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CCAAT/Enhancer-binding protein β promotes pathogenesis of EAE

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Abstract

The CCAAT/Enhancer Binding Protein β (C/EBPβ) transcription factor is activated by multiple inflammatory stimuli, including IL-17 and LPS, and C/EBPβ itself regulates numerous genes involved in inflammation. However, the role of C/EBPβ in driving autoimmunity is not well understood. Here, we demonstrate that *Cebpb*$^{-/-}$ mice are resistant to EAE. *Cebpb*$^{-/-}$ mice exhibited reduced lymphocyte and APC infiltration into CNS following EAE induction. Furthermore, MOG-induced Th17 cytokine production was impaired in draining LN, indicating defects in Th17 cell priming. *In vitro* Th17 polarization studies indicated that T cell responses are not inherently defective, instead supporting the known roles for C/EBPβ in myeloid lineage cell activation as the likely mechanism for defective Th17 priming *in vivo*. However, we did uncover an unexpected role for C/EBPβ in regulating *Il23r* expression in APCs. ChIP assays confirmed that C/EBPβ binds directly to the *Il23r* gene promoter in dendritic cells and Th17 cells. These data establish C/EBPβ as a key driver of autoimmune inflammation in EAE, and propose a novel role for C/EBPβ in regulation of IL-23R expression.

Keywords

C/EBPβ; transcription factor; EAE; IL-17; Th17; IL-23R

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Introduction

The NIH estimates that 5–8% of the US population suffers from an autoimmune disorder [1, 2]. Despite improvements in treatment due in part to anti-cytokine biologic drugs, the underlying etiology of autoimmunity remains poorly understood [3]. A major advance in understanding autoimmune pathogenesis came in 2005 with the discovery of Th17 cells, a new class of $CD4^+$ T helper cells. Characterized by expression of IL-17 (IL-17A), IL-17F, IL-22 and GM-CSF, these cells play a non-redundant function in immunity to extracellular microbes, particularly fungi [4]. However, Th17 cells are dysregulated in many autoimmune diseases. Th17 cells differentiate from naïve Th0 cells under conditions of inflammation, driven by signals from TGFβ, IL-6, IL-1 and IL-21 [5]. During differentiation, Th17 cells express the IL-23 receptor (IL-23R), and signals from IL-23 are necessary for these cells to be pathogenic [6–9]. Indeed, antibodies against IL-17A and IL-23 are effective in treating autoimmunity, particularly plaque psoriasis [10, 11]. These biologics are also under evaluation for ankylosing spondylitis, uveitis and multiple sclerosis [12–14].

The IL-17 cytokine family is the newest and least well-understood of the cytokine subclasses [15, 16]. IL-17 and IL-17F signal through a heterodimeric receptor composed of IL-17RA and IL-17RC, and the primary cellular targets of IL-17 are non-hematopoietic cells [16]. Upon engagement with IL-17, the receptor recruits the adaptor Act1 (also known as CIKS), which activates the classical NF-κB pathway, MAPK pathways, and a TRAF2/5-dependent cascade that promotes mRNA stability [3, 17]. In addition, IL-17 activates the CCAAT/Enhancer Binding Protein (C/EBP) family of transcription factors, particularly C/EBPβ [18–24], but the biological role of this pathway is not well defined. *Cebpb*$^{-/-}$ mice are highly susceptible to systemic *Candida albicans* and *Listeria monocytogenes* infections, clearance of which requires IL-17 [25, 26]. In contrast, *Cebpb*$^{-/-}$ mice were found to be resistant to oral candidiasis, showing increased susceptibility only under conditions of steroid-induced immunosuppression [27]. Hence, it does not appear that all immune responses are defective in the absence of C/EBPβ, and the requirement for C/EBPβ in IL-17 signaling is not absolute in all circumstances.

C/EBPβ plays diverse roles in inflammation through a variety of cell types [28, 29]. For example, C/EBPβ is a central regulator of adipocyte differentiation [30]. Additionally, studies in vivo have identified especially important functions in macrophage populations, [31] [26, 32]. However, to date its function in autoimmunity is not well defined.

The experimental autoimmune encephalomyelitis (EAE) model of multiple sclerosis is frequently employed for defining parameters of T cell-driven autoimmunity, particularly Th17 cells [33, 34]. Mice deficient in IL-17, IL-23 or the associated receptors are resistant to EAE [35]. IL-17 promotes expression of chemokines that recruit myeloid cells to the CNS, and also acts directly to damage CNS-resident cells such as oligodendrocytes. IL-23 also promotes Th17 production of GM-CSF that activates recruited macrophages as well as CNS-resident microglia to further promote tissue inflammation [36–38]. Indeed, macrophage recruitment and activation as well as DC presentation of myelin antigens are required for development of EAE [39]. Notably, the CCAAT/Enhancer Binding Protein (C/EBPβ) transcription factor (TF) is upregulated in human MS tissue samples [40]. A recent GWAS

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study of MS patients identified a risk allele in a C/EBPβ binding element in the promoter of CBL-B (Casitas B-lineage lymphoma proto-oncogene b) [41]. To date, however, the role of C/EBPβ) in EAE has not been assessed.

