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A dynamic equilibrium between TTP and CPEB4 controls

mRNA stability and inflammation resolution

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SUMMARY

Temporal control of inflammation is critical to avoid pathological developments, and is largely defined through the differential stabilities of mRNAs. While TTP-directed mRNA deadenylation is known to destabilize ARE-containing mRNAs, this mechanism alone cannot explain the variety of mRNA expression kinetics observed during inflammation resolution. Here we show that inflammation resolution requires CPEB4 expression, *in vitro* and *in vivo*. Our results identify that CPEB4-directed polyadenylation and TTP-mediated deadenylation compete during the resolutive phase of the LPS response to uncouple the degradation of pro-inflammatory mRNAs from the sustained expression of anti-inflammatory mRNAs. The outcome of this equilibrium is quantitatively defined by the relative number of CPEs and AREs in each mRNA, and further shaped by the coordinated regulation by the MAPK signalling pathway of the levels and activities of their trans-acting factors, CPEB4 and TTP. Altogether, we describe a temporal- and transcript-specific regulatory network controlling the extent of the inflammatory response.

KEYWORDS

Inflammation resolution; Innate immunity; mRNA stability; RNA Binding Protein; CPEBs; TTP; MAPK signalling;

INTRODUCTION

As part of the innate immune system, macrophages sense infectious pathogens and orchestrate inflammatory and antimicrobial immune responses. These immune reactions require tight temporal regulation of multiple pro- and anti-inflammatory factors, connected by negative feedback loops, which ultimately ensure inflammation resolution. Alterations of these dynamic and coordinately regulated gene expression programs cause pathological inflammation and lead to diseases such as auto-immune disorders and cancer (Carpenter et al., 2014). While changes in the rate of gene transcription are important for the initial inflammatory response, its duration and strength are determined mainly by the rate of mRNA decay (Rabani et al., 2011).

AU-Rich Elements (AREs) are key cis-acting elements that regulate mRNA deadenylation and the stability of transcripts involved in inflammation (Spasic et al., 2012). The role of the ARE-binding protein Tristetraprolin (TTP) has been widely characterized in macrophages stimulated by bacterial lipopolysaccharide (LPS) (Carpenter et al., 2014). During the late LPS response, TTP-mediated mRNA decay limits the expression of inflammatory genes, thereby establishing a post-transcriptional negative feedback loop that promotes the resolution of inflammation (Anderson, 2010; Spasic et al., 2012). However, early in the LPS response, TTP activity is counterbalanced by another ARE-binding protein, Hu-antigen R (HuR), which stabilizes its mRNA targets by competing with TTP for ARE occupancy (Tiedje et al., 2012). The competitive binding equilibrium between TTP and HuR is post-translationally regulated by the mitogen-activated protein kinase (MAPK) signalling pathways, whose activation is induced by LPS through Toll-like receptor 4 (TLR4) (Arthur and Ley, 2013; O'Neil et al., 2018).

Despite extensive work, ARE-mediated deadenylation and mRNA decay do not explain the variety of temporal expression patterns and destabilization kinetics needed to orchestrate inflammation resolution (Sedlyarov et al., 2016). Thus, additional mechanisms would be required to define the temporal expression patterns of pro- and anti-inflammatory genes. In early development, the cytoplasmic regulation of poly(A) tail length is not a unidirectional event but rather the reflection of a dynamic equilibrium between deadenylation and polyadenylation by ARE-binding proteins and cytoplasmic polyadenylation element binding proteins (CPEBs) (Belloc and Mendez, 2008; Nousch et al., 2019; Pique et al., 2008). CPEBs bind to the cytoplasmic polyadenylation elements (CPEs) present in the 3' UTR of some mRNAs and promote translation of these mRNAs by favouring the elongation of their poly(A) tails (Ivshina et al., 2014; Weill et al., 2012). The activities exerted by CPEBs are quantitatively defined by the number and position of CPEs present in their target transcripts, thus determining the polyadenylation kinetics and the transcript-specific temporal patterns of translation.

The global contribution of CPEBs to the regulation of mRNA expression during inflammatory processes has not been addressed. In this work, we show that myeloid CPEB4 is needed for the resolution of the LPS-triggered inflammatory response. We further show that the levels and activities of CPEB4 and TTP are sequentially regulated by LPS-induced MAPK signalling. In turn, the combination of CPEs and AREs in their target-mRNAs generate transcript-specific decay rates. These two opposing mechanisms allow destabilization of inflammatory transcripts while maintaining the expression of the negative feedback loops required for efficient inflammation resolution.

RESULTS

Inflammation resolution is impaired in *Cpeb4*KO macrophages

To determine a potential contribution of CPEBs to the regulation of inflammatory responses, we interrogated the expression of *Cpeb*-encoding mRNAs during the course of a systemic inflammatory response in sepsis patients. In two independent GEO datasets, we observed that *Cpeb4* mRNA levels were significantly upregulated in blood, whereas *Cpeb1-3* mRNAs did not present major expression changes **(Figure 1A)**. Monocytes/macrophages, which are enriched during sepsis, are the immune cell populations expressing higher *Cpeb4* mRNA levels **(Figure S1A, S1B)**. Moreover, deconvolution analysis suggested that myeloid cells express higher levels of *Cpeb4* mRNA during sepsis **(Figure S1C, S1D).** To further explore the significance of this correlation, we generated a myeloid-specific *Cpeb4* knockout mouse model (*Cpeb4*MKO) and challenged these mice with an intraperitoneal dose of LPS, the main membrane component of gram-negative bacteria. Upon LPS-induced endotoxic shock, *Cpeb4*MKO mice displayed lower survival rates than the wild-type controls **(Figure 1B)**. *Cpeb4*MKO animals also presented splenomegaly **(Figure 1C)** and increased splenic levels of cytokines *Il6*, *Tnf*, and *Il1a* **(Figure 1D)**. These results link CPEB4 ablation in myeloid cells to an exacerbated inflammatory response, which impairs survival from sepsis.

To further characterize CPEB4 function during the LPS response, we stimulated Bone Marrow-Derived Macrophages (BMDMs) with LPS. Time-course analysis showed that *Cpeb4* mRNA was transiently upregulated, peaking between 3 and 6 h after LPS stimulation, while the other *Cpeb* mRNAs remained virtually unaffected **(Figure 2A)**. This peak of *Cpeb4* mRNA expression was

115   followed by CPEB4 protein accumulation **(Figure 2B)**. The CPEB4 protein was

116   detected as a doublet with the slow migrating band corresponding to the

117   hyperphosphorylated and active form (Guillén-Boixet, 2016) **(Figure 2C)**.

118   Consistent with the observation that phosphorylated CPEB4 accumulated during

119   the late phase of the LPS response, comparative transcriptomic analysis between

120   wild-type and *Cpeb4*KO BMDMs showed that most differences appeared 6-9 h

121   after LPS stimulation, during the resolution phase of the inflammatory response

122   **(Figure 2D, Table S1)**. The transcriptomic profile of *Cpeb4*KO BMDMs included

123   increases in hypoxia, glycolysis and mTOR pathways, which have been linked to

124   pro-inflammatory macrophage polarization during sepsis (Shalova et al., 2015)

125   **(Figure S2A)**. We confirmed that HIF1α levels were increased, both at protein

126   and mRNA levels, after 9 h of LPS treatment **(Figure 2E, 2F)**. Conversely, anti-

127   inflammatory transcripts like *Il10* mRNA were reduced in the *Cpeb4*KO BMDMs

128   **(Figure 2G, Figure S2B).** All together, these results suggested that the absence

129   of myeloid CPEB4 disrupts the homeostatic switch towards inflammation

130   resolution.

131   **The p38α-HuR-TTP axis regulates *Cpeb4* mRNA stability**

132   We next addressed how CPEB4 expression was regulated in BMDMs treated

133   with LPS. Since the peak of *Cpeb4* mRNA followed similar kinetics to pro-

134   inflammatory ARE-containing mRNAs like *Il6* or *Il1a* **(Figure S3A)**, we searched

135   *Cpeb4* 3' untranslated region (3'-UTR) for AREs and found 17 repeats of the

136   AUUUA pentanucleotide **(Figure S3B)**. ARE-containing transcripts are stabilized

137   through HuR binding during the early phase of the LPS response and destabilized

138   at later times by TTP binding. The switch between these two ARE-binding

139   proteins is regulated by LPS-activated p38α MAPK (Tiedje et al., 2012). To

140   determine whether *Cpeb4* expression was controlled by the p38α-HuR-TTP axis,

141   we used BMDMs from myeloid-specific p38α KO mice (*p38αMKO*) (Youssif et

142   al., 2018) and found that *Cpeb4* expression levels were indeed reduced in LPS-

143   treated *p38αMKO* macrophages **(Figure 3A)**. This observation was confirmed by

144   treating wild-type BMDMs with the p38α inhibitor PH797804 **(Figure 3B)**. To

145   further define whether p38α regulates *Cpeb4* expression at the transcriptional or

146   post-transcriptional levels, we inhibited transcription in LPS-treated wild-type and

147   *p38αMKO* BMDMs, and found that *Cpeb4* mRNA levels decayed significantly

148   faster in the latter **(Figure 3C)**. Next, we assessed whether the stabilization of

149   *Cpeb4* mRNA by p38α was mediated through the differential binding of HuR and

150   TTP. By measuring *Cpeb4* mRNA co-immunoprecipitated with HuR from wild-

151   type and *p38αMKO* BMDMs, we observed that HuR binding to *Cpeb4* mRNA was

152   strongly enriched upon LPS treatment in a *p38α*-dependent manner. Similar

153   results were observed in *Tnf* mRNA as a control **(Figure 3D, Figure S3C)**. To

154   determine TTP binding to *Cpeb4* mRNA, we took advantage of a recent study

155   reporting TTP immunoprecipitation (iCLIP) followed by genome-wide analysis of

156   its associated mRNAs in LPS-stimulated BMDMs (Sedlyarov et al., 2016).

