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NADPH Oxidase 2 Interaction with TLR2 Is Required for Efficient Innate Immune Responses to Mycobacteria via Cathelicidin Expression

Chul-Su Yang,*† Dong-Min Shin,*† Ki-Hye Kim,*† Zee-Won Lee,§ Chul-Ho Lee,§ Sung Goo Park,∥ Yun Soo Bae,‡ and Eun-Kyeong Jo2*†

Gp91phox/NADPH oxidase (NOX) 2 is the main catalytic component of NOX, which mediates the phagocytic killing of ingested pathogens via the production of reactive oxygen species (ROS). However, Mycobacterium tuberculosis (Mtbc) is relatively resistant to the microbial effects of ROS. Thus, the exact roles of NOX2 in the innate immune control against Mtbc infection are not fully resolved. In this study, we show that NOX2 is essential for TLR2-dependent inflammatory responses and 1,25-dihydroxyvitamin D3 (1,25D3)-mediated antimicrobial effect against viable Mtbc through the modulation of cathelicidin expression in human macrophages. Treatment of NOX2 knocked down /H9010 with cathelicidin restored the 1,25D3-induced antimicrobial effect, suggesting that the NOX2-dependent induction of cathelicidin in macrophages is part of a defensive strategy against Mtbc. Furthermore, cathelicidin expression was required for the Mtbc-induced release of ROS and the production of proinflammatory cytokines/chemokines, indicating a positive circuit of inflammation in response to Mtbc. Our data collectively demonstrate a novel regulatory mechanism for TLR2-dependent innate responses to Mtbc involving crosstalk between NOX2 and TLR2 and the expression of cathelicidin. The Journal of Immunology, 2009, 182: 3696–3705.

Phagocytes generate reactive oxygen species (ROS)3 by using superoxide-generating NADPH oxidase (NOX) family proteins, including the catalytic subunit NOX2/gp91phox (1). NOX2, which is mainly expressed in neutrophils and macrophages, plays pivotal roles in host defense against bacterial and fungal pathogens (2). Activated NOX2 generates ROS such as superoxide and H2O2, which are vital components of the innate immune system, in phagosomes containing invasive microorganisms (3). Mutations in NOX2 result in chronic granulomatous disease (CGD), a group of inherited disorders in which the phagocytic cells fail to generate superoxide that are characterized by recurrent life-threatening bacterial and fungal infections (4). However, previous findings on the roles of NOX2 in the intracellular growth of mycobacteria have been controversial. In vitro studies on bacterial growth within phagocytes collected from patients with CGD indicated the failed destruction of intracellular bacillus Calmette-Guérin, revealing the mycobactericidal role of the respiratory burst (5). Patients with CGD are susceptible to tuberculosis (TB) and bacillus Calmette-Guérin-related complications (6), although the incidence of TB is not greatly increased among those with CGD (7). Indeed, reactive oxygen intermediates are unlikely to be involved in the direct killing of virulent strains of Mycobacterium tuberculosis (Mtbc) (8). Thus, the exact roles of NOX2 in the innate immune response to Mtbc are unclear.

Not only is NOX-derived ROS production involved in the regulation of biological activities such as host defense, it is also crucial for the regulation of intracellular signaling via MAPK and NF-κB (9–11). Previous studies revealed that TLR4/LPS stimulation induced the production of intracellular ROS, which are required for the formation of a complex between the adaptor molecule TNFR-associated factor (TRAF) 6 and apoptosis signal-regulating kinase 1 and the subsequent activation of innate immune responses (12). We also recently showed that the mycobacterial tuberculin purified protein derivative (PPD) activates TLR2-dependent proinflammatory signaling in monocytes/macrophages via intracellular ROS (13). Furthermore, the direct interaction of TLR4 with NOX4, a protein related to NOX2, is involved in TLR4-mediated ROS production and NF-κB activation (14). These data suggest the existence of a
link between NOX activation and TLR2 signaling during mycobacterial infection.

TB, which is caused by Mtb, is a worldwide problem and a leading cause of morbidity and death (15). The control of Mtb in phagocytes is mediated by the transfer of antimicrobial peptides from neutrophils to macrophages (16). Among these antimicrobial peptides, the 37-aa peptide cathelicidin (LL-37/human (h) CAP-18) (17) plays an important role in the killing of intracellular Mtb in TLR2/1-activated human macrophages, which express high levels of vitamin D receptor and vitamin D-1-hydroxylase (18). In addition, recent studies have shown that cathelicidin is required for antimicrobial activity against intracellular Mtb in human THP-1 cells following stimulation with the active vitamin D hormone 1,25-dihydroxyvitamin D$_3$ (1,25D$_3$) (19). Additional studies in several human cell types following mycobacterial infection have shown that cathelicidin is expressed in human alveolar macrophages, monocytes, neutrophils, and epithelial cells (20). Although cathelicidin comprises a distinct class of antimicrobial peptides in the innate immune system (21), little is known about its mechanism of induction and regulation in macrophage responses to Mtb.

In the present study, we investigated how NOX2 interacts with and coordinates the TLR2 signals required for efficient innate immune activation in response to Mtb. We found that NOX2 associates with TLR2 in response to Mtb and that this association is required for the activation of inflammatory responses and ROS production in macrophages. We also found that NOX2 is required for the intracellular control of Mtb via TLR2/1-mediated cathelicidin expression in 1,25D$_3$-treated human macrophages. Furthermore, cathelicidin enhanced both the Mtb-induced release of ROS and the production of IL-8, indicating an extended role for cathelicidin as an inflammatory mediator.

