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IL-18 Stimulates CSF-1 Expression *via* MyD88-dependent Pathway*

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Abstract Interleukin-18 (IL-18) is a new member of IL-1 cytokine family and known as IFN-γ-inducing factor previously. An increased secretion of IL-18 is generally a marker for many chronic inflammation and autoimmune disorders, implying its involvement in inflammatory responses. Here, it was showed that IL-18 stimulated the expression of CSF-1 in Jurkat T lymphocytes that might be a manifestation for IL-18 to play its biological functions in inflammatory pathogenesis. The signaling mechanism that contributes to IL-18-induced transcriptional activation of CSF-1 was investigated further. Combined data suggested a MyD88-NF-κB-CSF-1 signal pathway is involved in the up-regulated expression of CSF-1.

Key words IL-18, MyD88, NF-κB, CSF-1
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Inflammation is a defense-oriented pathophysiologic process, which is initiated by immune response to infectious agents or non-infectious tissue damage and is persisted or propagated by pro-inflammatory factors. The development of inflammatory response consists of the release of a series of mediators and the recruitment of leukocytes. Circulating leukocytes become activated at the inflammatory site and release more cytokines[1]. Different cytokines exert pro- or anti-inflammatory effects. There is a balance between pro-inflammatory and anti-inflammatory cytokines during inflammation development and this balance is crucial for health. Disruption of the balance can lead to exacerbation of inflammatory injury and cause disease[2].

Interleukin (IL)-18 is a pleiotropic cytokine. Recent studies have shown that IL-18 is a prototypic pro-inflammatory cytokine that contributes to both innate and adaptive immune responses. Accumulated studies demonstrate a convincing role of IL-18 in pathogenesis of many chronic inflammatory and autoimmune diseases such as rheumatoid arthritis (RA)[3–5], systemic lupus erythematosus (SLE)[6], type Ⅰ diabetes [7] and atherosclerosis [2]. Moreover, IL-18 is essential to promote the innate immunity by inducing IFN-γ and NO, and thereby trigger intracellular anti-microbial mechanisms for control of bacterial, fungal, and protozoan infections [8–10]. IL-18 was originally described as an IFN-γ-inducing factor (IGIF) for its properties to enhance production of interferon-γ in sera and livers of mice preconditioned with *Propionibacterium acnes* [11]. The human IL-18 gene is located on 11q23.1 and contains six exons over ~20.8 kb span [11]. Later studies identified that IL-18 shares structural and functional properties with IL-1β, but lacks binding to type Ⅰ IL-1 receptor, and was defined as a member of the IL-1 cytokine family[5, 12]. IL-18 is secreted by various types of immune cells and non-immune cells including lymphocytes, peripheral blood mononuclear cells, fibroblast, synovial macrophages, intestinal epithelial cells[11, 13–17].

A prominent biological function of IL-18, particularly in synergy with IL-12, is activation of IFN-γ production in T cells, B cells and NK cells [18].

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Additionally, IL-18 initiates a cytokine-expressing cascade of pro-inflammatory mediators such as TNF-α, IL-1β, GM-CSF, and NO in immunocompetent cells, augments the T and NK cells cytotoxicity and expression of chemokine receptors [19–21]. Thus, by serving as a potent pro-inflammatory cytokine, IL-18 is able to enhance the adaptive immunity mediated by Th1 and Th2 cells.

The activated immune cells secrete colony-stimulating factor-1 (CSF-1) that accelerates the activation and proliferation of mononuclear macrophages, promotes the adhesion of leukocyte to the vessel epithelia, and stimulates the production of cytokines and growth factors to regulate the inflammation [22–24]. CSF-1 is identified as a potential marker for inflammatory diseases, and it plays an important role in atherosclerosis and many other immune disorders [2, 23]. In our previous studies, we reported that the activated leukocytes secrete CSF-1 during the engagement of adhesion molecules that happens while leukocytes adhere to the activated endothelium [25–26]. In the present study, we investigated whether IL-18 participates in inflammation *via* promoting the expression of CSF-1, as well as the precise signaling mechanism. Our results showed that IL-18 up regulated the expression of CSF-1, activated the transcriptional activities of NF-κB and CSF-1 promoter *via* the MyD88-dependent signal pathway. The results of the present study provided an insight into the biological significance of IL-18 and the involved signal transduction mechanism from IL-18R.

