TRIL, a Functional Component of the TLR4 Signaling Complex, Highly Expressed in Brain


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TRIL, a Functional Component of the TLR4 Signaling Complex, Highly Expressed in Brain


TLR4 is the primary sensor of LPS. In this study, we describe for the first time TLR4 interactor with leucine-rich repeats (TRIL), which is a novel component of the TLR4 complex. TRIL is expressed in a number of tissues, most prominently in the brain but also in the spinal cord, lung, kidney, and ovary. TRIL is composed of a signal sequence, 13 leucine-rich repeats, a fibronectin domain, and a single transmembrane spanning region. TRIL is induced by LPS in the human astrocytoma cell line U373, in murine brain following i.p. injection, and in human PBMC. Endogenous TRIL interacts with TLR4 and this interaction is greatly enhanced following LPS stimulation. TRIL also interacts with the TLR4 ligand LPS. Furthermore, U373 cells stably overexpressing TRIL display enhanced cytokine production in response to LPS. Finally, knockdown of TRIL using small interfering RNA attenuates LPS signaling and cytokine production in cell lines, human PBMC, and primary murine mixed glial cells. These results demonstrate that TRIL is a novel component of the TLR4 complex which may have particular relevance for the functional role of TLR4 in the brain.

TRIL is a functional protein in the TLR4 complex that may have particular relevance for TLR4-mediated responses in brain.

Materials and Methods
Reagents
Anti-Flag Ab and anti-Flag-agarose were purchased from Sigma-Aldrich. Anti-β-actin Ab was purchased from Sigma-Aldrich. The β-actin Ab was a gift from Prof. R. Hay (University of Dundee, Dundee, U.K.). The polyclonal phospho-p38 Ab was obtained from Cell Signaling Technology. The anti-TRIL Ab was purchased from Imgenex. The anti-mouse IgG (whole molecule) peroxidase conjugate and anti-rabbit IgG (whole molecule) peroxidase conjugate Abs were all purchased from Jackson ImmunoResearch Laboratories. LPS (TLR4 grade) from Escherichia coli serotype EH100 was obtained from Alexis. rTNF-α as well as IL-6, IL-8, IL-1β, TNF-α, and RANTES ELISAs were obtained from R&D Systems. Human and murine cDNA panels were obtained from BD Clontech. Human protein tissue samples were obtained from BD Clontech. The human macrophage THP1 cell line, HEK293 cells, and U373 parental cells were obtained from the European Collection of Animal Cell Cultures. U373/CD14 cells were a gift from Prof. Doug Golenbock (University of Massachusetts, Boston, MA).

Homology modeling studies
Predicted structural domains in TRIL were identified using the Meta Server 3D-Jury. Alignments were manually adjusted and used as templates to generate models using Modeler 9v13 (12). The Lingo-1 ectodomain (pdb 2d5s) was the template for the LRR domain.

Animals
C57BL/6 mice (3–4 mo old) were purchased from Harlan. Animals had free access to food and water, were housed in groups of three to six in a controlled environment (temperature: 20–22°C, 12:12-h light/dark cycle), and were maintained under veterinary supervision for the duration of the experiment. All experiments were conducted under a license from the Department of Health and Children (Ireland) and with ethical approval from Trinity College Ethical Committee. For LPS induction experiments, C57BL/6 mice were injected with either saline or LPS (100 μg/kg E. coli serotype 0111:B4; Sigma-Aldrich) i.p. and 3 h later mice were killed by cervical dislocation and the brain was rapidly removed and stored snap frozen until required for analysis.

Ab characterization
Two rabbit polyclonal anti-TRIL Abs were raised against a peptide sequence in the C terminus of the protein, aa 797–811-SLRREDRLLQR FAD (Eurogentec/21st Century Biochemicals), one of which was affinity purified. Ab specificity was confirmed by peptide competition analysis. HEK293 cells were seeded at 1 × 105 cells/ml in 6-well plates. Cells were either mock transfected or transfected with 3 μg of TRIL. Samples were harvested and electrophoresed on 10% SDS-gel, transferred to polyvinylidene fluoride membrane, and blocked in 5% (w/v) dried milk in TBS-Tween 20 buffer. Samples were either probed directly using anti-TRIL Ab or the primary Ab was incubated at 30°C for 30 min with 20 μg/ml of its corresponding competing peptide.

