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# Identification and characterization of a unique leucine-rich repeat protein (LRRC33) that inhibits Toll-like receptor-mediated NF-κB activation

Jingyi Liu [a,b], Zhujun Zhang [a], Limin Chai [a], Yongzhe Che [a], Siping Min [a], Rongcun Yang [a,*]

[a] Department of Immunology, Nankai University School of Medicine, Nankai University, Nankai District, Weijing Road No. 94, Tianjin 300071, China
[b] Markey Cancer Center, The University of Kentucky, College of Medicine, Lexington, KY 40506, USA

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ABSTRACT

Toll-like receptors (TLRs) are important initiators in innate immune responses against pathogenic microbes such as viruses, intracellular bacteria or parasites. Although the innate immune system is designed to fight infectious pathogens, excessive activation of TLR signaling may lead to unwarranted inflammation with hazardous outcomes. Mechanisms of restraining excessive inflammation and controlling homeostasis for innate immunity are the focus of intense study. Here we showed that LRRC33, a novel member of leucine-rich repeat (LRR) protein family, plays a critical role in desensitizing TLR signaling. LRRC33 is TLR homolog that contains 17 putative LRRs in the extracellular region but lacks a cytoplasmic Toll/IL-1 receptor (TIR) domain. Expression of LRRC33 appears to be ubiquitous with high level of expression found in bone marrow, thymus, liver, lung, intestine and spleen. The LRRs of LRRC33 is required for the interaction with TLR and its inhibitory effect on NF-κB and AP-1 activation as well as cytokine production. Our study sheds new insight into the TLR signaling and inflammatory response in development and human diseases.



## 1. Introduction

The human body is under constant attack by various microbial pathogens. To cope with these threats, an elaborate system of innate receptors has evolved to guard the integrity of tissues, lumen, fluids, and cells. The toll-like receptor (TLR) family of innate receptors sense invading microbial pathogens and play crucial roles in the activation of innate and adaptive immunity [1,2]. TLRs are the most characterized pattern recognition receptors (PRRs), which detect conserved structures of pathogens called pathogen-associated molecular patterns (PAMPs) as well as damage-associated molecular patterns (DAMPs) from endogenous molecules [3]. TLRs consist of three domains: (1) extracellular ectodomain containing leucine-rich repeats, which have avidity for PAMPs; (2) a transmembrane segment; and (3) an intracellular Toll-interleukin (IL)-1 receptor (TIR) domain, which interacts with downstream adapters or signaling proteins. Twelve TLRs have been identified and they form homo- or heterodimers in recognizing various PAMPs in mammals.

Binding of ligands followed by dimerization of TLRs recruits TIR domain-containing adapter proteins such as myeloid differentiation factor 88 (MyD88), TIR-domain-containing adaptor protein-inducing IFN-β (TRIF), TIR-associated protein (TIRAP), and TRIF-related adaptor molecule (TRAM). Individual TLRs recruit distinctive combinations of these adapter molecules to elicit specific immune responses tailored to infectious pathogens [4]. MyD88 is recruited by almost all TLRs and associates with IL-1R-associated kinases (IRAKs) and TNFR-associated factor 6 (TRAF6), resulting in activation of canonical IKK and nuclear factor (NF)-κB. Activation of TRAF6 also results in the activation of MAPK, leading to the phosphorylaton of JNK and nuclear translocation of AP-1. Activation of both NF-κB and JNK pathways causes a robust production of pro-inflammatory cytokines that are crucial for host defensive responses against pathogens. However, aberrant over-activation of TLR signaling is responsible for the pathogenesis of autoimmune, chronic inflammatory and infectious diseases [5]. Several desensitization mechanisms have been identified to restrain or terminate TLR activation [6]. These include: (1) dissociation of adaptor complexes; (2) degradation of signal proteins; and (3) transcriptional regulation. These negative regulations of TLR signaling are important in controlling the homeostasis of innate immune responses.

The leucine-rich repeats (LRR)-containing domain is evolutionarily conserved in many proteins associated with innate immunity in plants, invertebrates and vertebrates [7,8]. LRRs enhance the formation of receptor/co-receptor complexes among LRR proteins. For example, TLRs and NOD-like receptors (NLRs), interacting through their LRR domain, determine molecular patterns from a structurally diverse set of bacterial, fungal, parasite and viral-derived components in the innate immune response. LRR proteins consist

* Corresponding author. Fax: +86 859 257 6030.
*E-mail address:* rongcuny@yahoo.com (R. Yang).