1 Materials and methods

1.1 Reagents and antibodies

Polyclonal antibodies against CSF-1 (H-300, sc-13103), actin (sc-1616) and MyD88 (HFL-296, sc-11356) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Monoclonal antibodies against flag (F-1804), and Monensin (a reagent for blocking the secretion of glycoprotein or proteoglycan) (M5273) were purchased from Sigma-Aldrich. Human recombinant protein IL-18 as well as the monoclonal antibody for neutralization of IL-18β receptor bioacitity (MAB1181) were purchased from R&D systems. Western blotting detection reagents ECL Plus (RPN2132) was purchased from Amersham Biosciences (Little Chalfont, Buckinghamshire, UK).

1.2 Cell culture

The human leukemic Jurkat T-cell line was cultured in RPMI medium 1640 (Gibco, GrandIsland, NY) containing 10% fetal calf serum. HeLa cells were maintained in IMDM (Gibco) supplemented with 10% fetal calf serum.

1.3 Recombinant DNA constructs

The luciferase reporter plasmid with a CSF-1 promoter, pREP4-CSF-1-luc, and the pREP7-Renilla-luc plasmid, were the gifts of Dr. ZHAO Ke-Ji (NIH, Bethesda, MD). IκBα-SR (producing a mutant IκBα that resists the degradation resulting from IKK activation) was kindly provided by Dr. YANG Yuan-Zheng (Department of Microbiology and Immunology, University of Texas Medical Branch, Galveston TX). The pcDNA3.0-based plasmid for expression of flag-tagged proteins was provided by Dr. PEI Gang (Institute of Biochemistry and Cell Biology, SIBS, CAS). The sequence encoding the full-length MyD88 (Acc#: NM_002468) was amplified by PCR using the human cDNA as a template.

1.4 Reverse transcription and real-time RT-PCR

Total RNA from the quiescent or the IL-18-treated Jurkat cells was isolated by using Trizol reagent (Invitrogen) according to the manufacturer's instruction. 2 μg of total RNA was reverse-transcribed, and the cDNA was used as template. Each real-time PCR reaction consisted of l μl cDNA, 12.5 μl SYBR Green PCR Master Mix (PE Applied Biosystems, Foster City, CA), and 500 nmol/L forward and reverse primers. Reactions were carried out on an ABI PRISM 7000 Sequence Detection System (AB Applied Biosystems). PCR cycling condition was as follows: 50℃ for 2 min, 95℃ for 10 min, 40 cycles at 94℃ for 15 s, and 60℃ for 1 min. The following primers were used, β-actin forward: 5′ ATGCCAGGGTACATGG-TGGT 3′, reverse: 5′ TCGTGCGTGACATTAAGG-AG 3′; CSF-1-var1 forward: 5′ ACGCTGAGGAGTG-AAAGAACC 3′, reverse: 5′ AACCAGCACAGGG-CACTTA 3′; CSF-1-var4 forward: 5′ CTAAGACCC-CTCACCATCCT 3′, reverse: 5′ AGCCAAATACAA-AAGAGCAG 3′.

1.5 Transient transfection and luciferase activity assay

For enhancing the transfection efficiency of suspension cells, an effective electroporator Nucleofector® Ⅱ (Amaxa Biosystems, MD, USA) was applied. Transient transfections were performed following the manufacturer's instructions. Jurkat cells were suspended in 100 μl nucleofector mixed solution (solution Ⅰ: 0.36 mol/L ATP-Na_2, 0.59 mol/L $MgCl_2$•

$6H_2O$; solution Ⅱ: 0.15 mol/L KH_2PO_4, 23.8 mmol/L $NaHCO_3$, 3.36 mmol/L glucose, solution Ⅰ/solution Ⅱ: 1/50, v/v) at a concentration of 10^6 cells/ml with 1 μg DNA added. Cells were incubated for 10 min at room temperature, electroporated by using program X-005, and transferred to 500 μl pre-warmed culture medium in 12-well plates. 24 h later, transfected Jurkat cells were stimulated with the recombinant IL-18 for 12 h, and then lysed in 20 μl lysis buffer. For the blocking experiments, neutralizing antibody against IL-18β receptor was added in the medium 30 min prior to IL-18 stimulation. Transcriptional activity was detected by using a luminometer with the dual-luciferase assay system (Promega) to measure both the firefly and renilla luciferase activity, and data expressed as fold increase relative to the basal activity of the un-stimulated cells.