Cell culture and stable cell line generation
HEK293 cells and U373 parental cells were maintained in DMEM and THP1 cells were maintained in RPMI 1640. All medium used was supplemented with 10% FCS and 1% penicillin-streptomycin solution (v/v). Cerebellar granule neurons were isolated from the dissociated cerebella from 5-day-old rats. Cells were gently dissociated with a Pasteur pipette using 0.4% trypsin and 0.1% DNase I at room temperature. Cells were maintained in DMEM/F12. Cells were also maintained in 10 μM cytosine-1-β-d-arabinofuranoside solution. Cells were maintained on poly-l-ornithine-coated plates. Cortical astrocytes were isolated from the cortex of 5-day-old rats and maintained in DMEM for 10 days before treatment. Human PBMC were isolated from human blood and maintained in IMDM. Primary murine mixed glia cells were obtained from 1-day-old neonatal brains. Cells were cultured in DMEM for 10 days before treatment. U373 parental cells were used for stable cell line generation. U373 cells were transfected by nucleofection (Cell Line Nucleofector Kit V, program T-020, Amaxa). Cells were maintained in DMEM supplemented with 10% FCS and 1% penicillin-streptomycin solution (v/v). Cells stably transfected with pcDNA3.1 TRIL were selected and cloned using 300 μg/ml neomycin analog G418 (InvivoGen) with or without 10 μg/ml blasticidin. Overexpression of TRIL was confirmed by immunoblotting and/or FACS analysis.

Stable Chines hamster ovary (CHO) cells were generated using the pCDNA5 vector from Invitrogen (V652020). Positive clones were selected using hygromycin antibiotics.

RT-PCR
For quantitative real-time PCR, cDNA was transcribed using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Primers and probes for human and mouse TRIL were purchased from Applied Biosystems (assay identification HS00274460_s1 and Mm00503080_s1, respectively).

Small interfering RNA (siRNA)
To test the biological function of TRIL, we purchased a RNA interference duplex (Qiagen). Oligonucleotide 1 sequence for human TRIL was GGCGUGAUCAUAGCGCAA (sense strand sequence for human TRIL). The additional oligonucleotide was tested was CUGGAGAACUCGGCGCAUU (Dharmacon). Sequence scrambled negative control oligonucleotide for TRIL was CGUCAUCUGUGGACAGAAGA. siRNAs were tested at a number of concentrations. Following optimization, 50 nM of siRNA oligonucleotide 1 (Qiagen) showed the most consistent knockdown in all cell types. U373/CD14 cells were seeded at 5 × 105 cells/ml in 6-well plates. Oligonucleotides were transfected using Oligofectamine (Invitrogen) in serum-free medium. After 6 h, 1 ml of medium containing 20% FCS and 2% t-glutamine was added to the cells. Cells were harvested after 48–72 h and used for further analysis. Human PBMC, THP1 cells, and neurons mixed glial cells were transfected with siRNA using an Amaza electroporator and a Cell Line Nucleofector Kit V, program V-01 (PBMC), S-019 (THP1), and T-020 (mixed glial cells), according to the manufacturer’s instructions. One × 106 cells/ml PBMC or THP1 cells were used per point for nucleofection. Cells were harvested after 72 h and used for further analysis.

