Inflammatory response is modulated by lincRNA Cox2 via the NF-κB pathway in macrophages infected by Mycobacterium tuberculosis

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Received November 19, 2019; Accepted February 26, 2020
DOI: 10.3892/mmr.2020.11053

Abstract. Long intergenic non-coding RNAs (lincRNAs) are long non-coding transcripts from the intergenic regions of annotated protein-coding genes. lincRNA cyclooxygenase 2 (Cox2) is an early-primary response gene regulated by the NF-κB signaling pathway in macrophages. It was found that lincRNA Cox2 was significantly increased in patients with the Mycobacterium tuberculosis (M. tuberculosis) H37Ra strain infection and macrophages, using reverse transcription-quantitative PCR (RT-qPCR), ELISA, western blotting and RT-qPCR results indicated that the inflammatory response factors tumor necrosis factor-α, interferon-γ, interleukin-6, Cox2 and inducible nitric oxide synthase were significantly increased in H37Ra infected macrophages. In addition, the inflammatory regulating proteins NF-κB and Stat3 were significantly increased in H37Ra infected macrophages but decreased in lincRNA Cox2 knockdown macrophages infected with H37Ra. Moreover, the knockdown of lincRNA Cox2 increased the apoptotic rate of H37Ra infected macrophages and facilitated the proliferation of H37Ra. Collectively, the present results suggested that lincRNA Cox2 may be required for the activation of NF-κB and Stat3, in order to regulate inflammatory responses involved in resistance to M. tuberculosis infection.

Introduction

Mycobacterium tuberculosis (M. tuberculosis) is the causative agent of tuberculosis (TB) and is a major public health threat associated with high morbidity and mortality rates worldwide (1). Currently, >30% of the population worldwide is infected with M. tuberculosis according to data from the World Health Organization (2). The host immune response against M. tuberculosis is complex and multifaceted, thus understanding the pathogenesis and host immune mechanisms against TB will facilitate the development of novel diagnostic and therapeutic strategies for TB (3). Macrophages are critical immune cells that play important roles in the host immune system by phagocytizing M. tuberculosis to help eliminate infection, as well as initiating the protective immune responses by presenting antigens to T lymphocytes (4,5). Macrophages can prevent the intracellular growth and persistence of M. tuberculosis during various phases of TB, from primary infection with bacillary dissemination (6). These previous studies have revealed the functions of macrophages in M. tuberculosis infection, but the underlying regulatory mechanisms are still not fully understood.

Long non-coding RNAs (lncRNAs) are transcripts ~200 nucleotides in length that regulate the expression of protein encoding genes at the transcriptional and post-transcriptional levels (7). By dysregulating target genes, lncRNAs can participate in the development and progression of various human diseases, including cancer, inflammation and autoimmune diseases (8‑10). The regulatory potential of lncRNAs in epigenetic reprogramming is emerging as a novel mechanism to explain functional plasticity and the diversity of immune cells including T cells, B cells, dendritic cells and macrophages. Previous studies have shown that lncRNAs also play an essential role in human infectious diseases such as TB (11,12). Pawar et al (13) showed that downregulation of the lncRNA maternally expressed 3 promotes the eradication of intracellular mycobacterium in M. bovis Bacille Calmette Guerin-infected macrophages.

The aim of the present study was to investigate the regulatory mechanisms of the inflammatory response modulated by long intergenic non-coding RNA cyclooxygenase 2 (lincRNA Cox2) in M. tuberculosis H37Ra infected macrophages.

Materials and methods

Samples collection and ethical statement. The peripheral blood samples (3 ml) were obtained from 56 patients with
active pulmonary TB (age range, 22-62 years; median age, 36 years; sex, male: female=3:5) and 60 healthy individuals (age range, 20-60 years; median age, 32 years; male:female=4:6) in the China-Japan Friendship Hospital from June 2015 to June 2017. Written informed consent was obtained from all donors prior to the study. This study was approved by the Ethics Committee of the China-Japan Friendship Hospital (grant no. ZRYYE/C/2015/27-2).

