

The Monocyte-Derived Exosomal *CLMAT3* Activates the CtBP2-p300-NF- κ B Transcriptional Complex to Induce Proinflammatory Cytokines in ALI

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Monocytes and macrophages are the two major cell types involved in innate immunity. Exosomes act as signaling molecules to regulate cell-to-cell communication by releasing proteins, mRNAs, microRNAs (miRNAs), and long noncoding RNAs (lncRNAs). However, it is still unclear whether monocyte-derived exosomes are involved in the communication between monocytes and macrophages. In this study, we analyzed the differentially expressed lncRNA profiles in monocytes isolated from blood samples of healthy controls and acute lung injury (ALI) patients. We focused our study on investigating the signaling downstream of *CLMAT3* (colorectal liver metastasis-associated transcript 3), a lncRNA that regulated proinflammatory cytokine genes. We revealed that *CLMAT3* specifically targeted *CtBP2* (C-terminal binding protein 2) and repressed its expression. Elevated CtBP2 acted as a coactivator to assemble a transcriptional complex with histone acetyltransferase p300 and NF- κ B (nuclear factor κ B) subunits. *In vitro* coculture and *in vivo* injection of ALI monocyte-derived exosomes increased the production of proinflammatory cytokines. Importantly, the administration of two CtBP2 inhibitors, NSC95397 and MTOB, could significantly reverse CtBP2-mediated transactivation. Collectively, our results support a model in which monocyte-derived exosomal *CLMAT3* activates the CtBP2-p300-NF- κ B complex to induce proinflammatory cytokines, thus contributing to the pathogenesis of ALI.

INTRODUCTION

Acute lung injury (ALI) and its severe form, acute respiratory distress syndrome (ARDS), are disorders of acute inflammation.¹⁻³ Clinical and experimental data suggest that proinflammatory cytokines such as interleukin (IL)-1 β , IL-6, IL-8, IL-18, and tumor necrosis factor alpha (TNF- α) are elevated during the pathogenesis of ALI, and the concentrations of these proinflammatory cytokines are associated with the morbidity and mortality of ALI.⁴⁻⁶ How the human immune system is activated when inflammation happens has been well characterized.⁷ In the injury sites, monocytes are initially recruited, and they can differentiate into macrophages and dendritic cells, thus composing the three major immune cell types involved in the innate immune system.⁸⁻¹⁰ These three types of cells have multiple func-

tions, mainly phagocytosis, antigen production, and cytokine production.⁸⁻¹⁰ According to the expression levels of CD14 (Cluster of differentiation 14) and CD16 on their cell surface, monocytes can be generally classified into three subclasses, including the classical CD14²⁺CD16⁻, the intermediate CD14²⁺CD16⁺, and the nonclassical CD14⁺CD16²⁺.^{11,12} Macrophages are present in almost all tissues, where they can sense and respond to pathogens and other external stimuli.^{7,13} When tissue injury and cell infection occur, the macrophages activate inflammatory signaling pathways, especially Toll-like receptor 4/nuclear factor κ B (TLR4/NF- κ B) axis signaling, which controls the induction of proinflammatory cytokine genes.^{7,13}

In the past several years, exosomes have been proven to play important roles in the regulation of cell-to-cell communication.^{14,15} Exosomes are small organelles secreted by almost all cell types with a diameter of ~30 to ~200 nm.^{16,17} Similar to cells, single-membrane exosomes also encompass messenger RNAs (mRNAs), microRNAs (miRNAs), long noncoding RNAs (lncRNAs), proteins, lipids, and glycoconjugates.¹⁴⁻¹⁷ These exosomal components can be released from the donor cells to the target cells, thereby triggering signaling pathways in the target cells.¹⁴⁻¹⁷ In particular, the dysregulation of miRNAs and lncRNAs in the exosomes of donor cells can regulate the expression of genes in the target cells, which has been found in a variety of inflammation-associated diseases, such as cancers, inflammatory bowel diseases (IBDs), kidney diseases, and rheumatoid arthritis (RA).¹⁸ The inflammatory microenvironment is critical for tumorigenesis, migration, invasion, and metastasis.¹⁸ Exosomes derived from tumor cells can activate the NF- κ B pathway in macrophages and increase the expression of proinflammatory cytokine genes such as *IL-6*, *TNFA*, *GCSF* (Granulocyte colony-stimulating factor), and *CCL2* (Chemokine C-C motif ligand 2), promoting tumor metastasis.¹⁹ In a mouse IBD model, the injection of exosomes from

Received 13 April 2020; accepted 29 July 2020;
<https://doi.org/10.1016/j.omtn.2020.07.040>

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the normal intestine can decrease the severity of mouse inflammation.²⁰ However, coculture of exosomes isolated from IBD patients with the human colonocyte cell line DLD-1 can result in an increase of IL-8.²¹ In acute and chronic kidney-injured mice, the number of exosomes with an elevated level of CCL2 is much more abundant than in the control mice.²² Mice injected with exosomes isolated from tubular epithelial cells (TECs) with a higher CCL2 level could experience tubular injury and renal inflammation.²² In the pathology of RA, exosomes obtained from RA patients contain abundant tumor necrosis factor alpha (TNF- α), and they can enter CD³⁺ T cells and cause the activation of AKT (protein kinase B) and NF- κ B signaling.²³ However, it is still unclear whether exosomes are involved in the pathogenesis of ALI by affecting the activation of macrophages.

The NF- κ B transcription factor family plays a critical role in immune and inflammatory responses by controlling the transcription of various genes, especially proinflammatory cytokine genes such as *IL-1B*, *IL-6*, *IL-15*, *IL-18* and *TNFA*.^{24,25} To transcribe these genes, the heterodimers of NF- κ B members, especially p50/p65, need to translocate from the cytoplasm to the nucleus, where they bind to the promoters of target genes.^{24,25} The transcription process also requires other transcription regulators, such as coactivators and corepressors.²⁶ The NF- κ B subunit p65 has been shown to interact with coactivator CREB-binding protein (CBP) and its partner p300, a histone acetyltransferase.^{27,28} Mechanically, CBP/p300 interacts with the transcriptional activation domain (TAD) of p65, which dimerizes with p50, and then the heterodimers bind to the promoters of proinflammatory cytokine genes to activate their expression.^{27,28} However, it is unclear whether other transcriptional regulators also participate in this transactivation process. Several studies have shown that CtBP1/2 (C-terminal binding proteins 1 and 2) can interact with p300 and HDACs (histone deacetylases) through the PXDLS motif.^{29–31} The current evidence suggests that CtBPs have both transrepression and transactivation roles in regulating the expression of genes, miRNAs, and lncRNAs.^{32–35} Generally, CtBPs interact with p300, HDACs, or transcription factors such as ZEB1 (zinc finger E-box-binding homeobox 1) and FOXP2 (Forkhead box protein P2).³⁵ However, there is no study showing that CtBPs can assemble a transcriptional complex with p300 (or HDACs) and NF- κ B to control proinflammatory cytokine gene expression.