Immunoprecipitation, glycosidase treatment, and LPS-binding assays
HEK293 cells were seeded at 2 × 105 cells/ml in 10-cm dishes. Flag-TRIL (pcDNA3.1) was transfected using Genejuice Transfection Reagent (No-gen). Cells were treated as outlined in the figure legends. Cells were washed in ice-cold PBS and lysed in 500 μl of high-stringency lysis buffer (50 mM HEPES (pH 7.5), 100 mM NaCl, 1 mM EDTA, 10% glycerol, and 1% Nonidet P-40). Supernatants were removed and added to the relevant precoated Ab. Fifty microliters of each lysate was retained to confirm expression of TRIL and TRIL-L. Samples were incubated overnight at 4°C. Following incubation, the immune complexes were washed twice with 1 ml of lysis buffer and once with ice-cold PBS. All supernatants were removed and beads were resuspended in 30 μl of 5× sample buffer. The samples were boiled for 5 min and SDS-PAGE analysis was performed as described previously.

TRIL was immunoprecipitated from TRIL-FLP-In CHO cells (~1–2 × 106 cells/point) using anti-TRIL-coupled protein A-Sepharose beads. The complex was washed twice with high-stringency lysis buffer and once with PBS before resuspending the beads in glycosidase reaction buffer. Beads were incubated with 2.5 U of PNGase F (PP0261–1KT, Sigma-Aldrich) for 40 min at 37°C. SDS-PAGE sample buffer was added and the samples were analyzed by Western blotting.

HEK293 cells were seeded at 2 × 105 cells/ml in 10-cm dishes. Cells were transfected with 4 μg of pcDNA-TRIL plasmid. After 24 h, lysates containing overexpressed TRIL were incubated with 2 μg/ml biotinylated LPS (InvivoGen) or 3 μg/ml biotinylated CpG (MWG) before precipitation on avidin beads (Pierce). The complexes were washed in high-stringency buffer and analyzed by SDS-PAGE. LPS pull-down experiments were also conducted in the presence and absence of nonbiotinylated LPS for 2 h at room temperature before precipitation with avidin-agarose for a further 2 h.

Results
Identification and characterization of TRIL
A microarray was conducted on embryonic stem cells to identify novel LPS-inducible genes. A previously uncharacterized gene was identified, which we named TRIL (GenBank accession no. NM_014817). The open reading frame of TRIL comprises 2436 bp and is predicted to encode a protein of 811 aa with a calculated molecular mass of 83 kDa. The chromosomal location has been mapped to chromosome 7q15.1 and the coding region consists of one exon. A number of LRR glycoproteins have evolved to encode their entire LRR cassette within one exon such as syntenin (13),
chondroadherin (14), and 5T4 oncofetal leucine-rich glycoprotein (15) in addition to TLR1, TLR2, and TLRs 5–10. The TRIL protein sequence is highly conserved across species and is shown for humans and mice in Fig. 1. Analysis of the TRIL sequence using Meta Server 3D-Jury structural homology software identified two distinct structured domains: an N-terminal LRR domain followed by a type III fibronectin domain before the putative transmembrane region (Fig. 2A). There was no predicted structural domain in the proposed intracellular portion of TRIL. The N terminus of the protein contains a 23-aa signal sequence as predicted by signal P, suggesting that the protein is directed to the endoplasmic reticulum and from there it could go to several types of vesicles or to the plasma membrane (16). It has a predicted isoelectric point of 9.7, which we have confirmed by two-dimensional electrophoresis (data not shown).

A homology model of the TRIL LRR region was generated using Modeler 9v3 (12) and has the characteristic curved solenoid of other LRR domains as shown in Fig. 2B. This model is based on LINGO-1 and is also the common structural element found in the TLRs and CD14. Altogether there are 13 LRR motifs and N- and C-terminal capping structures typical of an extracellular LRR. Each separate TRIL LRR is 24 residues long except for LRR7 which contains two single residue insertions (Fig. 2C). The asparagine ladder is strictly conserved throughout.

**TRIL expression profiling**

We conducted quantitative RT-PCR to assess the expression of TRIL. Results indicate that TRIL is most highly expressed in murine brain, spinal cord, day 11 embryo, and lung (Fig. 3A). All samples were normalized against GAPDH and expressed relative to the lowest detectable sample. Results from a human cDNA panel are consistent with the murine results and also indicate high levels of TRIL in the brain, ovary, small intestine, and spleen (Fig. 3B). Since high expression levels were found in brain, we investigated this further and found that TRIL is highly expressed on rat cortical astrocytes and on day 5 cerebellar granule neurons (Fig. 3C). Expression of TRIL was also observed throughout the brain in the hippocampus, cortex, and cerebellum at the protein level and also at the RNA level using laser capture microdissection (data not shown).

