Original Article

IncRNA-cox2 enhance the intracellular killing ability against mycobacterial tuberculosis via up-regulating macrophage M1 polarization/nitric oxide production

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Abstract: Objective: In the present study, we demonstrate a novel role of IncRNA-cox2 in regulating the anti-mycobacterial response. Methods: The expression profile of IncRNA-cox2 during mycobacterial infection was analyzed by using quantitative reverse-transcriptase polymerase chain reaction (qRT-PCR). Examination of IncRNA-cox2 function was performed by transient transfecting with IncRNA-Cox2 expression vector and small interfering RNA (siRNA). The potential target of IncRNA-cox2 was identified by RNA Immunoprecipitation (RIP) assay. Results: We found that knockdown of IncRNA-cox2 suppress the elimination of intracellular Bacille Calmette-Guerin, and this process was through inhibition of M1 polarization/nitric oxide production, which was determined by IncRNA-cox2 siRNA transfection, together with related inflammatory factors treatment. Further chromatin Immunoprecipitation (ChIP) assay demonstrated that IncRNA-cox2 could enhance the NF-κB p65 and p50 binding to the iNOS promoter, and therefore mediated nitric oxide production. Conclusion: These results indicated that the IncRNA-cox2 expression level plays a key role in immunity response against MTB and this might provide potential targets for the future treatment of tuberculosis infection.

Keywords: IncRNA, tuberculosis, polarization, NF-κB, nitric oxide

Introduction

Mycobacterium tuberculosis (MTB) is one of the most troublesome human pathogenic bacteria infecting over one third of the world’s population, which results in more mortality than any other infectious agent [1]. Macrophages play pivotal role in innate immune responses against wide range of pathogens including MTB [2]. Studies have elucidated part of the mechanism of macrophages in limiting the growth of mycobacteria [3]. However, MTB is able to arrest normal phagosome maturation, avoid fusion with lysosomes, and render the intraphagosomal environment more compatible with bacterial survival and replication. Thus, critical balance between the macrophage and mycobacteria’s interactions could be instrumental in determining the outcome of infection. During the coexistence with host, mycobacteria consist of a complex network within macrophages to precisely regulate the immune response [3].

The human transcriptome comprises not only large numbers of protein-coding messenger RNAs (mRNAs), but also a large set of non-protein coding transcripts that have structural, regulatory, or unknown functions. Noncoding RNAs are divided into long noncoding RNAs (lncRNAs) and short noncoding RNAs (microRNAs) according to their length. Long noncoding RNA (lncRNAs), tentatively defined as noncoding RNAs more than 200 nt in length, are characterized by the complexity and diversity of their sequences and mechanisms of action [4-6]. Lately, sporadic publications reported that IncRNA might play important roles during cellular development and their misregulation has been shown in various types of diseases such as cancers [7-10]. However, the report of potential role of IncRNAs in the immune response to an intracellular pathogen is rare. IncRNA-cox2, a recently identified long noncoding RNA located ~51 kb upstream of the protein-coding gene cyclooxygenase-2 (COX-2), has
been confirmed to play important role in immunity regulation [11-13]. For this reason, we supposed that IncRNA-cox2 might be involved in the intracellular TB clearance.

**Material and methods**

**Strains and CFU determination**

Bacille of Calmette-Guérin (BCG) were purchased from Beijing Institute for Tuberculosis Control and were cultured by Lowenstein-Jensen (LJ) medium before used. Cultured cells were homogenized and viable bacteria were grown on certain medium and enumerated by the pour plate method after serial dilution. Colonies for BCG were determined 21 days after incubation at 36.5°C.

**Cell culture and bacterial infection**

RAW 264.7 were seeded to the wells of a 12-well plate and grown to 80% confluency, and then were directly infected with BCG for 4 h at a ratio of 10 CFU per macrophage. Infected cells were then washed three times with RPMI to remove all the non-phagocytosed bacteria.

**Plasmid, siRNA and cell transfection**

The IncRNA-Cox2 expression vector and siRNA of IncRNA was constructed according to the previous report [13]. Transfections were performed using Lipofectamine 2000 (Invitrogen).

