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Involvement of multiple transcription factors in regulation of IL-β-induced MCP-1 expression in alveolar type II epithelial cells

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ABSTRACT

During acute lung injury, a large number of monocytes are recruited into the pulmonary tissue, which is mainly mediated by local production of monocyte chemotactic protein 1 (MCP-1). As an essential component of the lung tissues, alveolar type II epithelial cells are one of the major sources of MCP-1. Therefore, uncovering the mechanism whereby MCP-1 production is regulated in the alveolar type II cells will provide a pivotal theoretical basis for clinical intervention in acute lung injury. In the current study, we find that there is a κB binding site in the MCP-1 promoter region, and mutation of the site leads to reduced production of MCP-1 in alveolar type II epithelial cells. In contrast, overexpression of NF-κB p65 significantly increases MCP-1 expression. Furthermore, we elucidate that IKKα/β—NF-κB p65 signaling pathway and phosphorylation of serine 534 in NF-κB p65 are required for the maximal expression of MCP-1. Also, Activator protein 1 (AP-1) site in the promoter region and JNK1/2—c-Jun signaling are required for MCP-1 generation in alveolar type II epithelial cells. Moreover, a CCAAT/enhancer-binding protein (C/EBP) element is identified in the MCP-1 promoter region through the point mutation technique, and further experiments demonstrate that both C/EBPβ and C/EBPδ are involved in basic and IL-1β-mediated MCP-1 expression. Of note, specificity protein 1—Sp1 expression is not changed in alveolar type II epithelial cells incubated with IL-1β, but it still control MCP-1 production by binding to the consensus sequence in the promoter region. More importantly, we find that the results derived from the cell line—MLE-12 cells and primary cells are consistent. Taken together, our data provide insights into the molecular mechanism how MCP-1 expression in inflammatory alveolar type II epithelial cells is regulated at transcription level.

1. Introduction

In critically ill patients, significant morbidity is induced by acute lung injury (ALI) and its more severe form—acute respiratory distress syndrome (ARDS), causing 75,000 deaths per year in the United States (Rubenfeld et al., 2005). ALI/ARDS often presents with the breakdown of alveolar epithelial and endothelial barrier integrities, which is driven by acute inflammatory responses and subsequent accumulation of activated inflammatory cells (Yan et al., 2016, 2013b; Yan et al., 2012b). Furthermore, ample evidences show that accumulation of inflammatory monocytes in pulmonary tissues is essential for ALI/ARDS (Coates et al., 2018; Jiang et al., 2017). Actually, MCP-1 generated in the local is the predominant signal promoting migration of peripheral blood monocytes from blood vessels into lungs. Exceptfor its chemotactic function, MCP-1 can also participate in inflammation by promoting expressions of other inflammatory mediators. For example, MCP-1 stimulates IL-6 and ICAM-1 expressions in human tubular epithelial cells (Viedt et al., 2002). Thus, the understanding of MCP-1 expression and underlying mechanisms will provide us with potent tools for clinical intervention in ALI/ARDS.

In unstimulated cells, NF-κB is sequestered in the cytoplasm by IκB family proteins. Once certain inflammatory signal appears, IκB kinase (IKK) is activated, leading to degradation of IκB family proteins and subsequent NF-κB activation. It has been demonstrated that LPS-induced MCP-1 expression in human monocyte cell line THP-1 cells is dependent on NF-κB (Ueda et al., 1997), and the involvement of NF-κB

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in ALI has also been validated (Kang et al., 2001). AP-1 transcription factor family members consisting of c-Fos, c-Jun, ATF and JDP families play critical roles in inflammatory responses. There is a report that MCP-1 expression is absent in tumorigenic HPV 18 positive cervical epithelial cells, which is due to alterations in the heterodimerization pattern of AP-1 and its selective accessibility to opened chromatin, indicating the important role of AP-1 in MCP-1 transcription expression (Finzer et al., 2000). C/EBPβ and C/EBPδ are two members of C/EBP family, and their roles in ALI/ARDS have been extensive demonstrated in our previous studies (Yan et al., 2016, 2013a; Yan et al., 2017, 2013b; Yan et al., 2012b). Sp1 is a zinc finger transcription factor which interacts with GC-rich motifs of a variety of promoters, and is involved in many physiological processes, such as lung inflammation (Schrumpf et al., 2017), cell growth and apoptosis. Its activity is predominantly regulated at posttranslational levels. However, roles of all the above transcription factors in MCP-1 production in inflammatory alveolar type II epithelial cells are still blurry.

During ALI/ARDS, pulmonary inflammatory reactions could be initiated by interaction of alveolar macrophages with danger signals, leading to secretion of pro-inflammatory mediators including early-response cytokines that function as inflammatory amplifiers. IL-1β is one of the early-response cytokines with the highest biologically active in lung tissues of patients with ALI/ARDS. It has been demonstrated that IL-1β increases lung permeability *via* alphavbeta5 and alphavbeta6 integrin-dependent mechanisms (Ganter et al., 2008). Moreover, it can also elevate pulmonary inflammation by enhancing expressions of various downstream pro-inflammatory factors like MCP-1. One of the major targets of IL-1β is alveolar type II epithelial cells that are the important MCP-1 producers during ALI/ARDS. Previous study has demonstrated that IL-1β treatment leads to the inducible expression of MCP-1 at mRNA level and the followed secretion of biologically active MCP-1 in alveolar macrophages (Brieland et al., 1995). However, MCP-1 expression and the roles of various transcription factors including AP-1, NF-κB, C/EBP and Sp1 in its generation are largely unknown in alveolar type II cells treated with IL-1β. In the present study, we seek to investigate the influence of the above mentioned transcription factors on IL-1β induction of MCP-1 in alveolar type II epithelial cells. Here, we find that IKKα/β-NF-κB p65-κB element pathway, JNK1/2-c-Jun-AP-1 site signaling, interaction between C/EBPβ/δ and C/EBP binding site, and binding of Sp1 to its consensus sequence are all required for IL-1β-induced maximal production of MCP-1 in alveolar type II epithelial cells.

2. Materials and methods

2.1. Cell cultures and reagents

MLE-12 cells (murine alveolar type II epithelial cells) are purchased from American Type Culture Collection (Manassas, VA, USA). The cells are grown in DMEM/F12 (1:1) medium supplemented with 5% fetal bovine serum (Gibico), 1% L-glutamine (Gibico) and 1% penicillin/streptomycin (Gibico). Murine IL-1β is obtained from PeproTech and dissolved in cell culture-grade phosphate-buffered saline.

2.2. Evaluation of gene expressions by real-time PCR

RNAs are isolated from MLE-12 cells by using Trizol (Invitrogen) as per the manufacturer's protocol. Then 2 μg of total RNAs are reverse transcribed into cDNAs by virtue of PrimeScript™ RT reagent Kit from TaKaRa. To evaluate the gene expression, the following protocol is applied to real-time PCR: denaturation at 95 °C for 3 min which is followed by 40 cycles of 95 °C for 10 s and 60 °C for 30 s. All the primers used are listed as follows: MCP-1, 5′ primer, 5′-AGG TGT CCC AAA GAA GCT GT-3′ and 3′ primer, 5′-ACA GAA GTG CTT GAG GTG GT-3′; C/EBPβ, 5′ primer, 5′-CAA GAA GAC GGT GGA CAA GC-3′ and 3′ primer, 5′-AGC TGC TCC ACC TTC TTC TG-3′; C/EBPδ, 5′ primer, 5′-CTT TTC AGC CTG GAC AGC C-3′ and 3′ primer, 5′-CAT GGA GTC AAT GTA GGC GC-3′; c-Jun, 5′ primer, 5′-GAG TCT CAG GAG CGG ATC AA-3′ and 3′ primer, 5′-CTG TTC CCT GAG CAT GTT GG-3′; Sp1, 5′ primer, 5′-TCA GCA GAC ACA GCA ACA AC-3′ and 3′ primer, 5′-ATT CTG CAG CTG AAG GGT CT-3′; NF-κB p65, 5′ primer, 5′- CAC CGG ATT GAA GAG AAG CG-3′ and 3′ primer, 5′-AAG TTG ATG GTG CTG AGG GA-3′.