To investigate whether exosomes are involved in the communication between monocytes and macrophages in the pathogenesis of ALI, we isolated exosomes in monocytes derived from blood samples from healthy controls and ALI patients. After analyzing the lncRNA profiles of the exosomes, we identified 29 differentially expressed lncRNAs. We focused our investigation on revealing the mechanism of *CLMAT3* (Colorectal liver metastasis-associated transcript 3), the only lncRNA whose knock down (KD) or overexpression (OE) could change the expression of the proinflammatory cytokine genes. Our *in vitro* and *in vivo* results suggested that *CLMAT3* negatively regulated the expression of *CtBP2*. The release of *CLMAT3*-containing exosomes into macrophages caused the OE of *CtBP2*, whose encoded protein assembled a complex with p300 and NF- κ B to transactivate

the expression of proinflammatory cytokine genes, inducing an inflammation response.

RESULTS

Identification of Differentially Expressed lncRNAs in Exosomes Derived from ALI Monocytes

To determine whether lncRNAs are involved in the communication between monocytes and macrophages in the inflammatory response, we purified monocytes from blood samples of healthy controls and ALI patients using a combined Ficoll and anti-CD14 magnetic bead method (Figure 1A). After determining the purity of the monocytes with flow cytometry, we isolated exosomes from the two-sourced monocytes. Three independent exosomes isolated from the controls and ALI patients were further used for RNA extraction, followed by lncRNA-specific microarray analysis. We discovered 29 aberrantly expressed lncRNAs in the ALI-sourced monocytes, including 12 downregulated and 17 upregulated lncRNAs, respectively (Table S1). We included 20 of these differentially expressed lncRNAs in a heatmap (Figure 1B). To further determine whether the expression levels of these lncRNAs were changed in a larger population of exosomes from healthy controls and ALI patients, we randomly selected two upregulated lncRNAs (*AOC4P* [amine oxidase copper containing 4, pseudogene] and *BCAR4* [breast cancer anti-estrogen resistance 4]) and two downregulated lncRNAs (*CLMAT3* and *MIAT* [myocardial infarction associated transcript]) and then examined their expression levels. Consistent with the microarray results, we also observed the OE of *AOC4P* and *BCAR4* and the downregulation of *CLMAT3* and *MIAT* in 40 exosomes derived from ALI monocytes compared to control exosomes (Figures 1C–1F). These results suggested that we had identified a variety of lncRNAs that were differentially expressed in ALI-sourced exosomes.

ALI Monocyte-Sourced Exosomes Could Activate Macrophages

To investigate whether ALI monocyte-sourced exosomes could activate macrophages, we cocultured healthy monocyte-sourced and ALI monocyte-sourced exosomes with U937 cells (Figure 2A). We found that ALI monocyte-sourced exosomes caused over 80% of the U937 cells to become adherent, which suggested that they were activated. Given that the activation of macrophages resulted in the induction of proinflammatory cytokines, we also measured the mRNA levels of five proinflammatory cytokine genes—*IL-1B*, *IL-6*, *IL-15*, *IL-18*, and *TNFA*—in the cocultured cells. The quantitative real-time PCR results showed that all five of these genes were significantly induced in cells cocultured with ALI monocyte-sourced exosomes in comparison to cells cocultured with healthy monocyte-sourced exosomes (Figures 2B–2F). However, we did not observe any changes in the anti-proinflammatory cytokines *IL-4* and *IL-13* in either group (Figures 2G and 2H). In addition, we also measured the concentrations of these cytokines in the supernatant of the cell cultures by enzyme-linked immunosorbent assays (ELISAs). Consistent with their corresponding mRNA levels, we also observed a significant increase of IL-1 β , IL-6, IL-15, IL-18, and TNF- α in the supernatants of cell cultures of U937 and ALI monocyte-sourced exosomes compared to controls (Figures 2I–2M). Similar to their mRNA levels,

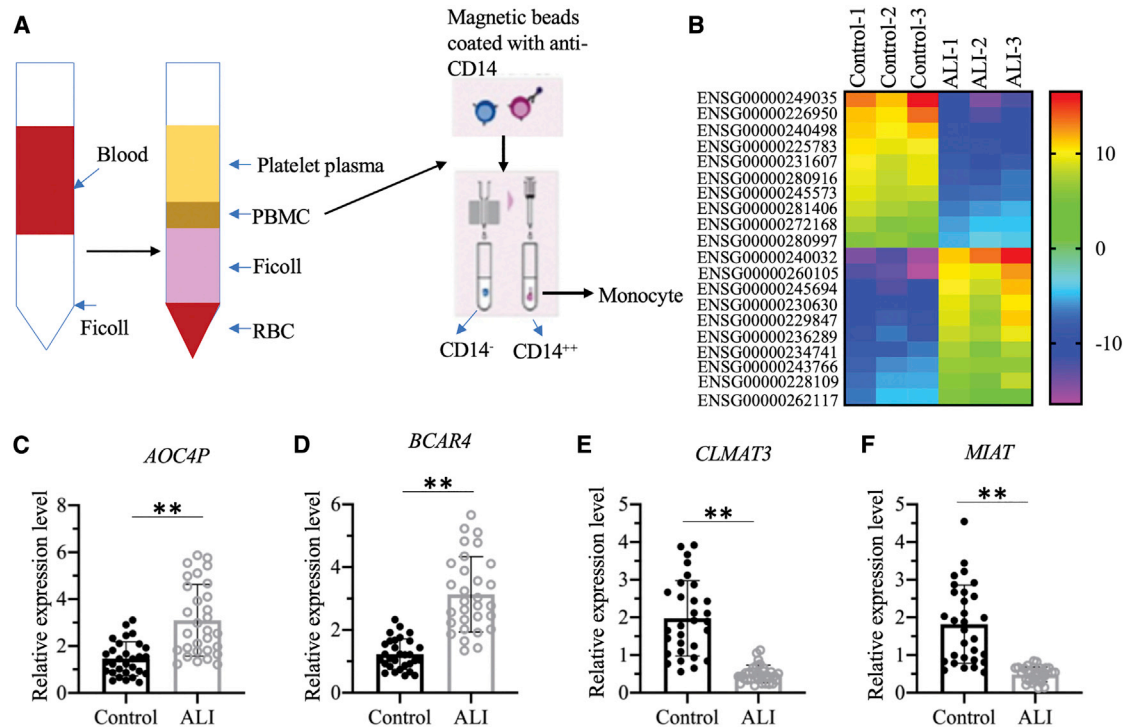


Figure 1. Identification of Differentially Expressed lncRNAs in Exosomes Isolated from ALI Monocytes

(A) A schematic diagram of monocyte isolation. PBMCs, peripheral blood mononuclear cells; RBCs, red blood cells. (B) The differentially expressed lncRNA profile in the ALI monocyte-sourced exosomes. (C–F) Verification of lncRNA levels in 40 exosomes. A total of 40 RNA samples from healthy and ALI-sourced exosomes were subjected to quantitative real-time PCR analyses to examine the expression levels of *AOC4P* (C), *BCAR4* (D), *CLMAT3* (E), and *MIAT* (F). **p < 0.01.