In this study, we assessed the role of C/EBPβ in Th17-dependent autoimmunity in EAE. We found that CNS in *Cebpb*$^{-/-}$ mice was associated with decreased infiltration of lymphocytes and APCs as well as reduced expression of Th17 cytokines in the CNS. LN expression of the IL-23 receptor (IL-23R) was reduced in *Cebpb*$^{-/-}$ mice during EAE, and ChIP analyses confirmed that C/EBPβ binds to the *Il23r* promoter in Th17 cells and bone marrow derived myeloid cells. Thus, we conclude that C/EBPβ contributes to Th17-dependent regulation of inflammation in EAE.

Materials and Methods

Mice

Cebpb^{tm1Vpo}/J$^{+/+}$ mice from The Jackson Laboratory (JAX, Bar Harbor ME) were bred to generate *Cebpb*$^{-/-}$ and *Cebpb*$^{+/+}$ littermate controls. C57BL/6 mice were also from JAX.

EAE

Cebpb$^{-/-}$ and littermates were immunized s.c. with 100 μg MOG peptide (residues 35–55) in CFA containing 500 μg heat killed *Mycobacterium tuberculosis* as described [42]. Mice received 100 ng pertussis toxin (List Biological Laboratories, Campbell CA) i.p. on days 0 and 2. Disease severity was evaluated using the the following scale: 1: flaccid tail; 2: impaired righting reflex and hindlimb weakness; 3: partial hindlimb paralysis; 4: complete hindlimb paralysis; 5: hindlimb paralysis with partial forelimb paralysis; 6: moribund. All procedures were approved by the University of Pittsburgh IACUC.

qPCR

RNA was isolated with RNAeasy Kits (Qiagen, Gaithersburg, MD), and cDNA was created with the SuperScript III First-Strand Synthesis System (Invitrogen, Carlsbad CA). Quantification was determined by real-time PCR with SYBR Green (Quanta BioSciences, Beverly MA) normalized to *Gapdh*. Primers were from Qiagen. Results were analyzed on a 7300 Real Time PCR System (Applied Biosystems, Carlsbad, CA).

Cell cultures, Flow cytometry, ELISAs

Bone marrow-derived myeloid cells were generated by cultured bone marrow cells with GM-CSF (10ng/ml) for 6-8 d. When indicated, BM-derived myeloid cells were stimulated with lipopolysaccharide (*lps*) (100ng/ml), TNFα (10ng/ml) or GM-CSF (10ng/ml) for 24 h. CD4$^+$ T cells were isolated from LN and spleen of naïve animals by positive selection using Miltenyi Biotec (San Diego, CA) CD4 (L3T4) MicroBeads and LS columns. 10^6 CD4$^+$ cells were stimulated with plate bound anti-CD3 (5ug/ml, Bio X Cell, Lebanon, NH) for 3 days. Differentiation conditions were: Th0, anti-IFNγ (10ug/ml); Th17, TGFβ (2.5ng/ml), IL-6 (20ng/ml), IL-1β (20ng/ml), IL-23 (50ng/ml), anti-IFNγ (10ug/ml) and anti-IL-2 (10ug/ml) unless otherwise described. Cytokines were from R&D Systems (Minneapolis, MN). Differentiated cells were stimulated with 50ng/ml PMA and 500ng/ml Ionomycin (Sigma, St

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Louis, MO) for 4 hours with Golgi Plug (BD Biosciences). After stimulation, cells were stained for CD4 (RPA-T4, RM4-5), IL-17 (TC11-18H10). Dead cells were excluded with Ghost Dye Violet 510 (Tonbo Biosciences, San Diego CA). Intracellular cytokine staining was performed with the Cytofix Cytoperm kit (BD Biosciences). CNS preparations were stained with the following Abs: CD45 (30-F11), CD4 (RPA-T4, RM4-5), Gr1 (RB6-8C5), CD11b (M1/70), CD11c (HL3), IA/IE (2G9), CD80 (16-10A1), CD86 (GL1). Data were acquired on a FACS ARIA II or BD Fortessa (BD Biosciences) and analyzed using FlowJo (Ashland, OR). For ELISAs, supernatants were analyzed in duplicate with kits from eBiosciences.

Promoter Analysis

Promoter sequences, defined as 1.5 kb upstream of the transcription start site (TSS) for the human and murine IL-23 receptor genes, were obtained from the UCSC genome browser (University of California, Santa Cruz, CA). Conservation was assessed using LALIGN and CLC Main Workbench 6 software (Qiagen). C/EBPβ binding sites were identified using Match software (Biobase Biological Databases, Qiagen).