**FIGURE 1.** Sequence analysis of TRIL. The amino acid sequence of human and mouse TRIL. Regions with identical amino acids are highlighted in black.

**FIGURE 2.** The TRIL LRR domain. A, Schematic depicting the domain topology of TRIL. The extracellular region (residues 1–697) contains both a LRR domain and type III fibronectin domain. The predicted transmembrane domain is shaded black. The intracellular section (residues 718–811; light gray) is predicted to lack specific structured domains. B, Homology model of the TRIL LRR domain using the LINGO-1 ectodomain (pdb 2id5) as a template. The LRR is shown as a cartoon representation inside the molecular surface. LRR1 is located at the top of the image and the insert containing LRR7 is highlighted in purple. C, Human TRIL LRR sequences. In the consensus sequence, L = leucine, isoleucine, valine, or phenylalanine; N = asparagine, cysteine, serine, or threonine; F = phenylalanine or leucine. For each TRIL LRR consensus sequence, residues are in bold and deviations from the consensus are highlighted. The high levels of deviation from the consensus in the latter half of LRR13 reflect the movement of the structure into the C-terminal cap.
TRIL is a LPS-inducible protein
Abs to TRIL were generated to measure endogenous protein levels. The peptide sequence to which the Abs were raised is highly conserved across a number of species. The specificity of the Ab was confirmed by peptide competition as shown in Fig. 4A for both overexpressed (lane 1 vs lane 3) and endogenous (lane 2 vs lane 4). As can be seen from this figure when TRIL is transiently overexpressed in 293 cells, it migrates as a doublet. A number of putative N-linked glycosylation sites can be identified in the TRIL sequence that may explain the different banding patterns observed.

TRIL is a component of the TLR4 signaling complex
We next sought to determine whether TRIL plays any role in LPS signaling given the nature of the extracellular domain and enhanced expression following LPS stimulation. As mentioned above, wild-type U373 cells respond poorly to LPS in the absence of CD14 although they express basal levels of both TLR4 and MD2. We compared LPS-induced cytokine production in wild-type U373 and U373 cells stably overexpressing TRIL. As can be seen in Fig. 5, stable overexpression of TRIL markedly increased the induction of IL-6, IL-8, and RANTES in response to LPS (Fig. 5, A–C, respectively). Given this ability of TRIL to markedly enhance LPS signaling, we next sought to address whether TRIL can interact with components of the TLR4 receptor complex. HEK293 cells were transfected with Flag-tagged TLR4 and stimulated with LPS for various times (Fig. 6A). TLR4 was found to interact with endogenous TRIL (Fig. 6, lane 1) and the
TRIL associates with TLR4 and binds LPS. A, U373 cells were transfected with 4 μg of Flag-TLR4 for 24 h before stimulation with 100 ng/ml LPS for the indicated time points. Interaction with endogenous TRIL was assessed following immunoprecipitation (IP; gel 1, lanes 4–6). Transfection with a scrambled oligonucleotide (Fig. 7A, gel 3, lanes 4–6). This result also further confirms the specificity of the TRIL Ab. Silencing of TRIL affected the LPS signaling pathway by delaying the degradation of IκBα in response to LPS (Fig. 7A, gel 2, lanes 4–6). This result was also confirmed with an additional siRNA oligonucleotide. We also found that TRIL knockdown inhibited LPS-induced phosphorylation of p38 (Fig. 7A, gel 3, lanes 4–6). Transfection with a scrambled version of the oligonucleotide (Fig. 7B, Neg. Ctl, gel 1, lanes 1–3) did not affect endogenous levels of TRIL nor did it ablate LPS-induced readouts. Importantly, silencing of TRIL did not affect the TNF-α signaling pathway and IκBα degradation occurred as normal in all samples in response to TNF-α (Fig. 7B, gel 2, lanes 4–6). TRIL expression was also knocked down in the human monocytic cell line THP1. This was confirmed at the protein level (Fig. 7C, inset) and cytokine production was monitored. Silencing of TRIL in these cells led to a decrease in IL-6 and TNF-α production following LPS stimulation (Fig. 7, C and D). To ensure that there were no off-target affects, endogenous levels of TLR4 were tested and found to be unaffected by knockdown of TRIL (data not shown).