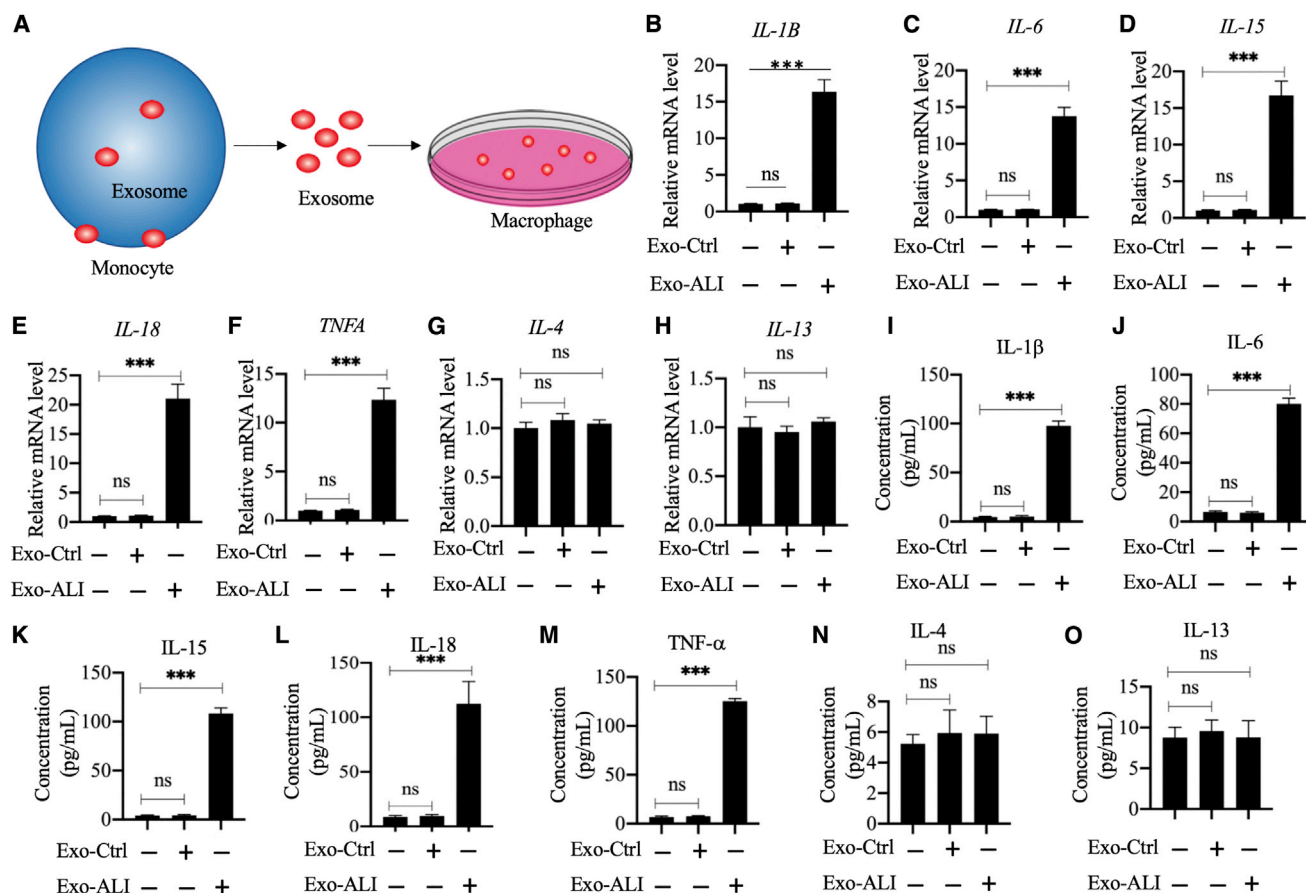
we also did not find any significant changes in the IL-4 and IL-13 concentrations in the supernatant of two different cell cultures (Figures 2N and 2O).

KD or OE of *CLMAT3* Changed the Expression of Proinflammatory Cytokine Genes and *CtBP2*

Our aforementioned results showed that 29 lncRNAs were dysregulated in ALI-sourced exosomes, and these kinds of exosomes could activate macrophages and induce the expression of proinflammatory cytokines. We next sought to investigate whether the activation of proinflammatory cytokines was controlled by these differentially expressed lncRNAs. For this purpose, we selected 11 lncRNAs—*DANCR*, *ANRIL* (antisense noncoding RNA in the INK4 locus), *AOC4P*, *BISPR* (BST2 interferon-stimulated positive regulator), *BCAR4*, *GAS5* (growth arrest-specific 5), *FOXCUT* (FOXC1 upstream transcript), *PTCSC3* (papillary thyroid carcinoma susceptibility candidate 3), *MIAT*, *XIST* (X-inactivate specific transcript), and *CLMAT3*—and then determined the effects of their KD and OE on the expression levels of proinflammatory cytokines. Our quantitative real-time PCR results indicated that KD or OE of *DANCR*, *ANRIL*, *AOC4P*, *BISPR*, *BCAR4*, *GAS5*, *FOXCUT*, *PTCSC3*, *MIAT*, and *XIST* could not change the expression of proinflammatory cytokine genes (Figures S1 and S2). Interestingly, we found that the downregulation of *CLMAT3* could induce the expression of proinflammatory

cytokine genes, while OE of *CLMAT3* caused the reverse effects (Figure 3A).

To investigate the direct targets of *CLMAT3*, we conducted a microarray analysis using *CLMAT3* KD and OE cell lines to identify genes regulated by *CLMAT3*. We obtained 15 aberrantly expressed genes, and most of these genes were NF- κ B targets (Figure 3B; Table S2), which suggested that *CLMAT3* might target NF- κ B and thus affect the expression of NF- κ B target genes. In addition, we also found that the expression of a transcriptional regulator *CtBP2* was controlled by *CLMAT3* (Figure 3B). To verify the correctness of our microarray results and also to determine whether *CLMAT3* could directly regulate the expression of NF- κ B subunits, we examined the expression levels of *S100A8* (S100 calcium-binding protein A8), *IFNG* (interferon gamma), *IL-15*, *IL-18*, *IL-23A*, *ICAM1* (intercellular adhesion molecule 1), *CtBP2*, *p50*, and *p65*. Interestingly, we found that the expression patterns of *S100A8*, *IFNG*, *IL-15*, *IL-18*, *IL-23A*, *ICAM1*, and *CtBP2* were similar; that is, they were upregulated in *CLMAT3*-KD cells but downregulated in *CLMAT3*-OE cells (Figure 3C). However, KD or OE of *CLMAT3* could not change the expression of *p50* and *p65* (Figure 3C). These results suggested that the regulation of *CLMAT3* in NF- κ B targets might be through an indirect mechanism. Given that *CtBP2* is a well-known transcriptional regulator, we speculated that it might be a direct target of *CLMAT3*. To verify this



possibility, we performed RNA immunoprecipitation (RIP) assays to determine the interaction of *CLMAT3* and CtBP2 using CtBP1, p50, and p65 as controls. Our results indicated that the enrichment of CtBP2 was significantly decreased in *CLMAT3-KD* cells but markedly increased in *CLMAT3-OE* cells (Figure S3A). However, we did not observe any binding of CtBP1, p50, and p65 to *CLMAT3* (Figures S3B–S3D). These results suggested that *CLMAT3* could directly target *CtBP2* and repress its expression.