157   Analysis of these datasets showed binding of TTP to the *Cpeb4* 3'-UTR 6 h after

158   LPS treatment **(Figure S3D)**, as well as significantly reduced decay rates of both

159   *Cpeb4* and *Tnf* mRNAs in TTPMKO macrophages **(Figure 3E)**. To further

160   analyse the contribution of p38α signalling to *Cpeb4* mRNA stability, we used

161   human osteosarcoma (U2OS) cells expressing the p38 MAPK activator MKK6

162   under the control of the TET-ON promoter(Trempolec et al., 2017). MKK6-

163   induced p38α activation was sufficient to increase *Cpeb4* mRNA levels in these

164   cells, and this effect was reversed by treatment with p38α chemical inhibitors

165 **(Figure 3F)** or by shRNA-mediated depletion of HuR **(Figure 3G, Figure S3E,**

166 **S3F, S3G)**. All together, these results indicate that *Cpeb4* mRNA is stabilized by

167 HuR and destabilized by TTP, and that the competitive binding of these two ARE-

168 binding proteins to *Cpeb4* mRNA is regulated by LPS-driven p38α activation.

169

170 **CPEB4 stabilizes mRNAs encoding negative feedback regulators of the**

171 **LPS response**

172 To address the functional contribution of CPEB4 to inflammation resolution,

173 we performed RIP-seq to identify CPEB4-bound mRNAs in untreated and LPS-

174 activated wild-type BMDMs, using *Cpeb4*KO BMDMs as controls **(Figure 4A)**.

175 We identified 1173 and 1829 CPEB4-associated mRNAs in untreated and LPS-

176 treated BMDMs, respectively **(Table S2)**. These included previously described

177 CPEB4 targets such as *Txnip* or *Vegfa*, while negative controls such as *Gapdh*

178 were not detected **(Figure 4B)**. The 3'-UTRs of the CPEB4 co-

179 immunoprecipitated mRNAs were enriched in canonical CPE motifs (Pique et al.,

180 2008) **(Figure 4C)** and also in CPEs containing A/G substitutions **(Figure S4A,**

181 **S4B)**. These CPE variants have been shown to be specifically recognized by the

182 *Drosophila* orthologue of CPEB2-4 (Orb2) (Stepien et al., 2016). Gene ontology

183 analysis of CPEB4 targets indicated an enrichment of mRNAs encoding for

184 components of the LPS-induced MAPK pathways **(Figure S4C).** These targets

185 included mRNAs that participate in anti-inflammatory feedback loops that

186 negatively regulate the LPS response, consistent with the phenotype observed

187 in *Cpeb4*KO BMDMs. Thus, *Dusp1*, *Il1rn, Socs1, Socs3, Zfp36* (encoding for

188 TTP) and *Tnfaip3* mRNAs specifically co-immunoprecipitated with CPEB4

189 **(Figure 4D)**. The binding of CPEB4 to *Txnip, Dusp1* and *Il1rn* mRNAs was further

validated by RT-qPCR, using *Gapdh* as negative control **(Figure 4E)**. The functional importance of CPEB4 association was confirmed by the observation that *Socs1* mRNA **(Figure 4F)** and SOCS1 protein **(Figure 4G)** levels were both reduced in LPS-treated *Cpeb4*KO BMDMs compared with wild-type BMDMs. Likewise, the levels of *Dusp1*, *Il1rn, Socs3, Tnfaip3* and *Zfp36* mRNAs were similarly reduced in LPS-treated *Cpeb4*KO BMDMs **(Figure 4H)**. To examine whether these changes in mRNA levels originated transcriptionally or post-transcriptionally, we measured the stability of *Socs1* and *Il1rn* mRNAs in wild-type and *Cpeb4*KO BMDMs stimulated with LPS and treated with actinomycin D. Both *Socs1* and *Il1rn* mRNAs displayed reduced stability in the absence of CPEB4 **(Figure 4I)**. To confirm that this effect was mediated by the presence of CPEs in the mRNA 3'-UTRs, we studied the stability of reporter mRNAs with or without CPEs in LPS-stimulated RAW264 macrophages. Indeed, after 6 h of LPS treatment, the stability of a reporter with CPEs was increased compared with the same mRNAs where the CPEs were inactivated by point mutations **(Figure 4J)**. Taken together, our results suggest that CPEB4 sustains the expression of anti-inflammatory factors at late times following LPS stimulation by binding to the corresponding mRNAs and promoting their stabilization.

The equilibrium between CPEB4/CPEs and TTP/AREs defines mRNA oscillation patterns

Given that CPEB4/CPEs and TTP/AREs have opposite effects on mRNA stability, we explored the coexistence of both elements in the same 3'-UTR. We found that CPEB4-bound mRNAs in BMDMs were enriched in AREs **(Figure 5A, Table S3)**. Conversely, 53% (102/193) of the TTP-bound mRNAs in

215 BMDMs(Sedlyarov et al., 2016) **(Table S4)** were also co-immunoprecipitated by

216 CPEB4 **(Figure 5B)**. Indeed, at genome-wide level, we observed a linear

217 correlation between the number of CPEs and the number of AREs present in the

218 same 3'-UTR **(Figure 5C, Table S3)**. To define how the coexistence of these two

219 elements impact on mRNA stability, we compared the expression kinetics of

220 mRNAs targeted by both CPEB4 and TTP, containing both CPEs and AREs but

221 at different ratios, and the levels of these transcripts in LPS-activated BMDMs

222 from the *Cpeb4*KO **(Figure 5D, Table S1)** and TTPMKO mice (Sedlyarov et al.,

223 2016) **(Figure 5E)**. The induction of *Socs1* and *Il1rn* mRNA was reduced in

224 *Cpeb4*KO macrophages, but the expression of *Cxcl1* or *Ptgs2* mRNA remained

225 unaffected. Conversely, in the absence of TTP, *Socs1* and *Il1rn* mRNAs did not

226 display major changes, while *Cxcl1* and *Ptgs2* mRNA levels increased. In a third

227 group, mRNA levels, such as those of *Ccl2* and *Cxcl2,* were affected (in opposite

228 directions) in both the *Cpeb4*KO and TTPMKO macrophages. Thus, despite

229 being all bound by both proteins, some mRNAs were more dependent on CPEB4-

230 mediated stabilization while others were more sensitive to TTP-mediated

231 destabilization. Interestingly, the 3'-UTRs of *Socs1* and *Il1rn* mRNAs were more

232 enriched in CPEs; those of *Cxcl1* and *Ptgs2* mRNA were enriched in AREs, while

233 those of *Ccl2* and *Cxcl2* mRNA presented an intermediate position.

234 Based on these observations, we classified the mRNAs that were targeted by

235 both TTP and CPEB4 according to the ratio between the number of AREs and

236 CPEs in their 3'-UTRs (ARE/CPE Score; **Figure 5F; Figure S5A)**. Thus, mRNAs

237 with more CPEs than AREs were named CPE-dominant (CPEd, ARE/CPE

238 score<0), and those with more AREs were classified as ARE-dominant (AREd,

239 ARE/CPE score>0) **(Figure 5G; Table S3)**. In an expression kinetics analysis

240   following LPS stimulation, we found that the decay rate at late times (6-9 h) was

241   more pronounced for AREd than for CPEd mRNAs, while no differences were

242   observed at early times (1-3 h) **(Figure 5H; Figure S5B, S5C; Table S5)**. When

243   the same mRNAs were classified only as a function of their number of AREs, we

244   found no differences in the decay rates **(Figure 5G, 5H; S5D)**. Next, we

245   generated a list of 1521 ARE- and CPE-containing mRNAs **(Table S6)** and

246   classified them according to their decay rate at late times after LPS stimulation,

247   between "downregulated" (fast decay) and "sustained" (slow decay) mRNAs.

248   Then, we interrogated if these mRNAs presented different ARE/CPE scores. We

249   confirmed that mRNAs with slower decay rates had a lower ARE/CPE score

250   **(Figure 5I)**. However, no differences were found when we only considered the

251   number of AREs **(Figure S5E)**. These results indicate that the ARE/CPE ratio,

252   but not the number of AREs alone, defines differential mRNA decay rates during

253   the resolutive phase of the LPS response.

254   To directly test this model, we expressed Firefly luciferase reporter mRNAs,

255   with different combinations of CPEs and AREs, in macrophages and measured

256   their levels after exposure to LPS. As transfection control, we used Renilla

257   luciferase with a control 3'-UTR (without CPEs or AREs). The parental Firefly

258   luciferase reporter included 5 AREs and 3 CPEs, which were inactivated by point

259   mutations to generate the following combinations: 5ARE/3CPE, 2ARE/3CPE,

260   0ARE/3CPE, 5ARE/0CPE, 2ARE/0CPE and 0ARE/0CPE **(Figure 5J)**.

261   Compared with the reporter without CPEs or AREs (0ARE/0CPE), the presence

262   of CPEs determined higher mRNA levels at late times after LPS stimulation and

263   these levels were progressively reduced by the addition of AREs (increasing

264   ARE/CPE score) **(Figure 5K)**. On the other hand, the number of AREs in the

265 absence of CPEs had no significant impact on the reporter expression levels

266 **(Figure 5K)**. No significant differences were found at early time points **(Figure**

267 **S5F)**. These results confirm that the combination of CPEs and AREs on mRNAs,

268 together with the regulation of their trans-acting factors CPEB4 and TTP,

269 provides a temporal- and transcript-specific regulatory network that controls

270 mRNA expression during the late phase of inflammatory processes.

271 **DISCUSSION**

272 Physiological inflammatory responses rely on both rapid induction and efficient

273 resolution to avoid the development of diseases. Differential regulation of mRNA

274 stability plays a pivotal role in the uncoupling between the expression of pro- and

275 anti-inflammatory mediators and is key to engage the negative feedback loops

276 that switch-off the inflammatory response (Schott et al., 2014). Our work shows

277 that the temporal control of inflammation is regulated by CPEB4-mediated mRNA

278 stabilization, which acts in a coordinated manner with the well-known TTP-driven

279 destabilization of mRNAs. The dynamic equilibrium between positive (CPEs) and

280 negative (AREs) cis-acting elements in the mRNA 3'-UTRs, together with the

281 regulation of their trans-acting factors CPEB4 and TTP, generates customized

282 temporal expression profiles, which fulfil the functions needed in each phase of

283 the inflammatory process.

284 Our results illustrate how CPEB4 and TTP levels and activities are integrated

285 during the LPS response through cross-regulation at multiple post-transcriptional

286 and post-translational layers. First, their activities are coordinately regulated by

287 MAPK signalling pathways. Upon LPS stimulation, p38 MAPK signalling

288 regulates TTP phosphorylation, causing a shift in the competitive binding

289 equilibrium between HuR and TTP towards HuR, that stabilizes *Cpeb4* mRNA

290   and promotes CPEB4 expression during the late phase of the LPS response.

