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C/EBPβ regulates the M2 transcriptome in β-adrenergic-stimulated macrophages

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Abstract

At the M2 terminal of the macrophage activation spectrum, expression of genes is regulated by transcription factors that include STAT6, CREB, and C/EBPβ. Signaling through β-adrenergic receptors drives M2 activation of macrophages, but little is known about the transcription factors involved. In the present study, we found that C/EBPβ regulates the signaling pathway between β-adrenergic stimulation and expression of *Arg1* and several other specific genes in the greater M2 transcriptome. β-adrenergic signaling induced *Cebpb* gene expression relatively early with a peak at 1 hour post-stimulation, followed by peak *Arg1* gene expression at 8 hours. C/EBPβ transcription factor activity was elevated at the enhancer region for *Arg 1* at both 4 and 8 hours after stimulation but not near the more proximal promoter region. Knockdown of *Cebpb* suppressed the β-adrenergic-induced peak in *Cebpb* gene expression as well as subsequent accumulation of C/EBPβ protein in the nucleus, which resulted in suppression of β-adrenergic-induced *Arg1* gene expression. Analysis of genome-wide transcriptional profiles identified 20

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additional M2 genes that followed the same pattern of regulation by β-adrenergic- and C/EBPβ-signaling. Promoter-based bioinformatic analysis confirmed enrichment of binding motifs for C/EBPβ transcription factor across these M2 genes. These findings pinpoint a mechanism that may be targeted to redirect the deleterious influence of β-adrenergic signaling on macrophage involvement in M2-related diseases such as cancer.

Keywords

adrenergic receptor; bioinformatics; functional genomics; macrophage; transcription factor; tumor immunology

1. Introduction

Macrophages can take on many forms over space and time in the course of an inflammatory process [1]. Although the M1-M2 distinction continues to serve as a useful heuristic for the study of macrophage activation in the inflammatory process, macrophage biologists understand that these phenotypes are best understood as extremes on a spectrum constituted by several possible variations [2]. At the M2 extreme, induction of *Arg1* by IL-4 requires transcription by STAT6 [3]. However, further research has shown that STAT6 is assisted by the transcription factor C/EBPβ, with both factors acting at an enhancer region ~3kb upstream of the *Arg1* locus [4]-[5]. More recently, it was shown that C/EBPβ is regulated by cAMP and CREB. Deletion of CREB binding sites from the *Cebpb* promoter down-regulated expression of *Arg1* and other M2-associated genes [6]. Moreover, stimulation of macrophages with cAMP increased C/EBPβ binding to the enhancer region for *Arg1* [7], but it is possible that the effect of cAMP may also be mediated independently of CREB [8].

We previously found that transcription factor binding motifs for C/EBPβ were enriched among up-regulated genes in β-adrenergic-stimulated macrophages using a transcriptome-wide bioinformatic approach [9]. Ligation of β-adrenergic receptors activates the $G_{\alpha s}$ guanine nucleotide-binding protein to stimulate adenylyl cyclase synthesis of cAMP, which signals CREB in a PKA-dependent manner [10]. Our interest in β-adrenergic-stimulated macrophages stems from the finding that β-adrenergic signaling can promote mammary cancer progression, in part, by increasing macrophage accumulation in the primary tumor along with increased expression of *Arg1* [11]. Also, several clinical studies have found reduced cancer progression in patients who are taking β-adrenergic antagonists (reviewed in [12]-[13]), although it is not clear from such studies that this protective effect is mediated through alterations in tumor-associated macrophages (TAMs). Nonetheless, these findings have spurred early phase clinical trials that seek to determine the utility of beta-blockers as an adjuvant treatment in cancer patients that can help prevent recurrence of malignancy [14]-[15]. Although *Arg1* expression can also be upregulated in M1 macrophages [16], TAMs in several cancer types often exhibit M2-like properties [17]. There can however be substantial heterogeneity in phenotype, even within the same tumor [18]. Similarly, although β-adrenergic-stimulated macrophages did not fit cleanly into any one of the pre-defined categories along the M1-M2 spectrum in our previous research, the set of up-regulated genes in β-adrenergic-stimulated macrophages that exhibited enrichment of binding motifs for C/

EBPβ were found to locate on the M2 side of the M1-M2 spectrum. However, it remains to be determined whether those genes were specifically or causally regulated by C/EBPβ. The present study addressed that issue by defining the extent to which C/EBPβ regulates the signaling pathway between β-adrenergic stimulation and specific genes in the greater M2 transcriptome.

2. Methods

2.1. Bone marrow-derived macrophage (BMDM) and RAW 264.7 cell culture models

Mice were handled under protocols approved by the Institutional Animal Use and Care Committee of the University of California, Los Angeles. Flushed bone marrow from female Balb/c mice (Charles River, 8–10 weeks) in RPMI-1640 with L-glutamine (Cellgro-Corning, Inc., #10–040-CV) was passed through a 30-μm cell strainer (Miltenyi, #130–041-407) and subjected to red blood cell lysis buffer (BD Biosciences, #555899). White blood cells were counted by hemocytometry and seeded at 1×10^6 cells per well in 1mL in 24-well polystyrene low attachment plates (Costar, #3473) for gene expression kinetics experiments or at 2.5×10^6 cells per well in 3mL in 6-well plates (Costar, #3471) for chromatin immunoprecipitation experiments. Bone marrow cells were cultured in RPMI-1640 with L-glutamine supplemented with 10% FBS (Atlanta Biologicals, #S11550H), 100 IU penicillin/mL, 100 μg streptomycin/mL (Cellgro-Corning, #30–002-CI), and 20 ng/mL of recombinant mouse M-CSF (Biolegend, #576402) in a humidified incubator at 37 °C, 5% CO_2, for 7 days (media replenished after 2 and 5 days) before initiating β-adrenergic stimulation with the non-selective β-adrenergic agonist, isoproterenol (Sigma, #I2760) and/or the β-adrenergic antagonist, *s*-propranolol (Sigma, #P8688), dissolved in cell culture media as vehicle.

The RAW 264.7 macrophage-like cell line, which was derived the Balb/c mouse congenic substrain, BAB/14, was acquired from the European Collection of Authenticated Cell Cultures (ECACC) through its U.S. distributor (Millipore-Sigma, #91062702–1VL) and cultured in DMEM with high glucose, Glutamax, and pyruvate (Gibco, #10569044), with 10% FBS for 2 days before being seeded at 0.25×10^6 cells per well in 2mL in 6-well plates (Corning, #353046). Following 18–24 hours incubation after seeding, cells were transfected with *Cebpb* siRNA (Thermo Fisher Silencer Select, #s63860) or negative control siRNA (#4390843) at a concentration of 25nM, using Lipofectamine RNAiMAX transfection reagent (#13778075). Following 24 hours of transfection, cells were then stimulated with isoproterenol. We conducted extensive experimentation with BMDMs to try to knock down *Cebpb* but found that these primary cells were not amenable to efficient transfection with siRNA. As such, we focused our siRNA studies on RAW 264.7 cells, which were amenable to transfection with siRNA.

