TL1, a Novel Tumor Necrosis Factor-like Cytokine, Induces Apoptosis in Endothelial Cells

ININVOLVEMENT OF ACTIVATION OF STRESS PROTEIN KINASES (STRESS-ACTIVATED PROTEIN KINASE AND p38 MITOGEN-ACTIVATED PROTEIN KINASE) AND CASPASE-3-LIKE PROTEASE

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TL1 is a recently discovered novel member of the tumor necrosis factor (TNF) cytokine family. TL1 is abundantly expressed in endothelial cells, but its function is not known. The present study was undertaken to explore whether TL1 induces apoptosis in endothelial cells and, if so, to explore its mechanism of action. Cultured bovine pulmonary artery endothelial cells (BPAEC) exposed to TL1 showed morphological (including ultrastructural) and biochemical features characteristic of apoptosis. TL1-induced apoptosis in BPAEC was a time- and concentration-dependent process (EC50 = 72 ng/ml). The effect of TL1 was not inhibited by soluble TNF receptors 1 or 2. TL1 up-regulated Fas expression in BPAEC at 8 and 24 h after treatment, and significantly activated stress-activated protein kinase (SAPK) and p38 mitogen-activated protein kinase (p38 MAPK). The peak activities of SAPK and p38 MAPK in TL1-treated BPAEC were increased by 9- and 4-fold, respectively. TL1-induced apoptosis in the BPAEC was reduced by expression of a dominant-interfering mutant of c-Jun (62.8%, p < 0.05) or by a specific p38 inhibitor, SB203580 (1–10 μM) dose-dependently. TL1 also activated caspases in BPAEC, and TL1-induced apoptosis in BPAEC was significantly attenuated by the caspase inhibitor, ZVAD-fluoromethyl-ketone. The major component activated by TL1 in BPAEC was caspase-3, which was based on substrate specificity and immunocytochemical analysis. These findings suggest that TL1 may act as an autocrine factor to induce apoptosis in endothelial cells via activation of multiple signaling pathways, including stress protein kinases as well as certain caspases.

TL1 is a novel protein with a molecular mass of 22 kDa identified recently by searching the Human Genome Sciences cDNA data base. TL1 is a type II membrane protein and exhibits about 30% sequence homology to human tumor necrosis factor α (TNFα)1 (1). This newly identified member of the TNF family has been demonstrated to be abundantly expressed in endothelial cells as well as in kidney, lung, and prostate. TL1 expression in HL-60 and THP1 cells was induced by phorbol 12-myristate 13-acetate treatment. Radiation hybrid mapping localized TL1 gene on chromosome 9q32, near CD30L. Because of its robust expression in endothelial cells, TL1 has been suggested to possibly play a role in vascular functions (1). However, no functional study with this novel protein has been reported so far. The present study was undertaken to explore whether TL1 induces endothelial cell apoptosis, a phenomenon suggested to be one cause of endothelial cell damage contributing to various inflammatory disorders and cardiovascular dysfunction (2, 3). To examine this possibility, we used bovine pulmonary artery endothelial cells (BPAEC) in which TNFα-induced apoptosis has been demonstrated (4). Apoptosis was detected on the basis of morphological (including ultrastructural) and biochemical characteristics including DNA fragmentation. In addition, we studied the effects of TL1 on the activity of stress kinases, p38 mitogen-activated protein kinase (p38 MAPK) and stress-activated protein kinase (SAPK/JNK), and certain caspases. Both signaling pathways are believed to be implicated in programmed cell death (5–8). The expression of Fas and Bcl-2 in TL1-stimulated BPAEC was also determined in view of the death-promoting effect of Fas and the anti-apoptotic effect of Bcl-2 (9, 10).

EXPERIMENTAL PROCEDURES

Materials

Ac-YVAD-AMC and Ac-DEVAD-AMC were purchased from American Peptide (Sunnyvale, CA). ZVAD-fmk and Ac-YVAD-CHO were obtained from Enzyme Systems (Dublin, CA) and Peptides International (Louisville, KY), respectively. Ac-DQMD-AMC, Ac-LEED-AMC, Ac-VEDAD-AMC, and anti-p38 MAPK monoclonal antibodies were provided by SmithKline Beecham (SB) Pharmaceuticals (King of Prussia, PA). Ac-IETD-AMC and mouse-anti-human JNK monoclonal antibodies were purchased from Biomol Research Laboratories (Plymouth Meeting, PA) and Pharmingen (San Diego, CA), respectively. Mouse soluble TNF receptor 1 (sTNFR1) and TNF receptor 2 (sTNFR2) were obtained from R&D Systems (Minneapolis, MN).

