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Liver X receptors and inflammatory-induced C/EBP□ selectively cooperate to control CD38 transcription

Estibaliz Glaría[1,2], Pol Rodríguez Martínez[1,2], Joan Font-Díaz[1,2], Juan Vladimir De la Rosa[3], Antonio Castrillo[3,4], Dylan J. Crawshaw[1,2], Jose Manuel Vidal Taboada[5,6,7]; Josep Saura[5,6], Jonathan Matalonga[1], Eduardo Nunes Chini[8], Carme Caelles[2,9], Annabel F. Valledor[1,2,*]

[1]Department of Cell Biology, Physiology and Immunology, School of Biology, University of Barcelona, Barcelona, Spain.

[2]Institute of Biomedicine of the University of Barcelona (IBUB), Barcelona, Spain.

[3]Unidad de Biomedicina (Unidad Asociada al CSIC), Instituto Universitario de Investigaciones Biomédicas y Sanitarias (IUIBS), Grupo de investigación Farmacología Molecular y Traslacional (BIOPharma), Universidad de Las Palmas de Gran Canaria, Las Palmas, Spain.

[4]Instituto de Investigaciones Biomédicas Sols-Morreale. Centro mixto CSIC-Universidad Autónoma de Madrid, Madrid, Spain.

[5]Department of Biomedical Sciences, Biochemistry and Molecular Biology Unit, School of Medicine, University of Barcelona, IDIBAPS, Barcelona, Spain.

[6]Institute of Neurosciences, University of Barcelona, Barcelona, Spain.

[7]Peripheral Nervous System, Brain & Mind and Behaviour eCORE, VHIR, Vall d'Hebron Research Institute, Barcelona, Spain.

[8]Signal Transduction and Molecular Nutrition Laboratory, Kogod Aging Center, Department of Anesthesiology and Perioperative Medicine, Kogod Center on Aging, Mayo Clinic College of Medicine, Jacksonville, FL, USA.

[9]Department of Biochemistry and Physiology, School of Pharmacy and Food Sciences, University of Barcelona, Barcelona, Spain.

***Correspondence:** Dr. Annabel F. Valledor, Departament Biologia Cel·lular, Fisiologia i Immunologia, Fac. Biologia, Universitat de Barceloan. Av. Diagonal, 643, pl 3. Barcelona 08028. Spain. Phone: +34-93-4039384; e-mail address: afernandezvalledor@ub.edu

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Abstract

Introduction. Macrophages abundantly express liver X receptors (LXRs), which are ligand-dependent transcription factors and sensors of several cholesterol metabolites. In response to agonists, LXRs promote the expression of key lipid homeostasis regulators. Crosstalk between LXRs and inflammatory signals exist in a cell type- and gene-specific manner. A common feature in the macrophage response to inflammatory mediators is the induction of CCAAT/enhancer-binding protein beta (C/EBPβ), a master transcriptional regulator and lineage-determining transcription factor in monocytes/macrophages. Methods. Quantitative real-time PCR in control and C/EBPβ-deficient macrophages was used to explore the role of C/EBPβ in the crosstalk between inflammatory mediators and the macrophage response to pharmacological LXR activation. The functional interaction between C/EBPβ and LXRs on selected genomic regions was further characterized by chromatin-immunoprecipitation (ChIP) and gene reporter studies. Results. Whereas inflammatory signaling repressed several LXR-regulated genes involved in lipid metabolism, these effects were conserved after deletion of C/EBPβ. In contrast, inflammatory mediators and LXRs synergistically induced the expression of the multifunctional protein CD38 in a C/EBPβ-dependent manner. C/EBPβ and LXRs bound to several regions with enhancer activity upstream and within the mouse *Cd38* gene and their functional cooperation in macrophages required intact binding sites for LXR and C/EBPβ. Conclusion. This study reveals positive crosstalk between C/EBPβ and LXRs during the macrophage inflammatory response, which selectively impacts CD38 expression.

Introduction

Macrophages are innate immune cells that play key roles in the host defense against insults of diverse origin, but they are also integral tissue components involved in the regulation of metabolism and homeostasis [1]. The diversity of activities performed by macrophages is, in part, the consequence of their great plasticity in phenotypically adapting to signals from the microenvironment [2]. In this regard, the integration of environmental signals impacts macrophage metabolism, which in turn modulates the characteristics of the response.

Liver X receptors (LXRs) are members of the nuclear receptor family of ligand-dependent transcription factors that play diverse roles in the crosstalk between inflammatory pathways and lipid metabolism in macrophages (reviewed in [3]). LXRs are sensors of natural cholesterol metabolites, but can also be pharmacologically activated by highly specific synthetic agonists, such as TO-901317 (T1317) and GW3965. Two LXR subtypes exist, namely LXRα and LXRβ, encoded by separate genes. While LXRβ is ubiquitously expressed in the body, LXRα predominates in tissues and cells with high metabolic activity, including macrophages (reviewed in [4]).

LXRs form heterodimers with another nuclear receptor, the retinoid X receptor (RXR). LXR-RXR heterodimers bind to LXR response elements (LXREs) on DNA and interact with corepressor complexes to repress gene transcription in the absence of an agonistic ligand [5,6]. Activation of LXR-RXR heterodimers with an LXR agonist promotes the exchange of corepressors for coactivators to induce gene transcription (reviewed in [7]). LXRs control the expression of key regulators of cholesterol, fatty acid and phospholipid homeostasis (reviewed in [3]), including the sterol transporters ATP binding cassette A1 (ABCA1) and G1 (ABCG1), which are sterol transporters that promote cellular cholesterol efflux [8,9], several apolipoproteins (Apo), such as ApoC1, involved in lipid transport [10], and sterol regulatory element-binding protein 1 (SREBP1c; encoded by the gene *Srebf1*), which activates the transcription of lipogenic genes [11,12].

In myeloid cells, LXRs also control the expression of molecules involved in immune responses, including the macrophage apoptosis inhibitory factor CD5L [13,14], the receptor MER tyrosine kinase (MERTK) with important roles in the phagocytosis of apoptotic bodies [15], and the multifunctional protein CD38 [16,17].

CD38 is expressed both as an integral membrane protein and as an extracellular soluble form, and is a therapeutic target in several types of cancer [18]. Membrane-bound CD38 has several functions as a receptor [19] or co-receptor in association with other immune protein complexes (reviewed in [20]), and as an enzyme (reviewed in [18]). The enzymatic activities of CD38 result in large consumption of nicotinamide adenine dinucleotide (NAD) to generate calcium-mobilizing second messengers, for which reason CD38 is considered to be the major regulator of NAD levels in mammalian tissues [21]. The loss of functional CD38 expression and/or activity is associated with impaired immune responses and metabolic and behavioral alterations [18,22–24].

Pieces of evidence support reciprocal negative crosstalk between pharmacologically-activated LXRs and inflammatory signals. LXR agonists antagonize pro-inflammatory gene expression through a combination of mechanisms, including trans-repression [25] and the modulation of genes involved in cholesterol metabolism [26,27]. In turn, pro-inflammatory signaling represses the induction of several LXR target genes involved in lipid homeostasis [28,29]. However, in contrast with these mutually opposing effects, LXRs and several inflammatory mediators, namely tumor necrosis factor alpha (TNFα), interferon gamma (IFNγ), and lipopolysaccharide (LPS), cooperated to synergistically increase the expression and NADase activity of CD38 in murine macrophages [17]. Cooperation with inflammatory signaling was observed when either synthetic (T1317 or GW3965) or natural (25-hydroxycholesterol) LXR agonists were used [17].

Despite the usage of different signal transduction modules, a common feature in the macrophage response to these inflammatory mediators is the induction of CCAAT/enhancer-binding protein beta (C/EBPβ), a transcription factor that acts as a master regulator of gene expression in monocytes/macrophages [30]. Members of the C/EBP family are characterized by a highly-conserved basic leucine zipper domain by which they dimerize and bind to palindromic α-helical recognition sequences in the DNA, and an effector domain that mediates transactivation or repression [31]. After protein synthesis, C/EBPβ rapidly translocates to the nucleus to regulate transcription by binding to DNA either as a homodimer or as a heterodimer with other transcription factors [32].

C/EBPβ is also considered a lineage-determining transcription factor (LDTF) [33,34], important for priming cis-regulatory elements required for macrophage identity [35]. Accumulated evidence supports a model in which combinations of LDTFs collaborate on selected enhancers providing cell type-specific sites of open chromatin that facilitate the binding of signal-dependent transcription factors [36]. This model was initially exemplified by the requirement of the LDTF PU.1 for the induction of LXR- and toll-like receptor (TLR)4-dependent gene expression in macrophages [35]. Based on the association of high C/EBPβ expression with the pro-inflammatory response of macrophages [37,38], in this work we have explored the relevance of this transcription factor in the crosstalk between inflammatory signals and the LXR transcriptional program in macrophages. Activation of macrophages with inflammatory mediators resulted in changes in the expression of LXR and RXR subtypes as well as in the transcriptional response to an LXR agonist. Whereas C/EBPβ was dispensable for most of these changes, the cooperative induction of CD38 by LXRs and inflammatory signaling fully required C/EBPβ expression. The cooperative effect between the LXR pathway and C/EBPβ was further supported by their binding profile in the proximity of the gene encoding for mouse *Cd38* and by functional collaboration in gene-reporter studies.