291   Then, ERK1/2 MAPK signalling (Guillén-Boixet, 2016), which is also activated by

292   LPS, controls the progressive accumulation of the active hyperphosphorylated

293   form of CPEB4. Moreover, CPEB4 regulates its own mRNA, generating a positive

294   feedback loop that contributes to the increase in CPEB4 levels and activity at the

295   late phase of the LPS response. Additionally, CPEB4 binds to the mRNA

296   encoding TTP (*Zfp36*), adding a negative feedback loop that restores TTP activity

297   during the late LPS response. These results underscore a superimposed layer of

298   coordination between the MAPKs signalling pathways in the regulation of both

299   CPEB4 and TTP levels and activities **(Figure 6A-B)**.

300   The regulatory circuit that we have uncovered provides tight temporal control

301   of the stability of TTP and CPEB4 co-regulated mRNAs, which is further

302   delineated by the cis-acting elements in the 3'UTR **(Figure 6A-B)**. In these

303   transcripts, the equilibrium between CPEs and AREs provides transcript-specific

304   behaviours that contribute to uncouple the expression of pro-inflammatory

305   mediators, which need to be rapidly silenced, from anti-inflammatory mediators,

306   whose synthesis need to be sustained longer. Accordingly, ARE-dominant

307   mRNAs are enriched in transcripts encoding pro- inflammatory cytokines, which

308   are expressed during the early phase of the LPS response (Anderson, 2010;

309   Spasic et al., 2012). In contrast, CPE-dominant mRNAs are enriched in

310   transcripts encoding factors that contribute to inflammation resolution during the

311   late phase of the response.

312   CPEB4 targets include negative regulators of MAPKs, thereby generating a

313   negative feedback loop that limits the extent of the inflammatory response.

314   Accordingly, we found that CPEB4 is overexpressed in patients with sepsis, and

myeloid CPEB4 KO mice have decreased survival upon septic shock associated with exacerbated inflammation. Other components of this circuit can also regulate analogous inflammatory phenotypes. Thus, TTP KO mice have exacerbated LPS-induced shock (Kafasla et al., 2014), HuR KO mice display inflammatory phenotypes (Kafasla et al., 2014), and p38α deletion in macrophages reduces the LPS response and renders mice more resistant to endotoxic shock (Kang et al., 2008).

CPEBs were originally identified as a mechanism to activate maternal mRNAs, during meiotic progression in oocytes. In that scenario, a similar unidirectional and self-sustained regulatory network generated sequential waves of polyadenylation, promoting a hierarchical translation of specific subpopulations of mRNAs at each meiotic phase through the coordinated actions of CPEBs and the ARE-binding protein C3H-4 (Belloc and Mendez, 2008; Igea and Mendez, 2010; Pique et al., 2008). Our results indicate that the resolution of inflammation is controlled by an analogous regulatory network, which is coordinated through MAPK signalling pathways, the levels and activities of ARE-BP and CPEBs, and the AREs/CPEs arrangement on inflammatory and anti-inflammatory mRNAs.

ACKNOWLEDGMENTS

AUTHOR CONTRIBUTIONS

348   CS performed all the studies and contributed to experimental design, data

349   analysis and interpretation, wrote the manuscript and assembled the figures. AS

350   performed part of the studies, contributed to all experimental design, data

351   analysis and interpretation and helped with manuscript preparation. RM

352   conceived, directed and discussed the study, wrote the manuscript and obtained

353   funding. JM contributed to *in vivo* mouse experiments. CLC performed the

354   experiments with ARE/CPE constructs. OR analysed the sepsis datasets and the

355   RIPseq. ID performed the genome-wide ARE/CPE analysis in the JD laboratory.

356   AC performed the RNAseq analysis. ER contributed to the p38MKO experiments.

357   VC contributed to the RIPseq experiments. ARN contributed to experimental

358   design and manuscript preparation and obtained funding.

DECLARATION OF INTERESTS

360   The authors declare no competing interests.

FIGURE LEGENDS

362   **Figure 1. CPEB4 expression in myeloid cells is required for survival from**

363   **sepsis. (A)** Differential expression of *Cpeb* mRNAs in the blood of sepsis

364   patients/healthy individuals. Statistics: limma moderated t-test. Pvadj (Benjamini-

365   Hochberg) is shown. **B-D** Wild-type and myeloid-specific *Cpeb4*KO mice

366   (*Cpeb4*MKO) were injected with an i.p. dose of LPS. **(B)** Kaplan-Meier survival

367   curves.   Results   represent   three   independent   experiments

368   (n>7/group/experiment). Statistics: Likelihood-ratio test-pv. **(C)** Spleen weights

369   normalized to total animal weight. Statistics: Two-way ANOVA. **(D)** Splenic total

370   mRNA was measured by RT-qPCR and referred to *Tbp* (n>5 animals/condition).

371   Statistics: Multiple t-test. **(C, D)** Data are represented as mean ± SD.

372   **Figure 2. Inflammation resolution is impaired in *Cpeb4*KO macrophages.**

373   **A-C** LPS-stimulated wild-type BMDMs. **(A)** *Cpeb1-4* levels were measured by

374   RT-qPCR (n=6). **(B)** (Left) CPEB4 immunoblot, α-Tubulin served as loading

375   control. (Right) Quantification (n=3). **(C)** (Left) CPEB4 immunoblot in protein

376   extracts treated with λPhosphatase when indicated. (Right) Quantification of P-

377   CPEB4 signal (n=3). **D-G** LPS-stimulated wild-type and *Cpeb4*KO BMDMs. **(D)**

378   Number of differentially expressed genes (pv<0.01) between genotypes. mRNA

379   levels were quantified by RNASeq (n=4). Statistics: Deseq2 R package. **(E)** (Left)

380   HIF1α immunoblot, vinculin served as loading control. (Right) Quantification,

381   signal intensity was normalized to wild-type 9 h LPS (n=3). **(F)** *Hif1a* levels

382   measured by RT-qPCR (n=6). **(G)** Differential mRNA expression measured by

383   RNASeq (n=4). Statistics: Deseq2 R package. **(A, F)** *Tbp* was used to normalize.

384   **(B-C, E-F)** Data are represented as mean ± SEM. **(E-F)** Statistics: Two-way

385   ANOVA. **(D, G)** See also Table S1.

386   **Figure 3. The p38α-HuR-TTP axis regulates *Cpeb4* mRNA stability. (A)**

387 *Cpeb4* levels in wild-type and *p38α*MKO BMDMs stimulated with LPS (n=3). **(B)**

388 *Cpeb4* levels in LPS-stimulated BMDMs treated with the p38 inhibitor PH or

389 DMSO as control (n=4). **(C)** *Cpeb4* mRNA stability was measured by treating with

390 actinomycin D (ActD) wild-type and *p38α*MKO BMDMs stimulated with LPS for 1

391 h (n≥2). Statistics: Multiple t-test. **(D)** *Cpeb4* mRNA levels in HuR RNA-

392 immunoprecipitates (IP) performed in wild-type and *p38α*MKO BMDMs,

393 stimulated with LPS when indicated. IgG IPs served as control. IP/Input

394 enrichment is shown, normalized to wild-type IP LPS (n=2). See also Figure S3C.

395 **(E)** *Cpeb4* and *Tnf* decay rates in wild-type and TTPMKO BMDMs stimulated for

396 6 h with LPS (data from(Sedlyarov et al., 2016)). See also Figure S3D. **F-G** U2OS

397 cells were treated with tetracyline to induce the expression of a constitutively

398 active MKK6, which induces p38 MAPK activation(Trempolec et al., 2017). **(F)**

399 *Cpeb4* levels upon p38 activation (+MKK6) or inhibition with p38α inhibitors (SB

400 or PH) (n=3). **(G)** *Cpeb4* levels in control or HuR-depleted U2OS cells, where the

401 p38 MAPK has been activated (+MKK6) or inhibited (SB) (n=2). See also Figure

402 S3F-S3G. **(A-D, F-G)** mRNA levels were quantified by RT-qPCR. *Gapdh* (a-b, g)

403 or *18S* **(C)** were used to normalize. **(A-B, D, F-G)** Data are represented as mean

404 ± SEM. **(A-B)** Statistics: Two-way ANOVA. **(F-G)** Statistics: One-way ANOVA.

405 **Figure 4. CPEB4 stabilizes mRNAs encoding negative feedback**

406 **regulators of the LPS response. A-D** CPEB4 RNA-Immunoprecipitation (IP)

407 and sequencing was performed in total lysates (Input) from wild-type and

408 *Cpeb4*KO BMDMs, untreated or stimulated with LPS for 9 h (n=1). **(A)** CPEB4

409 Immunoblot, vinculin served as a loading control. **(B)** Read coverage in Inputs or

410 IPs of selected mRNAs. Peak enrichments between wild-type/*Cpeb4*KO IP are

411 shown in blue. **(C)** CPE-containing transcripts [according to(Pique et al., 2008)]

412 in Inputs and CPEB4 IPs. Statistics: Fisher's exact test. **(D)** Read coverage in the

413 IPs of selected mRNAs. **(E)** CPEB4 IP and RT-qPCR was performed in wild-type

414 and *Cpeb4*KO BMDMs stimulated with LPS for 9 h. IP/Input enrichment is shown

415 (n=3). **(F)** *Socs1* mRNA levels in LPS-stimulated wild-type and *Cpeb4*KO

416 BMDMs. mRNA levels were measured by RT-qPCR normalizing to *Tbp* (n=6).

417 **(G)** Immunoblot of SOCS1 in wild-type and *Cpeb4*KO BMDMs treated with LPS.

418 Vinculin served as loading control. Quantification is shown (FC to wild-type 9 h

419 LPS) (n=3). **(H)** Differential expression between wild-type and *Cpeb4*KO BMDMs

420 treated with LPS measured by RNASeq (n=4). Statistics: Deseq2 R package. **(I)**

421 mRNA stability was measured by treating with actinomycin D (ActD) wild-type

422 and *Cpeb4*KO BMDMs stimulated with LPS for 3 h. Gene expression was

423 analysed by RT-qPCR, normalizing to *Gapdh* (n=3). **(J)** RAW 264,7

424 macrophages were transfected with a Firefly luciferase reporter under the control

425 of the cyclin B1 3'-UTR, either wild-type (CPE +) or with its CPE motifs mutated

426 (CPE -). The same plasmid contained Renilla luciferase reporter as a control.