1.6 Western blotting

For the detection of CSF-1 protein, Jurkat cells were stimulated with recombinant IL-18 plus Monensin (0～48 h). Cells were suspended in lysis buffer (50 mmol/L Tris pH 7.5, 150 mmol/L NaCl, 1 mmol/L EDTA, 1 mmol/L EGTA, 1% Nonidet P-40, 2.5 mmol/L sodium pyrophosphate, 10 mmol/L NaF, 1 mmol/L each of glycerophosphate, Na_3VO_4, aprotinin, leupeptin, and proteinase inhibitor PMSF) and incubated on ice for 25 min. The cell lysates were centrifuged at 4℃, 13 000 g for 20 min, and supernatants were collected and resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Proteins were transferred to the nitrocellulose membranes, and probed with appropriate primary antibody and secondary antibody. Protein expression was detected by using Western blotting reagents ECL Plus for the chemiluminesence. For the detection of expression level of exogenous fusion proteins (Flag-MyD88 and Flag-MyD88-TIR), transfected HeLa cells were subjected to Western blotting as described above by using anti-FLAG antibody.

1.7 Statistical analysis

All experiments were performed at least thrice for each determination. Data were statistically evaluated using analysis of variance (ANOVA). Significance was assigned at $P < 0.01$.

2 Results

2.1 IL-18 stimulates CSF-1 expression

Human CSF-1 has at least 4 variants of mRNA by alternative splicing [27]. The 3′-UTR of all variants is coded by two exons, of which, variant 1 and variant 4 possess one respectively. Thus, we designed two pairs of primers directed to the sequences of two 3′-UTR structure, aiming at that the PCR product reflects the situation of all variants. To detect if IL-18 up regulates the CSF-1 mRNA level, Jurkat cells were incubated with 10 μg/L IL-18 for different time intervals. The results of real-time PCR showed that IL-18 induced maximal (～5-fold) increase in variant 1 of CSF-1 mRNA at 12 h (Figure 1a). Likewise, incubation with IL-18 induced maximal (8-fold) increase in variant 4 of CSF-1 mRNA at 6 h (Figure 1b). All mRNA variants generate three isoforms of CSF-1 protein. For

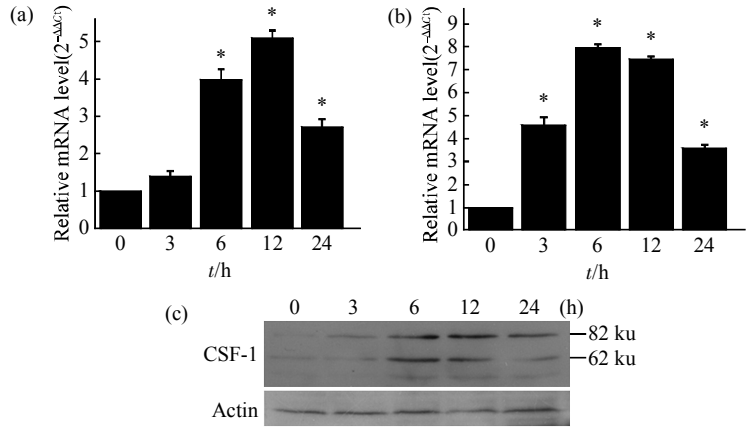

Fig. 1 IL-18 stimulates the expression of CSF-1

For examination of mRNA, Juekat cells were treated with 10 μg/L of IL-18 for different time intervals as indicated. Total RNA was isolated and real-time PCR was performed as described under "**Materials and methods**". After normalization to β-actin mRNA , relative increase in variant 1 (a) and variant 4 (b) of CSF-1 mRNA level in IL-18-treated cells was plotted in comparison with the untreated control ($P < 0.01$, * IL-18-treated on different time points versus basal). For the detection of CSF-1 protein, Jurkat cells were cultured and incubated with or without 10 μg/L of IL-18 (plus Monensin) as described under "**Materials and methods**". Cell lysate (40 μg of protein) were resolved by SDS-PAGE, and Western blotting was performed with antibodies against CSF-1 and β-actin(c).