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of 2–45 leucine-rich repeats, with each repeat about 20–30 residues long. These LRR domains adopt a horseshoe shape structurally, with the concave face consisting of parallel β-strands and the convex face representing a more variable region of secondary structures including helices [9]. Aside from TLRs and NLRs, there are about 375 human LRR proteins have been identified. Although mutations/defects are found in more than 30 LRR proteins that are associated with human diseases, functions of many of these LRR proteins remain unclear [10]. In this study, we identified a novel LRR protein, LRRC33 (leucine-rich repeat containing 33), that negatively regulates TLR signaling. LRRC33 is TLR homolog that lacks a TIR domain, but instead contains a short cytoplasmic tail of 21 amino acids. LRRC33 appears to be ubiquitously expressed, but with high levels of expression found in bone marrow, thymus, liver, lung, intestine and spleen. LRRC33 inhibited NF-κB activity and suppressed proinflammatory cytokines production induced by TLR signaling. The inhibitory function is mediated by its extracellular LRRs and appears to be general and uniform for various TLRs. Thus, LRRC33 represents the first identified LRR-containing protein that plays a critical role in desensitizing TLR-mediated innate immune responses.

## 2. Materials and methods

### 2.1. Cell culture

HEK293T and U937 cells were purchased from ATCC and maintained in DMEM and RPMI-1640 medium (Invitrogen), respectively, supplemented with 10% FBS (GIBCO Life Technologies)100 units/ml penicillin and streptomycin. Human monocyte-derived dendritic cells (DCs) were generated from monocytes isolated from peripheral blood of healthy donors. Monocytes were purified by density-gradient centrifugation on Ficoll-Paque, followed by plastic adherence, and were cultured for 5–7 days in 6-well tissue culture plates at $1 \times 10^6$/ml in RPMI-1640 medium supplemented with 10% FBS, IL-4, and recombinant human GM-CSF (20 ng/ml). After 5 days of culture, the outcoming population represented typical immature DC (iDCs). For the generation of mature DCs (mDCs), iDCs were cultured for additional 48 h with addition of 100 ng/ml lipopolysaccharide (LPS).

### 2.2. Plasmids

We first obtained the leucine-rich repeat of TLRs as a query protein sequence and performed a BLAST search of the human genome. This computational screening identified a potential transmembrane molecule LRRC33. Human LRRC33 cDNA clone (MGC:50789) was purchased from ATCC and the coding sequence of LRRC33 was amplified by PCR. Deletion mutants that lack extracellular, intracellular or LRR domain were constructed by PCR as described previously [11]. The PCR products were then ligated into the pcDNA3.1-V5 mammalian expression vector (Invitrogen). All sequences were verified by DNA sequencing. Primer sequences are available upon request.

### 2.3. RT-PCR

Total RNA was extracted from kidney, spleen, thymus, lung, liver and heart of adult mice. First-strand cDNA was generated from total RNA using oligo-dT primers and reverse transcriptase (TAKARA). Human LRRC33 cDNA was further amplified using the following primers: forward primer, 5′-GCCGGTACCATGCTCATTCTGGA TGCTAACC-3′ and reverse primer, 5′-GAAGCGGCCGCTTAGAAGAG ACCAGTGTCCACCTGT-3′. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control. PCR products were visualized on 1.0% (wt/vol) agarose gel.

### 2.4. In situ hybridization

Oligonucleotide probes were synthesized from published genomic sequence of human LRRC33 mRNA (GenBank Accession#: NM_198565) and were labeled with Digoxin (DIG). These probes recognize sequences conserved in the human LRRC33 mRNA sequence. The sense probes were used as negative control.

Mouse tissues were formaldehyde-fixed and paraffin-embedded materials following standard procedures. Briefly, 5 μm sections were deparaffinized, rehydrated with ethanol, and washed with 0.1% DEPC-treated water three times for 5 min. *In situ* hybridization for LRRC33 mRNA was done according to the manufacturer's protocol (TBD). Staining was completed with the Diaminobenzidine Staining Kit. All sections were counterstained with Mayer's hematoxylin and Canada balsum mount.

### 2.5. Transfection and reporter assays

HEK293T cells were transiently transfected with pNF-κB-SEAP (secreted alkaline phosphatase) (BD Clontech), pcDNA3.1-V5-LRRC33 (full length or different deletion mutants) with or without plasmid encoding TLR 2–4 using Lipofectamine 2000 (Invitrogen) in a 24-well plate. Empty vector (pcDNA3.1) was used as a negative control. HEK293T cells were grown in DMEM plus 2% FBS. After 24 h, cells were stimulated using 2 μg/ml peptidoglycan (PGN), 10 μg/ml poly I:C, 100 ng/ml lipopolysaccharide (LPS). The supernatants were collected and analyzed by the chemiluminescence secreted alkaline phosphatase assay according to the manufacturer's protocol (BD Clontech).

### 2.6. Immunofluorescence analysis

HEK293T cells were seeded on a coverslip in a 6-well plate. After 24 h of transfection with pcDNA3.1-V5-LRRC33, cells were rinsed twice with PBS and then fixed in 4% paraformaldehyde at 4 °C for 5 min, permeabilized for 5 min in PBS containing 0.1% Triton X-100, blocked with 10% goat serum in PBS for 10 min at room temperature, and then incubated with anti-V5-FITC antibody. Coverslips were mounted in slide. Images were collected by confocal microscopy (Fluoview FV300, Olympus Optical Corp). To examine the nuclear translocation of the p65, cells were fixed as described above. The cellular localization of p65 was analyzed with a specific antibody (1:200 dilutions for 1 h) from Santa Cruz Biotechnology. Immunofluorescence staining was visualized by a cy3-conjugated goat anti-rabbit antibody (Millipore) under fluorescence microscope.