CtBP2 Assembled a Transcriptional Complex with p300 and NF-κB Subunits

Our aforementioned results revealed that *CtBP2*, instead of NF-κB subunits, was a direct target of *CLMAT3*. One interesting result was that various NF-κB targets were also regulated by *CLMAT3*. These results raised the possibility that CtBP2 might assemble a transcriptional complex with NF-κB and then regulate the expression of NF-κB targets. To examine this possibility, we performed an immu-

noprecipitation (IP) assay using the anti-CtBP2 antibody in U937 cells activated by ALI monocyte-sourced exosomes. The CtBP2-associated protein complex members were determined by mass spectrometry, and we identified a total of 29 proteins in this complex (Figure 4A; Table S3). Among these proteins, we found two NF-κB subunits, p50 and p65, and p300 (Table S3). We then verified the existence of p300, p50, and p65 in the CtBP2-immunoprecipitated complex by western blotting assays (Figure 4B). Our microarray results shown in Figure 3B verified that the NF-κB targets were upregulated in *CLMAT3-KD* cells, which suggested that NF-κB was activated in these cells. To verify this activation, we examined the nuclear and cytoplasmic fractions of p50 and p65 in *CLMAT3-KD* cells. As expected, we found that the NF-κB subunits were activated and the nuclear:cytoplasmic ratios of these two subunits were significantly increased when *CLMAT3* was knocked down (Figure 4C). We then performed co-immunoprecipitation (coIP) assays to determine the direct interactions between CtBP2-p300, CtBP2-p50, CtBP2-p65,

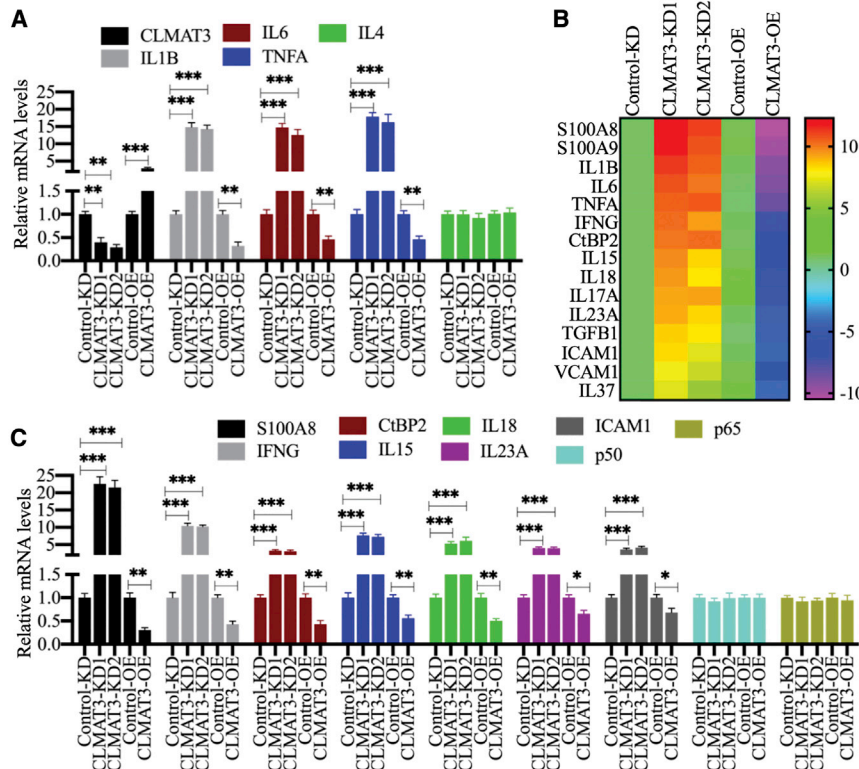


Figure 3. KD or OE of *CLMAT3* Affected the Expression of NF- κ B Targets

(A) The expression of NF- κ B targets in *CLMAT3*-KD and *CLMAT3*-OE cells. The quantitative real-time PCR analyses were performed to examine the expression of *CLMAT3*, *IL1B*, *IL-6*, *TNFA*, and *IL-4*. ** $p < 0.01$; *** $p < 0.001$. (B) Identification of differentially expressed genes dependent on *CLMAT3*. RNA samples used in (A) were applied to a microarray analysis to identify differentially expressed genes. The top 20 aberrantly expressed genes are indicated in the heatmap. (C) Verification of aberrantly expressed gene levels. The RNA samples used in (A) were applied to quantitative real-time PCR analyses to examine the expression levels of *S100A8*, *IFNG*, *CtBP2*, *IL-15*, *IL-18*, *IL23A*, *ICAM1*, *p50*, and *p65*. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

p300-p50, and p300-p65. The immunoblot results showed that CtBP2 could directly interact with p300, but not p50 and p65, and that p300 could directly interact with p50 and p65 (Figures 4D and 4E). These results suggested that p300 acted as a linker to assemble CtBP2 and NF- κ B subunits as a complex (Figure 4F).

ALI Monocyte-Sourced Exosomes Could Not Activate Macrophages when CtBP2 Was Deficient

Since the downregulation of *CLMAT3* in ALI monocyte-sourced exosomes could activate macrophages through regulating the CtBP2-p300-NF- κ B complex, we speculated that *CLMAT3* could not activate macrophages when CtBP2 was deficient. To evaluate this hypothesis, we generated two CtBP2-KD cell lines and then cocultured healthy monocyte-sourced exosomes and ALI monocyte-sourced exosomes with them. After coculture, we examined the expression levels of *CtBP2*, *IL-1B*, *IL-6*, and *TNFA*. As expected, we found that the expression levels of *IL-1B*, *IL-6*, and *TNFA* were significantly decreased with the downregulation of *CtBP2*, and coculture of ALI monocyte-sourced exosomes with CtBP2-KD cells also could not efficiently induce the expression of *IL-1B*, *IL-6*, and *TNFA* (Figure 5A). In addition, we treated U937 cells with two CtBP2 inhibitors, NSC95397 and MTOB, prior to coculture with exosomes. Similarly, we also observed the downregulation of *IL-1B*, *IL-6*, and *TNFA* in NSC95397- or MTOB-treated cells. ALI monocyte-sourced exosomes could not induce the expression of *IL-1B*, *IL-6*, and *TNFA* in NSC95397- or MTOB-treated cells to a level comparable to that in cells only treated with ALI monocyte-sourced exosomes (Figure 5B).

To determine how *CtBP2* KD or inhibition of CtBP2 caused the decrease of NF- κ B targets, we performed chromatin immunoprecipitation (ChIP) assays to measure the occupancies of CtBP2, p300, and p65 on the promoter of *IL-1B* as an example. The ChIP results indicated that KD of *CtBP2* or inhibition of CtBP2 decreased the occupancies of p300 and p65 on the promoter of *IL-1B* (Figures 5C and 5D). Although the inhibition of CtBP2 by NSC95397 and MTOB did not change the occupancy of CtBP2 (Figure 5D), it resulted in a decrease in p300 and p65 occupancies. The reason for this phenomenon might be because NSC95397 and MTOB treatments disrupted the function of CtBP2, which limited the interaction between CtBP2 and p300 and thus affected the assembly of the CtBP2-p300-NF- κ B complex.

