The metabolic cofactor Coenzyme A enhances alternative macrophage activation via MyD88-linked signaling

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Running Title: *CoA enhances alternative activation via MyD88*

ABSTRACT

Metabolites and metabolic co-factors can shape the innate immune response, though the pathways by which these molecules adjust inflammation remain incompletely understood. Here we show that the metabolic cofactor Coenzyme A (CoA) enhances IL-4 driven alternative macrophage activation [m(IL-4)] *in vitro* and *in vivo*. Unexpectedly, we found that perturbations in intracellular CoA metabolism did not influence m(IL-4) differentiation. Rather, we discovered that exogenous CoA provides a weak TLR4 signal which primes macrophages for increased receptivity to IL-4 signals and resolution of inflammation via MyD88. Mechanistic studies revealed MyD88-linked signals prime for IL-4 responsiveness, in part, by reshaping chromatin accessibility to enhance transcription of IL-4-linked genes. The results identify CoA as a host metabolic co-factor that influences macrophage function through an extrinsic TLR4-dependent mechanism, and suggests that damage-associated molecular patterns (DAMPs) can prime macrophages for alternative activation and resolution of inflammation.

INTRODUCTION

Macrophages are innate immune cells that execute a variety of functions such as detecting and removing foreign pathogens, instructing adaptive immune cell function, secreting cytokines, and maintaining tissue homeostasis. To fulfill these diverse roles, macrophages link the detection of distinct external cues with the engagement of specific transcriptional programs to support different functional states. This process is commonly termed macrophage activation or 'polarization'[1,2].

In vitro studies often consider macrophage polarization in two discrete states: classical (Type 1) and alternative (Type 2) activation[3]. Classical activation is associated with antimicrobial immunity, and occurs when macrophages detect pathogen-associated molecular patterns (PAMPs) such as lipopolysaccharide (LPS) or damage-associated molecular patterns (DAMPs). Alternative activation is associated with wound healing along with allergen and helminth immunity, and is initiated by the macrophage response to interleukin-4 (IL-4) ± IL-13.

Along with transcriptional programs that support core macrophage functions such as cytokine secretion and phagocytosis, changes in cellular metabolism are also important and perhaps essential for macrophage effector function[4-6]. Upon classical activation with LPS, macrophages increase glycolysis and repurpose mitochondria away from oxidative phosphorylation and towards the generation of metabolic signals thought to amplify the pro-inflammatory response[7-10]. Conversely, alternatively activated macrophages (AAMs) display increased rates of oxidative phosphorylation and fatty acid oxidation[11,12], though evidence is mixed as to whether these changes are essential for the macrophage IL-4 response [m(IL-4)][13-15]. The mechanistic links between how increased oxidative phosphorylation and/or fatty acid oxidation could specifically support m(IL-4) are also unclear. Proposed mechanisms, though, include changes in histone acetylation from enhanced acetyl CoA production[16] as well as transcriptional changes that respond to the mitochondrial membrane potential[17]

Previous work in our laboratory has associated intracellular levels of CoA with the macrophage IL-4 response[13,18]. When trying to identify why excess concentrations of the CPT-1 inhibitor etomoxir blocked m(IL-4) but genetic ablation of either *Cpt1* or *Cpt2* did not[14,19], we discovered that excess etomoxir disrupted macrophage CoA homeostasis. In support of this as a putative mechanism, provision of exogenous CoA restored both intracellular CoA levels as well as the expression of AAM-associated cell surface markers[13]. However, precisely how CoA instructs macrophage activation was not studied.

Here we demonstrate that CoA augments AAM function via weak toll-like receptor 4 (TLR4) agonism and myeloid differentiation primary response protein 88 (MyD88)-linked

signaling, a pathway commonly associated with classical or pro-inflammatory activation. In investigating the mechanism by which exogenous CoA regulates m(IL-4), we surprisingly discovered that CoA provision did not act either by changing intracellular CoA levels or by enhancing known metabolic hallmarks of the IL-4 response. Rather, pharmacologic and genetic approaches showed exogenous CoA is a weak TLR4 agonist and boosts m(IL-4) by activating MyD88-linked signaling. MyD88 agonism was sufficient to enhance *in vitro* and *in vivo* alternative activation and increase chromatin accessibility at the promoter regions of IL-4-target genes. The data show that (i) CoA is a TLR4 agonist, (ii) many of the metabolic hallmarks of the m(IL-4) do not always correlate with anti-inflammatory activation, and (iii) pro-inflammatory MyD88-linked signaling can support AAM function and resolution of inflammation. Furthermore, the results indicate CoA can act as a damage-associated molecular pattern (DAMP) that primes macrophages for the resolution of inflammation by an extrinsic TLR4-dependent mechanism.