Materials and Methods

Preparation of Mtb

Cultures of Mtb H37Rv (provided by Dr. R. L. Friedman, University of Arizona, Tucson, AZ) were prepared as described previously (22). The effective concentration of LPS was <50 pg/ml in those experiments with a bacterium-to-cell ratio of 10:1. Heat-killed Mtb (HK-Mtb) were obtained by heating for 30 min at 80°C.

Cell preparation and infection

This study was approved by the bioethics committee of Chungnam National University Hospital (Daejeon, South Korea), which oversees studies using samples from human subjects. Adherent monocytes were prepared from PBMCs donated by healthy subjects, as described previously (22). Human monocyte-derived monocytes (MDMs) were prepared by culturing peripheral blood monocytes for 4 days in the presence of 0.2 ng/ml human GM-CSF (Sigma-Aldrich), as described previously (23). Primary bone marrow-derived macrophages (BMDM) from NOX2 and TLR2 knockout (KO) (C57BL/6 background) and wild-type (WT) C57BL/6 mice or TLR4 KO mice (C3H/HeJ) and WT C3H/HeJ mice were purchased, as described previously (22). All preparations were transformed with the hostlike peptides at 20 to 50%, respectively (data not shown).

Reagents, DNA, and Abs

The LPS (Escherichia coli O111:B4) and 1,25D$_3$ used for the in vitro assay were purchased from Sigma-Aldrich. Peptidoglycan (Staphylococcus aureus) was obtained from Fluka. Bacterial lipoprotein (BLP), a synthetic bacterial triamylamylolipopeptide (PanCys-Ser-Lys$_2$) that mimics the acylated N terminus of bacterial LPS, was obtained from InvivoGen. N-Acetylcysteine (NAC), diphenyleneiodonium (DPI), and allopurinol were purchased from Calbiochem. DMSO (Sigma-Aldrich) was added to the cultures at 0.1% (v/v) as a solvent control. The cationic human host defense peptide LL-37 (LLGDFRKSSKEKIGKEFKRIVRQDRFLNLVRPTESS) was synthesized by solid-phase biochemistry and purified by reverse-phase HPLC (Cosmo Genetech).

Expression plasmids encoding the deletion mutants of hNOX2 (N terminus, Δ1-290; C terminus, Δ291-570) were created by inserting WT NOX2 cDNA into pcMV.SPORT6 and Δ1TR-ΔTLR2 cDNA (where TIR is Toll/IL-1R) into the WT-TLR2 sequence in pUNO using a QuikChange site-directed mutagenesis kit (Strategene) according to the manufacturer’s instructions. The hLL-37 plasmid was subcloned into pcDNA3.1 (+) (pcDNA-LL37). The psiRNA-h7SKGFP plasmids for hTLR2 short hairpin RNA were purchased from InvivoGen. Transfection of these plasmids into human monocytes was achieved using Nucleofector technology (Amaxa). The human and mouse NOX2 small interfering RNAs (siRNAs) were a pool of three target-specific siRNAs (20–25 nt in length) designed to knock down gene expression (Santa Cruz Biotechnology). Human cathelicidin (LL-37) and control siRNAs were purchased from Dharmacon. The cells were transfected using LipofectAMINE 2000 as indicated by the manufacturer (Invitrogen).

Specific Abs against ERK1/2, phospho-(Thr202/Tyr204)-ERK1/2, p38, phospho-(Thr180/Tyr182)p38, stress-activated protein kinase (SAPK)/JNK, phospho-(Thr183/Tyr185)-SAPK/JNK, phospho-IkB kinase (IKK) α/β, and Flag (M2) were purchased from Sigma-Aldrich, as described previously (22). For the sandwich ELISA, cell culture supernatants were analyzed for TNF-α, IL-6, and IL-8 using DuoSet Abs purchased from BD (Pharmingen) as described previously (22).

For Western blot analysis, primary Abs were used at a 1/1,000 dilution. The membranes were developed using a chemiluminescence assay (ECL; Amersham Pharmacia) and subsequently exposed to X-ray film. Each reaction was performed in triplicate, and the optical data were analyzed using the default and variable parameters available in the iCycler iQ Optical System (version 3.0a; Bio-Rad). The samples were normalized to the reference reporter β-actin.

ELISA, Western blotting, and coimmunoprecipitation

RAW264.7 cells and human monocytes were treated as indicated and processed for analysis by sandwich ELISA, Western blotting, and coimmunoprecipitation as previously described (22). For the sandwich ELISA, cell culture supernatants were analyzed for TNF-α, IL-6, and IL-8 using Duoset Abs purchased from BD Pharmingen as described previously (22).

For Western blot analysis, primary Ab were used at a 1/1,000 dilution. The membranes were developed using a chemiluminescence assay (ECL; Amersham Pharmacia) and subsequently exposed to X-ray film. Each reaction was performed in triplicate, and the optical data were analyzed using the default and variable parameters available in the iCycler iQ Optical System (version 3.0a; Bio-Rad). The samples were normalized to the reference reporter β-actin.