Cell culture. THP-1 cells were purchased from the American Type Culture Collection and cultured in RPMI-1640 (Gibco; Thermo Fisher Scientific, Inc.) with 10% FBS (Gibco; Thermo Fisher Scientific, Inc) and antibiotics (100 U/ml penicillin and 100 mg/ml streptomycin) at 37°C under the humidified atmosphere of 5% CO2. The attenuated M. tuberculosis strain H37Ra was provided by the China Center for Disease Control. M. tuberculosis H37Ra was cultured on Middlebrooks 7H9 plates. M. tuberculosis H37Ra culture and related experiments were performed in the BSL-3 (P3) Laboratory of China Center for Disease Control.

THP-1 cells were differentiated into macrophages using Phorbol ester (0.01 mM; Merck KGaA) for 24 h and were then incubated with H37Ra at a multiplicity of infection of 10 (10 bacteria:1 cell) for 24 h at 37°C.

Small interfering RNA (siRNA) transfection and luciferase reporter assay. For gene silencing, siRNAs for lincRNAcox2 were synthesized and purchased from Invitrogen (Thermo Fisher Scientific, Inc.). The following siRNAs were used: Mock siRNA sense, 5'-UAAGGCUAUAAGACUUAUCAU-3' and antisense 5'-GAUACUCAUCUAUAGGCUAUAU-3'; si-lincRNAcox2 siRNA sense, 5'-GCCUAUAAUGGUUGUUAUCAU-3' and antisense, 5'-ACAACCCACUAUAAUGGGCUA-3' and lincRNAcox2 sense, 5'-AGTATGGGATAACAGCTGAGGT-3' and antisense, 5'-GAATGCTGAGAGTGAGGAAATAAAG-3'. Macrophages were transfected with siRNAs (final concentration, 20 nM) using Lipofectamine RNAiMax reagent (cat. no. 13778030; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol for 24 h. The lincRNAcox2 expression vector was generated by reverse transcription-quantitative PCR (RT-qPCR) amplification of lincRNAcox2 cDNA using RNA from macrophages and cloned into a pcDNA3.3 vector (Thermo Fisher Scientific, Inc.). The promoter of lincRNAcox2 was amplified by PCR from human macrophage genomic DNA. Macrophages were transfected with each reporter construct for 24 h, followed by assessment of luciferase activity.

RT-qPCR and rapid amplification of cDNA ends (RACE) PCR. Total RNA was extracted with TRIzol solution (cat. no. R0016; Beyotime Institute of Biotechnology) according to the manufacturer's protocol. All primers for PCR analysis were designed and synthesized by Invitrogen (Thermo Fisher Scientific, Inc.). RT-qPCR was performed on a Master-cycler (Eppendorf Corp.) using the SYBR Green PCR Master Mix [cat. no. R101T; Takara Biotechnology (Dalian) Co., Ltd.]. The PCR conditions were as follows: Initial denaturation at 95°C for 25 sec, followed by 36 cycles at 95°C for 20 sec, 58°C for 25 sec and 72°C for 15 sec. U6 was used as an internal control and the relative expression of genes was calculated with the 2-ΔΔcq method (14). Primers used were as follows: lincRNAcox2 forward, 5'-AGTATGGGATAACAGCTGAGGT-3' and reverse, 5'-GAATGCTGAGAGTGAGGAAATAAAG-3'; and U6 forward, 5'-CGCTTCGGGCATACATACTA-3' and reverse, 5'-CGCTTCGAATTTGCGCTGCA-3'.

RACE PCR was used to identify the 5' and 3' ends of lincRNAcox2 to localize the transcriptional start site. The SMART RACE cDNA Amplification kit (cat. no. 634923; Clontech Laboratories, Inc.) was used and the primers for 5' and 3' ends of lincRNAcox2 RACE PCR analysis were designed and synthesized by Invitrogen (Thermo Fisher Scientific, Inc.).

