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Toll/IL-1 domain-containing adaptor inducing IFN-β (TRIF) mediates innate immune responses in murine peritoneal mesothelial cells through TLR3 and TLR4 stimulation

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ABSTRACT

Mesothelial cells are composed of monolayer of the entire surface of serosal cavities including pleural, pericardial, and peritoneal cavity. Although mesothelial cells are known to express multiple Toll-like receptors (TLRs) which contribute to trigger innate immune responses against infections, the precise molecular mechanism remains still unclear. In the present study, we investigated the role of Toll/IL-1 domain-containing adaptor inducing IFN-β (TRIF), one of the two major TLRs–adaptor molecules, on innate immune response induced by TLR3 and TLR4 stimulation in murine peritoneal mesothelial cells (PMCs). TRIF was strongly expressed in PMCs and its deficiency led to impaired production of cytokines and chemokines by poly I:C and LPS in the cells. Activation of NF-κB or MAPKs through poly I:C and LPS stimulation was reduced in TRIF-deficient PMCs as compared to the WT cells. TRIF was also necessary for optimal nitric oxide synthesis and gene expression of inducible nitric oxide synthase (iNOS) and IFN-β in PMCs in response to poly I:C and LPS. Furthermore, both *Escherichia coli* and *Pseudomonas aeruginosa* induced high level of IL-6, CXCL1, and CCL2 production in PMCs, which was significantly impaired by TRIF deficiency. These results demonstrated that TRIF is required for optimal activation of innate immune responses in mesothelial cells against microbial infections.

1. Introduction

Peritonitis is an inflammation of the peritoneum which lines the inner wall of the abdomen and covers and supports most of abdominal organs. The two major types of life-threatening peritonitis are: (i) primary spontaneous peritonitis, an infection that develops in the peritoneum [1]; and (ii) secondary peritonitis, which usually develops when an injury or infection in the abdominal cavity allows infectious organisms into the peritoneum [2,3].

Mesothelial cells are monolayer of specialized cells which extends over the entire surface of the three serosal cavity (pleural, pericardial, and peritoneal) and the organs contained within these cavities [4]. The predominant role of the mesothelium is to act as a protective barrier against physical damage and invading pathogens. Recent studies have shown many different roles of mesothelial cells, which include antigen presentation, tumor cell adhesion and growth, initiation and resolution of inflammation, and tissue repair [4]. Under physiologic conditions, the mesothelial cells secrete numerous glycosaminoglycans, proteoglycans, and phospholipids that constitute a glycocalyx surrounding the cells and provide a protective barrier against abrasion and a slippery non-adhesive surface for intracoelomic movement [5]. When faced with an infection, mesothelial cells express specific surface markers that enable them to promote the migration of neutrophils, to interact with extracellular matrix proteins, to present antigens to immune cells, and to produce biologically important molecules such as proinflammatory cytokines, chemokines, and nitric oxide (NO) [6,7].

Abbreviations: TLR, Toll-like receptor; NLR, Nod-like receptor; iNOS, inducible NO synthase; PMC, peritoneal mesothelial cell; poly(I:C), polyinosinic–polycytidylic acid; TIR, Toll/IL-1 receptor; TIRAP, TIR domain-containing adapter protein; TRIF, TIR domain-containing adapter inducing IFN-β; JNK, c-Jun N-terminal kinase; IRF, IFN regulatory factor; MAP, mitogen-activated protein.

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Toll-like receptors (TLRs) are type I transmembrane proteins and comprise an ectodomain, which contains leucine-rich repeats that mediate the recognition of pathogen-associated molecular patterns (PAMPs); a transmembrane region; and a cytosolic Toll/IL-1 receptor (TIR) domain that activates downstream signaling pathways [8]. Recognition of microbial components (e.g. LPS, lipoprotein, flagellin, and nucleic acids) by TLRs initiates inflammatory signal cascades via two distinct pathways: (i) myeloid differentiation primary response 88 (MyD88)-dependent; and (ii) Toll/IL-1 receptor domain-containing adaptor inducing IFN-beta (TRIF)-dependent pathways. MyD88 is an adapter molecule that triggers inflammatory signals commonly utilized by various TLRs with the exception of TLR3. Recruitment of MyD88 leads to the activation of nuclear factor-kappa B (NF-κB) and mitogen activated protein kinases (MAPKs) to regulate the pro-inflammatory cytokines genes. On the other hand, TRIF is recruited to TLR3 and TLR4 and activates an alternative pathway that triggers the activation of NF-κB, MAPKs, and IRF3. These signaling cascades lead to the production of proinflammatory cytokines, type I interferons (IFNs), chemokines, and antimicrobial peptides to remove the invading pathogens [9,10].

Mesothelial cells have been known to express multiple TLRs [11,12]. TLR1–6 are expressed in murine peritoneal mesothelial cells (PMCs) [12] and each ligand induce the production of chemokines such as CXCL1 and CCL2 [13]. In human PMCs, TLR3 is functionally expressed and its agonist poly I:C leads to the production of IL-6, CCL2, and CCL5 [14,15]. The role of TLR4 is somewhat controversial. Colmont et al. demonstrated that TLR4 was not expressed in primary cultured human PMCs and LPS also did not induce any inflammatory molecules tested [11], whereas it was strongly expressed in a cell line (HMrSV5 cells) and LPS-induced autophagy was dependent on TLR4 in the cells [16,17]. Bacterial and viral peritonitis can occur in patients with intestinal perforation or undergoing continuous ambulatory peritoneal dialysis (CAPD) [18,19]. Corona virus and herpes simplex virus can cause peritonitis in IFN-γ-deficient mice and diabetics patient [20,21]. In addition to its role in response to viral infection, TLR3 is involved in immune response to bacterial infection-induced tissue damage which is one of the major cause of peritoneal inflammation [22–24]. Therefore, TLR3 and TLR4-mediated signal pathways are considered to play central roles in activation of host immune system in response to various pathogenic microbes in PMC. Although TRIF is the only adaptor molecule mediating both TLR3 and TLR4 signaling pathways, investigations on the detailed function of TRIF in mesothelial cells have not been available. To address this limitation, in the current study, we characterized molecular mechanisms of TRIF-mediated innate immune response in murine PMCs.

