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# mTORC1 mediates peptidoglycan induced inflammatory cytokines expression and NF-κB activation in macrophages

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ABSTRACT

Peptidoglycan (PGN) is the major structural component of the bacterial cell wall, especially gram positive bacteria, which induces inflammatory responses. Mammalian target of rapamycin (mTOR) regulates the production of inflammatory cytokines induced by antigens, while the function of mTORC1 in peptidoglycan induced inflammatory response is unknown. This study aims to examine the role and the regulatory mechanism of mTOR signaling pathway in peptidoglycan induced cytokine expression in mouse macrophages. We observed that peptidoglycan upregulated the secretion of proinflammatory cytokines IL-6, TNF-α and anti-inflammatory cytokine IL-10 in a dose- and time-dependent manner. mTORC1 positively regulates IL-6 and TNF-α, but negatively regulates IL-10 secretion. mTORC1 regulates NF-κB p65 activation by degrading IκB-α in response to peptidoglycan. mTOR, NF-κB and STAT3 signaling pathways are involved in peptidoglycan induced inflammatory cytokines expression *via* a TLR1/TLR2-dependent mechanism in macrophages. Thus, mTORC1 pathway regulates the innate immune response to bacterial peptidoglycan.



## 1. Introduction

Peptidoglycan (PGN) is an essential and unique component of bacteria that provides rigidity and structure to the bacterial cell wall and pathogen-associated molecular pattern (PAMP) consist of molecules not found in the host, that is recognized by pattern recognition receptors (PRRs), resulting in innate immune responses [1]. It has been known for a long time that PGN promotes an inflammatory response [2]. PGN stimulates the production of inflammatory cytokines, such as interleukin (IL) 6, IL-1α/β, and tumor necrosis factor alpha (TNF-α) in monocytes, macrophages, human embryonic kidney, and epithelial cells [1,3]. Recognition of PAMPs by specific proteins called pattern recognition molecules (PRMs) activates inflammatory signaling pathways and immune response [4].

Toll-like receptors (TLRs) are known as PRRs and TLR2 is recognized as the major PGN receptor [5,6]. In general, TLRs interact with other different coreceptors as well as with each other for full ligand sensitivity [7,8], and TLR2 is involved in cooperation with other TLRs, particularly TLR1 and TLR6. TLR1 cooperates with TLR2 to form TLR1/TLR2 heterodimer on the cell surface to initiate signal transduction [9]. Moreover, TLR1/TLR2 heterodimer is responsible for recognizing gram-positive bacteria and mycoplasma triacyl lipopeptides [10] to initiate the release of proinflammatory cytokines, such as TNF-α and IL-6 [9,11,12]. IL-6 is a multifunctional cytokine that controls immune responses, inflammation, hematopoiesis, bone metabolism, and immunity [13] and TNF-α is a proinflammatory cytokine that regulates the inflammatory response [14]. On the contrary, interleukin 10 (IL-10) is an anti-inflammatory cytokine that modulates innate and adaptive immunity. IL-10 induction was TLR ligand selective, in that CpG DNA,

*Abbreviations:* mTORC1, Mammalian target of rapamycin complex 1; TNF-α, tumor necrosis factor α; IL-6, interleukin-6; IL-10, interleukin-10; PGN, Peptidoglycan; TLR, Toll-like receptor; PAMP, pathogen-associated molecular pattern; PRMs, pattern recognition molecules.

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imidazoquinolin, peptidoglycan, and zymosan led to IL-10 production, which depending on TLR signaling [15].

Stimulation of cells with PGN activates the protein kinase RICK (synonym of the Receptor-interacting serine-threonine-protein kinase 2), followed by recruitment of IKK (IκB kinase) complex, activating NF-κB (nuclear factor kappa-light-chain-enhancer of activated B cells) and MARK (mitogen activated protein kinase) signaling pathways [1,16]. PGN also activates a pro-inflammatory caspase, caspase-1, that acts to process pro-IL-1β and pro- IL-18 to active forms for secretion [16]. In addition, TLR2 appears to activate a signaling cascade composed of the Rho GTPase Rac1, the phosphatidylinositol-3 kinase (PI3K), and Akt that targets nuclear NF-κB transactivation independently of IκB-α degradation [17]. Signal transducer and activator of transcription 3 (STAT3) mediates the expression of a variety of genes in response to various cytokines and growth factors, which plays a key role in many cellular processes such as cell growth and apoptosis [18]. Recently studies showed an emerging role of STAT3 in antimicrobial defense and cancer progression [19,20].

Mammalian target of rapamycin (mTOR) is a serine-threonine kinase which mediates cell growth and proliferation, ribosome biogenesis, and cytoskeletal organization. mTOR exists in two multi-protein complexes, mTORC1 and mTORC2 [21–23]. mTORC1 has also been shown to promote the phosphorylation of their downstream targets such as ribosomal p70S6 kinase (p70S6K) and initiation factor 4E-binding protein 1 (4E-BP-1), and rapamycin is a specific inhibitor of mTORC1 [24]. mTORC1 signaling is involved in lipopolysaccharide (LPS) induced pro- and anti-inflammatory cytokine production in various cells, such as macrophages, monocytes, dendritic cells, and other immune cells [14,25,26]. However, the function of mTOR signaling pathway in PGN induced inflammatory response is unclear.