2.3. Western blot analysis of protein expressions

Total cellular proteins are extracted by lysing MLE-12 cells in RIPA buffer (Beyotime Biotechnology, China). 20 μg of lysates are then run on 12% SDA-PAGE gel at 100 V. Two hours later, the resolved proteins are transferred to PVDF membranes. The membranes are blocked with 5% milk dissolved in phosphate-buffered saline plus 0.1% Tween 20 (PBS-T) for one hour. The blocked membranes are incubated overnight at 4 °C with rabbit anti-p-NF-κB p65 (Ser536) (Cell Signaling Technology), rabbit anti-NF-κB p65 (Santa Cruz), mouse anti-p-JNK (Santa Cruz), mouse anti-JNK1/2 (Santa Cruz), goat anti-p-c-Jun (Ser63/73) (Santa Cruz), mouse anti-c-Jun (Santa Cruz), rabbit anti-C/EBPδ (Santa Cruz), rabbit anti-IKKα/β (Ser176/180) (Cell Signaling Technology), mouse anti-C/EBPβ (Proteintech) (Santa Cruz), rabbit anti-Sp1 (Proteintech), or rabbit anti-GAPDH (Cell Signaling Technology). After being washed three times in PBS-T, the blots are incubated with horseradish peroxidase-conjugated secondary antibodies. Finally, the immunoreactive proteins are visualized by using the Enhanced Chemiluminescence kit (Amersham Biosciences).

2.4. Plasmids

NF-κB p65, C/EBPβ, C/EBPδ, Sp1 and c-Jun overexpression vectors are constructed as described previously (Yan et al., 2018). Briefly, the coding regions of the above genes are amplified from the mouse lung cDNA library by the PCR technique. Then the resulting fragments are cut with the restriction enzymes, and inserted into pcDNA3.1-Myc-His (Invitrogen) to form pcDNA3.1-NF-κB p65-Myc-His, pcDNA3.1-C/EBPβ-Myc-His, pcDNA3.1-C/EBPδ-Myc-His, pcDNA3.1-Sp1-Myc-His, and pcDNA3.1-c-Jun-Myc-His, respectively. The Mut Express II Fast Mutagenesis Kit V2 (Vazyme, China) is used for constructions of two c-Jun mutants (Ser63 to Ala or Ser73 to Ala) and one NF-κB p65 mutant (Ser534 to Ala). pcDNA3.1-EGFP is generated by ligation of the EGFP coding region from pIRES-EGFP (Clontech) with the pcDNA3.1-Myc-His backbone. Different lengths of MCP-1 promoter, -441 to +64, -176 to +64, -120 to +64 and -45 to +64, are amplified from mouse genomic DNA by PCR, and are then cloned into pGL4-basic vector (Promega) to form distinct recombinant plasmids—pGL4/441, pGL4/176, pGL4/120 and pGL4/45. The MCP-1 promoter-luciferase construct (MCP-1 Luc) with the indicated transcription factor binding site mutation is produced by virtue of The Mut Express II Fast Mutagenesis Kit V2. AP-1 Luc—AP-1 reporter gene, is purchased from Beyotime Biotechnology (China). κB reporter gene (κB Luc) is obtained from Promega.

2.5. Lentivirus- or siRNA-mediated gene knockdown

All lentiviral particles including control, NF-κB p65, c-Jun, Sp1 and C/EBPδ shRNA, and control siRNA and C/EBPβ siRNA are obtained from Santa Cruz. MLE-12 cells are infected by the lentivirus at an IFU of 5 for 72 h, which is followed by real-time PCR assays of the gene knockdown efficiencies. For siRNA-triggered downregulation of gene expression, MLE-12 cells are transfected with control siRNA or C/EBPβ specific siRNA by using Lipofectamine 2000 (Invitrogen). 24 h later, RNAs are extracted and the gene production is detected by real-time PCR.

2.6. Luciferase measurement

The indicated plasmids are transfected into MLE-12 cells by using

Fugene®6 (Promega) according to the instruction provided by the manufacturer. One day later, the cells are treated with or without IL-1β for 6 h. Then the cells are lysed, and the Dual-Luciferase Reporter Assay System (Promega) is applied to measure the luciferase activities of the lysates. Firefly luciferase expression is first normalized to that of the renilla luciferase, and then set to 1 in control cells.

2.7. ELISA

The MLE-12 cells are incubated with IL-1β for 24 h. Then the supernatants are harvested and centrifuged at 3000 rpm for 5 min at 4 °C. The resulting cell-free supernatants are subjected to measurements of MCP-1 concentrations by using ELISA kit obtained from R&D.

2.8. Isolation of primary type II alveolar epithelial cells

All the animal experiments are conducted according to the protocol approved by Southeast University and the Animal Ethics number is 20160101003. Isolation of primary type II alveolar epithelial cells from C57BL/6 mouse lungs is performed as described previously (Yan et al., 2018, 2012a). Briefly, mouse lungs are harvested under sterile conditions, and the single cell suspension is prepared. The cell suspension is first incubated with biotinylated mouse anti-CD32 and anti-CD45 antibodies and then mixed with Streptavidin MagneSphere to remove lymphocytes from the suspension.

2.9. Statistical analysis

All values are represented by the mean ± S. E. M. $p < 0.05$ means significant difference. Data sets are analyzed by using Student's *t*-test or one-way ANOVA, with individual group means being compared with the Student-Newman-Keuls multiple comparison test.

3. Results

3.1. Multiple regulatory elements in the promoter region are required for MCP-1 expression in alveolar type II epithelial cells

MCP-1 is the most potent chemokine in the regulation of migration and infiltration of monocytes, and its expression is mainly regulated at the transcription level. In the lung tissues, the alveolar type II epithelial cell is one of the most important sources of MCP-1. To identify regulatory elements which are required for transcriptional regulation of MCP-1 in the promoter region, a 505 bp genomic fragment containing 441 bp of 5′-flanking region upstream of the gene is amplified from mouse genome and is cloned into the pGL4-basic vector to form MCP-1 promoter-luciferase construct (pGL4/441) (Fig. 1A). Then a series of 5′-deletion mutants of the promoter region are constructed based on pGL4/441, which are referred as pGL4/176, pGL4/120, and pGL4/45, respectively (Fig. 1A). The resulting chimeric plasmids are transfected into the alveolar type II epithelial cells—MLE-12 cells, and the luciferase activities are measured. When compared with MLE-12 cells transfected with pGL4/441 plasmids, the cells transfected with pGL4/176 plasmids demonstrate slightly increased luciferase expression, but there is no significant difference between the two groups (Fig. 1A), which indicates that there might be no crucial elements involved in MCP-1 transcription in the promoter region from -441 bp to -176 bp. In contrast, the cells transfected with pGL4/120 and pGL4/54 plasmids show decreased transcriptional activity of the MCP-1 promoter (43%, and 60%, respectively, compared with full length controls) (Fig. 1A), which means that there are critical transcription factor binding sites in the segment located between -176 bp and -45 bp upstream of the gene transcription start site. By virtue of transcription factor binding site prediction software—the Genomatix Gene2promoter analysis tool, we find several potential regulatory sites including κB site, AP-1 site, C/EBP site and Sp1 site in the region between -176 bp and -45 bp upstream of the transcription start site of the MCP-1 gene (Fig. 1B).

