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C/EBPβ is a transcriptional key regulator of IL-36α in murine macrophages

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ABSTRACT

Interleukin (IL)-36α – one of the novel members of the IL-1 family of cytokines – is a potent regulator of dendritic and T cells and plays an important role in inflammatory processes like experimental skin inflammation in mice and in mouse models for human psoriasis. Here, we demonstrate that C/EBPβ, a transcription factor required for the selective expression of inflammatory genes, is a key activator of the *Il36A* gene in murine macrophages. RNAi-mediated suppression of C/EBPβ expression in macrophages (C/EBPβ^{low} cells) significantly impaired *Il36A* gene induction following challenge with LPS. Despite the presence of five predicted C/EBP binding sites, luciferase reporter assays demonstrated that C/EBPβ confers responsiveness to LPS primarily through a half-CRE•C/EBP element in the proximal *Il36A* promoter. Electrophoretic mobility shift assays showed that C/EBPβ but not CREB proteins interact with this critical half-CRE•C/EBP element. In addition, overexpression of C/EBPβ in C/EBPβ^{low} cells enhanced the expression of *Il36A* whereas CREB-1 had no effect. Finally, chromatin immunoprecipitation confirmed that C/EBPβ but neither CREB-1, ATF-2 nor ATF4 is directly recruited to the proximal promoter region of the *Il36A* gene. Together, these findings demonstrate an essential role of C/EBPβ in the regulation of *Il36A* gene via the proximal half-CRE•C/EBP element in response to inflammatory stimuli.

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1. Introduction

C/EBP proteins are a group of transcription factors that belong to the bZIP class of DNA-binding proteins. This group of transcription factors currently consists of six members — C/EBPα, -β, -γ, -δ, -ε, and -ζ [1]. C/EBP proteins are expressed in a variety of cell types where they play important roles in tissue-specific gene expression, proliferation, differentiation, and inflammation. C/EBPβ was identified as a nuclear factor that binds to the promoter of the *Il6* gene [2]. The major forms of C/EBPβ (LAP*, LAP and LIP) are translated from a single exon mRNA species by alternative translation initiation [3]. C/EBPβ represents one of the key transcription factors involved in the regulation of inflammatory genes in macrophages [4,5]. Examples of genes regulated by C/EBPβ include the cytokines IL-6, TNF-α, IL-1β, G-CSF, and IL-12 p40 [6–10], the chemokine IL-8, macrophage inflammatory protein (MIP)-1α/β, monocyte chemoattractant protein-1 and chemokine (C–C motif) ligand 5 (Ccl5) [6,11–13] and the proinflammatory genes encoding lysozyme, cyclooxygenase (COX)-2 and inducible NO synthase (iNOS) [14–16]. C/EBP-mediated gene expression in general requires dimerization of C/EBPs and subsequent DNA binding to the consensus sequence $^{A}/_{G}$TTGCG$^{C}/_{T}$AA$^{C}/_{T}$ [17]. In addition to forming homodimers, C/EBP proteins can also heterodimerize with each other or with members of the CREB/ATF family by interactions via the bZIP domain [18]. For example, it has been shown that C/EBPβ and CREB/ATF bind to the non-symmetrical C/EBP•CRE composite site GTTAC•GTCAG in the human *IL1B* gene enhancer following LPS stimulation [19]. Further examples of genes regulated by C/EBP•CREB/ATF heterodimers via this non-symmetrical C/EBP•CRE composite site include the adenovirus E4 promoter and the rat phosphoenolpyruvate carboxykinase (*PCK1*) gene [20,21].

The interleukin-1 (IL-1) family of cytokines are central mediators of innate immunity and inflammation [22]. The two original members of the IL-1 family, IL-1α and IL-1β, induce proinflammatory signals upon binding to type I IL-1 receptor (IL-1RI) and subsequent complex formation with the co-receptor IL-1 receptor accessory protein (IL-1RAcP). The natural receptor antagonist, IL-1RA, exerts anti-inflammatory activities by competing with IL-1 for binding to the IL-1RI [23].

Abbreviations: BMDMs, bone marrow-derived macrophages; bZIP, basic leucine zipper; ChIP, chromatin immunoprecipitation; half-CRE, half-cAMP response element; IL-1Ra, IL-1R antagonist; IL-36Ra, IL-36R antagonist; IL-1RAcP, IL-1R accessory protein; qPCR, quantitative PCR; qRT-PCR, quantitative RT-PCR

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IL-36α (IL-1F6) together with IL-18, IL-33, IL-36β (IL1-F8) and IL-36γ (IL-1F9) belongs to the group of the more recently identified members of the IL-1 superfamily of cytokines [24,25]. IL-36 cytokines act as agonists of the IL-36R (IL-1Rrp2), a receptor of the IL-1R family. Similar to the classical IL-1 family ligands, the IL-36R also uses IL-1RAcP as a co-receptor to stimulate intracellular signals [26]. The receptor antagonist IL-36RA (IL-1F5) antagonizes stimulatory effects of IL-36 cytokines [27]. IL36 members lack a conventional signal sequence and do not contain a caspase cleavage site but N-terminal truncation of IL-36α, IL-36β, and IL-36γ leads to a dramatic increase in proinflammatory activity [26].