Chromatin immunoprecipitation

Cell suspensions were sonicated for 15 cycles at 15% amplitude (BM-myeloid cells) or 15–20 cycles at 30% amplitude ($CD4^+$ T cells) (30 secs ON/30 secs OFF) using the Epishear Probe Sonicator (Active Motif Carlsbad, CA). ChIP was performed using the EZ-Magna ChIP A/G Chromatin Immunoprecipitation Kit (EMD Millipore, Billerica, MA). Cross-linked chromatin was immunoprecipitated with anti-C/EBPβ Abs (C19 sc-150; Santa Cruz Biotechnology, Santa Cruz CA) or non-immune rabbit IgG. Primers at positions -284 (IL-23R 01A) and -1296 (IL-23R 02A) upstream of the TSS of the *Il23r* promoter (Qiagen) were used for qPCR.

Statistics

At least 2 independent replicates were performed for all experiments. Data were compared by unpaired Student's *t* test or ANOVA and Mann-Whitney using GraphPad Prism (v. 4). *P* values < 0.05 were considered significant.

Results

C/EBPβ deficient mice are resistant to EAE

Cebpb$^{-/-}$ and *Cebpb*$^{+/+}$ controls on a mixed 129S/SvEv-Gpi1 background were immunized with MOG(35–55) in CFA to induce EAE. *Cebpb*$^{+/+}$ mice developed a typical onset and clinical course of EAE, peaking at 13–14 days post immunization, with an incidence and disease severity similar to typical EAE in WT C57BL/6 mice (Fig 1A and data not shown). At peak of disease, *Cebpb*$^{-/-}$ mice showed markedly reduced EAE disease severity compared to WT *Cebpb*$^{+/+}$ littermate controls (Fig 1A). Concomitant with reduced disease scores, there was a significant reduction in the percentage and absolute numbers of $CD45^+$ infiltrating mononuclear cells and in the CNS of *Cebpb*$^{-/-}$ compared to WT mice at the peak of disease following EAE induction (Fig 1B). Of the infiltrating cell populations, both frequencies and absolute numbers of $CD4^+$ T cells (Fig 1C) and $CD45^{hi}CD11b^{hi}$

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macrophages were reduced (Fig 1D–F). Given that C/EBPβ is a key transcription factor for macrophage activation [31], it was perhaps surprising to note that expression of class II MHC and costimulatory molecules CD80 and CD86 by infiltrating macrophages in the CNS were not significantly different in *Cebpb*$^{-/-}$ mice (Fig 1G). Hence we concluded that *Cebpb* is required for full induction of the inflammatory response in autoimmune attack of the CNS during EAE and that reduced disease severity is associated with reduced recruitment of inflammatory cells to the CNS. Nonetheless, we have not ruled out a delayed onset of clinical signs in *Cebpb*$^{-/-}$ mice.

C/EBPβ$^{-/-}$ T cells have impaired induction of MOG-reactive Th17 responses

To determine whether defects in autoreactive Th17 cell priming contributed to the resistance of *Cebpb*$^{-/-}$ mice to EAE induction, we tested the LN MOG(35–55) response in immunized *Cebpb*$^{-/-}$ mice. MOG(35–55)-induced IL-17 production was markedly reduced in *Cebpb*$^{-/-}$ mice compared to WT littermates (Fig 2A). Although addition of IL-23 enhanced expression of IL-17 in WT cultures, this response was impaired in *Cebpb*$^{-/-}$ mice (Fig 2A). Production of IFNγ and GM-CSF showed a similar trend to reduced expression in *Cebpb*$^{-/-}$ compared to *Cebpb*$^{+/+}$ mice (Fig 2B–C). Thus, T cell effector cytokines are reduced with C/EBPβ deficiency.

We next evaluated gene expression associated with EAE to determine which, if any, aspects of Th17 cell development were impaired in the absence of C/EBPβ. Gene expression analysis from EAE LN cultures indicated that mRNA expression of putative Th17 transcription factors STAT3 and RORγt were not significantly different in absence of C/EBPβ (Fig 3A,B). It should be noted that gene expression does not necessarily indicate function of transcription factors, which depend on post-transcriptional modifications and nuclear localization for activity, and it is not known whether C/EBPβ could also regulate these. C/EBPδ has also been shown to contribute to EAE [43], and C/EBPδ regulates C/EBPβ expression in adipocytes [20, 30]. However, there was no difference in C/EBPδ expression (Fig 3C), indicating that this did not account for the impact of C/EBPβ in this setting. Unexpectedly, expression of *Il23r*, a receptor required for effector Th17 proliferation and function, was significantly reduced in *Cebpb*$^{-/-}$ cultures (Fig 3D). Collectively, these data indicate that MOG-reactive Th17 cell cytokine production is impaired in absence of C/EBPβ.