RESULTS

Exogenous CoA provision enhances alternative macrophage activation in vitro and in vivo

Prior work had shown that exogenous CoA could rescue the inhibition of m(IL-4) by etomoxir, but the mechanisms underlying this effect were not defined. To better understand how exogenous CoA influenced alternative macrophage activation, mouse bone marrow-derived macrophages (BMDMs) were treated with IL-4 alone or in combination with CoA for 48 hr. Gene expression studies revealed that CoA enhanced the expression of multiple IL-4-associated genes, including *Mgl2, Pdcd1gl2, Fizz1, Chil4, Ccl8, Arg1, and Mrc1* (Figure 1a and Supplemental Fig 1a)[17,20-22]. Flow cytometry analysis revealed a similar relationship when measuring IL-4-linked cell surface marker expression. CoA increased the mean fluorescence intensity of CD206 and CD301, as well as the frequency of $CD206^+/CD71^+$ and $CD206^+/CD301^+$ BMDMs (Figs. 1b-d). This effect was observed with CoA concentrations as low as 62.5μM (Supplemental Fig. S1b). Importantly, CoA itself did not stimulate the expression of IL-4-associated genes or cell surface markers (Figs. 1a-d), demonstrating it is not an IL-4 receptor agonist but rather acts cooperatively to enhance AAM differentiation.

Mannose receptor activity is essential for the initiation of the T_H2 response during the helminth infection[23,24]. Given the observed increase in its gene (*Mrc1*) and protein (CD206) expression, we measured the effect of exogenous CoA on activity of the mannose receptor. As expected, high-content imaging revealed CoA enhanced the cellular uptake of FITC-dextran, a fluorescently labeled polysaccharide and mannose receptor ligand[25] (Figs. 1e&f).

125 To assess whether CoA could enhance alternative activation *in vivo,* mice were injected
126 with IL-4 complex (IL-4c) i.p. in the presence or absence of CoA (40 mg/kg). After one day, the
127 peritoneum was flushed and the frequency of $CD206^{+}/CD71^{+}$ peritoneal macrophages assessed.
128 Indeed, CoA enhanced the fraction of AAMs co-expressing CD206 and CD71 (Figure 1g),
129 demonstrating that it enhances IL-4-mediated AAM differentiation *in vitro* and *in vivo*.
130
131 *CoA does not augment alternative macrophage activation by enhancing metabolic hallmarks of*
132 *M(IL-4)*
133 We next sought to identify the mechanism by which CoA enhances m(IL-4). Several
134 metabolic hallmarks of AAMs require CoA as a necessary cofactor, including enhanced
135 mitochondrial respiratory capacity[26-28], mitochondrial pyruvate oxidation[12,29], and *de novo* lipid
136 synthesis[30,31]. As such, we hypothesized that addition of exogenous CoA enhanced the IL-4
137 response by increasing intracellular CoA levels to support flux through these metabolic
138 pathways[32-34].
139 We first confirmed that exogenously added CoA could expand the cellular CoA pool.
140 Supplementing culture medium with CoA increased the steady-state abundance of both
141 intracellular CoA and acetyl CoA (Fig. 2a). Next, we investigated whether CoA provision
142 enhanced IL-4-driven increases in respiration and glycolysis induced by IL-4[12,26,30]. Similar to
143 other reports, we observed increases in ATP-linked respiration, maximal respiratory capacity, and
144 glycolysis with IL-4. However, addition of CoA did not further augment these metabolic changes,
145 and even limited the maximal respiratory capacity of AAMs (Figs. 2b-d).
146 After determining that CoA does not enhance alternative macrophage activation via an
147 expansion of bioenergetic capacity, we then examined whether CoA affected other IL-4-linked
148 metabolic alterations such as increased abundance of TCA cycle metabolites[29] or enhanced
149 pyruvate oxidation[12], glutamine oxidation[29], and *de novo* lipogenesis[31]. Indeed, we reproduced
150 previous reports that show IL-4 increases steady-state levels of select TCA cycle metabolites
151 (Figs. 2e), enrichment from glucose into the TCA cycle (Fig. 2f), and *de novo* lipid synthesis (Fig.
152 2g). However, as before, addition of CoA did not further increase these metabolic changes (Figs.
153 2e-g, S2a-c), and even blocked IL-4-stimulated increases in lipogenesis (Figs. 2g, S2d). As such,
154 the results show exogenous CoA augments alternative activation by a mechanism discrete from
155 reprogramming metabolism.
156
157
158