Methods

Reagents. The synthetic and high affinity LXR agonists T1317 and GW3965 were purchased from Cayman Europe and Tocris, respectively. The RXR agonist LG268 was synthetized as described [17,39]. Recombinant murine IFN or TNF were purchased from PeproTech. LPS from E. Coli 0127:B8 was obtained from Sigma-Aldrich.
Animals. C57BL/6 mice were purchased from Envigo and raised as a colony in our animal facility. LXR-deficient mice were initially donated by Dr. David Mangelsdorf (UT Southwestern Medical Center, Dallas, TX, USA) and backcrossed into C57BL/6 background for more than ten generations. Mice with myeloid C/EBPβ deficiency were generated by crossing transgenic mice expressing Cre-recombinase under the lysozyme M (LysM) promoter (B6.129P2-Lyz2tm1(cre)Ifo/J 004781, Jackson Laboratories) with mice carrying a C/EBPβ gene flanked by LoxP sites [40] kindly donated by Dr. Esta Sterneck (National Cancer Institute, Frederick, MD, USA). These mice were in C57BL/6 background and are referred to as LysMCre-C/EBPβ$^{fl/fl}$ hereafter. Control littermates were transgenic for C/EBPβ$^{fl/fl}$ but negative for the Cre-recombinase [38]. CD38-deficient mice correspond to JAX stock #003727 [41] backcrossed in the C57BL/6 background for more than ten generations.
Cells. Bone marrow-derived macrophages (BMDMs) were obtained from six to ten-week-old mice as described [42]. Briefly, bone marrow precursors were differentiated to macrophages during 7 days in DMEM supplemented with 20% heat inactivated fetal bovine serum (FBS) (Sigma-Aldrich) and 30% L929 conditioned media as a source of macrophage-colony stimulating factor (M-CSF).
RNA extraction, cDNA synthesis and quantitative real-time PCR analysis. Total RNA was extracted from cells or tissues using Trizol (Invitrogen) as recommended by the manufacturer. For cDNA synthesis, 1 g of RNA was reverse transcribed with M-MLV Reverse transcriptase RNase H Minus, Point Mutant, oligo(dT)$_{15}$ and PCR nucleotide mix (Promega). Quantitative real time PCR (qPCR) was carried out using the Power SYBR Green Reagent Kit (Applied Biosystems). Specific primers used in this study are shown in Supplemental Table I. The data are presented as mRNA levels relative to ribosomal *L14* expression.
Protein extraction and western blot analysis. The cells were placed on ice, washed with PBS, and lysed in RIPA lysis solution (50 mM Tris-HCl pH 7.4, 1 % Triton-X-100, 0.5 % Na-deoxycholate, 0.1 % SDS, 150 mM NaCl, 2m M EDTA, 50 mM NaF) supplemented with protease inhibitors (1 mM phenylmethylsulfonyl fluoride and a protease inhibitor cocktail (Santa Cruz Biotechnology)). Insoluble material was removed by centrifugation at 14,000 × g for 15 minutes at 4°C. Cell lysates (20 µg) were boiled for 5 minutes at 95°C in Laemmli SDS loading buffer. Proteins were separated by 10% SDS-PAGE and transferred to PVDF membranes (Immobilon-FL). The membranes were blocked in Odissey blocking buffer (Li-Cor) diluted 1:1 with TBS-0.05% Tween 20 (TBS-T) and later incubated with a rabbit polyclonal anti-C/EBP antibody (Santa Cruz Biotechnology, #sc-150) or a goat polyclonal anti-CD38 antibody (Santa Cruz Biotechnology, #sc-7049). Mouse monoclonal anti--tubulin (Life technologies, #13-8000) or rabbit polyclonal anti-β-tubulin (Cell Signaling, #2146) antibodies were used to monitor differences in protein loading. After incubation with primary antibodies, the membranes were washed three times in TBS-T and incubated for 1 h with peroxidase-conjugated secondary antibodies. After three washes with TBS-T, enhanced chemiluminescence detection was performed (GE Healthcare) and the membranes were exposed to X-ray films (Fujifilm) (in the case of CD38 detection) or quantified in an Odyssey Fc Imaging System (Li-Cor) with the Image Studio™ Lite software (Li-Cor) (to study relative C/EBP isoform expression).
ELISA. Supernatants were collected after treating macrophages with LPS (100 ng/ml) for different periods of time. The levels of secreted IL-6 and IL-12 were measured using specific ELISA kits (Preprotech, #900-K50 and #900-K97, respectively) and following the manufacturer’s recommendations.
Analysis of RNA sequencing data. Public RNA-sequencing (RNA-seq) data were obtained from the GEO NCBI database with accession number GSE90046 [38,43] (Supplemental Table II). RNA readings were aligned to the mm10 version of mouse genome with Rsubread [44]. The RNAseq count matrix was analyzed using DESeq2 R package, a method based on the negative binomial distribution with variance and mean linked by local regression [45]. Normalized whole read counts were used for the evaluation of changes in the expression of several genes of

interest in primary microglia from C/EBPβ$^{fl/fl}$ and LysMCre-C/EBPβ$^{fl/fl}$ mice stimulated with LPS or vehicle [38,43]. For data visualization, a heatmap was produced with Heatmapper (Wishart Research Group, University of Alberta, Canada).

Identification of enhancer regions. Public chromatin immunoprecipitation-sequencing (ChIP-seq) datasets were obtained from the GEO NCBI database (Supplemental Table III) as indicated below. ChIP-seq data was obtained from experimental approaches performed in BMDMs for various transcription factors and histone modifications. In more detail, ChIP-seq data for CEBPβ were obtained from accession number GSE109965 [46], using BMDMs from C57BL/6J mice, either left untreated or stimulated with KLA for 1 h. ChIP-seq data for RXR correspond to BMDMs, either control or treated with GW3965 for 1 h, from accession number SRP019970 [47]. ChIP-seq data for acetylated histone H3 on lysine 27 (H3K27ac) carried out in BMDMs, either control or stimulated with LPS for 2, 4, or 24 h, were obtained from accession numbers GSE56123 [48] and GSE38377 [49]. ChIP-seq for LXR was performed using a dual LXR/ antibody [50] in LXR-deficient BMDMs, either left untreated or treated with GW3965 in combination or not with LPS for 24 h. These data are available under accession numbers GSE200922 [51] and GSE275506. All sequencing data were mapped to the mm10 assembly of the mouse genome using Bowtie2. The data were then analyzed using HOMER [35], with each sequencing experiment normalized to 10^7 uniquely mapped tags. Sequencing experiments were visualized with IGV genome browser. Regions of interest with pronounced H3K27ac marks were scanned for LXRE and C/EBP binding motifs using public databases and motif matrices through R [52] and Bioconductor packages, including MotifDb [53] and Universalmotif [54].

Site-directed mutagenesis. We had previously cloned a 613 bp genomic region (using primers $^{5'}$ACCTGCTGGACTGTGTCCTT$^{3'}$ and $^{5'}$CCTTTGAGGGGTCCTTTCTC$^{3'}$) containing the major part of the mouse CD38 enhancer region R2 within a pGL3 promoter-luciferase plasmid (Promega) [17], hereafter named pGL3-R2. A modified version of that plasmid was also available containing four site mutations in an LXRE within R2 [17]. In the current study, specific mutations were also introduced into the C/EBP binding site identified within this region using a Quickchange Site-directed Mutagenesis Kit (Agilent Technologies). All the plasmids were sequenced to confirm correct cloning and mutations. Plasmid DNA was prepared using Maxi-prep columns (Jet Star 2.0 Kit from Genomed).

Reporter activity assays. Raw264.7 macrophages or COS-7 kidney cells were plated in 12-well plates ($2x10^5$ cells/well) and 24 h later the cells were co-transfected with 500 ng of reporter plasmid pGL3-R2, 300ng of either pcDNA3-LXRα or pcDNA3-LXR, 300 ng of pcDNA3-RXRα and/or 300 ng of pMSV-C/EBPβ. Alternatively, variants of pGL3-R2 containing four point-mutations in an LXRE [17] or in a C/EBPb binding site were used in some experiments. Empty vector (pcDNA3 and/or pMSV) was used in control transfections. To control transfection efficiency, all transfections included 100 ng of a renilla luciferase-expressing plasmid (pRL-TK). Transfections were carried out using Superfect Transfection Reagent (Qiagen) following the manufacturer's instructions. Luciferase activity was assessed using the Firefly & Renilla Luciferase Single Tube Assay Kit (Biotium) in an Infinite M200 luminometer (Tecan). Firefly luciferase activity values were normalized to those of renilla luciferase. In each experiment, all experimental conditions were evaluated in duplicates or triplicates.

Chromatin-immunoprecipitation assays (ChIP). Cell fixation and cross-linking were performed in two steps. First, 20×10^6 cells were cross-linked with 2 μM disuccinimidyl glutarate (ThermoFisher Scientific) diluted in PBS for 30 min. Then, the cells were washed with PBS and incubated with 1% formaldehyde (Merck) in PBS for 10 min. Cross-linking was quenched with 200 mM glycine (Merck) for 10 min. Chromatin was extracted with a two-step lysis method. First, the cells were swollen with hypotonic buffer (50 mM Tris-HCl pH 8, 85 mM KCl, 0.5% IGEPAL, and protease inhibitors) and then incubated with lysis buffer (50 mM Tris-HCl, pH 8, 10 mM EDTA, 1% SDS, and protease inhibitors). Chromatin was sonicated with a Sonopuls sonicator (Bandelin), yielding 300-1000 bp fragments. A fraction (10%) of the total volume was kept as input control. Immunoprecipitation was performed with 2 μg of rabbit anti-LXR/ IgG [55], 1:500 dilution of rabbit anti-RXR IgG (Invitrogen, # PA1-815) or 1 μg rabbit anti-C/EBP IgG (Santa Cruz Biotechnology, # sc-150) in 1 ml dilution buffer (10 mM Tris-HCl, pH 8, 2 mM EDTA, 1% Triton X-100, 150 mM NaCl, 5% glycerol, and protease inhibitors) during 15 h at 4°C. Antibody-bound complexes were recovered with protein G Dynabeads (Life Technologies). The complexes were sequentially washed once with buffer I (20 mM Tris-HCl, pH 8, 2 mM EDTA, 1% Triton X-100, 0.1% SDS, 150 mM NaCl), buffer II (20 mM Tris-HCl, pH 8, 2 mM EDTA, 1% Triton X-100, 0.1% SDS, 500 mM NaCl), and buffer III (10 mM Tris-HCl pH 8, 1% sodium deoxycholate, 1 mM EDTA, 1% IGEPAL, 250 mM LiCl), and twice with TE buffer (10 mM Tris-HCl pH 8, 1 mM EDTA). Reverse cross-linking of protein-DNA fragments was performed by incubating the samples in reverse cross-linking buffer (1% SDS, 0.1 M $NaHCO_3$) during 6 h at 65 °C. DNA was recovered with a QIAquick PCR purification kit (Qiagen) following the manufacturer's reccomendations and quantified by qPCR. The primers shown in Supplemental Table IV were used to amplify genomic regions of interest.

