival of immune cells, including macrophages, should be fundamentally important for immune regulation. Identification and characterization of the ligand for CD300LF is essential

to clarify the in vivo role of CD300LF-mediated cell death as well as its inhibitory function in immune responses.

References

1. Ravetch, J. V., and L. L. Lanier. 2000. Immune inhibitory receptors. *Science 290:84-89.*

2. Lanier, L. L. 2001. Face off--the interplay between activating and inhibitory immune receptors. *Curr Opin Immunol 13:326-331.*

3. Takai, T., and M. Ono. 2001. Activating and inhibitory nature of the murine paired immunoglobulin-like receptor family. *Immunol Rev 181:215-222.*

4. Martin, A. M., J. K. Kulski, C. Witt, P. Pontarotti, and F. T. Christiansen. 2002. Leukocyte Ig-like receptor complex (LRC) in mice and men. *Trends Immunol 23:81-88.*

5. Zola, H., B. Swart, I. Nicholson, B. Aasted, A. Bensussan, L. Boumsell, C. Buckley, G. Clark, K. Drbal, P. Engel, D. Hart, V. Horejsi, C. Isacke, P. Macardle, F. Malavasi, D. Mason, D. Olive, A. Saalmueller, S. F. Schlossman, R. Schwartz-Albiez, P. Simmons, T. F. Tedder, M. Uguccioni, and H. Warren. 2005. CD molecules 2005: human cell differentiation molecules. *Blood 106:3123-3126.*

6. Clark, G. J., B. Cooper, S. Fitzpatrick, B. J. Green, and D. N. Hart. 2001. The gene encoding the immunoregulatory signaling molecule CMRF-35A localized to human chromosome 17 in close proximity to other members of the CMRF-35 family. *Tissue Antigens 57:415-423.*

7. Yotsumoto, K., Y. Okoshi, K. Shibuya, S. Yamazaki, S. Tahara-Hanaoka, S. Honda, M. Osawa, A. Kuroiwa, Y. Matsuda, D. G. Tenen, A. Iwama, H. Nakauchi, and A. Shibuya. 2003. Paired activating and inhibitory immunoglobulin-like receptors, MAIR-I and MAIR-II, regulate mast cell and macrophage activation. *J Exp Med 198:223-233.*

8. Okoshi, Y., S. Tahara-Hanaoka, C. Nakahashi, S. Honda, A. Miyamoto, H. Kojima, T. Nagasawa, K. Shibuya, and A. Shibuya. 2005. Requirement of the tyrosines at residues 258 and 270 of MAIR-I in inhibitory effect on degranulation from basophilic leukemia RBL-2H3. *Int Immunol 17:65-72.*

9. Nakahashi, C., S. Tahara-Hanaoka, N. Totsuka, Y. Okoshi, T. Takai, N. Ohkohchi, S. Honda, K. Shibuya, and A. Shibuya. 2007. Dual assemblies of an activating immune receptor, MAIR-II, with ITAM-bearing adapters DAP12 and FcRgamma chain on peritoneal macrophages. *J Immunol 178:765-770.*

10. Nakano, T., S. Tahara-Hanaoka, C. Nakahashi, I. Can, N. Totsuka, S. I. Honda, K. Shibuya, and A. Shibuya. 2008. Activation of neutrophils by a novel triggering immunoglobulin-like receptor MAIR-IV. *Mol Immunol 45:289-294.*

11. Chung, D. H., M. B. Humphrey, M. C. Nakamura, D. G. Ginzinger, W. E. Seaman, and M. R. Daws. 2003. CMRF-35-like molecule-1, a novel mouse myeloid receptor, can inhibit osteoclast formation. *J Immunol 171:6541-6548.*