159 *Alterations in intracellular CoA levels are not sufficient to alter M(IL-4)*

160 We then questioned whether changes in intracellular CoA levels, in fact, shape the IL-4
161 response. To answer this, we utilized two compounds with opposing impacts on intracellular CoA
162 levels. To decrease steady-state CoA levels, we treated IL-4-stimulated BMDMs with
163 cyclopropane carboxylic acid (CPCA), which decreases the abundance of "free" CoA as its
164 cognate thioester CPC-CoA is formed[18,35] (Fig. 2h). To increase steady-state CoA levels, we
165 treated IL-4-polarized BMDMs with PZ-2891, a pantothenate kinase agonist which relieves
166 inhibition of CoA biosynthesis[36] (Fig. 2h). As expected, CPCA decreased intracellular levels of
167 CoA and acetyl CoA, while PZ-2891 increased their abundance (Fig. 2i, S2e). Surprisingly,
168 despite altering steady-state intracellular CoA levels, neither compound impacted alternative
169 activation (Fig. 2j). The results show that although exogenous CoA provision augments the
170 macrophage IL-4 response, the mechanism cannot be attributed to changing intracellular levels
171 of CoA and acetyl CoA.

172

173 *Exogenous CoA induces a macrophage pro-inflammatory response in vitro and in vivo*

174 Neither metabolic alterations nor intracellular CoA levels could explain why CoA provision
175 enhanced m(IL-4). Therefore, we sought a more complete understanding of how CoA impacts the
176 transcriptome of AAMs by conducting bulk RNA sequencing (RNA-seq) on naïve macrophages
177 alongside those that were stimulated with either IL-4 or IL-4 with CoA. As expected, cells
178 stimulated only with IL-4 increased the expression of genes associated with alternative activation
179 and decreased expression of genes associated with classical activation relative to vehicle
180 controls. In line with qPCR studies (Fig. 1a), CoA provision further increased the expression of
181 IL-4-linked genes associated with alternative activation (Fig. 3a, *right*). Unexpectedly, however,
182 CoA addition also increased the expression of genes associated with classical activation that
183 were not associated with the IL-4 response (Fig. 3a, *right*). Subsequent Gene Set Enrichment
184 Analysis (GSEA) showed that genes associated with TLR signaling pathways were upregulated
185 upon co-treatment with CoA and IL-4 (Fig. 3b).

186 This unbiased approach suggested that CoA may elicit a pro-inflammatory response in
187 BMDMs along with its ability to enhance m(IL-4). To confirm this, we assessed whether CoA itself
188 could induce expression of pro-inflammatory genes in the absence of IL-4. Indeed, exogenous
189 CoA induced expression of *Il1b*, *Tnf*, *Nos2*, and *Irg1* (Fig. 3c), genes linked to the TLR adaptor
190 protein MyD88, as well as the interferon-stimulated gene (ISG) *Mx1* (Fig. 3d). As *Irg1* encodes
191 the enzyme generating the anti-microbial metabolite itaconate, we also observed a ~10-fold
192 increase in itaconate synthesis upon CoA provision (Fig. 3e). Lastly, i.p. administration of CoA in

