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Cutting Edge: Lymphocyte Inhibitor of TRAIL (TNF-Related Apoptosis-Inducing Ligand): A New Receptor Protecting Lymphocytes from the Death Ligand TRAIL[1]

Juthathip Mongkolsapaya, Alison E. Cowper, Xiao-Ning Xu, Gwilym Morris, Andrew J. McMichael, John I. Bell, and Gavin R. Screaton[2]

Apoptosis can be triggered by the engagement of cell surface receptors by their ligands. A growing number of receptors belonging to the TNF receptor family have been identified that contain a conserved cytoplasmic death domain. These include Fas, TNF-R1, lymphocyte-associated receptor of death (LARD), DR4, and TNF-related apoptosis-inducing ligand receptor inducer of cell killing-2 (TRICK2). The latter two are receptors for the cytotoxic ligand TNF-related apoptosis-inducing ligand (TRAIL), and one of the paradoxes raised by the cloning of these molecules was why do most cells not die upon contact with the widely expressed TRAIL molecule? This is a particular problem for lymphocytes that express DR4 and TRICK2 and are in constant circulation through TRAIL-expressing tissues. We have cloned LIT (lymphocyte inhibitor of TRAIL), which lacks a death domain. LIT is expressed predominantly on PBL, where it can competitively inhibit TRAIL-induced apoptosis through DR4/TRICK2, and may function to modulate lymphocyte sensitivity to TRAIL. ***The Journal of Immunology,*** **1998, 160: 3–6.**

Programmed cell death or apoptosis is a crucial biologic process involved in the development and function of a variety of biologic systems (1, 2). The central importance of apoptosis in immune function is exemplified by Fas, which plays a role in CTL killing, activation-induced death of lymphocytes, and possibly thymic selection, although the finding that mice deficient in Fas develop a normal T cell repertoire implies the existence of other pathways (3, 4). Murine mutations in Fas (*lpr*) or Fas-L[3] (*gld*) lead to a failure of activation-induced death, with consequent lymphadenopathy, splenomegaly, and autoimmune disease (5).

Until recently, two cell surface receptors, TNF-R1 and Fas, had been identified that could promote apoptosis when contacted by their ligands, TNF and Fas-L (5). Recently discovered death receptors include LARD/DR3/Wsl-1/Apo-3/TRAMP (TNF receptor-related apoptosis-mediating protein), which is expressed on lymphocytes and regulated by alternative splicing (6–10), and the receptors for TRAIL/Apo-2L (11, 12), DR4, and TRICK2 (13, 14).

DR4 and TRICK2 are expressed on PBL, but the expression of TRAIL on a wide variety of tissues provides a potential problem for circulating lymphocytes. We have cloned a new TRAIL receptor, LIT (lymphocyte inhibitor of TRAIL), which we believe can competitively inhibit TRAIL to protect resting lymphocytes from inappropriate apoptosis.

Materials and Methods

Cloning and expression patterns of LIT

The EST subset of the NCBI (National Center for Biological Information) database was screened for homology to TRICK2 and DR4; four clones were identified and sequenced. Multiple tissue human Northern blots (Clontech, Palo Alto, CA) were hybridized with a ^{32}P random-primed probe encompassing the nerve growth factor receptor (NGFR) domains of LIT.

$CD4^+$, $CD8^+$, and B cells were purified by absorption onto magnetic beads coated with Abs to CD4, CD8, and CD19. RT-PCR (30 cycles) was conducted on cDNA produced from these samples with primer pairs spanning around 400 bp of the extracellular domains of LIT, TRICK2, and DR4 or 600 bp of TRAIL.

TRAIL apoptosis assay

293 T cells were cotransfected by calcium phosphate precipitation with pCDNA3 TRAIL (40 μg/15-cm plate) and CD8α cDNA (10 μg/plate). Cotransfected cells were isolated using anti-CD8-coated magnetic beads (Dynal, Chantilly, VA) at 48 h. Cells (10^5/well) were seeded into 96-well plates with 5×10^3 ^{51}Cr-labeled Jurkat T cells and soluble Fc fusion proteins, used at 20 μg/ml. Supernatants were harvested at 12 h and counted by scintillation on a beta plate counter. The percentage of specific release was calculated as ((experimental − background)/(maximum − background)) × 100. Fusions between the IgG1 constant domain and extracellular domains of DR4 amino acids 104 to 217, Fas amino acids 17 to