12. Kumagai, H., T. Oki, K. Tamitsu, S. Z. Feng, M. Ono, H. Nakajima, Y. C. Bao, Y. Kawakami, K. Nagayoshi, N. G. Copeland, D. J. Gilbert, N. A. Jenkins, T. Kawakami, and T. Kitamura. 2003. Identification and characterization of a new pair of immunoglobulin-like receptors LMIR1 and 2 derived from murine bone marrow-derived mast cells. *Biochem Biophys Res Commun 307:719-729.*

13. Izawa, K., J. Kitaura, Y. Yamanishi, T. Matsuoka, T. Oki, F. Shibata, H. Kumagai, H. Nakajima, M. Maeda-Yamamoto, J. P. Hauchins, V. L. Tybulewicz, T. Takai, and T. Kitamura. 2007. Functional analysis of activating receptor LMIR4 as a counterpart of inhibitory receptor LMIR3. *J Biol Chem 282:17997-18008.*

14. Luo, K., W. Zhang, L. Sui, N. Li, M. Zhang, X. Ma, L. Zhang, and X. Cao. 2001. DIgR1, a novel membrane receptor of the immunoglobulin gene superfamily, is preferentially expressed by antigen-presenting cells. *Biochem Biophys Res Commun 287:35-41.*

15. Shi, L., K. Luo, D. Xia, T. Chen, G. Chen, Y. Jiang, N. Li, and X. Cao. 2006. DIgR2, dendritic cell-derived immunoglobulin receptor 2, is one representative of a family of IgSF inhibitory receptors and mediates negative regulation of dendritic cell-initiated antigen-specific T-cell responses. *Blood 108:2678-2686.*

16. Fujimoto, M., H. Takatsu, and H. Ohno. 2006. CMRF-35-like molecule-5 constitutes novel paired receptors, with CMRF-35-like molecule-1, to transduce activation signal upon association with FcRgamma. *Int Immunol 18:1499-1508.*

17. Kitamura, T., M. Onishi, S. Kinoshita, A. Shibuya, A. Miyajima, and G. P. Nolan. 1995. Efficient screening of retroviral cDNA expression libraries. *Proc Natl Acad Sci U S A 92:9146-9150.*

18. Shibuya, K., L. L. Lanier, J. H. Phillips, H. D. Ochs, K. Shimizu, E. Nakayama, H. Nakauchi, and A. Shibuya. 1999. Physical and functional association of LFA-1 with DNAM-1 adhesion molecule. *Immunity 11:615-623.*

19. Vermes, I., C. Haanen, H. Steffens-Nakken, and C. Reutelingsperger. 1995. A novel assay for apoptosis. Flow cytometric detection of phosphatidylserine expression on early apoptotic cells using fluorescein labelled Annexin V. *J Immunol Methods 184:39-51.*

20. Munafo, D. B., and M. I. Colombo. 2001. A novel assay to study autophagy: regulation of autophagosome vacuole size by amino acid deprivation. *J Cell Sci 114:3619-3629.*

21. Biederbick, A., H. F. Kern, and H. P. Elsasser. 1995. Monodansylcadaverine (MDC) is a specific in vivo marker for autophagic vacuoles. *Eur J Cell Biol 66:3-14.*

22. Ron, D. 2002. Translational control in the endoplasmic reticulum stress response. *J Clin Invest 110:1383-1388.*

23. Calfon, M., H. Zeng, F. Urano, J. H. Till, S. R. Hubbard, H. P. Harding, S. G. Clark, and D. Ron. 2002. IRE1 couples endoplasmic reticulum load to secretory capacity by processing the XBP-1 mRNA. *Nature 415:92-96.*

24. Levine, B., and J. Yuan. 2005. Autophagy in cell death: an innocent convict? *J Clin Invest 115:2679-2688.*

25. Tsujimoto, Y., and S. Shimizu. 2005. Another way to die: autophagic programmed cell death. *Cell Death Differ 12 Suppl 2:1528-1534.*