CSF-1's biological and antibody-binding activities, all isoforms require glycosylation and homologous dimerization, which explains the observed variation in the molecular mass of CSF-1 longest isoform between 47～76 ku in diverse types of cell[28-29]. When Jurkat cells were subjected to the insult of IL-18, Monensin, a reagent for blocking the secretion of glycoproteins, was added in the medium simultaneously for facilitating the detection of protein expression of CSF-1 in whole cell lysates by Western blotting. Jurkat cells (1×10^7) were stimulated with 10 μg/L IL-18 for different time intervals. Western blotting assay showed a weak expression of CSF-1 in the quiescent cells, whereas, the expression of CSF-1 reached the maximum between 6 to 12 h (Figure 1c). The lower bands around 62-ku might be two shorter isoforms of CSF-1. The results demonstrated that IL-18 stimulates gene expression of the inflammation mediator CSF-1 in leukocyte at both mRNA and protein levels.

2.2 NF-κB mediates the activation of CSF-1 promoter triggered by IL-18

NF-κB regulates the expression of a wide-range of genes related to immunity and inflammation. To investigate whether IL-18 stimulates CSF-1 expression through the action of activated NF-κB on the transcription of CSF-1, we performed a series of reporter gene assays. The activity of luciferase driven by CSF-1 promoter increased about 2-fold if the transfected cells were incubated with 10 μg/L IL-18 for 12 h(Figure 2a). Likewise, the transient transfection assays by using a luciferase reporter gene controlled by two NF-κB binding sites also showed that