Il23r expression is reduced in innate cells from Cebpb deficient mice

As C/EBPβ is mostly thought to act in myeloid lineage cells, it was intriguing that Th17 responses and particularly IL-23R were defective, raising the question of T cell intrinsic activities of C/EBPβ. To determine whether C/EBPβ was acting directly in T cells, CD4^{+} T cells were isolated from *Cebpb*$^{-/-}$ or *Cebpb*$^{+/+}$ littermates and differentiated in vitro with anti-CD3 Abs in the absence of APCs. Induction of IL-17-producing cells under these conditions occurred independently of C/EBPβ (Fig 4A). There was a trend but no significant decrease in IL-23R in Th17 cells activated by anti-CD3 Abs (Fig 4B). In total splenocyte cultures, which contain myeloid lineage cells including DCs and macrophages, there was also a non-significant trend towards reduced IL-17 levels in *Cebpb*$^{-/-}$ T cells (Fig 4C). Addition of anti-CD28 to the cultures boosted IL-17 but did not change the overall trend

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towards decreased IL-17 in *Cebpb*$^{-/-}$ cells (Fig 4D). However, there was a clear and significant reduction in IL-23R mRNA expression in *Cebpb*$^{-/-}$ splenocyte cultures compared to WT controls (Fig 4E). In contrast to Th17 conditions, differentiation of *Cebpb*$^{-/-}$ Th1 cells by culture with IL-12 showed no difference from WT cells (Fig 4F), suggesting that global T cell differentiation is not impaired in *Cebpb*$^{-/-}$ cells. Finally, analysis of *Cebpb* expression in CD4^{+} T cells compared to splenocytes confirmed that C/EBPβ is not highly expressed in T cells (Fig 4E). Rather, total splenocyte cultures showed high levels of C/EBPβ (Fig 4E), consistent with a requirement for C/EBPβ for optimal IL-23R expression in that population. Albeit indirect evidence, these data suggest a novel role for C/EBPβ in regulating IL-23R, in addition to its known functions in myeloid cell activation.

C/EBPβ binds directly to the Il23r promoter

C/EBPβ has multiple activities that could lead to regulation of IL-23R expression. Using computational analysis, we identified specific C/EBPβ binding sites within the human and mouse *Il23r* promoter sequences, with the promoter defined as the first 1.5 kb upstream of the predicted transcriptional start site (Fig 5A). These promoters are 48% identical and indeed encode several putative C/EBPβ DNA binding elements (Fig 5A). Since IL-23R expression was clearly defective in cultures that contained innate myeloid cells, we first confirmed that bone-marrow derived myeloid cells express IL-23R in response to pro-inflammatory cytokines present in Th17 cultures: TNF and GMCSF. They also induced IL-23R in response to LPS (Fig 5B). Chromatin immunoprecipitation (ChIP) was performed with anti-C/EBPβ Abs, and genomic DNA was analyzed by qPCR for the conserved proximal promoter region of the IL-23R that contains C/EBPβ recognition elements (IL-23R-01A)) (primer position indicated in Fig 5A). The *Il23r* promoter sequence was enriched following C/EBPβ ChIP in LPS-stimulated myeloid cells compared to untreated cells (Fig 5C). Similarly, C/EBPβ associated with the *Il23r* promoter in Th17 cells more than in Th0 cells (Fig 5D). Together, these data indicate that C/EBPβ can occupy the proximal *Il23r* promoter, suggesting a direct role in regulating IL-23R expression.

Discussion

Cebpb mRNA is upregulated in the brain tissue of MS patients [40]. In relapsing-remitting patients, enhanced binding of C/EBPβ to a risk-associated CBL-B allele is observed, correlating with reduced CBL-B expression in CD4^{+} T cells [41]. However, no mechanistic studies to date have assessed the contribution of C/EBPβ to CNS inflammation. The C/EBP transcription factors regulate numerous genes impacting inflammation and immunity [29]. Our prior observations indicated that C/EBPβ is regulated by IL-17 in multiple ways [3]. For example, *Cebpb* is part of the characteristic IL-17-induced gene signature [20, 22, 44, 45]. IL-17 promotes alternative translation of C/EBPβ from its dominant “LAP” (liver activated protein) isoform into the LAP* and LIP (liver inhibitory protein) species [44]. Additionally, C/EBPβ is phosphorylated following IL-17 stimulation, influencing its transcriptional activity [21, 24].

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These connections led us to evaluate the role of C/EBPβ in IL-17-driven autoimmunity in the mouse model of multiple sclerosis, EAE. Indeed, we observed a reduced susceptibility of *Cebpb*$^{-/-}$ mice to EAE. The associated defect in MOG-reactive Th17 cell priming suggested a role for C/EBPβ in APC-mediated Th17 cell activation. This was further supported by the finding that T cells isolated from *Cebpb*$^{-/-}$ mice were able to differentiate Th17 cells to a similar level as WT cells when stimulated with anti-CD3 and exogenous cytokines in the absence of APC; these data also confirmed that T cells from these mice are not inherently defective. C/EBPβ is already known to regulate expression of several factors that contribute to Th17 priming, including IL-6 [28, 46, 47] hence to some extent these observations are in line with previously published actions of C/EBPβ and we did not focus our studies on these mechanisms.

