THE ROLE OF TOLL-LIKE RECEPTOR (TLR) 2 IN *MYCOBACTERIUM TUBERCULOSIS* H37RV-INDUCED GENERATION OF INTRACELLULAR REACTIVE OXYGEN SPECIES (ROS) IS DEPENDENT

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SUMMARY

Mycobacterium tuberculosis is a pathogenic bacterial species and the causative agent of most cases of tuberculosis. *Tuberculosis* has a long history and still is a major infectious disease in most parts of the world. *Mycobacterium tuberculosis* H37Rv (Mtb) was an important respiratory pathogen which was controlled primarily by cytokine-activated macrophage. The recent studies were to focus on the role of specific pattern-recognition receptor (PRR)-microbial interaction for the host defense against mycobacterial infections and the intracellular mechanisms activated by PRRs during infections with Mtb and their specific ligands. The recent discovery of novel classes of receptors, including Toll-like receptor (TLR) is challenging the crucial role of innate immune system for recognition of Mtb The interaction of Mtb with TLRs triggers the intracellular signaling cascades culminating the activation of NFκB and mitogen-activated protein kinases, thus mediates the proinflammatory responses against mycobacterial infection Besides, reactive oxygen species (ROS) is important in controlling Mtb during primary pulmonary infection. However, the role of TLR-2 in the regulation of mycobacteria pathogenesis-induced ROS is poorly understood. In this study, we investigated the role of TLR2 in intracellular ROS generation during macrophage with tuberculosis infection. To extend these studies, we examined Nicotinamide adenine dinucleotide phosphate (NADPH) oxidase activities were differentially modulated in macrophages from WT and $TLR2^{-/-}$ mice in response to *Mtb* infection. Moreover, ROS-generating NADPH oxidase activities are dependent on TLR2. Collectively, these data demonstrated the pivotal role of reactive oxygen species (ROS) signaling through TLR-2-mediated innate responses during mycobacterial infection.

Keywords: *Mycobacterium tuberculosis H37Rv, reactive oxygen species (ROS), Nox2, TLR2*

INTRODUCTION

Mycobacterium tuberculosis (Mtb) is the causative agent of pulmonary tuberculosis (TB), a disease that affects nearly one-third of the world's population (Flynn, Chan, 2001; Van Crevel *et al.*, 2002). Mtb is an intracellular pathogen capable of infecting and surviving within macrophages. Mtb is an important respiratory pathogen and the growth of which is controlled primarily by cytokine-activated macrophages (Yang *et al.*, 2006; Yang *et al.*, 2007a). Toll-like receptors (TLR) recognize specific structural motifs of various pathogens, known as pathogen-associated molecular patterns, and are critical in provoking innate immune responses (Akira *et al.*, 2006). Recent studies have shown that the differential pro-inflammatory immune responses to mycobacterial infections are dependent on the activation and modulation of mitogen activated protein kinase (MAPK) signaling, which play an important role in promoting anti- mycobacterial activity and production of proinflammatory mediators, including tumor nuclear factor (TNF)-α, interleukin (IL)-6, IL-1, and IL-12, chemokine and nitro oxide (Yang *et al.*, 2007a; Schorey, Cooper, 2003; Yadav *et al.*, 2006).

Nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (Nox)-generated reactive oxygen species (ROS) can participate in immune function in a variety of ways. Nox is a multicomponent complex, composed of membrane-bound protein known as flavocytochrome b_{558}, the cytosolic proteins p40phox, p47phox, p67phox, and two small GTPase, Rac1/2. Cytochrome b_{558}, the redox core of the respiratory burst oxidase, consists of a large glycoprotein gp91phox or Nox2 and the small protein p22phox (Vignais, 2002; Babior, 2004). ROS are produced in

mammalian cells in response to the activation of various cell surface receptors (Biberstine et al., 2001) and involved in regulation in diverse receptor-ligand intracellular signaling pathways, such as MAPK or NF-κB pathways (Nathan, 2003; Dusi et al, 1998; Aschnoune et al., 2004). However, cellular mechanisms that direct the production of Nox-derived superoxide to selectively influence certain receptor signaling pathways remain poorly understood.

Given the importance of ROS in regulating host response to the TLR agonists and the lack of knowledge about how ROS-dependent inflammatory signaling is regulated in human TB, the present study investigated the molecular mechanisms that regulate the ROS-mediated inflammatory responses in macrophages activated by *Mycobacterium tuberculosis* H37Rv (*Mtb*). In the present study, macrophages respond to *Mtb* through ROS-dependent signaling via TLR2.

