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The Role of Apoptosis Signal-regulating Kinase 1 in Lymphotoxin-β Receptor-mediated Cell Death*

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LIGHT (homologous to lymphotoxins, shows inducible expression, and competes with herpes simplex virus glycoprotein D for herpesvirus entry mediator, a receptor expressed by T lymphocytes) is a member of the tumor necrosis factor superfamily that can interact with lymphotoxin-β receptor (LTβR), herpes virus entry mediator, and decoy receptor (DcR3). In our previous study, we showed that LIGHT is able to induce cell death via the non-death domain containing receptor LTβR to activate both caspase-dependent and caspase-independent pathway. In this study, a LIGHT mutein, LIGHT-R228E, was shown to exhibit similar binding specificity as wild type LIGHT to LTβR, but lose the ability to interact with herpes virus entry mediator. By using both LIGHT-R228E and agonistic anti-LTβR monoclonal antibody, we found that signaling triggered by LTβR alone is sufficient to activate both caspase-dependent and caspase-independent pathways. Cross-linking of LTβR is able to recruit TRAF3 and TRAF5 to activate ASK1, whereas its activity is inhibited by free radical scavenger carboxyfullerenes. The activation of ASK1 is independent of caspase-3 activation, and kinase-inactive ASK1-KE mutant can inhibit LTβR-mediated cell death. This suggests that ASK1 is one of the factors involved in the caspase-independent pathway of LTβR-induced cell death.

Lymphotoxin-β receptor (LTβR)[1] is a member of the tumor necrosis factor receptor (TNFR) superfamily and is ubiquitously expressed on the surface of most cell types, except T and B lymphocytes (1, 2). It has been reported that LTβR interacts specifically with two ligands: lymphotoxin LTα1/β2 (3, 4) and LIGHT (5, 6). There is ample evidences to demonstrate that LTβR plays an essential role in the development of lymphoid organs. Lymphoid nodes are deficient in LTα gene-deleted ($LT\alpha^{-/-}$) mice (7), and the impairment of lymph node development as well as the loss of splenic architecture was also observed in LTβ knockout mice (8). Furthermore, LTβR-deficient mice are shown to lack Peyer's patches, colon-associated lymphoid tissues, and all lymph nodes (9). Interestingly, the administration of agonistic antibody to LTβR can induce lymph node development in $LT\alpha^{-/-}$ mice (10). In addition to its role in lymphoid organ formation, LTβR is also involved in host immune responses to foreign antigens. Blockade of LTβR with LTβR-Fc not only prevents germinal center formation in spleen but also results in impaired IgG antibody responses to sheep red blood cells (11). Moreover, administration of LTβR-Fc is shown to enhance host survival after virus challenge (12) and is effective in preventing the onset of Th2 cell-mediated colitis (13).

The cytoplasmic domains of TNFR families function as docking sites for downstream signaling molecules. Signaling occurs mostly through two classes of cytoplasmic adaptor proteins: death domain-containing molecules and TNFR-associated factors (TRAFs). The death domain-containing molecules or TRAFs are recruited to the cytoplasmic domain of members of TNFR after engagement with ligands. The cytoplasmic domain of LTβR does not contain consensus sequences characteristic of death domain; thus, LTβR-transduced signaling is mainly mediated by TRAFs. TRAF molecules consist of amino-terminal RING finger domain, central zinc finger loop, and carboxyl-terminal TRAF domain. The TRAF domain mediates interactions between TRAF proteins and both their upstream and downstream effectors, whereas the RING finger domain is reported to be necessary for TRAF effector activation (14). Among the six TRAF proteins, TRAF2, TRAF3, and TRAF5 are found to associate with LTβR (15–17). Further study has indicated that TRAF3 plays an important role in mediating LTβR-in-

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[1] The abbreviations used are: LTβR, lymphotoxin-β receptor; ASK1, apoptosis signal-regulating kinase 1; IFN-γ, interferon-γ; HVEM, herpes virus entry mediator; TRAF, tumor necrosis factor receptor-associated factor; ROS, reactive oxygen species; C_{60}, carboxyfullerenes; HA, influenza hemagglutinin; TNFR, tumor necrosis factor receptor; LTα, lymphotoxin-α; MEKK5, mitogen-activated protein kinase/extracellular signal-regulated kinase kinase 5; JNK, c-Jun N-terminal kinase; mAb, monoclonal antibody; MEF, mouse embryonic fibroblast; MBP, myelin basic protein; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; X-gal, 5-bromo-4-chloro-3-indolyl-β-D-galatopyranoside; PIPES, 1,4-piperazinediethanesulfonic acid; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid; z-VAD-FMK, benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone; hLTβR, human LTβR.

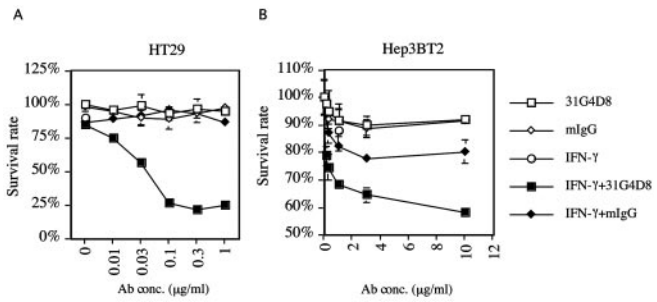


FIG. 1. **Cytotoxic effects of anti-LTβR monoclonal antibody 31G4D8.** HT29 cells (*A*) or Hep3BT2 cells (*B*) were seeded in culture plates precoated with a series of diluted 31G4D8 mAb in conjunction with 50 units/ml IFN-γ (for HT29 cells) or 100 units/ml IFN-γ (for Hep3BT2 cells) and were incubated for 72 h. Cell viability was determined by MTT assays, whereas the percentage of cell survival was determined by measurement of A_{280} for cells treated with cytokines *versus* cells cultured in medium alone.

duced cell death (15, 16, 18, 19), whereas TRAF2 and TRAF5 have been shown to be involved in the activation of NF-κB (17). Moreover, two serine/threonine protein kinases (p50 and p80) are reported to be associated with cytoplasmic region of LTβR (20), but their roles in LTβR-mediated signaling have not been elucidated yet.

Apoptosis signal-regulating kinase 1 (ASK1), also called mitogen-activated protein kinase/extracellular signal-regulated kinase kinase 5 (MEKK5), can be activated in response to various stress signals, including genotoxic stress (21), oxidative stress, reactive oxygen species (ROS) (22), and laminar flow (23). Furthermore, the kinase-inactive mutant of ASK1 inhibits cell death induced by tumor necrosis factor, Fas ligation, anti-cancer drugs, or withdrawal of neurotrophic factors (21, 24–27). ASK1 functions as an upstream component of the kinase cascades and interacts with a variety of molecules involved in stress-induced signaling pathways (21, 24). ASK1 phosphorylates and activates MKK4/7, which then activates the c-Jun NH_2-terminal protein kinases (JNKs), also known as the stress-activated protein kinases. JNK activation requires phosphorylation at a specific motif (TPY). Moreover, ASK1 phosphorylates and activates MKK3 and MKK6, leading to activation of the p38 mitogen-activated protein kinases (24, 28, 29). It has been reported that JNK and p38 activations are abolished in *ASK1*$^{-/-}$ embryonic fibroblasts (28).