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AF039390. § To whom correspondence should be addressed: Dept. of Cardiovascular Pharmacology, SmithKline Beecham Pharmaceuticals, King of Prussia, PA 19406-0939. Tel.: 610-270-5313; Fax: 610-270-5080; E-mail: tian-li.yue@phhrd.com.

1 The abbreviations used are: TNF, tumor necrosis factor; PBS, phosphate-buffered saline; PAGE, polyacrylamide gel electrophoresis; SAPK, stress-activated protein kinase; MAPK, mitogen-activated protein kinase; SB, SmithKline Beecham; sTNFR, soluble TNF receptor; GST, glutathione S-transferase; BPAEC, bovine pulmonary artery endothelial cells; AMC, 7-amino-4-methylcoumarin; CHO, aldehyde; fmk, fluoromethylketone; JNK, c-Jun N-terminal protein kinase; TUNEL, in situ end labeling.
Expression and Purification of Bacterially Expressed TL1

An expression vector containing a 24-amin acid deletion from the N-terminal sequence of the predicted full-length protein (GenBank™ accession number AF039830) was constructed. The DNA sequence encoding TL1 was amplified using polymerase chain reaction oligonucleotide primers specific to the coding sequence of the TL1 gene. Additional nucleotides containing restriction sites to facilitate cloning were added to the 5' and 3' sequences, respectively. The 5' oligonucleotide 5'-GCCATGGTGAAGACAATTCCTCCACAG-3' contained a NcoI restriction site, followed by 18 nucleotides of corresponding coding sequence. The 3' primer has the sequence 5'-CGCAAGCTTCTATAGTAAGGCT- CC-3' containing a HindIII restriction site followed by 18 nucleotides complementary to the last 15 nucleotides of the coding sequence and the stop codon. The amplified TL1 DNA was cloned into vector pQE60 (Qiagen) after digestion with restriction enzymes and ligation. The ligation mixture was transformed into competent Escherichia coli cells (strain M15/rep4). Expression of the TL1 protein was induced by isopropyl-1-thio-β-D-galactopyranoside, and protein was purified from the inclusion bodies into 8 M urea. The solubilized protein was passed over a PD-10 column in 2× phosphate-buffered saline (PBS), thereby removing the urea, and refolding the protein. To remove endotoxin, the protein was passed over a polynymin column. The resulting TL1 preparation was found to be more than 90% pure by SDS-PAGE and N-terminal sequencing. Automated N-terminal sequencing was carried out using a model ABI-494 sequencer (Perkin-Elmer/Applied Biosystems, Inc.) and the Gas-phase Blot cycles. Endotoxin levels were assayed using the Amebocyte Lysate Test (Bio-Whittaker) and were <10 EU of endotoxin/mg of protein (11).

Cell Cultures

BPAEC were obtained from the American Type Culture Collection (Rockville, MD). The cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% heat-inactivated fetal calf serum in a humidified environment of 5% CO₂, 95% air at 37 °C as described previously (12). Cells at a subconfluent density were used. Before experiments, the medium was changed to Dulbecco’s modified Eagle’s medium containing 2% fetal calf serum. BPAEC from passages 17–20 were used in all studies.

Morphological Assessment and Quantification of Apoptosis

To quantify cells undergoing apoptosis, cell monolayers were fixed and stained with Hoechst 33342 (Molecular Probes, Eugene, OR) as described previously (12). The morphological features of apoptosis (cell shrinkage, chromatin condensation, blebbing, and fragmentation) were monitored by fluorescence microscopy. Transmission electron microscopy study was done as reported previously (12).