One unexpected finding was reduced expression of IL-23R in *Cebpb*$^{-/-}$ cells, uncovering an additional novel mechanism through which C/EBPβ can contribute to inflammatory responses in a Th17 setting. IL-23R is not expressed on naïve T cells, but is induced by inflammatory signals from cytokines on T cells, including IL-6, IL-1, and enhanced by IL-23 itself. In the EAE model, IL-23 is required to promote late effector Th17 cell generation, including production of GM-CSF[48]. We also observed a decrease in IFNγ production. Although historically associated with Th1 responses in the EAE model, fate-tracking reporter mice have demonstrated that the majority of IFNγ in EAE actually is produced by cells of Th17 origin[49]. Furthermore, conversion to IFNγ producing cells actually requires signaling from IL-23[8, 49, 50]. For innate cells, there are a paucity of studies on the roles of IL-23R in macrophages and dendritic cells, although it has been shown to be upregulated in models of EAE, psoriasis and atopic dermatitis [51–53]. Accordingly, the regulation and function of IL-23R expression in myeloid cells is less clear than for T cells, but it is also likely to be induced by inflammatory stimuli through both cytokines and also pattern recognition receptors as we have shown here for LPS. In a model of atopic dematitis, IL-23 was shown to stimulate IL-23R^{+} skin dendritic cells to enhance their production of IL-23 in a feed-forward loop that potentiated local inflammation through activation of Th22 cells[53]. In EAE, IL23R^{+} myeloid cells were reported to express Th17-associated factors including RORγt[51–53], although the functional significance of this finding has not been clarified. Our study indicates a direct role for C/EBPβ in promoting *Il23r* gene expression, through binding to the proximal region of the *Il23r* promoter following inflammatory signals from LPS (dendritic cells) and cytokines (Th17 cells). In Th17 cells, deficiency of C/EBPβ did not appear to strongly affect the early cytokine-driven IL-23R expression in vitro, which would be expected since other transcription factors including STAT3 are strongly activated by these signals and known to also directly regulate *Il23r* gene expression [54]. However, this does not rule out a role for CEBPβ-23R expression in later effector Th17 cells.

It is likely that there are additional roles for C/EBPβ acting downstream of IL-17 signaling in the CNS, for example to promote expression of chemokines for recruitment of inflammatory myeloid cells, which contribute to the resistance of *Cebpb*$^{-/-}$ mice to EAE. Since these events occur after Th17 cell activation, which was also defective in the absence of C/EBPβ, the experiments performed here do not provide a conclusive answer to this question. Because *Cebpb*$^{-/-}$ mice have developmental defects that include low viability of

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pups, they have to be maintained on a mixed genetic background [26, 27]. Although more representative of an outbred human population, this mixed background made it impossible to perform the 'typical' cross-transfer experiments that one would perform to distinguish whether immune cells (and which ones) versus tissue-resident cells require C/EBPβ for EAE. Nevertheless, they provide further basis for consideration of C/EBPβ functions in Th17-mediated disease.

In addition to its impact on C/EBPβ, IL-17 induces expression of the related transcription factor C/EBPδ. In mesenchymal cell types, C/EBPβ and C/EBPδ redundantly activate target genes downstream of IL-17 such as *Il6* and *Cxcl5* [20, 55]. However, such functional overlap does not appear to occur in EAE, as *Cebpb*$^{-/-}$ and *Cebpδ*$^{-/-}$ mice are both resistant to disease [43]. During adipogenesis, C/EBPβ and C/EBPδ co-regulate one another [23]; however, the current study showed that *Cebpd* expression is normal in *Cebpb*$^{-/-}$ mice subjected to EAE, suggesting that the resistant phenotype in C/EBPβ$^{-/-}$ mice is not secondary to C/EBPδ-deficiency.

In conclusion, our data reveal that C/EBPβ plays a vital role in the development of CNS inflammation in the EAE model, with impaired development of myelin-reactive Th17 cells and reduced recruitment of inflammatory infiltrating cells to the CNS in *Cebpb*$^{-/-}$ mice. These results are in accordance with previous studies highlighting the role of C/EBPβ in promoting a cycle of inflammatory responses to promote recovery from infection, but have not been shown before for autoimmune disease. Furthermore, we have uncovered an unexpected role for C/EBPβ in regulating expression of IL-23R, a key lynchpin in Th17 autoimmune disease, and raise the possibility of a pathogenic role for C/EBPβ in other IL-23-dependent autoimmune settings.