2.2. RT-qPCR

To quantify gene expression from BMDMs and RAW 264.7 cells, total RNA from RLT lysates was extracted (Qiagen RNeasy Mini Kit, #74104), cleared of contaminating DNA with on-column DNase digestion (Qiagen RNase-Free DNase Set, #79254), and quantified by spectrophotometry (NanoDrop ND-1000, Thermo Scientific). We did not include a

control for the efficiency of on-column DNase digestion. Gene transcripts were examined by RT-qPCR with the CFX96 Touch Real-Time PCR Detection System (Bio-Rad), using one-step assay reagents (Qiagen Quantitect Probe RT-PCR, #204443) and TaqMan Gene Expression Assay primer-probes for mouse *Cebpb* and *Arg1* (Thermo Fisher, Mm00843434_s1 and Mm00475988_m1, respectively). Following reverse transcription of RNA template for 30 minutes at 50°C, resulting product underwent an initial activation step at 94°C for 15 minutes followed by 50 amplification cycles of 15 seconds of strand separation at 94°C and 60 seconds of annealing and extension at 60°C. Triplicate determinations of each biological replicate were quantified by threshold cycle analysis of FAM fluorescence intensity using CFX Manager software (Bio-Rad), normalized to values of beta-actin mRNA amplified in parallel (*Actb*, #Mm00607939_s1).

2.3. Chromatin immunoprecipitation (ChIP) and qPCR

ChIP was performed on BMDMs using reagents from a commercially available kit (Active Motif, #53042). Isoproterenol-stimulated cells (or control cells) were removed from culture, fixed in 1% formaldehyde, and washed. Fixed cells were lysed via 1 flash freeze-thaw cycle followed by incubation with lysis buffer on ice and vortexing at highest speed with detergent. Chromatin was then sheared by immersion of a sonicator probe set to the highest allowable amplitude without inducing foam (Fisher Scientific Model 100 Sonic Dismembrator) to produce fragments ranging between 200 and 1200 bp in size. Input DNA was prepared from reverse cross-linked chromatin through phenol-chloroform extraction and analyzed for quality and quantity through spectrophotometry (Nanodrop). ChIP reactions began with overnight incubation of 20–35 μg of chromatin and 10 μg of antibody per reaction (Abcam, Anti-CEBP Beta antibody [1H7]-ChIP Grade, #AB15050). Antibody-bound protein/DNA complexes were then captured with Protein G agarose beads, and chromatin was eluted, reverse cross-linked, and DNA purified. DNA near the *Arg1* locus was quantified with the CFX96 Touch Real-Time PCR Detection System (Bio-Rad), using Taqman Genotyping Master Mix (Thermo Fisher, #4371355) and TaqMan Copy Number primer-probes for mouse *Arg1* and the enhancer region ~3kb upstream (NCBI GeneBank #AY074883.1) of the transcription start site for *Arg1* (Thermo Fisher, Mm00735717_cn and custom assay ID ayforrev_cdkaj3j9, respectively; custom assay forward 5'-CAAGAAATACAGTTTACACATATGTCTGCAT-3', reverse 5'-CCTTCTGCTCTCTGACTTCCTTATT, probe 5'-CTCGTCGGCTACCACCCTC-3'). Initial activation was at 95°C for 10 minutes, followed by 50 amplification cycles of 15 seconds of strand separation at 95°C and 60 seconds of annealing and extension at 60°C. Triplicate determinations of each biological replicate were quantified by threshold cycle analysis of FAM fluorescence intensity using CFX Manager software (Bio-Rad). ChIP-ed DNA was normalized to the amount of input DNA assayed in parallel. Positive and negative control antibodies and primer sets were utilized to test for defects in ChIP assay performance (Active Motif, #53027) and indicated that our chromatin preparation and immunoprecipitation procedures worked correctly (Supplementary Figure 1).

2.4. Flow cytometry

Flow cytometry was used to test for differences in expression of arginase I protein by BMDMs stimulated with isoproterenol. Following 8 and 12 hours of stimulation, cells were

collected, treated with blocking solution (Innovex, #NB309), and then washed with cold FACS buffer (PBS, 2% FBS) before being fixed and permeabilized with an intracellular staining kit (Thermo Fisher, #88–8824-00). Cells were then incubated with fluorescence-conjugated monoclonal antibodies against arginase I, clone A1exF5 (Thermo Fisher, #17–3697-82) and washed. Cytometry was performed with a Becton Dickinson LSRFortessa X-20 SORP flow cytometer, using a 40mW solid state 640nm red laser to excite the antibody's APC fluorophore. Forward and side scatter dot plot was used to identify intact cells versus debris. Side scatter and pulse width were used to exclude doublets from single cell measurement. Unlabeled cells were used to select the photomultiplier tube voltages required to detect baseline autofluorescence, and unlabelled cells from each experimental condition (control vs. isoproterenol) were measured. Relative quantification of arginase I protein for each sample was then accomplished by calculating the mean fluorescence intensity of a given sample's fluorophore-positive cells, which were identified from orthogonal gating to that of unlabeled autofluorescent cells (no difference found in autofluorescence between conditions). Isotype control antibody-labeled cells (Thermo Fisher, #17–4321-81) from each experimental condition were also measured to test for difference in mean fluorescence intensity that was due to unspecific binding.