427 Macrophages were stimulated with LPS for 3 h and then ActD was added. mRNA

428 levels were measured by RT-qPCR. **(B, D)** Integrated genomic viewer (IGV)

429 images. **(E-G)** Data are represented as mean ± SEM. **(F-G, I-J)** Statistics: Two-

430 way ANOVA. See also Table S1 and Table S2.

431 **Figure 5. The equilibrium between CPEB4/CPEs and TTP/AREs defines**

432 **different mRNA oscillation patterns. (A)** ARE-containing transcripts in the

433 Inputs and CPEB4 IPs from Fig. 4a-d. Statistics: Fisher's exact test. **(B)** Overlap

434 between CPEB4 and TTP targets(Sedlyarov et al., 2016). **(C)** Genome wide

435 correlation between AREs and CPEs motifs in the 3'-UTRs. Black line shows the

436 linear regression trend line. R^2=0,6364. **(D)** mRNA levels in wild-type and

437   *Cpeb4*KO BMDMs were measured by RT-qPCR, normalizing to *Tbp* (n=6).

438   Statistics: Two-way ANOVA. *Socs1* data are also shown in Fig. 4f. **(E)** mRNA

439   expression in wild-type and TTPMKO BMDMs treated with LPS. Statistics:

440   DESeq2 software [data from(Sedlyarov et al., 2016)]. **(F)** Common TTP and

441   CPEB4 target mRNAs were classified according to the ARE/CPE score as ARE-

442   dominant (AREd, red) or CPE-dominant (CPEd, blue). See also Figure S5A. **(G-**

443   **H)** CPEB4 and TTP target mRNAs were plotted according to the number of AREs

444   and CPEs in the 3'-UTR. (Left) The dashed line separates AREd and CPEd

445   mRNAs. (Right) mRNAs were classified according only to the number of AREs in

446   their 3'UTR. The dashed lines separates ARE^{high} (>4 AREs; yellow) from ARE^{low}

447   ($\leq$4 AREs; navy) mRNAs. **H-I** Wild-type BMDMs were stimulated with LPS and

448   mRNA levels were quantified by RNAseq (n=4). **(H)** Common CPEB4 and TTP

449   target mRNAs were classified as AREd/CPEd (left) or ARE^{high}/ARE^{low} (right). For

450   each mRNA, the levels after 9 h of LPS treatment was normalized by its

451   expression at 6 h LPS. **(I)** 1521 CPE- and ARE-containing mRNAs were classified

452   as Sustained>0,5 or Downregulated<0,5 according to their expression after 9 h

453   of LPS treatment, normalized by its peak of expression throughout LPS response.

454   For each mRNA, its ARE/CPE score was calculated. **J-K** RAW 264,7

455   macrophages were transfected with a Firefly luciferase reporter under the control

456   of a chimeric 3'-UTR combining Ier3 and Cyclin B1 AREs and CPEs motifs,

457   respectively. Inactivating specific CPE or ARE motifs, six different 3'-UTRs with

458   distinct ARE/CPE scores were generated. The same plasmid contained

459   Renilla luciferase reporter as a control. **(J)** Scheme of the six constructs used for

460   the dual luciferase reporter assay. Inactivated motifs are shown in grey. **(K)** RAW

461   264,7 macrophages were stimulated with LPS for 6 h and Firefly/Renilla levels

462 were measured by RT-qPCR. Values were normalized to the 0CPEs/0AREs

463 construct. Statistics: One-way ANOVA Friedman Test. All significant differences

464 are shown except 5AREs/0CPEs against 3CPEs/0AREs (**) and 3CPEs/2AREs

465 (*). **(D, H, K)** Data are represented as mean ± SEM. **(H-I)** Statistics: Mann-

466 Whitney t test. See also Tables S2, S3, S4, S5, and S6.

467 **Figure 6. Dynamic equilibrium between TTP and CPEB4-mediated**

468 **regulation of mRNAs during inflammation resolution. (A)** LPS stimulates the

469 MAPK signalling cascades downstream of TLR4. p38α controls TTP

470 phosphorylation, causing a shift in the competitive binding equilibrium between

471 HuR and TTP towards HuR, which stabilizes ARE-containing mRNAs. The

472 p38α/HuR/TTP axis also regulates *Cpeb4* mRNA stability and promotes CPEB4

473 expression during the late phase of the LPS response. CPEB4 then accumulates

474 in its active state, which involves phosphorylation by ERK1/2 MAPK signalling.

475 During the resolutive phase of the LPS response, CPEB4 and TTP compete to

476 stabilize/destabilize mRNAs containing CPEs and AREs. The equilibrium

477 between these positive and negative cis-acting elements in the mRNA 3'-UTRs

478 generates customized temporal expression profiles. CPEB4 stabilizes CPE-

479 dominant mRNAs, which are enriched in transcripts encoding negative regulators

480 of MAPKs that contribute to inflammation resolution. **(B)** Immunoblot of the

481 indicated proteins in wild-type BMDMs treated with LPS for 0-9 h. Vinculin and

482 Ponceau staining served as loading control.

483 **METHODS**

484 **Human Sepsis Microarray Analysis.** Datasets used (sepsis patients/healthy

485 subjects): GSE65682 (n=760/42), GSE57065 (n=28/25). Affymetrix U219 arrays

486 from the GSE65682 cohort were downloaded from NCBI GEO and processed

487 using RMA background correction and summarization with R and Bioconductor

488 (Gentleman et al., 2004). Technical variable assessment and adjustment was

489 performed using the Eklund metrics (Eklund and Szallasi, 2008), adjusting for

490 RMA IQR, PM IQR, RNA Degradation and PM Median. Differentially expressed

491 genes were determined using limma (Ritchie et al., 2015), adjusting by gender

492 and the selected Eklund metrics, and using a |FC|>2 and Benjamini-Hochberg p-

493 value lower than 0.05. For volcano plots, for each gene, the probeset with highest

494 log2RMA Interquartile Range (IQR) is shown; and the y-axis represents the –

495 log10 Benjamini-Hochberg adjusted p-value computed via limma moderated t-

496 test. Gene expression deconvolution of blood samples in order to obtain cell

497 proportion estimates was performed using the CellMix package (Gaujoux and

498 Seoighe, 2013) using the DSA built-in blood signature database (Abbas et al.,

499 2009) and the DSA algorithm with default options. The same process was done

500 for Affymetrix U133Plus2 arrays from GSE57065 cohort. Cpeb4 mRNA

501 expression in human immune cell lineages was obtained from Haemosphere

502 website (Choi et al., 2019).

503 **Animal Studies**. To generate a Myeloid-specific *Cpeb4* KO mice

504 (*Cpeb4*MKO), conditional *Cpeb4* animals (*Cpeb4*[lox/lox(Maillo et al., 2017)]) were crossed

505 with LysM-Cre[T/+] (Clausen et al., 1999) transgenic animals obtained from

506 Jackson Laboratory. Offspring was maintained in a C57BL/6J background.

507 Routine genotyping was performed by PCR. Bone Marrow-Derived Macrophages

508 (BMDMs) were obtained from wild-type and Myeloid-specific $p38\alpha$ KO mice

509 (*p38α*MKO) (Youssif et al., 2018) and full CPEB4 KO mice (Maillo et al., 2017).

510 The mice were given free access to food and water and maintained in individually

511 ventilated cages under specific-pathogen-free conditions (unless otherwise

512 specified). All experimental protocols were approved by the Animal Ethics

513 Committee at the University of Barcelona.

514 **LPS-induced Endotoxic Shock**. Wild-type and *Cpeb4*MKO mice were

515 injected intraperitoneally with LPS (10 mg/kg; Santa Cruz SC-3535, *E. coli*

516 0111:B4). Animals were monitored and samples were collected at the indicated

517 times. Mice between 2 and 5 months of age were used, matched for age and sex.

518 **Cell culture**. BMDMs were isolated from the femurs of adult mice as

519 previously described(Celada et al., 1996). Bone marrow cells were differentiated

520 for 7 days on bacteria-grade plastic dishes (Nirco, Ref. 140298) in DMEM

521 supplemented with 20% (vol/vol) FBS, penicillin (100 units/ml), streptomycin (100

522 mg/ml), L-glutamine (5 mg/ml) and 20% L-cell conditioned medium as a source

523 of M-CSF. The medium was renewed on day 5 when BMDMs were plated for the

524 experiment. On day 7, media were changed to DMEM containing 10% FBS,

525 penicillin (100 units/ml), streptomycin (100 mg/ml), L-glutamine (5 mg/ml). On day

526 8, BMDM were primed with LPS (10 ng/ml, *E. coli* 0111:B4, Santa Cruz SC-3535)

527 for the indicated time points. To inhibit p38 MAPK, BMDMs were treated with

528 PH797804 (1 uM) for 1 h prior to LPS treatment.

529 Osteosarcoma cells (U2OS) were grown as in (Trempolec et al., 2017). Briefly,

530 U2OS cells expressing the MKK6 construct were cultured in DMEM containing

531 10% FBS, penicillin (100 units/ml), streptomycin (100 mg/ml), L-glutamine (5

532 mg/ml) in the presence 4 µg/ml Blasticidin S HCl (A11139-03, Invitrogen) and

533 35 µg/ml Zeozin (R250-01, Invitrogen). To activate the p38 signaling pathway,

534 cells were treated with 1 µg/ml tetracycline (Sigma 87128-25 G) or the

535 corresponding amount of ethanol (1:1000 dilution). For the inhibition of p38α, we

536 used 1 µM PH-797804 (Selleckchem, S2726) or 10 µM SB203580 (Axon

537 MedChem).

538 **Infection with shHuR-expressing lentivirus.** To generate short hairpin

539 RNAs (shRNAs) in a lentivirus delivery system, 293 T cells (60% confluent) were

540 transfected with 2 different shRNA-encoding DNA, pLKO.1-shHuR03 and -

541 shHuR04 (5 µg) (IRB Barcelona Genomic Facility), VSV-G (0.5 µg) and ∆89

542 packaging DNA (4.5 µg) using the calcium chloride method. After overnight

543 incubation, the media were replaced, and 48 h later, the media containing the

544 virus were collected and passed through PVDF filters. For cell infection, the virus-

545 containing media were diluted 1:10 in buffer containing 6 mg/l of of polybren,

546 incubated for 10 min and then placed on the U2OS cell monolayer. The following

547 day media were replaced, and 24 h later the cells were subjected to selection with

548 puromycin (2 µg/ml) for 24–48 h. Selected cell populations were cultured in media

549 with puromycin (2 µg/ml) and used for further analysis.