We also knocked down TRIL expression in human PBMC. Delivery of a FITC-tagged TRIL siRNA oligonucleotide was confirmed by FACS analysis and transfection efficiency as achieved by Amaxa electroporation was found to be close to 100% (data not shown). Knockdown was confirmed at the protein level (Fig. 8A, inset). In agreement with results mentioned above, silencing of TRIL affected LPS signaling by delaying IκBα degradation and inhibiting the phosphorylation of p38 (data not shown). At the

Knockdown of TRIL affects LPS signaling

We next performed siRNA knockdown experiments to determine whether TRIL functions in the TLR4 signaling pathway. TRIL knockdown was confirmed at the protein level in U373/CD14 cells (Fig. 7A, gel 1, lanes 4–6). This result also further confirms the specificity of the TRIL Ab. Silencing of TRIL affected the LPS signaling pathway by delaying the degradation of IκBα in response to LPS (Fig. 7A, gel 2, lanes 4–6). This result was also confirmed with an additional siRNA oligonucleotide. We also found that TRIL knockdown inhibited LPS-induced phosphorylation of p38 (Fig. 7A, gel 3, lanes 4–6). Transfection with a scrambled version of the oligonucleotide (Fig. 7B, Neg. Ctl, gel 1, lanes 1–3) did not affect endogenous levels of TRIL nor did it ablate LPS-induced readouts. Importantly, silencing of TRIL did not affect the TNF-α signaling pathway and IκBα degradation occurred as normal in all samples in response to TNF-α (Fig. 7B, gel 2, lanes 4–6). TRIL expression was also knocked down in the human monocytic cell line THP1. This was confirmed at the protein level (Fig. 7C, inset) and cytokine production was monitored. Silencing of TRIL in these cells led to a decrease in IL-6 and TNF-α production following LPS stimulation (Fig. 7, C and D). To ensure that there were no off-target affects, endogenous levels of TLR4 were tested and found to be unaffected by knockdown of TRIL (data not shown).

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cytokine level, knockdown of TRIL led to a decrease in the production of TNF-α and IL-6 in response to LPS (Fig. 8, A and B). We were unable to test the effect of knockdown of TRIL on the production of cytokines by TNF as a control, as TNF could only induce minimal responses in PBMC; however, knockdown of TRIL did not affect CpG-induced IL-6 production (Fig. 8C). Interestingly, knockdown of TRIL also inhibited the induction of TNF-α by Pam3Cys4 as shown in Fig. 8D. Finally, we also looked at an additional readout in murine mixed glial cells that produce RANTES in response to LPS stimulation. Approximately 50% knockdown was achieved as shown at the protein level in Fig. 8E (inset). Silencing of TRIL in these cells led to a decrease in RANTES production following LPS stimulation (Fig. 8E). Taken together, these results indicate that TRIL is a key part of the TLR4 complex and is important for LPS responses.

Discussion

TLR signaling in response to LPS requires a number of proteins that function together as part of a complex to optimally induce proinflammatory responses. We have identified a novel component of this complex, which we have named TRIL. This protein contains 13 LRRs, a fibronectin domain, and a putative transmembrane domain. The predicted domain organization of TRIL is consistent with that of Dolan et al. (17) who grouped TRIL (referred to as KIAA0644) with the TLRs following sequence analysis of a number of LRR-containing proteins. Furthermore, the consensus sequence of the TRIL LRRs broadly matches that of the TLR family assigned by Matsushita et al. (18) comprising LxxLxLxx-NxLxxLxxxxF/LxxLxx. Consistent with other LRR proteins, TRIL can be modeled with the characteristic curved solenoid shape, contains N- and C-terminal cysteine caps and a conserved asparagine backbone.