### 2.7. Western Blotting analysis

Western Blotting was performed as described previously [12]. Hybridizations with primary antibodies were carried out for one hour at room temperature in blocking buffer. The protein–antibody complexes were detected using peroxidase-conjugated secondary antibodies (Boehringer Mannheim) and enhanced chemiluminescence (Amersham). Antibodies against Flag, V5 and β-actin were purchased from Abmart and Invitrogen, respectively.

### 2.8. ELISA

After 24 h of transfection, HEK293T cells were stimulated with TLR ligands for additional 24 h. Cell-free supernatants were collected and assayed for IL-8 production. Commercial enzyme-linked immunosorbent assay (ELISA) kits were used for quantitation of IL-

8 and TNFα (R&D Systems). The optical density at 450 nm of each sample was measured with a SpectraMax 190 ELISA plate reader. Cytokine levels were quantified from two to three titrations with standard curves and expressed as the number of picograms per milliliter.

## 3. Results

### 3.1. Tissue distributions of LRRC33

Using either SMART or Pfam with several transmembrane-prediction and motif-detection programs [13,14], we identified a novel transmembrane molecule LRRC33, which resides in chromosome 3q29 (Fig. 1A). This gene contains 3 exons and encodes a protein of 692 amino acids, with a putative signal peptide (SP) for cell membrane translocation, an extracellular domain, a transmembrane segment (TM), and a short intracellular domain. A putative protein kinase C (PKC) phosphorylation site was found at the intracellular domain of LRRC33. Because the extracellular region of LRRC33 contains 17 LRRs, along with a pattern of juxtamembrane cysteines that is conserved among Toll and TLR family, LRRC33 is thus categorized into LRR_Tollkin group (containing a cytoplasmic TIR domain or clustering with the Toll proteins) [7,8]. Human LRRC33 shares more than 80% amino acid sequence homology with a murine protein (data no shown), suggesting that LRRC33 is evolutionarily conserved.

To examine the tissue distributions of LRRC33, we measured the levels of LRRC33 mRNA in different tissues and organs from BALB/c mice. Although it is ubiquitous expressed in all tissues examined, LRRC33 was highly expressed in bone marrow, thymus, liver, lung, intestine, and spleen (Fig. 1B). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH), serving as a control, did not reveal differences among these tissues. Using LRRC33 mRNA specific probes for *in situ* hybridization, we found that LRRC33 mRNA was expressed in human renal proximal tubular epithelial cells (HRPTEpiC) and human kidney tissues (left two panels, Fig. 1C). However, control experiment using LRRC33 sense probe was negative, suggesting that LRRC33 mRNA expression in HRPTEpiC and human kidney tissue was specific. We further examined the expression of LRRC33 mRNA in adult tissues from C57BL/6 mice. In line with RT-PCR results, we found that LRRC33 mRNA was highly expressed in the thymus, liver, lung, kidney, spleen and heart by *in situ* hybridization (right panels, Fig. 1C). Control incubations with LRRC33 sense probe were negative (data not shown). These results suggest that LRRC33 plays a critical role in the development and maintenance of homeostasis of these organs.

We also analyzed LRRC33 mRNA expression in different cell lines. LRRC33 was highly expressed in a human myelomonocytic leukemia cell line U937 (Fig. 1D). LRRC33 mRNA also expressed in monocytes; however, its level gradually decreased during GM-CSF-mediated cellular differentiation from monocytes to matured dendritic cells (mDcs); these data imply that LRRC33 is important