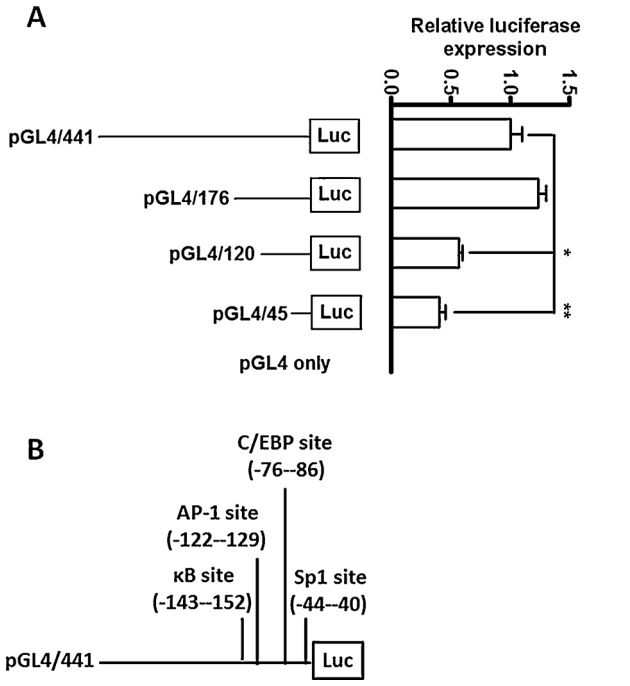

Fig. 1. Identification of possible transcription factor binding sites in the MCP-1 promoter region in alveolar type II epithelial cells. A. 5′-truncated mutants of the MCP-1 promoter are constructed by progressive deletion of base pairs at the 5′ ends and ligation with the reporter plasmids—pGL4-basic vectors. Then MLE-12 cells are transiently transfected with a series of deletion mutants of the MCP-1 promoter. 24 h later, the cells are lysed and luciferase activities are measured. B. The potential transcription factor binding sites are predicated by the Genomatix Gene2promoter analysis tool. Data are expressed as mean ± SEM (N = 3). * and ** indicate $p < 0.05$, and 0.01, respectively.

3.2. Involvement of the κB site and the IKK-NF-κB p65 signaling pathway in IL-1β induction of MCP-1

The software prediction has found a κB site in the MCP-1 promoter region. However, the existence of the site should be further demonstrated by experiments. So MCP-1 promoter reporter plasmids with NF-κB binding site mutation are constructed. When transfected into MLE-12 cells, the luciferase production by MCP-1 reporter genes with κB site mutation is only 43% of the wild type control (Fig. 2A), indicating that the NF-κB binding site plays a crucial role in MCP-1 transcription. Moreover, in the presence of NF-κB p65 overexpression plasmids, the luciferase expressions driven by the wild type MCP-1 promoters are significantly elevated (Fig. 2B). Our previous paper has proven the critical role for phosphorylated serine 534 in NF-κB p65 in IL-6 transcription (Yan et al., 2018), so we plan to examine its effect on MCP-1 expression in MLE-12 cells. When compared with its wild type controls, NF-κB p65 with the mutation of serine to alanine cannot stimulate MCP-1 promoter-mediated luciferase activities anymore (Fig. 2C). To further confirm the influence of endogenous NF-κB p65 on MCP-1 reporter genes, NF-κB p65 expression in MLE-12 cells is firstly downregulated by using the specific shRNA (Fig. 2D). As shown in Fig. 2E, NF-κB p65 specific shRNA treatment decreases MCP-1 promoter-mediated luciferase generation to 31% of that in control shRNA-treated MLE-12 cells.

To further determine if NF-κB signaling is required for endogenous MCP-1 expression in MLE-12 cells in an inflammatory environment. We first treat MLE-12 cells with different concentrations of IL-1β for 2 h to find out if MCP-1 expression is affected by the pro-inflammatory mediator. As shown in Fig. 3A, when the concentration of IL-1β is in the range of 0–5 ng/ml, MCP-1 transcription is dose-dependently increased

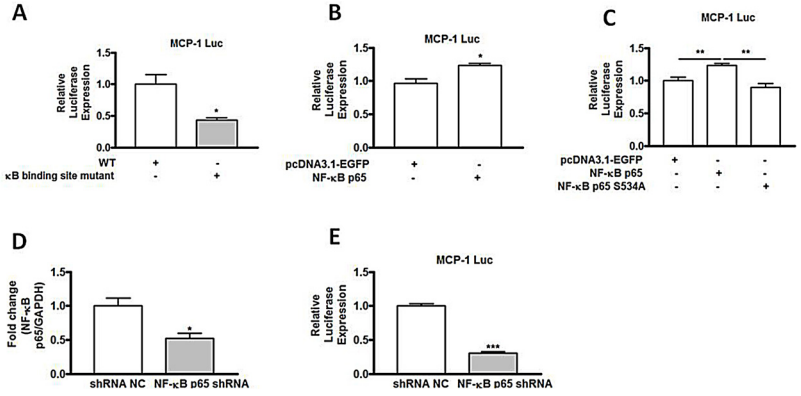

Fig. 2. Crucial roles of κB site and NF-κB p65 in MCP-1 promoter-driving luciferase production in alveolar type II epithelial cells. A, B and C. MLE-12 cells are transiently transfected with the indicated plasmids. 24 h later, the cell lysates are used to evaluate luciferase activities. -D. MLE-12 cells are infected with lentivirus expressing control shRNA and NF-κB p65 specific shRNA, respectively. The knockdown efficiency is estimated by virtue of real-time PCR. E. The cells constructed in Fig. 2D are transiently transfected with the indicated constructs. 24 h later, the cells are lysed and luciferase activities are determined. Data are expressed as mean ± SEM (N = 3). *, ** and ** indicate $p < 0.05$, 0.01, and 0.001, respectively.

by the stimulus, however, its expression cannot be further amplified when the concentration of IL-1β is elevated to 10 and even 20 ng/ml. Therefore, the concentration of IL-1β used in the subsequent experiments is 5 ng/ml. Then we stimulate the MLE-12 cells with IL-1β for different time points, and real-time PCR techniques are applied to estimate MCP-1 generation at the transcription level. We observe that after 2 h treatment with IL-1β, MCP-1 production is sharply increased to more than 200 folds, and then gradually decreased from 4 h to 24 h (Fig. 3B). To analyze the importance of NF-κB signaling for IL-1β-stimulated MCP-1 expression, the effect of IL-1β on NF-κB-mediated transcription activity is measured. As shown in Fig. 3C, the luciferase expression induced by IL-1β treatment is more than twice that of the control. We further investigate the upstream signaling that might be involved in NF-κB activation, and confirm that IKKα/β phosphorylation