DNA Fragmentation Analysis

DNA Ladder—Cells treated with vehicle or TL1 were lysed in lysis buffer containing 100 mM NaCl, 10 mM Tris-HCl, pH 8.0, 2.5 mM EDTA, 0.5% SDS, and 100 μg/ml protein kinase K. The lysates were incubated at 55 °C for 16 h. After incubation, the lysates were gently extracted three times with phenol/chloroform/isoamyl alcohol, precipitated in ethanol, and subjected to N-terminal sequencing analysis. The purity of the protein was >90%, which was determined by SDS-PAGE.

In Vitro Transfection of Dominant-interfering Mutant of c-Jun in BPAEC

The cells were plated in two-chamber slides. The cells were cotransfected with 0.5 μg/ml PEG-f-p1 (CLONTECH) (15) as a fluorescent marker of transfected cells together with 1 μg/ml amounts of either the empty cloning vector pCDNA1 (control) or the dominant-interfering c-Jun mutant pCDNA1-Flg9169 (5) using the Calphos Maximizer transfection kit (CLONTECH) according to the manufacturer’s recommendation. Following transfection, the cells were allowed to recover in complete medium for 24 h. The cells were treated with TL1, and the number of apoptotic cells was assessed by nuclear staining after fixation as described under Morphological Assessment and Quantification of Apoptosis.

Caspase Activity Assay

The cells were treated with vehicle or TL1 for a time period indicated in the figure legends. Caspase activity assays were performed with five different substrates under the condition optimal for each caspase as reported previously (13). Levels of released 7-amino-4-methylcoumarin (AMC) were measured with a Cytosensor-4000 fluorescent plate reader (Perseptive Biosystems) at excitation and emission wavelengths of 360 and 460 nm, respectively.

Immunohistochemical Analysis for Fas, Bcl-2, and Caspase-3 Expression

The cells were cultured in two-chamber slides. After treatment with vehicle or TL1, the cells were fixed with 4% paraformaldehyde for 30 min at 4 °C and then washed with cold PBS, and the nonspecific immunoglobulin binding sites were blocked with nonfat great serum (Vector Laboratories) for 1 h at room temperature. The cell samples were incubated with the primary antibody: mouse anti-human Fas (Upstate Biotechnology), mouse anti-human Bcl-2 (DAKO), or rabbit anti-human CPP32 p17 peptide polyclonal antisera (provided by Dr. K. Kikly, SmithKline Beecham), for 1 h at room temperature. As a negative control, the cell samples were incubated with nonimmune IgG (for Bcl-2 and CPP32) or IgM (for Fas) instead of the primary antibody. After incubation with the primary antibody, cells were washed with PBS and then incubated for 30 min with a secondary antibody conjugated to fluorescein isothiocyanate. Cells were washed, treated with Vectashield mounting medium (Vector Laboratories), and viewed by fluorescence microscopy (Olympus IX70).

RESULTS

Expression and Purification of Bacterially Expressed TL1—The predicted amino acid sequence of TL1 shares 20–30% homology to other members of the TNF family as reported previously (1). Hydrophobicity analysis of the protein predicts a hydrophobic region of 10–12 amino acid near the N terminus of the protein following 10–15 non-hydrophobic amino acids and a membrane-anchoring region at the C-terminal region, suggesting that TL1 is a characteristic type II membrane-bound protein. Therefore, we constructed an expression vector containing a 24-amino acid deletion from the N-terminal sequence of TL1. The recombinant TL1 was expressed, purified, and subjected to N-terminal sequencing analysis. The purity of the protein was >90%, which was determined by SDS-PAGE.

TL1 Induces Apoptosis in BPAEC—BPAEC exposed to TL1 showed plasma membrane blebbing and retracted from their neighboring cells, whereas their cytoplasm appeared condensed. Cells stained with Hoechst 33342 and assessed by fluorescence microscopy demonstrated condensed chromatin, fragmented nuclei, and blebbing of the plasma membrane (Fig. 1A, panel B). The study with transmission electron microscopy revealed that TL1-treated BPAEC displayed morphologic alterations characteristic of apoptosis including condensation of chromatin and appearance of apoptotic bodies (Fig. 1B, panel B). The characteristic degradation...
of DNA into oligonucleosomal-length fragmentation was observed when the cells were exposed to TL1 (30–300 ng/ml) for 24 h (Fig. 1C). DNA fragments in situ were further visualized by the TUNEL method (Fig. 1D, panel B). A considerable fraction of endothelial cells treated with TL1 showed positive staining; no positively stained cells were found in the vehicle-treated cultures (Fig. 1D, panel A).