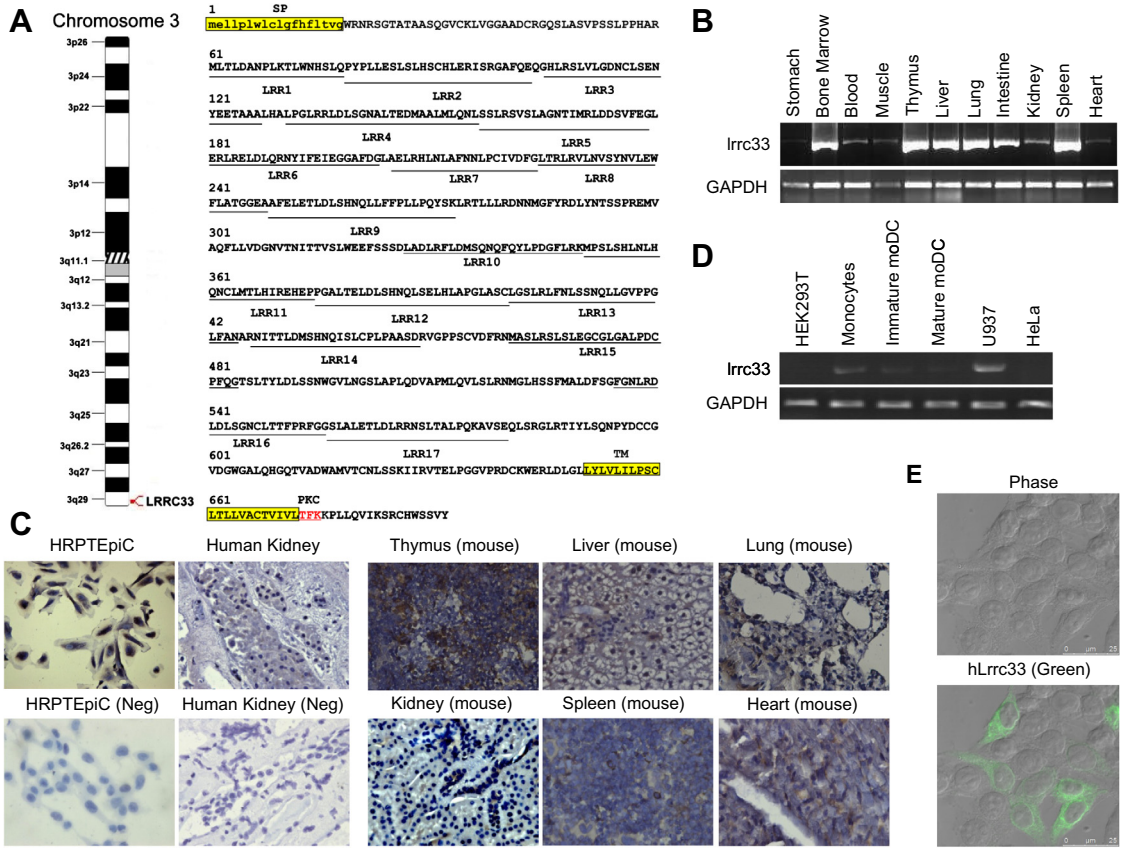


**Fig. 1.** Structure and expression patterns of LRRC33. (A) Ideogram showing lrrc33 resides in chromosome 3q29 (left panel). Schematic diagram showing the primary sequence and structure of LRRC33 (right panel). The signal peptide and the transmembrane segment are highlighted yellow. LRRs are numbered sequentially from 1 to 17 as indicated and underlined. The putative protein kinase PKC site is indicated in red. (A) Expression of Lrrc33 mRNA in tissues and organs from BALB/c mice was determined by RT-PCR analysis. GAPDH was used as an internal control. (A) Expression of Lrrc33 mRNA is examined by *in situ* hybridization using the anti-sense probe of Lrrc33 in human cell line, human kidney specimens, and mouse tissues. (B) Expression of lrrc33 mRNA in various cells was examined by RT-PCR analysis. GAPDH was used as an internal control. (C) HEK293T cells were transfected with pcDNA3.1-V5-Lrrc33. Cells were stained by FITC labeled anti-V5 antibody. Images were captured by confocal microscopy. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

in cellular differentiation and stem-cell maintenance. LRRC33 mRNA was absent in differentiated epithelial HEK293T and HeLa cells (Fig. 1D). When human LRRC33 was ectopic expressed in HEK293T cells, we found that LRRC33 was mainly localized in the cell membrane (Fig. 1E). Taken together, these results indicate that LRRC33 is a novel LRR-containing transmembrane protein that has unique tissue and cell line expression pattern.

### 3.2. LRRC33 interacts with TLRs and inhibits TLR-mediated NF-κB activation

To understand the relationship of LRRC33 within the LRR protein superfamily, a phylogenetic tree analysis of LRRC33 together with known LRR members was performed using the ClustalW and ClustalX programs (Fig. 2A) [7]. As presented in the phylogenetic tree, LRRC33 has the shortest distance to TLR11 and TLR12. Tertiary structure analysis also showed a high similarity of LRRC33 with other TLRs (data not shown). However, the intracellular region of LRRC33 contains a short cytoplasmic tail with 21 amino acids and lacks a TIR domain, which is highly conserved across the TLRs as well as in the receptors for IL-1 and IL-18. Thus, LRRC33 may have genetic similarities but different biological functions with other TLRs.