RNA extraction and quantitative real-time PCR

RNA was isolated using an RNaseasy mini kit (Qiagen) and treated with RNase-free DNase as per the manufacturer’s instructions (Qiagen). The LL-37 primers and reaction conditions used for quantitative real-time RT-PCR were as described previously (19). Each reaction was performed in triplicate, and the optical data were analyzed using the default and variable parameters available in the iCycler iQ Optical System (version 3.0a; Bio-Rad). The samples were normalized to the reference reporter β-actin.

Immunofluorescence analysis of NF-κB p65, NOX2, TLR2, and LL-37

The cells were fixed on coverslips with 4% (v/v) paraformaldehyde in PBS and then permeabilized for 10 min using 2% (v/v) Triton X-100 in PBS at 25°C. NF-κB p65, NOX2, TLR2, or LL-37 was detected using a 1/100 dilution of the primary Ab for 1 h at 25°C. Rabbit Abs (anti-human TLR2 Ab; H-175) were preabsorbed overnight at 4°C with 2 mg/ml crude Mtb protein A-Sepharose by incubation at 4°C for 18 h on a rotator. The samples were subsequently solubilized in SDS sample buffer and separated by SDS-PAGE for Western blot analysis.
or ANOVA for multiple comparisons and are presented as the mean ± SD. The nuclei were visualized following a 15-min incubation with 1 μg/ml 4,6-diamidino-2-phenylindole (Sigma-Aldrich). Plasma membranes were visualized following a 15-min incubation with 20 nM Alexa Fluor 350 wheat germ agglutinin (Invitrogen). The slides were examined using laser-scanning confocal microscopy (model LSM 510; Zeiss).

Measurement of intracellular ROS and determination of NADPH oxidase activity

The intracellular ROS levels were measured using dihydroethidium (DHE; Calbiochem) (22). The cells were incubated with 20 μM DHE for 15 min at 37°C under 5% CO2, and examined by laser-scanning confocal microscopy (model LSM 510). The mean relative fluorescence intensity for each group of cells was measured using a Zeiss vision system (LSM 510, version 3.2). NADPH oxidase activity was measured using a lucigenin (bis-N-methylacridinium nitrate) chemiluminescence assay with 5 × 10−7 M lucigenin (Sigma-Aldrich) in the presence of its substrate NADPH (10−4 M; Sigma-Aldrich) as described previously (26). The values are expressed as relative light units per 1 × 105 cells.

Quantification of mycobacterial growth

To ensure the reliable quantification of intracellular Mtb, we used two independent methods (27). The first method used CFUs. After various periods of incubation, cells were lysed with 0.3% saponin (Sigma-Aldrich) to release the intracellular bacteria. The lysates of the infected cells were then resuspended vigorously, transferred to screw-capped tubes, and sonicated in a preheated 37°C water bath sonicator (Elma) for 5 min. Aliquots of the sonicates were then diluted 10-fold in 7H9 medium. Four dilutions of each sample were plated separately on 7H10 agar plates and incubated at 37°C with 5% CO2 for 21 days.

The second method involved [3H]uracil incorporation. At the end of the incubation period, the cells were lysed and then transferred to 96-well round-bottom plates and incubated in the presence of 1 μCi [3H]uracil (Amersham Pharmacia). After 24 h, the mycobacteria were killed by treatment with paraformaldehyde (final concentration of 4%) for 30 min. [3H]Uracil incorporation was measured using a beta counter (Berthold). Background radioactivity in the uninfected cells was <500 cpm in all experiments. To document the suitability of our quantification methods for detecting antimycobacterial activity, we performed experiments in the presence of the cationic human host defense peptide LL-37.

Statistical analysis

All data were analyzed using Student’s t test with a Bonferroni adjustment or ANOVA for multiple comparisons and are presented as the mean ± SD. Differences were considered significant at p < 0.05.

Results

Mtb induces NOX2-derived ROS production in macrophages via TLR2

Previously, we showed that a tuberculin PPD could inhibit the activation of inflammatory signaling by TLR2 in murine BMDMs and human primary monocytes (13). In the current study, we extended this observation to demonstrate that Mtb-mediated ROS production is largely dependent on NOX2 and TLR2. Mtb stimulation led to a robust burst of ROS production in macrophages; however, the effect was significantly abrogated in BMDMs taken from NOX2 KO mice (Fig. 1A, left). Similar patterns of NOX2 dependency were observed in BMDMs stimulated with HK-Mtb, as seen in those infected with viable Mtb (Fig. 1A, right). In addition, BLP-induced ROS production was substantially reduced in NOX2-null cells, whereas no significant difference in the LPS-mediated release of ROS was detected between WT and NOX2 KO cells (Fig. 1A, right). Similar results were obtained using NOX2 siRNA-transfected RAW264.7 cells (data not shown). Thus, NOX2 appears to be responsible for activating TLR2/Mtb-dependent ROS production in macrophages.

We next examined whether NOX activity and superoxide production in response to Mtb depends on TLR2. As shown in Fig. 1, B–D, NOX activity and superoxide production were markedly inhibited in BMDMs from TLR2 KO but not TLR4 KO mice stimulated with Mtb or BLP, a synthetic TLR2 agonist. However, similar levels of superoxide production and NADPH oxidase activity were observed in LPS-stimulated macrophages from TLR2 KO mice and WT cells, whereas they were dramatically abrogated in TLR4-deficient cells (Fig. 1, B–D). These data indicate that TLR2 is essential for the Mtb-induced production of ROS in macrophages.