2.5. Liquid chromatography and tandem mass spectrometry (LC-MS/MS)

Liquid chromatography was used with tandem mass spectrometry to quantify arginase activity-dependent urea levels in BMDM cell culture media. Urea (Fisher Scientific, #BP169) was used to make calibrator standards in duplicate at concentrations of 1000, 500, 100, 50, 10 and 0 pg/uL. The urea chemical analogue, glycinamide (Sigma, #G6104), was used as an internal standard for all calibrators and samples. Samples were prepared in duplicate from cell culture media supernatants following centrifugation of cell culture solutions. Blank samples from unused cell culture media were also prepared. For each replicate of a calibration standard or sample, 100 μL was added to a microcentrifuge tube followed by 10 μL of internal standard at 10 mM and vortexed briefly. Protein was then precipitated by adding 400 μL of acetonitrile/water/formic acid (90/10/0.1, v/v/v) and vortexing before centrifugation at 16,000 × *g* for 5 minutes to pellet the precipitate. Supernatant (100 μL) was transferred to liquid chromatography (LC) injector vials. Aliquots of the replicate solutions (10 uL) were injected onto a HILIC column (Waters Acuity UPLC® BEH HILIC, 150 × 3 mm, 1.7 um particle size) equilibrated with 10% eluant A (0.1% formic acid in water) and 90% eluant B (0.1% formic acid in acetonitrile) and eluted (300 uL/min) with increasing concentration of eluant B (min/% B; 0/90, 4/90, 21/10, 24/10, 25/90, and 35/90). The effluent from the column was directed to an Agilent Jet Stream electrospray ionization (ESI) source connected to a triple quadrupole mass spectrometer (Agilent 6460) operating in the positive ion tandem mass spectrometric multiple reaction-monitoring (MRM) mode in which the intensity of the urea (m/z 61→44, retention time 4.1 min) and glycinamide (m/z 75→30, retention time 5.0 min) parent to fragment transitions were recorded using previously optimized instrument settings (fragmentor 50 and 35, collision energy 13 and 9, for urea and glycinamide, respectively). The amount of urea in each sample was then calculated by interpolation from the curves constructed using the data from the calibrator standards.

2.6. Immunofluorescent staining

For analysis of cellular C/EBPβ protein and transcription factor localization in nuclei, RAW 264.7 cells were cultured on glass cover slips in 6-well plates before fixation with 1% formaldehyde and permeabilization with 0.25% Triton X-100. Cells were treated with blocking solution (Innovex, #NB309) before overnight incubation at 4°C with antibodies against C/EBPβ (Abcam, Anti-CEBP Beta antibody [1H7]-ChIP Grade, #AB15050) at ¼00 dilution, followed by incubation with fluorescent Alexa 594-conjugated secondary antibody F(ab')2 fragments (Thermo Fisher, #A-11020) at 1/500 dilution and DAPI nuclear counter stain solution (Molecular Probes #R37606). Coverslips were then mounted onto glass slides using antifade fluorescent mounting media (Agilent, #S302380–2). Fluorescence was visualized with a Zeiss inverted Axio Observer Z1 microscope and fluorescence filters, using a 20× Plan Apo objective with NA = 0.8. Digital images were acquired with the system's AxioCam MRm monochromatic camera and Axiovision software (version 4.8.2.0), with gain and exposure time held constant across conditions of a given experiment. To quantify the relative amount of cellular C/EBPβ protein, 5 random images from each biological replicate were processed as 8-bit tagged image format files to accomplish in situ assay via pixel threshold analysis with ImageJ software [19]-[20]. To quantify relative amount of C/EBPβ localization in nuclei, JACoP plugin [21] was utilized, which calculates Mander's Colocalization Coefficients [22], indicating the percentage of thresholded pixels in the blue channel that are occupied by corresponding thresholded pixels in the red channel.

2.7. Whole transcriptome analysis

Total RNA (2.5 μg per biological replicate) was converted to cDNA (Lexogen QuantSeq 3' FWD) and sequenced using an Illumina HiSeq 4000 instrument in the UCLA Neuroscience Genomics Core Laboratory. Low-level sequencing data was mapped to the RefSeq mouse genome sequence and normalized to counts per million mapped reads (CPM) using the Illumina BaseSpace cloud computing platform. Gene expression data from the present study are deposited in the National Center for Biotechnology Information Gene Expression Omnibus (GEO; accession No. GSE119707). Empirically-defined transcriptome-wide M2 genes from our previous study (9) were used as a reference set to identify M2 genes in the current study that exhibited up-regulation by β-adrenergic-signaling with inhibition by *Cebpb* knockdown. The reference set was defined as genes exhibiting an M2 diagnosticity score > 3 (i.e., > 3 standard deviations above the mean score, indicating greater expression of that gene by IL-4-stimulated M2 macrophages relative to IFN-γ-stimulated M1 macrophages; see reference (9) for full details). The orthogonal pattern of regulation was examined for M1 genes, using empiricallydefined M1 genes (i.e., diagnosticity score < −3) as a reference set from the same previous study. Genes constituting these M2 and M1 reference sets, with their corresponding diagnosticity scores, are listed in Dataset S1. Differential regulation was defined by ≥ 20% difference in mean log2-transformed CPM values in RAW 264.7 cells treated with isoproterenol vs. controls. Genes differentially expressed by isoproterenol were identified based on biological effect size (i.e., difference = mean isoproterenol – mean control) rather than statistical effect size (e.g., t statistic or P value), because previous research has shown that biological effect size-based criteria yield more replicable results than do statistical effect size criteria (e.g., t statistics, P values, or false discovery rate q values) [23]-[24]. To test for a significant difference in the prevalence

of transcription factor binding motifs (TFBMs) for C/EBPβ in the promoters of genes that exhibited up-regulation or down-regulation by β-adrenergic-signaling, with inhibition or rescue by *Cebpb* knockdown, respectively, we conducted differential expression analysis with the Transcription Element Listening System (TELiS; www.telis.ucla.edu) as previously described [25]. The V$CEBPB_02 binding motif definition for C/EBPβ was retrieved from the TRANSFAC database. Analyses averaged results derived from nine parametric variations of promoter length (300 bp relative to RefSeq transcription start site, 600 bp, and 1000 bp to + 200) and target TFBM match stringency (MatSim = 0.80, 0.90, 0.95).

2.8. Statistical analysis

Data are reported as mean ± standard error of the mean. Analyses were conducted in the R environment, version 3.1.3 [26]. For Figure 1, Student's t-test was used to analyze the effect of β-adrenergic stimulation on gene expression results at each time point in the context of a repeated measures univariate analysis of variance; factorial analysis of variance was used to examine the effects of isoproterenol and propranolol on gene expression results, with Tukey's adjustment for multiple comparisons. For Figure 2, ChIP results, arginase I protein expression results, and urea results were analyzed with Student's t-test. For Figure 3, factorial analysis of variance was used to examine the effects of β-adrenergic signaling and C/EBPβ inhibition on gene expression results and protein colocalization, with Tukey's adjustment for multiple comparisons. For Figure 4, the mean value of all possible V$CEBPB_02 binding motif prevalence ratios was tested for significant deviation from a null population mean ratio of 1 with single-sample t-test for differentially-regulated genes vs. the reference genome in TELiS.