To investigate the potential function of LRRC33 and its relationship with TLRs, we co-expressed LRRC33 with TLR2, TLR3, TLR4, or TLR9 or Myd88 in HEK293T cells and performed a co-immunoprecipitation experiment (Fig. 2B). After immunoprecipitation with LRRC33, we identified the associated TLRs. The interaction between LRRC33 and TLRs is specific, as the control IgG, that failed to immunoprecipitated LRRC33, showed no association with TLRs. Notably, in contrast with TLRs, LRRC33 did not interact with MyD88 (Fig. 2C). Strikingly, expression of LRRC33 inhibited TLR-mediated basal NF-κB activation, and this inhibitory effect was significantly enhanced when cells were stimulated with PGN and LPS, ligands for TLR2 and TLR4, respectively (Fig. 2D). Consistent with the inhibitory effect of LRRC33, production and secretion of IL-8, a major cytokine induced by the TLR-mediated NF-κB activation, was greatly reduced when TLRs were co-expressed with LRRC33. To further confirm the inhibition of NF-κB, TLR2 was co-expressed with either vector or LRRC33 in HEK293T cells followed by PGN stimulation. We found that p65 (subunit of NF-κB) was shuttled inside the nucleus in cells expressed TLR2. However, co-expression of LRRC33 completely blocked the nuclear translocation of p65 (Fig. 2E). Together, these data clearly indicate that LRRC33 interacts with TLRs and inhibits TLR-mediated NF-κB activation.

### 3.3. LRRs of LRRC33 are responsible for the interaction with TLRs and the inhibition of NF-κB activation

The N-terminal LRRC33 contains 17 LRRs and its C-terminus has a short intracellular domain containing 21 amino acids (Fig. 3A). To identify the region that is responsible for the LRRC33 interaction