**Signal inhibitors**

NF-κB (BAY-117082; cat. no. YE1330), MYD-88 (ST2825; cat. No. YE2035), p38 MAPK (SB203580; cat. no. YE0411), ERK1/2 (U0126; cat. no. YE1161) and JNK (SP600125; cat. no. YE0021) inhibitors were purchased from Amquar Biological Technology Co., Ltd. (Shanghai, China). The macrophages were incubated in 6-well plates (10⁶ cells/well). The inhibitors BAY-117082 (20 μM), ST2825 (20 μM), SB203580 (20 μM), U0126 (10 μM) and SP600125 (20 μM) were added to the appropriate well and incubated at 37°C for 45 min. The cells were subsequently incubated at 37°C for 24 h prior to being harvested.

**In vivo infection**

Mice were aerosol infected with approximately 100 CFU of bacteria/lung using an intratracheal injection, according to previous report [14]. At given time points, organs were harvested and homogenized, and serial dilutions of tissue homogenates were plated on 7H11 agar plates supplemented with oleic albumin dextrose catalase (OADC). Additionally, lung samples were processed for RNA extraction, histology, and cytokine determination. The animal study protocols were approved by the Institutional Animal Care and Use Committees of PLA 309 hospital.

**Quantitative RT-PCR**

RNA was extracted with an RNeasy kit (Qiagen, Dusseldorf, Germany), and cDNA was synthesized using SuperScript III RT (Invitrogen, Carlsbad, CA, USA). An ABI 7900 real-time PCR system was used for quantitative PCR, with primer and probe sets obtained from Applied Biosystems. Results were analyzed using SDS 2.1 software. The expression of each target gene is presented as the “fold change” relative to that of control samples. The mRNA level of β-actin was used as an internal control. The sequences of primers used are shown in Table 1.

**Western blot**

Cells were washed with ice-cold phosphate-buffered saline (PBS) and then lysed with protein lysis (Pierce, Rockford, IL, USA). After centrifugation at 5,000 × g for 15 min at 4°C, the protein concentration was measured with a bicinchoninic acid (BCA) protein assay kit (Pierce). Fifty microgram aliquots of lysates were loaded on a sodium dodecylsulfate (SDS) polyacrylamide 10% gradient gel and transferred to a polyvinylidene difluoride membrane. The membranes were blocked with 5% nonfat dry milk in Tris-buffered saline, pH 7.4, containing 0.05% Tween 20, and were incubated with primary antibodies (1:200; Santa Cruz, Delaware Avenue, CA, USA) and horseradish peroxidase-conjugated secondary antibodies (1:5000; Santa Cruz) in accordance with manufacturer’s instructions. The protein of interest was visualized using an enhanced chemiluminescence (ECL) Western blotting substrate (Pierce) and the Chemidoc XRS Gel Documentation System (BioRad).

**Flow cytometry**

For flow cytometry method analysis of cell-surface markers, cells were stained with antibodies in PBS containing 0.1% (wt/vol) BSA and
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Flow cytometry data were acquired on a FACSCalibur (Becton Dickinson) or a Beckman Coulter Epics XL benchtop flow cytometer (Beckman Coulter, Brea, CA, USA), and data were analyzed with FlowJo Software (TreeStar, Ashland, OR, USA). Cell numbers of various populations were calculated by multiplying the percentage of interested cells with the total cell number.

Enzyme-linked immunosorbent assay (ELISA) and Griess assay

Supernatants from the indicated cells were harvested, and IL-6, IL-12p40 and TNF-α protein levels were tested using ELISA kit (BD Bioscience) following the manufacturer’s instructions. Nitric oxide production was determined by measuring its stable end product nitrite, using a Griess reagent (Promega Corporation, Madison, WI) according to manufacturer’s protocol.

RNA immunoprecipitation (RIP) assay

The formaldehyde crosslinking RIP was performed as described [13]. Briefly, cells in culture were treated with formaldehyde at a final concentration of 0.3% (v/v) at room temperature for 10 min. Crosslinking reactions were quenched by the addition of glycine (pH 7.0) to a final concentration of 0.25 M. The cells were then harvested and nuclei pellets were collected. The crosslinked complexes were then incubated with the specific antibody-coated beads. Formaldehyde cross-links were reversed by incubation at 65°C with rotation for 4 h. Presence of RNA was measured by quantitative, strand-specific RT-PCR using the iCycler iQ Real-time detection system (BioRad).