3. Results

3.1. Kinetic profile of β-adrenergic-induced Cebpb and Arg1 gene expression

To investigate the connection between C/EBPβ and M2 gene expression in macrophages following β-adrenergic stimulation, we first determined the kinetics of isoproterenol-induced gene expression for C/EBPβ (*Cebpb*) and Arginase 1 (*Arg1*) in bone marrow-derived macrophages (BMDMs). *Cebpb* expression peaked at 1 hour (2.9-fold ± 0.2; $P < 0.001$) and remained significantly elevated above the control condition at 2 hours (2.0-fold ± 0.2; $P < 0.001$) and 4 hours (2.8-fold ± 0.2; $P = 0.006$) post stimulation (Figure 1A). In contrast, *Arg1* expression did not peak until the 8-hour time point (6.3-fold ± 0.9; $P < 0.001$), although significant elevation above controls appeared as early as 2 hours post stimulation and remained elevated through 24 hours (P values < 0.05; Figure 1B). Blockade of β-adrenergic receptors with propranolol significantly abrogated peak *Cebpb* expression at 1 hour (mean suppression: 97% ± 2.5%; $P < 0.001$; Figure 1C) and peak *Arg1* expression at 8 hours (mean suppression: 97% ± 1.9%; $P < 0.001$; Figure 1D). This temporal precedence of peak *Cebpb* expression before peak *Arg1* expression suggested that β-adrenergic signaling might shift the macrophage transcriptome to the M2 side of the M1-M2 spectrum through its more immediate up-regulation of C/EBPβ. To further confirm that possibility, we conducted additional studies as follows.

3.2. C/EBPβ protein binding near the Arg1 locus after β-adrenergic stimulation

To determine whether β-adrenergic-induced *Cebpb* expression gives rise to greater level of C/EBPβ protein binding and transcription factor activity near the *Arg1* locus at the later time points associated with peak expression, we conducted chromatin immunoprecipitation experiments at 4 and 8 hours after isoproterenol stimulation. Using real-time quantitative PCR of purified DNA from C/EBPβ-bound chromatin, we found that the *Arg1* enhancer region was significantly more enriched in isoproterenol-treated cells vs. control cells at both 4 hours (2.6-fold ± 0.4; $P = 0.009$; Figure 2A) and 8 hours (2.3-fold ± 0.7; $P = 0.031$; Figure 2B). Subsequently, isoproterenol-treated cells exhibited significantly greater arginase I protein levels (8% ± 0.2%; $P = 0.024$; Figure 2C; Supplementary Figure 2) and marginally greater enzyme activity (12% ± 4%; $P = 0.07$; Figure 2D). In contrast, *Arg1* DNA proper in C/EBPβ-bound chromatin exhibited no reliable increase at either 4 hours ($P = 0.13$) or 8 hours ($P = 0.58$) (Supplementary Figure 3). These results strongly suggested that β-adrenergic-induced C/EBPβ transcription factor activity up-regulates *Arg1* expression and does so by binding the distal enhancer of *Arg1* rather than its more proximal promoter area.

3.3. Effects of C/EBPβ inhibition on β-adrenergic-induced transcription factor localization in the nucleus

To further define β-adrenergic-induced C/EBPβ regulation of the M2 transcriptome, we conducted RNA interference experiments with RAW 264.7 cells. Twenty-four hours after transfection with *Cebpb* siRNA, cells were stimulated with isoproterenol for 1 hour. Similar to BMDMs, β-adrenergic signaling significantly increased *Cebpb* expression in RAW 264.7 cells (2.2-fold ± 0.2; $P < 0.001$; Figure 3A). Knockdown of *Cebpb* with siRNA (mean knockdown: 85%; Figure 3A) significantly inhibited β-adrenergic induction (mean suppression: 77% ± 2%; $P < 0.001$; Figure 3A). To determine the degree to which these results translated into suppression of C/EBPβ protein level and transcription factor localization in the nucleus, we used immunostaining to visualize C/EBPβ protein at 4 hours post stimulation. β-adrenergic signaling significantly increased C/EBPβ protein staining in the nucleus (1.9-fold ± 0.3; $P = 0.006$; Figure 3B,3D). *Cebpb* knockdown translated into knockdown of C/EBPβ protein (mean: 70%; Supplementary Figure 4) and significantly inhibited the β-adrenergic effect on C/EBPβ protein staining in the nucleus (mean suppression: 94% ± 22%; $P < 0.001$) and, in turn, suppressed β-adrenergic-induction of *Arg1* expression at 8 hours post stimulation (mean suppression: 76% ± 17%; $P = 0.002$; Figure 3C). These results confirmed that β-adrenergic induction of *Arg1* is regulated by C/EBPβ.

3.4. C/EBPβ regulation of M2 and M1 genes modulated by β-adrenergic signaling

To identify other β-adrenergic-induced M2 genes that are regulated by C/EBPβ, we screened RNAseq results from RAW 264.7 macrophages for previous empirically-defined M2 genes in the whole transcriptome (9) that followed the *Arg1* pattern of up-regulation by β-adrenergic-signaling with inhibition by *Cebpb* knockdown. This analysis identified 20 additional M2 genes, including several that encode known protumoral mechanisms in tumor-associated macrophages (Figure 4A). TELiS differential expression analysis confirmed that transcription factor binding motifs for C/EBPβ (TRANSFAC V$CEBPB_02) were

significantly enriched among these genes (mean fold difference [MFD] = 2.49, $P < 0.001$; Figure 4B). Because our previous study found that β-adrenergic signaling shifts the macrophage transcriptome to the M2 side of the M1-M2 spectrum, in part, through suppression of M1 transcripts (9), we also screened RAW 264.7 macrophages for empirically-defined M1 genes that exhibited the orthogonal pattern of *Arg1* regulation (i.e., down-regulation by β-adrenergic signaling, with rescue of this effect by *Cebpb* knockdown). This latter screen identified eight M1 genes whose suppression by β-adrenergic signaling is regulated by C/EBPβ (Figure 4C). However, in contrast to the M2 geneset, TELiS differential expression analysis found no significant difference in prevalence of V $CEBPB_02 binding motifs among these M1 genes (MFD = 0.74, $P = 0.27$; Figure 4D). These results suggested that while suppression of M1 genes by C/EBPβ in β-adrenergic-stimulated macrophages is likely achieved through an indirect mechanism, C/EBPβ regulation of select M2 genes in β-adrenergic-stimulated macrophages likely involves direct transcription factor activity by the protein.