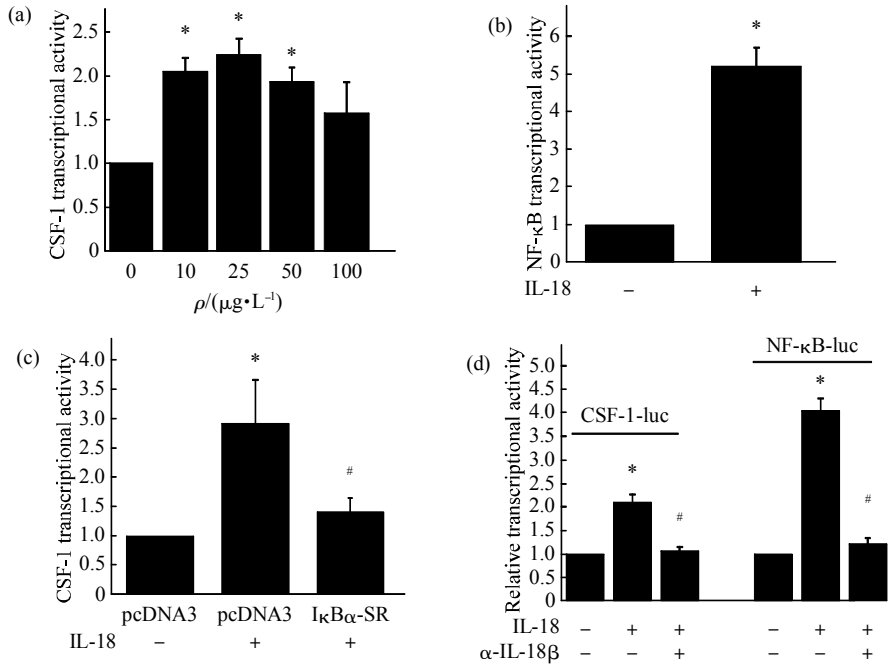

Fig. 2 NF-κB mediates the transcriptional upregulation of CSF-1

(a) Jurkat cells were transfected with CSF-1-luc plasmid, and then were cultured normally or in the presence of different concentrations of IL-18 as indicated for 12 h. The relative CSF-1 transcriptional activity was measured by dual-reporter assay. The transcriptional activity of CSF-1 in normal cells was considered as the basal level and valued 1 or 100%. ($P < 0.01$, *basal versus IL-18-treated). (b) Jurkat cells were transfected with NF-κB-luc plasmid (with two NF-κB-binding sites), and then cultured normally or in the presence of 10 μg/L of IL-18 for 12 h. The relative NF-κB transcriptional activity was measured by dual-reporter assay. The transcriptional activity of NF-κB in normal cells was considered as the basal level and valued 1 or 100%. ($P < 0.01$, * basal versus IL-18-treated). (c) Jurkat cells were co-transfected with CSF-1-luc plasmid plus pcDNA3 or IκBα-SR, and then cultured normally or in the presence of 10 μg/L of IL-18 for 12 h. The relative CSF-1 transcriptional activity was measured by dual-reporter assay. The transcriptional activity of CSF-1 in cells transfected with pcDNA and without IL-18 treatment was considered as the basal level and valued 1 or 100%. ($P < 0.01$, *basal versus IL-18-treated; #NF-κB-activated versus NF-κB-inhibited). (d) Jurkat cells were transfected with CSF-1-luc plasmid or NF-κB-luc plasmid, and then cultured normally or incubated with 10 μg/L of IL-18 (with or without pre-incubation of IL-18β receptor neutralization antibody) for 12 h. The transcriptional activity of CSF-1 or NF-κB in normal cells was considered as the basal level and valued 1 or 100% respectively ($P < 0.01$, *basal versus IL-18-treated; #IL-18-treated versus IL-18R bioactivity blocking). All data ($\bar{x} \pm s$) were representative of three independent experiments.

transcriptional activity of NF-κB dramatically increased in the cells stimulated with 10-μg/L IL-18 for 12 h (Figure 1b). To address the contribution of activated NF-κB to the transcriptional up regulation of CSF-1 promoter, we further utilized an expression construct named IκBα super repressor (IκBα-SR), which produces a mutant IκBα and is resistant to IKK-induced degradation, and thereby, inhibits the transcription activity of NF-κB. Compared with the cells co-transfected with pcDNA3.0, the transcriptional up regulation of CSF-1 promoter induced by IL-18 was dramatically inhibited by co-transfection of IκBα-SR (Figure 2c), suggesting NF-κB mediated the activation of CSF-1 promoter triggered by IL-18. Neutralizing antibody of IL-18β blocked the transcriptional activation of NF-κB and CSF-1 further confirmed the function of IL-18 (Figure 2d).

2.3 Transcriptional activation of CSF-1 induced by IL-18 is MyD88-dependent

Among the cytoplasm adapters of IL-1 receptor/ Toll like receptor (TLR) super-family, MyD88 is essential for mediating the transduction of extracellular signals. After activation of IL-18R, MyD88 homo-dimer binds to IL-18R by using its TIR domain, recruits IRAK and TRAF6 by using its DD domain, and then activates downstream transcription factors such as NF-κB or AP-1 [30]. Besides this canonical MyD88-dependent pathway, recent studies suggested a PI-3K/Akt pathway that was initiated from the cytoplasm tails of TLRs without the involvement of MyD88 [31–32]. To detect the contribution of MyD88 to the activation of NF-κB as well as the transcriptional up regulation of CSF-1 in the present study, we generated two constructs expressing Flag-MyD88 and Flag-MyD88-TIR fusion proteins (Figure 3a, b). Co-transfection of expression plasmids for fusion proteins with luciferase reporter gene constructs showed that over-expression of the full-length MyD88 moderately increased the transcriptional activities of NF-κB and CSF-1 promoter. In comparison, truncated mutant MyD88-TIR inhibited the transcriptional activities of both reporter genes to basal level (Figure 3c, d) indicating that over-expressed truncated mutant MyD88-TIR competitively blocked signal delivery, and the absence of DD domain caused the disability of MyD88 to recruit downstream molecules. These