Signaling mediated by death domain-containing receptors, such as TNFRI and Fas, could be inhibited efficiently by caspase inhibitors. However, caspase inhibitor has only a partial effect to prevent LIGHT/IFN-γ-induced cell death (30). In contrast, free radical scavenger carboxyfullerenes (C_{60}) can completely inhibit LIGHT/IFN-γ-induced cell death (30), indicating the important roles of ROS in LIGHT/IFN-γ-induced cell death (30). Since ROS are key mediators to activate ASK1, which contributes to progression of cell death (22, 31), we investigated the role of ASK1 in LIGHT-LTβR-induced cell death. Here we report that activation of LTβR alone, without the necessity to trigger HVEM activation, by either agonistic anti-LTβR mAb or a LIGHT mutein (LIGHT-R228E) incapable of HEVM binding, could induce the production of free radicals and the activation of ASK1. Blockade of ASK1 activation by free radical scavenger C_{60} could inhibit LTβR-mediated cell death. Thus, in addition to caspase activation, the activation of ASK1 also contributes to LTβR-mediated apoptotic pathways.

EXPERIMENTAL PROCEDURES

Cell Culture—The human hepatoma cells (Hep3BT2), human cervical carcinoma cells (HeLa), human embryonic kidney (HEK293) cells, and *traf* knockout mouse embryonic fibroblasts (MEFs) were maintained in Dulbecco's modified Eagle's medium (Invitrogen), supplemented with 10% (v/v) heat-inactivated fetal bovine serum (Invitrogen) at 37 °C in 5% (v/v) CO_2.

Plasmids and Transfection—Plasmids containing the hLTβR and hLTβR-CD proteins have been described (19). The hemagglutinin (HA)-tagged expression constructs of ASK1, catalytically inactive ASK1-KE-HA, were kindly provided by Dr. Wen-Chen Yeh (32). The dominant negative TRAF mutants were provided by Dr. Wen-Chen Yeh (TRAF2 mutant) and Dr. Bharat B. Aggarwal (TRAF3, -5, and -6 mutants) vector. All of the TRAF mutants contained the c-Myc tag except TRAF6 mutant. For DNA transfection, cells were plated and grown for 16 h and transfected with expression vectors by the calcium phosphate method or by using LipofectAMINE™ (Invitrogen).

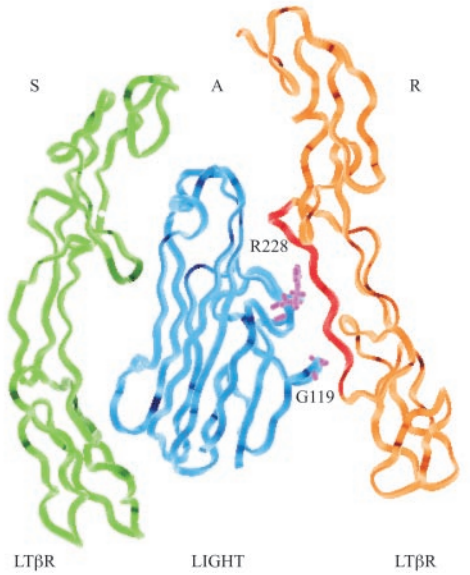
Generation of Anti-LTβR Monoclonal Antibody—Monoclonal antibodies were prepared by immunizing Balb/c mice with recombinant human lymphotoxin β receptor-Fc (hLTβR-Fc) protein (6). Spleen cells were fused with NS-1 cells, and hybridomas were screened by enzyme-linked immunosorbent assay. Anti-hLTβR monoclonal antibodies were selected by their specific binding to hLTβR but not to the Fc portion of human IgG1.

Generation of LIGHT Mutein—The cDNA of extracellular region of LIGHT was cloned into pIZ/V5-His-FLAG (Invitrogen). Substitution of Arg^{228} by glutamic acid was performed by overlap extension using polymerase chain reaction (33). The primers used for polymerase chain reaction were designed to introduce an *Xho*I site as described in the followings: 5′-GAGGATGGTACCCGGTCTTACTTC-3′ (sense) and 5′-GAGTCGAACCAGGCGTTCATC-3′ (antisense). The PCR products were ligated at the *Xho*I site of pIZ/V5-His-FLAG-LIGHT to create pIZ/V5-His-FLAG-LIGHT(R228E). The construct was autosequenced

TABLE I
Kinetics of ligand binding to receptors determined by surface plasmon resonance

Values are the means of at least five measurements over a ligand concentration range of 100–800 nM. K_a, association rate constant; K_d, dissociation rate constant; K_D, equilibrium dissociation constant (from K_d/K_a). Under the table is a homology-derived model of LIGHT (blue) in complex with LTβR (two units, one in orange the other in green), showing both the A–R interface (blue-orange) and the A–S interface (blue-green). The two amino acids whose mutants exhibited selective binding of HVEM and not LTβR (glycine 119) (35) and vice versa (arginine 228) (this work) are labeled (pink).

Ligand	LTβR-Fc			HVEM-Fc		
	Ka $10^4 M^{-1}S^{-1}$	Kd S^{-1}	KD nM	Ka $10^4 M^{-1}S^{-1}$	Kd S^{-1}	KD nM
LIGHT	2.99±0.24	2.61±0.88x10^{-4}	8.72±3.21	1.91±0.09	1.69±0.55x10^{-4}	8.81±3.2
LIGHT-R228E	0.93±0.06	7.26±0.64x10^{-4}	77.8±41		ND	