Statistical Analysis. GraphPad Prism 6.0 software was used to perform all statistical analyses. The data was analyzed using either one- or two-way ANOVA, or two-tailed Student's *t*-test for data with normal distribution, or the non-parametric Kruskal Wallis-Dunn's test or Mann–Whitney U test for data not following normal distribution. To make different experiments comparable, the data were normalized using the following procedure. The intensity of each experiment (ie) was calculated by determining the mean value of gene expression between the negative and positive controls. The intensities of separate experiments were normalized by the mean intensity value of all the experiments (im) and, for each experiment, the resulting normalization factor (im/ie) was multiplied by the expression levels of all the samples in that experiment.

Results

Inflammatory signals modulate the macrophage response to pharmacological LXR activation

Previous work demonstrated that TLR-3 and -4 ligands, as well as IFN, compromised the capacity of pharmacological LXR agonists to induce key regulators of lipid metabolism [28,29]. To gain further insight into the crosstalk between inflammatory signals and the LXR pathway, we performed time-course analysis of the expression of LXR and RXR subtypes and of a battery of their well-established transcriptional targets in BMDM stimulated with the high affinity LXR agonist T1317 and/or the inflammatory mediators TNF, IFN or LPS (Figure 1A-C and Supplemental Figure 1). Inflammatory signaling resulted in potent and sustained induction of *Lxra* expression, particulary in response to TNF or LPS, whereas *Lxrb* expression was transiently upregulated by IFN and LPS. The effects on *Lxra* are in agreement with recent work demonstrating increased expression of this transcription factor upon prolonged exposure to TLR ligands [51]. In contrast with the effects on LXR subtypes, inflammatory mediators downregulated the expression of the heterodimeric partner *Rxra* without significantly affecting the levels of *Rxrb* (Figures 1A-C). As expected, the LXR agonist did not impact the expression of LXR/RXR subtypes, but instead induced the expression of their transcriptional targets, such as *Abca1*, *Abcg1*, *Srebp1c*, *Cd5l* and others, in a time-dependent manner (Figure 1 and Supplemental Figure 1). Notably, despite potently increasing *Lxra* expression, inflammatory mediators impacted negatively the induction of some LXR targets. The most drastic effects were observed on *Cd5l*, *ApoC1* and *Mertk*, although the induction of *Abca1* was also compromised in the presence of IFN (Figure 1). Other genes, namely *Abcg1* and *Srebp1c*, were not significantly affected by inflammatory mediators (Supplemental Figure 1), which suggests that negative functional crosstalk between inflammatory signals and the LXR pathway is gene-dependent.

Notably, and in sharp contrast with the negative effects described above, inflammatory signals cooperated positively with the LXR pathway on the induction of one particular target, the gene encoding CD38 (Figures 2A-C). This positive crosstalk increased upon prolonged stimulation (Figures 2-D) and translated into strong expression of the CD38 protein (Figure 2E), in line with our previous work [17].

C/EBPβ is a key regulator of macrophage CD38 expression

Considering the evidences supporting the involvement of C/EBPβ in the pro-inflammatory response in macrophages [37,38], we next explored the crosstalk between C/EBPβ and the LXR pathway. First, we evaluated whether pharmacological activation of LXRs impacts C/EBPβ expression in BMDMs. The levels of *Cebpb* mRNA were measured at different time points following macrophage stimulation with TNFα, IFNγ, or LPS, either in the presence or in the absence of the LXR agonist T1317. As a control, the expression pattern of *Cebpa* was also determined. As shown in Figures 3A-C, inflammatory stimuli resulted in opposite effects on *Cebpa* and *Cebpb* expression: whereas inflammatory mediators induced a transient increase in *Cebpb* expression, the levels of *Cebpa* were sharply reduced under such conditions. Notably, LXR activation did not influence the expression pattern of *Cebpb* (or *Cebpa*) in response to inflammatory signaling (Figures 3A-C). The lack of effect of the LXR pathway was further supported by the fact that inflammatory signals induced similar levels of *Cebpb* expression in wild-type (WT) and LXR-deficient macrophages (Figures 3D-F).

We next performed immunoblotting to better understand how inflammatory signaling affects the expression of the C/EBPβ protein in BMDMs (Figures 3G-H). Three isoforms have been described for C/EBPβ, which arise from alternative translation initiation sites [26]. Two of the isoforms, liver-enriched activating protein* (LAP*, Full or C/EBPβ1, ~38KDa) and LAP (LAP or C/EBPβ2, ~34KDa), are transcriptional activators, whereas liver-enriched inhibitory protein (LIP or C/EBPβ3, ~20KDa) lacks the transactivation domain and is a dominant-negative isoform. The main isoform detected in differentiated BMDMs was LAP, and its expression increased progressively in response to TNF⍰, IFN⍰ or LPS (Figures 3G-H). Of note, a concomitant increase in the levels of the inhibitory LIP isoform was also observed upon inflammatory signaling (Figures 3G-H), consistent with a role for this molecule as a negative feedback mechanism to avoid excessive C/EBPβ activity [56].

In view of the strong upregulation of the active C/EBPβ LAP isoform during the macrophage response to different inflammatory mediators, we evaluated whether the activity of this transcription factor is important for the regulatory actions that inflammatory signals exert on the LXR pathway. As a first approach, we analyzed publicly available RNA-seq data comparing the response to LPS in control (C/EBPβ$^{fl/fl}$) and C/EBPβ-deficient (LysM-Cre-C/EBPβ$^{fl/fl}$) brain macrophages [38]. We placed our focus on the effects of LPS on the expression of components of the LXR pathway, namely LXRs and RXRs and the set of transcriptional targets already studied in Figure 1. LPS signaling upregulated the expression of LXR⍰ and ⍰, while repressing the expression of RXR⍰ and, to a lesser extent, RXR⍰ (Figures 4A-B), which highly resembles the scenario observed in BMDMs (Figure 1). In addition, LPS potently downregulated the expression of *Abca1* in brain macrophages (Figures 4A and C). All these changes, however, occurred regardless of C/EBPβ expression. Strikingly, LPS induced a strong increase in the expression of CD38, which was fully dependent on functional C/EBPβ (Figures 4A-B).

C/EBPβ and pharmacological LXR activation cooperate to induce CD38 expression

We next extended the analysis and evaluated the consequences of C/EBPβ-deficiency in cells undergoing prolonged pharmacological activation of the LXR pathway. For this, BMDMs were obtained from C/EBPβ$^{fl/fl}$ and LysM-Cre-C/EBPβ$^{fl/fl}$ mice and then treated with the LXR agonist T1317 for 24 h either alone or in combination with pro-inflammatory mediators (Figure 5). As in cells exposed to shorter time-courses (Figures 1A-C), the LXR agonist did not affect the expression of LXR and RXR subtypes, but it did induce the expression of their transcriptional targets as expected, including *Abca1*, *Abcg1*, *Srebp1c* and others. Consistent with the effects of LPS in brain macrophages (Figure 4), stimulation of BMDMs with LPS or TNF⍰ increased LXR⍰ expression, while reducing the levels of RXR⍰, and these effects were independent of C/EBPβ (Figure 5). In the same line, the inhibitory actions of inflammatory mediators on specific targets of the LXR pathway, namely *ApoC1*, *Mertk* and *Cd5l*, were not reverted in the absence of C/EBPβ (Figure 5). However, the induction of *Cd38* by inflammatory signals either alone or in combination with the LXR agonist was drastically impaired in C/EBPβ-deficient cells (Figure 5). These observations indicate that C/EBPβ is required for the cooperative actions that LXRs and inflammatory signaling selectively exert on CD38 expression. Of note, C/EBPβ deficiency resulted in increased basal expression of *Cd38* in unstimulated BMDMs (Figure 5), but not in microglia (Figure 4), which is suggestive of basal de-repression of this gene in BMDMs lacking functional C/EBPβ.

Accumulated evidence suggests that genomic regions functioning as transcriptional enhancers are enriched in closely spaced binding sites for LDTFs and signal-dependent transcription factors and are primarily responsible for transcriptional responses to external stimuli [36]. By analyzing publicly available ChIP-Seq experiments for H3K27ac occupancy [48,49], a histone modification that marks active enhancers [57], we identified potential transcriptional enhancers in the vicinity of the *Cd38* gene that are activated in BMDMs treated with LPS (Figure 6A, orange tracks), hereafter named regions R1, R2 and R3. Two of these regions (R1 and R2) are located upstream of the *Cd38* gene, whereas region R3 is intronic. Interestingly, several putative binding motifs for LXR-RXR heterodimers (LXREs) and for C/EBP⍰ were identified in close proximity within these three regions (Suplemmental Figure 2).

We next analyzed the binding of LXR⍰ to these enhancers by ChIP-Seq. In these experiments, BMDMs were treated for 24h with an LXR agonist (GW3965) either alone or in combination with LPS, whereas control cells were left untreated. Binding of LXR⍰ increased in regions R1 and R3 in response to the LXR agonist (Figure 6A, green tracks). Interestingly, the combination of the LXR agonist and LPS further enhanced the occupancy by LXR⍰⍰in all three enhancer regions (Figure 6A, green tracks). The binding of its heterodimeric partner RXR to these regions was also evident when we analyzed publicly available ChIP-Seq data from macrophages treated with the LXR agonist for 1 h [47] (Figure 6A, grey track). Altogether, these data suggest that LXR-RXR heterodimers are able to bind to at least three enhancer regions that show responsiveness to LPS in the vicinity of the *Cd38* gene.

Next, we used available ChIP-Seq data on C/EBPβ occupancy [46] to evaluate whether C/EBPβ has the potential to bind to the enhancer regions identified in this study. The tracks analyzed here correspond to BMDMs from C57BL/6 mice either left untreated or stimulated for 1h with Kdo2-Lipid A (KLA), an LPS substructure with endotoxin activity analogous to that of native LPS. The occupancy of R2 by C/EBPβ was rather discrete under the conditions tested in these experiments, however considerable binding of C/EBPβ to regions R1 and R3 was observed under basal conditions and further enhanced upon stimulation with KLA (Figure 6A, red tracks).