550 **RNA stability in BMDMs.** Cells were stimulated with LPS (10 ng/ml, *E. coli*

551 0111:B4, Santa Cruz SC-3535) for the indicated times, and control cells (time 0)

552 were collected. Fresh medium and Actinomycin D (5-10 µg/ml, Sigma Aldrich

553 A9415) were added, and cells were collected at the indicated times. Total RNA

554 was isolated and cDNA was synthesized to perform RT-qPCR analysis. For each

555 timepoint, the remaining mRNA was normalized to *Gapdh* mRNA levels. The

556 value at time 0 was set as 100%, and the percentage of remaining mRNA was

557 calculated for the rest of timepoints.

558 **RNA analysis.** Total RNA was either extracted by TRIzol reagent (Invitrogen),

559 using phenol-chlorofom, or using Maxwell following the manufacturer's

560 instructions (Maxwell 16 LEV SimplyRNA Cells Kit, Promega). Next, 1 µg of RNA

561   was reverse-transcribed with oligodT and random primers using RevertAid

562   Reverse Transcriptase (ThermoFisher) or the SuperScript™ IV First-Strand

563   Synthesis System (Thermo Fisher), following the manufacturer's

564   recommendations. Quantitative real-time PCR was performed in a QuantStudio

565   6flex (ThermoFisher) using PowerUp SYBR Green Master (ThermoFisher). All

566   quantifications were normalized to an endogenous control (*Tbp*, *Rpl0*, *Gapdh*,

567   *18S*). All the oligos used for RT-qPCR are listed in Table S6. Values are

568   normalized by the median expression in untreated wild-type animals/cells.

569   **Cell transfection.** Murine RAW 264,7 macrophages were cultured in DMEM

570   supplemented with 10% fetal bovine serum, 2 mM L-glutamine and 1%

571   penicillin/streptomycin at 37ºC and 5% CO_2. Cells were seeded in a p6

572   wells/plate at a concentration of 270,000 cells/well; 24 h later cells were

573   transiently transfected with SuperFect Transfection Reagent (Qiagen Ref.

574   301305), following the manufacturer's instructions, for 2.5 h and with 1.5 µg of

575   plasmid. Cells were washed twice with PBS and allowed to rest at 37ºC for 40 h.

576   Transfected cells were then treated with LPS (10 ng/ml, *E. coli* 0111:B4, Santa

577   Cruz SC-3535) for the indicated times. For cells used in the stability experiments,

578   Actinomycin D (5 µg/ml, Sigma Aldrich A9415) was added for 0-60 min and cells

579   were collected to extract total RNA. Firefly was normalized to Renilla, and time 0

580   of ActD was used as reference for all timepoints.

581   **Plasmid construction.** Firefly luciferase gene with downstream Ier3 ARE

582   fragments were purchased from GeneArt (Thermo Fisher); three fragments

583   containing 0, 2 or 5 AREs were cloned with BamHI and HindIII restriction

584   enzymes in pCMV-lucRenilla plasmid (Villanueva et al., 2017) upstream of the

585   3'-UTR of cyclin B1 mRNA. The three CPEs of cyclin B1 3'-UTR were mutated

586   (5'-TTTTAAT-3'→5'-TTgggAT-3'; 5'-TTTTACT-3'→5'-TTggACT-3'; 5'-TTTAAT-

587   3'→5'-TTggAAT-3') using the QuikChange Lightning Multi Site-Directed

588   Mutagenesis Kit (Agilent).

589   **Immunoblotting.** Protein extracts were quantified by DC Protein assay (Bio-

590   Rad), and equal amounts of proteins were separated by SDS-polyacrylamide gel

591   electrophoresis. After transfer of the proteins onto a nitrocellulose membrane

592   (GE10600001, Sigma) for 1 h at 100mV, membranes were blocked in 5% milk,

593   and specific proteins were labeled with the following antibodies: CPEB4 (Abcam

594   Ab83009 / clone 149C/D10, monoclonal homemade); HuR (3A2, sc-5261 Santa

595   Cruz); HIF1a (Cayman 10006421); Phospho-p44/42 (Erk1/2) (Thr202/Try204)

596   (Cell Signaling Clon E10, 9106); SOCS1 (Abcam Ab9870); Phospho-p38

597   (Thr180/Y182) (cell signaling, 9211S); p38alpha (C-20)-G (Santa Cruz, sc-535-

598   G); Phospho-MAPKAPK2 (Thr222) (Cell Signaling, 3044 S), Tristetraprolin (TTP,

599   D1I3T) (Cell Signaling, 71632) and Vinculin (Abcam Ab18058). Quantification

600   was done with ImageStudioLite software, and protein expression was normalized

601   by loading control signal.

602   **Lambda protein phosphatase assay (λ-PPase).** BMDMs were lysed in l-

603   PPase reaction buffer (New England BioLabs, Ipswich, MA) supplemented with

604   0.4% NP-40 and EDTA-free protease inhibitors (Sigma-Aldrich). λ-PPase (New

605   England BioLabs) reactions were performed following the manufacturer's

606   instructions.

607   **RNASeq and data analysis. Pre-processing and normalization.** Reads

608   were aligned to the mm10 genome using STAR 2.3.0e (Dobin et al., 2013) with

609   default parameters. Counts per genomic feature were computed with the function

610   featureCounts R (Liao et al., 2014) of the package Rsubread (Liao et al., 2013).

611 Counts data were normalized using the rlog function of the DESeq2 R package

612 (Love et al., 2014). **Differential expression at gene level.** Differential

613 expression between genotype conditions (*Cpeb4*KO vs. wild-type) was

614 performed using the DESeq2 R package (Love et al., 2014) on the original counts

615 data. This was done independently for every LPS time point. To study expression

616 patterns over time for wild-type samples, we first back-transformed the average

617 (over the four samples) rlog expression to obtain normalized counts. These

618 were further scaled by the maximum value obtained throughout the LPS

619 response for each gene. **Gene set analysis.** Genes were annotated to Hallmark

620 terms (Liberzon et al., 2015) using the R package *org.Hs.eg.db* Marc Carlson

621 (2018). Gene set analysis was then performed using the regularized log

622 transformed matrix, in which genes with low expression (genes with less than an

623 average count of 5 reads) were filtered out. The rotation-based approach for

624 enrichment (Wu et al., 2010) implemented in the R package limma (Ritchie et al.,

625 2015) was used to represent the null distribution. The maxmean enrichment

626 statistic proposed in (Efron B, 2007), under restandardization, was considered for

627 competitive testing. **Visualization.** To obtain a measure of the pathway activity

628 in the transcriptomic data, we summarized each Hallmark as z-score gene

629 signature (Lee et al., 2008). To this end, rlog normalized expression values were

630 centered and scaled gene-wise according to the mean and the standard deviation

631 computed across samples, which were then averaged across all genes included

632 in that Hallmark term. In addition, a global signature was computed for all the

633 genes in the expression matrix and then used for a-priori centering of Hallmark

634 scores. This strategy has proven useful to avoid systematic biases caused by the

635 gene-correlation structure present in the data and to adjust for the expectation

636 under gene randomization, i.e., the association expected for a signature whose

637 genes have been chosen at random(Efron B, 2007; Lee et al., 2008). All statistical

638 analyses were carried out using R and Bioconductor (Sharma et al., 2020).

639 **RNA-immunoprecipitation (RIP).** *Cpeb4*KO and wild-type primary BMDMs,

640 untreated or stimulated for 9 h with LPS (10 ng/ml), were cultured in DMEM

641 supplemented with 10% FBS, rinsed twice with 10 ml PBS, and incubated with

642 PBS 0.5 % formaldehyde for 5 min at room temperature under constant soft

643 agitation to crosslink RNA-binding proteins to target RNAs. The crosslinking

644 reaction was quenched by addition of glycine to a final concentration of 0.25 M

645 for 5 min. Cells were washed twice with 10 ml PBS, lysed with a scraper and

646 RIPA buffer [25 mM Tris-Cl pH7.6, 1% Nonidet P-40, 1% sodium deoxycholate,

647 0.1% SDS, 100 mM EDTA, 150 mM NaCl, protease inhibitor cocktail, RNase

648 inhibitors], and sonicated for 10 min at low intensity with Standard Bioruptor

649 Diagenode. After centrifugation (10 min, max speed, 4ºC), supernatants were

650 collected, precleared, and immunoprecipitated (4 h, 4ºC, on rotation) with 10 µg

651 anti-CPEB4 antibody (Abcam), and 50 µl Dynabeads Protein A (Invitrogen).

652 Beads were washed 4 times with cold RIPA buffer supplemented with Protease

653 inhibitors (Sigma-Aldrich), resuspended in 100 µl Proteinase-K buffer with 70 µg

654 Proteinase-K (Roche), and incubated for 60 min at 65ºC. RNA was extracted by

655 standard phenol-chloroform, followed by Turbo DNA-free Kit (Ambion) treatment.

656 **Sequencing and analysis.** Samples were processed at the IRB Barcelona

657 Functional Genomics Facility following standard procedures. Libraries were

658 sequenced by Illumina 100bp single-end and FastQ files for Input and IP samples

659 were aligned against the mouse reference genome mm10 with Bowtie2 version

660 2.2.2 (Langmead and Salzberg, 2012) in local mode accepting 1 mismatch in the

661 read seed and using default options. Alignments were sorted and indexed with

662 sambamba v0.5.1(Tarasov et al., 2015). Putative over-amplification artifacts

663 (duplicated reads) were assessed and removed with the same software.

664 Coverage tracks in TDF format for IGV were made using IGVTools2 (Tarasov et

665 al., 2015). Quality control assessment for unaligned and aligned reads was done

666 with FastQC 0.11 (Brown et al., 2017). Further quality control steps

667 (Gini/Lorenz/SSD IP enrichment, PCA) were performed in R with the htSeqTools

668 package version 1.12.0 (Planet et al., 2012). rRNA contamination was assessed

669 using the mm10 rRNA information available in the UCSC genome browser tables.