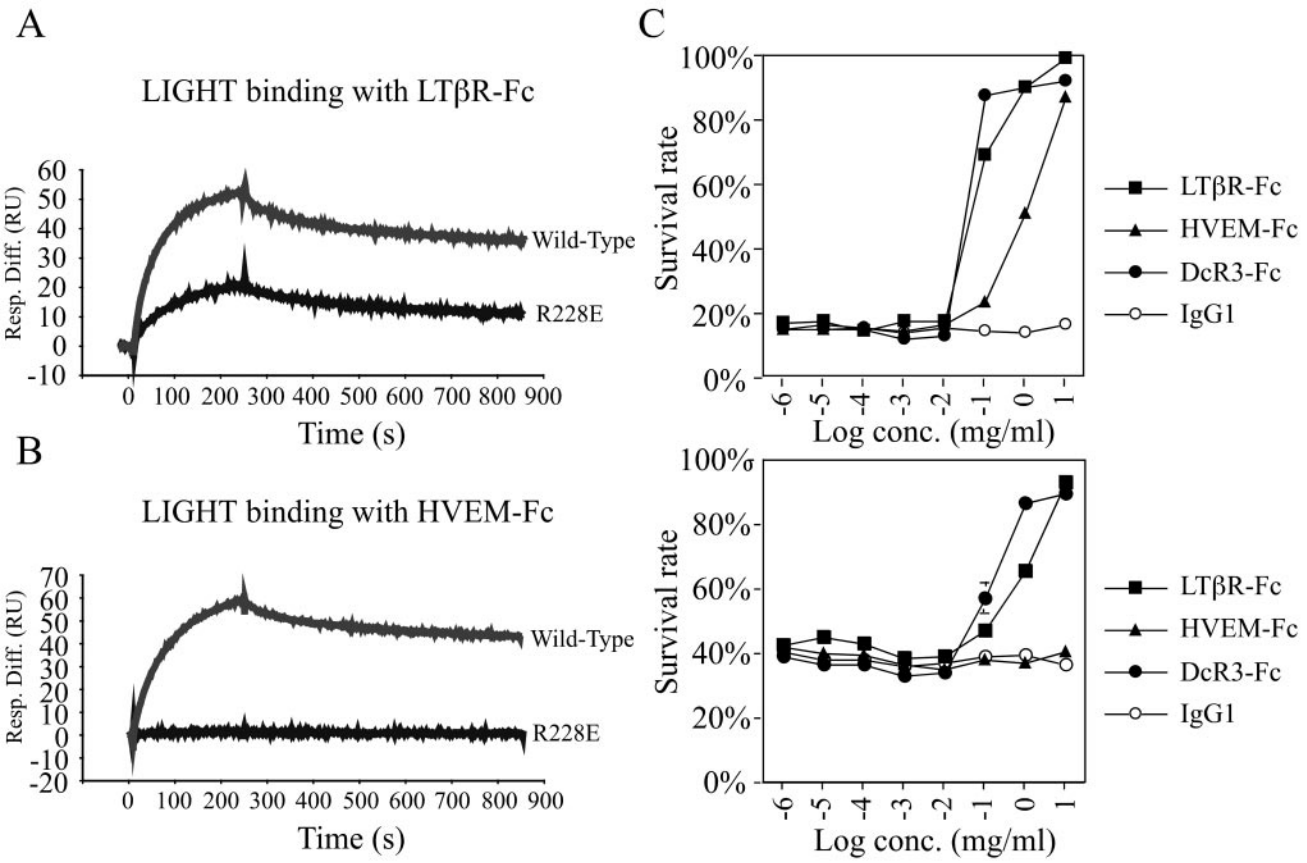


FIG. 2. **Binding specificity of LIGHT-R228E mutant.** *A* and *B*, kinetic analysis for the interaction of LIGHT and LTβR-Fc and HVEM-Fc by surface plasmon resonance. Human IgG1 or OPG-Fc was first mobilized on channel one of a CM5 chip as the blank to determine the bulk effect of injection itself, whereas HVEM-Fc and LTβR-Fc were immobilized on channel two and three, respectively, for analysis of its kinetic interaction with wild type LIGHT or LIGHT-R228E mutant. LIGHT or LIGHT-R228E, as the analyte, was injected from 100 to 800 nM, respectively. The interaction between LIGHT or LIGHT-R228E with LTβR-Fc (*A*) or HVEM-Fc (*B*) was determined by surface plasmon resonance using a BIAcore 2000. *C*, Hep3BT2 cells were incubated with 50 ng/ml wild type LIGHT (*upper panel*) or LIGHT-R228E (*lower panel*) in conjunction with IFN-γ (100 units/ml). The LTβR-Fc, HVEM-Fc, DcR3-Fc, and human IgG1 (ranging from 10^{-6} to 10 μg/ml) were added to culture medium, respectively, and incubated for 72 h to determine their inhibitory effect on LIGHT and LIGHT-R228E-mediated cell death.

(MB Mission Biotech) for verification of the mutation. The pIZ/V5-His-FLAG-LIGHT(R228E) construct was transfected into Sf21 cells by Lipofectin™ (Invitrogen). Stable transfectants were selected with 500 μg/ml Zeocin (Invitrogen). Protein was purified by agarose beads conjugated with anti-FLAG antibody (M2) and followed by dialysis in phosphate-buffered saline as described (30).

Generation of ASK1-KE Stable Transfectants—ASK1-KE DNA construct (a gift from Dr. Wen-Chen Yeh) was transfected into Hep3BT2 using LipofectAMINE™ (Invitrogen) as suggested by the vendor. Stable transfectants were selected with G418 (800 μg/ml Geneticin; Sigma), followed by immunoblot analysis to confirm the expression of ASK1-KE.

Antibodies and Other Reagents—The expression of ASK1-HA and TAK1-HA was detected by using anti-HA mAb (clone 3F10; Roche Molecular Biochemicals) or anti-human ASK1 antibodies (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). The expression of c-Myc-tagged TRAF2-DN, TRAF3-DN, and TRAF5-DN was detected by using anti-c-Myc tag polyclonal antibody (Upstate Biotechnology, Inc.). Rabbit polyclonal antibody against TRAF6 was obtained from Santa Cruz Biotechnology. Recombinant human IFN-γ was purchased from Roche Molecular Biochemicals.

Immunoblot Analysis—Cell lysates were prepared by the addition of lysis buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 1% (v/v) Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, 2 μg/ml leupeptin, and 2 μg/ml aprotonin). Equal amounts of protein were subjected to electrophoresis, transferred onto nitrocellulose membrane (Hybond-C extra, Amersham Biosciences), and reacted with appropriate antibodies in phosphate-buffered saline containing 5% nonfat dry milk, 0.02% Tween 20. Blots were then incubated with horseradish peroxidase-conjugated secondary antibodies and reacted with enhanced chemiluminescence reagents subsequently (Amersham Biosciences).

Surface Plasmon Resonance—Association and dissociation rates of the interaction of LIGHT or LIGHT-R228E with human LTβR-Fc or HVEM-Fc were determined by surface plasmon resonance using a BIAcore® 2000 biomolecular interaction analysis system (BIA-core Inc., Piscataway, NJ). The Fc fusion proteins (50 μg/ml) were coupled to a CM5 sensor chip by amine coupling at pH 7.0. The sensor surface was equilibrated with phosphate-buffered saline, and sensorgrams were collected at 25 °C and a flow rate at 30 μl/min. A 120-μl injection of LIGHT or LIGHT-R228E was passed over the sensor surface. After the association phase, 600 s of dissociation data were collected. The sensor surface was regenerated after each cycle with a 15-μl pulse of 10 mM glycine (pH 2.0) twice with a 30-s interval. Sets of eight analyte concentrations, 100–800 nM, were collected and analyzed.

Immunocomplex Kinase Assay—To measure the activity of ASK1 in cell extracts, the immune complex was incubated at 30 °C for 30 min with 2 μg of substrates (such as myelin basic protein (MBP)) in 30 μl of solution containing 20 mM Tris-HCl (pH 7.5)/10 mM $MgCl_2$/0.5 μCi of [γ-^{32}P]ATP. Reactions were stopped by the addition of Laemmli sample buffer. Samples were then fractionated by SDS-PAGE, and proteins were visualized by Coomassie Blue staining. Phosphorylated proteins were identified by autoradiography and quantified by a densitometer (Amersham Biosciences).

Determination of Cell Death—Cell death induced by overexpression of LTβR was determined by β-galactosidase-based cell morphology assay, and the killing effect of LIGHT/IFN-γ treatment was detected by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. For the β-galactosidase-based cell morphology assay, HeLa cells were co-transfected with *lacZ* expression vector, pBKCMV-lacZ. After 24 h of transfection, cells were fixed and then were stained with 5-bromo-4-chloro-3-indolyl-β-D-galatopyranoside (X-gal) to determine the percentage of apoptotic cells as described previously (19). The survival rate of Hep3BT2 cells was determined by MTT assay. Briefly, cells were seeded in 96-well flat bottom plates at a density of 5×10^3 cells/well.

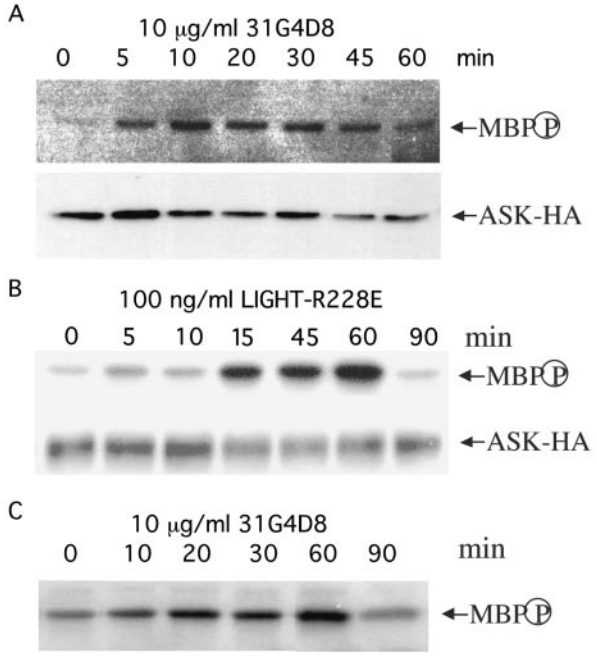


FIG. 3. **Activation of ASK1 induced by cross-linking of LTβR.** HeLa cells were transfected with HA-tagged ASK1, followed by incubation with 10 μg/ml anti-LTβR antibody 31G4D8 (*A*) or 100 ng/ml LIGHT-R228E (*B*) for various time intervals. ASK1 was immunoprecipitated by anti-HA antibody, whereas the ASK1 kinase activity contained in the immunocomplex was determined by incubation with MBP as a substrate by *in vitro* kinase assay. *C*, the endogenous ASK1 of HeLa cells was precipitated by polyclonal anti-ASK1 antibody after treatment with 10 μg/ml 31G4D8 mAb, whereas its activity was determined by incubation with MBP as a substrate by *in vitro* kinase assay.