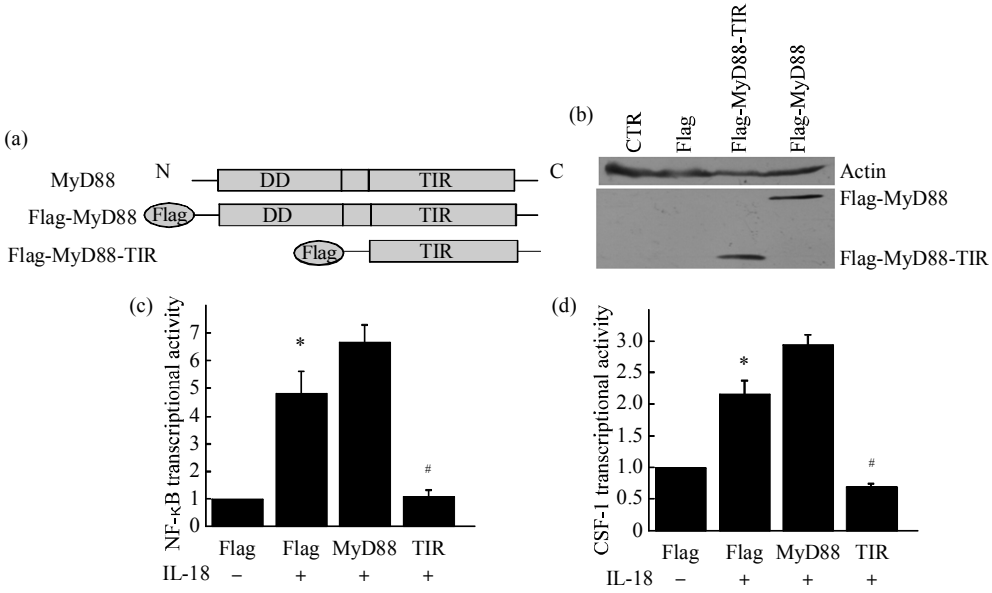

Fig. 3 The transcriptional activation of NF-κB and CSF-1 induced by IL-18 is MyD88-dependent

(a) Schematic diagram for constructs of different fusion proteins (Flag-MyD88 and Flag-MyD88-TIR). (b) Normal HeLa cells and the cells transfected with different constructs mentioned above or empty Flag-tag vector were harvested and lysed. Cell lysate (40 μg of protein) were resolved by SDS-PAGE, and Western blotting was performed with antibodies against flag and β-actin. (c) Jurkat cells were co-transfected with NF-κB-luc plasmid plus empty Flag-tag vector or constructs producing different fusion proteins, and then cultured normally or in the presence of 10 μg/L of IL-18 for 12 h. The relative NF-κB transcriptional activity was measured by dual-reporter assay. The transcriptional activity of NF-κB in cells transfected with empty Flag-tag vector and without IL-18 treatment was considered as the basal level and valued 1 or 100%. Data ($\bar{x} \pm s$) were representative of three independent experiments (P <0.01, *basal versus IL-18-treated; #MyD88-TIR versus flag-vector). (d) Dual-reporter assay for measure the effects of fusion proteins on CSF-1 transcription was performed as described in (c) by using CSF-1-luc instead of NF-κB-luc.

results suggested that IL-18 induce activation of NF-κB and CSF-1 promoter might in a MyD88-dependent manner.

3 Discussion

Once activated, leukocytes(monocytes, and B cell, T lymphocytes) secrete CSF-1, which promotes monocyte/macrophage proliferation and activation. It has been demonstrated that CSF-1 mediates the inflammation triggered by various stimuli such as TNF-α[33], LPS[34] and IL-4[35]. Whether IL-18, a novel cytokine detected in various organs and tissues during chronic inflammation, stimulates CSF-1 expression was investigated in our present study. We used real time RT-PCR and Western blotting to detect CSF-1 expression at both mRNA and protein levels. Our data showed that recombinant IL-18 significantly increased the mRNA and protein level of CSF-1 in Jurkat cells (Figure 1, 2), and provided indication of mechanistic role of IL-18 in mediating inflammation.