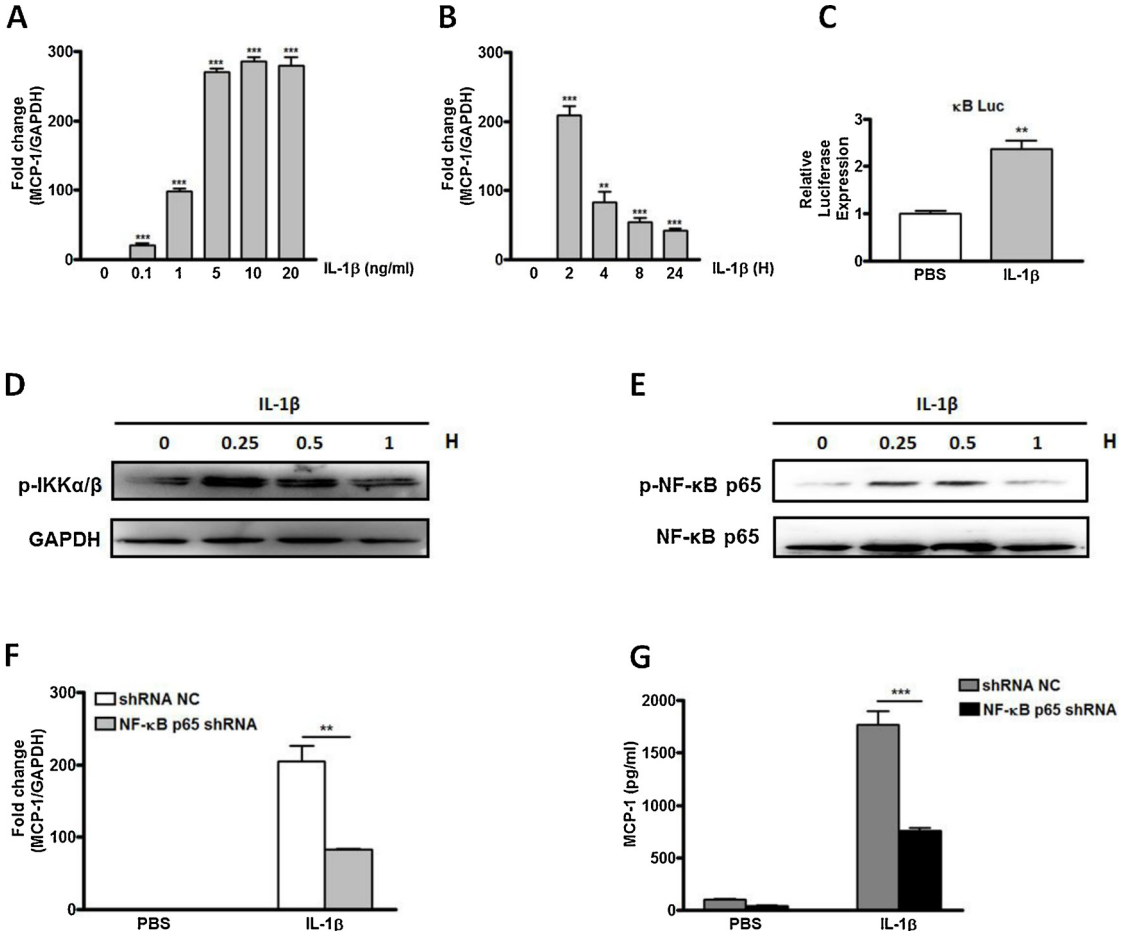

Fig. 3. Positive regulation of endogenous MCP-1 expression by IKKα/β-NF-κB p65 signaling pathway in alveolar type II epithelial cells. A. MLE-12 cells are incubated with different concentrations of IL-1β for 2 h. Then RNAs are extracted and real-time PCR is performed to measure the relative generation of MCP-1 (N = 3). B. MLE-12 cells are stimulated by 5 ng/ml of IL-1β, and total RNAs are harvested at distinct time points. MCP-1 levels are measured by using real-time PCR technique (N = 3). C. MLE-12 cells receiving the indicated treatment are lysed and subjected to luciferase assays (N = 3). D and E. MLE-12 cells are treated by 5 ng/ml of IL-1β for the indicated time periods. Total cellular proteins are then extracted and subjected to Western blot assays. The following antibodies are used: anti-IKKα/β, anti-p-NF-κB p65, anti-NF-κB p65 and anti-GAPDH. MLE-12 cells receiving control shRNA or NF-κB p65 shRNA treatment are incubated with PBS or 5 ng/ml of IL-1β. Then RNAs and cell-free supernatants are harvested separately, and MCP-1 generation is measured at the RNA (F, N = 3) and protein (G, N = 5) levels, respectively. Data are expressed as mean ± SEM. ** and ** indicate $p <$ 0.01, and 0.001, respectively.

is greatly enhanced 15 min after addition of IL-1β to the medium (Fig. 3D). Moreover, activation of IKKα/β could be detected even an hour after IL-1β stimulation (Fig. 3D). Our previous experiment has proven the critical role of Ser534 in NF-κB p65 in MCP-1 transcription, so we examine if the amino acid residue is phosphorylated in MLE-12 incubated with IL-1β. The Western blot assays show that as early as 15 min after treatment with IL-1β, the phosphorylation of the serine residue is markedly induced and then -remains at a high level (Fig. 3E). Furthermore, we determine the influence of NF-κB p65 on IL-1β-induced MCP-1 transcription, and demonstrate that in the presence of IL-1β, downregulation of NF-κB p65 expression causes MCP-1 production to drop to 40% of the control (Fig. 3F). Also, IL-1β-mediated MCP-1 expression at the protein level is decreased to less than 50% of the control due to suppression of NF-κB p65 expression (Fig. 3G).

3.3. Critical effects of AP-1 element and JNK/c-Jun on IL-1β-stimulated MCP-1 expression

Our previous work has predicted an AP-1 site in the MCP-1 promoter region. To further confirm our prediction, mutations are introduced into the AP-1 binding site in the MCP-1 promoter region, and the influence of AP-1 site mutation on the gene transcription is measured. As shown in Fig. 4A, AP-1 binding site mutation results in a decrease of the luciferase generation to around 10% of the control group. However, the exogenous expression of c-Jun leads to a 44% increase in the luciferase production-driven by the wild type promoter sequence, compared to the control (Fig. 4B). We next intend to investigate the importance of serine 63 and serine 73 of c-Jun for its transcription activity in MCP-1 expression. As shown in Fig. 4C, ectopic expression of wild type c-Jun, c-Jun S63 A, and c-Jun S73 A, causes the luciferase activity to increase to 139%, 120%, and 116% of the control, respectively, which means that mutation of serine 63 or 73 in c-Jun does not totally abolish its transcription activity, but either mutation indeed attenuates the activity of the wild type protein. To further elucidate the effect of endogenous c-Jun on the luciferase expression driven by the MCP-1 promoter, we inhibit c-Jun expression by infecting alveolar type II epithelial cells with lentivirus producing c-Jun specific shRNA (Fig. 4D), which results in the luciferase production to drop by nearly 50% (Fig. 4E).

Next, we plan to clarify if endogenous MCP-1 expression is affected by AP-1 in alveolar type II epithelial cells treated with IL-1β. The activation of AP-1 under IL-1β treatment is firstly examined, and we find that AP-1-meidated luciferase expression is increased by the stimulus to 195% of the control group treated by PBS (Fig. 5A). Normally, JNK phosphorylation is the upstream signal of AP-1 activation. As expected, phosphorylation of JNK1/2 is obviously augmented in alveolar type II epithelial cells 15 min after IL-1β stimulation, and then the phosphorylation level is slowly decreased (Fig. 5B). Moreover, the elevated activity of JNK1/2 leads to enhanced activation of the downstream signaling molecule—c-Jun (Fig. 5C), which proves that AP-1 signaling is activated. Then the influence of AP-1 signaling on IL-1β-stimulated endogenous MCP-1 transcription is illustrated by interfering with the expression of c-Jun. As shown in Fig. 5D, IL-1β treatment dramatically increases MCP-1 transcription to 147 folds of the control stimulated by PBS. However, in the presence of c-Jun specific shRNA, IL-1β-stimulated gene expression is reduced by 49% (Fig. 5D). Furthermore, ELISA techniques are applied to evaluate the effect of c-Jun knockdown on MCP-1 expression in IL-1β-treated alveolar type II epithelial cells at the protein level. We observe that in the presence of IL-1β, MCP-1 protein level is sharply increased, and downregulation of c-Jun expression leads to a reduction of MCP-1 secretion to 57% of the control (Fig. 5E).

3.4. Roles of C/EBP binding site and C/EBPs in regulation of IL-1β-stimulated MCP-1 expression

The software analysis has found a C/EBP binding site in the MCP-1 promoter region. To validate if the element is important for promoting MCP-1 production, we construct the MCP-1 promoter reporter gene with mutations in C/EBP binding site and estimate the effect of C/EBP element on the gene production. We observe that C/EBP element mutation leads to a 50% decrease in luciferase production when compared with the control (Fig. 6A). Our previous studies have demonstrated that C/EBPβ plays an essential role for IL-6 expression in alveolar type II epithelial cells (Yan et al., 2012a). So we try to explore whether the C/EBP family member is also critical for MCP-1 expression in alveolar type II epithelial cells. As shown in Fig. 6B, ectopic C/EBPβ expression increases MCP-1 promoter-driving luciferase production by 40% compared to the control. We further analyze the role of endogenous C/EBPβ in generation of MCP-1 promoter-mediated luciferase activity. As shown in Fig. 6C, C/EBPβ specific siRNA treatment reduces the