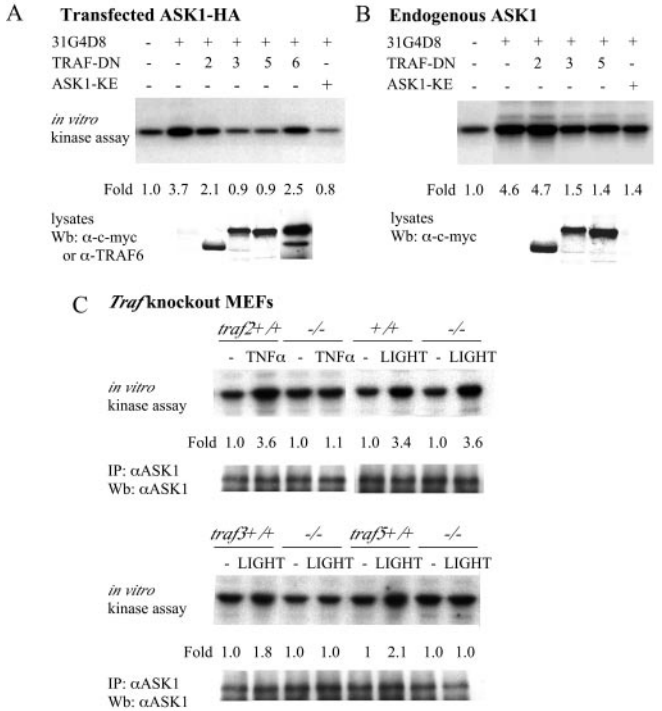


FIG. 4. **Inhibition of ASK1 activity by dominant negative mutant of TRAF (TRAF-DN) proteins and impairment of ASK1 activation in *traf*-deficient MEFs.** HeLa cells were transfected with HA-tagged ASK1 in conjunction with TRAF-DNs and ASK-KE for 24 h, followed by incubation with 10 μg/ml 31G4D8 for 30 min. To determine the ASK1 activity, cells were incubated with anti-HA antibody (*A*) or polyclonal anti-ASK1 antibody (*B*) to precipitate HA-tagged ASK1 (*A*) or endogenous ASK1 (*B*), followed by incubation with MBP to determine their activities by *in vitro* kinase assay. Expression of TRAFs in the transfectants was detected by Western blot analysis using anti-Myc and anti-TRAF6 antibodies. Data shown are representative of three independent experiments. *C*, *traf*-deficient and wild type MEF cells were stimulated with TNFα (200 ng/ml) for 15 min or with LIGHT (200 ng/ml) for 45 min, and cell lysates were collected and incubated with polyclonal anti-ASK1 antibody to precipitate endogenous ASK1 for an *in vitro* kinase assay.

After treatment, 10 μl of 5 mg/ml MTT per well was added and incubated at 37 °C for 4 h. Cells were then lysed by the addition of 50 μl of 10% SDS in 0.4 N HCl per well and incubated at 37 °C for another 16 h. The optical density of each sample was determined by measuring the absorbance at 570 *versus* 650 nm using an enzyme-linked immunosorbent assay reader (TECAN; RainBow) (30).

Measurement of Caspase Activity—Cytosolic extracts were prepared by freezing and thawing of cells in extraction buffer (50 mM PIPES-NaOH, pH 7.0, 50 mM KCl, 5 mM EGTA, 2 mM $MgCl_2$, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin, 1 μg/ml pepstatin A) as described (34). Cell lysates (50 μg) were diluted with 500 μl of ICE standard buffer (100 mM HEPES-KOH buffer, pH 7.5, 10% sucrose, 0.1% CHAPS, 10 mM dithiothreitol, 0.1 mg/ml ovalbumin) and incubated at 30 °C for 60 min with 20 μM fluorescent substrates. Fluorescence intensity was measured using a fluorescence spectrophotometer (Hitachi F-4500) at an excitation wavelength of 325 nm and emission wavelength of 392 nm.

RESULTS

Characterization of Agonistic Anti-LTβR-mAb (31G4D8) and LIGHT-R228E Mutein—Cross-linking of cell surface receptor by ligand or by agonistic antibodies can trigger signal transduction, and members of the TNFR superfamily are reported to be activated by agonistic antibodies, such as anti-human Fas antibody (CH11) and anti-mouse Fas antibody (Jo2). To study the signaling transduced by LTβR, monoclonal antibodies against human LTβR were raised. One of the selected clones, 31G4D8, is found to bind to LTβR specifically. Anti-LTβR mAb 31G4D8 does not have any cytotoxic effect to Hep3BT2 or HT29, which are sensitive to LIGHT/IFN-γ-mediated cell death. However, in conjunction with IFN-γ, 31G4D8 mAb is able to induce cell death with similar extent as that induced by wild type LIGHT (Fig. 1). This observation is in agreement with the previous observation that overexpression of LTβR is able to induce cell death (19), and LIGHT mutein incapable of binding to LTβR loses its ability to induce cell death (35).

To further confirm this argument, we designed a recombinant LIGHT mutein to bind LTβR but not HVEM, using a strategy of molecular modeling. A three-dimensional model for the interaction of LIGHT and its receptors (LTβR, HVEM, and DcR3) was generated by homology modeling (Molecular Simulation Inc., San Diego, CA) based on the crystallographic complex structure of LTα and TNFRI (Protein Data Bank code 1TNR) (36–38). Residues of the receptor-binding sites of this system, conventionally denoted as the A-R interaction domain and the A-S interaction domain, were identified. A few charge or polar residues were chosen for site-specific mutagenesis with the prediction that their mutations would, depending on the type of receptor, either enhance or interrupt receptor binding through altered electrostatic interactions. One of the LIGHT muteins that we have substantially characterized, the mutation at amino acid 228 from arginine to glutamic acid (LIGHT-R228E) at the A-R interaction domain (see the model in Table I), met the modeling objective of the present study.

