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TNF-α induction of IL-6 in alveolar type II epithelial cells: Contributions of JNK/c-Jun/AP-1 element, C/EBPδ/C/EBP binding site and IKK/NF-κB p65/κB site

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ABSTRACT

Although participation of IL-6 in lung inflammation has been widely elucidated, the transcriptional regulation of its generation in alveolar type II cells stimulated by TNF-α remain unclear. Here, we find that TNF-α significantly induces IL-6 production, and TNF-α induction of IL-6 is mainly regulated at transcriptional level. Upon stimulated by TNF-α, Activator Protein-1 (AP-1)-mediated transcriptional activity is apparently increased in alveolar type II epithelial cells, which might be derived from elevated phosphorylation of JNK and subsequent activation of c-Jun. Either down-regulation of c-Jun or the AP-1 site mutation leads to significant reduction of IL-6 expression. In contrast, ectopic expression of c-Jun notably increases IL-6 generation. So, c-Jun, one of the AP-1 family members, plays a pivotal role in TNF-α-induced IL-6 generation. CCAAT/enhancer binding protein δ (C/EBPδ) expression is significantly amplified by TNF-α, which may contribute to the rise of C/EBP activity in alveolar type II cells. C/EBPδ shRNA treatment results in attenuation of IL-6 expression in the cells, which is consistent with data by introduction of mutations into the C/EBP site in the promoter. However, overexpression of C/EBPδ greatly increases the IL-6 promoter activity. In addition, data regarding another transactivator in the family—C/EBPβ show that it does not affect IL-6 production. We also find that the IKK/NF-κB p65 pathway is activated in TNF-α-treated alveolar type II epithelial cells, and plays an essential role in positive regulation of IL-6 expression in TNF-α-treated alveolar type II epithelial cells *via* knockdown or forced expression of NF-κB p65, or elimination of κB sites in the IL-6 promoter. Notably, IL-6 promoter-driven luciferase production in primary alveolar type II epithelial cells can also be increased by the ectopic expression of c-Jun, C/EBPδ, and NF-κB p65, respectively. Collectively, our data provide insights into molecular mechanism involved in IL-6 expression in alveolar type II epithelial cells on TNF-α treatment, which provides a theoretical basis for specific inhibition of IL-6 production at the transcriptional level.

1. Introduction

Ample evidences indicate that alveolar type II epithelial cells may take participation in the lung inflammatory responses by secretion of a variety of inflammatory/anti-inflammatory mediators (Sallenave et al., 1994; Maniscalco et al., 1995; Thorley et al., 2005). For example, type II epithelial cells relieve bacterial pneumonia by generation of leukemia inhibitory factor (Traber et al., 2017). However, the potential role of type II epithelial cells in production of inflammatory/anti-inflammatory mediators, and the corresponding molecular mechanism remain largely unclear. During the early stage of acute respiratory distress syndrome (ADRS), abundant TNF-α could be recovered from patients' bronchoalveolar lavage (BAL) fluids. As one of the most active pro-inflammatory mediators, TNF-α could exert effects on inflammatory reactions by initiating expressions of a series of downstream cytokines and chemokines such as IL-6. It appears that the principal mechanism whereby TNF-α induction of IL-6 is transcriptionally driven, as the abundance of IL-6 mRNA is increased under different inflammatory conditions (Yan et al., 2016; Tang et al., 2014). Although AP-1, C/EBP and NF-κB have been implicated in regulation of inflammatory

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responses (Eickelberg et al., 1999; Smart et al., 2001; Mann et al., 2002), the significance of these transcription factors and related signaling pathways in TNF-α-induced IL-6 production in alveolar type II epithelial cells has not been identified.

AP-1 is a family of transcription factors consisted of hetero- and homodimers, which are comprised of activating transcription factor (ATF), Jun dimerization protein (JDP), c-Fos and c-Jun. The family members form dimers through a leucine zipper and associate with the conserved DNA sequences through an adjacent basic region. AP-1 mediates gene expressions in response to a plethora of stimuli such as cytokines produced in a variety of inflammatory processes (Hess et al., 2004). For instance, TNF-α-stimulated CD44 expression in human monocytic cells is *via* activation of c-Jun (Mishra et al., 2005). JNK which belongs to the mitogen-activated protein kinase family is initially determined as kinases that bind to c-Jun and make it phosphorylation on Ser63 and Ser73 within its N-terminal transcriptional activation domain. Increasing evidences suggest the critical roles of JNK—c-Jun axis in gene expressions (Sato-Matsubara et al., 2017; Arous et al., 2015; Liu et al., 2013), but its effect on TNF-α-mediated IL-6 expression in alveolar type II epithelial cells remains enigmatic.

C/EBP family is consisted of six members: C/EBPα, -β, -δ, -ε, -γ, and -ζ. They share a highly conserved, basic-leucine zipper (bZIP) domain at C-terminal, which is responsible for dimerization and DNA binding. Binding of C/EBP heterodimers or homodimers to the consensus sequence 5′-T(T/G)NNGNAA(T/G)-3′ may initiate gene transcription. Although each family member may be involved in transcriptional regulation of distinct portfolios of genes, C/EBPβ and C/EBPδ appear to be mainly transcriptional stimulators of inflammation, because they have been extensively implicated in stimulating inflammation-associated gene induction (Yan et al., 2012a; Yan et al., 2013; Yan et al., 2010; Yan et al., 2012b). In the current work, we plan to examine if IL-6 expression is regulated by C/EBPs in TNF-α-treated alveolar type II epithelial cells, and which family member might be involved in induction of IL-6.