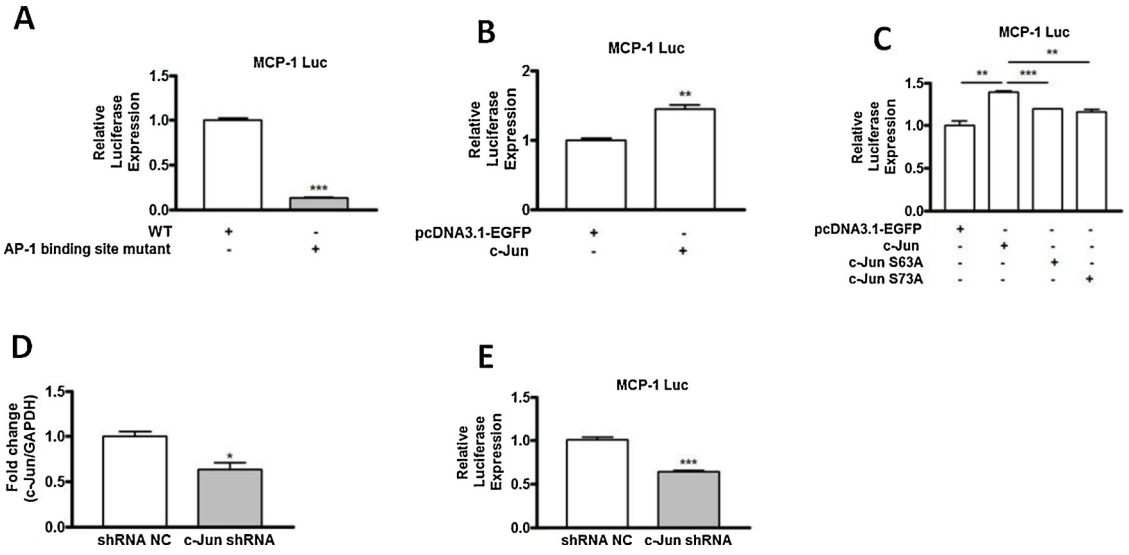

Fig. 4. Involvement of AP-1 element and c-Jun in production of MCP-1 promoter reporter plasmids in alveolar type II epithelial cells. A, B and C. The indicated plasmids are transfected into MLE-12 cells. 24 h later, the cells are lysed and subjected to evaluation of luciferase production. D. RNAs are extracted from MLE-12 cells treated with lentivirus expressing control shRNA or c-Jun specific shRNA. Real-time PCR techniques are applied to measure c-Jun expressions. E. The cells constructed in Fig. 3F are transiently transfected with the indicated constructs. 24 h later, the cell lysates are used to determine the luciferase activities. Data are expressed as mean ± SEM (N = 3). *, ** and *** indicate $p < 0.05$, 0.01, and 0.001, respectively.

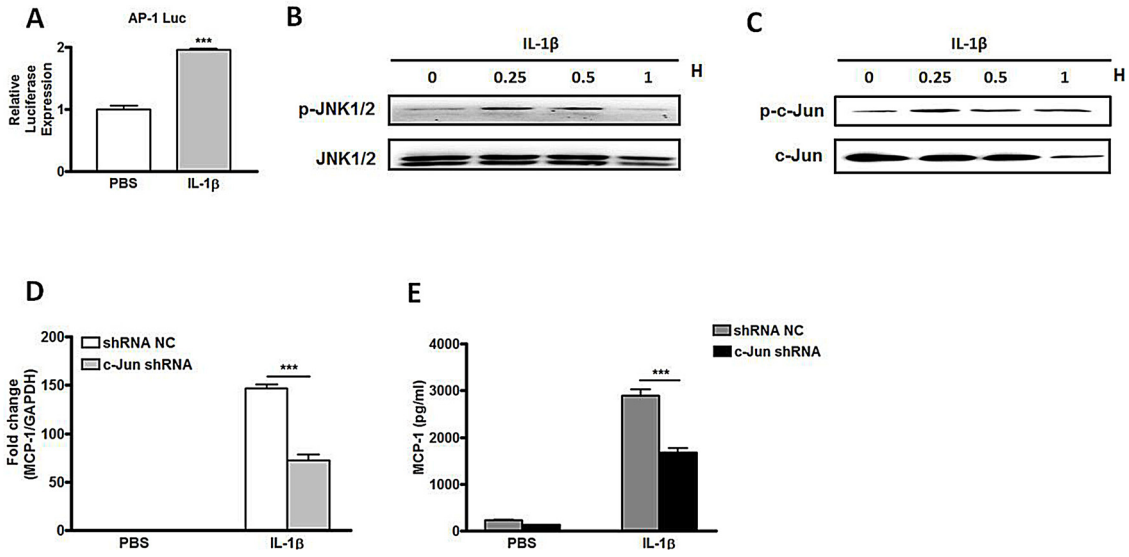

Fig. 5. Influence of c-Jun and the related signaling pathway on IL-1β induction of MCP-1 in alveolar type II epithelial cells. A. MLE-12 cells are transiently transfected with the AP-1 reporter plasmids (AP-1 Luc). 24 h later, the cells are treated with PBS and 5 ng/ml of IL-1β, respectively. Then the cell lysates are used to determine the luciferase expression (N = 3). B and C. MLE-12 cells are incubated with 5 ng/ml of IL-1β for different time periods, and then total cellular proteins are extracted. The cellular proteins are analyzed by using antibodies against p-JNK1/2, JNK1/2, p-c-Jun, and c-Jun, respectively. D (N = 3) and E (N = 5), Cells constructed in Fig. 4D are treated with PBS or 5 ng/ml of IL-1β. Then total RNAs and cell-free supernatants are harvested separately, and real-time PCR and ELISA are used to determine MCP-1 production at the RNA and protein levels, respectively. Data are expressed as mean ± SEM. *** indicate $p < 0.001$.

endogenous gene expression by about half, and downregulation of C/EBPβ expression leads to a 23% reduction in the luciferase production (Fig. 6D).

We then elucidate whether endogenous MCP-1 expression is regulated by C/EBPβ in IL-1β-treated alveolar type II epithelial cells. We find that C/EBPβ mRNA level in MLE-12 cells is significantly increased after two hours of IL-1β stimulation and then slowly decreased (Fig. 6E), which is almost consistent with the Western blot analysis (Fig. 6F). The above data indicate that C/EBPβ might be involved in regulation of IL-1β-stimulated MCP-1 production. Indeed, IL-1β-mediated MCP-1 expression is significantly inhibited at both mRNA and protein levels by specific siRNA targeting C/EBPβ (Fig. 6G and H).

An abundance of evidences show that another C/EBP family member—C/EBPδ is a critical transcription factor involved in expressions of pro-inflammatory factors. Therefore, we examine the influence of C/EBPδ on MCP-1 production. As shown in Fig. 7A, C/EBPδ-mediated production of MCP-1 promoter reporter gene is elevated to 1.18 folds that of the control, and suppression of C/EBPδ expression by shRNA treatment (Fig. 7B) results in a 19% decrease in the luciferase activity compared with the normal group (Fig. 7C). Further experiments show that C/EBPδ production in MLE-12 cells is obviously stimulated by IL-1β at both mRNA and protein levels (Fig. 7D and E), which contributes to IL-1β-triggered MCP-1 generation (Fig. 7F and G).

3.5. Indispensable roles of Sp1 element and Sp1 in IL-1β induction of MCP-1 in alveolar type II epithelial cells

The software scan finds a Sp1 binding site in the MCP-1 promoter region. To prove the prediction, we introduce mutants into the Sp1 element in the promoter region, which is followed by luciferase assays. We observe that the mutant promoter-mediated luciferase production is reduced by 88% compared with the wild type control (Fig. 8A). In addition, exogenous Sp1 leads to 2.42-fold raise in the luciferase generation driven by the MCP-1 promoter when compared with the control (Fig. 8B). We then illustrate if downregulation of Sp1 affect production of MCP-1 promoter reporter genes by treatment of MLE-12 cells with Sp1 specific shRNA (Fig. 8C). As shown in Fig. 8D, endogenous suppression of Sp1 expression causes the wild type promoter-mediated luciferase expression by more than 50%.

We further elucidate the influence of Sp1 on IL-1β-induced MCP-1 expression in alveolar type II epithelial cells. As shown in Fig. 8E and F, Sp1 production is rarely changed in IL-1β-treated MLE-12 cell. However, infection of MLE-12 cells with Sp1 shRNA-expressing lentiviruses results in a 33% decrease in MCP-1 transcription when compared with the normal control (Fig. 8G). Furthermore, MCP-1 protein production in Sp1 shRNA-treated cells is also reduced to 65% of that in the control group (Fig. 8H).