ELISA assay. ELISA kits were used to detect the levels of the cytokines tumor necrosis factor (TNF)-α (cat. no. EK0526; Wuhan Boster Biological Technology, Ltd.), interferon (IFN)-γ (cat. no. EK0373; Wuhan Boster Biological Technology, Ltd.) and interleukin (IL)-6 (cat. no. EK0411; Wuhan Boster Biological Technology, Ltd.) in the plasma of patients with TB infection and in cell supernatant of macrophages infected with H37Ra, according to the manufacturer's protocol. Cytokine concentrations were calculated using a standard curve.

Western blotting. Cell lysates were obtained with RIPA lysis buffer (cat. no. P0013B; Beyotime Institute of Biotechnology) and were quantified using a bicinchoninic acid kit (cat. no. P0012S; Beyotime Institute of Biotechnology). Proteins (30 µg) were run on 10% SDS-PAGE and transferred to PVDF membranes (EMD Millipore). After blocking with 5% non-fat milk for 1 h at room temperature, the membranes were incubated overnight at 4°C with the following antibodies according to the manufacturer's protocol: Cox2 (cat. no. sc1745; Santa Cruz Biotechnology, Inc.; 1:800), inducible nitric oxide synthase (iNOS; cat. no. sc-7271; Santa Cruz Biotechnology, Inc.; 1:1,200), Stat3 (cat. no. sc-8019; Santa Cruz Biotechnology, Inc.; 1:600), NF-κB (cat. no. sc-8414; Santa Cruz Biotechnology, Inc.; 1:1,000) and GAPDH (cat. no. sc-47724; Santa Cruz Biotechnology, Inc.; 1:2,000). Then, membranes were incubated with the goat anti-rabbit immunoglobulin G-horseradish peroxidase (cat. no. sc-2004; Santa Cruz Biotechnology, Inc; 1:4,000). Specific protein bands were detected using the Chemiluminescent Substrate system (Thermo Fisher Scientific, Inc.). The band intensity was measured using ImageJ 1.47 (National Institutes of Health).

Analysis of the apoptotic rate of H37Ra infected macrophages. Macrophages transfected with si-lincRNAcox2 or lincRNAcox2 were infected with 1x105 colony forming units (CFU) of H37Ra for 24 h at 37°C and then cultured for 24 h in medium at 37°C. Then, cells were harvested and incubated with Annexin-V/PI (cat. no. C1062L; Beyotime Institute of Biotechnology) for 30 min in the dark on ice at 4°C. Apoptotic cells were detected using a DxFLEX flow cytometer (Beckman Coulter, Inc.) and analyzed using CytExpert version 2 software (Beckman Coulter, Inc.), according to the manufacturer's protocol.

Proliferation analysis of H37Ra. Macrophages were transfected with si-lincRNAcox2 or lincRNAcox2 for 24 h, then
were infected with H37Ra (1x10^5 CFU) for 24 h. For titration of intracellular H37Ra, macrophages were lysed with 0.025% SDS, neutralized with 0.5% albumin and grow on Middlebrooks 7H9 plates (cat. no. G0Y-PYJ0567; Shanghai Guyan Biotechnology Company). Then, H37Ra titration was determined after 3, 6, 9, 12 and 15 days to analyze their proliferative capacity by bacteria plate count.

Statistical analysis. Data are presented as the mean ± SEM from ≥3 independent experiments. Data were compared with an unpaired Student's t-test or one-way analysis of variance with Tukey's post hoc test. All statistical tests were performed with GraphPad Prism 6.0 (GraphPad Software, Inc.). P<0.05 was considered to indicate a statistically significant difference.