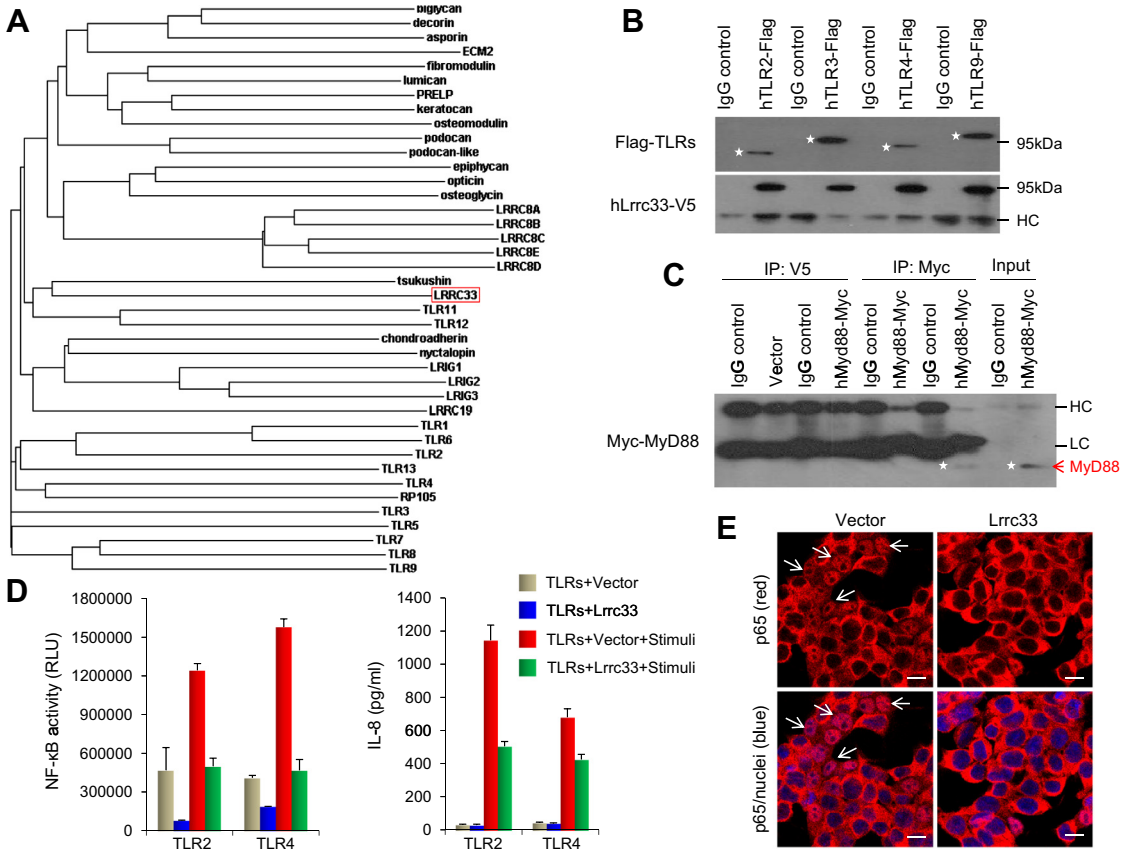


**Fig. 2.** LRRC33 interact with TLRs and inhibit TLR-mediated NF-κB activation. (A) Phylogenetic tree analysis indicates the relationship of LRRC33 with other LRR-containing proteins. (B) V5-tagged Lrrc33 was co-expressed Flag-tagged TLR2, 3, 4, or 9 in HEK293T cells. After 24 h, cells were stimulated with PGN, polyI:C, LPS or bacterial DNA for additional 24 h. Lysates were immunoprecipitated with V5 antibody, the associated TLRs was detected by Western Blotting using Flag antibody. (C) V5-tagged Lrrc33 was co-expressed Myc-tagged MyD88 in HEK293 cells. After 48 h, lysates were immunoprecipitated with either V5 or Myc antibody. The associated MyD88 was examined by Western blotting using Myc antibody. (D) TLR2 or TLR 4 was co-expressed with either vector or Lrrc33 along with NF-κB reporter in HEK293T cells. After stimulated with PGN and LPS for 24 h, NF-κB reporter alkaline phosphatase activities and IL-8 production were measured. (E) TLR2 was co-expressed with either vector or Lrrc33 in HEK293T cells. After cells were stimulated with PGN for 24 h, the cellular localization of p65 (red) was examined by immunofluorescent staining. Nuclei are stained with Dapi (blue) (scale bar = 15 μM). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

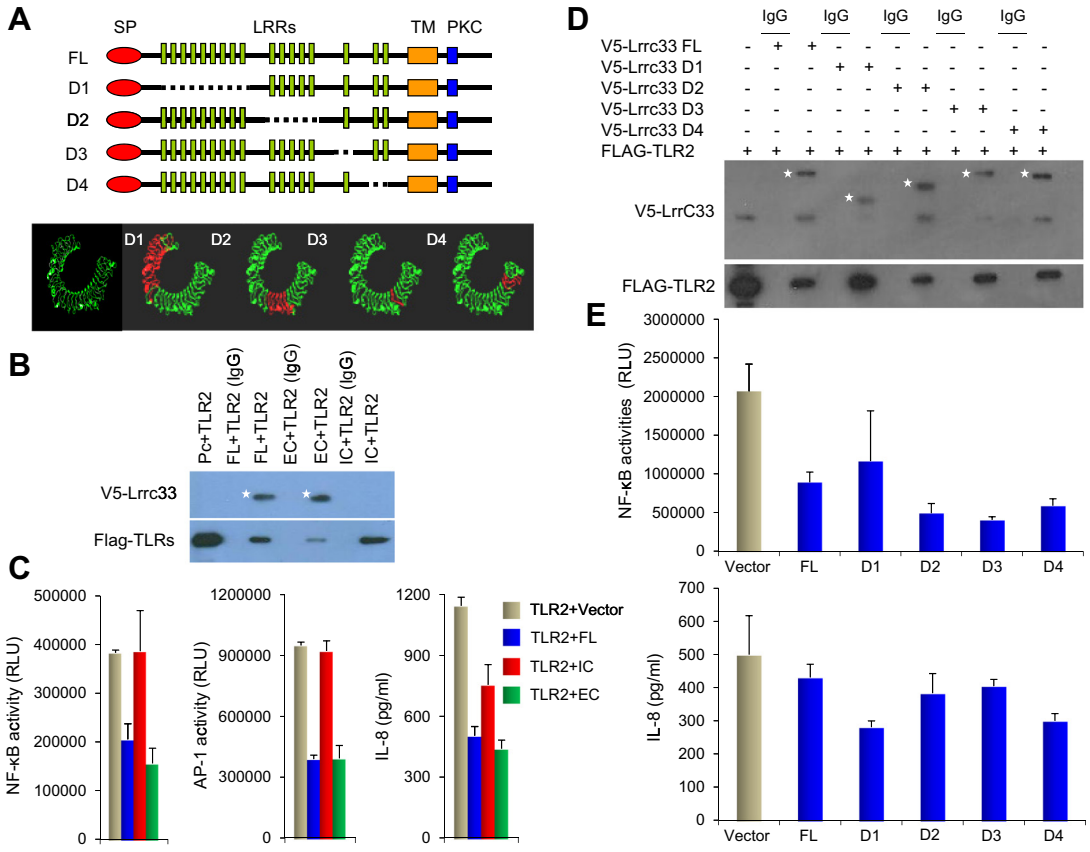


**Fig. 3.** LRRs of LRRC33 are responsible for the interaction with TLRs and the inhibition of NF-κB activation. (A) Schematic diagram showing the structure of LRRC33 and deletion constructs used (top panel). A predicted tertiary structure of LRRC33 (composed using PyMOL Molecular Graphics System, http://www.pymol.org) showing position of deleted LRRs in different deletion mutants (bottom panel). (B) V5-tagged full-length (FL), extracellular domain (EC), or intracellular domain (IC) of LRRC33 was co-expressed with Flag-tagged TLR2 in HEK293T cells. Lysates were immunoprecipitated with Flag antibody, bound LRRC33 was examined by Western blotting using V5 antibody. (C) TLR2 was co-expressed with either vector, FL, EC, or IC of LRRC33 along with NF-κB reporter in HEK293T cells. After stimulated with PGN, the NF-κB reporter activities and IL-8 production in the culture medium were analyzed. (D) Flag-tagged TLR2 was co-expressed with FL or different LRR deletion mutants of LRRC33 in HEK293T cells. Lysates were immunoprecipitated with Flag antibody, and bound LRRC33 was detected by Western Blotting using V5 antibody. (E) TLR2 was co-expressed with either FL or different LRR deletion mutants of LRRC33 in HEK293T cells. After stimulated with PGN for 24 h, the NF-κB reporter activities and IL-8 production in the culture medium were analyzed.