The association and dissociation rates of wild type LIGHT and LIGHT-R228E to LTβR and HVEM were determined by surface plasmon resonance. As shown in Fig. 2 and Table I, the binding affinity of wild type LIGHT to both HVEM (K_D = 8.81 ± 3.2 nM) and LTβR (K_D = 8.72 ± 3.21 nM) is similar, whereas the binding affinity of LIGHT-R228E to HVEM is almost undetectable, and its binding affinity to LTβR (K_D = 77.8 ± 41 nM) is reduced from that of the wild type but is clearly evident (Fig. 2*B*). The reduction in affinity of R228E for

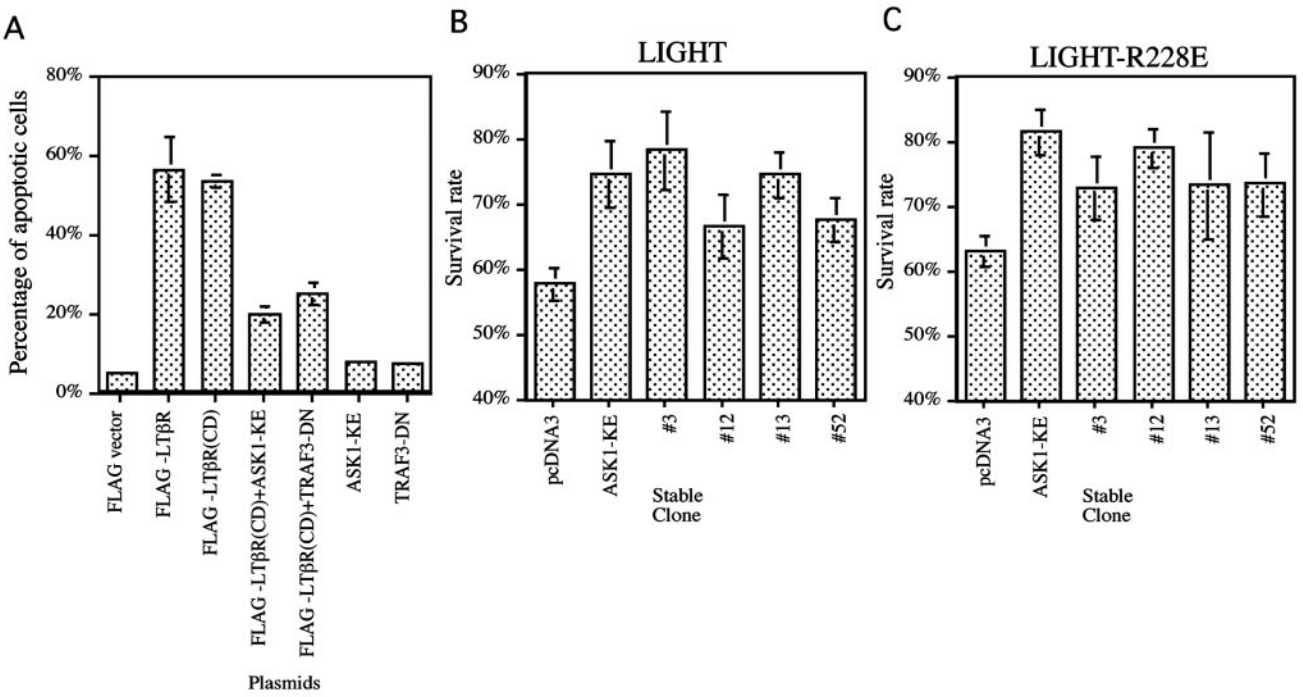


FIG. 5. **Effects of ASK1 dominant negative mutant (ASK1-DN) on LT-βR-mediated cell death.** *A*, HeLa cells were co-transfected with DNA constructs expressing full-length LTβR (pFLAG-LTβR) or its cytoplasmic domain (FLAG-LTβR-CD), in conjunction with dominant mutants of ASK1, TRAF3, and pCMV-lacZ in a ratio of 7:1:7. Cells were stained with X-gal at 24 h after transfection, followed by examination under a phase-contrast microscope. The percentage of apoptotic cells was calculated as the number of blue cells with apoptotic morphology divided by the total number of blue cells. At least 1000 blue cells were counted for each sample. The data shown here are the averages ± S.D. of triplicate experiments. *B* and *C*, Hep3BT2 cells overexpressing ASK1-KE (clones 3, 12, 13, and 52) and control vector were treated with LIGHT (50 ng/ml)/IFN-γ (100 units/ml) (*B*) or LIGHT-R228E (50 ng/ml)/IFN-γ (100 units/ml) (*C*) for 72 h. Cell viability was determined by MTT assays, whereas the percentage of survival rate was determined by measurement of A_{280} for cells treated with cytokines compared with cells cultured in medium alone.

LTβR-Fc was due to a decrease in association rate and an increase in dissociation rate (Table I). The binding of LIGHT-R228E to LTβR and the lack of it to HVEM were further confirmed by a competition analysis using LTβR-Fc or HVEM-Fc to inhibit wild type LIGHT and LIGHT-R228E-mediated cell death (Fig. 2*C*). Namely, wild type LIGHT/IFN-γ-induced cell death could be blocked by either LTβR-Fc or HVEM-Fc in a dose-dependent manner (Fig. 2*C*, *upper panel*), whereas LIGHT-R228E/IFN-γ-induced cell death was only blocked by LTβR-Fc and not by HVEM-Fc (Fig. 2C, *lower panel*). These observations provided direct evidence that the amino acid arginine 228 is essential for the interaction between LIGHT and HVEM, and LTβR alone is sufficient for LIGHT-mediated cell death.

Activation of ASK1 by 31G4D8 mAb and LIGHT-R228E—Oxidative stress was reported to disrupt the ASK1-thioredoxin complex and thereby to activate ASK1 (39). It has been shown that ROS play essential roles in LIGHT/IFN-γ-induced cell death (30); thus, we ask whether signaling through LTβR alone is enough to activate ASK1 activation to induce cell death. To address this question, HeLa cells were transfected with HA-tagged ASK1, followed by incubation with agonistic 31G4D8 mAb (Fig. 3*A*) or LIGHT-R228E (Fig. 3*B*) to test their ability to activate HA-tagged ASK1 by *in vitro* kinase assay. As shown in Fig. 3*A*, a rapid increase of ASK1 activity was observed at 5 min after 31G4D8 treatment and observed to last for at least 60 min (Fig. 3*A*). LIGHT-R228E had a similar effect as 31G4D8 mAb in ASK1 activation but with distinct kinetics. ASK1 activity increased at 15 min, peaked at 60 min, and returned to basal level at 90 min when stimulated with LIGHT-R228E. The kinetics of endogenous ASK1 activation in Hep3BT2 was similar to that of transfected HA-tagged ASK1 after 31G4D8 mAb stimulation (Fig. 3*C*). This demonstrated that ASK1 could be activated by LTβR-transduced signaling.

Inhibition of ASK1 Activation by TRAF Mutants—ASK1 has been implicated in transmitting TRAF-dependent signaling (32, 40). In order to investigate the roles of TRAFs on ASK1 activation induced by LTβR, we examined the effects of TRAF dominant negative (TRAF-DN) mutants in ASK1 activation. To address this question, HeLa cells were transfected with ASK1-HA in conjunction with TRAF-DN mutants. It was obvious that TRAF3-DN and TRAF5-DN, but not TRAF2-DN and TRAF6-DN mutants, effectively inhibited transfected ASK1 activation in the *in vitro* kinase assay (Fig. 4*A*). The endogenous ASK1 activity was also inhibited by TRAF3-DN and TRAF5-DN, but not by TRAF2-DN, to the same extent as catalytic inactive ASK1-KE mutant (Fig. 4*B*).

To further confirm this observation, we investigated the endogenous ASK1 activation induced by LIGHT in *traf2*$^{-/-}$, *traf3*$^{-/-}$, and *traf5*$^{-/-}$ MEFs. The ASK1 activation induced by TNFα is impaired in *traf2*$^{-/-}$ MEFs, which is consistent with previous report that TNFα-induced ASK1 activation is TRAF2-dependent (32). In contrast, the activation of ASK1 by LIGHT is not affected in *traf2*$^{-/-}$ MEFs (Fig. 4*C*, *upper panel*). However, the activation of endogenous ASK1 is inhibited in either *traf3*$^{-/-}$ or *traf5*$^{-/-}$ MEFs (Fig. 4*C*, *lower panel*), suggesting that LTβR-mediated ASK1 activation is via TRAF3 and TRAF5 but not TRAF2.