Chromatin immunoprecipitation (CHIP) assay

CHIP analysis was carried out according to the Simple ChiP Enzymatic Chromatin IP Kit protocol (Cell Signalling). After infection and treatment, RAW 264.7 cells (4 × 10⁶) were submitted to CHIP assay as described [13]. The chromatin was immunoprecipitated with anti-p50 (Millipore 06-886) antibodies at 4°C under rotation for 16 h. The DNA isolated from immunoprecipitated material was amplified by real-time PCR for iNOS promoter: forward 5’-ACAAGACTAGGAGTGATCATG-3’ and reverse 5’-ACAAAGACCCAAGGCTCC-3’.

Statistical analysis

The results are expressed as means ± SD from at least 3 separate experiments performed in triplicate. The differences between groups were determined using two-tailed Student’s t-test or one-way analysis of variation (ANOVA), using SPSS software (Armonk, NY, USA). P values of less than 0.05 were considered significant.

Results

LncRNA-cox2 significantly increased in BCG challenged murine macrophage

Using the BCG infected RAW 264.7 cells, we found that lncRNA-cox2 expression was increased under Mycobacterial challenging in a time-dependent manner (Figure 1A) and dosage dependent manner (Figure 1B). The same tendency was also observed in H37Ra challenging (Figure 1C, 1D). We pretreated RAW 264.7 cells respectively with NF-κB, MYD88, p38 MAPK, ERK1/2 or JNK inhibitors, followed by exposure to BCG for 24 h. As shown in Figure 1E, the NF-κB inhibitor (BAY-117082) showed the strongest suppression in the lncRNA-cox2 expression, followed by JNK inhibitor (SP600125), p38MAPK inhibitor (SB203580) and MYD88 inhibitor (ST2825), suggesting that the involved pathway was important in regulating lncRNA-cox2 expression (Figure 1E). Since the

Table 1. Sequences of oligonucleotides used in this article

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence</th>
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<tbody>
<tr>
<td>Mus β-actin forward</td>
<td>5’-GGACTCCTATGTTGGGCGAGG-3’</td>
</tr>
<tr>
<td>Mus β-actin reverse</td>
<td>5’-GGGAGACGACCATCGCTGATG-3’</td>
</tr>
<tr>
<td>Mus lncRNA-cox2 forward</td>
<td>5’-AGAAGACTTGCGGTGTGA-3’</td>
</tr>
<tr>
<td>Mus lncRNA-cox2 reverse</td>
<td>5’-GAAGAGCTAGAGCTTATATG-3’</td>
</tr>
<tr>
<td>Mus TNF-α forward</td>
<td>5’-CTCGTGAACGACACACAGA-3’</td>
</tr>
<tr>
<td>Mus TNF-α reverse</td>
<td>5’-TTGAAGAGAAGACCTTGAGAGACA-3’</td>
</tr>
<tr>
<td>Mus IL-6 forward</td>
<td>5’-TGAGAATCTGAAATGAGAG-3’</td>
</tr>
<tr>
<td>Mus IL-6 reverse</td>
<td>5’-CTCTGAAAGACTCTGCTGTTG-3’</td>
</tr>
<tr>
<td>Mus β-actin forward</td>
<td>5’-GACATCAGTCAAGAGTTCTAGAT-3’</td>
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<td>Mus β-actin reverse</td>
<td>5’-AGGAAGCTTGTITTTGAATAATTTTAA-3’</td>
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</table>
Function of lncRNA-cox2 against TB infection

Mouse model represents a tractable in vivo tool for investigating TB pathogenesis in molecular terms, we examined the expression of lncRNA-cox2 during experimental aerogenic TB in mice. We found that expression of lncRNA-cox2 was enhanced rapidly 2 d after BCG infection (P<0.05), reached its highest levels by 5 d, and slowly decreased by 14 d (Figure 1F).

Figure 1. lncRNA-cox2 expression is induced after mycobacterial infection in cultured cells. (A and B) RAW 264.7 cells were infected with (A) BCG or (B) H37Ra at an MOI of 10 for the indicated time points. The expression levels of lncRNA-cox2 were examined by realtime PCR. ANOVA was used to determine the significance of the differences. (C and D) RAW 264.7 cells were infected with (C) BCG or (D) H37Ra at indicated MOI for 24 h. The expression levels of lncRNA-cox2 were examined by realtime PCR. ANOVA was used to determine the significance of the differences. (E) RAW 264.7 cells were infected with BCG plus indicated target inhibitors for 24 h. Student t-test was used to determine the significance of the differences between the inhibitors treatment and control. (F) C57BL/6 mice were aerosol infected with low dose BCG for 14 days. The lungs were harvest and the expression levels of lncRNA-cox2 were examined by realtime PCR. ANOVA was used to determine the significance of the differences. *P<0.05; **P<0.01; ***P<0.001.