Recently, biological functions and the in vivo effects of IL-36 have been defined. IL-36α mRNA is highly expressed in internal epithelial tissues, particularly in keratinocytes and also in LPS-stimulated monocytes [25,28]. IL-36 plays an important role in the initiation and control of inflammatory responses in mouse experimental skin inflammation and human psoriasis [29–32]. Moreover, increased levels of IL-36α were detected in mouse models of chronic kidney disease [33], and it was shown that IL-36α is involved in pro-inflammatory processes in the lung of mice [34]. Two recent reports demonstrated, that IL36 receptor ligands are potent regulators of dendritic and T cells [35,36]. Nevertheless, the transcriptional regulation of the IL-36 members themselves in response to inflammatory stimuli is completely unknown.

In this study we generated and characterized a murine C/EBPβ knock-down macrophage cell line. Quantitative analysis of the expression of genes involved in inflammatory processes led to the identification of a group of genes whose transcriptional induction was reduced in the knock-down cells including a novel C/EBPβ target, the *Il36A* gene. By using luciferase reporter- and band-shift assays as well as chromatin immunoprecipitation, we demonstrate that C/EBPβ binds to a half-CRE•C/EBP element in the promoter and activates transcription of the *IL36A* gene.

2. Materials and methods

2.1. Reagent and antibodies

Media used for macrophage cell culture, Lipofectamine LTX, and Zeocin were obtained from Life Technologies (Darmstadt, Germany). Purified LPS was purchased from Sigma (*Salmonella enterica*; Taufkirchen, Q4 Germany) and Invivogen (ultra-pure LPS from *Escherichia coli* 0111:B4; Toulouse, France), respectively. G418 and nitrocellulose were obtained from Roth (Karlsruhe, Germany). If not stated otherwise all other reagents were from Sigma (Taufkirchen, Germany). Antibodies against CREB-1/ATF-1 (sc-270), ATF-2 (sc-187), CREB-2/ATF-4 (sc-200), C/EBPα (sc-61), C/EBPβ (sc-150), and C/EBPδ (sc-151) were purchased from Santa Cruz (Heidelberg, Germany). The antibody against GAPDH (D16H11) was obtained from Cell Signalling (Frankfurt am Main, Germany) and rat anti-DYKDDDDK tag epitope (FLAG tag) was from BioLegend (London, UK).

2.2. Plasmid construction

The plasmid encoding a short hairpin interfering RNA sequence against murine C/EBPβ (5′-GAGCGACGAGTACAAGATG-3′) cloned into plasmid mU6pro was a gift from J. Schwartz and described previously [37]. To generate a C/EBPβ version expressing LAP*, LAP and LIP resistant to the shRNA, silent mutations were introduced using the oligonucleotides 5′-GGACAAGCTGTCCGATGAATACAAGATGCG-3′, 5′-CGCATCTTGTATTCATCGGACAGCTTGTCC-3′ (mutated nucleotides underlined) and pMSV-C/EBPβ (kindly provided by J. Papaconstantinou) as template. The resulting plasmid harbouring the mutation was *Bam*HI digested and the C/EBPβ fragment was subcloned into pcDNA3.1 Zeo (−) (Life Technologies Darmstadt, Germany). The plasmid encoding rat CREB-1 was obtained from Addgene (Plasmid 22968; Cambridge, MA, USA). To generate the pGL3-*IL36A* promoter constructs the following primers were used to amplify the respective regions: PromIL1F6_for$_{-1120}$ 5′-GCGCTAGCACCCACTAAGGTATGAAAGTGA-3′, PromIL1F6_for$_{-779}$ 5′-GCGCTAGCAAATGTGAGTGGTTCATCAG-3′, PromIL1F6_for$_{-590}$ 5′-GCGCTAGCTTCTTTCCTCCATCAGGTTA-3′, PromIL1F6_for$_{-356}$ 5′-GCGCTAGCTCCCTTTCCAATGTTTCCTA-3′, PromIL1F6_for$_{-258}$ 5′-GCGCTAGCTACTCATTCTAGCCCACTCTAC-3′, PromIL1F6_for$_{-189}$ 5′-GCGCTAGCTGCACTTCCTGTAGGTTC-3′, and PromIL1F6_rev$_{+1}$ 5′-GCAAGCTTGACAAGCTCTACTAAGGTCCAAG-3′. Resulting PCR fragments were cloned into pGL3-basic (Promega, Mannheim, Germany) via *Hind*III/*Nhe*I restriction sites. Point mutations in putative binding motifs were introduced by site-directed mutagenesis. At least two nucleotides (underlined) were mutated in each of the motifs using the following primers designed with the Genomatix software (Munich, Germany) without introducing new TF binding sites: Q5