4. Discussion

These data confirm that β-adrenergic signaling induces an M2-like transcriptional profile in macrophages via induction of *Cebpb* gene transcription and subsequent recruitment of C/EBPβ protein to an array of M2-related gene loci, including *Arg1.* β-adrenergic signaling induced gene expression for C/EBPβ transcription factor (*Cebpb*) well in advance of peak *Arg1* gene expression. Consequently, ChIP experiments showed that C/EBPβ transcription factor activity was elevated at the enhancer region for *Arg1* during its peak expression, but not at the proximal promoter area. Additional C/EBPβ blocking experiments were performed through RNAi, which inhibited β-adrenergic induction of *Cebpb*, along with subsequent nuclear localization of C/EBPβ and *Arg1* expression, further confirming that β-adrenergic induction of *Arg1* is regulated by C/EBPβ. A screen of global gene expression results from RNAseq in the C/EBPβ blocking experiments identified 20 other β-adrenergic-induced M2 genes and eight β-adrenergic-suppressed M1 genes that are regulated by C/EBPβ, with bioinformatic indication that transcription factor binding motifs for C/EBPβ were over-represented in the M2 gene set but not in the M1 gene set. Together, these results show that C/EBPβ mediates the signaling pathway between β-adrenergic stimulation and expression of *Arg1* and several other specific genes in the greater M2 transcriptome.

The M2 effects produced by β-adrenergic signaling in our study parallel the M2 effects produced by epinephrine and norepinephrine at concentrations ranging from 1nM to 1μM in a previous study [27]. That study also found that epinephrine and norepinephrine were acting through β_2-adrenergic receptors rather than α-adrenergic receptors. Thus, given that both human [28] and rodent [29] stressor tasks have been shown to increase epinephrine into the nM range and norepinephrine levels to the near-μM range (0.1μM) in circulation, with presumably greater levels of norepinephrine in tissue microenvironments because of direct innervation (12), it seems plausible that the data from the present macrophage models may reflect how stress-induced β-adrenergic signaling could affect macrophage polarity in peripheral tissues.

C/EBPβ expression has been found to associate with greater progression in several tumor types, including colon [30], glioma [31], ovarian [32], and renal [33], although it is not clear that macrophages are the source in these cancer patient studies. Nonetheless, given its apparent involvement in malignancy, C/EBPβ has emerged as a target for the development of small-molecule inhibitors [34]-[35]. Similarly, early-phase clinical trials are beginning to target β-adrenergic receptors (14–15), given the sizeable body of pharmaco-epidemiological data that shows reduced cancer progression in patients who are taking β-adrenergic antagonists (reviewed in 12–13). The results from the current study underscore the notion that intervening at opportune points along the β-adrenergic-C/EBPβ pathway may inhibit malignancy by down-regulating M2 polarization in TAMs that can otherwise facilitate tumor progression.

Arginase I production by macrophages reduces the amount of L-arginine substrate available for nitric oxide production and, thus, is thought to reduce macrophage tumoricidal capacity [36]. However, the best defined mechanism by which arginase I has protumor effects may come from a line of studies that show arginase I enzyme activity depletes extracellular L-arginine, which in turn down-regulates expression of CD3ζ, cyclin D3, and cyclin dependent kinase 4 in neighboring T-cells, arresting them in the G0-G1 phase of the cell cycle and, thus, contributing to the well-known T-cell anergy that cancer immunotherapy seeks to reverse (reviewed in [37]). Interestingly, anti-tumor T-cell responses have also been found to be regulated by type I interferons from dendritic cells [38], which are up-regulated by lymphotoxin-β receptor (LTβR) signaling [39]. In this regard, LTβR signaling was recently found to enhance anti-tumor immunity in models of pancreatic, breast, and brain cancer [40]. Thus, the down-regulation of gene expression for the LTβR ligand subunit, *Ltb*, in the present study, alongside increased *Arg1* expression, suggests the potential for a multifaceted immunosuppresive mechanism by which β-adrenergic-C/EBPβ signaling in TAMs could compliment direct inhibitory effects on T-cells by β-adrenergic signaling [41] in the promotion of tumor progression.

In addition to immunosuppression, other M2 properties contribute to malignant progression via tissue remodeling effects, which enable tumor angiogenesis, invasion, and metastasis (17). In the present study, we found that the β-adrenergic-C/EBPβ pathway up-regulates *Csf2rb*. This gene encodes the common beta chain of the receptor for GM-CSF, which has a well-established role for regulating matrix metalloproteinase (MMP) production by macrophages [42], and elevated MMP activity in the stroma surrounding breast cancer has been found to facilitate tumor invasion [43]. MMPs support malignant invasion and metastasis by degrading the extracellular matrix (ECM), which releases encapsulated cancer cells and facilitates neoangiogenesis and lymph-angiogenesis in the expanding tumor [44]-[45]. Thus, β-adrenergic-C/EBPβ signaling in macrophages may also contribute to cancer progression by promoting degradation of the ECM.

In addition to malignancy, these results may be relevant for other pathologies. For example, it is well-documented that β-adrenergic signaling primes the specialized population of macrophages in the brain, i.e., microglia, to respond with enhanced proinflammatory signaling molecules and bactericidal activity when stimulated with an immune challenge (reviewed in [46]). In turn, an amplified and prolonged neuroinflammatory response

mediated by primed microglia is associated with cognitive and behavioral deficits and progressive neurodegenerative disease such as Alzheimer's [47]. However, complicating the picture of neurodegeneration are recent reports that have found β-adrenergic signaling associates with a reduction in alpha-synuclein, the protein that accumulates to form Lewy bodies in the brains of most patients with Parkinson's disease [48], with potential beneficial involvement of microglia in this effect (reviewed in [49],[50],[51]. Given the extensive role that C/EBPβ plays in myeloid cell development and function [52]), we speculate that this transcription factor may be involved in such effects. However, further research is needed to fully address the question.

It should be noted that extrapolation from murine in vitro models, like those in the present study, is necessarily limited. For several reasons, the genomic and protein markers that characterize a stimulated macrophage in one model may not always correlate well with phenotype in another macrophage model that uses the same stimulus (reviewed in [53]). Species differences between models can play a role in this. Compared to human macrophages, mouse macrophages can have faster response times to a given stimulus [54] and generally have higher sensitivity to the standard M1 stimulus, LPS (53). A larger factor affecting model differences may be the ontogeny of the macrophage model. For example, *in vivo* macrophages obtained from a participant's specific tissue site have been shown to respond differently than the participant's *in vitro* monocyte-derived macrophages when encountering the same stimulus [55],[56]. However, in the context of wound healing pathology (e.g., burn victims), which is characterized by M2 macrophage dynamics, circulating monocytes have been found to display M2 markers in parallel with macrophages situated at the site of tissue damage [57]. Nonetheless, further research with human macrophages is needed to determine the full relevancy of the present findings in mice.