193 mice increased expression of *Il1b* and *Tnf* in peritoneal leukocytes and increased the abundance
194 of IL-1B, TNF-α, IL-6, and CXCL1 in the peritoneal lavage fluid (Figure 3f&g). Taken together, the
195 results demonstrate that CoA, in addition to enhancing the IL-4 response, elicits a pro-
196 inflammatory response *in vitro* and *in vivo*.
197
198 *CoA is a weak TLR4 agonist*
199 We then hypothesized that CoA could act as an agonist for a specific TLR. Since CoA
200 stimulated the expression of multiple pro-inflammatory genes linked to MyD88 – a signaling
201 adaptor protein necessary for full activation of several murine toll-like receptors[37] (Fig. 3c) – we
202 examined whether the pro-inflammatory response from CoA could persist in BMDMs lacking
203 MyD88. BMDMs harvested from *Myd88*$^{-/-}$ mice significantly reduced the expression of pro-
204 inflammatory genes induced by CoA, but marginal expression of *Il1b*, *Tnfa*, and *Irg1* persisted
205 (Fig. 4a). The result suggested both MyD88-dependent and -independent signaling cascades
206 underlie the pro-inflammatory response.
207 We therefore hypothesized that CoA was a TLR4 agonist. Toll-like receptor 4 (TLR4)
208 elicits its inflammatory response by activating both MyD88-dependent and -independent signaling
209 arms[38]. The MyD88-dependent pathway of TLR4 causes increased expression of cytokines such
210 as *Il1b*[39], while the TIR-domain-containing adapter-inducing interferon-β (TRIF) pathway is
211 independent of MyD88 and increases expression of ISGs and production of type 1 interferons[40,41].
212 Indeed, previous results showed CoA supplementation stimulated expression of the ISG *Mx1*
213 (Fig. 3d).
214 To determine if CoA is a TLR4 agonist, we utilized a reporter cell line which secretes
215 alkaline phosphatase in response to TLR agonism[42]. Addition of CoA activated a cell line
216 expressing human TLR4 (hTLR4) (Fig. 4b, c) but no effect was observed in cells expressing
217 hTLR2 or hTLR7, other Myd88-linked TLRs (Fig. 4c, S3a). Interpolation of an LPS standard curve
218 showed that 1mM CoA had a comparable effect to 0.1ng/mL LPS (Fig. 4b), indicating CoA is a
219 relatively weak TLR4 agonist. BMDMs harvested from *Tlr4*$^{-/-}$ mice further confirmed that CoA acts
220 via TLR4, as CoA did not increase expression of pro-inflammatory genes (Fig. 4d) or production
221 or itaconate (Fig. 4e) in TLR4-deficient macrophages.
222 It was next essential to confirm that the pro-inflammatory response was due to CoA itself
223 rather than an impurity from the >85% pure, yeast-derived CoA used in this study. In support of a
224 direct effect of CoA, cells treated with 99% pure, synthetically-derived CoA elicited a more potent
225 pro-inflammatory response relative to biologically-derived CoA (Fig. S3b,c). Furthermore, both
226 the yeast-derived and synthetically-derived CoA were free of endotoxin as determined by a

227 Limulus test (Fig. S3d). In total, the data demonstrate that CoA directly induces an inflammatory
228 response by acting as a weak TLR4 agonist.
229
230 *Myd88-linked TLR agonists enhance the IL-4 response*
231 Next, we asked which signaling cascade downstream of TLR4 was mediating the
232 enhanced AAM differentiation. Although previous studies have shown that exposure to LPS and
233 interferon gamma (IFN-γ) inhibits the acquisition of m(IL-4)[26,43], much of this work has used high
234 concentrations of LPS that correspond to effects that are orders of magnitude greater than that of
235 CoA (calibrated to 0.1 ng/mL; Fig. 4b) and activate both MyD88 and TRIF.
236 We therefore activated macrophages with IL-4 and other TLR ligands that activate either
237 MyD88- or TRIF-dependent signaling. Co-treatment with the MyD88-linked TLR2 agonist
238 Pam3CSK4 (Pam3) increased the population of $CD206^+/CD301^+$ BMDMs, whereas this
239 population was decreased upon co-treatment with the TRIF-linked TLR3 agonist Poly (I:C) (Figs.
240 5a&b). We also stimulated cells with IL-4 in combination with 0.1ng/mL LPS, and indeed observed
241 increased expression of IL-4 dependent cell-surface markers (Figs. 5a&b). The TLR5 agonist
242 flagellin and the TLR7 agonist imiquimod, both of which are upstream of MyD88, also increased
243 expression of these IL-4-linked cell surface markers.
244 Indeed, LPS (0.1 ng/mL) and Pam3 also increased expression of IL-4-associated genes
245 (Fig. 5c) and mannose receptor activity (Fig. 5d). As with cell surface marker expression, Poly
246 (I:C) co-treatment lowered the expression of IL-4-stimulated genes. To definitively show that
247 MyD88-linked signaling could affect AAM differentiation, we examined whether a low
248 concentration of LPS or Pam3 could enhance the IL-4 response in the absence of MyD88. As
249 expected, the effect of LPS and Pam3 on IL-4-associated cell-surface markers (Fig. 5e) and
250 genes (Fig. 5f) was lost in BMDMs isolated from *Myd88*$^{-/-}$ mice. Unexpectedly, both CoA and
251 imiquimod still enhanced the IL-4 response even in the absence of MyD88 (Fig. S4). However,
252 both compounds contain adenine-like moieties, and may interact with additional plasma
253 membrane receptors that affect the IL-4 response independently of MyD88-linked signaling.
254 Lastly, we determined whether MyD88 agonists could improve M(IL-4) *in vivo* using two
255 independent approaches. First, intraperitoneal injections of either 125µg of LPS or 25µg Pam3
256 prior to IL-4 complex increased the number of $CD206^+/CD301^+$ cells harvested from the peritoneal
257 cavity, whereas no difference was observed with Poly I:C. (Fig. 5g). As further proof-of-concept,
258 we leveraged a tumor model where alternative macrophage activation supports the growth of
259 implanted B16 melanoma tumors[15,44]. In line with our previous results, co-treatment of IL-4-
260 stimulated BMDMs with Pam3 resulted in significantly larger tumors when mixed with B16