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[3] Abbreviations used in this paper: Fas-L, Fas ligand; LARD, lymphocyte-associated receptor of death; TRICK2, tumor necrosis factor-related apoptosis-inducing ligand receptor inducer of cell killing-2; TRAIL, tumor necrosis factor-related apoptosis-inducing ligand; LIT, lymphocyte inhibitor of tumor necrosis factor-related apoptosis-inducing ligand; NGFR, nerve growth factor receptor; RT-PCR, reverse transcription-polymerase chain reaction.

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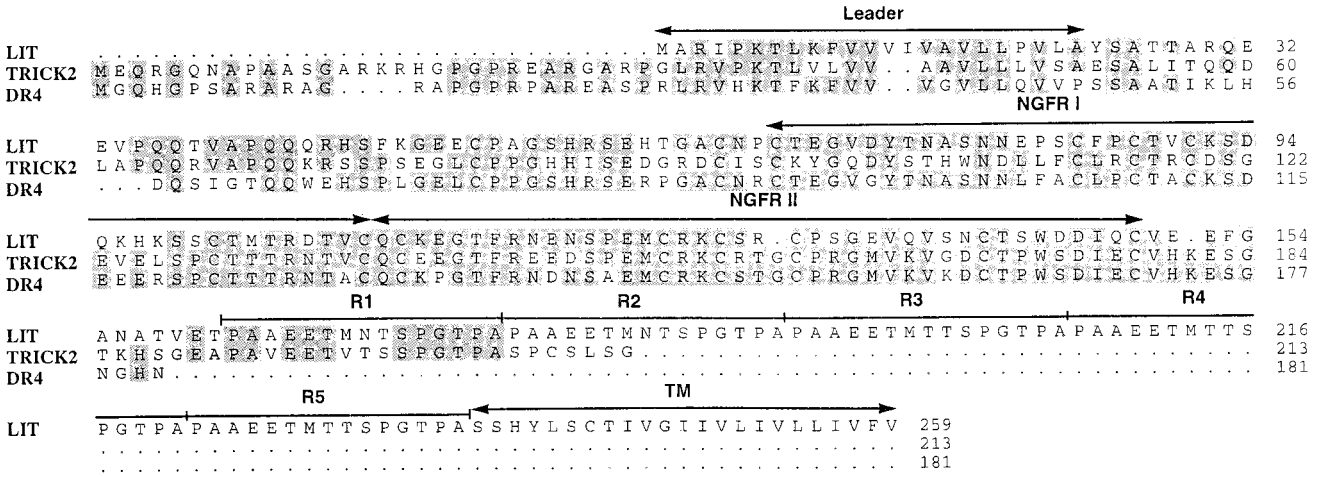

FIGURE 1. *A,* Alignment of the predicted amino acid sequence of LIT with the extracellular domains of TRICK2B and DR4. The predicted leader, NGFR repeats, five tandem repeat sequences (R1 through R5), and transmembrane domain (TM) are marked. The sequence of LIT has been deposited at GenBank, access number AFO33845.

173, TNF-R1 amino acids 24 to 210, TRICK2 amino acids 57 to 183, and LIT amino acids 35 to 152 were produced in 293T cells.

Results and Discussion

Cloning of LIT

Searching the expressed sequence tag subset of the NCBI database for the sequences of TRICK2 and DR4 revealed a novel sequence with a high degree of homology in the extracellular domain. Four clones were sequenced, and the predicted amino acid sequence of LIT is shown in Figure 1 aligned with the extracellular domains of TRICK2 and DR4. LIT is a type I membrane protein and has a 23-amino acid signal sequence, a 213-amino acid extracellular domain, and a 23-amino acid transmembrane domain. The mature protein has a predicted molecular mass of 24.9 kDa and has five consensus sites for *N*-linked glycosylation. The extracellular domain has two and a half NGFR repeats that have 54 and 69% amino acid identity to the repeats of TRICK2 and DR4, respectively. During the preparation of this manuscript, reports describing the cloning of an identical molecule TRID/DcR1 have appeared (15, 16).