26. von Gunten, S., S. Yousefi, M. Seitz, S. M. Jakob, T. Schaffner, R. Seger, J. Takala, P. M. Villiger, and H. U. Simon. 2005. Siglec-9 transduces apoptotic and nonapoptotic death signals into neutrophils depending on the proinflammatory cytokine environment. *Blood 106:1423-1431.*

27. Li, C., E. Capan, Y. Zhao, J. Zhao, D. Stolz, S. C. Watkins, S. Jin, and B. Lu. 2006. Autophagy is induced in CD4+ T cells and important for the growth factor-withdrawal cell death. *J Immunol 177:5163-5168.*

28. Strasser, A., L. O'Connor, and V. M. Dixit. 2000. Apoptosis signaling. *Annu Rev Biochem 69:217-245.*

29. Kaufmann, S. H., and M. O. Hengartner. 2001. Programmed cell death: alive and well in the new millennium. *Trends Cell Biol 11:526-534.*

30. Jaattela, M., and J. Tschopp. 2003. Caspase-independent cell death in T lymphocytes. *Nat Immunol 4:416-423.*

31. Mateo, V., L. Lagneaux, D. Bron, G. Biron, M. Armant, G. Delespesse, and M. Sarfati. 1999. CD47 ligation induces caspase-independent cell death in chronic lymphocytic leukemia. *Nat Med 5:1277-1284.*

32. Mateo, V., E. J. Brown, G. Biron, M. Rubio, A. Fischer, F. L. Deist, and M. Sarfati. 2002. Mechanisms of CD47-induced caspase-independent cell death in normal and leukemic cells: link between phosphatidylserine exposure and cytoskeleton organization. *Blood 100:2882-2890.*

33. Pettersen, R. D., K. Hestdal, M. K. Olafsen, S. O. Lie, and F. P. Lindberg. 1999. CD47 signals T cell death. *J Immunol 162:7031-7040.*

34. Johansson, U., K. Higginbottom, and M. Londei. 2004. CD47 ligation induces a rapid caspase-independent apoptosis-like cell death in human monocytes and dendritic cells. *Scand J Immunol 59:40-49.*