In the present study, we treated mouse macrophages with PGN in the presence or absence of rapamycin, and then measured inflammatory gene expression and activation of transcription factors. Our data suggest that mTOR positively regulates IL-6, TNF-α expression, but negatively regulates IL-10 expression induced by PGN. We further demonstrate that mTOR regulates NF-κB activation *via* IκB-α to mediate PGN induced inflammatory cytokines expression in macrophages.

## 2. Materials and methods

### 2.1. Cell culture conditions

The Ana-1 mouse macrophage cell line was purchase from Cell Bank of Chinese Academy of Sciences. The cells were cultured in completed medium (RPMI 1640, containing 10% heat-inactivated fetal calf serum, 2 mM L-glutamine, 100 U/ml penicillin, and 100 mg/ml streptomycin) at 37 °C in a 5% $CO_2$ humidified atmosphere.

### 2.2. Reagents and antibodies

Purified peptidoglycan from *S. aureus* was purchased from Sigma Aldrich (St. Louis, MO, USA). Rapamycin was purchased from Gene Operation (Ann Arbor, Michigan, USA). The antibodies used for immunoblotting were purchased from the manufacturers listed below and used according to the manufacturer's protocol. Antibodies to phospho-S6 (Ser240/244), S6, phospho-4E-BP1 (Thr37/46), IκB-α, NF-κB p65, phospho-STAT3 (Tyr705), and STAT3 were purchased from Cell Signaling (Beverley, MA, USA). Antibodies to 4E-BP1, phospho-mTOR (Ser2448), mTOR, and phospho-NF-κB p65 (Ser536) were purchased from Abcam (Cambridge, UK). Antibody to β-actin was purchased from Sigma Chemical (St. Louis, MO, USA). Antibodies to TLR1, TLR2 were purchased from Santa Cruz Biotechnology (Dallas, Texas, USA). Cytokine ELISA kits were obtained from eBioscience (San Diego, CA, USA).

### 2.3. Cytokine ELISA

Cell culture supernatants were collected, centrifuged to remove cellular precipitates, and assayed immediately or stored at −80 °C until analysis. Cytokines were measured according to the ELISA kit manufacturer's instructions, and absorbance was read at 450 nm and 570 nm on a Varioskan Flash Multimode Reader (Thermo Fisher Scientific, Pittsburgh, PA, USA). All measurements were made in triplicate, and the mean values of the three independent measurements were used for the statistical analysis.

### 2.4. Western blot analyses

Ana-1 cells were treated with peptidoglycan in the presence or absence of rapamycin and washed two times with ice-cold PBS (pH 7.4). Cells were lysed in lysis buffer that contained 25 mM Tris-HCl (pH 7.6), 150 mM NaCl, 1% Nonidet P-40, 1% sodium deoxycholate, 0.1% SDS, protease inhibitor mixture, and phosphatase inhibitors (Sigma Chemicals, St. Louis, MO, USA) and then placed on ice for 10 min. Next, the cells were collected by scraping and centrifuged at 4 °C for 10 min at 13,000 rpm. Equal amounts of cell lysates were separated by SDS-PAGE on 10% polyacrylamide gels and transferred to PVDF membranes, which were immunoblotted with the designated primary antibodies. The membranes were then treated with horseradish peroxidase-conjugated goat anti-rabbit IgG or goat anti-mouse IgG (GE Healthcare, UK) and detected using the ECL detection (Thermo Fisher Scientific, Pittsburgh, PA, USA) by exposure to X-ray film. The resolved bands were quantified using Gel-Pro Analyzer 4.0 (Media Cybernetics, USA). Three independent experiments were performed for western blots and analyzed by statistical analyses.

### 2.5. Cell viability assay

Ana-1 cells were seeded onto 96-well plates at $6 \times 10^3$ cells per well for culture. After 24 h, the cells were divided into four groups: cells treated with peptidoglycan (25 μg/ml) only; cells treated with rapamycin (100 nM) only; cells pretreated with rapamycin (100 nM) for 3 h before stimulation with 25 μg/ml peptidoglycan (10 mg/ml); cells treated with an equal volume of vehicle (absolute ethanol) were used as a mock. After 6 h treatment, cell proliferation was measured by methyl thiazolyl tetrazolium (MTT) assay. Briefly, 0.02 ml MTT solution (5 mg/ml in PBS) was added to each well and incubation for 4 h at 37 °C, after which the media was replaced with 0.15 ml dimethyl sulfoxide, followed by a 10 min incubation. Then, the optical density was measured at 490 nm with a Varioskan Flash Multimode Reader (Thermo Fisher Scientific, Pittsburgh, PA, USA). The means of the three independent measurements were used for the statistical analysis.

### 2.6. TLR1 and TLR2 antibodies blocking assay

Ana-1 cells were seeded onto 6-well plates at $7.8 \times 10^5$ cells/well in 2 ml medium per well. The Ana-1 cells were pretreated with TLR1, TLR2 antibody alone or both of them for 1 h and stimulated with 25 μg/ml peptidoglycan for 6 h. Cells treated with peptidoglycan alone were used as a positive control and an equal volume of vehicle (absolute ethanol) were used as a negative control. After treatment, cell culture supernatants were gathered, centrifuged to remove cellular precipitates, and assayed by ELISA. Cells were lysed, and total protein was collected for western blot analysis.