Following the NGFR repeats, LIT has five almost perfect repeats of the sequence PAAEETMNTSPGTPA. This repeat is homologous to a membrane-proximal insertion in the alternatively spliced variant of TRICK2, TRICK2B. This raises the possibility that the 15 amino acid repeats are encoded by an alternatively spliced exon or retained intron, but we were unable to find any evidence of alternative splicing of this region in PBL (data not shown). A termination codon is found at the end of a 23-amino acid predicted transmembrane domain. Thus, LIT has no cytoplasmic domain and cannot signal apoptosis.

Of the death receptors described to date, TNF-R1 and LARD have four NGFR repeats; Fas has three; DR4, TRICK2, LIT, and the chicken death receptor CAR1 (17) have two. The NGFR repeats in LIT are most similar in structure to domains 2 and 3 of TNF-R1. These two repeats make contacts with the ligand molecule in the TNF/TNF-R1 crystal structure (18). Thus, the two domain receptors contain the core binding surface, and the function of the extra repeats found in TNF-R1 and LARD is unclear, although the N-terminal repeat may help to prevent self aggregation (19).

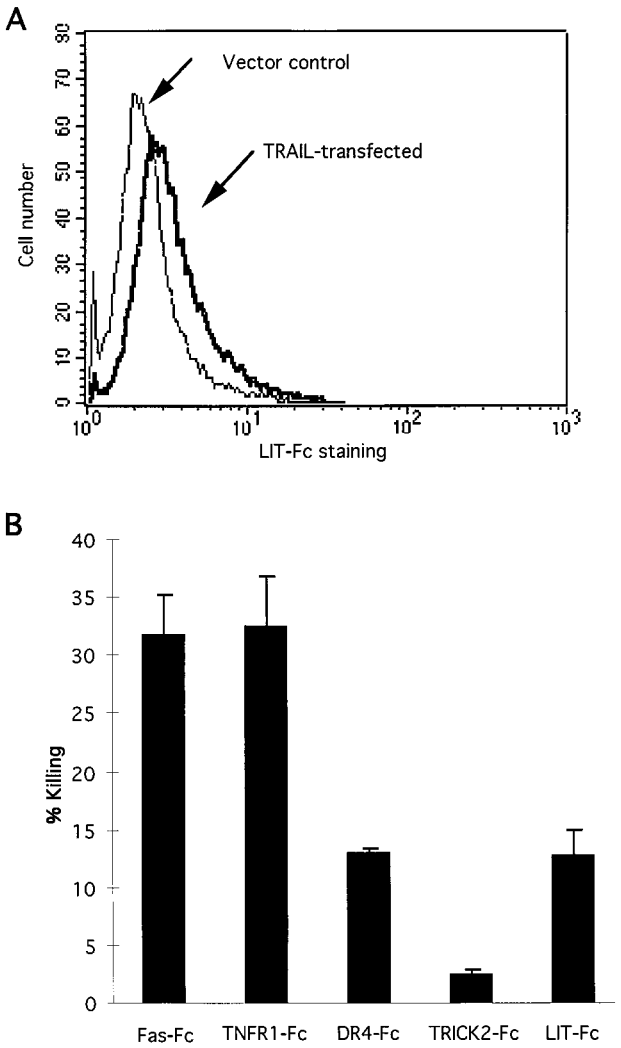

FIGURE 2. *A,* LIT-Fc fusion protein binds to TRAIL-transfected 293T cells. Biotinylated LIT-Fc was used to stain cells transfected with CD8/TRAIL or CD8/control vector. Cotransfected cells were gated with CD8-phycoerythrin, and the histogram represents staining with streptavidin-FITC. *B,* Soluble LIT inhibits TRAIL killing of Jurkat cells.