Figures

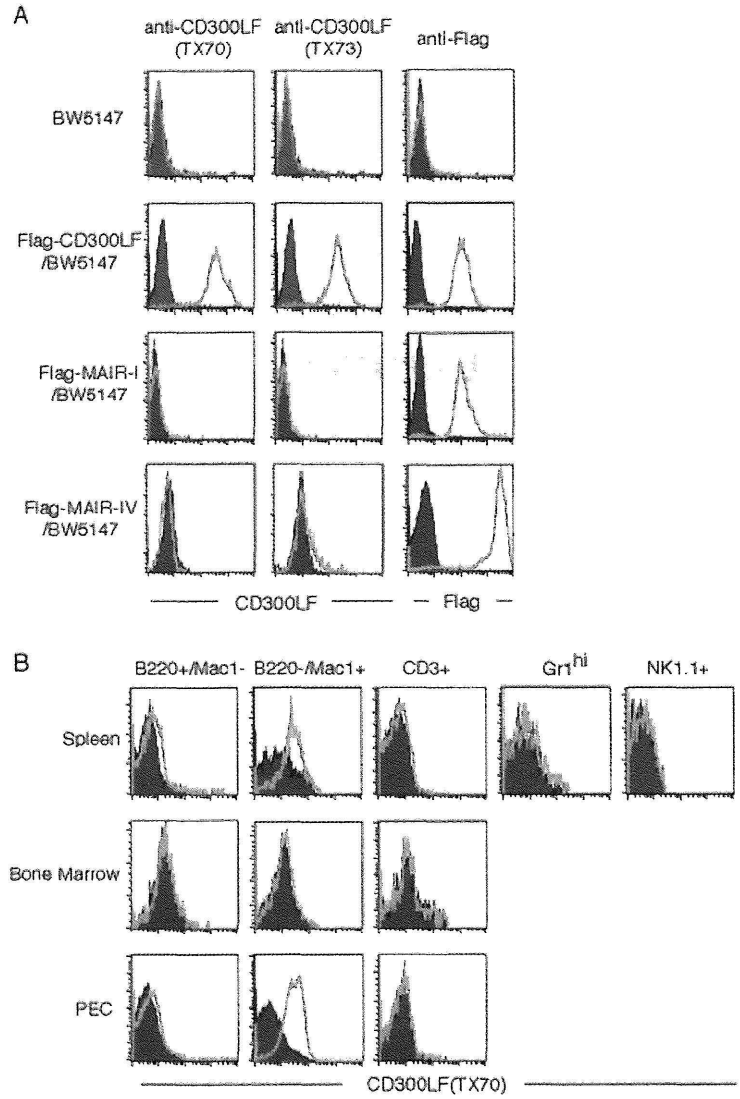

Figure 1. Expression of CD300LF on hematopoietic cells.

A. BW5147 parent cells or transfectants expressing the indicated Flag-tagged MAIR proteins were stained with biotin-conjugated anti-CD300LF (TX70, TX73) mAb (open histograms) or anti-Flag mAb (open histograms), followed by staining with APC-conjugated streptavidin. Shaded histograms shows negative controls with isotype-matched control IgGs.

B. Spleen, bone marrow (BM), and peritoneal exudate cells (PEC) from C57BL/6 mice were stained with PE-conjugated TX70 mAb (open histograms) or isotype-matched control antibodies (shaded histograms) in combination with the indicated lineage-specific mAbs. Cells were gated according to lineage markers expression by flow cytometry.