Independent validation studies were performed by ChIP in order to further compare, under the same conditions, the binding of the LXR-RXR heterodimer and C/EBPβ to the three regions of interest (Fig 6B). In these studies, WT BMDMs were treated with the LXR agonist T1317 and/or LPS for 24 h. To monitor LXR binding we used a polyclonal antibody recognizing both LXR and , previously validated for ChIP assays [29,51], whereas RXR recruitment was determined with an antibody against the RXR subtype. The binding of C/EBP was evaluated with specific antibodies against this isoform. Interestingly, C/EBP recruitment was observed in the three regions R1, R2 and R3 under the effect of LPS (Figure 6B), in line with the augmented expression of this transcription factor in LPS-treated macrophages (Figure 3). Notably, the levels of occupancy by C/EBP were very high in region R3, in line with the data from ChIP-Seq (Figure 6A), which can be explained by the presence of several C/EBP binding sites in this region (Supplemental Figure 2). The combination of LXR agonist and LPS did not further increase C/EBP recruitment to any of the regions evaluated. In contrast, the combination of signals did result in a significant enrichment of LXRs and RXR on the three enhancer regions as compared to their binding in basal conditions (Figure 6B), thus validating the results observed for LXR in ChIP-Seq (Figure 6A). Of note, LPS treatment alone also promoted LXR/RXR binding to the R2 region. Taken together, these results suggest that combined treatment with the LXR agonist and LPS helps stabilize the LXR/RXR heterodimer in enhancer regions co-occupied by C/EBP upstream or within the *Cd38* gene.
To further evaluate the functional cooperation between C/EBP and the LXR/RXR heterodimer we performed gene reporter studies overexpressing both pathways in the absence of inflammatory signals. In these studies we focused on region R2 for several reasons. First, we had already identified a functional LXRE within this region that was important for the cooperation between LPS signaling and the LXR pathway [17]. Second, region R2 contains only one putative C/EBP binding site, as opposed to the presence of several sites in regions R1 and R3, simplifying the process of introducing inactivating mutations. In a first approach, the consequences of overexpressing C/EBPβ and/or LXR/RXR were evaluated in Raw264.7 macrophages. The cells were co-transfected with the luciferase reporter plasmid pGL3-R2 along with plasmids overexpressing C/EBPβ, LXR, LXR or RXR. Control cells were transfected with an empty plasmid (not encoding these transcription factors). Overexpression of LXR/RXR heterodimers alone resulted in a moderate increase in reporter activity, but a pharmacological agonist (GW3965) was not sufficient to upregulate this response (Figure 7A). On the other hand, overexpression of C/EBPβ alone substantially increased reporter activity, however this activity was further enhanced in cells co-expressing LXR/RXR heterodimers (Figure 7A), indicating that both pathways functionally cooperate with each other to promote reporter activity. Interestingly, pharmacological activation of LXRs further increased the reporter activity only in cells co-overexpressing C/EBPand LXR/RXR. For this reason, subsequent studies were carried out with overexpression of these molecules.
We next evaluated the consequences of mutating specific response elements. Mutations in a *bona fide* LXRE blunted both the response to C/EBPβ and the cooperative effect between C/EBPβ and the LXR pathway (Figure 7B). Likewise, a mutated version of the C/EBP binding site impaired the response to C/EBP when acting alone (Figure 7C) or in combination with activated LXR/RXR (Figure 7D), indicating that synergistic cooperation between C/EBPand LXR/RXR require binding sites for both pathways. Similar results were obtained when LXR (Figures 7B and D) or LXR (Figure 7E) were used in these studies. In contrast with the results obtained in Raw264.7 cells, C/EBPβ overexpression was not sufficient to promote reporter activity in COS-7 kidney cells (Figure 7F), suggesting that lineage-dependent factors are required for the activity of C/EBPβ on the R2 enhancer.
Taken together, these results strongly suggest that C/EBPβ and LXR/RXR bind to several regions with enhancer activity upstream or within the *Cd38* gene. In addition, gene-reporter assays further support the existence of functional synergistic cooperation between these two pathways in macrophages to control *Cd38* transcription.

CD38 activity is involved in the fine-tuning of cytokine production in macrophages
Based on the essential role of C/EBPβ in the regulation of CD38 expression in macrophages, we investigated whether these two molecules are coordinately involved in the macrophage response to LPS. To answer this question we re-analyzed the RNA-Seq data from *Cebpb*$^{fl/fl}$ and LysM-Cre-*Cebpb*$^{fl/fl}$ brain macrophages [38] in search of genes induced by LPS that are highly dependent on C/EBPβ. To this end, validated protein-coding genes were filtered based on two conditions: 1) expression induced by LPS at least 3-fold (log2 fold change > 1.58; adjusted p-value < 0.001) in control *Cebpb*$^{fl/fl}$ macrophages, and 2) expression drastically reduced in LPS-treated LysMCre-*Cebpb*$^{fl/fl}$ macrophages compared to LPS-treated control *Cebpb*$^{fl/fl}$ macrophages (log2 fold change < -3; with an adjusted p-value < 0.001). A list of C/EBPβ-dependent genes is displayed in Figure 8A, ordered by their log2 fold change between LPS-treated C/EBPβ-deficient macrophages and LPS-treated control cells. In addition,

genes encoding for cytokines and other classical mediators of the macrophage response to LPS were included in the analysis (Figure 8B). One of these genes codes for cholesterol 25-hydroxylase (*Ch25h*), which catalyzes the production of 25-hydroxycholesterol (25-HC), a natural endogenous LXR ligand. With the exception of (*Il*)*12b*, the genes analyzed here showed a tendency for downregulation in C/EBPβ-dependent cells (Figure 8B).
We then determined whether C/EBPβ-dependent genes also showed dependency on CD38 expression. For this, BMDMs were obtained from WT and CD38-deficient mice and treated with LPS for different periods of time (Figure 8C). The expression of *Il6* and *Il12b* was significantly reduced in CD38-deficient macrophages (Figure 8C), which translated in reduced secretion of these cytokines (Figures 8D-E). These observations are in line with previous data from our group showing decreased cytokine expression in CD38-deficient macrophages undergoing bacterial infection [17]. However, while these results support a pro-inflammatory role for CD38 in contributing to fine-tuning the production of certain cytokines by macrophages, the majority of the C/EBPβ-dependent genes analyzed here were not affected by the absence of functional CD38 (Figure 8C), suggesting that CD38 is not required for the overall C/EBPβ transcriptional response.

Discussion

CD38 displays receptor and enzymatic activities that facilitate the establishment of an effective immune response during infection [20]. However, recent studies also suggest the involvement of the enzymatic activity of CD38 in immunosuppression, for example within the tumor microenvironment [58]. Several of its activities use large amounts of nicotinamide adenine dinucleotide (NAD) as a substrate, which translates into CD38 being the main NAD-degrading enzyme in mammalian tissues [21]. Therefore, the modulation of the levels and/or activity of CD38 offers therapeutic potential, especially in diseases associated with NAD decline [59].
The study presented here contributes to the understanding of the mechanisms governing the expression of CD38 by uncovering the essential role of C/EBPβ in the transcriptional regulation of the *Cd38* gene. In this sense, the induction of CD38 by inflammatory mediators (TNFα, IFNγ, and LPS), either alone or in combination with pharmacological LXR activation, was severely impaired in C/EBPβ-deficient macrophages. Members of the C/EBP family bind to common elements on DNA and, in some cases, their functions overlap, as it is the case of C/EBPβ and C/EBP on a number of LPS-induced genes [60]. However, our results indicate that, in macrophages, C/EBPβ is indispensable for the induction of CD38 by inflammatory mediators and this role is not compensated by other members of the family. In addition, overexpression of C/EBPβ in Raw 264.7 macrophages (in the absence of inflammatory signals) is sufficient to induce transcriptional activity from an enhancer region containing a C/EBP binding site located upstream of the *Cd38* gene.
Despite reciprocal negative interactions between LXRs and inflammatory signals in different settings [26], the induction of CD38 expression stands out as being cooperatively targeted by both pathways. In this regard another important conclusion from this study is the functional cooperation between C/EBPβ and the LXR pathway in mediating CD38 transcription, which is supported by the finding of several regions with enhancer activity near the *Cd38* gene to which both transcription factors are able to bind. C/EBP family members are known to establish pioneering functions in determining enhancer selection in macrophages [35]. A hierarchical model has been proposed in which LDTFs, including PU.1 and C/EBPα/β in macrophages, collaborate in enhancer selection and priming by cooperatively interacting at genomic regions containing closely spaced binding motifs for each factor. In response to stimuli, signal-dependent transcription factors regulate gene expression in a cell type-specific manner by binding to primed enhancers [36]. Based on the pioneering roles described for C/EBPβ in macrophages [35] we anticipated that C/EBPβ deficiency would impact broadly the macrophage response to pharmacological activators of the LXR pathway. To our surprise, however, except for the case of CD38, the rest of LXR target genes evaluated here (including canonical targets such as *Abca1*, *Abcg1* and *Srebp1c*) were induced by an LXR agonist at similar levels in control and C/EBPβ-deficient macrophages. We cannot discard here that C/EBP might be able to compensate for the lack of C/EBPβ and facilitate LXR-mediated transcription of these genes, especially in the absence of inflammatory signals (when C/EBP is highly expressed). In this sense, differentiation of specific populations of resident peritoneal and lung macrophages is highly sensitive to the absence of functional C/EBPβ, but other tissue macrophages develop normally in C/EBPβ-deficient mice [61], indicating that C/EBP is dispensable at the steady state in many macrophage populations.
C/EBP and C/EBP were indeed oppositely regulated in response to inflammatory mediators, which is in line with previous observations [62], but neither activation nor deletion of LXRs impacted their expression profile. Likewise, basal expression of LXR or RXR isoforms in macrophages was not substantially affected by C/EBP-deficiency, indicating that cooperation between these transcription factors to activate CD38 transcription does

not require reciprocal regulation of their expression levels. The results shown here may also have physiological implications when LXR agonists are produced endogenously. Indeed, several evidences demonstrate accumulation of the natural LXR agonist 25-HC (as a consequence of the increase in CH25H expression) during the inflammatory response (reviewed in [63]). In fact, in our previous work we showed synergistic effects between inflammatory signaling and 25-HC on *Cd38* expression in an LXR-dependent manner [17]. In the gene reporter studies presented in this work, the mere overexpression of LXR/RXR heterodimers (in the absence of pharmacological LXR activation) resulted in moderate induction of the enhancer activity of region R2 (Figure 7A). This activity was further enhanced upon addition of C/EBPβ to the system, which suggests that endogenous agonists of the LXR/RXR pathway might be produced during transfection of Raw264.7 cells.