MATERIALS AND METHODS

Reagent and Abs

The following reagents were purchased from Calbiochem (San Diego, CA): antioxidant *N*-acetyl-L-cysteine (NAC), an inhibitor of NADPH oxidase (DPI), a xanthine oxidase inhibitor (allopurinol), an inhibitor of mitochondrial electron transfer chain subunit I (rotenone). Dimethyl sulfoxide (DMSO; Sigma) was added to cultures at 0.1% (vol/vol) as a solvent control. gp91phox (Nox_2), and actin antibody was from Santa Cruz Biotechnology.

Preparation of *M. tuberculosis*

Cultures of *M. tuberculosis* H37Rv (kindly provided by Dr. Eun-Kyeong Jo, University of Chungnam, Korea) were prepared as described previously (Yang et al., 2007a). Mycobacteria were grown in roller bottles to mid-log phase in Middlebrook 7H9 liquid medium supplemented with oleic acid/albumin/dextrose/catalase (Difco, Becton-Dickinson, Palo Alto, CA), The cells were collected by centrifugation, washed and resuspended with basal RPMI 1640, and centrifuged at 150 g for 5 min to remove any clumps. Aliquots of the upper bacterial suspension were kept frozen at -80 °C until used. Before infection, the thawed bacterial aliquot was dispersed using a bath sonicator and centrifuged at 150 g for 2 min. The bacteria remaining in suspension were used for the infection. Quantification was performed by assessing the CF after plating serial 10-fold dilutions of t supernatant on Middlebrook 7H10 agar.

Isolation and culture of murine bone marro derived macrophages

Murine marrow-derived macrophages (BMDM from TLR2 Knock-out (KO) mice of C57BL background and C57BL/6 mice were prepared fro these mice, as previously described (Yang et a 2007a). All animals were maintained in a pathoge free environment. All experimental procedures we reviewed and approved by the Institutional Anim Care and Use Committee (IACUC) in Chungna National University.

Cell culture and infection of Mtb

Mouse macrophage cell line was maintained complete medium [DMEM (Gibco-BRl Gaithersburg, MD) with 10% fetal bovine seru (Gibco-BRL), sodium pyruvate, non-essential amir acids, penicillin G (100 IU/ml), and streptomyci (100 μg/ml)]. BMDMs were infected with *Mtb* (Tl multiplicity of infection (MOI) = 1) and incubat for the indicated times at 37°C under 5% CO2. Aft the time allowed for phagocytosis, cells were wash three times with fresh phosphate-buffered saline remove extracellular bacteria and then incubate again with complete DMEM without antibiotics f indicated times. Cultures of uninfected cells we maintained under the same conditions during t entire time of the assays.

Western blot assay

BMDMs were treated as indicated and process for analysis by, Western blot, as previously describ (Yang et al., 2007a). For Western analysi antibodies for phosphorylated gp91phox, and act antibody was used at a 1:1,000 dilution. Membran were developed using a chemiluminescence ass (ECL; Pharmacia-Amersham, Freiburg, German and subsequently exposed to chemiluminescen film (Pharmacia-Amersham).

Measurement of intracellular ROS

Intracellular ROS levels were measured by DH assays, as previously described (Yang et al., 2007 Briefly, BMDM cells were stimulated with M DPI for 30 min. The cells were incubated with e 2 μM dihydroethidium (DHE) (Calbiochem) for 1 min at 37°C in 5% CO_2.

Determination of NADPH oxidase activity

NADPH oxidase activities were measured by lucigenin (*bis-N*-methylacridinium nitrate) chemiluminescence assay (5×10^{-6} mol/L lucigenin, Sigma) in the presence of its substrate NADPH (10^{-4} mol/L, Sigma) as described previously (Griendling *et al.*, 1994).

In brief, BMDMs were incubated with Mtb for 30 min in the presence or absence of ROS inhibitors. Lucigenin-enhanced chemiluminescence assay was performed to analyze the level of superoxide production as previously reported (Griendling *et al.*, 1994) The cells were transferred into scintillation vials containing Krebs-HEPES buffer (100 mM NaCl, 4.7 mM KCl, 1.9 mM $CaCl_2$, 1.2 mM $MgSO_4$, 1.03 mM K_2HPO_4, 25 mM $NaHCO_3$, 20 mM Na-HEPES, pH 7.4) with 5 µM lucigenin. The chemiluminescence, which occurred over the ensuing 1 min in response to the addition of 100 µM NADPH, was recorded using a luminometer (Lumet LB9507; Berthold Technologies, Bad Wildbad, Germany). The emitted light units, after subtracting a blank, were used as a measure of superoxide production. Values were expressed as relative light units per 1×10^{7} cells.

Statistical analysis

For statistical analysis, data obtained from independent experiments are presented as the mean ± SD and they were analyzed using a Student's *t* test with Bonferroni adjustment or ANOVA for multiple comparisons. Differences were considered significant for $p < 0.05$.