NF-κB is ubiquitously expressed in almost all mammalian cells, and involved in a number of cellular processes such as proliferation, apoptosis and inflammation. Under normal conditions, NF-κB is present mainly as an inactivated form due to sequestration in the cytoplasm by IκB family proteins (Bhoj and Chen, 2009). Upon certain stimulation signal such as inflammatory stimulus appears, rapid phosphorylation of IκB is induced *via* activation of the IκB kinase (IKK) composed of two catalytic subunits, IKKα and IKKβ, and an essential regulatory subunit called IKKγ. Phosphorylated IκB is then degraded through ubiquitin-dependent pathway resulting in the liberation of active NF-κB, which is followed by its recruitment to nucleus, where it binds to the promoter regions of targets and induce the corresponding gene transcription (Bhoj and Chen, 2009). Though the influence of NF-κB on inflammation has been widely demonstrated (Leu et al., 2017; Kapoor et al., 2010; Kim et al., 2011), the impact of IKK—NF-κB p65 signaling pathway on TNF-α induction of IL-6 in alveolar type II epithelial cells remains unclear.

In the current study, we prove that the JNK/c-Jun/AP-1 site, C/EBPδ/C/EBP binding site, and IKK/NF-κB p65/κB element are all involved in positive regulation of IL-6 expression in alveolar tyep II epithelial cells treated with TNF-α. However, C/EBPβ does not affect TNF-α induction of IL-6 in type II cells. Our data provide a theoretical basis for specific interruption of IL-6 expressions at the transcriptional level, which is beneficial for treatment of lung diseases induced by excessive IL-6 secretion from alveolar type II epithelial cells.

2. Materials and methods

2.1. Cell cultures and reagents

Murine alveolar type II epithelial cells (MLE-12) are purchased from American Type Culture Collection and maintained in DEME/F12 culture medium containing 5% fetal bovine serum (Gibico). Murine TNF-α purchased from PeproTech is dissolved in sterile PBS.

2.2. RNA extraction and real-time PCR

Total cellular RNAs are isolated from MLE-12 cells by using Trizol (Invitrogen), and are subjected to reverse transcription by using the PrimeScript™ RT reagent Kit from TaKaRa according to the manufacturer's guidelines. To measure the gene expression at mRNA level, real-time PCR is carried out by using the following protocol: an initial step at 95 °C for 3 min followed by 40 cycles of 95 °C for 5 s and 60 °C for 30 s. The primers used are as follows: IL-6, 5′ primer, 5′-AGT TGC CTT CTT GGG ACT GA-3′ and 3′ primer, 5′-TCC ACG ATT TCC CAG AGA AC-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′; C/EBPβ, 5′ primer, 5′-CAA GCT GAG CGA CGA GTA CA-3′ and 3′ primer, 5′-AGC TGC TCC ACC TTC TTC TG-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

Total cellular proteins are extracted from MLE-12 cells by using RIPA buffer (Beyotime Biotechnology, China). 30 μg of proteins are electrophoresed on SDS-PAGE gel, and are then transferred to PVDF membranes. The membranes are blocked in 5% non-fat milk, which is followed by overnight incubation with the 1st antibodies. Then the membranes are incubated with the corresponding 2nd antibodies, and are examined by using the Enhanced Chemiluminescence kit purchased from Amersham Biosciences UK. The 1st antibodies are as follows: rabbit anti-C/EBPδ (Santa Cruz), rabbit anti-C/EBPβ (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-p-NF-κB p65 (Ser536) (Cell Signaling Technology), rabbit anti-NF-κB p65 (Santa Cruz), rabbit anti-IKKα/β (Ser176/180) (Cell Signaling Technology), and rabbit anti-GAPDH (Cell Signaling Technology).

2.4. Plasmids

c-Jun, C/EBPδ and NF-κB p65 encoding regions are amplified from mouse lung-derived cDNA by using PCR. The obtained fragments are then inserted into pcDNA3.1-Myc-His (Invitrogen). The generated overexpression plasmids are termed pcDNA3.1-c-Jun-Myc-His, pcDNA3.1-C/EBPδ-Myc-His, and pcDNA3.1-NF-κB p65-Myc-His, respectively. c-Jun overexpression plasmids with mutation of Ser63 to Ala or Ser73 to Ala, and NF-κB p65 overexpression plasmids with mutation of Ser534 to Ala are generated by using the Mut Express II Fast Mutagenesis Kit V2 (Vazyme, China). pcDNA3.1-EGFP is generated by insertion of the amplified EGFP encoding segment from pIRES-EGFP (Clontech) into pcDNA3.1-Myc-His. The full length IL-6 promoter region (-271 to +59), and 5′-truncted forms (-221 to +59 and -119 to +59) are amplified from mouse genomic DNA by using PCR. Then the recombinant constructs are generated by ligating the purified fragments with the pGL4-basic vector (Promega), and are termed pGL4/271, pGL4/221, and pGL4/119, respectively. The reporter genes with AP-1 or C/EBP or κB site mutation in the full length IL-6 promoter region are produced by using the Mut Express II Fast Mutagenesis Kit V2 (Vazyme, China). Unless specifically stated otherwise, IL-6 Luc in the study indicates pGL4/271. The AP-1 reporter genes (AP-1 Luc) are obtained from Beyotime Biotechnology (China). The C/EBP reporter genes (C/EBP Luc) and NF-κB reporter plasmid (κB Luc) are obtained from Promega.