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HIGHLIGHTS

- *Cebpb*$^{-/-}$ mice are resistant to EAE
- In vivo Th17 responses are impaired in absence of C/EBPβ
- Th17 differentiation is normal in APC-free cultures
- C/EBPβ binds to the IL-23R in myeloid and Th17 cells

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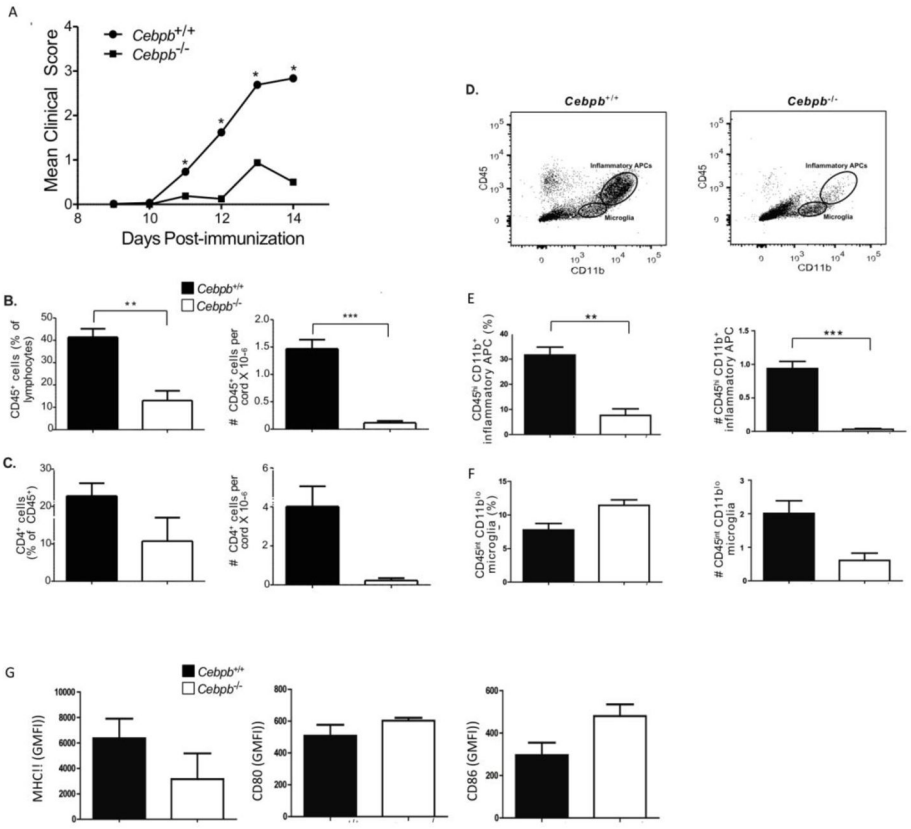

Figure 1. *Cebpb*$^{-/-}$ mice are resistant to EAE

Cebpb$^{+/+}$ and *Cebpb*$^{-/-}$ mice were immunized with MOG to induce EAE. **A.** Clinical EAE scores of *Cebpb*$^{+/+}$ (n =52) and *Cebpb*$^{-/-}$ (n=9) mice, data are pooled from multiple experiments, statistical significance of EAE scores was assessed by Mann Whitney separately for each timepoint (* P <0.05). **B**. Percentage (left) and absolute numbers (right) of CD45^{+} cells gated from FSC/SSC lymphocyte gate in the CNS on day 14, *Cebpb*$^{+/+}$ (n=31), *Cebpb*$^{-/-}$ (n=6). **C.** Percentage (left) and absolute numbers (right) of CD4^{+} cells in the CNS on day 14. *Cebpb*$^{+/+}$ (n=15), *Cebpb*$^{-/-}$ (n=2). (**D**) Representative plots showing gating strategy to identify microglia and infiltrating macrophages from the CNS of C/EBPβ mice on day 14; (**E**) percentage (left) and absolute numbers (right) of CD45^{high}CD11b^{+} infiltrating macrophages and (**F**) percentage (left) and absolute numbers (right) of CD45^{int}CD11b^{int} microglia. (**G**) MFI of MHC Class II (IA/IE), CD80, CD86 in CD45^{high}CD11b^{+} macrophages, *Cebpb*$^{+/+}$ (n=23–31) and *Cebpb*$^{-/-}$ (n=3–5). Data pooled from more than 3 independent experiments, except C is pooled from 2 experiments. Bars represent mean and SEM. ** P<0.005 and *** P<0.0005 by student unpaired t-test.