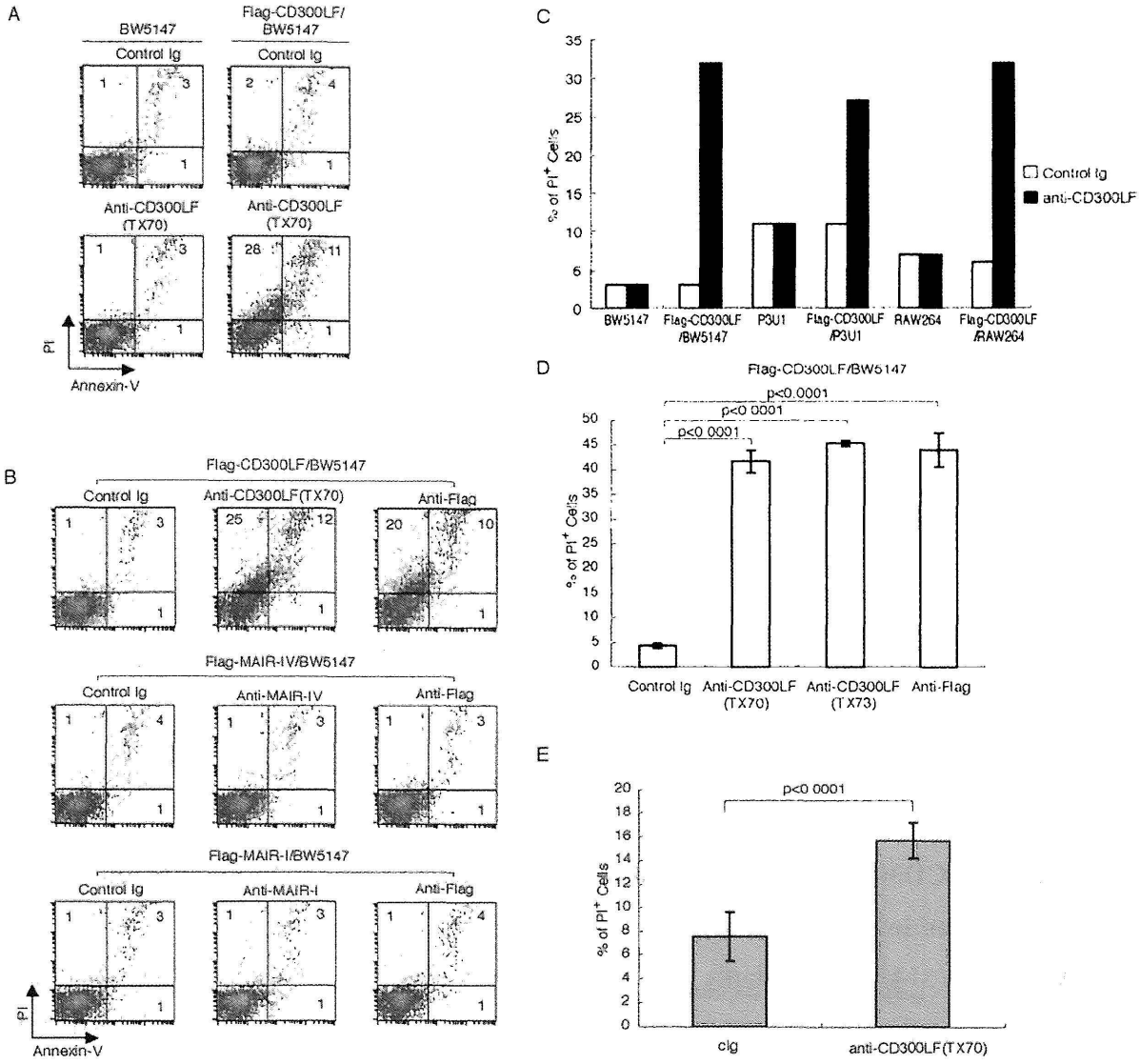

Figure 2. CD300LF mediates cell death.

BW5147, P3U1 or RAW264 parent cells or transfectants expressing the indicated Flag-tagged MAIR proteins were stimulated with the indicated immune complex of monoclonal antibodies for 30 min., stained with PI and PE-conjugated annexin-V, as described in the Methods, and then analyzed by flow cytometry. The representative profiles on flow cytometry are shown (A, B). The ratios of PI-positive cells calculated based on the data of flow cytometry are shown (C-E). Data are representative from 3 to 7 independent experiments (A~D). Peritoneal macrophages derived from mice 48 hours after i.p. injection with thyoglycolate were stimulated with the immune complex of anti-CD300LF (TX70) mAb and then analyzed as described above (E) (n=7).

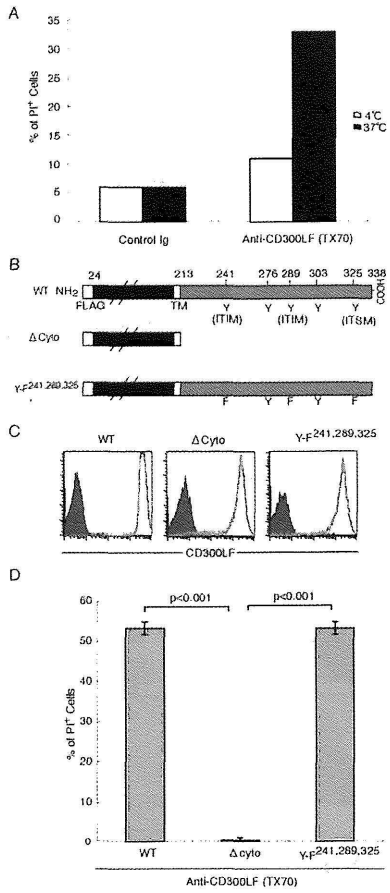

Figure 3. CD300LF-mediated cell death was dependent n the cytoplasmic region, but independent of ITIM or ITSM.

A. D. BW5147 transfectants expressing Flag-tagged WT or mutant CD300LF were stimulated with the immune complex of control Ig or anti-CD300LF (TX70) at 4°C or 37°C for 30 min., stained with PI and PE-conjugated annexin-V, as described in the Methods, and then analyzed by flow cytometry. The ratios of PI-positive cells calculated based on the data of flow cytometry are shown. Data are representative of two independent experiments (A) and the means + S.D. (n=3) (D).