TL1-induced endothelial cell apoptosis was a time- and concentration-dependent process with an EC_{50} value of 72 ng/ml (Fig. 2). A significant increase in the number of cells with apoptotic morphology was apparent 6–8 h after exposure of the cells to TL1. Under similar conditions, TNF-α, at 10 ng/ml, induced apoptosis in BEAPC by 16.7 ± 3.2% (n = 4).

**Effects of sTNFR1 and sTNFR2 on TL1-induced Apoptosis in BPAEC**—Neither sTNFR1 nor sTNFR2 at 30 μg/ml showed effect on TL1 (0.1 μg/ml)-induced apoptosis in BPAEC. Under the same condition TNFα-induced apoptosis in BPAEC was reduced by sTNFR1 significantly (data not shown).

**Regulation of Fas and Bcl-2 Expression in Endothelial Cells by TL1**—Immunocytochemical analysis of Fas and Bcl-2 proteins was determined at 8 and 24 h after treatment with TL1. Fas antigen was not detectable at resting BPAEC (Fig. 3A). However, a significant number of cells expressing Fas receptor were detected at 8 and 24 h after stimulation (Fig. 3B and C). When mouse IgM was substituted for the primary antibody, positive Fas immunoreactivity was not detected. In contrast, Bcl-2 expression was not detected in either unstimulated or TL1-treated BPAEC (data not shown).

**Activation of SAPK/JNK and p38 MAPK**—The effects of TL1 on SAPK/JNK activity in BPAEC are shown in Fig. 4. Exposure

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**Fig. 1. TL1-induced apoptosis in BPAEC.** A, fluorescence photomicrographs showing nuclear morphology of BPAEC treated with vehicle (A) or TL1 (50 ng/ml) (B) for 24 h and stained with Hoechst 33324 (original magnification, ×400). B, transmission electron micrographs of BPAEC cultures treated with vehicle (a) and TL1 (50 ng/ml) (b) for 8 h (Bar = 2.5 μm). C, electrophoretic analysis of internucleosomal DNA fragmentation in TL1-treated BPAEC. Cells were treated with vehicle (lane 5) or TL1 at 30 (lane 4), 100 (lane 3), and 300 (lane 2) ng/ml for 24 h. Lane 1, DNA size markers. D, in situ detection of DNA fragments. BPAEC were cultured in two-chamber slides and treated with vehicle (A) or TL1 (50 ng/ml) (B) for 24 h. Cells were washed in PBS, fixed, permeabilized, and then labeled with fluorescent dUTP and analyzed by fluorescent microscopy.
of endothelial cells to TL1 induced a rapid activation of SAPK/JNK. A significant increase in SAPK/JNK activity was detected 20 min after stimulation, peaked at 40 min, and then returned to the basal levels after 60 min (Fig. 4A). As shown in Fig. 4B, TL1-induced activation of SAPK/JNK in endothelial cells was a concentration-dependent process. The SAPK/JNK activity was increased by 5.6 ± 1.4-fold (p < 0.05, n = 4) and 9.1 ± 1.8-fold (p < 0.01, n = 6) over the basal level in the presence of 50 and 300 ng/ml TL1, respectively.

The effects of TL1 on p38 MAPK activity are shown in Fig. 5. TL1 activated p38 MAPK in BPAEC with a similar time course as SAPK/JNK but a lesser extent. The peak of p38 MAPK activity was increased by 3.1 ± 0.5- and 3.8 ± 0.4-fold over the basal level in the presence of 100 and 300 ng/ml TL1, respectively.

Effects on TL1-induced Apoptosis by Expression of Dominant-interfering Mutant of c-Jun in BPAEC or by the p38 MAPK Inhibitor, SB203580—To investigate the role of SAPK/JNK in TL1-induced apoptosis in BPAEC, we transfected BPAEC with a dominant-interfering mutant of c-Jun, pCDNA1-FlagΔ169, in which a deletion in the N-terminal transactivation domain that includes the binding site for JNK (5). As shown in Fig. 6, expression of dominant-interfering c-Jun construct in BPAEC reduced TL1-induced apoptosis by 62.8% (p < 0.05). The reduction in apoptosis in BPAEC was also attenuated by a specific p38 MAPK inhibitor, SB203580 (23), in a concentration-dependent manner as shown in Fig. 7. In the presence of 3 and 10 μM SB203580, TL1-induced BPAEC apoptosis was reduced by 33% (p < 0.05) and 51% (p < 0.01), respectively. No further inhibition was observed when the concentration of SB203580 was increased.