3.6. IL-1β-induced MCP-1 expression in primary alveolar type II epithelial cells is promoted by NF-κB p65, c-Jun, C/EBPβ/δ and Sp1

To further investigate the regulatory roles of NF-κB p65, c-Jun, C/EBPβ/δ and Sp1 in MCP-1 production, luciferase assays are conducted in primary alveolar type II epithelial cells. As shown in Fig. 9, MCP-1 promoter-mediated luciferase generation is significantly upregulated by IL-1β, and NF-κB p65, c-Jun, C/EBPβ, C/EBPδ and Sp1 further increase IL-1β-triggered MCP-1 promoter activity to 2.12 times, 2.59 times, 1.53 times, 1.58 times, and 2.78 times, respectively. Thus, the data obtained from primary alveolar type II epithelial cells clarify the essential roles of the above five transcription factors in IL-1β induction of MCP-1, which is consistent with the results from the cell line.

4. Discussion

The number of alveolar macrophages is dramatically elevated during ALI/ARDS (Brieland et al., 1987), which is at least partially derived from the influx of activated peripheral blood monocytes (Denholm et al., 1989). Recruitment of active monocytes into the pulmonary space during inflammatory lung injury is due to chemokines generated at the inflammatory focus (Brieland et al., 1987; Denholm et al., 1989; Yoshimura et al., 1989). There are increasing evidences showing that MCP-1, upon binding to its receptor CCR2, becomes one of the most biologically active chemokines controlling activation and the followed recruitment of monocytes into inflammatory lung tissues. The alveolar type II epithelial cells play essential roles in the induction of acute lung inflammation. Our recent data indicate that following

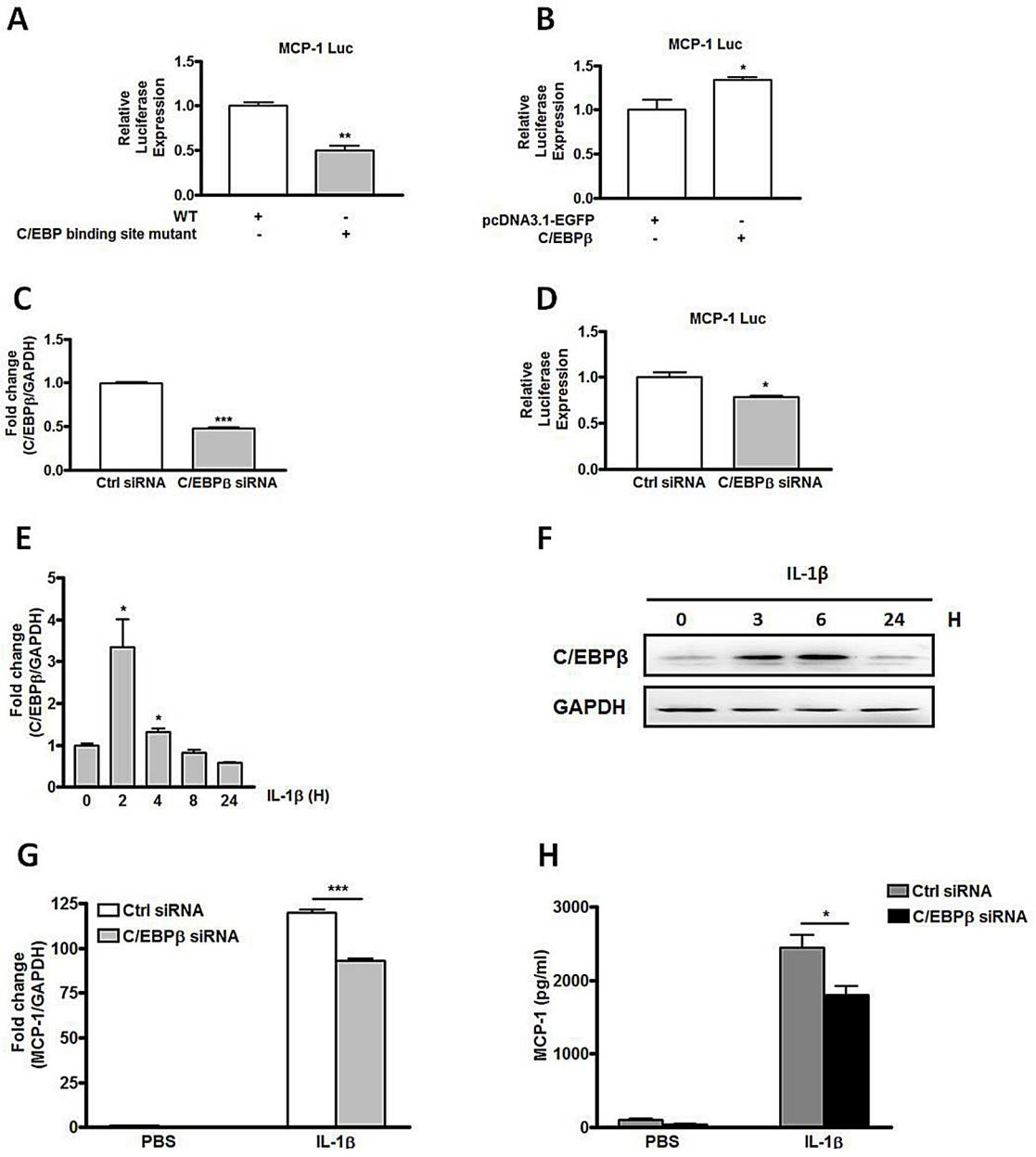

Fig. 6. Involvement of C/EBPβ in MCP-1 production in alveolar type II epithelial cells stimulated by IL-1β. A and B. The indicated recombinant plasmids are transfected into MLE-12 cells. 24 h later, the cells are lysed and the luciferase activity is measured (N = 3). C. MLE-12 cells are transfected with control siRNA or C/EBPβ siRNA. Then total cellular RNAs are extracted and RT-qPCR is performed to evaluate C/EBPβ generation (N = 3). D. MLE-12 cells are handled with the indicated treatment. 24 h later, cell lysates are used to measure the luciferase activities (N = 3). MLE-12 cells are treated with 5 ng/ml of IL-1β for different time periods. Then total RNAs or whole cellular proteins are harvested, and C/EBPβ production is examined at both RNA (E, N = 3) and protein levels (F). MLE-12 cells are transfected with control siRNA or C/EBPβ siRNA. 24 h later, the cells are stimulated with PBS or 5 ng/ml of IL-1β. Then total RNAs and cell-free supernatants are harvested, and MCP-1 generation is evaluated by RT-qPCR (G, N = 3), and ELISA (H, N = 5), respectively. Data are expressed as mean ± SEM. * and ** indicate $p < 0.05$, and 0.001, respectively.

induction of inflammatory response in alveolar type II cells by either TNF-α or IL-1β, expression and secretion of IL-6 could be observed (Yan et al., 2018, 2012a). However, inflammatory mediators, present *in vivo* during the acute pulmonary inflammation, which are involved in regulation of MCP-1 expression by alveolar type II cells have not been totally identified. In the current study, we carry out a series of *in vitro* experiments to investigate the influence of IL-1β on MCP-1 generation in the alveolar type II cells, and the corresponding mechanisms.

IL-1β could be recovered from pulmonary tissues of patients early in ALI/ARDS (Meduri et al., 1995). As one of the early-response cytokines, IL-1β would exacerbate ALI/ARDS by aggravating pulmonary inflammatory responses through stimulation of downstream pro-inflammatory mediators' production (Kolb et al., 2001; Lentsch et al., 1998; Olman et al., 2002). Our previous studies have shown that IL-6 expression at both mRNA and protein levels is significantly induced by IL-1β treatment (Yan et al., 2012a). Moreover, the deleterious effects of IL-6, which is a pleiotropic cytokine generated in response to a variety of inflammatory stimuli by different types of cell populations, on ALI/ARDS have been widely proven (Cuzzocrea et al., 1999; Hierholzer et al., 1998). However, the role of IL-1β in production of other inflammatory mediators during ALI/ARDS is not totally determined. Here, we observe that MCP-1 transcription in alveolar type II epithelial cells is significantly upregulated by IL-1β, which leads to high levels of the protein release.