The strong increase in C/EBPβ expression in response to inflammatory mediators, in particular the active LAP isoform, has been shown to be important for the upregulation of specific subsets of inflammatory genes in activated macrophages [30,64]. In line with this notion, mice with C/EBPβ-deficient myeloid cells showed robust attenuation of the clinical signs associated to experimental autoimmune encephalitis [38]. In this work we analysed RNA-Seq data to identify genes strongly induced by LPS in a C/EBPβ-dependent manner. Some of these genes were also shown to be C/EBPβ-dependent in a previous study [64]. Comparative analysis of LPS-induced gene expression in WT and CD38-deficient macrophages indicated that CD38 activity contributes to the inflammatory response by fine-tuning the production of specific cytokines, namely IL-6 and IL-12. IL-6, but not IL-12, is also a target of C/EBPβ ([64] and Figure 8B). Our data does not support a major role for CD38 in the overall C/EBPβ transcriptional response, but impaired expression of CD38 in mice with C/EBPβ-deficient microglia may influence cytokine production and contribute to the ameliorated inflammatory phenotype of these mice.

In conclusion, the study presented here identifies C/EBPβ as an essential mechanism by which inflammatory signals and the LXR pathway synergistically upregulate the expression of the multifunctional protein CD38 in macrophages. These observations raise potential novel targets that may provide therapeutic opportunities, either alternatively or in combination with anti-CD38 antibodies, for diseases in which CD38 has pathological implications.

Statements

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Statement of Ethics

The study was approved by the Bioethics Comission of the University of Barcelona (Institutional Review Board IRB00003099). All the protocols requiring animal manipulation were approved by the Institutional Animal Care and Use Committees from Parc Científic de Barcelona (approved by the Government of Catalonia (Generalitat of Catalunya) with number 9672) and University of Barcelona (number 222/19), following the standard ethical regulations and meeting quality and experimental requirements of current applicable National (RD 53/2013, article 38) and European legislation (RD 53/2013 Council Directive; 2010/63/UE; Order 214/1997/GC).

Conflict of Interest Statement

E.G. is currently an employee at OneChain Immunotherapeutics. J.M. is currently an employee at Avidity Biosciences. E.N.C. holds a patent on the use of CD38 inhibitors for metabolic diseases that is licensed by Elysium Health. E.N.C. is a consultant for TeneoBio, Calico, Mitobridge and Cytokinetics. E.N.C. is on the advisory board of Eolo Pharma. E.N.C. owns stocks in TeneoBio. Dr. Chini is the head of the external research advisory board for Neolaia Bio. Research in the Chini laboratory has been conducted in compliance with the Mayo Clinic conflict of interest policies. C.C. is a consultant for Aromics. The rest of the authors declare no potential conflicts of interest.

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Author Contributions

Conception and design: EG, AFV; Development of methodology: EG, PRM, JFD, JVD, AC, DJC, JM, AFV; Acquisition of data: EG, JFD, PRM, JVD, AC, AFV; Analysis and interpretation of data: EG, JFD, PRM, JVD, AC, JMVT, JS, CC, AFV; Writing-original draft: EG, AFV; Material support: AC, JS, ENC, CC; Study supervision: AFV.

Data Availability Statement

All data generated or analyzed during this study are included in this article. As part of the study, the authors have analyzed publicly available RNA-Seq and ChIP-Seq datasets. Further inquiries can be directed to the corresponding authors.

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Legends to Figures

Figure 1. Inflammatory signals modulate LXR transcriptional responses. BMDMs were treated with the LXR agonist T1317 (1 µM) (A-C), TNFα (20 ng/ml) (A), IFNγ (5 ng/ml) (B) or LPS (100 ng/ml) (C) or with the combination of both agonist and inflammatory signal for the indicated periods of time. Cells not incubated with the LXR agonist were treated with vehicle (DMSO). The expression of LXR and RXR subtypes and of LXR-RXR target genes was determined by qPCR and normalized by the expression levels of L14. Mean ± SD of n=4 biological replicates obtained through 2 independent experiments. Two-way ANOVA-Tukey. *, p<0.05; **, p<0.01; ***, p<0.001; ****, p<0.0001 *vs* unstimulated cells (time 0). Significant effects of inflammatory signals on the macrophage response to the LXR agonist are also indicated; ##, p<0.01; ###, p<0.001; ####, p<0.0001. For each of the genes analyzed, the time course data for the LXR agonist is identical in panels A-C and is used as reference.

Figure 2. Inflammatory signals and activation of the LXR/RXR pathway cooperatively induce CD38 mRNA and protein expression. A-C) BMDMs were treated with the LXR agonist T1317 (1 µM) (A-C), TNFα (20 ng/ml) (A), IFNγ (5 ng/ml) (B) or LPS (100 ng/ml) (C) or with the combination of both agonist and inflammatory signal for the indicated periods of time. D) BMDMs were treated for 24 h with the same stimuli as in A and C. In A-D, cells not incubated with the LXR agonist were treated with vehicle (DMSO). The expression of *Cd38* was determined by qPCR and normalized by the expression levels of L14. For each of the genes analyzed, the time course data for the LXR agonist is identical in panels A-C. Mean ± SD; n=4 biological replicates obtained through 2 independent experiments (A-C); n=3 independent experiments (D). Two-way ANOVA-Tukey. *, p<0.05; **, p<0.01; ****, p<0.0001 *vs* unstimulated cells (time 0). Additional relevant comparisons are also indicated, #, p<0.05; ##, p<0.01; ####, p<0.0001. E) BMDMs were treated for 24 h with agonists for LXR and RXR (T1317 and LG268, 1 µM each, respectively), TNFα (20 ng/ml), IFNγ (5 ng/ml) or LPS (10 ng/ml) or with simultaneous combinations of LXR/RXR agonists and inflammatory signals. Control cells were treated with vehicle (DMSO). The expression of CD38 was analyzed by Western blotting. As a control of protein loading, the expression of -tubulin was also monitored.

Figure 3. Inflammatory signals induce *Cebpb* expression in an LXR-independent manner. A-C) BMDMs were treated with the LXR agonist T1317 (1 M) (A), TNFα (20 ng/ml) (A), IFNγ (5 ng/ml) (B) or LPS (100 ng/ml) (C) or with simultaneous combinations of the LXR agonist and the inflammatory mediator (A-C) for the indicated periods of time. Control cells were treated with vehicle (DMSO). *Cebpb* and *Cebpa* mRNA levels were determined by qPCR and normalized by the expression levels of L14. Mean ± SD of n=4 biological replicates obtained through 2 independent experiments. Two-way ANOVA-Tukey. ****, p<0.0001 *vs* unstimulated cells (time 0). ns, non-significant differences between time-courses. The results from the LXR agonist time-course are only displayed in the panels in A. D-F) WT or LXRα/β-deficient (LXR/-/-) BMDMs were treated with TNFα (20 ng/ml, 3 h) (D), IFNγ (5 ng/ml, 6 h) (E) or LPS (100 ng/ml, 6 h) (F). Gene expression was measured by qPCR. Mean ± SD of n=3 biological replicates. Two-way ANOVA-Tukey. ****, p<0.0001. G-H) Inflammatory signals strongly increase the expression of C/EBP LAP. BMDMs were treated with TNFα (20 ng/ml), IFNγ (5 ng/ml) or LPS (100 ng/ml) for 6 or 12 h. Control cells were left unstimulated. The expression of C/EBP isoforms and β-tubulin was determined by immunoblotting. G) Representative immunoblot of n=3 independent experiments that showed similar results. H) Relative protein expression for the two C/EBPβ isoforms detected, LAP (34 kDa) and LIP (20 kDa). The relative protein expression of each C/EBPβ isoform was calculated using the Image Studio Lite software and normalized to the expression values of β-tubulin. The graphics display the quantification data of the immunoblot shown in G.

Figure 4. C/EBP is a key regulator of CD38 expression in brain macrophages. Analysis of RNAseq data from primary microglial cell cultures from *Cebpb*$^{fl/fl}$ and LysMCre-*Cebpb*$^{fl/fl}$ mice treated with vehicle (VEH) or LPS (100 ng/mL) for 6 h. A) Heatmap of normalized expression values of LXR and RXR subtypes, and of LXR-RXR target genes. As a control, the expression of *Cebpb* is also included in the analysis. For better visualization of the results, expression values of LXR and RXR subtypes (B) and of selected LXR-RXR targets (C) are also represented as graphics. Two-way ANOVA-Tukey post hoc test. Comparisons between the indicated conditions: *, p<0.05; **, p<0.01 ***, p<0.001. Comparisons *vs* the same treatment in *Cebpb*$^{fl/fl}$ cells: #, p<0.05; ##, p<0.01; ###, p<0.001.