Additionally, we performed transient transfection to check the transcriptional activity of CSF-1 promoter in Jurkat cells. Dual luciferase reporter assay showed that IL-18 significantly increased the transcriptional activity of CSF-1 promoter, indicating the up regulation of CSF-1 expression can be achieved, at least partly, at transcription level (Figure 2a). NF-κB regulates the expression of a wide-range of genes related to innate immunity, adaptive immunity, inflammation, apoptosis, proliferation, stress response and cancer[36]. Several transcription factor binding sites exist in the human CSF-1 promoter ranging from TSS (transcription start site) upstream to –565 nt that contain two NF-κB sites, κB-1 and κB-2 at the region from –406 nt to –344 nt[37–38]. In HL-60 cells, the κB-1 sequence in CSF-1 regulatory elements was demonstrated to be necessary for the TNF-α-mediated transcription of CSF-1 gene[39]. In resting state, NF-κB forms a protein complex with its inhibitory protein IκBs, including three subunits, namely IκBα, IκBβ and IκBε. The nuclear localization signal (NLS) of NF-κB is blocked by IκBs, so NF-κB stays in an inactive form in cytoplasm in the absence of external activation. Once stimulated, IκB is rapidly phosphorylated by the multi-protein complex IKK, followed by the 26S proteasome-mediated proteolysis, leading to the activation of NF-κB[40]. To test whether IL-18 up regulates the transcription of CSF-1 *via* NF-κB, we utilized an expression construct named IκBα-SR, which produces the mutant IκBα resisting the degradation by IKK. The results showed the inhibitory construct dramatically diminished the transcriptional activity of CSF-1 promoter in the cells stimulated by IL-18 (Figure 2b, c).

The multi-biological functions of IL-18 are mediated by the interaction between IL-18 and its receptor IL-18R belonging to the IL-1 receptor/Toll like receptor (TLR) super-family. IL-18R is a heterodimeric receptor consisting of IL-18Rα and β chains, both of which are required for signaling[41–42]. Upon IL-18 binding, IL-18R forms a hetero-dimer with right conformation, interacts with the TIR domain of the MyD88 homo-dimer, then recruits the downstream signaling molecules including IRAK and TRAF6, and finally activates NF-κB and MAPK, promoting the expression of other inflammatory factors. This is the most canonical signaling pathway called MyD88-dependent pathway[43]. Myeloid differentiation factor MyD88 is a critical homodimeric adaptor protein in the intracellular signaling elicited by IL-1R super-family and most if not all of the TLRs[44]. MyD88-deficient mice lack the ability to induce pro-inflammatory cytokines resulting in infection or inflammation and were also impaired in their response to IL-1 and IL-18[43]. MyD88 contains an amino-terminal death domain (DD) and a carboxyl-terminal highly conserved TIR domain separated by a short intermediate domain. Function of TIR domain of MyD88 is to dimerize with the IL-1R and favor recruitment of IRAK family members through interaction *via* the DD domain[30]. However, cytosolic tail of IL-1R and TLR family members contain many other domains in addition to TIR domain, and their specific functions remain unclear. It has been found that no MyD88 was involved in an IL-1β-mediated signaling pathway; when IL-1R intracellular domain is phosphorylated, MyD88 cannot bind to the receptor. Instead, p85 subunit of PI3K kinase directly interacts with the receptor, thus induces the activation of Akt[31–32]. In the present study, over-expression of full-length MyD88 did not substantially enhance the transcriptional activities of NF-κB and CSF-1 promoter induced by IL-18, thus, suggesting that the endogenous MyD88 is adequate for the mobilization to

the receptor. Notably, over expression of MyD88 TIR domain inhibited the activation of NF-κB and CSF-1 promoter to the basal level (Figure 3c, d), indicating that the DD-domain-deficient mutant competitively inhibited the binding of endogenous MyD88 to the receptor and prevented the recruitment of downstream molecules, thus blocking the IL-18 signaling pathway. Our results suggested that IL-18 regulated CSF-1 gene expression *via* MyD88-dependent signaling pathway.

In summary, we have shown that IL-18 up regulates the expression of CSF-1, which might be one of the approaches of IL-18 to participate in inflammation. IL-18 promotes the transcriptional activation of CSF-1 might *via* MyD88-NF-κB pathway.

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IL-18 通过 MyD88 依赖信号途径调控 CSF-1 表达 *

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摘要 IL-18 是 IL-1 家族的一个成员，最初发现被命名为 IFN-γ 诱导因子．现在研究表明，IL-18 是炎症反应的重要标志，参与许多慢性炎症和自身免疫性疾病炎性反应．证明了 IL-18 在 Jurkat T 淋巴细胞的炎症反应中能够调节 CSF-1 的表达，其信号机制是 IL-18 通过 MyD88-NF-κB-CSF-1 信号途径上调 CSF-1 的基因转录．

关键词 白介素 18(IL-18)， MyD88，核因子 κB，集落刺激因子 1
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