**Results**

Expression of lincRNAcox2 and inflammatory responses were analyzed in patients with TB. lincRNAcox2 is a critical gene for regulating the expression level of NF-κB in macrophages and NF-κB is an important protein regulating the inflammatory pathway especially in the process of tuberculosis infection. Thus the expression of lincRNAcox2 was investigated in TB patients. The expression levels of lincRNAcox2 were determined by RT-qPCR in the plasma (Fig. 1A) and mononuclear cells of patients with TB (Fig. 1B). It was found that lincRNAcox2 was significantly increased in plasma and mononuclear cells of patients with TB compared with healthy individuals. Moreover, the expression levels of TNF-α, IFN-γ and IL-6 in the plasma of patients with TB were significantly...
increased compared with healthy individuals (Fig. 1C). In addition, the expression levels of Cox2 and iNOS in mononuclear cells of patients with TB were significantly elevated both at mRNA (Fig. 1D) and protein levels (Fig. 1E and F).

**Expression of lincRNA>Cox2 and inflammatory responses in H37Ra infected macrophages.** Previous studies have shown that lincRNA>Cox2 increased significantly in TB patients and TB infection macrophages in vivo and in vitro, so the function of lincRNA>Cox2 in an in vitro cell experiment was assessed. In vitro cell experiments, RT-qPCR and western blotting were performed to assess the expression level of lincRNA>Cox2 in macrophages after exposure to H37Ra. It was demonstrated that lincRNA>Cox2 was increased in H37Ra infected macrophages (Fig. 2A). Furthermore, the inflammatory factors TNF-α, IFN-γ and IL-6 were increased in the supernatant of H37Ra infected macrophages, as determined by ELISA (Fig. 2B). Moreover, the mRNA and protein expression levels of Cox2 and iNOS were significantly increased in H37Ra infected macrophages compared with the control group (Fig. 2C-E).

**lincRNA>Cox2 knockdown in macrophages.** The expression of lincRNA>Cox2 was enhanced in TB patients and TB infection macrophages in vivo and in vitro, then the effects of the knockdown of lincRNA>Cox2 in TB infected host cells will be studied. The siRNA in the present study was designed to target lincRNA>Cox2 and lincRNA>Cox2 was transfected into macrophages to induce its overexpression. lincRNA>Cox2 was knocked down using an RNA interference approach and the expression level of lincRNA>Cox2 was measured by RT-qPCR. RACE PCR analysis results identified a transcript of lincRNA>Cox2 in macrophages using a luciferase reporter vector (Fig. 3A). Furthermore, it was found that the expression level of lincRNA>Cox2 was significantly decreased when macrophages were transfected with si-lincRNA>Cox2 (Fig. 3B). However, H37Ra infection promoted the expression level of lincRNA>Cox2 in si-lincRNA>Cox2 transfected macrophages (Fig. 3C).

**Inflammatory responses in lincRNA>Cox2 knockdown macrophages are induced by H37Ra.** As lincRNA>Cox2 plays a...
Figure 3. Luciferase reporter assay of lincRNACox2 promoter infected macrophages. (A) Upstream sequence of lincRNACox2 (3 kb) was cloned and inserted into the pGL3-luciferase construct. (B) Luciferase activity assay results indicated that macrophages infected with si-lincRNACox2 significantly increased luciferase activity. (C) Expression level of lincRNACox2 in H37Ra infected macrophages transfected with siRNA were determined by RT-qPCR. Data are presented as the mean ± standard error of the mean from three independent experiments, *P<0.05 vs. Mock siRNA. RT-qPCR, reverse transcription-quantitative PCR; siRNA, small interfering RNA; lincRNACox2, long intergenic non-coding cyclooxygenase 2 RNA.