261 melanoma cells relative to IL-4 alone (Fig. 5h). In total, these results indicate that activation of the
262 MyD88 pathway enhances alternative macrophage activation *in vitro* and *in vivo*.
263
264 *MyD88 alters the chromatin accessibility of alternatively activated macrophages*
265 Recent studies have highlighted the critical role of epigenetic remodeling when
266 macrophages are exposed to a mix of pro- and anti-inflammatory ligands[45,46]. We therefore
267 hypothesized that one mechanism by which MyD88 signaling could augment m(IL-4) was by
268 increasing chromatin accessibility in the promoter regions of IL-4 target genes. To test this, we
269 conducted an Assay for Transposase-Accessible Chromatin with high-throughput sequencing
270 (ATAC-seq) analysis on IL-4-stimulated BMDMs with or without co-stimulation with Pam3. We
271 first generated a list of the 10,878 genomic regions which had increased accessibility following
272 IL-4 stimulation [$\log_2$ fold change (LFC) >1, false discovery rate (FDR) <0.01] relative to vehicle
273 controls. These regions were localized mainly in intergenic and intronic regions (Fig. 6a, S5a).
274 We next assessed whether these IL-4-induced regions had increased accessibility upon
275 co-treatment with Pam3 by creating 10 equal bins in increasing order of LFC values (Fig. 6a,
276 S5b). The analysis revealed over 30% of the IL-4-induced regions were more accessible with
277 TLR2 co-treatment (Bins 8-10; Fig. 6b). To identify potential transcription factors that may mediate
278 the ability of MyD88 to increase alternative activation, we then conducted HOMER transcription
279 factor motif analysis[47]. The analysis indicated that these IL-4-induced regions with increased
280 accessibility following Pam3 co-treatment (Bins 8-10) were enriched for STAT6 and Jun/AP-1
281 binding motifs (Fig. 6b). Moreover, when we expanded our analysis to consider all regions
282 significantly increased by Pam3 (LFC >0.5 and FDR <.05, n = 1766), we noted that these regions
283 were enriched with motifs for the AP-1 subunit *Fra1*, *Stat6* and *Egr2*. (Fig. 6c). Consistent with
284 our hypothesis, the promoter regions of *Chil4* and *Ccl8,* genes we previously associated with the
285 IL-4 response (Fig. 1a), were significantly more accessible upon exposure to Pam3 (Fig. 6d).
286 Additionally, both had significantly more accessible Jun/AP-1 binding motifs following Pam3 co-
287 treatment. Other genes associated with alternative activation such as *Pdcd1gl2* and *Arg1* also
288 had consistent, though not statistically significant, increases in accessibility of their promoter
289 regions following Pam3 co-treatment (Fig. S5c). In total, ATAC-Seq analysis shows that MyD88
290 activation regulates chromatin accessibility in AAMs. Further, the data identify the Jun/AP-1 family
291 of transcription factors as candidates that may mediate the synergy between the MyD88 pathway
292 and the IL-4 response.
293
294

295 **DISCUSSION**

296 Our results demonstrate that the ubiquitous metabolic cofactor CoA enhances m(IL-4) in
297 *in vitro* and *in vivo*. Genetic and pharmacologic proof-of-concept studies show, surprisingly, that
298 CoA is a TLR4 agonist and augments alternative activation via MyD88-linked signaling. This
299 discovery and associated data have implications for the metabolic instruction of alternative
300 macrophage activation, the plasticity of macrophage polarization, and the breadth of intracellular
301 metabolites and co-factors that can act as DAMPs.