In summary, the present study demonstrates that C/EBPβ mediates the signaling pathway between β-adrenergic stimulation and expression of *Arg1* and several other elements of the M2 transcriptome. Although gene expression panels are asserted to be one of the clearest ways to distinguish the polarization state of macrophages (1), we note herein the caution that biological function cannot be definitively inferred from such profiles. Nonetheless, the current findings identify C/EBPβ signaling as a potential molecular target for inhibiting the effects of β-adrenergic signaling on macrophage activation in cancer and other macrophage-related diseases.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Highlights

- β-adrenergic-induced C/EBPβ transcription factor activity up-regulated *Arg1* expression in macrophages
- Knockdown of *Cebpb* inhibited β-adrenergic induction of C/EBPβ protein localization in the nucleus and subsequent *Arg1* expression
- Analysis of RNAseq results identified 20 additional M2 genes and eight M1 genes that were regulated by β-adrenergic-C/EBPβ signaling

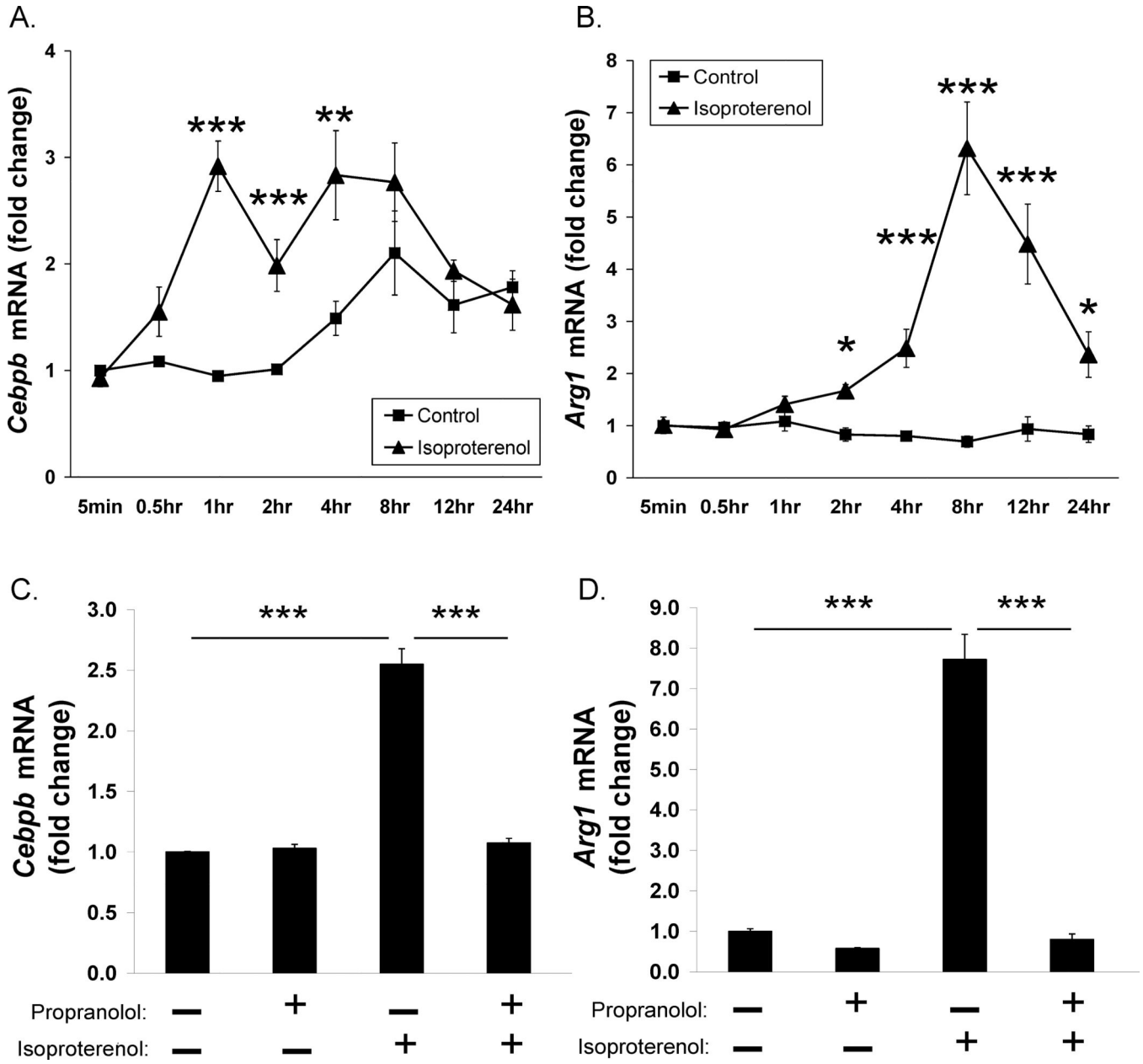

Figure 1.

Kinetic profile of β-adrenergic-induced *Cebpb* gene expression (A) and *Arg1* gene expression (B) in BMDMs relative to initial control level at first time point. Abrogation of β-adrenergic-induced *Cebpb* gene expression at 1 hr (C) and *Arg1* gene expression at 8 hrs (D) with the β-adrenergic antagonist, propranolol. Isoproterenol at 1μM. Propranolol at 10μM. Data represent mean ± SE of three independent experiments. ***$P < 0.001$, **$P < 0.01$, *$P < 0.05$ vs. control at corresponding time point (A and B) or vs. no propranolol / no isoproterenol control condition (C and D).