Activation of Caspases in BPAEC by TL1—As shown in Fig. 8, TL1-induced BPAEC apoptosis was attenuated by ZVAD-fmk, an irreversible cell-permeable inhibitor of caspase (16), added to the culture medium 1 h prior to TL1 treatment. Under the same conditions, the addition of Ac-YVAD-CHO, a relatively specific inhibitor of caspase-1 (17), up to 100 μM showed no effect in enhancing BPAEC rescue (data not shown). To further determine which of the caspase family members are activated in the TL1-induced apoptotic process in the endothelial cells, we examined cell extracts for proteolytic activity. The relative rates of AMC formation were measured with a series of defined peptide sequence variants that are relatively specific for caspase 1, 3, 4, 7, or 8 under the optimal conditions as described previously (13) and presented in Fig. 9A. Similar results were observed from three repeated experiments. Cell extracts from TL1-treated BPAEC were highly active on Ac-DEVD-AMC and to a lesser extent on Ac-DQMD-AMC, but not
active on the remaining three substrates which are more specific for caspase 1, 4, and 8. The proteolytic activity appeared at 6 h after the cells were treated with TL1, peaked at 24 h, and gradually returned to basal levels within 48 h. Fig. 9B shows a comparison of the relative velocities of four substrate hydrolysis rates by the TL1-treated cell extracts and the recombinant caspase-3. The relative velocities of the two enzyme sources on four substrates were very similar.

To further confirm the activation of caspase-3 in TL1-stimulated BPAEC, immunocytochemical detection of its enzymatically active form, the 17-kDa subunit, was performed. The antibody used was raised against a peptide derived from the C-terminal portion of the p17 subunit. The neoepitope antibody only binds caspase-3 following specific cleavage between the p10 and p20 subunits (13). As shown in Fig. 10, the 17-kDa subunit of caspase-3 was detected in TL1-treated but not vehicle-treated BPAEC, and was localized with fragmented nuclei within the cells.

**DISCUSSION**

The studies presented in this paper demonstrate that TL1, a novel TNF-like cytokine and a type II transmembrane protein, induces intensive apoptosis in cultured endothelial cells as reflected by morphological and biochemical criteria. Under our experimental conditions, spontaneous BPAEC death rate was
approximately 2–4%, well in accord with a previous observation (4). The effect of TL1 was concentration-dependent with an EC50 value of 72 ng/ml (3.5 nM), and a significant number of apoptotic cells were detected 6–8 h after treatment. Moreover, the expression of pro-apoptotic gene, Fas, was demonstrated in TL1-treated BPAEC, which is consistent with that observed in apoptotic endothelial cells reported previously (12).

The receptor(s) mediating TL1 activity has not been identified as yet. To examine whether TL1 acts via distinct receptor(s) or shares the known TNFR1 or TNFR2, we tested the effects of sTNFR1 and sTNFR2 on TL1-induced apoptosis in BPAEC. These two TNFRs have been shown previously to block the cell surface TNFR1 and TNFR2 mediated TNF bioactivities on responsive cell lines (data from R&D Systems). However, neither sTNFR1 nor sTNFR2 inhibited the effect of TL1 on BPAEC. In contrast, TNFα-induced apoptosis in BPAEC was significantly reduced by sTNF1. The results suggest clearly that TL1-induced cell death is independent of TNFR1 or TNFR2.