Figure 4. Inflammatory responses of H37Ra infected macrophages with lincRNACox2 knockdown. (A) Levels of TNF-α, IFN-γ and IL-6 in the supernatant of H37Ra infected macrophages with lincRNACox2 knockdown were determined by ELISA. (B) mRNA expression levels of Cox2 and iNOS in H37Ra infected macrophages with lincRNACox2 knockdown were detected by reverse transcription-quantitative PCR. (C) Protein expression levels and (D) analysis of Cox2 and iNOS in H37Ra infected macrophages with lincRNACox2 knockdown were determined by western blotting. Data are presented as the mean ± standard error of the mean from three independent experiments. *P<0.05 and **P<0.01 vs. Mock siRNA. lincRNACox2, long intergenic non-coding cyclooxygenase 2 RNA; TNF, tumor necrosis factor; IFN, interferon; IL, interleukin; iNOS, inducible NO synthase.
very important role in the inflammatory response pathway, so the inflammatory response of TB infected macrophages in which lincRNAcoX2 was knocked down was studied. After lincRNAcoX2 was silenced, macrophages were infected with H37Ra. It was demonstrated that macrophages transfected with si‑lincRNAcoX2 had significantly decreased expression levels of the inflammatory factors TNF‑α, INF‑γ, and IL‑6 compared with other groups (Fig. 4 a). Moreover, knockdown of lincRNAcoX2 reduced the mRNA and protein expression levels of COX2 and iNOS in H37Ra infection macrophages (Fig. 4B-D). Therefore, the present results suggested that the knockdown of lincRNAcoX2 suppressed the inflammatory response in H37Ra infected macrophages.

Inflammatory regulatory proteins Stat3 and NF‑κB are regulated by lincRNAcoX2. lincRNAcoX2 regulates the expression of inflammatory cytokines and inflammatory regulating proteins, so the inflammatory signaling pathways such as Stat3 and NF‑κB associated with lincRNAcoX2 were investigated. It was found that the knockdown of lincRNAcoX2 significantly inhibited the protein expression levels of NF‑κB and Stat3 in macrophages (Fig. 5A and B). Moreover, H37Ra significantly suppressed the expression levels of the inflammatory regulatory proteins Stat3 and NF‑κB in lincRNAcoX2 knockdown macrophages (Fig. 5C and D). Thus, the present results indicated that the NF‑κB pathway may be triggered by lincRNAcoX2 in macrophages. However, H37Ra is dependent on the expression level of lincRNAcoX2 to activate the NF‑κB signaling pathway to induce a cellular inflammatory response.

**Effect of lincRNAcoX2 on the apoptosis of H37Ra infected macrophages and the proliferation of H37Ra.** lincRNAcoX2 could regulate the inflammatory response of TB infection macrophages through Stat3 and NF‑κB signaling pathways, however the influences of lincRNAcoX2 on the apoptosis of H37Ra infected macrophages and the proliferation of H37Ra remains unknown. Macrophages transfected with si‑lincRNAcoX2 or lincRNAcoX2 were infected with H37Ra and the effect of lincRNAcoX2 on the apoptosis and proliferation of H37Ra infected macrophages was investigated. It was demonstrated that knockdown of lincRNAcoX2 significantly increased the apoptotic rate of H37Ra infected macrophages. However, the apoptotic rate of H37Ra infected macrophages with lincRNAcoX2 overexpression was significantly decreased compared with control groups (Fig. 6A). Moreover, knockdown of lincRNAcoX2 promoted the proliferation of H37Ra extracted from macrophages, but the overexpression of lincRNAcoX2 in H37Ra infected macrophages could suppress H37Ra proliferation via various mechanisms, including cytokine production and oxygen/nitrogen metabolism (Fig. 7).
Discussion

Previous studies have showed that IncRNAs play significant roles in various biological processes, such as the immune response, tumorigenesis and cellular infectious diseases (17-20). Interactions between macrophages and \textit{M. tuberculosis} can alter genetic material expression profiles, including circular RNAs expression profiles and IncRNA expression profiles. Examining these changes can improve the understanding of the regulation of anti-mycobacterium immunity in macrophages (21,22). Furthermore, previous studies have demonstrated IncRNAs can be used as biomarkers or therapeutic targets for various diseases (23,24). Several IncRNAs, such as miR3945HG-V1 and miR3945HG-V2, are differentially expressed in H37Ra or H37Rv infected macrophages, and could be used as novel diagnostic biomarkers for TB. Thus, this would provide insight into the mechanisms of \textit{M. tuberculosis} macrophage interactions and identify potential targets for the diagnosis and treatment of TB (25). LncRNA-TNF and HNRNPL related immunoregulatory IncRNA in THP-1 macrophages can modulate the expression levels of TNF-\textalpha{} and other inflammatory genes to regulate inflammation (26). Moreover, lncRNA-exopolysaccharide can regulate the inflammatory response in macrophages exposed to microbial ligands via the regulation of interferon regulated gene expression (27). Furthermore, lncRNA-Cox2 can mediate both the activation and repression of different immune genes.