B. C. Schematic diagram of wild type (WT) and mutant CD300LF tagged with Flag at the N-terminus. WT CD300LF contains 5 tyrosines (Y) indicated in the cytoplasmic region. ΔCyto and Y-$F^{241, 289, 325}$ are mutant CD300LF, in which the cytoplasmic region is deleted and the tyrosines at residues 241, 289 and 325 are replaced with phenylalanine (F), respectively (B). These transfectants were stained with Alexa-647-conjugated anti-CD300LF (TX70) mAb and analyzed by flow cytometry (C).

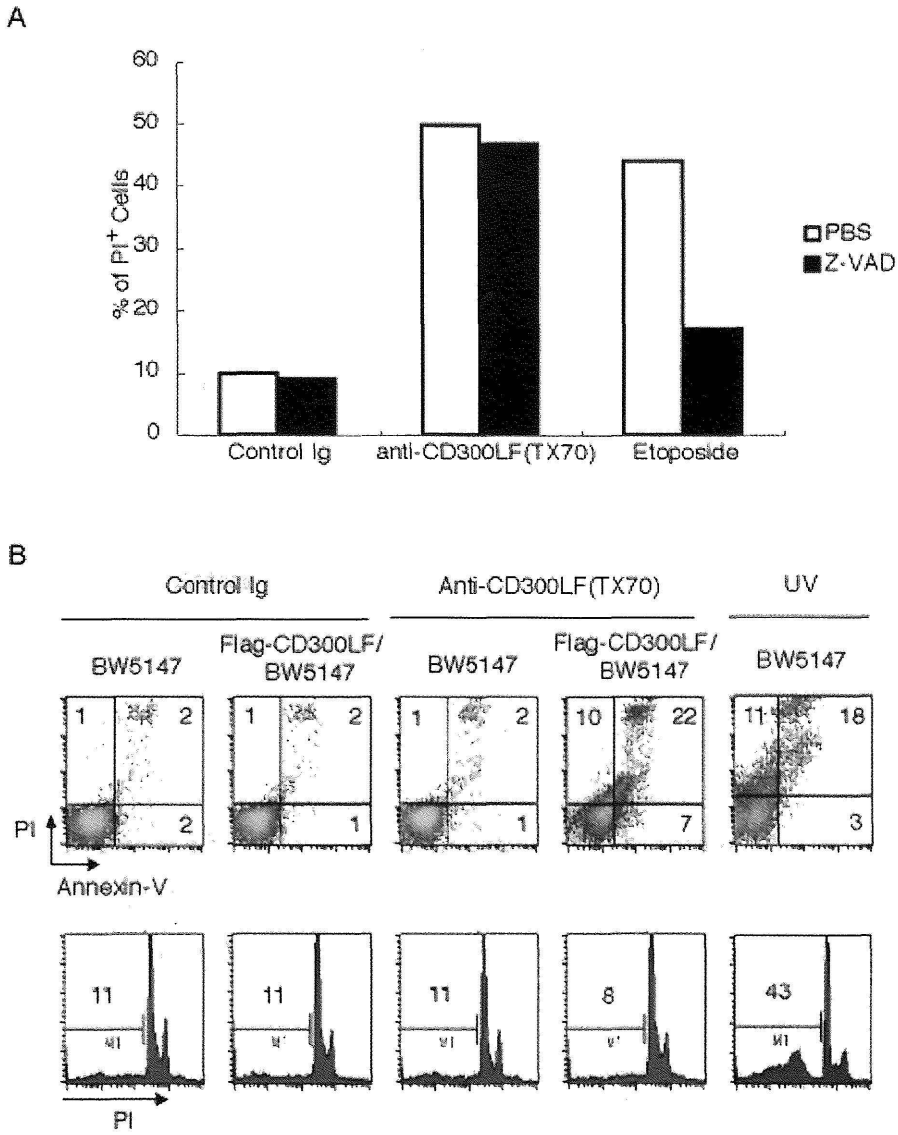

Figure 4. CD300LF-mediated cell death was independent of caspases.