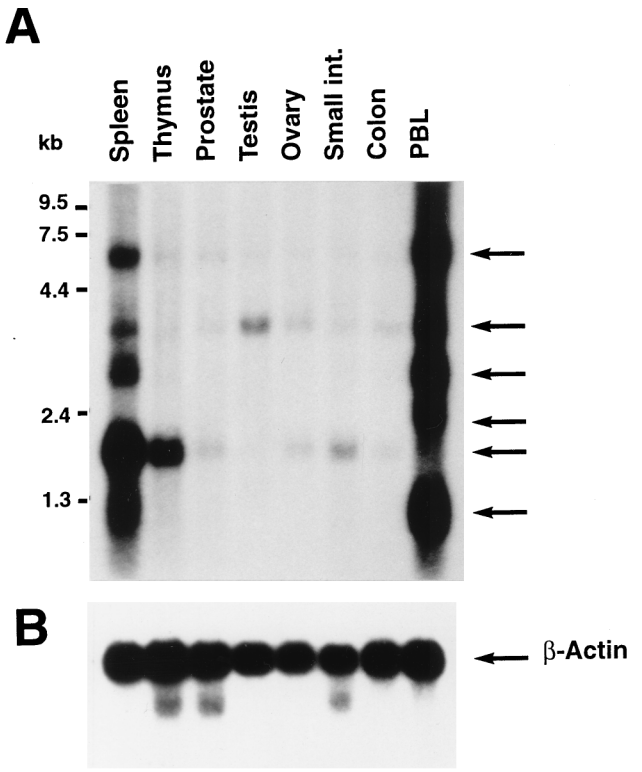

FIGURE 3. Northern blots of multiple human tissues probed with LIT (*A*) or a control β-actin probe (*B*).

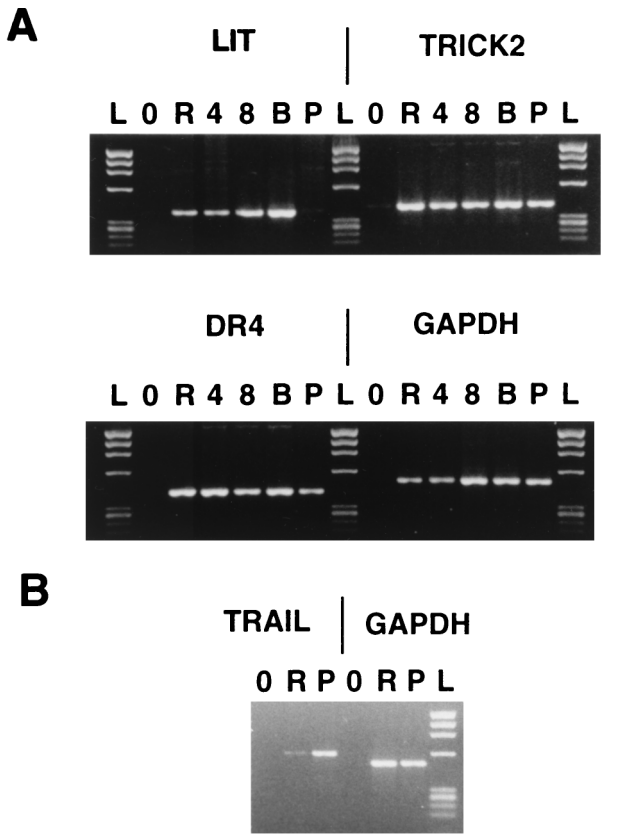

FIGURE 4. *A*, PCR amplification of cDNA prepared from fractionated lymphocyte subsets and PHA blasts for LIT, TRICK2, DR4, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) control. L, ladder; 0, control PCR, no cDNA; R, resting PBL; 4, $CD4^+$ T cells; 8, $CD8^+$ T cells; B, B cells; P, PHA blast PBL. *B*, PCR amplification of TRAIL and GAPDH control from cDNA isolated from resting and PHA blast PBL.

LIT is a receptor for TRAIL

Biotinylated LIT-Fc fusion protein was used to stain 293T cells cotransfected with CD8/TRAIL or CD8/empty vector (Fig. 2*A*). This allowed us to gate for TRAIL-transfected cells using an anti-CD8 mAb. Cells transfected with TRAIL, but not with an irrelevant second vector, stain positively with LIT-Fc, demonstrating that LIT is a receptor for TRAIL.

To determine whether LIT-Fc could inhibit TRAIL-mediated killing, we developed a bioassay to examine TRAIL cytotoxicity. This assay is similar to previous assays for Fas-L activity (20). 293T cells expressing TRAIL are used to kill ^{51}Cr-labeled Jurkat cells that express DR4 and TRICK2 but lack LIT (RT-PCR data not shown). Soluble TNF-R1 and Fas had no inhibitory effect, whereas LIT, in common with DR4 and TRICK2, competitively inhibited TRAIL-induced killing of Jurkat (Fig. 2*B*).