302 Unexpectedly, addition of exogenous CoA did not augment alternative activation by
303 increasing flux through metabolic pathways linked with m(IL-4). In fact, hallmarks of the IL-4
304 response such as increased respiratory capacity[17,26,27] and *de novo* lipid synthesis[31] were
305 significantly reduced by CoA provision. In fact, other work using exogenously added
306 prostaglandins shows AAM markers can further increase beyond what is induced by IL-4 while
307 simultaneously decreasing mitochondrial oxidative metabolism[17]. The result suggests there is
308 flexibility in the metabolic phenotypes that can support alternative macrophage activation, and
309 provides an additional data point to the mixed results regarding whether healthy oxidative
310 phosphorylation is obligatory for m(IL-4)[13,27].

311 Additionally, gain- and loss-of-function experiments with chemical modulators of
312 intracellular CoA levels revealed that altering CoA levels do not adjust alternative macrophage
313 activation. The rationale for examining this hypothesis arose from studying the effects of the CPT-
314 1 inhibitor etomoxir. High, off-target concentrations of the drug block the macrophage IL-4
315 response and deplete intracellular CoA, and both phenotypes were rescued upon addition of
316 exogenous CoA[13]. The findings presented here, however, show the link between intracellular CoA
317 levels and alternative macrophage activation is likely associative, and suggest the effects of high
318 concentrations of etomoxir are rescued by MyD88-linked signaling rather than restoration of
319 steady-state CoA levels. Although several independent lines of evidence show that etomoxir
320 blocks the IL-4 response via a mechanism independent of fatty acid oxidation[13-15,48,49] it remains
321 unclear why high concentrations of this lipophilic, reactive epoxide block alternative activation.

322 Various cellular metabolites, including lipids, ATP, and uric acid can function as DAMPs
323 to elicit an *in vitro* pro-inflammatory response[50-52]. Others, such as the complex lipid prostaglandin
324 E2 and adenosine, can enhance alternative activation[17,53]. Here we show that CoA is a putative
325 DAMP that primes macrophages for alternative activation and supports resolution via extrinsic,
326 weak agonism of pro-inflammatory TLR4 and MyD88 signaling.

327 This aligns with recent reports that show CoA increases the expression of pro-
328 inflammatory genes such as *Il1b*, *Tnf* and *Nos2* in mouse and human macrophages, an effect

329 lost in mice with simultaneous genetic ablation of TLR2, TLR4, and the TLR chaperone protein
330 Uncb93b1[54]. The data presented here that CoA itself is a TLR4 agonist likely explains the effect,
331 rather than CoA having an indirect effect on the pro-inflammatory response via altered
332 mitochondrial metabolism. As CoA consists of an adenosine diphosphate group linked to a
333 phosphopantetheine moiety[32], CoA is chemically distinct from many well characterized TLR4
334 agonists[55]. Interestingly, nucleoside analogues such as imiquimod (a guanosine analogue) and
335 CL264 (an adenine analogue) are potent TLR7/8 agonists. However, CoA did not activate hTLR7
336 reporter cells, and additional work is required to understand the structural specificity that enables
337 CoA to specifically activate TLR4.

338 TLR4 is well characterized for its ability to respond to ligands that are derived from
339 microbes, and its role in sensing endogenous ligands is increasingly appreciated[56]. Release of
340 intracellular proteins including tenascin 1 and high-mobility group box 1 (HMGB1) can induce a
341 TLR4-dependent inflammatory response[42,57,58], but the capacity for intracellular metabolites and
342 metabolic co-factors to activate TLR4 is less established[59]. Here we show with genetic and
343 pharmacologic proof-of-concept studies that CoA is an endogenous metabolic co-factor that can
344 extrinsically activate TLR4 at physiologically relevant concentrations. Intracellular CoA
345 concentrations can reach over 100 μM in the cytosol and up to 5 mM in the mitochondrial
346 matrix[32,34,60]. As such, cell injury or death could release sufficient CoA to trigger a TLR4-mediated
347 inflammatory response in nearby innate immune cells, particularly in regions where DAMPs can
348 accumulate such as poorly vascularized areas. Other intracellular metabolites function as DAMPs
349 following their cell death-induced release[61,62], and given that CoA contains an ADP moiety, the
350 findings are broadly consistent with the finding that adenosine can enhance the macrophage IL-
351 4 response via the A2 adenosine receptor[53].