![FIGURE 1. Mtb rapidly induces intracellular ROS production and NADPH oxidase activity in a TLR2-dependent manner. A, BMDMs from WT and NOX2 KO mice were stimulated with viable Mtb (MOI = 10), HK-Mtb (MOI = 10), BLP (100 ng/ml), or LPS (100 ng/ml) for 30 min. The cells were then incubated with DHE for 15 min, washed thoroughly, and immediately analyzed for superoxide production under a confocal microscope. The images shown are representative of three independent experiments. B, C, and D, The DHE fluorescence intensities of BMDMs from WT and TLR2 KO mice were stimulated with Mtb, BLP, or LPS for the times indicated. The data are the means ± SD of five experiments. C and D, The DHE fluorescence intensities of BMDMs from WT and TLR2 KO mice (C) or WT and TLR4 KO mice (D) stimulated with Mtb, BLP, or LPS for the times indicated were measured by laser-scanning microscopy with an LSM 510 software system and are presented in terms of the amount of ROS produced. The quantitative data shown for DHE (for superoxide) fluorescence are the means ± SD of three experiments.](http://www.jimmunol.org/Downloadedfrom)
NOX2 plays essential roles in Mtb-induced proinflammatory cytokine expression and the activation of MAPK and NF-κB signaling

Recent studies have shown that ROS play indispensable roles in cell signaling as second messengers (28, 29). The decrease in superoxide production in NOX2-deficient cells following Mtb stimulation suggested that NOX2 expression may be necessary for Mtb-dependent inflammatory responses. As shown previously (22), the engagement of TLR2 by Mtb in macrophages leads to inflammatory cytokine production and the activation of MAPKs and NF-κB (Fig. 2). To determine the role of NOX2 in Mtb-induced inflammatory responses, NOX2 was inhibited in RAW264.7 cells using RNA interference. RAW264.7 cells transfected with NOX2 SMARTpool siRNA and treated with viable or HK-Mtb showed significantly reduced levels of TNF-α and IL-6 (p < 0.001) compared with cells exposed to scrambled siRNA (Fig. 2A). Similar kinetics of proinflammatory cytokine productions were observed in RAW264.7 cells stimulated with HK-Mtb, as seen in those infected with viable Mtb (Fig. 2A). Similarly, NOX2-deficient BMDMs treated with viable Mtb, HK-Mtb, or BLP showed a significant reduction in TNF-α and IL-6 expression compared with WT cells (Fig. 2B).

Mtb increased the phosphorylation of MAPKs and IKKα/β and the degradation of IκBα with WT cells (Fig. 2C). In addition, the Mtb-induced localization of p65 into the nucleus was abolished in NOX2-deficient macrophages as shown by immunofluorescence (Fig. 2D). No inhibitory effects on the TLR4/LPS-mediated nuclear entry of p65 were observed in NOX2-deficient BMDMs (data not shown). Taken together, these data suggest that NOX2 specifically regulates TLR2/Mtb-dependent inflammatory responses and signaling activation.

NOX2 interacts with TLR2 after Mtb stimulation

The above data suggest the existence of cross talk between NOX2 and TLR2 in ROS-dependent signaling in response to Mtb, and TLR4 was previously shown to associate with the C-terminal region of NOX4 (14). Thus, we took a more direct approach to investigate the potential association of NOX2 with TLR2 in ROS-dependent signaling. As NOX2 is mainly expressed in macrophages, the interaction between endogenous NOX2 and TLR2 was identified using cell lysates after Mtb stimulation through immunoprecipitation experiments with anti-TLR2 and anti-NOX2 Abs. As shown in Fig. 3A, Mtb treatment triggered the biphasic association of TLR2 and NOX2 (within 1 min and after 30 min; Fig. 3A). Endogenous TLR2 was able to specifically pull down NOX2 from cell lysates of RAW264.7 macrophages stimulated with Mtb. Similarly, TLR2 was also detected in the immunoprecipitates of endogenous NOX2. However, this was not found following stimulation with other TLR ligands, such as TLR3, TLR4, or TLR9 (data not shown), which suggests that TLR2 specifically interacts with NOX2 in vivo.

We further evaluated the colocalization of NOX2 and TLR2 in response to Mtb stimulation by using immunofluorescence. At defined times following incubation with Mtb, the cells were fixed, permeabilized, and dually stained for TLR2 (red) and NOX2 (green) as described in Materials and Methods. The intracellular localization of the two proteins was then assessed by immunofluorescence and confocal microscopy. As shown in Fig. 3B, both TLR2 and NOX2 had membranous and cytosolic distributions un-
TLR2 and NOX2 colocalize at the plasma membrane by using specific tracking (Fig. 3C). NOX2 colocalization was visualized using anti-TLR2 (H-175) in red (TLR2-tetramethylrhodamine isothiocyanate) or anti-NOX2 in green (NOX2-Cy2) Abs (bar for B, 10 μm). The plasma membranes in THP-1 cells were further visualized with 20 nM Alexa Fluor 350 (bar for C, 20 μm). The cells were examined under a confocal laser scanning microscope and analyzed with LSM 510 software. The data are representative of four independent experiments with similar results. U, Uninfected. D, Interaction of TLR2 with NOX2 in HEK293T cells. HEK293T cells were transfected with Flag-NOX2-WT, Flag-NOX2-ΔN (Δ1–290), Flag-NOX2-ΔC (Δ291–570), HA-TLR2-WT, or HA-TLR2 ΔTIR. Cell lysates were then prepared and immunoprecipitated (IP) with anti-HA or anti-Flag Abs. The total cell lysates (input control for Flag or HA) and immunoprecipitated proteins were detected by immunoblotting with anti-Flag, or anti-HA Abs. The data are representative of four independent experiments with similar results.