Expression of LIT

Northern blot analysis of LIT expression is shown in Figure 3. Several mRNA species can be seen on this blot, running at 1.4, 2, 2.4, 3, 3.8, 4, and 6 kb. Expression is particularly marked in PBL, where the 1.4-kb band predominates; this corresponds to the LIT clones we sequenced. The 1.8-kb message predominates in spleen and is also found in thymus. We were not able to isolate any of the larger LIT transcripts from cDNA libraries, and they probably represent alternative 3′ untranslated sequences, although the possibility does exist that some may encode isoforms of LIT possessing a cytoplasmic domain.

To further characterize LIT expression on lymphocytes we performed RT-PCR on fractionated and PHA-treated PBL (Fig. 4*A*). LIT is expressed in roughly equal amounts on $CD4^+$ and $CD8^+$ T cells and B cells. This expression pattern is similar to that seen with DR4 and TRICK2. However, upon lymphocyte activation with PHA, LIT expression is almost completely lost, while expression of the death domain-containing molecules, DR4 and TRICK2, is maintained. LIT expression was also lost on the lymphocyte tumor cell lines Jurkat, CEM, and Raji (data not shown). Interestingly, TRAIL is induced by lymphocyte stimulation (Fig. 4*B*).

TRAIL is expressed on many tissues, which contrasts with the expression of Fas-L, which is restricted to activated lymphocytes and sites of immune privilege such as testis and retina (5). Early analysis of TRAIL indicated that most normal cells were resistant to its cytotoxic effect. These included PBL, which are resistant in the resting state but become sensitive to the action of TRAIL/Apo-2L following stimulation with IL-2 (21). This contrasts completely with the sensitivity of tumor cell lines, particularly the lymphocyte cell lines, which are exquisitely sensitive to TRAIL (11). It was therefore puzzling to find that the two death domain-containing receptors, DR4 and TRICK2, are also ubiquitously expressed and, in particular, are found at high levels on PBL. This means that PBL that continually circulate through the tissues will come into contact with cells expressing TRAIL. We believe that LIT has evolved to circumvent this problem encountered by PBL by acting as a competitive inhibitor of TRAIL at the lymphocyte surface. It is believed that the trimeric TNF family members will only signal when they bind three death receptor molecules. By lacking a death domain, LIT can regulate TRAIL apoptosis in a dominant negative fashion.

Although TRAIL and its death receptors are quite widely expressed, these molecules may have important immunoregulatory

roles. The up-regulation of TRAIL upon lymphocyte activation mirrors the rise in Fas-L expression and may provide another weapon to the armory of cytotoxic T cells. The down-regulation of LIT upon lymphocyte activation exposes activated cells to apoptosis and may thus play a role in activation-induced death of lymphocytes. Finally, the majority of cell in the thymus are immature lymphocytes, most of which will die by apoptosis; it may be that the low expression of LIT on thymocytes exposes them to TRAIL apoptosis during negative selection.

Fas-L expression by HIV-infected cells and malignant tumors (22, 23) may deliver a death signal to cytotoxic T cells expressing Fas, protecting the diseased cells from immune attack. It is therefore rather puzzling that tumors that would normally be supposed to develop mechanisms to evade apoptosis seem to lose expression of the protective LIT molecule and become exposed to TRAIL killing.

A complex regulatory mechanism needs to be in place to protect lymphocytes that express a set of apoptosis receptors from an untimely death. Several mechanisms have evolved to mediate this protection, such as tight regulation of ligand expression in the case of Fas, alternative pre-mRNA splicing to exclude the death domain in LARD, expression of antiapoptotic proteins, such as Bcl-2, and interfering with the signaling cascade by molecules such as Fas-associated protein-1 and the recently described cellular FLICE inhibitory proteins (FLIPs) (24–26). Our finding of LIT, a molecule without a cytoplasmic domain, represents an additional mechanism to control apoptosis signaled by cell surface receptors. A more detailed analysis of the expression and function of the TRAIL/DR4/TRICK2/LIT molecules in the immune system awaits the generation of an mAb panel and knockout animals.

Acknowledgments

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