Involvement of ASK1 in LTβR-induced Cell Death—We further asked whether activation of ASK1 is involved in LTβR-induced cell death. It has been shown that overexpression of LTβR could induce HeLa cell death (19); thus, we co-transfected ASK1-KE, LTβR, and β-galactosidase to test its effect in LTβR-mediated cell death. At 24 h after transfection, the percentage of cell death in cells overexpressing full-length LTβR or cytoplasmic LTβR was ~56.6 and 53.7%, respectively, whereas the co-expression of ASK1-KE reduced the percentage of apo-

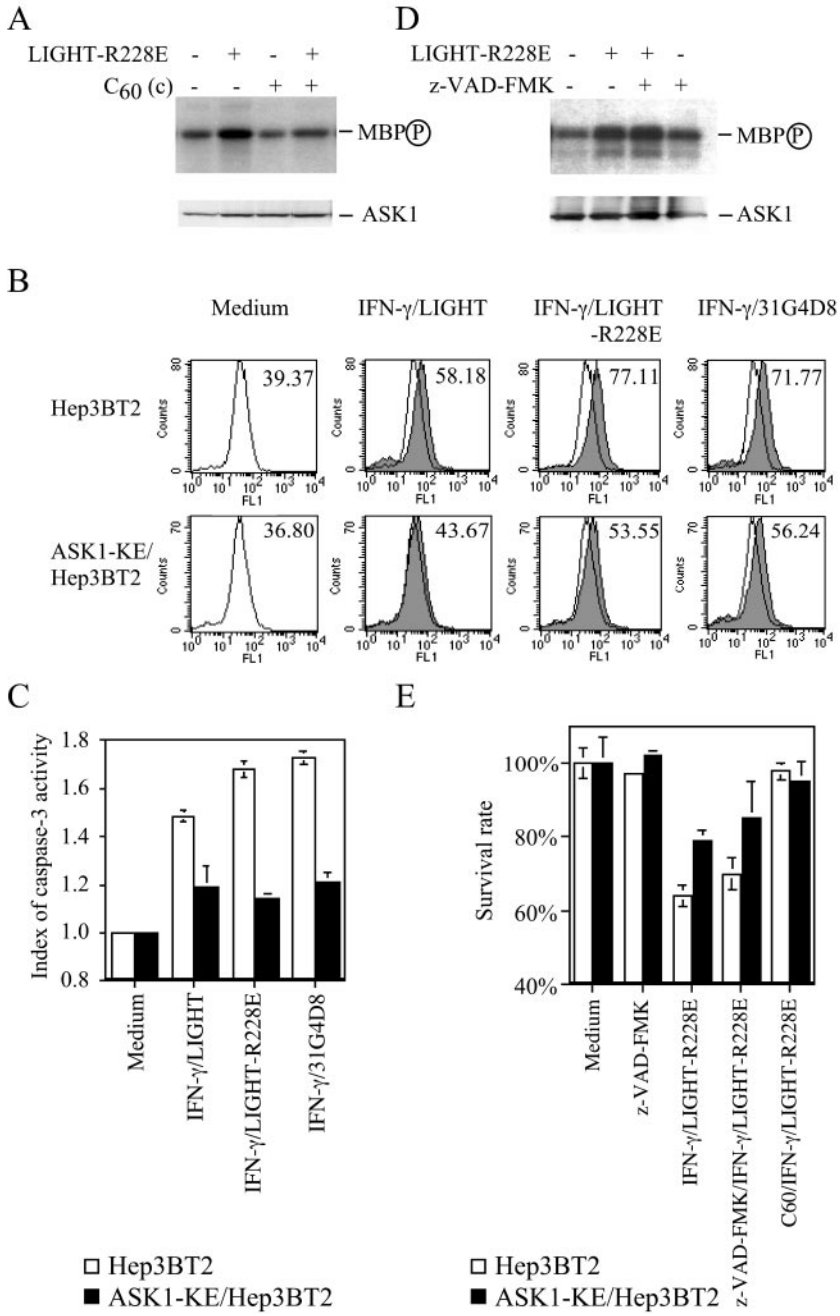


FIG. 6. **ASK1 activation triggered by LTβR cross-linking is regulated by ROS.** *A*, Hep3BT2 cells pretreated with 50 μM C_3 isoform of carboxyfullerene were incubated with 100 ng/ml LIGHT-R228E for 30 min, and the endogenous ASK1 activity was determined by immunoprecipitation using polyclonal anti-ASK1 antibody, followed by incubation with MBP as a substrate by an *in vitro* kinase assay. *B*, generation of ROS in wild type Hep3BT2 cells (*upper panel*) or Hep3BT2 cells overexpressing ASK1-KE (*lower panel*). After incubation with 100 ng/ml LIGHT, 100 ng/ml LIGHT-R228E, or 10 μg/ml 31G4D8 in conjunction with 100 units/ml IFN-γ for 6 h, Hep3BT2 cells or ASK1-KE/Hep3BT2 cells were stained with 5 μM 2′,7′-dihydrodichlorofluorescein diacetate at 37 °C for 15 min, followed by flow cytometry analysis to determine their fluorescence intensity. *Line*, medium; *shadow*, LIGHT/IFN-γ or 31G4D8 mAb; mean fluorescence intensity is indicated. *C*, Hep3BT2 cells or ASK1-KE/Hep3BT2 were incubated with 100 ng/ml LIGHT, 100 ng/ml LIGHT-R228E, or 10 μg/ml 31G4D8 in conjunction with 100 units/ml IFN-γ, and caspase activities were determined by incubating the cell lysates with fluorescence substrate MCA-DEVD.APK (7-methoxycoumarin-4-yl)acetyl-Asp-Glu-Val-Asp-Ala-Pro-Lys(2,4-dinitrophenyl)-OH). *D*, Hep3BT2 cells pretreated with 100 μM z-VAD-FMK were stimulated with 100 ng/ml LIGHT-R228E for 30 min, followed by immunoprecipitation using polyclonal anti-ASK1 antibody to determine endogenous ASK1 activity by an *in vitro* kinase assay. *E*, failure of caspase inhibitors to protect ASK1-KE/Hep3BT2 cells form LIGHT-R228E/IFN-γ-mediated cell death. Hep3BT2 and ASK1-KE/Hep3BT2 cells were pretreated with 100 μM z-VAD-FMK or 20 μM C_{60} (C3 form) for 1 h, followed by incubation in medium supplemented with 100 units/ml IFN-γ and 50 ng/ml LIGHT-R228E for 72 h. Cell viability was determined by MTT assay.

ptotic cells to 19% (Fig. 5*A*). This suggests that ASK1 is involved in LTβR-mediated cell death. ASK1-KE and TRAF3-DN are not toxic to HeLa cells under the same condition.

We further examined the relationship between ASK1 activation and cell death induced by LIGHT/IFN-γ. Hep3BT2 cells stably expressing ASK1-KE were incubated with IFN-γ in conjunction with LIGHT (Fig. 5*B*) or LIGHT-R228E (Fig. 5*C*) to test its resistance to cell death. Compared with cells transfected with control plasmid pcDNA3 (survival rate 57%), Hep3BT2 cells stably expressing ASK1-KE are relatively resistant to LIGHT/IFN-γ killing (survival rate 67–78%) or LIGHT-R228E/IFN-γ-mediated apoptosis (survival rate 73–81%). Thus, ASK1 is clearly involved in LTβR-mediated cell death.