FIGURE 4. Mtb induces the 1,25D3-mediated enhancement of cathelicidin expression in a TLR2-dependent manner. A, THP-1 cells were treated with Mtb (MOI = 10), 1,25D3 (20 nM), Mtb after 24 h of treatment with 1,25D3 (1,25D3 + Mtb), or infected with Mtb and then treated with 1,25D3 in medium containing human serum for 18 h (Mtb + 1,25D3); cathelicidin expression was then assessed by RT-PCR. The data are the means ± SD of three experiments. B, Effects of hTLR2 silencing on Mtb-stimulated cathelicidin mRNA expression in human monocytes cultured in medium containing human serum and 1,25D3. At 24 h after transfection, the cells were stimulated with viable Mtb or HK-Mtb (MOI = 10) for 18 h and treated with 1,25D3, for 3 h. The cells were then harvested and subjected to semiquantitative RT-PCR for cathelicidin. The β-actin mRNA level was used as loading controls. The data are representative of five independent experiments with similar results (top). Densitometric analysis of data for five experiments (means ± SD) is shown (bottom). The densitometry values for LL-37 mRNA were normalized to the β-actin levels (data not shown). We also found that the association between TLR2 and NOX2 occurs at the plasma membrane by using specific tracker (Fig. 3C, 1 min). We next examined the interaction of TLR2 with NOX2 in HEK293T cells (Fig. 3D). Subsequent immunoprecipitation resulted in a loss of immunoreactivity for the full-length NOX2-associated TLR2 ΔTIR-domain (Fig. 3D). Therefore, the C-terminal region of NOX2 likely binds the TIR domain of TLR2. Collectively, these data show that TLR2 colocalizes and physically interacts with NOX2.

Mtb stimulation synergistically induces 1,25D3-induced cathelicidin expression by human macrophages via TLR2

Previous studies showed that TLR2/1 activation up-regulates the expression of vitamin D receptor and vitamin D-1-hydroxylase and induces the production of the antimicrobial peptide cathelicidin (18), which is required for 1,25D3-triggered antimicrobial activity against intracellular Mtb (19). In our initial experiments, THP-1 cells were infected with viable Mtb H37Rv (at an MOI of 10), treated with 1,25D3, and infected with viable Mtb after 24 h of treatment. Upon stimulation with Mtb for 1 min, both proteins were recruited to the plasma membrane, with evidence of TLR2-NOX2 colocalization indicated by the yellow color in the overlay image (Fig. 3B, 1 min, lower left). After 30 min of Mtb treatment, TLR2 and NOX2 were colocalized in the cytosol as a rod-like shape (Fig. 3B, 30 min). Either NOX2 or TLR2 is present with FITC-labeled Mtb in the region of phagosomes at 30 min.
NOX2 is required for 1,25D$_3$-induced cathelicidin mRNA and protein expression in response to Mtb

We further examined the role of NOX2 in the expression of cathelicidin in human THP-1 cells using siRNA technology to knock down NOX2 transcription. THP-1 cells were transfected with siRNA specific for NOX2 (siNOX2) or a nonspecific control oligo (siNS). The transfection of 20 nM siNOX2 almost completely inhibited the mRNA expression of NOX2, whereas siNS had little effect (Fig. 5A and data not shown). THP-1 cells were transfected with siNOX2 or siNS and then treated with viable or killed Mtb and cultured with 1,25D$_3$ for the times indicated (Fig. 5B). In the presence of 1,25D$_3$, the cells transfected with siNS showed a time-dependent increase in cathelicidin expression in response to Mtb (Fig. 5, B and C, for mRNA and protein expression, respectively). Early induction of cathelicidin was observed from 15 to 30 min (Fig. 5B and data not shown). The cathelicidin expression levels at 1 h were comparable to the levels of cathelicidin mRNA at 3 h of stimulation. However, little induction of cathelicidin occurred in the cells transfected with siNOX2 in response to Mtb (Fig. 5, B and C). Furthermore, Mtb-induced cathelicidin expression in the 1,25D$_3$-treated THP-1 cells was modulated by treatment with the antioxidants NAC or DPI, whereas it was unaffected by treatment with the xanthine oxidase inhibitor allopurinol (Fig. 5D). Thus, NOX2 and NOX-derived ROS may selectively control the Mtb-induced expression of cathelicidin in 1,25D$_3$-treated macrophages.