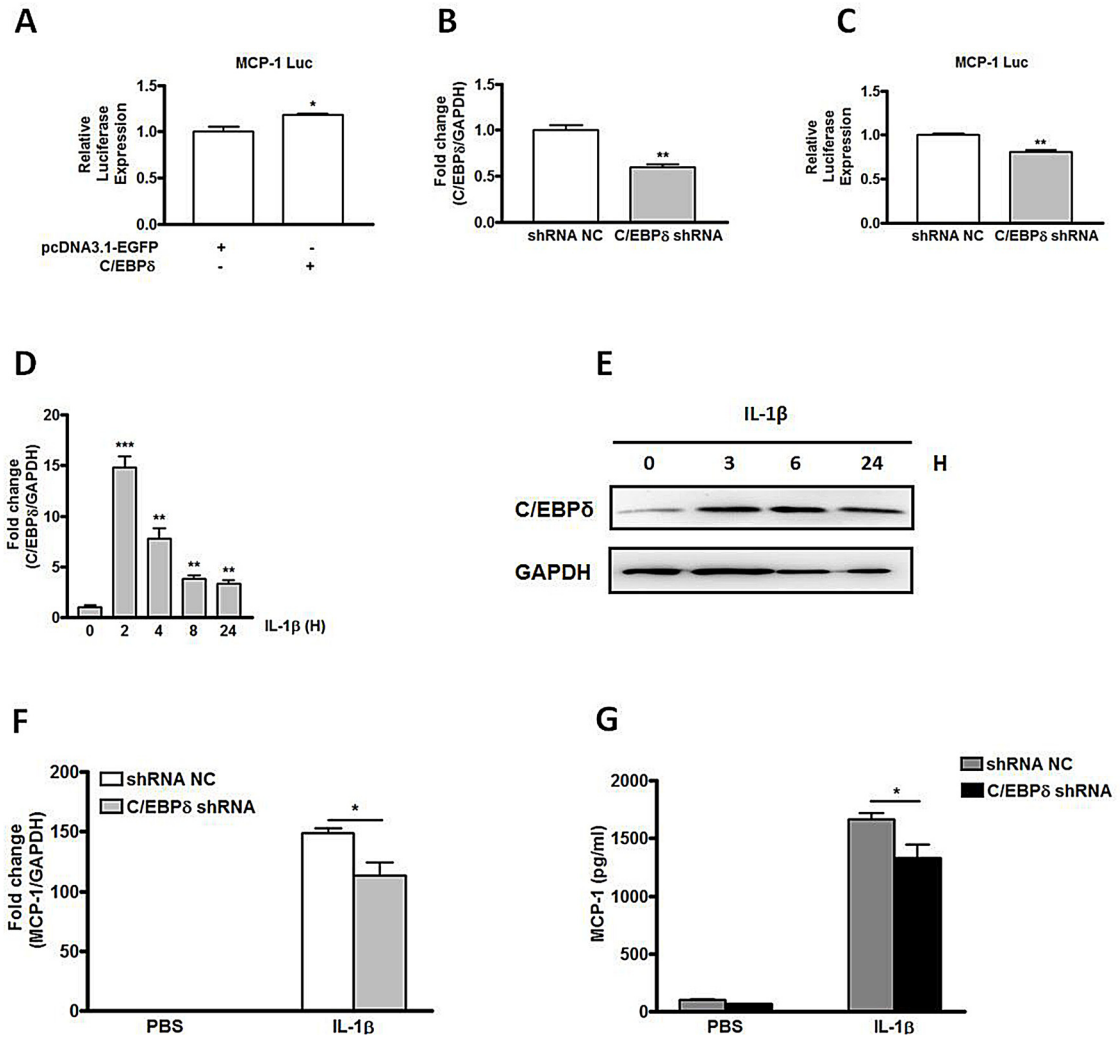

Fig. 7. Critical role of C/EBPδ in the MCP-1 production in IL-1β-treated alveolar type II epithelial cells. A. MLE-12 cells are transfected with the indicated recombinant genes, and the cell lysates are subjected to luciferase analysis 24 h after transfection (N = 3). B. MLE-12 cells are infected with lentiviruses expressing negative control or C/EBPδ shRNA, and the target gene generation is detected by RT-qPCR (N = 3). C. MLE-12 cells treated with control or C/EBPδ shRNA are transfected with MCP-1 promoter reporter genes. 24 h later, cell lysates are utilized to perform luciferase assays (N = 3). MLE-12 cells are incubated with 5 ng/ml of IL-1β for distinct time points, and then the total RNAs and proteins are extracted separately. RT-qPCR (D, N = 3) and Western blot assays (E) are used to detect C/EBPδ generation. MLE-12 cells are subjected to the indicated treatment. Then MCP-1 production is analyzed at both RNA (F, N = 3) and protein levels (G, N = 5). *, ** and ** indicate $p < 0.05$, 0.01, and 0.001, respectively.

During ALI/ARDS, the alveolar epithelium, composed of alveolar type I and II cells, is one of the major targets of IL-1β. In quantity, the ratio of alveolar type I to II cells is 1:2, but the alveolar type I cells cover about 95% of the alveolar surface by virtue of their thin squamous cell extensions (Crapo et al., 1982). So they are the main components of the alveolar epithelium. However, the cuboidal alveolar type II cells also play crucial roles in supporting the normal functions of pulmonary tissues by secretion of surfactants including SP-A, SP-B, SP-C and SP-D, and renewal of aging and injured type I cells. In addition, there are sufficient evidences that alveolar type II epithelial cells are themselves able to participate in pulmonary inflammatory responses through production of a series of pro-inflammatory mediators, resulting in activation and recruitment of immune cells like monocytes/macrophages. For example, the type II pneumocytes synthesize and secrete components of the classical and alternative complement activation pathways (Strunk et al., 1988). Additionally, other studies have reported that the alveolar type II epithelial cells might take part in fighting against bacterial infections by secreting chemokines, and the significance of MCP-1, one of the most active chemokines acting on dendritic cells and alveolar macrophages, has also been determined (Kannan et al., 2009; Sato et al., 2002; Thorley et al., 2005; Vanderbilt et al., 2003). However, the transcriptional mechanisms underlying MCP-1 expression are largely unknown.

The pivotal roles of NF-κB in the transcription of sorts of pro-inflammatory mediators have been widely demonstrated. Here, we identify that κB site in the promoter region is indispensable for IL-1β-induced MCP-1 expression in alveolar type II epithelial cells *via* using the point mutation technique. Furthermore, we find that the signaling pathway—IKKα/β-NF-κB p65 is activated by the stimulus and is required for the chemokine generation. Interestingly, NF-κB p65-mediated transcription of MCP-1 is significantly suppressed with the mutation of serine 534 into alanine. Replacement of the serine residue in NF-κB p65 with alanine prevents the amino acid from phosphorylation, indicating that phosphorylated serine 534 exerts positive effect on the transcription activity of the protein. However, the upstream kinase involved in adding phosphoric acid group to the serine residue remains an open question in alveolar type II epithelial cells. c-Jun belongs to AP-1 family consisted of c-Jun, c-Fos, Jun dimerization protein (JDP) and activating transcription factor (ATF) (Hess et al., 2004). AP-1 transcription factor has been reported to regulate a wide range of cellular