ASK1 Is Inhibited by ROS Scavenger but Not Caspase Inhibitor—It has been shown that ROS can induce dimerization of ASK1 and cause its activation in TNFα signaling (22), and inhibition of ROS production by C_{60} can inhibit LIGHT/IFN-γ-mediated cell death (30); thus, we are interested to know whether C_{60} can inhibit LTβR-mediated ASK1 activation. As shown in Fig. 6*A* (*upper panel*), pretreatment of C_{60} completely inhibits ASK1 activation in Hep3BT2 cells treated with LIGHT-R228E. This indicates that LTβR-mediated ASK1 activation is regulated by ROS. Moreover, the production of ROS induced by LTβR activation is not affected by ASK1-KE mutant; this further suggests that production of ROS is upstream to ASK1 activation in LTβR-mediated signaling (Fig. 6*B*).

It has been reported that ASK1-mediated cell death is via either a caspase-dependent or caspase-independent pathway (41, 42); thus, we ask whether caspase-3 activation is dependent on ASK1 activation induced by IFN-γ/LIGHT, IFN-γ/LIGHT-R228E, or IFN-γ/31G4D8. In Hep3BT2 cells stably expressing ASK1-KE, activation of caspase-3 by IFN-γ/LIGHT, IFN-γ/LIGHT-R228E, or IFN-γ/31G4D8 is partially inhibited (50%) (Fig. 6*C*), but ASK-KE does not have any effect on

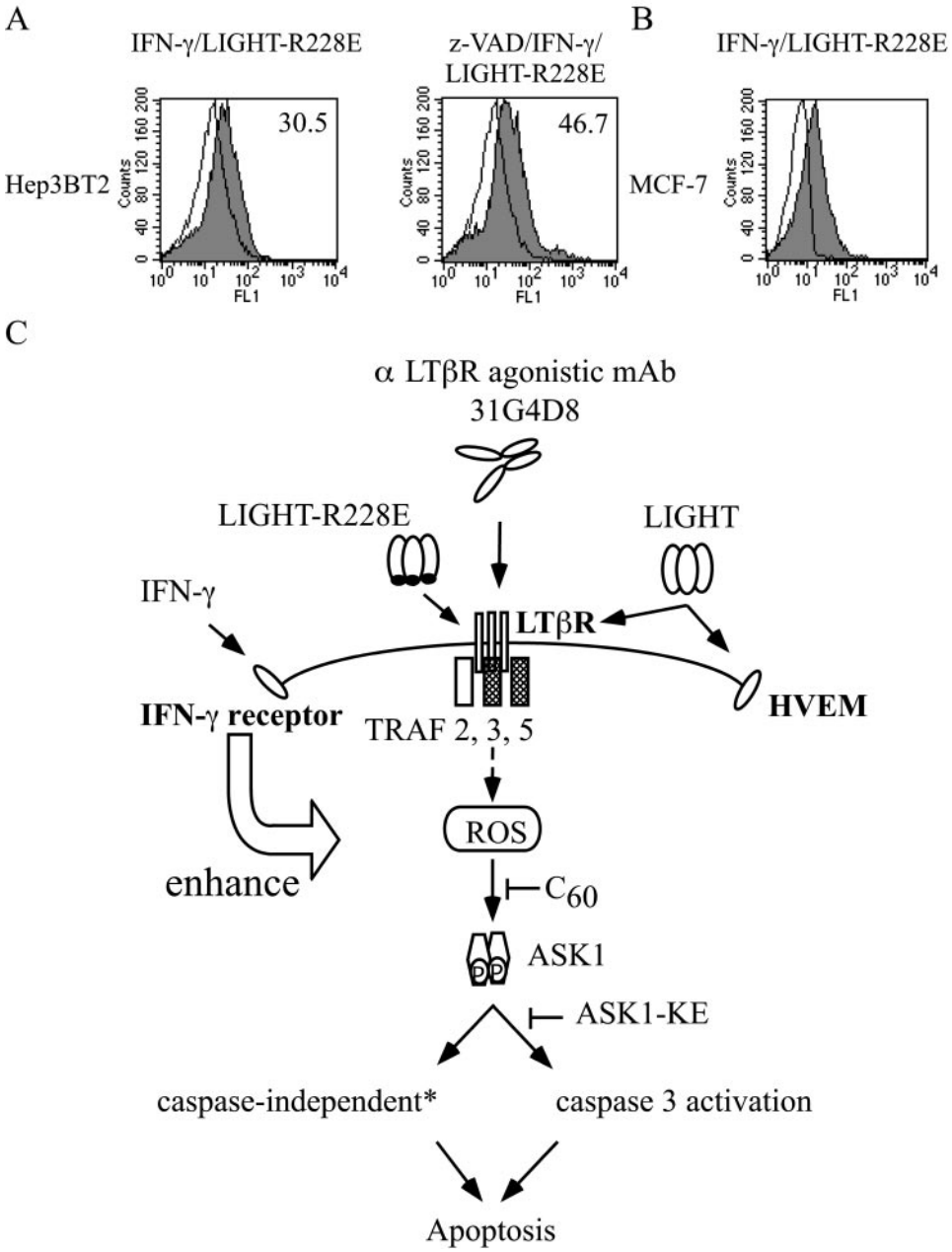


FIG. 7. **ROS release induced by LTβR is not regulated by caspases.** Hep3BT2 cells (*A*) or MCF-7 (*B*) pretreated with 100 μM z-VAD-FMK were incubated with 100 ng/ml LIGHT-R228E in conjunction with 100 units/ml IFN-γ for 8 h; Hep3BT2 cells or MCF-7 cells were stained with 5 μM 2′,7′-dihydrodichlorofluorescein diacetate at 37 °C for 15 min, followed by flow cytometry analysis to determine their fluorescence intensity. *Line*, medium; *shadow*, IFN-γ/LIGHT-R228E or z-VAD-FMK/IFN-γ/LIGHT-R228E; mean fluorescence intensity is indicated. *C*, putative model of LTβR-mediated apoptotic pathway. Activation of LTβR by agonistic mAb 31G4D8 or LIGHT-R228E induces the production of ROS, which is enhanced by IFN-γ. ROS initiate both caspase-3-dependent and caspase-independent pathways to induce cell death. One of the caspase-3-independent pathways is the activation of ASK1, via the recruitment of TRAF3 and TRAF5, to LTβR. *, caspases insensitive to z-VAD-FMK cannot be ruled out.

caspase activation induced by transforming growth factor-β1 (data not shown). This demonstrated the important role of ASK1 for caspase-3 activation in LTβR-mediated signaling pathway. Moreover, caspase-3 inhibitor does not have any effect on ASK1 activation in Hep3BT2 cells when treated with LIGHT-R228E, suggesting that ASK1 is upstream to caspase-3 activation (Fig. 6*D*). To further determine the role of ASK1 in LTβR-mediated cell death, wild type Hep3BT2 and Hep3BT2/ASK-KE cells were incubated with LIGHT-R228E in the presence or absence of caspase inhibitor z-VAD-FMK. Compared with wild type Hep3BT2 cells, cells overexpressing ASK-KE (Hep3BT2/ASK-KE) are more resistant to LIGHT-R228E-mediated cell death (Fig. 6*E*). Moreover, the addition of z-VAD-FMK provides partial protective effect in both wild type Hep3BT2 and Hep3BT2/ASK-KE cells. The protective effect of caspase inhibitor z-VAD-FMK is less than the ASK1-KE dominant negative mutant, indicating that ASK1 plays a more important role than caspase-3 activation in LTβR-mediated cell death. In contrast, C_{60} could fully protect both wild type Hep3BT2 and Hep3BT2/ASK-KE cells from LTβR-mediated cell death. Since the activation of ASK1 is regulated by free radicals, we conclude that ASK1 is one of the factors activated by free radicals contributing to LTβR-induced cell death.