NOX2 is required for 1,25D$_3$-triggered antimicrobial activity against viable Mtb through the induction of cathelicidin

Previously, cathelicidin was shown to be required for 1,25D$_3$-mediated antimicrobial activity against intracellular Mtb in THP-1 cells by using siRNAs specific for cathelicidin (19). THP-1 cells transfected with siNOX2 or siNS as well as untransfected THP-1 cells were infected at MOI values of 0.1, 1, or 10. After 4 h of contact with the macrophages, all extracellular bacteria were removed by three washes with warm PBS. The cells were then treated with 1,25D$_3$ for 3 days and the intracellular bacteria were harvested and assayed for viability by CFU and $[^3]$H]uracil uptake. The viability of the intracellular bacteria was reduced by 30–40% in the untransfected and siNS-transfected cells (Fig. 6, A and B, both CFU and $[^3]$H]uracil uptake, respectively), confirming the previously demonstrated antimicrobial effects of 1,25D$_3$ (19). Note that transfection with siNOX2 significantly blocked 1,25D$_3$-mediated antimicrobial activity when compared with siNS-transfected or untransfected cells (Fig. 6, A and B; $p < 0.001$; siNOX2 vs siNS). Moreover, pretreatment with the NOX inhibitor DPI significantly inhibited the antimycobacterial activity of 1,25D$_3$-treated human MDMs compared with control cells without DPI.

FIGURE 5. Mtb induces 1,25D$_3$-mediated cathelicidin expression in a NOX2-dependent manner. A, Efficacy of the siRNA targeting of NOX2 with mRNA loading normalized by β-actin in THP-1 cells. The cells were transfected with NOX2 siRNA using LipofectAMINE 2000. At 24 h after transfection, the cells were harvested for NOX2-specific semiquantitative RT-PCR. The data are representative of three independent experiments with similar results. B, THP-1 cells were transfected with NOX2 siRNA or a nonspecific control using LipofectAMINE 2000. At 24 h after transfection, Mtb (MOI = 10) was added to the cultures with 20 nM 1,25D$_3$ in medium containing human serum and incubated for the times indicated. The cells were then harvested and analyzed by semiquantitative RT-PCR (top) and real-time PCR (bottom) for the expression of LL-37 and cathelicidin, respectively. The results shown are from at least three separate experiments. M, media; D, DMSO control.
FIGURE 6. 1,25D$_3$ exerts antimicrobial activity against Mtb via cathelicidin expression in a NOX2-dependent manner. A and B, THP-1 cells were transfected with siNOX2 or siNS using LipofectAMINE 2000. At 24 h after transfection, the cells were infected with Mtb (MOI = 0.1, 1, or 10) for 18 h and then treated with 20 nM 1,25D$_3$, in medium containing human serum for 3 days. Afterward, the intracellular bacteria were harvested and assayed for metabolic activity and viability based on the number of CFUs (A) and $[^{3}H]$uracil uptake (B). Non si, No siRNA. A, Bacterial viability was calculated as the 1,25D$_3$-treatment divided by the day 0 value multiplied by 100. The CFU data shown are the average of four individual experiments (***, $p < 0.001$; siNOX2 vs siNS). The data were normalized according to the relative CFUs proportionate to $10^4$ CFUs in the day 0 samples. B, The data shown for $[^{3}H]$uracil uptake represent the average percentage of viability from four individual experiments (***, $p < 0.001$; siNOX2 vs siNS). The data are representative of four independent experiments with similar results. C and D, Human MDMs were pretreated with 20 μM DPI for 45 min and then infected with Mtb (MOI = 1) for 18 h and treated with 20 nM 1,25D$_3$, in medium containing human serum for 3 days (***, $p < 0.001$; DPI vs DMSO); the experimental conditions were as outlined in A and B. The data are representative of three independent experiments with similar results. Un, Untreated. E, THP-1 cells were transfected with siNOX2 or siNS using LipofectAMINE 2000. At 24 h after transfection, LL-37 was added at 100 μg/ml and the cells were incubated for 6 h before being infected with Mtb (MOI = 1) for 18 h. The cells were then treated with 20 nM 1,25D$_3$, in medium containing human serum for 3 days. F, LL-37 was added to human MDMs pretreated with 20 μM DPI for 45 min at 50 μg/ml and incubated with Mtb (MOI = 1) for 18 h. The cells were then treated with 20 nM 1,25D$_3$, in medium containing human serum for 3 days. G, THP-1 cells were transfected with LL-37 or empty vector for 24 h. Cells were pretreated with 20 μM DPI for 45 min and incubated for 6 h before the addition of Mtb (MOI = 1). After 18 h, the cells were treated with 20 nM 1,25D$_3$, in medium containing human serum for 3 days; the experimental conditions were as outlined in A and C. The data are representative of four independent experiments with similar results. Top, Lysates of $5 \times 10^5$ cells for each transfectant (Mock, Mock control; LL-37, LL-37 transfected) were analyzed by Western analysis for LL-37. The data included in the subfigures (E–G) are the median, interquartile range, and 95% confidence interval.

Cathelicidin is required for the Mtb-induced release of ROS and the production of proinflammatory cytokines and chemokines

Several studies have shown that LL-37 is capable of stimulating the secretion of IL-8, a neutrophil chemoattractant, by lung and skin epithelial cells (30–32), but little is known about the roles of LL-37 in mycobacterial infection. We thus investigated the role of LL-37 in the induction of proinflammatory cytokines and chemokines and intracellular ROS production. Transfection with siRNAs specific to human cathelicidin (siCat) knocked down the 1,25D$_3$-induced mRNA expression of cathelicidin in THP-1 cells, whereas siNS had little effect (Fig. 7A, lower right). THP-1 cells were transfected with siCat or siNS infected with Mtb and then treated with 1,25D$_3$, in medium containing human serum for 18 h, and the supernatants were collected for the assessment of TNF-α, IL-6, and IL-8 production. As shown in Fig. 7A, Mtb-induced TNF-α, IL-6, and IL-8 production was significantly inhibited in the cells transfected with siCat compared with the siNS-transfected controls ($p < 0.001$ for three cytokines).