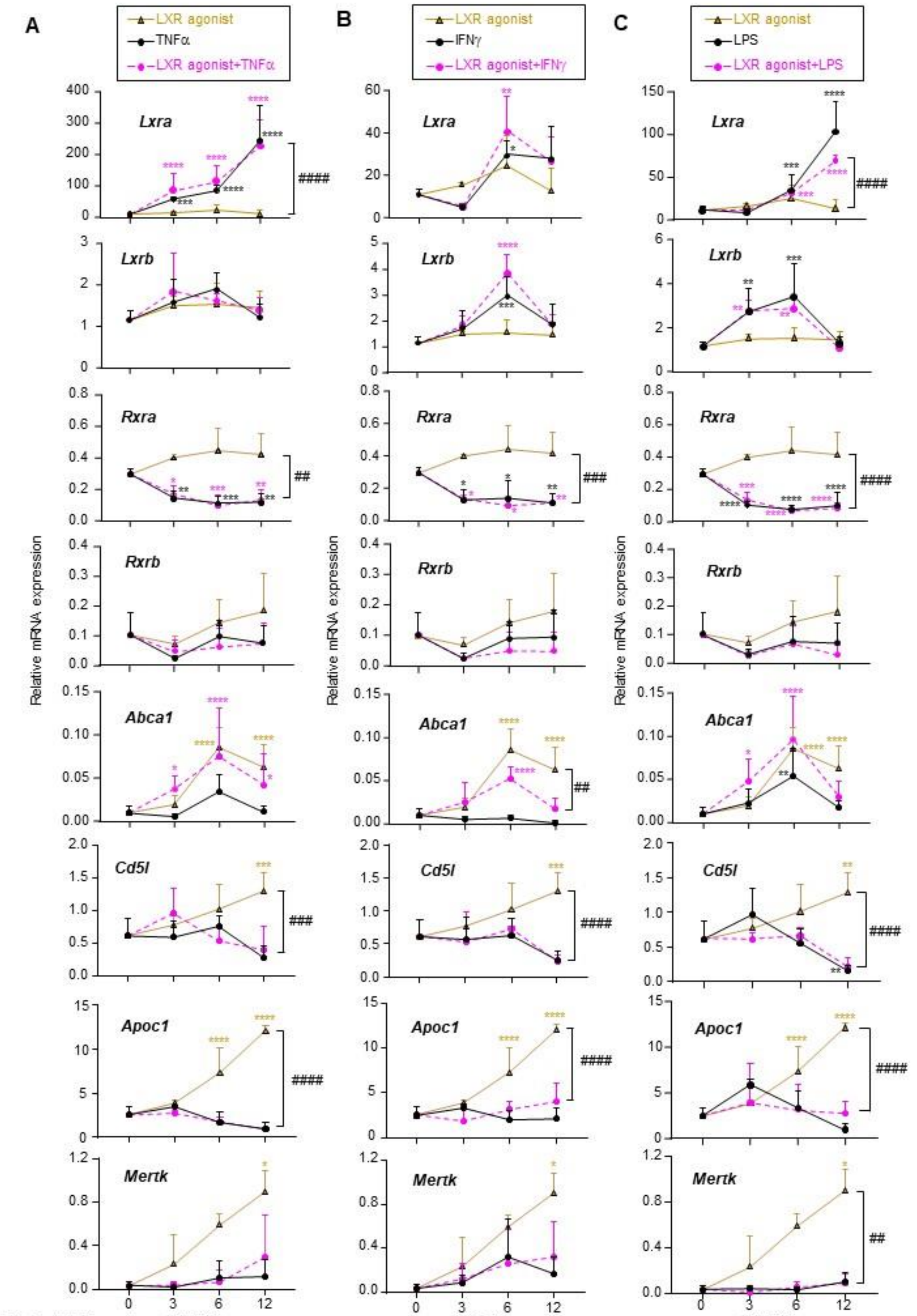
Figure 5. C/EBPβ mediates the cooperative effects between inflammatory signals and LXRs on Cd38 induction. *Cebpb*$^{fl/fl}$ and LysMCre-*Cebpb*$^{fl/fl}$ BMDMs were treated with vehicle (DMSO) or an LXR agonist (T1317, 1 µM) and/or an inflammatory stimulus, TNFα (20 ng/ml), IFNγ (5 ng/ml) or LPS (100 ng/ml) for 24 h. The expression of selected genes was measured by qPCR and normalized by L14 expression. Mean ± standard deviation (SD) of n=4

independent experiments performed with biological duplicates or triplicates. Two-way ANOVA-Tukey (for datasets with normal distribution and homogenous variance); Kruskal-Wallis-Dunn's test (for datasets without normal distribution). Comparisons between the indicated conditions: *, p<0.05; **, p<0.01; ***, p<0.001. Comparisons vs the same treatment in *Cebpb*$^{fl/fl}$ cells: #, p<0.05; ##, p<0.01; ###, p<0.001. For some genes, the effect of the LXR agonist was also compared using a Student's T-test (if normal distribution) or a Mann-Whitney test (otherwise): ^, p<0.05; ^^, p<0.01; ^^^, p<0.001.

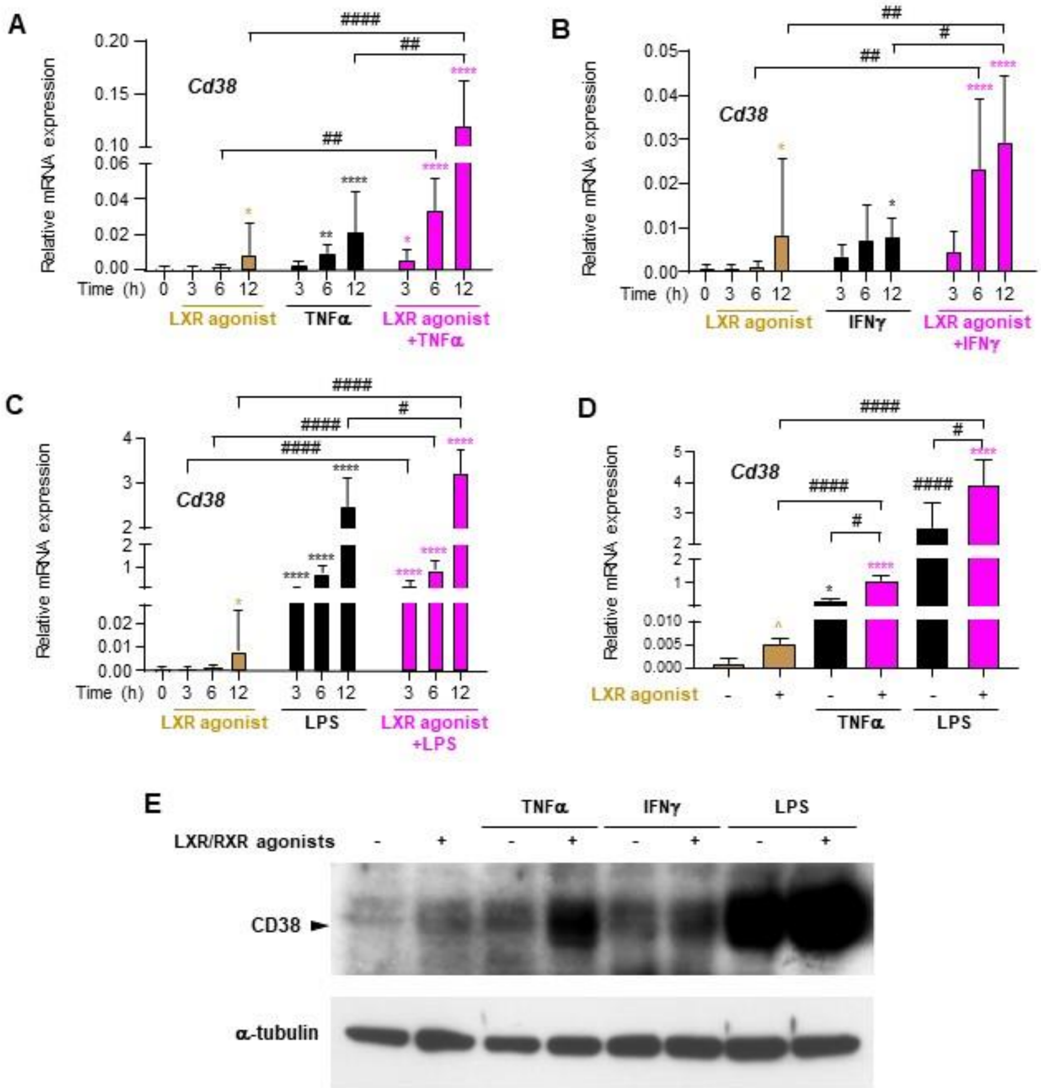
Figure 6. C/EBPβ and LXR co-occupy several regions with enhancer activity upstream and within the *Cd38* gene. A) Analysis of ChIP-seq data to test chromatin occupancy of H3K27ac (orange), LXR⍰⍰(green), RXR (grey) and C/EBPβ (red) in the vicinity of the *Cd38* gene. The occupancy of H3K27ac was analyzed in BMDMs treated with LPS for the indicated times (2 h and 4 h in database GSE56123 and 24h in database GSE38377). LXR⍰ ChIP-seq was carried out using an antibody against LXR⍰/⍰ in LXR⍰-deficient BMDMs stimulated for 24 h with the LXR agonist GW3965 (1 ⍰M) either alone or in combination with LPS (100 ng/ml) (database GSE200922). Binding events of RXR were determined in BMDMs treated with an LXR agonist (GW3965) for 1 h (database SRP019970). The occupancy of CEBPβ was analyzed in BMDMs from C57BL/6 mice stimulated with KLA for 1 h (database GSE109965). Control cells were left untreated in all datasets. Three regions (R1, R2 and R3) with pronounced H3K27ac marks and LXRE and C/EBP⍰ binding motifs are displayed. B) BMDMs were treated with an LXR agonist (T1317, 1 ⍰M), LPS (100 ng/ml) or both stimuli simultaneously during 24 h. Control cells were treated with vehicle (DMSO). The binding of LXRs, RXR⍰ and C/EBP⍰ to enhancer regions R1, R2 and R3 was measured by ChIP assay. One way-Anova; *, p<0.05; **, p<0.01; ***, p<0.001 between the indicated conditions.

Figure 7. C/EBPβ and LXRs functionaly cooperate in a genomic region with enhancer activity. A-F) Luciferase reporter studies using a pGL3 luciferase reporter vector containing the enhancer region R2 (pGL3-R2). Raw264.7 macrophages (A-E) or COS-7 kidney cells (F) were transiently co-transfected with pGL3-R2 containing either a WT sequence (A-F) or a mutated (MUT) LXRE (B, E-F) or C/EBP⍰ binding site (C-E). In addition, some transfections included pMSV-C/EBPβ (for C/EBPβ overexpression) (A-F), and/or a combination of pcDNA3-RXRα and either pcDNA3-LXR⍰ (A, E) or pcDNA3-LXRβ (A-B, D, F) (for LXR/RXR overexpression). In A, control cells were transfected with pGL3-R2 and an empty plasmid (empty pcDNA3 without overexpression of transcription factors). All transfections included pRL-TK (for constitutive Renilla expression) and the total amount of plasmid DNA was equilibrated using empty pcDNA3. The cells were then stimulated with an LXR agonist (GW3965, 1 µM) or vehicle for 24 h. The enhancer activity is represented as luciferase activity normalized by renilla activity. Mean ± SD of biological triplicates (A, C, F) or of n=3 independent experiments each performed with biological duplicates or triplicates (B, D-E). Two way-Anova (A-F); *, p<0.05; **, p<0.01; ***, p<0.001 between the indicated conditions; In addition, #, p<0.05; ##, p<0.01; ###, p<0.001 in comparison with the same condition in control cells not overexpressing transcription factors (empty plasmid) (A) or in comparison with the same condition in cells transfected with the WT pGL3-R2 sequence (B, D-F).