with TLRs, we generated LRRC33 domain-deletion mutants and co-expressed them with TLR2 in HEK293T cells (Fig. 3B). Immunoprecipitation of TLR2 revealed the association with full length LRRC33. We found that the extracellular domain of LRRC33 (EC) retained the ability to interact with TLR2. However, the intracellular domain of LRRC33 (IC) was incapable of interacting with TLR2. These results indicate that the N-terminal region of LRRC33 is required for the interaction of LRRC33 with TLR2. Consistent with this observation, EC of LRRC33 maintained, whereas IC of LRRC33 lost, the ability to inhibit NF-κB activation as well as IL-8 production in cells (Fig. 3C). Notably, LRRC33 also inhibited JNK activation as exemplified by the suppression of AP-1 activity. EC but not IC retained the ability to inhibit AP-1 activity (Fig. 3C). These results indicate that the interaction of LRRC33 with TLRs is critical in restraining inflammatory response.

To further define the region required for the interaction of LRRC33 with TLRs, we systematically deleted LRRs in LRRC33 by constructing several deletion mutants, these include D1 (delete 1–9 LRRs), D2 (delete 10–14 LRRs), D3 (delete 15 LRR), and D4 (delete 16–17 LRRs) (Fig. 3A). When these deletion mutants of LRRC33 were co-expressed with TLR2, immunoprecipitation of TLR2 was found to associate with the full-length structure, as well as with each of the deletion mutants of LRRC33 (Fig. 3D). These data suggest that these LRRs have no structural specificity in their association with TLRs. In addition, these deletion mutants maintained, at least partially, the ability to inhibit NF-κB activation and the suppression of IL-8 production (Fig. 3E). Together, these data indicate that LRRs of LRRC33 interact with TLRs and that this interaction is required for the suppressive effect of LRRC33 in inhibiting NF-κB activation and IL-8 production.

### 3.4. Knockdown of LRRC33 increases NF-κB and JNK activities

To further establish the causal relationship between LRRC33 and NF-κB/AP-1 activation, we knocked down the expression of endogenous LRRC33 in the dendritic cells (DCs). As expected, ectopic expression of LRRC33 suppressed LPS-induced NF-κB activation (left panel, Fig. 4A). However, knockdown of endogenous LRRC33 greatly increased NF-κB activation even in the absence of LPS stimulation in DCs. Further stimulation with LPS slightly enhanced NF-κB activation. Consistent with this finding, knockdown of endogenous LRRC33 increased the production of TNFα, a major cytokine secreted by DCs (right panel, Fig. 4A). The secretion of TNFα was drastically elevated by LPS stimulation. Interestingly, knockdown of LRRC33 also induced the duration of LPS-mediated JNK and p38 activation in DCs (Fig. 4B).

## 4. Discussion

In this study, we demonstrated that LRRC33 interacts with TLRs to inhibit TLR-mediated NF-κB activation (Fig. 4C). Our study has