We have found that TRIL is expressed at high levels in the brain and particularly in astrocytes. Although the protein is expressed basally, we have also found that levels of TRIL increase upon LPS stimulation as shown for U373s, murine BMDM, and human PBMC. TRIL was also induced by LPS in vivo in mouse brain possibly by LPS penetrating the blood-brain barrier or via induction of cytokines such as IL-1. TRIL induction may therefore help prolong LPS signaling. An immediate question that arises relates to the role of TRIL in relation to CD14, given that both proteins possess LRR domains and can bind LPS. Indeed, there is significant structural similarity between the LRR regions of TRIL and CD14; however, at the amino acid level, the sequence identity is 17%. In addition, both proteins can enhance responses to LPS when stably expressed in U373 cells. TRIL may therefore be a functional homolog of CD14. CD14 exists as a GPI-anchored protein and also as soluble factor which functions to transfer LPS to the TLR4 receptor complex. Interestingly, we have observed a soluble form of TRIL in FCS. This form was identified by mass spectroscopic analysis; however, this is preliminary data and we are currently investigating this further. One possibility is that TRIL acts a substitute for CD14 in cells where the latter may be expressed at low levels. Alternatively, TRIL may be acting as part of the LPS transfer mechanism that exists between accessory molecules and TLR4. In our studies, LPS treatment of cells increased the association of TRIL with TLR4. In addition, we observed that TRIL could specifically associate with LPS. Additional experiments will determine whether both TRIL and CD14 are required for optimal TLR4 responses.

TRIL could also have a role similar to RP105, an LRR-containing protein with no apparent signaling domain. RP105 binds to MD1 to positively or negatively regulate TLR4 signaling in a cell-type-dependent manner (3, 11). We are currently exploring whether TRIL can bind to MD1 and/or MD2 as well as examining the effects of TRIL in additional cell types.

A number of accessory proteins have also been shown to be required for TLR2 signaling. CD14 and vitronectin facilitate the transfer of lipopeptides to the TLR2 receptor complex (19, 20). It therefore appears that multiple accessory proteins are required by both TLR2 and TLR4 for optimum responses to lipid-based agonists. We have preliminary data to suggest that TRIL could also be involved in TLR2 signaling since knockdown of TRIL affected Pam3Cys4-induced TNF-α production. Further work will enable us to determine whether TRIL influences additional TLR signaling pathways.

The relatively high expression of TRIL in the brain and the in vivo induction of TRIL by LPS in this tissue is also of interest. It appears that inflammation following brain injury can have a protective or destructive role depending on which cytokines are produced (21, 22). TLR4-deficient mice have been examined following experimentally induced stroke and were found to have lower infarct volumes and lower expression of proteins associated with brain damage such as IRF-1, NO synthase, cyclooxygenase 2, and matrix metalloproteinase 9 (23, 24). TLR4 has also been shown to mediate innate immunity to bacterial infection in the CNS along with TLR2 (25). Furthermore, TLR4 has been shown to modulate...
hippocampal neurogenesis with TLR4-defective mice showing enhanced neuronal proliferation and differentiation (26). TRIL may participate with TLR4 in these various responses. Given their structural similarity, there may also be a relationship between TRIL and the NOGO and LINGO family of proteins, which similar to TLR8 are inhibitors of neurite outgrowth (27, 28). Neuroinflammation has also been associated with neurodegenerative diseases such as Alzheimer’s disease (29, 30). Interestingly, we have preliminary data which suggest that TRIL expression is increased in the brains of patients with Alzheimer’s disease. Further studies will need to be conducted to determine whether TRIL plays a role in the pathogenesis of this condition.

In conclusion, we have identified a novel component of the TLR4 receptor complex which could present a new therapeutic target to limit dysregulated TLR4 signaling and may have particular relevance in the context of neuroinflammation.

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Disclosures

The authors have no financial conflict of interest.

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