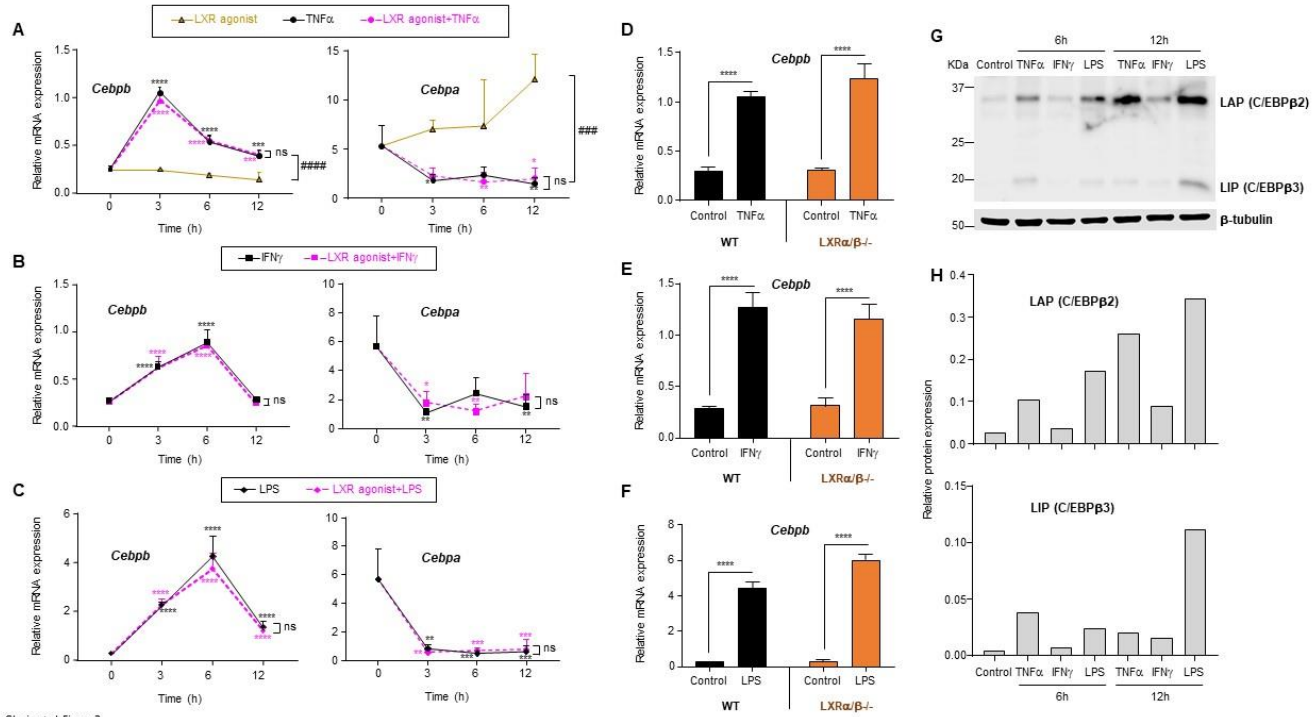
Figure 8. CD38 is dispensable for the induction of C/EBPβ-dependent genes, but contributes to fine-tuning macrophage cytokine production in response to LPS. A) RNAseq data from *Cebpb*$^{fl/fl}$ and LysMCre-*Cebpb*$^{fl/fl}$ brain macrophages treated with vehicle or LPS (100 ng/mL) for 6 h was analyzed to identify LPS-induced genes that are highly dependent on C/EBP⍰ expression. Heatmap displaying a list of filtered genes ordered by the log2 fold change between LPS-treated LysMCre-*Cebpb*$^{fl/fl}$ *vs* LPS-treated *Cebpb*$^{fl/fl}$ macrophages. B) Graphic representing log2 fold changes in the expression of selected cytokines and inflammatory mediators in LPS-treated LysMCre-*Cebpb*$^{fl/fl}$ *vs* LPS-treated *Cebpb*$^{fl/fl}$ macrophages. C-D) BMDMs obtained from WT or CD38-deficient mice were treated with LPS (100 ng/ml) during the indicated periods of time. In C, the expression of selected genes, including C/EBP⍰-dependent genes identified in A was analyzed by qPCR. In D, the levels of secreted IL-6 and IL-12 were measured by ELISA. Mean ± SD; n=3 experiments. Two-way ANOVA-Tukey. *, p<0.05; **, p<0.01; ***, p<0.001; ****, p<0.0001 *vs* unstimulated cells (time 0). Significant changes between WT and CD38-deficient cells are also indicated; #, P<0.05; ##, p<0.01; ###, p<0.001.



Glaria et al. Figure 1



Glaria et al. Figure 2



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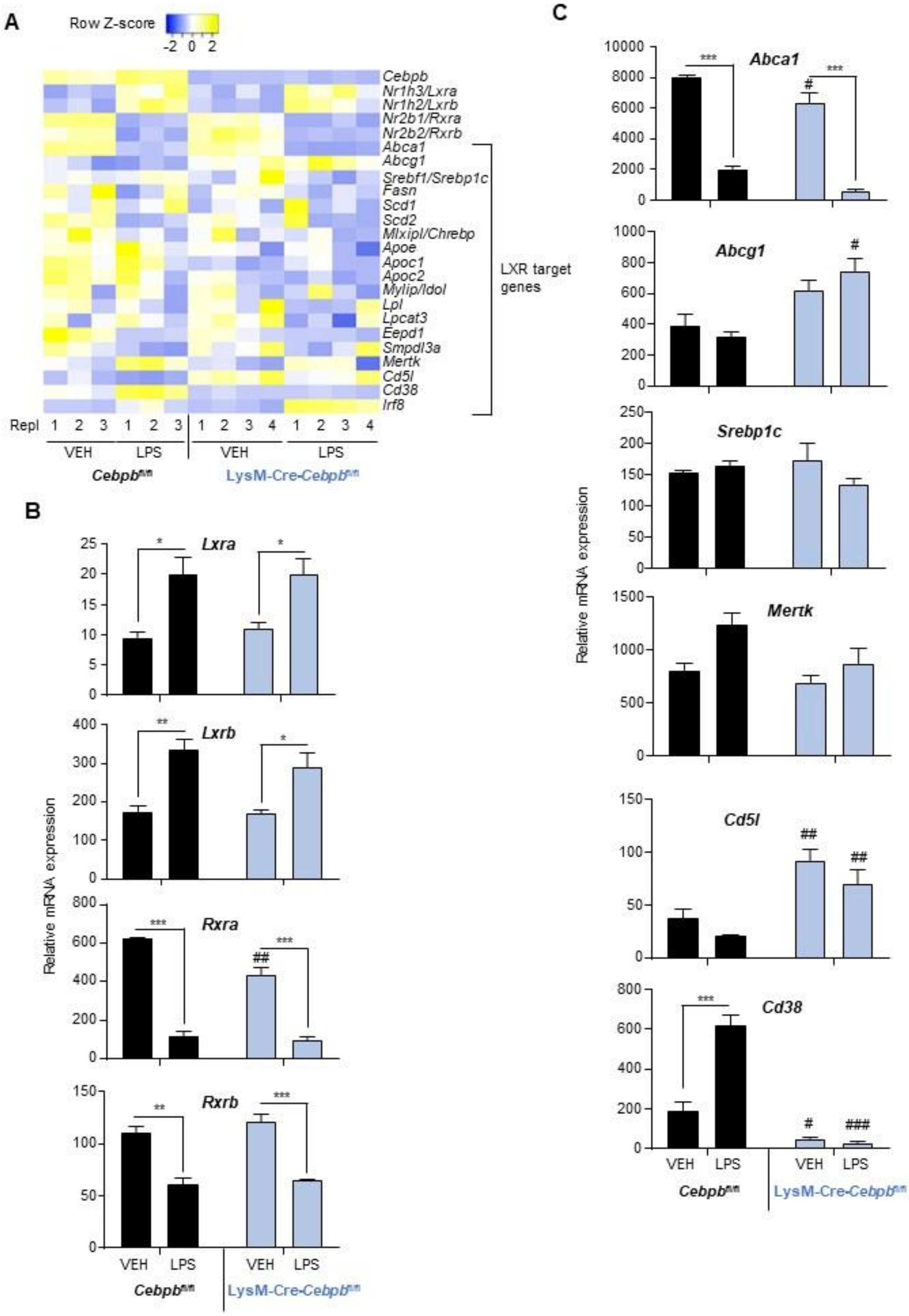