670 A preliminary peak calling process was performed for duplicate filtered

671 sequences with the MACS 1.4.2 software (Zhang et al., 2008) using default

672 options to assess overall enrichment in the IP sequenced samples against their

673 respective Input controls. The identified peaks were annotated against the mouse

674 reference genome mm10 annotation using the ChIPpeakAnno package version

675 2.16 (Zhu et al., 2010). Additionally, log2RPKM quantification for mm10 UCSC

676 3'-UTR genomic regions (Biomart ENSEMBL archive February 2014) in all

677 samples was obtained using custom R scripts, and potentially enriched 3'-UTRs

678 between wild-type and *Cpeb4*KO IPs were identified using the enrichedRegions

679 function from the htSeqTools package using default options and reporting

680 Benjamini-Yekutielli adjusted p-values (pvBY). Then, for each analyzed 3'-UTR

681 genomic region, the maximum peak score for overlapping MACS peaks was

682 retrieved (MaxScore). 3'-UTR regions without any overlapping MACS reported

683 peak were given a MaxScore value of 0. Finally, CPEB4-associated mRNAs were

684 defined as those with either (I) pvBY<0.02 and $log2_{RPKM}FC>0.5$; (II) pv<0.02 and

685 $log2_{RPKM}FC>1.2$ or (III) MaxScore>500 and $log2_{RPKM}FC>0.5$. Gene ontology

686 analysis was performed using the DAVID Functional Annotation Bioinformatics

687 Microarray Analysis (Dennis et al., 2003).

688 **RNA-immunoprecipitation and RT-qPCR.** RIP was performed as for RIP-

689 Seq but the obtained RNA was reverse-transcribed and analyzed by RT-qPCR

690 (see RNA analysis). The following antibodies were used: mouse monoclonal

691 CPEB4 (ab83009 Lot. GR95787-2, Abcam); HuR antibody (3A2, sc-5261

692 SantaCruz); and normal mouse IgG polyclonal antibody (12-371 Sigma). Protein

693 A/G beads were used in each case (Invitrogen).

694 **CPE-containing mRNAs.** For the analysis of CPE-A-containing mRNAs, the

695 script developed by Piqué et al. was run over mm10 3'-UTR reference sequences

696 (Biomart ENSEMBL archive February 2014). mRNAs containing a putative 3'-

697 UTR with a CPE-mediated repression and/or activation prediction were

698 considered CPE-containing mRNAs. For CPE-G-containing mRNAs, the same

699 script was adapted to mRNAs containing a putative 3'-UTR with the TTTTGT

700 motif within the optimal distances to the polyadenylation signal (PAS) established

701 by (Pique et al., 2008). As a control for the enrichment in CPE-containing mRNAs

702 in CPEB4 target lists, the BMDM transcriptome was defined as those transcripts

703 with > 10 reads in the corresponding wild-type input (untreated/LPS-stimulated

704 BMDMs).

705 **ARE-containing mRNAs**. For each gene, the reference sequence of the

706 longest 3'-UTR was selected. The number of AREs was calculated by scanning

707 the corresponding 3'-UTR and counting the number of occurrences of the ARE

708 motif (ATTTA) and overlapping versions (ATTTATTTA) up to 5 overlapping

709 motifs. Note that k overlapping motifs count as k ARE motif. ARE-containing

710 mRNAs were considered those with 2 or more ATTTA motifs.

711   **ARE/CPE score**. For computation of the ARE/CPE score, the reference

712   sequence of the longest 3'-UTR per gene was selected. Similarly to the number

713   of AREs, the number of CPEs was calculated by scanning the corresponding 3'-

714   UTR and counting the number of occurrences of all the CPE motifs ('TTTTAT',

715   'TTTTAAT', 'TTTTAAAT', 'TTTTACT', 'TTTTCAT', 'TTTTAAGT', 'TTTTGT'). A

716   version without considering the last motif yielded no significant differences. The

717   optimal distances to the polyadenylation signal (PAS) established by (Pique et

718   al., 2008) were not considered for this analysis.

719   **TTP and HuR target mRNAs.** Data were obtained from TTP and HuR PAR-

720   iCLIP experiments in LPS-stimulated BMDMs (Sedlyarov et al., 2016). For TTP,

721   mRNAs bound after 3 h or 6 h of LPS stimulation were considered. For HuR-

722   bound mRNAs, PAR-iCLIP data corresponded to 6 h of LPS stimulation. Only

723   mRNAs with HuR/TTP binding in the 3'-UTR were considered.

724   **ARE- and CPE-containing mRNAs.** The list of 1521 ARE- and CPE-

725   containing mRNAs contained HuR, TTP or CPEB4 target mRNAs that had at

726   least one ARE motif and one CPE motif in their 3'-UTR. mRNAs containing only

727   AREs or only CPEs were excluded.

728   **mRNA levels/stability in TTPMKO BMDMs.** Data were obtained from

729   (Sedlyarov et al., 2016). The statistical analysis of the publication was also

730   considered.

731   **Statistics.** Data are expressed as means±SEM. Dataset statistics were

732   analyzed using the GraphPad Prism software. For two group comparisons,

733   column statistics were calculated and based on Standard Deviation,

734   d'Agostino&Pearson normality test, parametric t test (assuming or not same SD)

735   or non-parametric t-test (Mann-Whitney) was performed. For multiple

comparisons, one-way ANOVA Kruskal Wallis test or two-way ANOVA followed by the Bonferroni post-hoc test was used. To assess mouse survival, Kaplan-Meier survival curves were computed with R2.15 and the survival package version 2.37-2 (Pique et al., 2008) using the likelihood-ratio test, adjusting by Gender, Weight and LPS dose. Fisher's exact test was used for contingency analysis. Differences under $p<0.05$ were considered statistically significant (*pv<0.05, **pv<0.01, ***pv<0.001; ****pv<0.0001). Differences under pv<0.1 are indicated as +. For animal studies, every effort was made to include more than five mice in each group. Animals were grouped in blocks to control for experimental variability. Furthermore, wild-type and knockout animals were bred in the same cages whenever possible and the experimenter was blinded until completion of the experimental analysis. Experiments were repeated independently with similar results, as indicated in the figure legends.

Data availability. RIP-seq and RNA-seq datasets are available in GEO (accession number GSE160191 and GSE160346, respectively). Correspondence and requests for materials or supporting raw data should be addressed to R.M. (raul.mendez@irbbarcelona.org).

SUPPLEMENTAL INFORMATION

Figure S1. Gene expression deconvolution analysis of blood samples from septic individuals and healthy patients. (A) Gene expression deconvolution was performed to estimate cell proportion contribution of blood samples from septic patients and healthy subjects. **(B)** *Cpeb4* mRNA expression in human immune cell lineages (Haemosphere data). **(C)** Volcano plots showing differential expression of Smith/Abbas myeloid and lymphoid signature genes (grey) and *Cpebs* (colored) in Septic patients vs. Healthy subjects. Horizontal red

761 dashed lines represent a pvadj of 0.05. Vertical grey dashed lines represent

762 log2FC of -1 or 1 thresholds. **(D)** p-values for *Cpeb4* differential expression

763 between septic patients and healthy subjects. Gene expression deconvolution

764 was performed to adjust for the different estimated cell proportions between

765 septic and healthy individuals.

766 **Figure S2. Inflammation resolution is impaired in *Cpeb4*KO**

767 **macrophages. (A)** Z-score signature of the indicated pathways in wild-type and

768 *Cpeb4*KO BMDMs stimulated with LPS. mRNA levels were quantified by

769 RNASeq (n=4). Statistics: rotation gene set enrichment analysis. See also Table

770 S1. **(B)** *Il10* mRNA levels in wild-type and *Cpeb4*KO BMDMs stimulated with LPS.

771 mRNA levels were measured by RT-qPCR, normalizing to *Tbp* (n=6). Statistics:

772 Two-way ANOVA.

773 **Figure S3. The p38α-HuR-TTP axis regulates *Cpeb4* mRNA stability. (A)**

774 BMDMs were stimulated with LPS and mRNA levels were measured by RT-

775 qPCR, normalizing to *Tbp* (n=6). *Cpeb4* mRNA values are also shown in Fig. 2a.

776 **(B)** Schematic representation of the *Cpeb4* 3'-UTR showing ARE domains. **(C)**

777 HuR immunoprecipitation (IP) was performed in wild-type and p38αMKO BMDMs

778 stimulated with LPS for 3 h when indicated. IgG IP was used as control. **(D)** TTP

779 PAR-iCLIP was performed in BMDMs treated with LPS for 6 h. Coverage plots

780 represent the number of crosslink sites (CL) detected in each position of *Cpeb4*

781 mRNA. For binding sites located in the *Cpeb4* 3'UTR, distance to the

782 Transcription Start Site (TSS) and their scores are indicated (data from Sedlyarov

783 et al., 2016). **E-G** U2OS cells, infected with shHuR (03, 04) or shCTR (-), were

784 treated with tetracyline to induce the expression of a constitutively active MKK6,

785 which induces p38 MAPK activation[2]. **(E)** HuR mRNA expression was measured

786 by RT-qPCR, normalizing to *Gapdh* (n=1). **(F)** HuR immunoblot, vinculin served

787 as loading control. **(G)** CPEB4 and HuR immunoblot after p38 MAPK activation

788 (+MKK6) in cells infected with shHuR or shCTR. Vinculin served as loading

789 control. Quantification is shown.

790 **Figure S4. CPEB4 stabilizes mRNAs encoding negative feedback**

791 **regulators of the LPS response. A-C** CPEB4 RNA-Immunoprecipitation (IP)

792 and sequencing was performed in total lysates (Input) from wild-type and

793 *Cpeb4*KO BMDMs, untreated or stimulated with LPS for 9 h (n=1). **(A)** CPE-G-

794 containing transcripts in Inputs and CPEB4 IPs. The script from Piqué et al., 2008,

795 was modified to consider TTTTGT as a CPE motif. Statistics: Fisher's exact test.