RESULTS

Mtb induces ROS generation in BMDM

ROS act as an intracellular second messenger, which can regulate various intracellular signal transduction cascades and transcription factors (Forman, Torres, 2002; Gwinn, Vallyathan, 2006). We investigated whether the *Mtb* induced ROS production in BMDMs from wild type (WT) mice. As shown in Fig 1, *Mtb*-induced superoxide generation was significantly in BMDMs. The effect was nearly abolished in BMDMs was incubated with DPI (an NADPH oxidase inhibitor), as measured by fluorescent microscope based on DHE, respectively. These results indicate that *Mtb* induced ROS generation in macrophages.

Figure 1. Intracellular ROS generation induced by Mtb BMDMs from WT mice were stimulated with *Mtb* (MOI=1) in the presence or absence of DMSO or DPI (20 µM) after 30 min DHE fluorescence image shown are representative of three independent experiments with similar results Bar, 50 µm. TB Rv, Mycobacterium tuberculosis Rv37.

Mtb-induced activation of NADPH oxidases is required TLR2

The data (Fig.1) suggest that superoxide production contributed to *Mtb* -induced ROS mediates signaling. To extend these studies, we evaluated *Mtb*-induced NADPH oxidase activity in BMDMs from WT and TLR2 KO mice, as shown in Fig. 2, the NADPH oxidase activity was markedly increased in BMDMs from WT stimulated with *Mtb*. However, significant inhibition of NADPH oxidase activity was recorded after pretreatment with various antioxidant (NAC, a general ROS scavenger; DPI, an NADPH oxidase inhibitor; Rotenone, a mitochondrial electron transfer chain subunit 1 inhibitor), except a xanthine oxidase inhibitor allopurinol in BMDMs from WT mice. These results suggested the activation of NADPH oxidases induced by *Mtb* via TLR2.

Nox2 expression is required for TLR2

We also examined whether Nox2 was differentially modulated in BMDMs from WT and TLR2 KO mice in response to *Mtb* infection. The expression of Nox2 was up-regulation in WT mice

by 18h after *Mtb* infection, but no differences in TLR2 KO mice (Fig.3). The robust Nox2 induction in WT mice and sustained in TLR2 KO mice indicate that TLR2 activation is necessary for *Mtb*-induced Nox2 expression. These results indicate that TLR plays an indispensable role for *Mtb*-induced ROS generating NADPH oxidase activation and Nox expression.

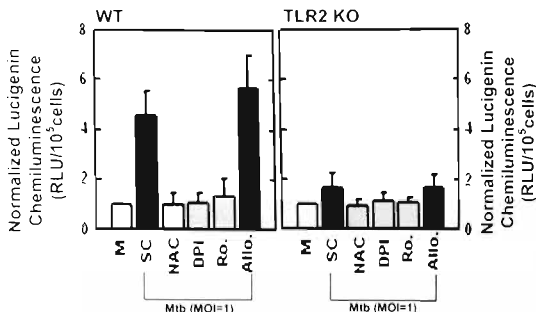

Figure 2. NADPH generation induced by Mtb is dependent on the toll-like receptor (TLR) 2-dependent manner. NADPH oxidase activity was quantified by measuring the production of ROS using a lucigenin-derived chemiluminescence assay. BMDMs from WT and TLR2 KO mice were 30 min of stimulated with Mtb (MOI=1) in the presence or absence of NAC (30 mM), DPI (20 μM), Rotenone (10 μM), or Allopurinol (0.1 mM). Data from one of four independent experiments are shown. Significant differences (*** $P<0.001$) compared with cultures of media control. M, media control; SC, solvent control.

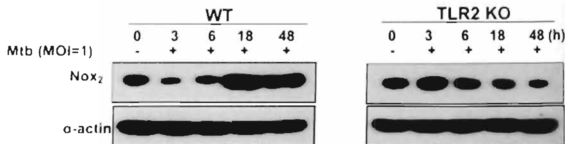

Figure 3. Nox2 expression induced by Mtb is dependent on the toll-like receptor (TLR) 2-dependent manner. BMDMs from WT and TLR2 KO mice were stimulated with *Mtb* (MOI=1) for the times indicated. The cells were harvested and subjected to Western blot analysis for Nox2. The same blots were washed and blotted for total α-actin as the loading controls. Data are representative of three independent experiments with similar results.

DISCUSSION

Despite the fact that the *M. tuberculosis* H37Rv is the most widely used antigen for mycobacterial research, little is known about the nature of recognition and signaling pathway activation that can activate and enhance the ROS generation induced by *Mtb* stimulation. The interaction between *Mtb* and various TLRs is not fully understood, but it appears that whole mycobacteria or distinct mycobacterial components may interact with different members of the TLR family. The present

data demonstrate that the *Mtb*-induced ROS generation is exclusively dependent on TLR2 (Fig.2). Elucidating the mycobacterial protein recognition and signaling pathway activation will reveal the key immunological processes induced by this important human pathogen and help in the rational design of more effective vaccines and adjuvants (Jo *et al.*, 2007).