2. Methods

2.1. Reagents and bacterial strains

Polyinosinic–polycytidylic acid (poly(I:C)) and ultrapure LPS were purchased from InvivoGen (San Diego, CA). *Escherichia coli* O111:B4 and *Pseudomonas aeruginosa* were grown overnight in Luria Bertoni (LB) broth grown anaerobic conditions at 37 °C. The culture was centrifuged at 3000 rpm for 15 min, and the cell pellet was washed twice with cold PBS. The pellet was suspended in one-tenth the original volume in PBS and the OD_{600} nm was adjusted to give the approximate desired inocula. The inoculum concentrations were verified by serial 10-fold dilutions of the bacterial suspensions. The bacterial concentrations of the suspensions were adjusted to 1×10^9 CFU/ml. The bacteria were diluted to the desired concentration and used in subsequent experiments.

2.2. Preparation of murine PMCs and BMDMs

TRIF-deficient mice on a C57BL/6 background were kindly provided by Dr. Shizuo Akira (Osaka University, Japan). Wild-type (WT) C57BL/6 mice were purchased from Koatech (Pyeongtaek, Korea). Bone marrow-derived macrophages (BMDMs) were prepared as previously described [25]. Mesothelial cells were prepared from the peritoneum and external surfaces of the liver, spleen, and kidney of adult mice as previously described [13]. Briefly, samples of peritoneum and intact organs were obtained from sacrificed mice and digested with 0.25%-trypsin–EDTA (Invitrogen, Grand Island, NY, USA) solution for 50 min at 37 °C. Intact tissues and tissue debris were discarded and the cell suspension was centrifuged at 90 g for 5 min. The pellet was resuspended in Dulbecco's modified Eagle's medium supplemented with 15% heat-inactivated FBS and 1% penicillin/streptomycin and cultured overnight. The next day, floating cells were removed by washing twice with PBS and adherent cells were cultured for five additional days. Mesothelial cells were used between passages 2 and 4.

2.3. RT-PCR

Total RNA was extracted from the cells using easyBLUE (Intron Biotechnology, Daejeon, Korea) according to the manufacturer's instruction. One microgram of total RNA was reverse transcribed into cDNA and PCR was performed using the Power cDNA Synthesis kit (Intron Biotechnology) and One-step RT-PCR with AccuPower®HotStart PCR PreMix (Bioneer, Daejeon, Korea). The following primer sets were used. Mouse TLR2 forward, 5′-GTGGTA CCTGAGAATGATGTGGG-3′; mouse TLR2 reverse, 5′-GTTAAGGAAGT CAGGAACTGGGTG-3′; mouse TLR3 forward, 5′-AGGTACCTGAGTTT GAAGCGAGC-3′; mouse TLR3 reverse, 5′-GAGCATCAGTCTTT GAAGGCTGG-3′; mouse TLR4 forward, 5′-CTGGGTGAGAAAT GAGCTGG-3′; mouse TLR4 reverse, 5′-GATACAATTC CACCTGCTGCC-3′; mouse TRIF forward, 5′-ATGGATAACC CAGGGCCTT-3′; mouse TRIF reverse, 5′-TTCTGGTCACTGCAGGG GAT-3′; mouse iNOS forward, 5′-CAGCCCAACAATACAAGATGACCC-3′; mouse iNOS reverse, 5′-CAGTTCCGAGCGTCAAAGACCTGC-3′; mouse IFN-β forward, 5′-ATGAACTCCAGCAGACAG-3′; mouse IFN-β reverse, 5′-ACCACCATCCAGGCGTAGC-3′; mouse GAPDH forward, 5′-GTCGGAGRCAACGGATT-3′; mouse GAPDH reverse, 5′-AAGCTTCC CGTTCTCAG-3′. The PCR reaction condition included predenaturing at 94 °C for 30 s, then 35–40 cycle of 56 °C for 30 s, 72 °C for 1 min. PCR products were then electrophoresed on a 1.5% agarose gel and visualized using a gel documentation system.

2.4. Measurement of the production of cytokines and nitric oxide (NO)

The concentration of IL-6 and CXCL1, CCL2 in the culture supernatants was determined using a commercial ELISA kit (R&D Systems, Minneapolis, MN, USA). NO synthase activity in the supernatant of cultured cells was assayed for nitrite accumulation by the Griess reaction [26].

2.5. Western blot

PMCs (5×10^4/well) and BMDMs (2×10^6/well) were plated in 35 mm culture dishes. The cells were treated with polyI:C (100 μg/ml) or LPS (100 ng/ml) were lysed in buffer containing 1% Nonidet-P40 supplemented with a complete protease inhibitor cocktail (Roche Diagnostics Ltd, Mannheim, Germany), and 2 mM dithiothreitol. Lysates were resolved by 10% SDS–PAGE, transferred onto a polyvinylidene fluoride (PVDF) membrane, and immunoblotted with primary antibodies against regular- and phospho-IκB-α, JNK (Cell Signaling Technology Inc., Beverly, MA, USA), p38, ERK, β-actin, Calretinin (Santa Cruz Biotechnology, Santa