The present results suggested that lncRNA-Cox2 was increased in patients with TB and H37Ra infected macrophages, and that the inflammatory factors TNF-\textalpha{}, IFN-\gamma{}, IL-6, Cox2 and iNOS were increased by lncRNA-Cox2 in vivo and in vitro. Thus, lncRNA-Cox2 may be a critical mediator for the development of inflammatory responses in both microglia and macrophages. The present study knocked down the expression of lncRNA-Cox2 in H37Ra infected macrophages and found this could suppress the inflammatory factors TNF-\textalpha{}, IFN-\gamma{}, IL-6, Cox2 and iNOS in these macrophages, which indicated that lncRNA-Cox2 may regulate the inflammatory response of H37Ra infected macrophages.

lncRNA-Cox2 expression is influenced by NF-\textkappa{}B signaling and serves as a coactivator of transcriptional factors to regulate inflammatory responses (28). NF-\textkappa{}B is composed of homo- or heterodimeric complexes of NF-\textkappa{}B subunits including p65, RelB, c-Rel, p50 and p52 in humans, and is an important regulator of inflammatory and immune responses (29). However,
the dysregulation of the NF-κB signaling pathway is linked to cancer, inflammation, autoimmune diseases and infectious diseases (30,31). Furthermore, numerous acute and late proinflammatory genes, critical to the pathogenesis of sepsis, are controlled by the NF-κB-gene (32). A nuclear NF-κB paradox has been identified during severe systemic inflammation, as defined by a disassociation between cytosolic NF-κB activation and nuclear NF-κB transcriptional processes (33). Previous studies have demonstrated the inflammatory response is induced in macrophages due to lipopolysaccharide stimulation via the NF-κB signaling pathway (34,35).

In the present study, the inflammatory response related signaling pathway involving Stat3 and NF-κB was analyzed in H37Ra infected macrophages transfected with si-lincRNA-Cox2. The present results suggested that the expression of lincRNA-Cox2 is induced by the NF-κB signaling pathway, potentially via binding of NF-κB subunits to the promoter region of the gene locus. The induction of lincRNA-Cox2 in patients with TB and H37Ra infected macrophages, along with the expression of the inflammatory factors TNF-α, IFN-γ, IL-6, Cox2 and iNOS, suggested that NF-κB-mediated lincRNA-Cox2 expression and its subsequent impact on the transcription of inflammatory genes may occur in vivo during systemic inflammation. In addition, knockdown of lincRNA-Cox2 promoted the proliferation of H37Ra via the inflammatory pathway or oxygen/nitrogen metabolism, thus indicating that lincRNA-Cox2 may have a central role in M. tuberculosis infection.

In conclusion, the present results suggested that lincRNA-Cox2 may be used as a novel biomarker for the diagnosis and therapeutic target of TB. However, the function of lincRNA-Cox2 in regulating the immune response of macrophages infected with H37Ra is still not fully understood.

Acknowledgements

Not applicable.

Funding

No funding was received.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors’ contributions

DL and YZ designed the experiments; CG and LZ performed the experiments and analyzed the data; DL and YZ drafted and revised the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The present study was approved by the Ethics Committee of the China-Japan Friendship Hospital (grant no. ZRYYEC/2015/27-2). Written informed consent was obtained from all donors prior to the study.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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