LTβR-induced ROS Release Is Not Affected in Caspase-3-deficient Cells or by z-VAD-FMK—After confirming the role of ROS in ASK1 activation, we further ask whether caspase acti-

vation lies upstream or downstream to ROS production. To address this question, Hep3BT2 cells were pretreated with general caspase inhibitor z-VAD-FMK, followed by incubation with IFN-γ/LIGHT-R228E to determine its effect on LTβR-induced ROS release by flow cytometry using 2′,7′-dihydrodichlorofluorescein diacetate as probe. As shown in Fig. 7*A*, the addition of z-VAD-FMK did not suppress mean fluorescence intensity, suggesting that the release of ROS is not affected, indicating that ROS release is not suppressed by general caspase inhibitor. In caspase-3-deficient MCF-7 cells, the mean fluorescence intensity is still increased after IFN-γ/LIGHT-R228E treatment. This suggests that ROS release is not dependent on the activation of caspase-3 and other caspases (such as caspase-1, -3, -5, -6, -7, -8, and -9), which are sensitive to z-VAD-FMK (43).

DISCUSSION

In a previous study, we have demonstrated that LIGHT/IFN-γ can induce the production of free radicals, which in turn induce cell death via both caspase-dependent and -independent pathways (30). Moreover, signaling triggered by LTβR overexpression or agonistic anti-LTβR mAb is shown to be sufficient for LIGHT/IFN-γ-mediated cell death (19, 35). However, it is unclear whether signaling triggered by LTβR is still able to activate both caspase-dependent and caspase-independent pathways to induce cell death. Previously we have demonstrated that the caspase-dependent pathway plays a minor role in LIGHT/IFN-γ-mediated cell death, since caspase inhibitor z-VAD-FMK only provides partial protective effect to LIGHT/IFN-γ-induced cell death. In this study, we further ask whether signaling triggered by LTβR alone is enough to activate a caspase-dependent pathway and/or caspase-independent pathway to induce cell death. To clarify this issue, LIGHT muteins and agonistic antibody against LTβR were generated to test the questions raised above. Among the LIGHT-muteins generated, we find that the amino acid arginine 228 is crucial for LIGHT-HVEM interaction, since mutation of arginine 228 to glutamic acid 228 abolished the interaction between LIGHT and HVEM (Fig. 2). It has been shown that amino acid glycine 119 is critical for LIGHT-LTβR interaction (35); in complementation, we showed here that amino acid arginine 228 is essential for LIGHT-HVEM interaction. According to the homology model (shown in Table I), both glycine 119 and arginine 228 interact with the receptor in the A-R interaction domain, but from different regions of LIGHT; whereas glycine 119 is located in the N-terminal A-A′ loop of LIGHT, arginine 228 is located in the G-H loop of the C-terminal. It will be of interest, and also of considerable use, for further studies to identify amino acid residues that are essential for LIGHT-DcR3 interactions.

Moreover, we further demonstrate that both LIGHT-R228E and agonistic antibody against LTβR still have the ability, like wild type LIGHT, to induce the production of free radicals and activate both caspase-dependent and -independent pathways to induce cell death. We find that LTβR-transduced signaling is able to activate ASK1 via the induction of free radicals (Fig. 6*A*), and activation of ASK1 also contributes to LTβR-mediated cell death (Fig. 6*E*); this observation thus reveals one of the mechanisms of LTβR-mediated caspase-independent pathway to induce cell death. Although ASK1 activity is not required in the caspase-independent cell death in the ASK1 overexpression system (42), the kinase activity of ASK1 is essential for LTβR-mediated cell death, since the kinase-inactive ASK1-KE can inhibit the cell death triggered by LTβR activation (Fig. 6*E*). Previous study has shown that kinase-inactive mutant of ASK1 is capable of inhibiting cell death induced by genotoxic stress, Fas, and tumor necrosis factor α overexpression (21, 24, 25); this implies that catalytic active ASK1 may contribute to a kinase-dependent, but caspase-independent, mechanism to cell death triggered by various cell death-inducing signals. In our recent study, we also demonstrate that signaling transduced by LTβR induces the secretion of IL-8 in HEK 293 via the activation of ASK1-MKK4/MKK7-JNK1/2-AP1 and NIK-IKK-NF-κB signaling cascades (44). Since activation of JNK/stress-activated protein kinase also contributed to cell death (28, 45, 46), the ASK-1-dependent cell death in our model system might be mediated by a JNK/stress-activated protein kinase signaling cascade.

The ROS has been demonstrated to play a crucial role in stress-activated mitogen-activated protein kinase kinase kinase signaling pathway (22, 39), and the activation of ASK1 by LTβR activation further provides an example of how free radical-regulated mitogen-activated protein kinase kinase kinase can mediate cell death. Recently, thioredoxin, a redox-sensing protein, has been shown to associate with ASK1 in its reduced form. Tumor necrosis factor can stimulate the production of ROS to activate ASK1 via the dissociation of ASK1 from thioredoxin, followed by binding to TRAF2 to form a TNFR-TRAF2-ASK1 complex (47). In our study, we find that the LTβR-mediated ASK1 activation is dependent on TRAF3 and TRAF5 but not on TRAF2 and TRAF6 (Fig. 4). This is consistent with the previous finding that the LTβR-mediated signaling cascade is transduced by TRAF3 and TRAF5 (17, 18) and that the dominant negative mutant of TRAF3 provides partial protection to LTβR-mediated cell death (19).

Although TRAF2 is essential for TNF-induced ASK1 activation (47), LIGHT-induced ASK1 activation is apparently independent of TRAF2. It has been shown that overexpression of TRAF2 or TRAF5, but not TRAF3, is able to activate ASK1 directly (40). However, we found that ASK1 activation is impaired not only in *traf5*$^{-/-}$ MEF cells but also in *traf3*$^{-/-}$ MEF cells (Fig. 4*C*). This suggests that even TRAF3 could interact with ASK1 directly (40), but TRAF3 alone is not enough to activate ASK1. Therefore, TRAF3-dependent ASK1 activation after LTβR activation might be via its interaction with TRAF5 to recruit ASK1, and further investigation is needed to clarify this question.

In a previous study, we demonstrated that activation of LTβR can trigger both a caspase-3-dependent and -independent pathway to induce cell death (30). Moreover, free radical scavenger C_{60} can completely inhibit LTβR-mediated cell death, whereas general caspase inhibitor z-VAD-FMK has only a partial protective effect, suggesting the important role of ROS in LTβR-mediated cell death (30). Here we provide further evidence that LTβR-induced ROS release is apparently independent from caspase-3 and other caspases that are sensitive to z-VAD-FMK, such as caspase-1, -3, -5, -6, -7, -8, and -9 (43). Whether caspase-2, -4, and -10 or other newly identified caspases affect LTβR-induced ROS needs to be tested in the future.

Unlike ROS inhibitor, ASK1 only provides a partial effect on LTβR-mediated cell death, although ASK1-KE is more potent than caspase inhibitor z-VAD-FMK. This indicates that a caspase-independent or z-VAD-sensitive caspase-independent pathway distinct from ASK1 activation is also responsible for LTβR activation. Fig. 7*C* summarizes our current understanding to LTβR-mediated cell death; HVEM apparently is dispensable for LIGHT-mediated free radical production as well as the activation of ASK1 and caspase-3. The recruitment of TRAF3 and TRAF5 to LTβR induces the production of free radicals to activate both caspase-3-dependent and z-VAD-sensitive caspase-independent pathways. Since IFN-γ enhances LTβR-mediated cell death, an IFN-γ-regulated pathway dis-

tinct from ASK1 activation might be one of the major pathways responsible for LTβR-mediated cell death. Identification of an IFN-γ-regulated pathway distinct from ASK1 activation might be very helpful to elucidate the caspase-independent pathway transduced by LTβR and other members of the TNFR superfamily.

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