CEBP1mut_f 5′-GGAAAAGGCATCAAAATCATCGCCTCGGTTTCCTTTC-3′, CEBP1mut_r 5′-ATGATTTTGATGCCTTTTCCTGTATAGGAG-3′, CEBP2mut_f 5′-GACCTTCCTGCTCCCCTCTCAGGCAAAACCATC-3′, CEBP2mut_r 5′-GAGAGGGGAGCAGGAAGGTCAGCATTTGATG-3′, CEBP3mut_f 5′-TACAGCCACCTTGTGTCTCTCTCCGGCTTAATAAA-3′, CEBP3mut_r 5′-AGAGACACAAGGTGGCTGTATCTGAAATGG-3′, CEBP4mut_f 5′-CTGTGCCTAATAGAGACTCATTCAGGAGCACCC-3′, CEBP4mut_r 5′-CTCTATTAGGCACAGATCACACCCAAAC-3′, CEBP5mut_f 5′-TACTGCTCTCACAGGACTACCTCACCCTCCCTTTC-3′, CEBP5mut_r 5′-CCTGTGAGAGCAGTAATCAGGAAATAGG-3′, NFκB2mut_f 5′-TAGTCCAGTCATCCATCCTGCTCAAATCCCATG-3′, NFκB2mut_r 5′-CAGGATGGATGACTGGACTAGGTGACGTAAG-3′, NFkB1mut_f 5′-AATATTGGCTGGGAGAAGGTATAATATCCACTG-3′, NFkB1mut_r 5′-ACCTTCTCCCAGCCAATATTCACGTTGCTTC-3′, CREB1mut_f 5′-AGTACTTCATCTTACGACTCCTAGTCCAG-3′, CREB1mut_r 5′-CGTAAGATGAAGTACTAGAATATTGC-3′. Deletion of the internal region was introduced by inverse PCR using the primers: PromIL1F6Δ$_{-469-262}$_f 5′-GCACTAGTTTCTTTCCTCCATCAGGTTA-3′, PromIL1F6Δ$_{-469-262}$_r 5′-GCACTAGTCTTCTCCACCGCAAGTTC-3′ and pGL3-*IL36A*-1120-Luc as template. All constructs were sequenced before use and purified with the Endofree plasmid maxi kit (Qiagen, Hilden, Germany).

2.3. Macrophage cell culture

The mouse macrophage cell lines RAW264.7 and J774A.1 were maintained in DMEM supplemented with 10% FCS, 1% glutamine, 100 units ml^{-1} penicillin, 100 μg ml^{-1} streptomycin (referred to as complete medium) at 37 °C and 8% CO_2.

For isolation of primary BMDMs, C57BL/6 mice were euthanized by CO_2 inhalation. Femurs und tibias were excised, and the bone marrow was flushed out with PBS. Cells from multiple animals were pooled, suspended, and filtered through a cell strainer (Partec, Münster, Germany). BMDMs were differentiated by culturing bone marrow cells in complete medium and 20% L929 conditioned medium for 7 days (fresh medium was added on day 3) or were frozen and stored at −80 °C. Cells were harvested on day 7 by washing with cold PBS and used for experiments on day 8.

All animal experiments were approved by the appropriate ethical board (Niedersächsisches Landesamt für Verbraucherschutz und Lebensmittelsicherheit, Oldenburg, Germany).

2.4. Generation of stable RAW264.7 cell lines

RAW264.7 cells were cultured in 37 °C, 8% CO_2 in 100 mm tissue culture dishes using complete medium. 7.5 μg per dish of C/EBPβ shRNA vector and 2.5 μg of pTK-neo plasmid (Merck Millipore, Schwalbach, Germany) diluted in Opti-MEM were transfected using Lipofectamine LTX reagent. Transfected cells were incubated in standard complete medium for 24 h and were thereafter maintained under selective pressure in complete medium supplemented with 400 μg/ml G418 for selection of C/EBPβ^{low} cells. For re-expression of C/EBPβ in C/EBPβ^{low} cells 5 μg pcDNA-C/EBPβ$_{resc}$ was transfected into C/EBPβ^{low} clone 7x as described

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