796 **(B)** CPE-A- or CPE-G-containing transcripts in Inputs and CPEB4 IPs. Statistics

797 with Fisher's exact test. **(C)** Top 10 Gene Ontology KEGG categories enriched in

798 CPEB4 target mRNAs in wild-type BMDMs stimulated with LPS for 9 h. *Mus*

799 *musculus* transcriptome was used as background. Statistics: Benjamini-

800 Hochberg adjusted p-value is shown. See also Table S2.

801 **Figure S5. The equilibrium between CPEB4/CPEs and TTP/AREs defines**

802 **mRNA oscillation patterns. (A)** ARE/CPE score definition. ARE and CPE motifs

803 used to calculate the score are specified. **B-E** Wild-type BMDMs were treated

804 with LPS and mRNA levels were quantified by RNASeq (n=4). **B-D** Common

805 TTP and CPEB4 target mRNAs were considered. **(B)** ARE-dominant (AREd, red)

806 and CPE-dominant (CPEd, blue) mRNA levels after 3 h of LPS stimulation,

807 normalized for its expression at 1 h. Statistics: Mann-Whitney t test. **(C)** Mean

808 expression profile of CPEd and AREd mRNAs in LPS-stimulated BMDMs. For

809 each mRNA, values were normalized to its peak of expression. Statistics: Two-

810 way ANOVA. **(D)** mRNAs were plotted according to the number of AREs in the

811 3'-UTR as ARE^{high} (>4 AREs) or ARE^{low} ($\leq$4 AREs) mRNAs. Mean mRNA

812 expression profile of ARE^{high} and ARE^{low} mRNAs in LPS-stimulated BMDMs. For

813 each mRNA, values were normalized to its peak of expression. **(G)** 1521 CPE-

814 and ARE-containing mRNAs were classified as Sustained>0.5 or

815 Downregulated<0.5, on the basis of their expression after 9 h of LPS treatment,

816 normalized by their peak of expression during the LPS response. For each

817 mRNA, the number of AREs in its 3'-UTR was calculated.   **(H)** RAW 264,7

818 macrophages were transfected with a Firefly/Renilla luciferase reporter under the

819 control of six different 3'-UTR with distinct ARE/CPE scores (See Figure 5J-K).

820 Then, macrophages were stimulated with LPS and mRNA expression was

821 analyzed by RT-qPCR. Heatmap shows the Firefly/Renilla expression ratio.

822 Statistics: Two-way ANOVA vs. 3CPEs/0AREs. **(B-C, E-F)** Data are represented

823 as mean ± SEM. See also Tables S2, S3, S4, S5, and S6.

824 **SUPPLEMENTARY TABLES**

825 **Table S1. RNAseq Wild-Type_vs_*Cpeb4*KO BMDMs.** Wild-type and

826 *Cpeb4*KO BMDMs were stimulated with LPS and mRNA levels were quantified

827 by RNASeq (n=4). Differential expression between genotype conditions was

828 performed with Deseq2 R package. Wild-type and *Cpeb4*KO samples were

829 compared for each time point independently.

830 **Table S2. RIPseq defined CPEB4 target mRNAs.** Wild-type and *Cpeb4*KO

831 BMDMs were left untreated or stimulated with LPS for 9 h. Immunoprecipitation

832 (IP) with anti-CPEB4 antibody was then performed, and RNA was extracted and

833   analyzed by RNAseq. CPEB4 targets were defined based on the enrichment

834   between wild-type and *Cpeb4*KO IPs.

835   **Table S3. Genome-wide AREs, CPEs and ARE/CPE Score.** For each gene,

836   the reference sequence of the longest 3'-UTR was selected. The number of AREs

837   and CPEs was calculated by scanning the corresponding 3'-UTR and counting

838   the number of occurrences of each motif. The ARE/CPE score was calculated as

839   the log2 transformed ratio between the number of ARE and CPE motifs.

840   **Table S4. TTP and HuR target mRNAs.** Data were obtained from TTP and

841   HuR PAR-iCLIP experiments in LPS-stimulated BMDMs[1]. Only mRNAs with

842   HuR/TTP binding in the 3'-UTR were considered. For TTP, mRNAs bound after

843   3 h or 6 h of LPS stimulation were considered. For HuR-bound mRNAs, PAR-

844   iCLIP data corresponded to 6 h of LPS stimulation.

845   **Table S5. RNAseq Wild Type BMDMs.** Wild-type BMDMs were stimulated

846   with LPS, and mRNA levels were quantified by RNASeq (n=4). Wild-type and

847   *Cpeb4*KO samples were compared for each time point independently. The

848   expression pattern over time for wild-type samples was analyzed (differential

849   expression results against consecutive time points and Rlog data normalized by

850   maximum).

851   **Table S6. ARE- and CPE-containing mRNAs.** ARE- and CPE-containing

852   mRNAs were defined as all mRNAs regulated by CPEB4, TTP or HuR in LPS-

853   stimulated BMDMs (See also Table S2 and Table S4).

854

855

RT-qPCR primers	Forward	Reverse
dtVNN		ccccccccccccccccccVNN
Firefly 1	gggacgaagacgaacacttc	ggaaagacgatgacggaaaa
Firefly 2	ctcgcatgccagagatccta	ctgaggggatcgtaaaaacagc
hCpeb4	aggtgtttgtaggcggattg	aacagcaggaatgcatagcc
hElavl1 (HuR)	ccgtcaccaatgtgaaagtg	tcgcggcttcttcatagttt
hGapdh	cgctctctgctcctcctgtt	ccatggtgtctgagcgatgt
m18S	cgcttccttacctggttgat	gagcgaccaaaggaaccata
mArg1	acaagacagggctcctttcag	ggcttatggttaccctcccg
mCcl2	agctgtagttttttgtcaccaagc	gtgctgaagaccttagggca
mCpeb1	cgctgcaaagaggtacaggt	caaggcaccaacaaacacc
mCpeb2	tggagcaaccatcagaacag	cgagtgaatttcggtggtg
mCpeb3	cgtttgtacggtggtgtttg	ccgtttgtcaatgtcgttgt
mCpeb4	ccagaatggggagagagtgg	cggaaactagctgtgatctcatct
mCxcl1	acccaaaccgaagtcatagcc	ttgtcagaagccagcgttc
mCxcl2	tttgccttgaccctgaagcc	ctttggttcttccgttgaggga
mDusp1	gcgctccactcaagtcttct	ctgtttgctgcacagctcag
mGapdh	cttcaccaccatggaggaggc	ggcatggactgtggtcatgag
mHif1a	tttggcagcgatgacacag	aagtggctttggagtttccg
mIl10	ggttgccaagccttatcgga	gagaaatcgatgacagcgcc
mIl1a	gagagccgggtgacagtatc	tgacaaacttctgcctgacg
mIl1rn	tccgcttctgacagtggaac	tcccagattctgaaggcttgc
mIl6	gccttcttgggactgatgct	tgccattgcacaactcttttct
mPtgs2	tgagtaccgcaaacgcttct	cagccatttccttctctcctgt
mRplp0	ccgcaggggcagcagtggt	aagcgcgtcctggattgtct
mSocs1	cgctccttggggtctgtt	gagatcgcattgtcggct
mSocs3	ttcgggactagctccccgggat	tttggagctgaaggtcttga
mTbp	agaacaatccagactagcagca	gggaacttcacatcacagctc
mTnf	ctatggcccagaccctcacactc	gctggcaccactagttggttgtctt
mTxnip	ggacgatgtggacgactctc	ggggcatgaactggaactca
Renilla	accagatttgcctgatttgc	gataactggtccgcagtggt

856

857   **Table S7. Oligos Suñer *et al*.** Primers used for RT-qPCR analysis.

858

859

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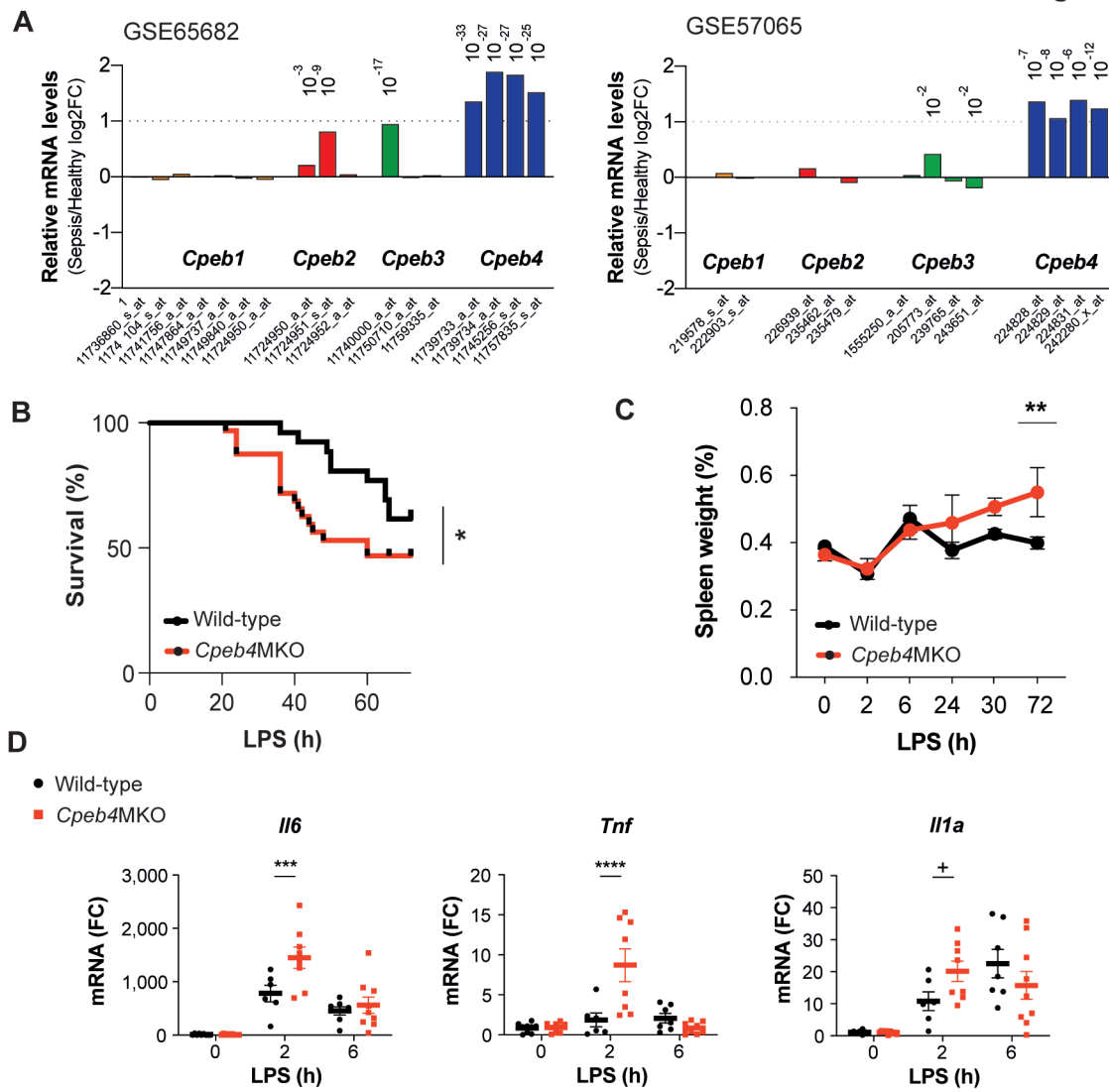Youssif, C., Cubillos-Rojas, M., Comalada, M., Llonch, E., Perna, C., Djouder, N., and Nebreda, A.R. (2018). Myeloid p38alpha signaling promotes intestinal IGF-1 production and inflammation-associated tumorigenesis. EMBO molecular medicine *10*, e8403.