We next examined whether the knockdown of cathelicidin affects ROS production using the oxidative fluorescent dyepretreatment (Fig. 6, C and D). These data suggest that NOX2 is required for 1,25D$_3$-mediated antimicrobial activity against intracellular Mtb in human macrophages.

To determine whether the drop in antimicrobial activity was due to a decreased concentration of LL-37, we examined the NOX2-mediated regulation of antimicrobial activity in the presence of LL-37. In siNOX2-transfected THP-1 cells treated with 100 μg/ml LL-37 for 6 h, the viability of the intracellular bacteria was reduced by 30–40%, as shown in the siNS-transfected cells (Fig. 6E). Similarly, pretreatment with DPI and exposure to 50 μg/ml LL-37 reduced the viability of the intracellular bacteria by 30–40% in human MDMs, as shown in the solvent control-treated cells (Fig. 6F). Moreover, the viability of the intracellular bacteria was reduced by 25–35% in THP-1 cells transfected with a LL-37 plasmid, compared with those transfected with the mock control, in the presence of DPI (Fig. 6G). The immunoreactive band corresponding to LL-37 was detected in the cells overexpressing LL-37 (Fig. 6G, top). These data suggest that the NOX2-dependent induction of cathelicidin is at least part of a macrophage defensive strategy against Mtb.
The present study is the first to demonstrate an association between TLR2 and NOX2 at 1 min and 30 min (see Fig. 3A). Previously, it was demonstrated that NOX2 interacts with TLR2 at lipid rafts and its early association with NOX2 may contribute to the robust ROS release that is crucial for proinflammatory responses in macrophages. Moreover, the current data demonstrate that a late interaction at 30 min occurred for the Mtb in the region of phagosomes where NOX2 is present along with TLR2 (see Fig. 3B). Previously, it was demonstrated that NOX2 is recruited to immature dendritic cell phagosomes where it causes active, sustained phagosome alkalization, thereby playing a critical role in giving dendritic cells the ability to function in Ag cross-presentation to CD8⁺ T cells (37). Therefore, the NOX2 interaction with TLR2 at plasma membrane and phagosomal membranes may contribute to both innate immunity through ROS generation and to adaptive immunity through the regulation of Ag cross-presentation by dendritic cells to CD8⁺ T cells.

Receptor-mediated ROS production is coupled with NOX isozymes (e.g., NOX1, NOX3, NOX4, and NOX5), novel homologues of NOX2 in phagocytic cells (38, 39). Previous studies demonstrated that the TIR domain of TLR4 associates with the C-terminal region of NOX4 in HEK293 cells (14). This association is functionally important, because the knockdown of NOX4 inhibits LPS-mediated NF-κB activation and ROS production (14). Recent studies have shown that this interaction involves aa 451–530 of NOX4 and the TIR domain of TLR4 (40). However, no mechanistic link between TLR2 and NOX2 has been reported. The present study is the first to demonstrate an association between the C-terminal region of NOX4 and the TIR domain of TLR2. The TIR domain of TLR2 functions in heterodimerization and as the binding site for other cytosolic adaptor proteins containing a TIR domain such as MyD88 and TIRAP to the receptor, leading to the formation of the IRAK and TRAF6 complexes (33). These proteins are involved in the TLR-mediated regulation of MAPK activation and immune responses. The knockdown of NOX2 significantly attenuated TLR2/MyD88-mediated ROS production and inflammatory signaling activation. Moreover, TLR2/MyD88-induced ROS production and NOX activation in macrophages were significantly dependent on TLR2, indicating a functional interaction between TLR2 and NOX2. The functional link between TLR2 and NOX2 appears to be involved in the regulation of inflammatory signaling pathways leading to the activation of innate immune responses against Mtb infection.

Before the availability of anti-TB drugs, vitamin D was used in the treatment of patients with cutaneous TB and was reported to have dramatic effects (41). Recent studies have demonstrated that antimicrobial activity against intracellular Mtb is mediated by 1,25D₃-induced cathelicidin expression in human macrophage-like THP-1 cells (19). However, the molecular mechanisms regulating

**Discussion**

Mycobacteria and its Ags recognize distinct TLRs, resulting in the rapid activation of the cells in the innate immune system that leads to MyD88-dependent activation of proinflammatory and antibacterial effector pathways and the production of proinflammatory cytokines such as TNF, IL-12, chemokines, and NO (33, 34). The regulation of the amplitude and the qualitative nature of TLR-induced inflammatory responses are fine tuned by crosstalk among several signaling pathways (33, 35). The molecular mechanisms underlying the signal transduction network between TLRs and other signaling pathways are largely unknown, and they may be regulated in a dynamic manner. Elucidating the regulatory mechanisms involved in this crosstalk may offer a molecular basis for therapeutic approaches to mycobacterial infection. In this study, we investigated how NOX2 interacts with and coordinates the TLR2 signals required for efficient innate immune responses and the intracellular control of Mtb through the induction of cathelicidin.