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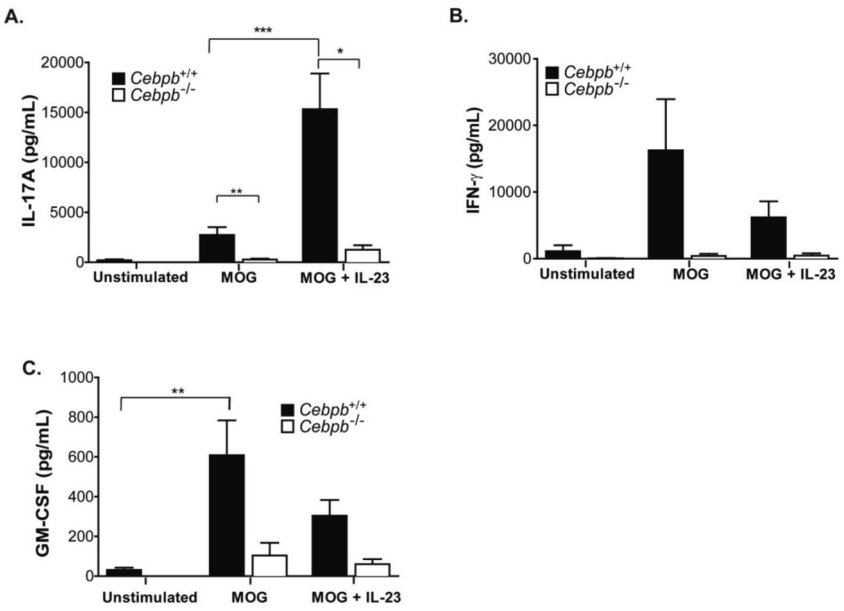

Figure 2. MOG-reactive IL-17 responses are reduced in *Cebpb*$^{-/-}$ LN during EAE

Cebpb$^{+/+}$ and *Cebpb*$^{-/-}$ mice were immunized with MOG to induce EAE, and day 14 draining LN cells were cultured with MOG(35–55) with or without IL-23 for 3 days before supernatants were assessed by ELISA for IL-17A (**A**), IFN-γ (**B**), and GM-CSF (**C**). Data are from 5 independent experiments, with *Cebpb*$^{+/+}$ n=17–22, *Cebpb*$^{-/-}$ n=5. Bars show mean and SEM. **P*<0.05, ***P*<0.005 and ****P*<0.0005 by one-way ANOVA.

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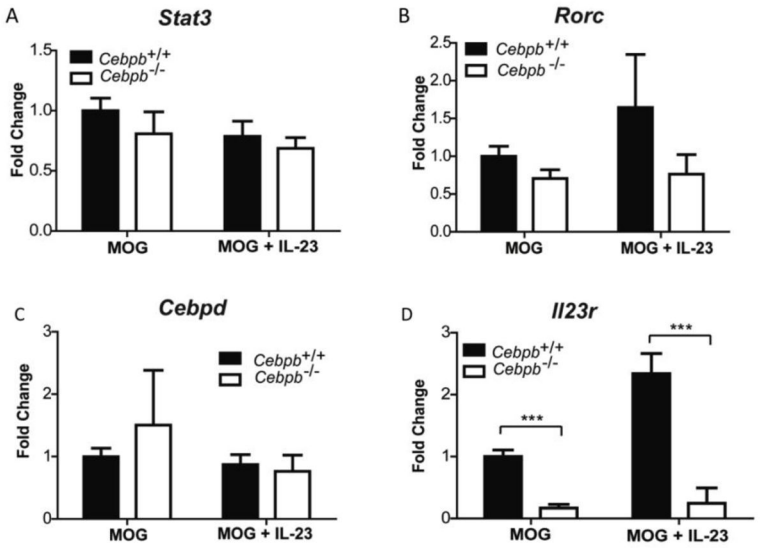

Figure 3. *Il23r* is reduced in *Cebpb*$^{-/-}$ LN during EAE

Cebpb$^{+/+}$ and *Cebpb*$^{-/-}$ mice were immunized with MOG to induce EAE, and draining LN cells were isolated and stimulated as described for Figure 2. Gene expression of (**A**) *Stat3* (**B**) *Rorc* (**C**) *Cebpd* and (**D**) *Il23r* were assessed by qPCR in triplicate. Data are presented as fold change compared to C/EBPβ$^{+/+}$ stimulated with MOG(35–55). Data are pooled from 3 independent experiments, with *Cebpb*$^{+/+}$ (n=7), *Cebpb*$^{-/-}$ (n=3). Bars show mean and SEM. ***P<0.0005 by one-way ANOVA.

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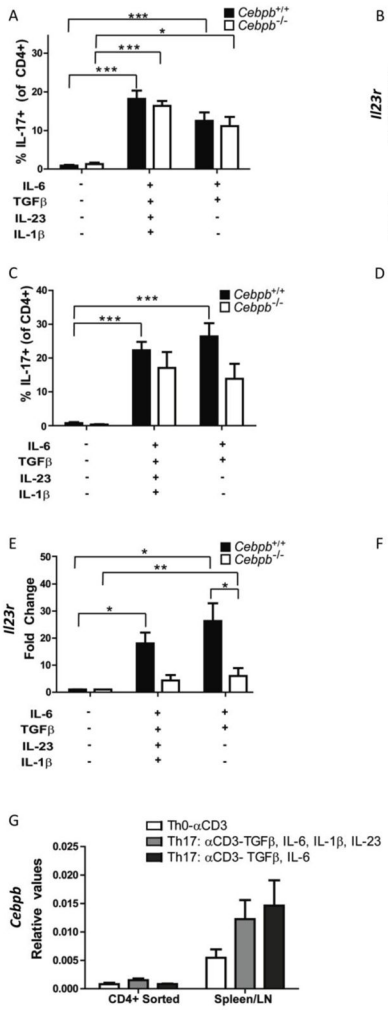