Inhibition of CtBP2 Significantly Decreased the Inflammatory Response in Mice Injected with ALI Monocyte-Sourced Exosomes

Since ALI monocyte-sourced exosomes could activate macrophages *in vitro* and were ineffective in CtBP2-deficient macrophages, we speculated they would also have a similar inflammation response *in vivo*. To examine this possibility, we injected healthy monocyte-sourced exosomes and ALI monocyte-sourced exosomes into wild-type mice. Mice in the ALI monocyte-sourced exosome group were further injected with NSC95397 or MTOB (Figure 6A). The concentrations of proinflammatory cytokines in the mouse blood were determined, and the results indicated that injection of ALI monocyte-sourced exosomes alone significantly induced the production of IL-1 β , IL-6, IL-15, and TNF- α but not IL-4 and IL-13 (Figures 6B–6E). Additional administration of NSC95397 and MTOB resulted in a dramatic decrease of proinflammatory cytokines (Figures 6B–6E). In addition, we also examined the mRNA levels of *IL-1B*, *IL-6*, *IL-15*, *IL-18*, *TNFA*, *IL-4*, and *IL-13* in the lung tissues from the different groups of mice. The quantitative real-time PCR results showed that proinflammatory cytokine genes but not

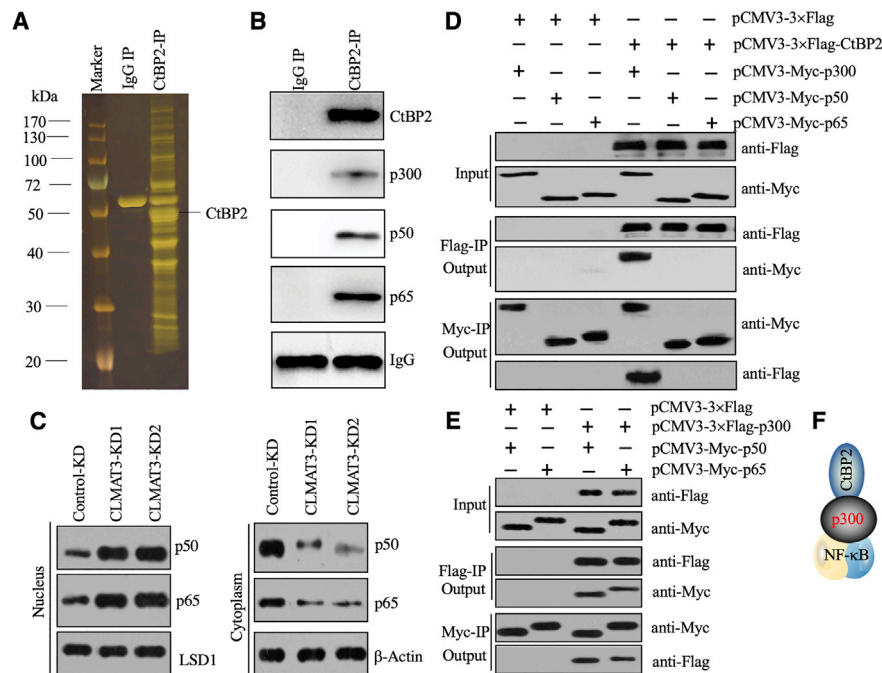


Figure 4. CtBP2 Assembled a Complex with p300 and NF- κ B Subunits *In Vivo* and *In Vitro*

(A) The CtBP2-associated protein complex in cells cocultured with ALI-monocyte-sourced exosomes with silver staining. (B) CtBP2 could pull down p300, p50, and p65 *in vivo*. The protein complexes purified in (A) were applied to western blotting to examine the protein levels of CtBP2, p300, p50, and p65. (C) The nuclear and cytoplasmic fractions of p50 and p65 in *CLMAT3*-KD cells. (D) CtBP2 directly interacted with p300 but not NF- κ B subunits *in vitro*. The U937 cells were transfected with different FLAG-tagged and Myc-tagged plasmids as indicated in the figure, followed by coIP assays to identify direct protein interactions. (E) p300 directly interacted with NF- κ B subunits *in vitro*. The U937 cells were transfected with different FLAG-tagged and Myc-tagged plasmids as indicated in the figure, followed by coIP assays to determine direct protein interactions. (F) A schematic diagram of the CtBP2-p300-NF- κ B complex.

anti-proinflammatory cytokine genes were significantly induced when mice were injected with ALI monocyte-sourced exosomes (Figure S4A). Treatments with NSC95397 and MTOB significantly reversed this induction (Figure S4A). Detecting the occupancies of CtBP2, p300, p50, and p65 in the promoter of *IL-1B* as a representative gene, we also found that their occupancy patterns were similar (Figure S4B). Their occupancies were significantly enriched in tissues from mice injected with ALI monocyte-sourced exosomes but dramatically decreased when mice were administrated with NSC95397 or MTOB (Figure S4B). These results suggested that inhibition of CtBP2 might be a therapeutic strategy to reduce the inflammation response during the treatment of ALI.

DISCUSSION

The activation of macrophages is a fundamental process in the regulation of the inflammation response.¹⁶ Exosomes have been shown to play important roles in cell-to-cell communication.^{14,15} In the present study, we revealed that *CLMAT3* was downregulated and that its target *CtBP2* was upregulated in exosomes derived from ALI monocytes. The ALI monocyte-sourced exosomes entered into macrophages and released CtBP2, which functioned as a coactivator to assemble a complex with p300 and NF- κ B. This transcriptional complex bound to the promoters of proinflammatory cytokine genes to

activate their expression, resulting in inflammation and contributing to the pathogenesis of ALI (Figure 7).

Monocytes originate from bone marrow, and they are present in the bloodstream under normal conditions.³⁶ Once inflammation occurs, circulating monocytes gather in the injured tissues, where they differentiate into macrophages and dendritic cells in response to the inflammation.³⁶ Although it is well known that monocytes can differentiate into macrophages during the inflammation process,^{11,12,36} the differentiation mechanism is not fully understood. In recent years, emerging evidence has revealed that exosomes are important vesicles that traffic signals between cells.^{14,15} The release of exosomes from donor cells to the target cells has been revealed to play important roles in triggering signaling transduction and in the regulation of gene, miRNA, and lncRNA expression.^{14,15}

In this study, we investigated the lncRNA profile in ALI monocyte-sourced exosomes and then found that only *CLMAT3* expression changes could affect the expression of proinflammatory cytokines. We revealed the mechanism of how *CLMAT3* contributed to the activation of macrophages and triggered inflammation. Although a few studies have found that some inflammatory exosomes can contribute to the pathogenesis of inflammatory diseases such as cancers, IBD,