LncRNA-cox2 re-programmed M1 macrophage differentiation to enhance the killing ability against mycobacteria pathogenesis

We next generated RAW 264.7 cell lines, in which lncRNA-Cox2 was silenced by siRNA, following with BCG treatment. We found that the decreased lncRNA-cox2 group had higher intracellular bacteria counting, which means a decreasing intracellular killing ability against BCG (Figure 2A). The mRNA expression of the M1 marker genes (proinflammation), including TNF-α, IL-6 and IL-12p40 were significantly decreased in lncRNA-cox2 knockdown macrophages compared with that of control cells (Figure 2B). Flow cytometry indicated that iNOS⁺ and IL-12p40⁺ cells were decreased in lncRNA-cox2 knockdown group (Figure 2C). Additional, iNOS protein expression (Figure 2D) and NO concentration (Figure 2E) were down-regulated in lncRNA-cox2 knockdown group.

NO plays a key role in lncRNA-cox2 induced intracellular mycobacteria killing

Considering that lncRNA-cox2 knockdown decrease the expression of IL-6, IL-12p40 and iNOS, we ask that weather lncRNA-cox2 affect the macrophage killing ability against BCG through regulating these cytokines. We pre-treated the infected cells with additional IL-6, IL-12 and SNAP (NO donor), following with lncRNA-cox2 knockdown and BCG challenge. We found that all these factors could reverse the intracellular killing ability by lncRNA-cox2 knockdown (Figure 3A-C). Interestingly, SNAP treatment could decrease BCG CFU counting of the lncRNA-cox2 knockdown macrophages and control cases to the same level (Figure 3A), whereas there remained
Figure 2. Decreasing M1 macrophage signature gene expression in IncRNA-cox2 knockdown macrophage triggered by BCG infection. RAW 264.7 cells were transiently transfected with control or IncRNA-cox2 siRNA for 24 h and then infected with BCG for another 24 h. (A) Intracellular bacterial CFUs was determined by the pour plate method after serial dilution. (B) mRNA expression of indicated genes was determined by qPCR; (C, D) Cells were stained for intracellular (C) iNOS and (D) IL-12p40 and analyzed by flow cytometry. up: Representative flow cytometric plot; down: quantification of iNOS+ or IL-12p40+ cells; (E) iNOS expression was determined by western blotting; up: Representative western blotting band; down: quantification of iNOS expression. (F) NO production were determined by Griess assay. Student t-test was used to determine the significance of the differences between the IncRNA-cox2 siRNA treatment and control. *P<0.05; **P<0.01; ***P<0.001.

Figure 3. Snap, IL-6 and IL-12p40 treatment altered the survival of intracellular bacterial. RAW 264.7 cells were transiently transfected with control or IncRNA-cox2 siRNA, together with (A) NO donor SNAP treatment; (B) IL-6 treatment; (C) IL-12 treatment for 24 h and then infected with BCG for another 24 h. Intracellular bacterial CFUs was determined by the pour plate method after serial dilution. Student t-test was used to determine the significance of the differences between indicated two groups. *P<0.05; **P<0.01; ***P<0.001. NS not significant.
significant differences of the CFU counting between the lncRNA-cox2 knockdown macrophage and control in treatment with IL-6 (Figure 3B) and IL-12 (Figure 3C). These evidences indicated that lncRNA-cox2 affect intracellular bacterial killing might partly through regulating macrophage NO production.

**Discussion**

Macrophages are the first line of defense against pathogens, and the mode of their activation will determine the success or failure of the host response to pathogen aggression. Based on limited numbers of markers, activated macrophages can be classified as M1 macrophages that support microbicidal activity or M2 macrophages that are not competent to eliminate pathogens [16]. M1 polarization play an important role in elimination of intracellular mycobacteria, and this functional phenotype has been reported to be regulated by various of short non-coding RNAs (for example, microRNAs) [17]. LncRNA were regarded as "transcriptional noise" before, but recently, many studies have reported that these non-coding RNAs partly through the NF-κB activity.