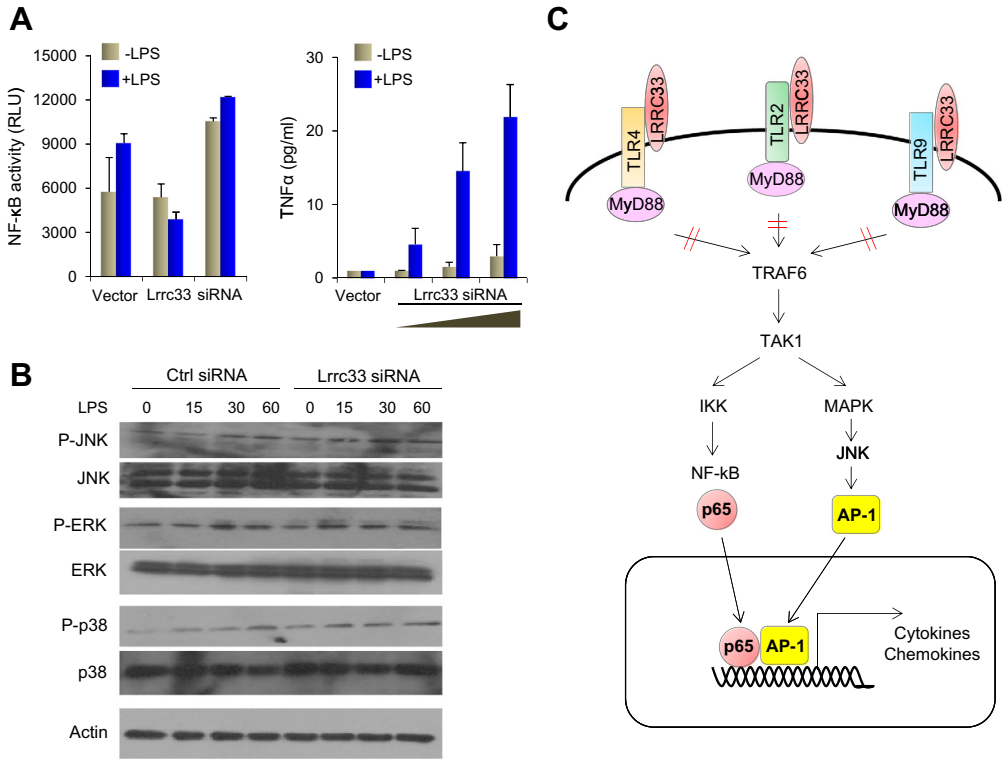


**Fig. 4.** LRRC33 is negative regulator of TLR signaling pathway. (A) Dendritic cells (DC) were established from human monocytes isolated from the peripheral blood of healthy individuals as described in Section 2. DCs were either ectopic expressed LRRC33 or siRNA against LRRC33 (knocked down endogenous LRRC33) along with NF-κB reporter, the NF-κB reporter activities was measured from culture medium (left panel). DCs were also expressed with increasing dosage of siRNA against LRRC33. After stimulated with or without LPS, the production of TNFα was measured by ELISA. (B) DCs were expressed with control or LRRC33 siRNA followed by LPS stimulation for different time intervals. Phosphorylation of JNK, p38 and ERK was analyzed by Western blotting. (C) A proposed model to illustrate LRRC33 interaction with TLRs and the suppression of TLR-mediating NF-κB and JNK activation.

revealed several novel insights into the regulation of the innate immune response. First, LRRC33 represents a general negative regulator that modulates the activation of TLR signaling. Activation of TLR signaling by pathogenic products elicits inflammatory responses, host-defense pathways and adaptive immunity. However, excessive inflammation causes cell and tissue damage, leading to many acute and chronic diseases. Coincident with activation of TLR signaling, diverse anti-inflammatory pathways and mechanisms are used, evolutionarily developed, to restrain excessive inflammation until tissue repair is complete. Most studies are focused on the identification of signaling components immediately downstream to the TLRs, and little is known of the regulation of TLRs themselves in desensitizing inflammatory response. We found that LRRC33 is a new TLR-interacting partner that restrains the inflammatory signaling. Similar to TLRs, LRRC33 has a conserved extracellular LRR domain and a TLR-like pattern of juxtamembrane cysteines. Different from TLRs, LRRC33 contains a short cytoplasmic tail and lacks a TIR domain. LRRC33 is ubiquitously expressed in most tissues with preferential high expression in bone marrow, thymus, liver, lung, intestine and spleen. LRRC33 interacted with the majority of TLRs examined and inhibited NF-κB activation and cytokine production mediated by various TLR ligands, such as PGN (for TLR2), LPS (for TLR4), and bacterial DNA (for TLR9). Our results suggest that LRRC33 plays a widespread and important role in controlling the homeostasis of innate immunity.

Second, our study indicates that the function of LRRC33 is dependent on its interaction with TLRs. LRRC33 contains 17 LRRs in the extracellular domain. Interaction of LRRC33 with TLRs is mediated through the extracellular domain of the LRRs. Deletion of extracellular domain abolishes, whereas remove of cytoplasmic tail retains, this interaction, and thus results in the loss of inhibitory effect on TLR-mediated NF-κB activation and the consequent IL-8 production. We have systematically deleted these 17 LRRs of LRRC33; surprisingly, remove several adjacent LRRs did not compromise the association of LRRC33 with TLRs, suggesting that these LRRs have a redundant role in maintenance of the tight association between LRRC33 and TLRs.

Third, our study indicates that LRRC33 not only inhibits TLR-mediated NF-κB activation, but also suppresses JNK activation (Fig. 4C). Most cytokines and chemokines are predominantly regulated by transcription factors NF-κB and AP-1/JNK during inflammation, because multiple NF-κB and AP-1 binding sites are commonly found in the promoters of inflammatory cytokines and chemokines [4,15,16]. We found that expression of LRRC33 inhibited the activation of both NF-κB and AP-1/JNK pathways, whereas knockdown of LRRC33 elevated these two signaling pathways in controlling the expression of IL-8. These results support our notion that LRRC33 is a major and general regulator in restraining inflammation in innate immunity.

In summary, our study highlights the importance of LRRC33 in desensitizing TLR-mediated immune response. To our knowledge, LRRC33 represents the first identified LRR-containing molecule in the negative regulation of TLR signaling. Our study sheds a new insight into the understanding of TLR signaling and inflammatory response in development and human diseases.

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