A. BW5147 transfectants expressing CD300LF were stimulated with the IC of control Ig, TX70 anti-CD300LF, or etoposide in the presence or absence of a caspase inhibitor, Z-VAD-fmk. Cells were then stained with PI and PE-conjugated annexin-V and analyzed by flow cytometry.

B. BW5147 cells or the transfectants expressing Flag-tagged CD300LF were stimulated with the immune complex of control Ig or anti-CD300LF (TX70) or irradiated with UV light for 30 min. These cells were then stained with PI and PE-conjugated annexin-V and analyzed by flow cytometry (upper panel) or incubated overnight in a hypotonic buffer (0.1% sodium citrate, 0.1% Triton X-100) containing 50 μg/ml PI and analyzed by flow cytometry (lower panel).

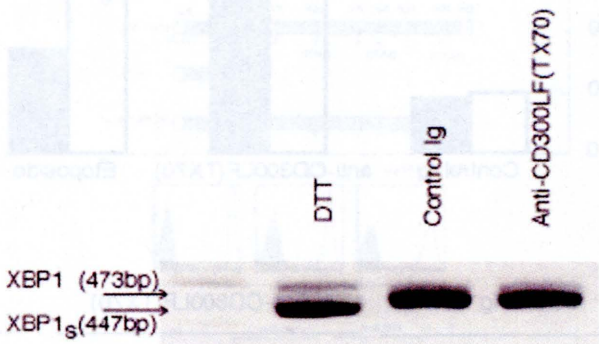

Figure 5. CD300LF-mediated cell death was independent of ER stress.
BW5147 transfectants expressing CD300LF were stimulated with the IC of control Ig, TX70 anti-CD300LF or 2 uM DTT, and then subjected to RT-PCR, as described in the Methods.

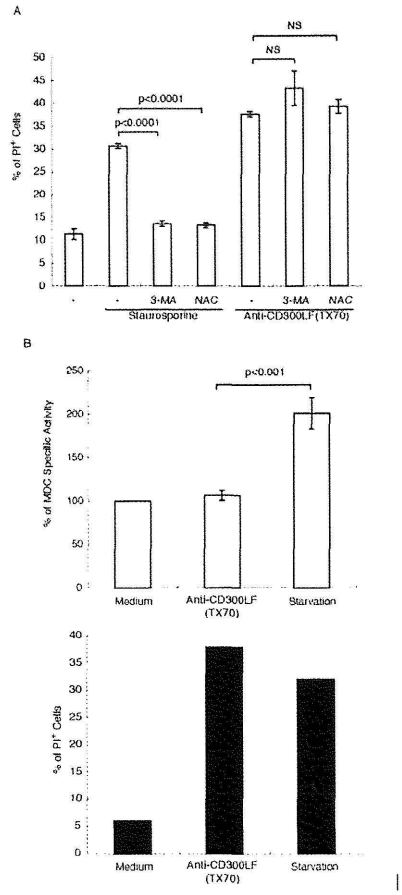

Figure 6. CD300LF-mediated cell death was independent of autophagy

A. BW5147 transfectants expressing CD300LF were pretreated or not with 3-MA (10 mM) or NAC (10 μM) for 12 hours and then stimulated with staurosporine (1 μM) or TX-70 anti-CD300LF. Cells were stained with PI and PE-conjugated annexin-V, as described in the Methods, and then analyzed by flow cytometry. The ratios of PI-positive cells calculated based on the data of flow cytometry are shown.