Previous studies have demonstrated that lncRNA-Cox2 was assembled to the SWI/SNF complex under LPS stimulation [13]. Our results demonstrated that there was also a detectable presence of lncRNA-cox2 in the RIP assay from BCG-infected RAW 264.7 cells by anti-Brg1, a known component of the SWI/SNF complex (Figure 5A). Additional, we detected a significant increase of lncRNA-Cox2 in the immunoprecipitates from BCG-stimulated cells using antibodies NF-κB p50 and p65 (Figure 5B). Considering that the expression of iNOS is tightly regulated by the binding of NF-κB on the iNOS promoter [15], we further evaluated the effect of lncRNA-cox2 on the binding of NF-κB on iNOS promoter by ChIP assay. The results showed that lncRNA-cox2 siRNA treatment for 48 hours markedly inhibited the binding of NF-κB p50 and p65 subunits to the iNOS promoter (Figure 5C), whereas transfection with lncRNA-cox2 over-expression plasmid got the opposite outcomes (Figure 5D).
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have a series of important regulatory potential both in transcription and post transcription [18, 19]. We reasonably hypothesized that there might be numbers of IncRNAs in regulating macrophage activation, and therefore play an important role in MTB infection control. IncRNA-cox2 has been identified as a long non-coding RNA mediates immune response [11-13], so we asked whether it was involved in bacterial clearance, and how could it functioned. In the present study, we found that IncRNA-cox2 knockdown significantly decrease the killing ability of macrophage against intracellular BCG. Additional, we clearly demonstrated that IncRNA-cox2 regulated NF-κB pathway to regulate anti-TB immune response. This conclusion was supported by the following observations: A) mRNA of TNF-α, IL-6 and IL-12 were significantly decreased in IncRNA-cox2 knockdown group; B) protein expression of iNOS was down-regulated in IncRNA-cox2 knockdown group; C) flow cytometry assay demonstrated that iNOS producing cells and IL-12 producing cells were cut down in IncRNA-cox2 knockdown group.

It is well known that NO derived from iNOS in macrophages and other innate immune cells is pro-inflammatory and an essential component of host defenses against various pathogens including tuberculosis [20]. Our findings demonstrated that iNOS was down-regulation together with NO decreasing in IncRNA knockdown group. We found that NO donor significantly decreases the CFU counting, and could put the intracellular bacterial killing to the same level in IncRNA-cox2 knockdown and control group, suggesting that NO might be the key factor to mediate IncRNA-cox2 function against intracellular bacterial.

Our another finding in this work is that mycobacterial enhanced the IncRNA-cox2 expression depending on the NF-κB pathway, and the IncRNA-cox2 also regulated NF-κB pathway to regulate anti-TB immune response. This result indicated that there would be a IncRNA-cox2 involved feedback pathway that play an important role in bacterial clearance. Previous reports have identified several targets for
IncRNA-cox2. Carpenter et al. found that IncRNA-cox2 could bind hnRNP-A/B and hnRNP-A2/B1 [11]. Hu et al. demonstrated that IncRNA-cox2 is assembled into the switch/sucrose nonfermentable (SWI/SNF) complex in cells after LPS stimulation [13]. hnRNPs are multifunctional nuclear RNA binding proteins involved in various aspects of RNA biology and has been linked to transcriptional repression of some genes [21]. SWI/SNF complex has been demonstrated in the transcription of late-primary and secondary response genes following NF-κB activation [22]. Our results demonstrated that IncRNA-cox2 affected the transcription of iNOS by regulating the binding of NF-κB on the iNOS promoter, and this function might be mediated by directly interaction between IncRNA-cox2 and NF-κB p50 subunit. Previous report have elucidated that p50 lacks a transcription activation domain. When bound p50-p50 as a homodimer to iNOS gene promoters, it blocks binding of active p65-p50 NF-κB and thus inhibits gene expression [23]. Our results showed that IncRNA-cox2 overexpression increased the p65-p50 binding to the iNOS promoter, so we supposed that interaction between IncRNA-cox2 and NF-κB p50 subunit might play an important role in regulating the balance of p50-p50 and p65-p50 NF-κB. Further study should be performed to elucidate our hypothesis of this point.

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Disclosure of conflict of interest

None.

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