352 Although macrophage polarization is often bifurcated into classical or alternative
353 activation, the physiological and pathological induction of an innate immune response involves
354 heterogeneity in activation signals and functional state[3,63,64]. For example, healing processes such
355 as muscle and skin repair are characterized by both an initial influx of pro-inflammatory
356 macrophages to stem infection, as well as a subsequent increase in the presence of alternatively
357 activated macrophages to promote resolution and tissue repair[65,66]. This plasticity can be
358 important for proper function. For example, inhibition of the initial pro-inflammatory response
359 dampens the future expression of alternative activation markers and decreases wound healing[67].

360 Cooperativity between classical and alternative macrophage activation has been
361 established for more than 20 years, with studies demonstrating that IL-4 can enhance the
362 expression of LPS-induced pro-inflammatory genes [68]. Recent studies have corroborated these

363 findings by demonstrating that IL-4 epigenetically primes macrophages to enhance the
364 transcriptional response to pro-inflammatory stimuli[46]. Indeed, our work suggests that pro-
365 inflammatory MyD88-linked signaling enhances the IL-4 response, at least in part, by altering
366 chromatin accessibility.

367 Recent work provides strong support that MyD88-linked signaling via TLR4 agonism is a
368 plausible mechanism by which CoA can augment m(IL-4)[69-72]. For example, the fungal effector
369 protein CLP-1, a TLR4 agonist, fails to enhance alternative activation in MyD88$^{-/-}$ BMDMs.
370 Moreover, other work has associated AAMs with IL-33[73], which is upstream of MyD88. Others
371 have also shown that interferon-β decreases the expression of cell surface markers and genes
372 associated with alternative activation[45,74,75], further supporting our findings that TLR4 agonism by
373 CoA enhances m(IL-4) via MyD88 rather than TRIF. The ATAC-Seq analysis presented here
374 furthers this work by identifying Jun/AP-1 signaling as a candidate pathway downstream of MyD88
375 that may mediate the enhanced IL-4 response.
376

377 **MATERIALS AND METHODS**

378 *Animals*

379 All animal protocols and procedures were approved and performed in accordance with the
380 NIH Guide for the Care and Use of Laboratory Animals and the UCLA Animal Research
381 Committee (ARC).
382

383 *Reagents*

384 Unless otherwise specified, yeast-derived CoA that was purchased from Sigma-Aldrich
385 (C4780; ≥85% purity) was used. Synthetic CoA was purchased from Avanti Polar Lipids
386 (870700P; >99% purity). IL-4 was purchased from PeproTech (214-14), and IL-4-reactive
387 monoclonal antibodies for *in vivo* studies (IL-4 MAb) were purchased from BioXCell (BE0045). All
388 TLR agonists were purchased from Invivogen: Pam3CSK4 (TLR2 ligand; tlrl-pms), Poly(I:C)
389 (TLR3 ligand; tlrl-pic), LPS (TLR4 ligand; tlrl-smlps), Flagellin (TLR5 ligand; tlrl-stfla), and
390 imiquimod (TLR7 agonist; tlrl-imqs-1).
391

392 *Isolation and differentiation of mouse BMDMs*

393 BMDMs from wild-type (000664), *Myd88*$^{-/-}$ (009088), and *Tlr4*$^{-/-}$ (029015) mice were
394 isolated as previously described from male mice aged between 6-12 weeks [76]. Briefly, bone
395 marrow cells were first isolated by flushing the femurs and tibiae with phosphate buffered saline