ROS are mainly produced by leukocytes and by the respiratory mitochondrial chain; they are essential for both cell signaling, and bacterial defence. Elevated ROS are thought to serve as a second messenger to control a broad range of physiological and pathological processes, including cell proliferation, inflammation and apoptosis (Yodoi *et al.*, 2001; Martindale, Holbrook, 2002). Although some studies have shown an increase in ROS production in vascular inflammatory response is dependent on TLR2 activation (Shishido *et al.*, 2006) and others have shown ROS-dependent ASK1-p38 pathway is crucial for TLR4-mediated proinflammatory cytokine induction (Into, Shibata, 2005), and ROS generation-p38 induced by stimulation with mycoplasmal lipoproteins or staphylococcal peptidoglycans is dependent on TLR2 (Lambeth, 2002), the role of ROS in mycobacterial signaling and its regulatory mechanisms remain unclear. The current data clearly demonstrate that the intracellular ROS signaling is dependent on the TLR2 (Fig.2).

Recently, various reports have suggested that receptor-mediated ROS generation is coupled with Nox isozymes (Nox1, Nox3, Nox4, and Nox5), novel homologues of Nox2 of NADPH oxidase in phagocytic cells (Bokoch, Knaus, 2003). We also found that TLR2 activation governs the Nox2 induction and NADPH oxidase activity (Fig.2 and Fig.3). To elucidate the reason why the NADPH oxidase activity was abolished in TLR2 KO, assembly of the oxidase complex was studied.

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VAI TRÒ CỦA THỤ THỂ TLR2 ĐỐI VỚI QUÁ TRÌNH TẠO RA GỐC TỰ DO OXY HÓA BỞI VI KHUẨN LAO (*MYCOBACTERIUM TUBERCULOSIS* H37RV)

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TÓM TẮT

Mycobacterium tuberculosis là một loài vi khuẩn gây bệnh và nguyên nhân chính của các trường hợp bị bệnh lao. Bệnh lao đã có từ rất lâu và vẫn là bệnh lây nhiễm chủ yếu ở hầu hết các nước trên thế giới. Vi khuẩn lao *Mycobacterium tuberculosis* H37Rv (Mtb) là một mầm bệnh quan trọng của đường hô hấp. Mầm bệnh này được kiểm soát ban đầu bằng các đại thực bào kích hoạt cytokines. Những nghiên cứu gần đây đã trọng tâm vào vai trò của quá trình tương tác giữa thụ thể nhận dạng mầm bệnh (PRR) đặc hiệu với vi khuẩn trong quá trình bảo vệ của vật chủ chống lại quá trình lây nhiễm của mycobacteria và những cơ chế hoạt động của tế bào bằng PRR trong quá trình nhiễm Mtb và những phối tử đặc hiệu của chúng. Việc khám phá về một lớp thụ thể mới, bao gồm thụ thể Toll-like (TLR), đang là thách thức vai trò chủ yếu của hệ thống miễn dịch đối với quá trình nhận dạng Mtb. Quá trình tương tác của Mtb cùng với TLRs tạo ra những lớp tín hiệu bên trong tế bào đến mức kích thích hoạt động của NFkB và các protein kinase do chất kích thích phân bào hoạt hóa (MAPKs), thực sự là truyền những đáp ứng tiền viêm chống lại quá trình nhiễm của mycobacteria. Ngoài ra, các gốc tự do ôxy hóa (ROS) có nhiệm vụ quan trọng trong quá trình kiểm soát *Mtb* trong giai đoạn đầu của bệnh lao phổi. Tuy nhiên, vai trò của thụ thể TLR2 (toll-like receptor 2) trong quá trình điều khiển ROS được sinh ra bởi quá trình nhiễm mầm bệnh vi khuẩn lao vẫn còn chưa rõ. Để làm rõ nghiên cứu này, chúng tôi đã chứng minh rằng hoạt động của Nicotinamide adenine dinucleotide phosphate (NADPH) bị điều khiển khác nhau trong macrophages được tách ra từ chuột bình thường và chuột bị bất hoạt thụ thể TLR2. Hơn nữa, hoạt động của NADPH tạo ra gốc ôxy hóa là phụ thuộc vào thụ thể TLR2. Những kết quả trên đây chỉ ra vai trò chủ yếu của tín hiệu ROS là thông qua những đáp ứng miễn dịch bẩm sinh được truyền đi bằng thụ thể TLR2 trong quá trình nhiễm mycobacteria.

***Từ khóa:** Vi khuẩn lao H37Rv, gốc tự do oxy hóa (ROS), Nox2, thụ thể TLR2*

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