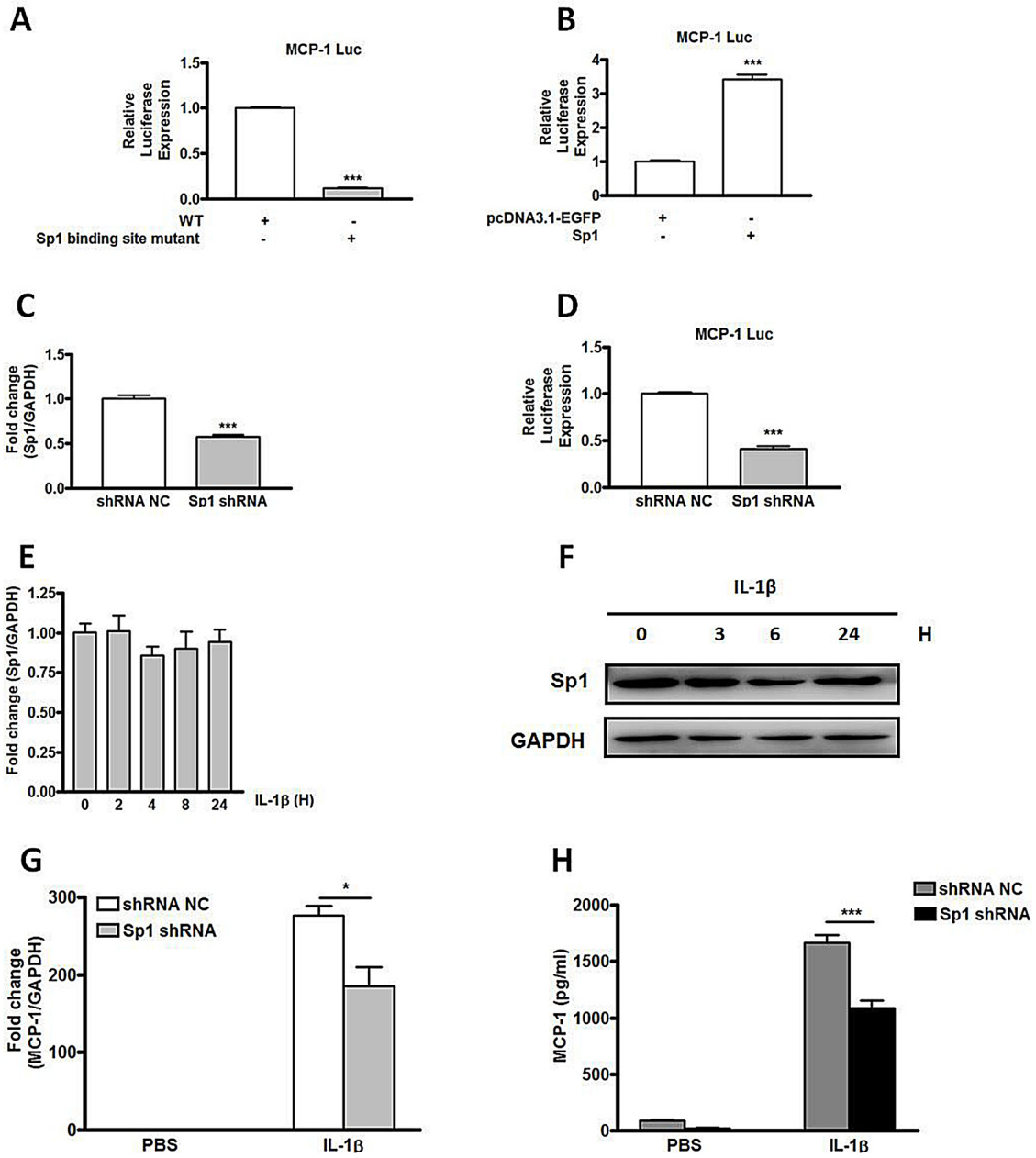

Fig. 8. Effect of Sp1 on IL-1β-induced MCP-1 expression in alveolar type II epithelial cells. A and B. MLE-12 cells are transfected with the indicated plasmids. 24 h later, the cells are lysed and luciferase production is measured (N = 3). C. MLE-12 cells are treated with control or Sp1 shRNA, and Sp1 expression is detected through RT-qPCR technique (N = 3). D. MLE-12 cells receive the indicated treatment. Then the cell lysates are subjected to luciferase analysis (N = 3). MLE-12 cells are stimulated by 5 ng/ml of IL-1β for different time points. Then the cells are harvested, and RNAs and proteins are extracted. Sp1 expressions are analyzed by RT-qPCR (E, N = 3), and Western blot, respectively (F). MCP-1 generation at both RNA (G, N = 3) and protein (H, N = 5) levels is detected in MLE-12 receiving the indicated treatment. * and ** indicate $p < 0.05$, and 0.001, respectively.

processes including inflammatory responses (Bhargava et al., 2012; Murakami et al., 2013; Sun et al., 2016). In the present paper, we observe that AP-1 binding site mutation in MCP-1 promoter region leads to a significant decrease in the gene expression. Further experiments show that phosphorylation of serine 63 and 73 in c-Jun by activated JNK1/2 is important for IL-1β induction of MCP-1 in alveolar type II epithelial cells. Except for the serine 63 and 73, other unknown phosphorylation sites and even distinct forms of posttranslational modification like ubiquitination, which may affect its transcriptional activity, are still elusive. C/EBPβ and C/EBPδ are C/EBP family members that can associate with the identical conserved DNA sequence (Ramji and Foka, 2002). Our previous study shows that C/EBPβ is activated in IL-1β-treated alveolar type II epithelial cells, which contributes to IL-6 transcription (Yan et al., 2012a). However, the mechanism involved in C/EBPβ activation is still enigmatic. Here, we find that IL-1β-mediated C/EBPβ activation is at least partly due to its increased expression. Moreover, C/EBPδ production at both RNA and protein levels is also increased in the cells stimulated by IL-1β. It is worth noting that both C/EBP family members play critical roles in MCP-1 generation under the inflammatory condition. Our previous study has reported that C/EBPδ but not C/EBPβ promotes IL-6 transcription in TNF-α-stimulated alveolar type II epithelial cells (Yan et al., 2018). Therefore, in-depth research should be conducted to find out the internal factors controlling the different phenomena. Sp1 belongs to the Sp/KLF family, and is a zinc finger transcription factor that binds to GC-rich motifs of a series of promoters (O'Connor et al., 2016). Of interest, its expression is hardly

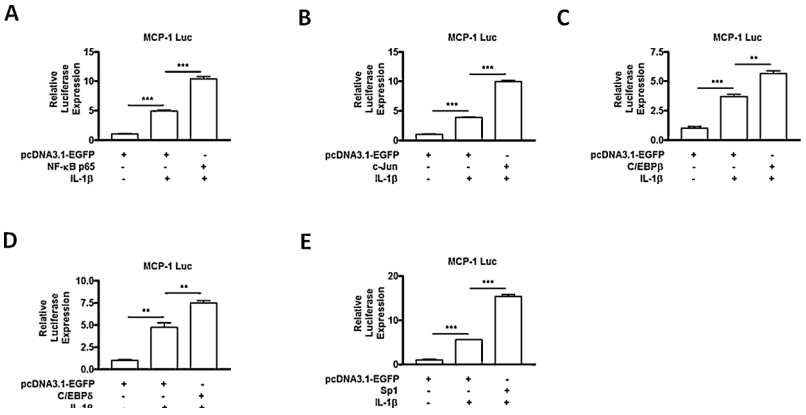

Fig. 9. Regulatory roles of NF-κB p65, c-Jun, C/EBPβ/δ and Sp1 in MCP-1 expression in IL-1β-treated primary alveolar type II epithelial cells. Primary alveolar type II epithelial cells are transfected with the indicated recombinant genes. 24 h later, the cells are stimulated by PBS or 5 ng/ml IL-1β for 6 h. Then the cells are lysed and subjected to luciferase assays. Data are expressed as mean ± SEM (N = 3). ** and *** indicate $p < 0.01$, and 0.001, respectively.

changed in alveolar type II epithelial cells incubated with IL-1β, however, it still plays an important for IL-1β-triggered MCP-1 expression through interaction with its consensus sequence in the gene promoter region, which indicates that posttranslational modifications might be involved in its activity.

In conclusion, our current study demonstrates that IKK/NF-κB, JNK/c-Jun, C/EBPβ, C/EBPδ and Sp1 are all required for IL-1β-induced MCP-1 expression in alveolar type II epithelial cells, and knockdown of any of these transcription factors results in a significant reduction in the expression of the chemokine. The above mentioned transcription factors are responsible for a diverse array of immune reactions, but the current study proves for the first time that multiple transcription factor binding sites containing κB, AP-1, C/EBP and Sp1 elements in the promoter region are involved in IL-1β-mediated MCP-1 expression in alveolar type II epithelial cells.

Acknowledgements

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