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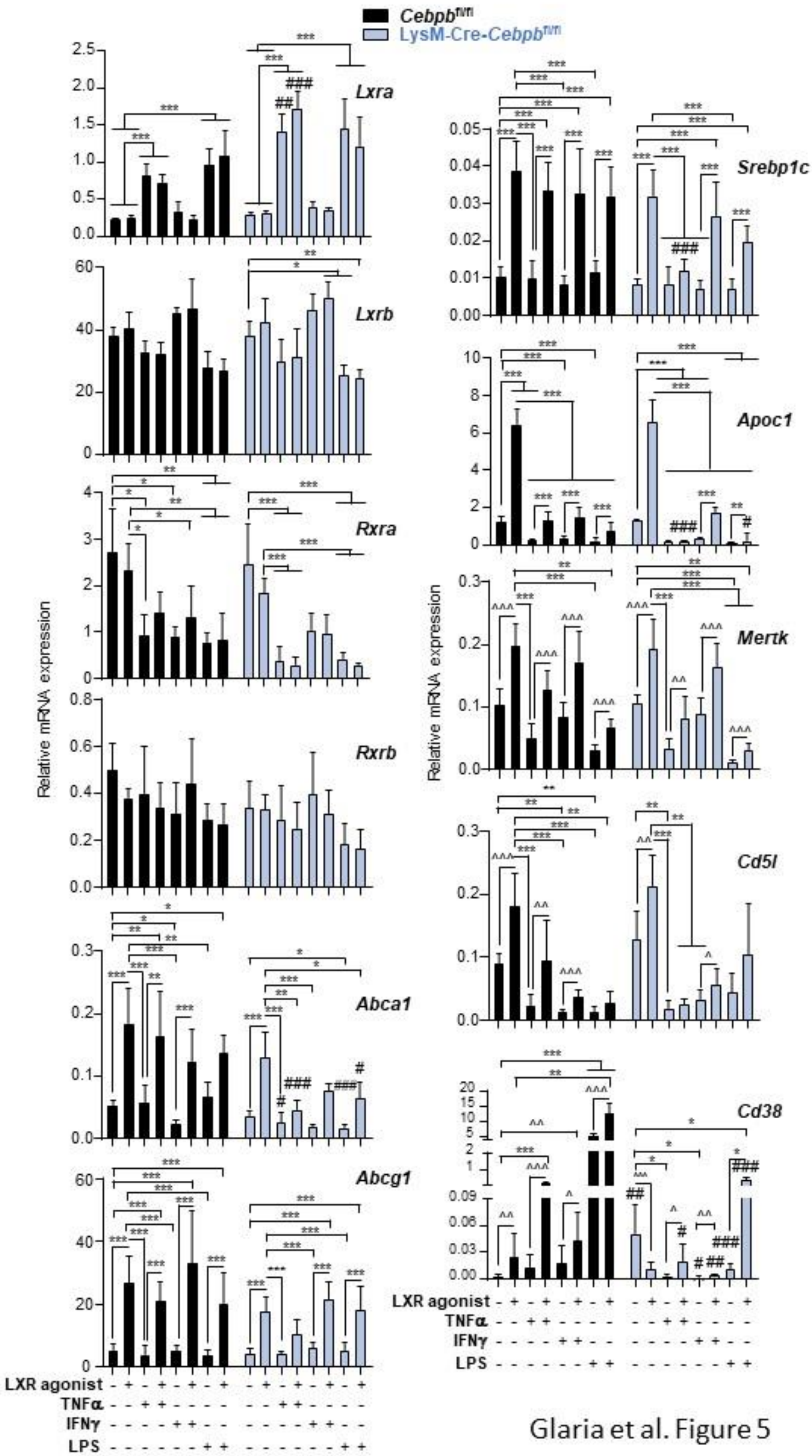

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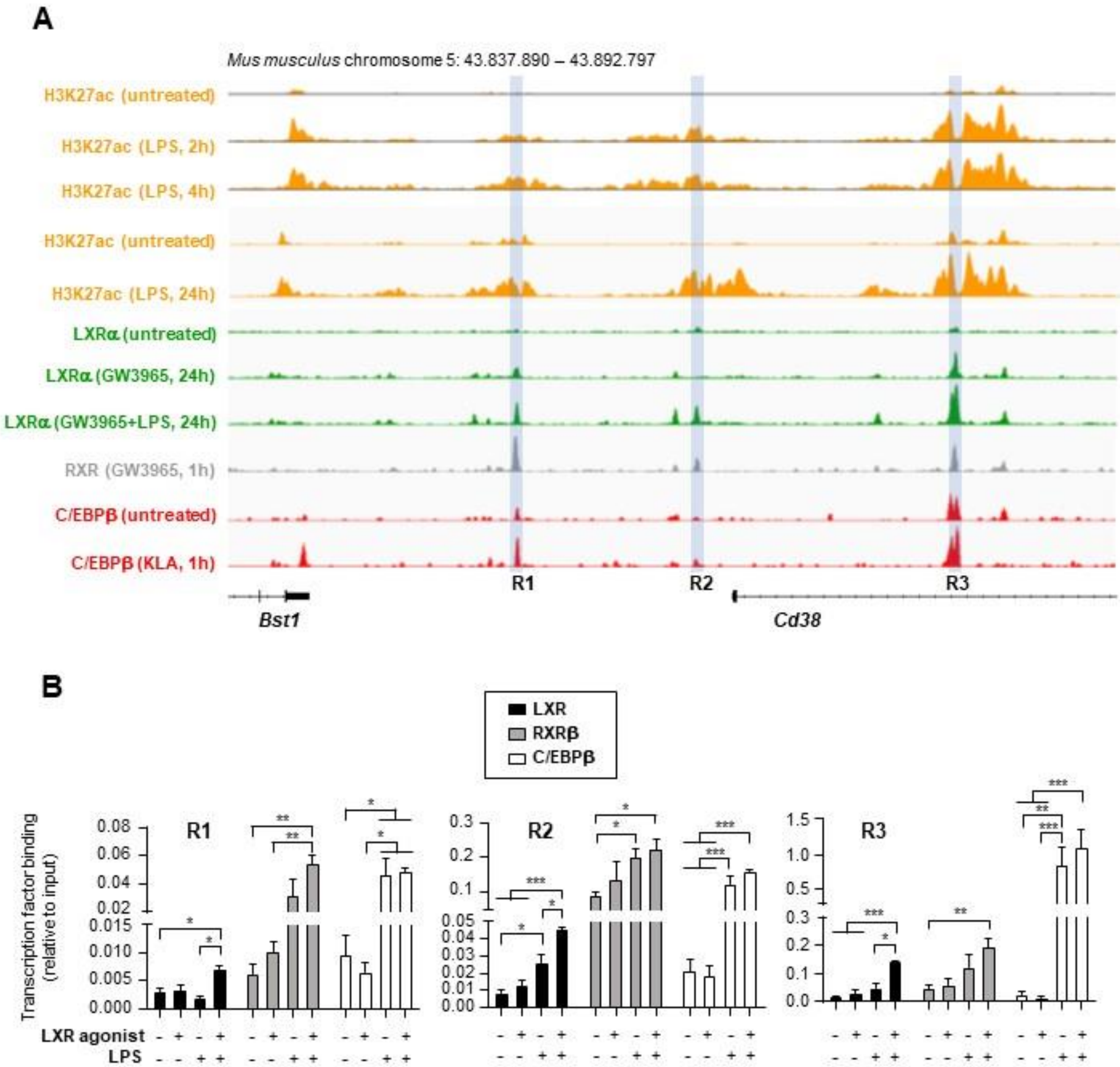

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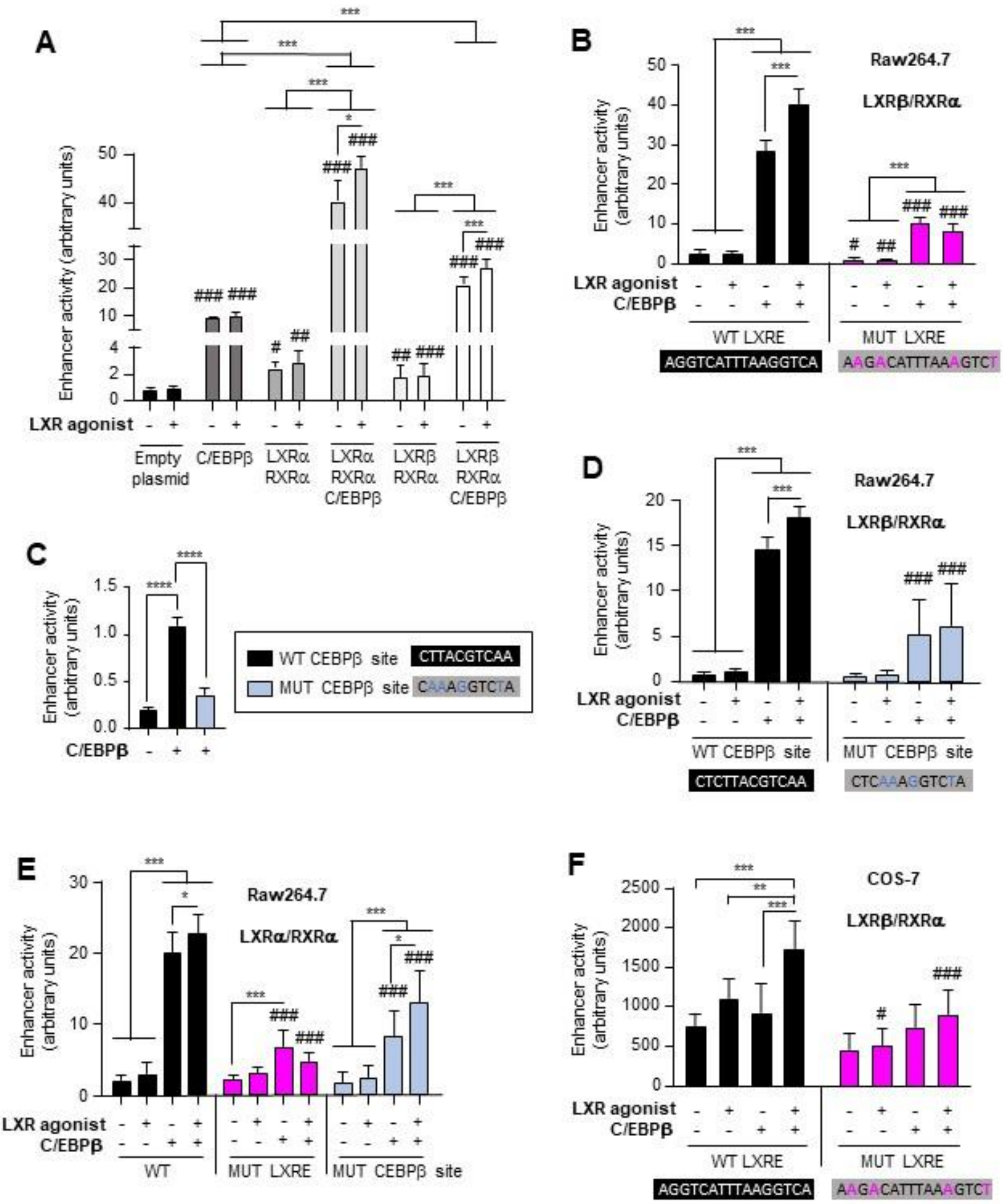

Glaria et al. Figure 7