Recent research efforts on TNF family members have demonstrated that TNFα and Fas activate stress protein kinases, SAPK/JNK and p38 MAPK, in a variety of cell types (18, 19); however, the effects of other members of this family on SAPK and p38 MAPK have not been well studied. Moreover, controversies regarding the role of SAPK/JNK and p38 MAPK in TNFα- or Fas-mediated cell death have been reported. For example, TNFα-induced apoptosis is dependent on JNK activity in U937 cells (6, 20) but not in fibroblasts (21), indicating that the consequences of JNK activation vary considerably among cell types. Fas-mediated JNK activation occurs with a
shown to specifically inhibit p38 MAPK activity and was tested. This inhibitor has been shown to specifically inhibit p38 MAPK activity in vitro, with no effect on a variety of kinases tested, including JNK-1 and ERK-1 (23). As shown in Fig. 7, TL1-induced apoptosis in BPAEC was also reduced by SB203580 in a concentration-dependent manner, indicating that p38 MAPK signaling pathway is involved in TL1-mediated BPAEC apoptosis. This effect is different from that observed in Fas-mediated apoptosis in Jurkat cells, in which SB203580 had no protective effect (19). Moreover, TL1-induced p38 MAPK activation occurs with much faster kinetics in BPAEC than that observed in Jurkat cells, in which the peak of p38 MAPK activation was at 2–4 h after stimulation by Fas, indicating TL1 and Fas most likely activate p38 MAPK through a different mechanism with a different outcome. Our data further suggest that different members of the TNF family may have different signaling pathways to mediate cell death or have different effects in different cell types.

Recent work has supported a central role for the caspase family members, as effectors of apoptosis (24). However, the role of caspases in endothelial cell apoptosis has not been sufficiently explored. Among the caspase family, caspase-3 (CPP32) has been considered as a central component of the proteolytic cascade during apoptosis and plays a key role in this family (25, 26). TL1-induced BPAEC apoptosis was inhibited by ZVAD-fmk, indicating a potential role for the caspase family in this effector pathway for apoptosis. To determine which of the caspase family members are involved, we examined the substrate specificity of proteolytic activity in the extracts from TL1-activated BPAEC by measuring the relative rate of AMC formation from six different substrates, which are relatively specific for caspases 1, 3, 4, 7, and 8 (27). Treatment of BPAEC with TL1 resulted in a significant increase in proteolytic activity towards DEVD-AMC mainly and DQMD-AMC to some extent, both of which show the relative specificity for caspase-3 (13, 27). There was no induction in proteolytic activity in TL1-activated cell extracts when Ac-YVAD-AMC, LEED-AMC, or VETD-AMC were used as the substrate, indicating that caspases 1, 4, and 8 might not be involved. Moreover, comparison of the substrate specificity of the extracts from TL1-treated BPAEC with the recombinant caspase-3 showed a similar pattern, further suggesting that caspase-3 may be the predominant member in the caspase family activated by TL1. Furthermore, immunocytochemical studies detected the active form of caspase-3 in TL1-treated BPAEC. It was reported that multiple caspase homologues were found in both the cytoplasm and nucleus in etoposide-induced apoptosis in HL-60 cells (28). Interestingly, in TL1-induced apoptotic BPAEC, the immunoreactive 17-kDa subunit of caspase-3 was only localized with fragmented nuclei, further indicating a role of caspase-3 in TL1-induced apoptosis. Whether this active caspase-3 was transported into the nucleus or the inactive caspase-3 is already in the nucleus awaiting activation promoted by TL1 requires further investigation. Taken together, these results suggest that caspase-3 was activated by TL1 in BPAEC and may mediate TL1-induced cell apoptosis. However, our results cannot exclude other members of this family, especially those closely related to caspase-3, such as caspase-7, in mediating TL1-induced cell apoptosis.

In summary, the present studies have demonstrated that TL1, a novel member of TNF cytokine family, causes endothelial cell apoptosis. TL1 appears to act through a receptor that is distinct from TNF receptors 1 or 2. The effect of TL1 is via activation of the stress protein kinases, SAPK/JNK and p38 MAPK, and the caspases, mainly caspase-3-like proteases. Apoptotic programmed cell death has been suggested to be a cause of endothelial cell damage contributing to various inflammatory disorders and cardiovascular injury (29). Moreover, endothelial cell apoptosis may be an important mechanism involved in a balance between antiangiogenic and proangiogenic processes, and loss of this balance will lead to a variety of diseases such as solid tumor metastasis and retinopathy (30, 31). Therefore, the biological significance and the potential roles of TL1 in these pathophysiological conditions, and the mechanism for regulation of TL1 production in endothelial cells require further studies.

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