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Suñer et al. **Figure 2**
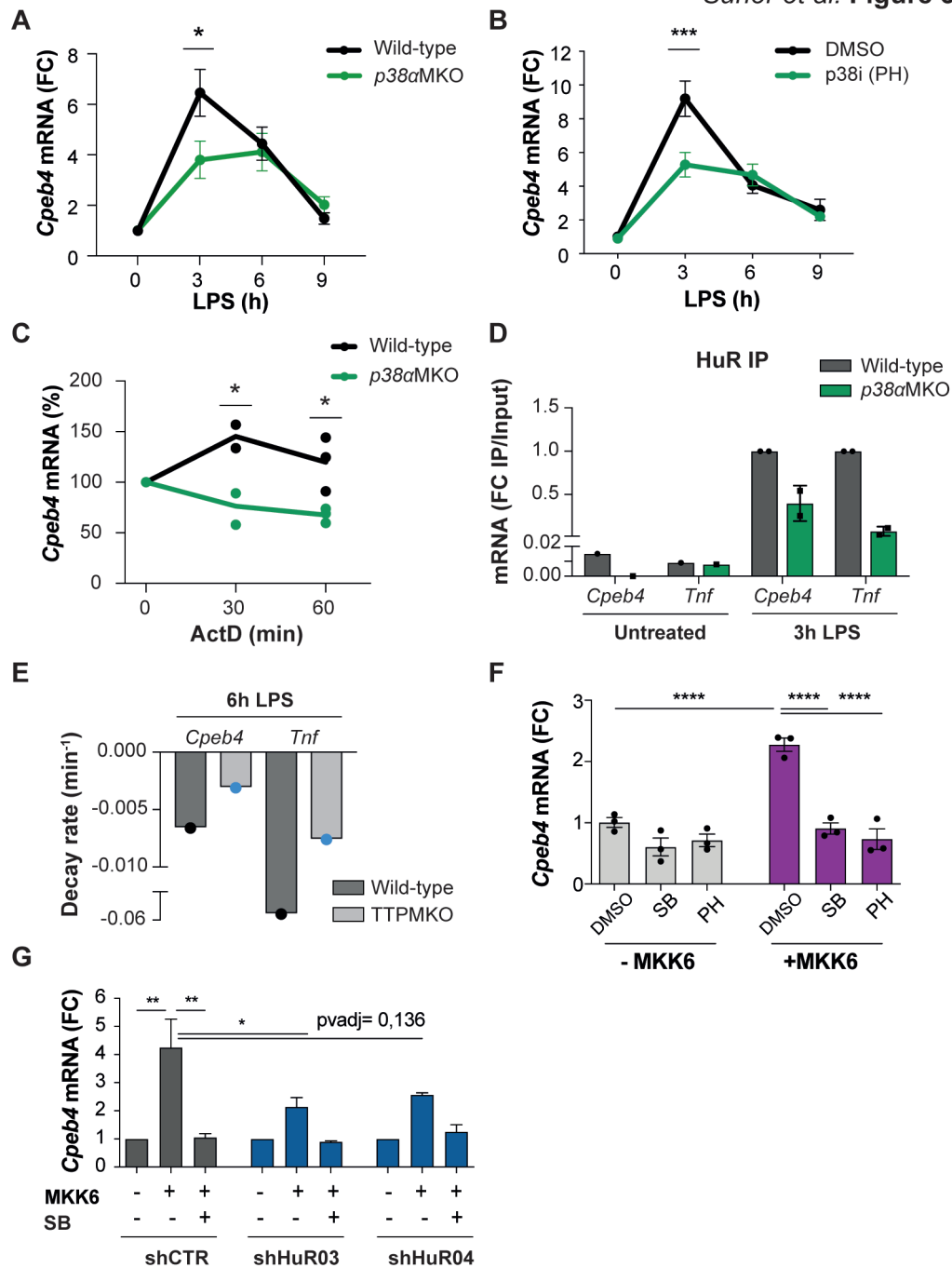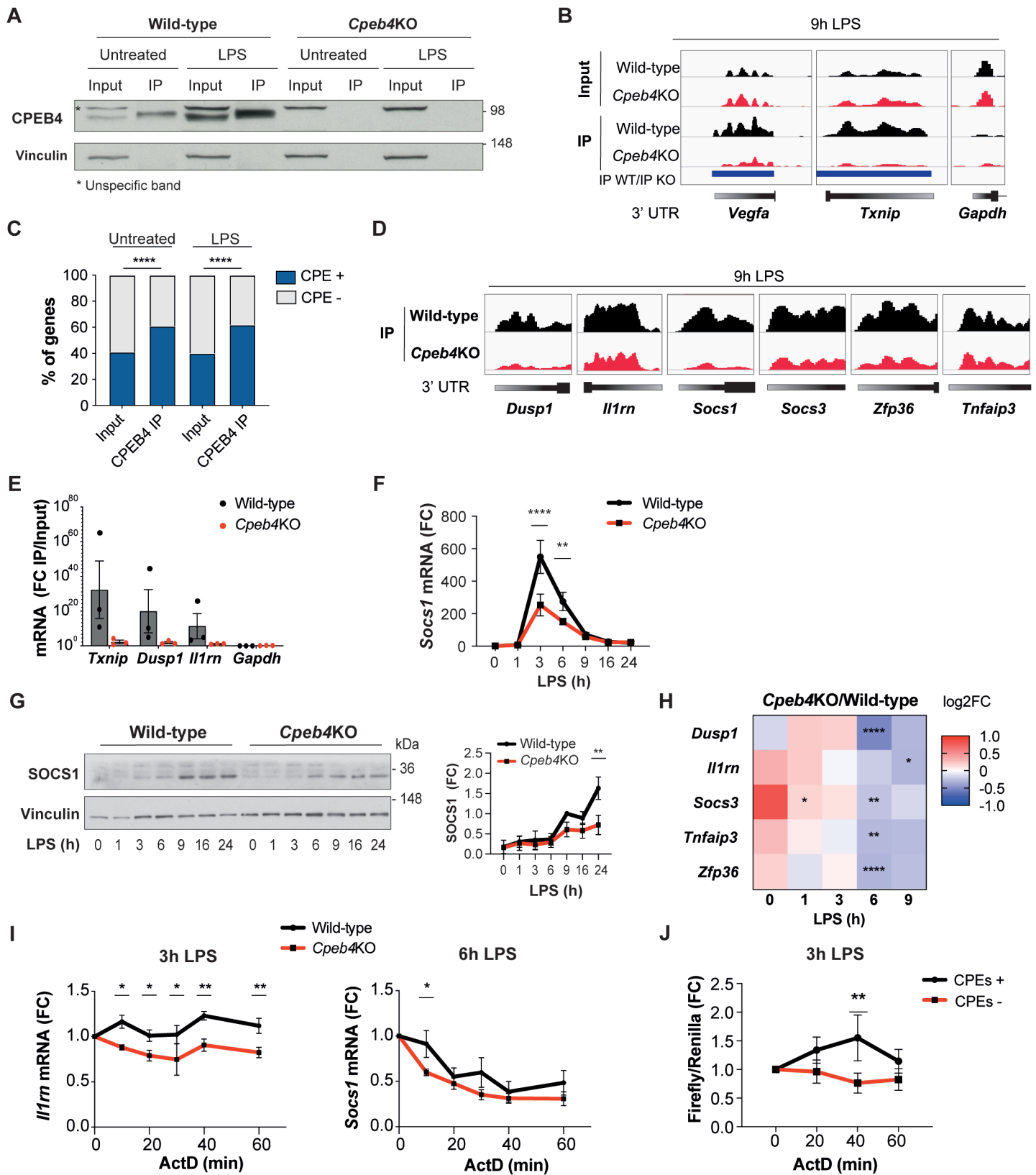
A

B

* Unspecific bands

A

GSE65682

GSE57065

Healthy subjects / Sepsis patients

B

Haemosphere data

Cpeb4 mRNA expression (FC to average)

C

Myeloid probes

GSE65682 / GSE57065

Cpeb1 / *Cpeb2* / *Cpeb3* / *Cpeb4*

Lymphoid probes

GSE65682 / GSE57065

D

Cell type	pvadj	
	GSE65682	GSE57065
Th	<2.2e-16	<2.2e-16
Tc	<2.2e-16	<2.2e-16
B	<2.2e-16	<2.2e-16
PC	<2.2e-16	<2.2e-16
NK	<2.2e-16	<2.2e-16
NK act	<2.2e-16	<2.2e-16
Mono	<2.2e-16	10^{-16}
Mono act	<2.2e-16	<2.2e-16
DC	<2.2e-16	<2.2e-16
DC act	<2.2e-16	<2.2e-16
Neutro	<2.2e-16	<2.2e-16

A

Untreated   LPS

B

Untreated   LPS

C CPEB4-associated mRNAs

A

ARE/CPE Score = $\log_2$(nº of AREs/nº of CPEs)

AREs: ATTTA

CPEs: TTTTAT   TTTTACT   TTTTGT
          TTTTAAT   TTTTCAT
          TTTTAAAT TTTTAAGT

B

C

D

E

F