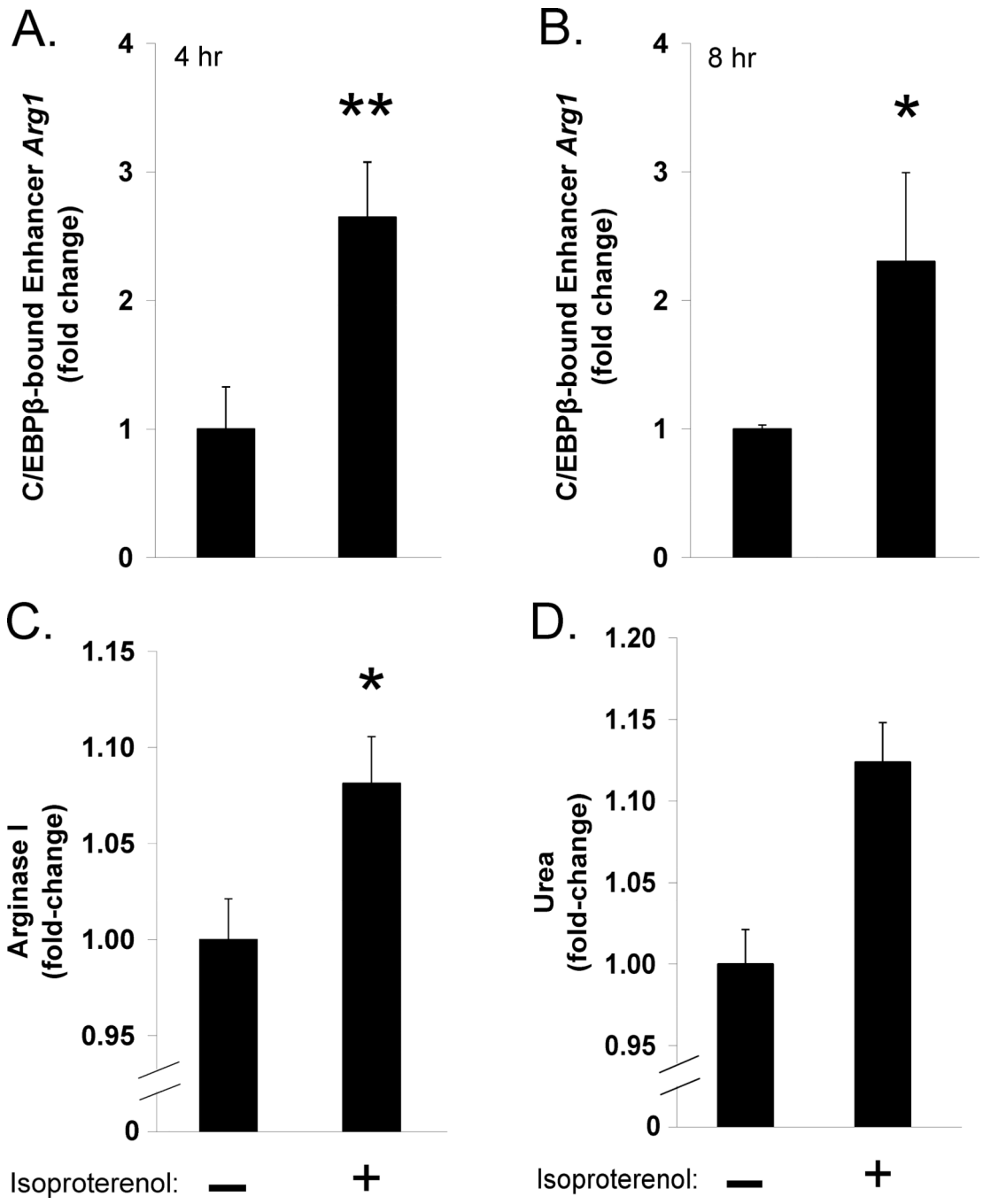

Figure 2.

C/EBPβ binding near the *Arg1* locus with subsequent arginase I protein expression and enzyme activity in BMDMs after β-adrenergic stimulation. Real-time qPCR of enhancer region DNA for *Arg1* from anti-C/EBPβ immunoprecipitated chromatin at 4 hours (A) and 8 hours (B) after stimulation. Arginase I protein expression at 8–12 hours after stimulation (C) and arginase enzyme activity-dependent urea levels in culture media at 24 hours after stimulation (D). Isoproterenol at 1μM. Data for each ChIP time point in (A) and (B) represent mean ± SE of two independent experiments with three ChIP reactions per group in

each experiment. Three experiments for (C) and two experiments for (D). $^{**}P < 0.01$, $^{*}P < 0.05$ vs. control.