B. Cell death of BW5147 transfectants expressing CD300LF were induced or not by stimulation with TX-70 anti-CD300LF or amino acid starvation. Cells were then incubated with 0.05 mM MDC in PBS at 37°C for 10 min. Intracellular MDC was measured by fluorescence photometry and normalized by the number of cells present in each well (upper panel), as described in Materials and Methods. Data are the means ± S.D. (n=3). For analysis of cell death, cells were also stained with PI and PE-conjugated annexin-V, and then analyzed by flow cytometry. The ratios of PI-positive cells calculated based on the data of flow cytometry are shown (lower panel).

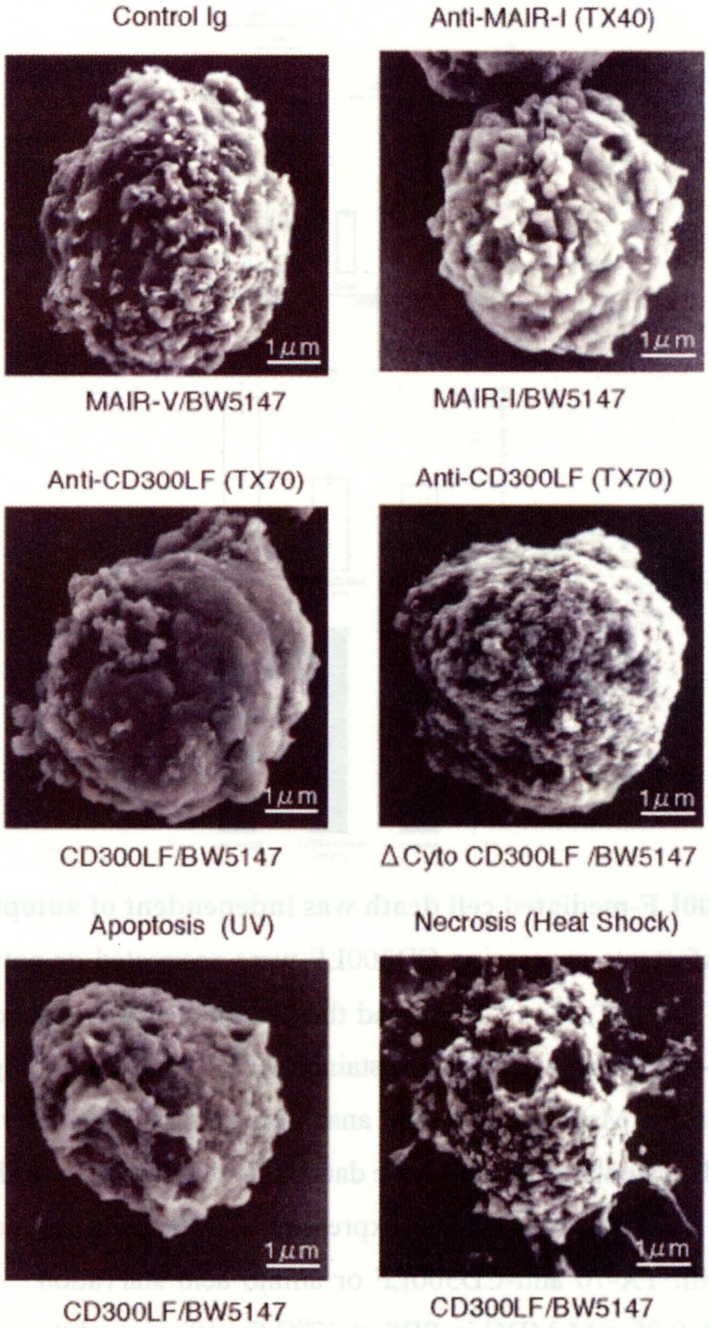

Figure 7. Morphology of CD300LF-mediated cell death

BW5147 transfectants expressing MAIR-I or WT or mutant (Δcyto) C 0LF were stimulated with the immune complex of each indicated antibody, irradiated with UV light for 30 min, or heated at 56°C. These cells were analyzed by scanning electron microscope.