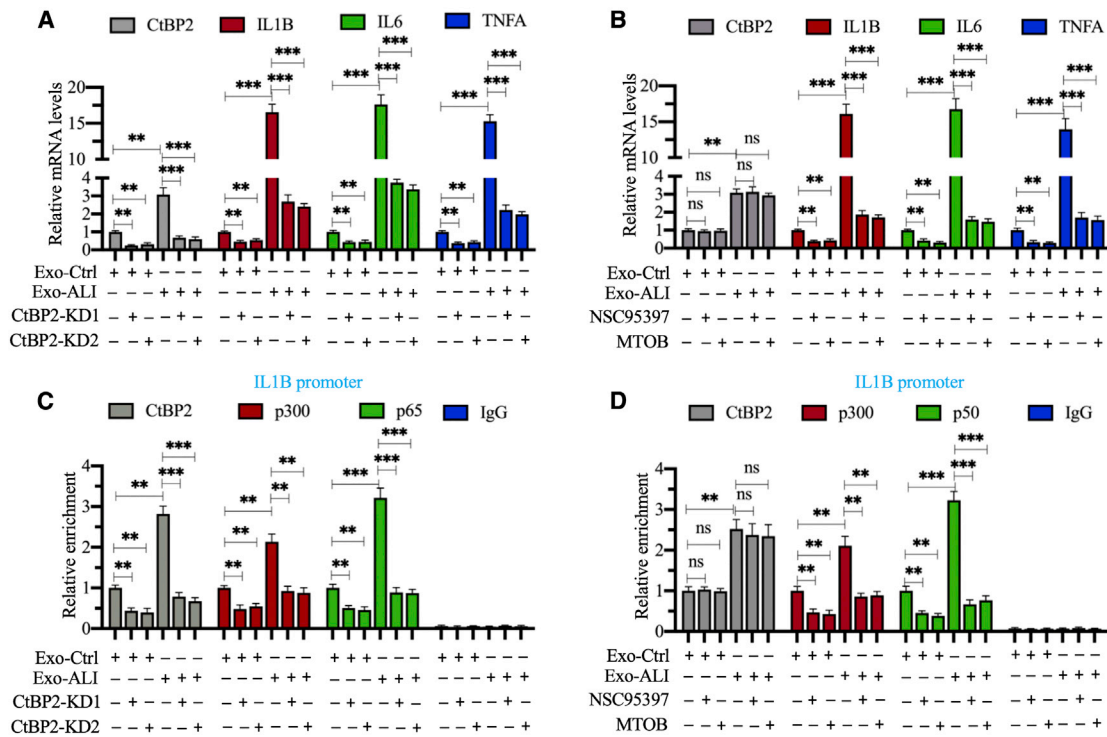


Figure 5. KD or Inhibition of CtBP2 Impaired the Activation of Macrophages Mediated by ALI-Sourced Exosomes

(A) The mRNA levels of *CtBP2*, *IL-1B*, *IL-6*, and *TNFA* in *CtBP2*-KD cells cocultured with Exo-Ctrl or Exo-ALI. ** $p < 0.01$; *** $p < 0.001$. (B) The mRNA levels of *CtBP2*, *IL-1B*, *IL-6*, and *TNFA* in *CtBP2*-deficient cells cocultured with Exo-Ctrl or Exo-ALI. ** $p < 0.01$; *** $p < 0.001$; ns, no significant difference. (C and D) The occupancies of CtBP2, p300, and p65 on the promoter of *IL-1B*. Cells used in (A) and (B) were subjected to ChIP assays using anti-CtBP2, anti-p300, anti-p65, and IgG antibodies, followed by quantitative real-time PCR analyses to examine their occupancies on the promoter of *IL-1B*. ** $p < 0.01$; *** $p < 0.001$.

kidney diseases, and RA through regulating NF- κ B signaling,^{19–22} it is still unclear whether exosomal lncRNAs participate in these pathologies.

Our investigation reports, for the first time, that an exosomal lncRNA secreted by monocytes can contribute to the activation of macrophages. In addition to our observation, *CLMAT3* was previously found to be overexpressed in colorectal cancer (CRC).³⁷ KD of *CLMAT3* could induce the expression of *CDH1* (Cadherin 1) and cause the accumulation of p27, thus affecting the cell-cycle pathway.³⁷ However, there is no previous study showing that *CLMAT3* is involved in the inflammation response. Our results provide a new avenue showing how exosomal *CLMAT3* derived from monocytes activates macrophages and causes inflammation, which will deepen our understanding of ALI pathology and open new insights into its therapy.

The activation of NF- κ B signaling is a fundamental signal in the process of inflammation.¹⁶ Although the upstream signaling and the downstream targets of NF- κ B have been well characterized, the mechanism of how NF- κ B coordinates with other transcriptional regulators to activate proinflammatory cytokine genes is still not fully understood. Herein, we revealed that NF- κ B could recruit p300 and CtBP2 to assemble a transcriptional complex and that CtBP2 func-

tions as a coactivator instead of a corepressor to regulate the expression of proinflammatory cytokine genes. NF- κ B signaling can be activated by many stimuli, such as lipopolysaccharides (LPSs), proinflammatory cytokines, and growth factors.^{24,25,38} These harmful stimuli trigger multiple pathways, especially TLR4-mediated signaling, and cause the translocation of NF- κ B from the cytoplasm to the nucleus, where the NF- κ B-associated transcriptional complex initiates transcription.^{24,25,38} In this study, we did not measure the activation of NF- κ B upstream signaling after exosome incubation, because it should have been activated due to the induction of NF- κ B targets. One important and interesting result of this study is that KD or inhibition of CtBP2 can decrease the expression and production of proinflammatory cytokines, alleviating the inflammation response, which provides a new therapeutic strategy for the treatment of ALI. Based on the notion that the activation of NF- κ B signaling is a basic phenomenon in inflammatory diseases, we speculate that CtBP2-mediated transactivation should be ubiquitous. Thus, targeting CtBP2-mediated transactivation may be a promising strategy to decrease proinflammatory cytokine levels during the treatment of inflammatory diseases.

In summary, the data presented in this study demonstrate a novel role for exosomal lncRNA *CLMAT3* derived from monocytes in the activation of macrophages through affecting the CtBP2-p300-NF- κ B

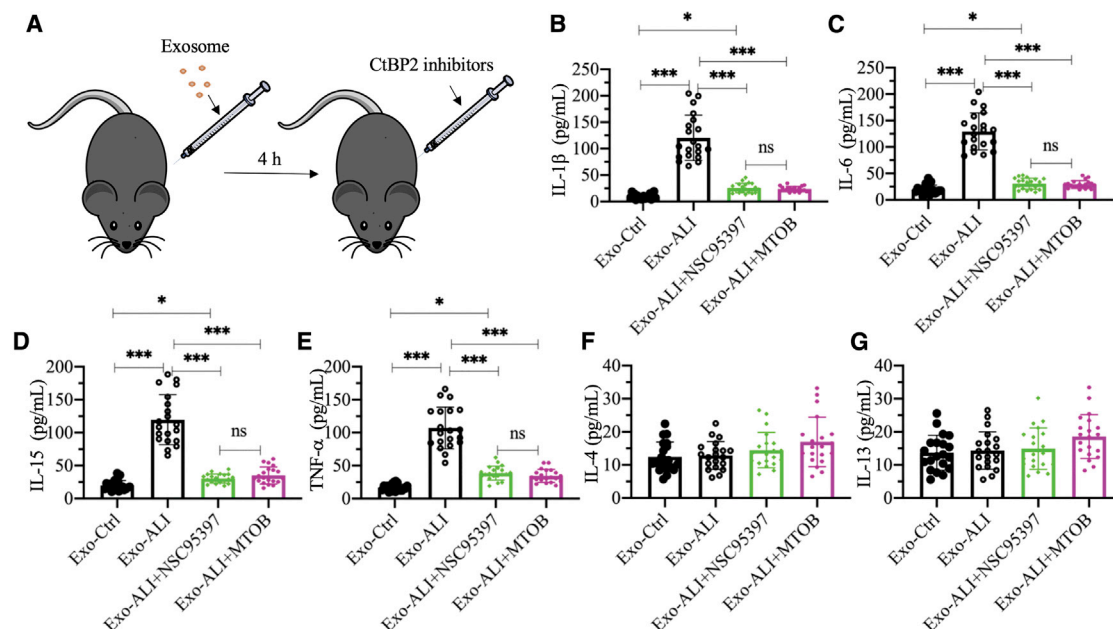


Figure 6. Treatments with NSC95397 and MTOB Alleviated the Inflammation Response In Vivo