Figure 4. *Il23r* expression is reduced in C*ebpb* deficient mice

A–B: CD4$^+$ T cells were isolated from *Cebpb*$^{+/+}$and *Cebpb*$^{-/-}$ mice and activated with anti-CD3 in presence or absence of Th17-inducing cytokines for 3 days before analysis of IL-17A by flow cytometry (**A**) and expression of *Il23r* by qPCR (**B**). **C–D:** Total splenocytes/lymph node cells from *Cebpb*$^{+/+}$and *Cebpb*$^{-/-}$ mice were activated with anti-CD3 in presence or absence of Th17-inducing cytokines for 3 days (**C**) or Th17-inducing cytokines and anti-CD28 (**D**) before analysis of IL-17A by flow cytometry and expression of *Il23r* by qPCR (**E**). **F:** Total splenocytes/lymph node cells from *Cebpb*$^{+/+}$and *Cebpb*$^{-/-}$ mice were activated with anti-CD3 in presence or absence of IL-12 to induce Th1 cells for 3 days before analysis of IFN**γ** by flow cytometry. **G**: *Cebpb* expression in WT T cells and total splenocyte/lymph node cell cultures. Data are pooled from 2 independent experiments. Bars on graphs show mean and SEM. *P<0.05, **P<0.005 and ***P<0.0005 by ANOVA.

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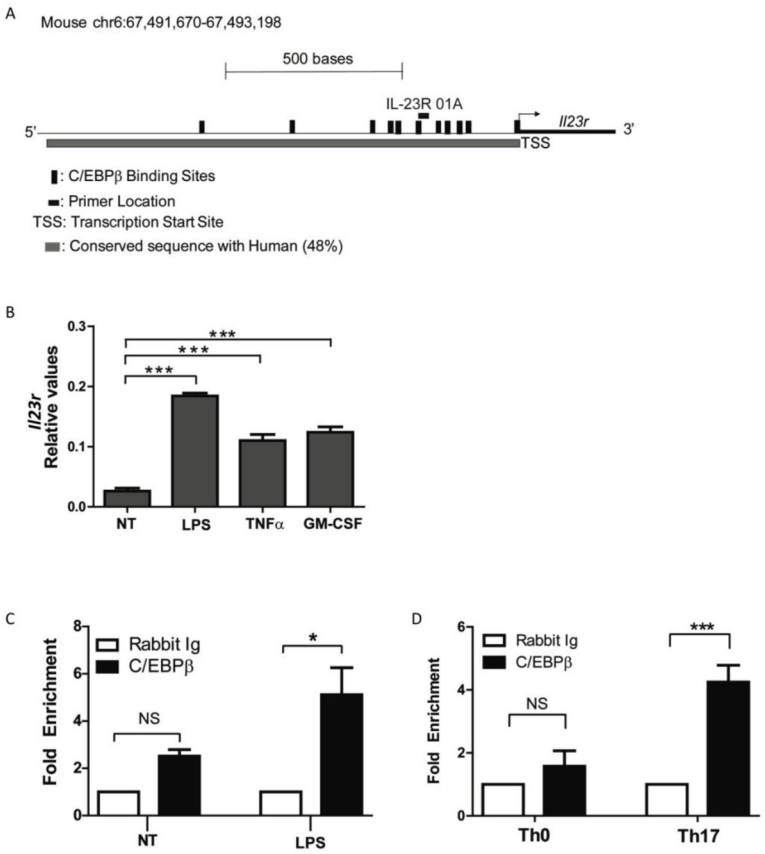

Figure 5. C/EBPβ occupies the *Il23r* promoter following inflammatory stimuli

(**A**) Diagram of mouse *Il23r* promoter. Predicted C/EBPβ binding sites and location of the *Il23r* qPCR primer are indicated. (**B**) BM-derived myeloid cells from WT mice were stimulated with lps, TNFα or GM-CSF for 24 hours. *Il23r* expression was assessed by qPCR in triplicate. Data are representative of 2 independent experiments. (**C–D**) ChIP with Abs to C/EBPβ (black bars) or IgG (white bars) was performed on genomic DNA from BM-myeloid cells from WT mice treated with *lps* for 24 hours (**C**) and from T cells differentiated for 3 days in presence or absence of Th17-inducing cytokines (**D**). Fold enrichment was calculated as $2^{-(\text{C/EBP}\beta\ \text{IP}) - (\text{Isotype IP}))}$. Data are pooled from 2 (**A,C**) and 4 (**D**) independent experiments. Bars show mean + SEM. **P*<0.05, and ****P*<0.0005 by unpaired student t-test and ANOVA.