TLR2 is essential for Mtb-induced ROS production and NOX activation in macrophages. These data concur with our previous findings that tuberculin PPD-induced ROS production is dependent on TLR2 in monocytes/macrophages (13). In addition, our study shows that Mtb treatment triggered biphasic association of TLR2 and NOX2 at 1 min and 30 min (see Fig. 3A). Early association between TLR2 and NOX2 was detected at the plasma membranes of cells. We previously demonstrated that the Mtb 19-kDa lipoprotein, a TLR2/1 agonist, results in the translocation of TLR2 to lipid rafts in the plasma membranes of cells within 1 min (36). Together with our previous data (36), the recruitment of TLR2 to lipid rafts and its early association with NOX2 may contribute to the robust ROS release that is crucial for proinflammatory responses in macrophages. Moreover, the current data demonstrate that a late interaction at 30 min occurred for the Mtb in the region of phagosomes where NOX2 is present along with TLR2 (see Fig. 3B). Previously, it was demonstrated that NOX2 is recruited to immature dendritic cell phagosomes where it causes active, sustained phagosome alkalization, thereby playing a critical role in giving dendritic cells the ability to function in Ag cross-presentation to CD8⁺ T cells (37). Therefore, the NOX2 interaction with TLR2 at plasma membrane and phagosomal membranes may contribute to both innate immunity through ROS generation and to adaptive immunity through the regulation of Ag cross-presentation by dendritic cells to CD8⁺ T cells.

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cathelicidin expression in human macrophages are largely unknown. Notably, NOX2-derived ROS production was required for the mRNA expression of cathelicidin and the subsequent antimycobacterial activities induced by 1,25D$_3$-treated macrophages. Previous studies showed that the reactive oxygen intermediate scavenger 4-hydroxy-TEMPO, degradative enzymes, and polyethylene glycol coupled to superoxide dismutase or catalase abrogated 1,25D$_3$-induced antimycobacterial activity, suggesting that 1,25D$_3$-induced antimycobacterial activity is mediated by NOX in phagocytes (42). In human dendritic cells, NOX-derived oxygen radicals are not required for dendritic cell differentiation, maturation, cytokine production, or the induction of T cell proliferation; however, they are essential for intracellular bacterial killing (43). Our data partly concur with and extend the recent study (44) demonstrating that 1,25D$_3$ strongly up-regulates the cathelicidin gene and protein expression via a NOX2-dependent manner (see Fig. 5). Moreover, either the addition of active peptide LL-37 or the overexpression of LL-37 ROS signaling may play an essential role in the antimycobacterial effects through 1,25D$_3$-induced cathelicidin expression in human macrophages.

Recently, LL-37 was shown to not only have antimicrobial effects against a variety of pathogens, but also to play roles in the regulation of inflammatory and immune responses, the chemotraction of immune or inflammatory cells to sites of infection/inflammation, and the acceleration of angiogenesis (45). Recent studies have also shown that LL-37 enhances IL-8 production under the control of p38 MAPK and ERK1/2, which are phosphorylated by LL-37 (46). That same study also revealed that LL-37 stimulates ROS production, probably via NOX activation and intracellular Ca$^{2+}$ mobilization in human peripheral blood neutrophils (46). In addition, LL-37 activates airway epithelial cells through the activation of ERK and increases the release of IL-8 (32). Moreover, LL-37 can selectively reinforce specific immune responses in the presence of immune mediators such as IL-1β and GM-CSF (47). Consistent with these data, we showed the immunomodulatory role of cathelicidin in Mtib-induced ROS release and the production of proinflammatory cytokines and IL-8 in human macrophages. Of note, the transcription of the cathelicidin gene seems to be triggered at a very early time (see Fig. 5B), supporting a role for cathelicidin as a second messenger in inflammatory signaling. These data partly concur with previous studies in which cathelicidin gene transcription was increased significantly after 1 h of infection with Mtib or Salmonella typhi (20). Therefore, our data suggest that cathelicidin is one of the mediators through which macrophages regulate the activation of ROS and inflammatory immune responses to Mtib in macrophages.

In summary, our data demonstrate the crucial role of NOX2 in the induction of TLR2-mediated innate immune responses and the intracellular control of mycobacteria through the modulation of cathelicidin expression in macrophages. Our findings suggest the molecular mechanisms underlying the induction and expression of the antimicrobial peptide cathelicidin and imply that the peptide is required for 1,25D$_3$-mediated antimicrobial activity against intracellular Mtib in THP-1 cells (19). In addition, our data indicate that the induction of cathelicidin provides a feedback loop for Mtib/TLR2-induced ROS production and inflammatory responses in primary human macrophages. Because HK-Mtb showed results comparable with those induced by viable Mtib, the observed effects can be induced by TLR2 ligation and are independent of macrophage infection. Overall, our results illuminate a novel crosstalk between NOX2 and TLR2, which is critical for the initiation of inflammatory responses and 1,25D$_3$-mediated antimicrobial activity against Mtib via cathelicidin expression. A better understanding of signaling pathways for antimycobacterial peptides may suggest a novel mechanism for enhancing therapeutic modalities for TB by promoting an efficient innate response to this nefarious pathogen.

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Disclosures

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