(A) A schematic diagram of exosome and CtBP2 inhibitor injection. Mice were injected with Exo-Ctrl ($n = 20$) or Exo-ALI ($n = 60$). The Exo-ALI group was further divided into three subgroups, which were injected with PBS, NSC95397, and MTOB, respectively. $n = 20$ for each subgroup. (B–G) The cytokine concentrations in mice treated with NSC95397 and MTOB. Blood samples from the four groups of mice described in (A) were used for measuring serum concentrations of proinflammatory cytokines, including IL-1 β (B), IL-6 (C), IL-15 (D), TNF- α (E), IL-4 (F), and IL-13 (G). * $p < 0.05$; *** $p < 0.001$.

transcriptional complex. Our results not only enhance our understanding of how monocytes activate macrophages when inflammation occurs but also provide evidence that targeting CtBP2 might alleviate inflammatory responses in the therapy of ALI.

MATERIALS AND METHODS

Blood Sample Collection

Venous blood samples were collected from 40 healthy controls (including 30 men and 10 women; average age, 56.7 ± 7.6 years) and 40 ALI patients (including 27 men and 13 women; average age, 51.4 ± 6.5 years) who were treated in the Department of Critical Care Medicine, Jiangxi Provincial People's Hospital, during 2015–2018. After collection, the blood samples were immediately stored in EDTA-coated tubes (367861; BD Biosciences, San Jose, CA, USA). All participants signed the informed consent reviewed by the Ethics Board of Jiangxi Provincial People's Hospital.

Monocyte Isolation from Blood Samples

The monocyte isolation procedure was the same as in a previous method.³⁹ In brief, blood samples were diluted in saline at a 1:10 ratio, and then the mixture was further incubated with an OptiPrep gradient medium (07820; StemCell Technologies, Cambridge, MA, USA) containing Ficoll. After centrifuging at $700 \times g$ for 20 min, the peripheral blood mononuclear cells (PBMCs) were collected. The CD14²⁺ monocytes were prepared from PBMCs with magnetic beads coated with anti-CD14 antibody (11149D; Thermo Fisher Sci-

entific, Waltham, MA, USA). The purity of the monocytes was determined using flow cytometry with an anti-CD14 antibody (12014942; Invitrogen, Carlsbad, CA, USA).

Cell Culture

Both the CD14²⁺ monocytes and the U937 macrophage cell line (CRL-1593.2; American Type Culture Collection [ATCC], Manassas, VA, USA) were incubated in RPMI 1640 medium (11875085; Thermo Fisher Scientific) containing 10% fetal bovine serum (FBS; 16000044; Thermo Fisher Scientific) and 100 IU/mL penicillin/streptomycin antibiotic solution (10378016; Thermo Fisher Scientific). The cells were cultured in a 37°C incubator containing 5% CO₂ with a medium change every 3 days.

Expression Vector Construction

The full length of 11 lncRNAs, including *DANCR*, *ANRIL*, *AOC4P*, *BISPR*, *BCAR4*, *GAS5*, *FOXCUT*, *PTCSC3*, *MIAT*, *XIST*, and *CLMAT3*, were cloned into the pCDNA3 empty vector. The coding sequences of *CtBP2*, *p300*, *p50*, and *p65* were amplified and cloned into the pCMV-3 \times FLAG and pCMV-Myc empty vectors, respectively. The constructed vectors were sequenced to ensure that the constructs were correct.

Cell Transfection

Cells under 80% confluence were used for transfection following a previous protocol.³³ In brief, plasmids and small interfering RNAs

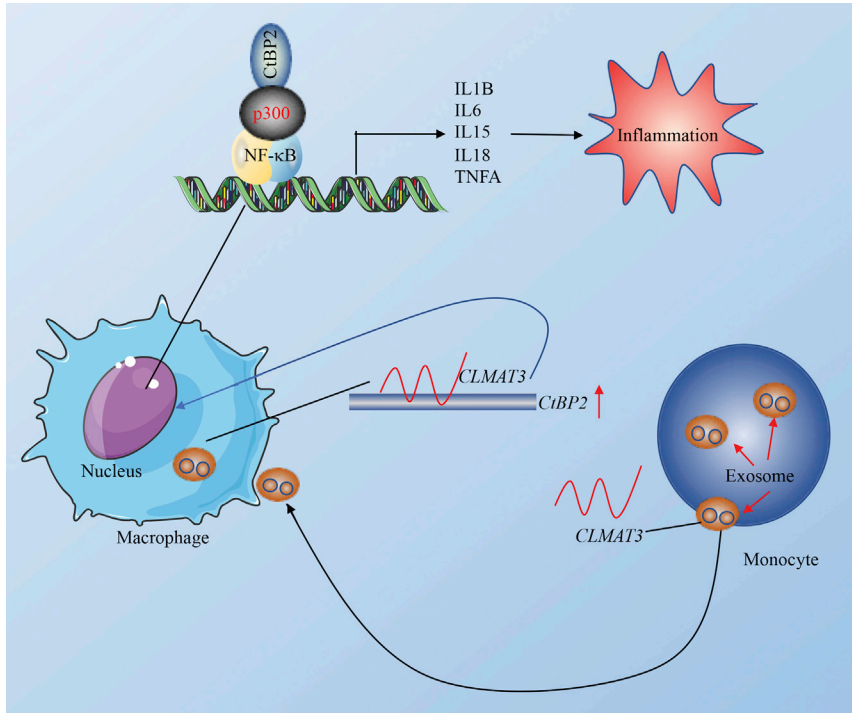


Figure 7. A Schematic Diagram of Exosomal *CLMAT3* Derived from Monocytes Activating Macrophages

The monocytes of ALI patients secrete exosomes in which the lncRNA *CLMAT3* is downregulated. *CLMAT3* specifically targets *CtBP2*, and its downregulation causes the accumulation of *CtBP2* in exosomes. Once exosomes integrate into macrophages, they release *CtBP2*, which assembles a transcriptional complex with the p300 and NF- κ B subunits to activate the expression of proinflammatory cytokine genes. The induction of proinflammatory cytokines promotes the inflammation response, contributing to the pathogenesis of ALI.

matory cytokines (IL-4 and IL-13), in the supernatant of the cell culture and serum were measured using ELISAs according to the methods provided by the manufacturer. All ELISA kits were purchased from Thermo Fisher Scientific, and they included IL-1 β (BMS2242-human and BMS6002-mouse), IL-6 (EH2IL6-human and BMS6032-mouse), IL-15 (BMS2106-human and BMS6023-mouse), TNF- α (BMS2234-human and BMS607HS-mouse), IL-4 (BMS2252-human and BMS613-mouse), and IL-13 (BMS2313-human and BMS6015-mouse).