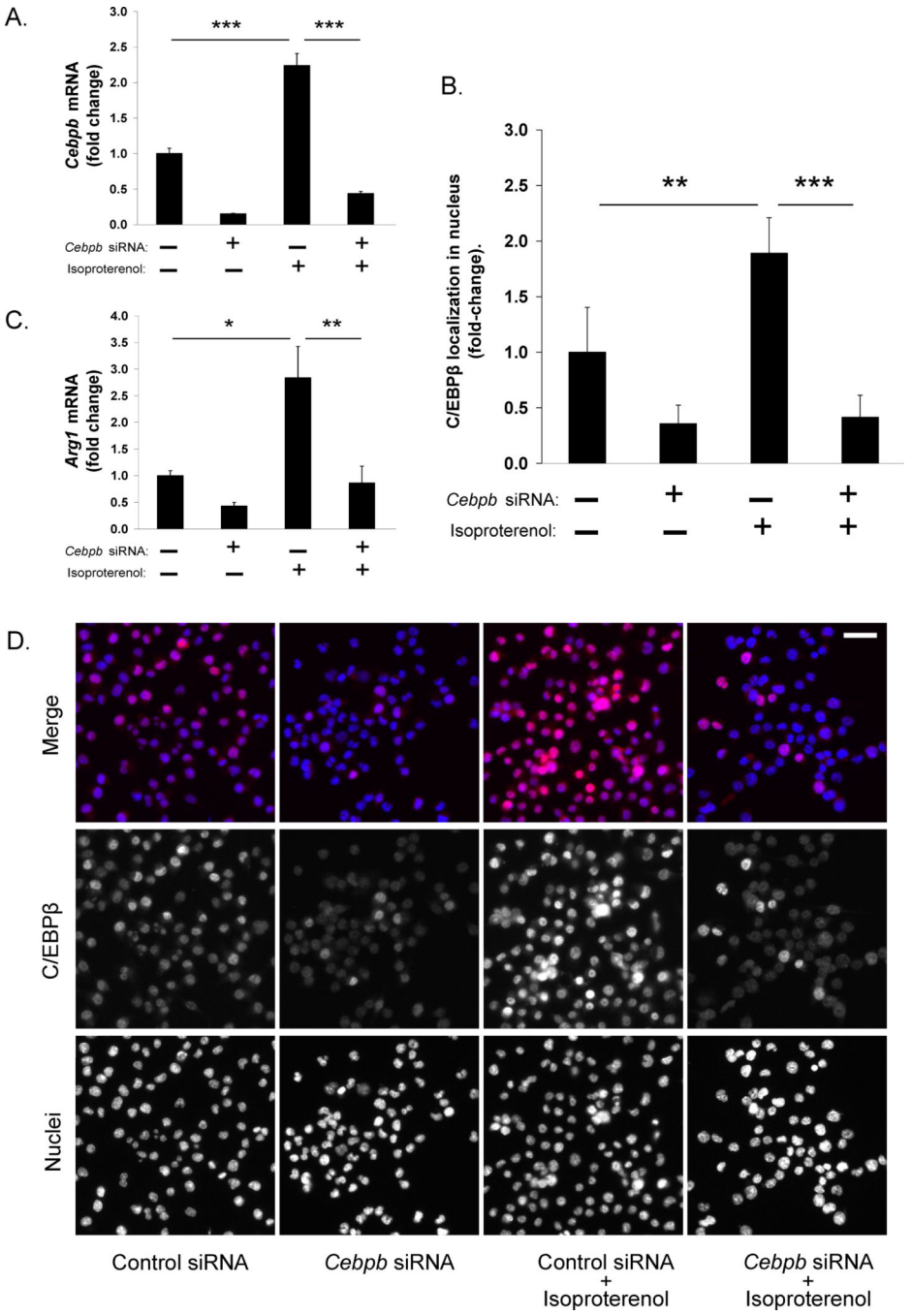

Figure 3.

Effects of C/EBPβ inhibition on β-adrenergic-induced transcription factor localization in the nucleus and *Arg1* gene expression in RAW 264.7 cells. (A) *Cebpb* gene expression following 24 hours transfection with siRNA for *Cebpb* (or control siRNA) and 1 hour of stimulation with isoproterenol. (B) Percentage of nuclear stain co-localized with C/EBPβ stain at 4 hours following isoproterenol stimulation. (C) *Arg1* gene expression following 24 hours transfection with siRNA for *Cebpb* (or control siRNA) and 8 hours of stimulation with isoproterenol. (D) Representative images of single channel gray scale and merged

pseudocolored immunofluorescent staining across conditions; in merged images, blue signal is nucleus and red signal is C/EBPβ. Scale bar 100μm. Isoproterenol at 1μM. Data represent mean ± SE of three independent experiments. *** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$ between indicated pairs.

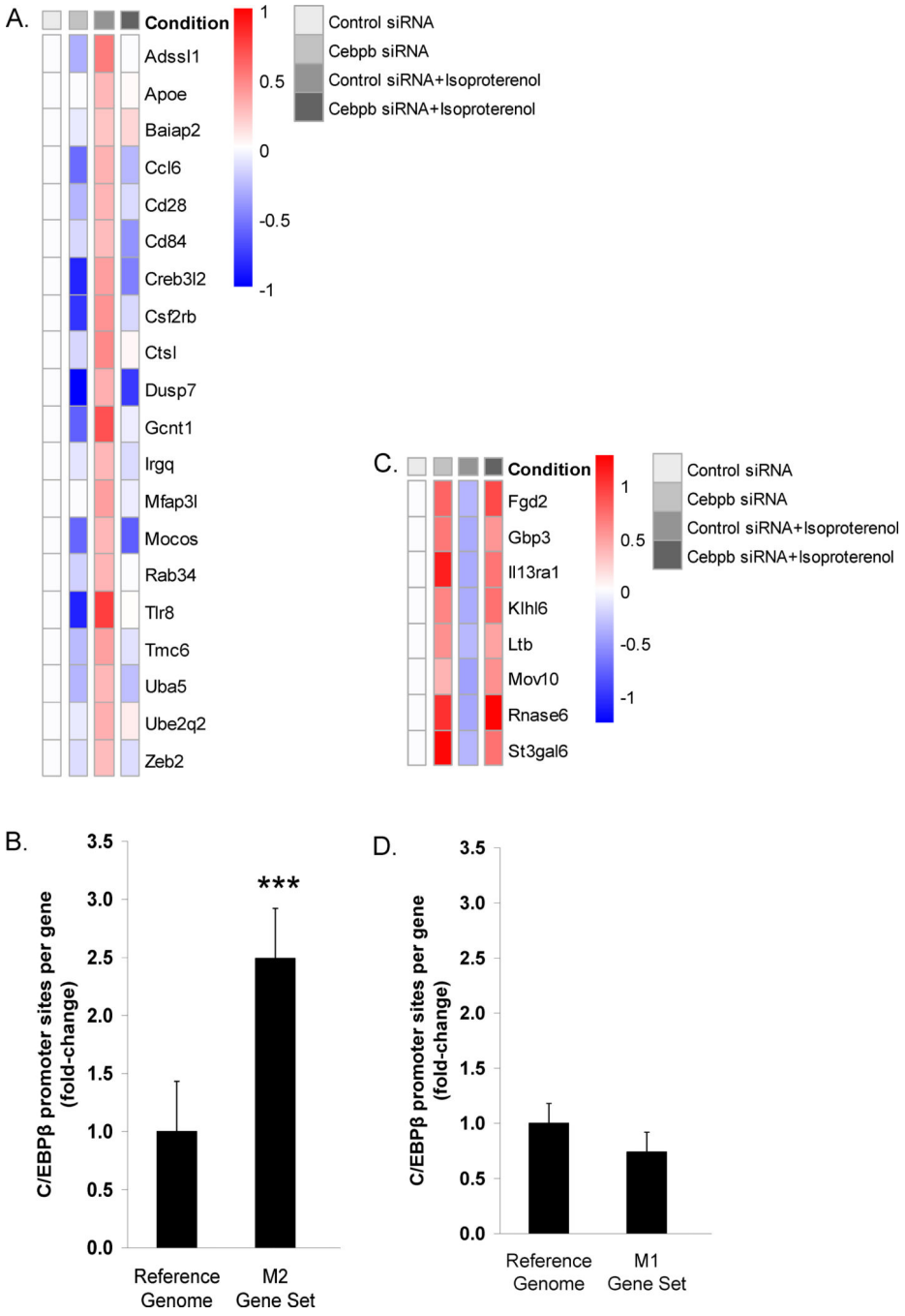

Figure 4.

C/EBPβ regulation of M2 and M1 genes modulated by β-adrenergic signaling in RAW 264.7 cells. (A) Isoproterenol up-regulated M2 genes that were suppressed by C/EBPβ inhibition in global gene expression profiles; red = up-regulation and blue = down-regulation, as mean fold change from control group fixed at 0 on log2 scale for each given gene. (B) TELiS differential expression analysis of transcription factor binding motif prevalence for C/EBPβ in M2 genes from panel A. (C) Isoproterenol down-regulated M1 genes that were rescued by C/EBPβ inhibition in global gene expression profiles. (D) TELiS

differential expression analysis of transcription factor binding motif prevalence for C/EBPβ in M1 genes from panel B. *** $P < 0.001$ vs. reference genome.