(siRNAs)—including *siDANCR* (n272703 and n505292), *siANRIL* (n272157 and n509344), *siAOCAP* (n258873 and n258868), *siBISPR* (n515269 and n515270), *siBCAR4* (n270275 and n270270), *siGAS5* (n332778 and n272336), *siFOXCUT* (n515875 and n515873), *siPTCSC3* (n513200 and n513202), *siMIAT* (n504881 and n504882), *siXIST* (n272633 and n272634), *siCLMAT3* (n510595 and n510596), and *siCtBP2* (s3701 and s3703)—were transfected into U937 cells with Lipofectamine 2000 Reagent (11668030; Thermo Fisher Scientific). All siRNAs were purchased from Thermo Fisher Scientific. The transfected cells were incubated at 37°C for 30 h, followed by extraction of their RNA and protein.

RNA Isolation, Microarray, and Quantitative Real-Time PCR Analyses

RNA was extracted from exosomes, cultured cells, and mouse lung tissues using TRIzol Reagent (15596018; Invitrogen) following a previous protocol.³³ In total, 500 ng RNA from each individual sample was used for the microarray analyses to identify the differentially expressed lncRNAs and genes with a Human lncRNA Array Kit v.5.0 (AS-S-LNC-H; Arraystar, Rockville, MD, USA) and a Human GE 4 \times 44K Microarray Kit, v.2 (G4845A; Agilent, Santa Clara, CA, USA), respectively. The expression of genes and lncRNAs was determined using quantitative real-time PCR analyses following a standard procedure as described previously.³³ The primers are listed in Table S4.

ELISAs

The concentrations of six cytokines, including four proinflammatory cytokines (IL-1 β , IL-6, IL-15, and TNF- α) and two anti-proinflam-

IP, Mass Spectrometry, CoIP, and Immunoblot Assays

The U937 cells cocultured with exosomes isolated from the ALI monocytes were lysed in RIPA lysis and extraction buffer (89900; Thermo Fisher Scientific) supplemented with a protease inhibitor cocktail (78439; Thermo Fisher Scientific). The cell lysates were immunoprecipitated with anti-CtBP2 (612044; BD Biosciences, San Jose, CA, USA) and immunoglobulin G (IgG) (negative control), respectively. The immunoprecipitated protein complexes were separated on protein gels and then stained with a kit (24600; Thermo Fisher Scientific). After cutting the gel into small pieces (<0.5 mm) and digesting with a trypsin kit (60109101; Thermo Fisher Scientific), the proteins were applied to mass spectrometry. For the coIP assay, the FLAG-tagged and Myc-tagged plasmids—including pCMV3-3 \times FLAG + pCMV3-Myc-p300, pCMV3-3 \times FLAG + pCMV3-Myc-p50, pCMV3-3 \times FLAG + pCMV3-Myc-p65, pCMV3-3 \times FLAG-CtBP2 + pCMV3-Myc-p300, pCMV3-3 \times FLAG-CtBP2 + pCMV3-Myc-p50, pCMV3-3 \times FLAG-CtBP2 + pCMV3-Myc-p65, pCMV3-3 \times FLAG + pCMV3-Myc-p50, pCMV3-3 \times FLAG + pCMV3-Myc-p65, pCMV3-3 \times FLAG-p300 + pCMV3-Myc-p50, and pCMV3-3 \times FLAG-p300 + pCMV3-Myc-p65—were cotransfected into U937 cells. After determining the successful transfection of these plasmids, the cells were then lysed and subjected to immunoprecipitation using anti-FLAG agarose (A4596; Sigma-Aldrich, Shanghai, China) and anti-Myc-agarose (Sigma-Aldrich, A7470), respectively. The purified FLAG- and Myc-associated protein complexes were used to examine protein-protein interactions by

immunoblots. The western blotting procedure was the same as in a previous study.³³ The antibodies, including anti-FLAG (F3165), anti-Myc (SAB4700447), anti-p300 (SAB4500771), anti-p50 (ZRB2082), anti-p65 (SAB4502615), and anti-LSD1 (ABE365) were all purchased from Sigma-Aldrich. The protein signals were visualized by films.

Coculture of Exosomes with Cells and Injection of Exosomes into Mice

The same amounts of exosomes isolated from healthy control-sourced and ALI-sourced monocytes were added into a 12-well plate to coculture with U937 cells. After incubation at 37°C for 24 h, the adherent cells were collected for RNA and protein isolation. Similarly, the same amounts of two sourced exosomes were also injected into 6-week-old C57BL/6 mice (n = 20 for the control exosome group, and n = 60 for the ALI-sourced exosome group). Four hours after the injection, mice in the ALI-sourced exosome group were further randomly divided into three groups. The first group of mice was injected with PBS, the second group was injected with NSC95397 (5 mg/kg) (N1786; Sigma-Aldrich), and the third group was injected with MTOB (5 mg/kg) (82890; Sigma-Aldrich). The treated mice were left for 24 h, followed by blood and lung tissue collection to evaluate their inflammatory response.

Cell Treatments

The U937 cells were treated with or without 2 μM NSC95397 and MTOB for 8 h, respectively, followed by coculturing with exosomes isolated from healthy control-sourced and ALI-sourced monocytes for another 12 h. The resulting cells were collected for RNA and protein extraction.

ChIP and RIP Assays

The cells (5×10^7) were rinsed twice with PBS and then treated with 1% formaldehyde (F8775, Sigma-Aldrich) for 12 min at room temperature. Following this crosslinking, the reaction was terminated using 1 M glycine for 5 min. After rinsing twice with PBS at room temperature, the cells were collected for ChIP and RIP assays with their corresponding kits (ChIP, 17-295; RIP, 17-700; Millipore Sigma, Burlington, MA, USA) according to the methods provided by the manufacturer. The antibodies used for the ChIP and RIP assays included anti-CtBP2, anti-p300, anti-p50, and anti-p65. IgG was used as a negative control. The purified DNA and RNA were used for quantitative real-time PCR analyses with the primers included in Table S5.

Statistical Analysis

All experiments were independently repeated at least three times. The results are presented as the mean ± standard deviation (SD) from a representative experiment. Comparisons between groups were performed using a two-tailed Student's t test, and the significance was set at *p < 0.05, **p < 0.01, and ***p < 0.001, respectively.

SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at <https://doi.org/10.1016/j.omtn.2020.07.040>.

AUTHOR CONTRIBUTIONS

Q.-G.L. and Z.-M.Q. designed the experiments. Z.C. performed most of the experiments, including exosome isolation; identification of lncRNA and gene profiles; and IP, coIP, and ChIP assays. W.-H.D. performed some of the experiments, including monocyte isolation, cell culture, and KD and OE of genes and lncRNA. Q.-G.L., Z.-M.Q., and Z.C. analyzed the data, tested the statistics, coordinated the figures, and wrote the manuscript. The other authors revised the article.

CONFLICTS OF INTEREST

The authors declare no competing interests.

ACKNOWLEDGMENTS

This study was supported by the Key Research and Development Project of Jiangxi Province (Social Development Field, no. 20202BBGL73059).

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