396 (PBS). Cells were then pelleted by centrifugation at 365g for 7 mins at room temperature.
397 Following the removal of red blood cells with RBC Lysis Buffer (Sigma, R7757) and centrifugation,
398 bone marrow cells were differentiated for 6 days at 37°C in a humidified 5% CO_2 incubator in
399 'BMDM differentiation medium'. BMDM differentiation medium consisted of DMEM (Gibco
400 #11965) supplemented with 10% (v/v) fetal bovine serum (FBS; Hyclone), 2mM L-glutamine,
401 500μM sodium pyruvate, 100 units/mL, 100 mg/mL penicillin/streptomycin,. Additionally, the
402 medium was further supplemented 10% (v/v) with the conditioned medium from CMG-14-12 cells
403 as a source of macrophage colony stimulate factor (M-CSF).
404
405 *In vitro BMDM activation and stimulation of Toll-like receptors*
406 After six days of differentiation, BMDMs were scraped with a cell lifter, counted, and
407 replated at the listed cell densities into the relevant assay format (e.g., six-well tissue culture dish,
408 Seahorse XF96-well plate, etc.) in differentiation medium. After two days, cells were stimulated
409 with compounds as indicated below and in the figure legends. Unless otherwise specified, all
410 measurements of the macrophage IL-4 response were conducted 48 hr. after treatment. When
411 assessing the effect of CoA on the pro-inflammatory response, BMDMs were treated for 4 hr.
412 (gene expression) or 24 hr. (itaconate abundance). Unless otherwise indicated in the figures and
413 legends, effector compounds were used at the following concentrations alongside matched
414 vehicle controls: IL-4 (20 ng/mL), CoA (1 mM), Pam3CSK4 (5 ng/mL), Poly(I:C) (1μg/mL), LPS
415 (0.1 ng/mL), flagellin (100ng/mL), and imiquimod (10μM).
416
417 *In vivo activation of peritoneal macrophages*
418 To induce alternative activation *in vivo*, mice were treated with an IL-4 complex (IL-4c)
419 consisting of a 2:1 molar ratio of IL-4 and anti-IL-4 mAb or PBS as a control. Each IL-4c-treated
420 mouse received 5μg IL-4 and 25μg of anti-IL-4 mAb. To test the effect of CoA on alternative
421 activation *in vivo* (Fig. 1g), mice were pretreated with an injection of either PBS or 40 mg/kg CoA
422 for 6 hr. prior to IL-4c administration according to the scheme in the figure. After 24 hr. peritoneal
423 macrophages were collected by rinsing the peritoneal cavity with 5mL PBS, and alternative
424 activation markers were assessed using flow cytometry.
425 To determine if CoA could induce a pro-inflammatory response *in vivo* (Figs. 3f&g), mice
426 were treated with either PBS or 40 mg/kg CoA for 6 hr. After treatment, peritoneal macrophages
427 were collected by rinsing the peritoneal cavity with 5mL of PBS. Cells were then pelleted, with
428 supernatant used to measure cytokine levels with the LEGENDplex multiplex ELISA kit
429 (Biolegend, 740848), while gene expression was measured in the peritoneal exudate cells..

430 To determine if TLR agonists could enhance the IL-4 response of peritoneal macrophages
431 *in vivo* (Fig. 5g), Mice were treated with either PBS or the indicated TLR agonist at the following
432 doses: Pam3CSK4 (50µg), Poly:IC (200µg), or LPS (125µg). After 24 hr. IL-4c was administered
433 as before, and the number of cells double-positive for CD206 and CD301 was assessed by flow
434 cytometry.
435
436 *B16 melanoma growth*
437 Prior to *in vivo* co-injection, *in vitro* BMDMs were either stimulated with vehicle control, IL-4 , or
438 IL-4 in combination with Pam3CSK4 for 48 hrs. On the day of implantation, a 1:1 mixture of
439 $1x10^5$ B16-F10 cells and $1x10^5$ BMDMs were suspended in PBS and injected into the rear right
440 flanks of 12 week old Male C57BL/6 mice[15]. Mice were sacrificed 20 days post injection and
441 subcutaneous tumors were excised, blotted dry, and weighed.
442
443 *Gene expression analysis*
444 Gene transcript levels were measured using qPCR. On Day 6 of differentiation, BMDMs
445 were seeded at $3.0 x10^5$ cells/well in 12-well plates. After activating cells with the concentrations
446 and durations as described earlier, RNA was extracted using the RNeasy Mini Kit (Qiagen, 74106)
447 and cDNA was synthesized using high-capacity cDNA reverse transcription kit (Applied
448 Biosystems, 4368814) according to the manufacturers' protocol. qPCR was performed with
449 PowerUp SYBR green master mix (Applied Biosystems, A25743) on a QuantStudio 5 RT-PCR
450 (Applied Biosystems). Relative gene expression values were calculated using the delta-delta Ct
451 method, with the ribosomal protein *Rplp0* used as a control for normalization.
452
453 *Flow cytometric analysis*
454 Cell surface marker expression was measured using flow cytometry. On Day 6 of
455 differentiation, BMDMs were seeded at $3.0 x10^5$ cells/well in 12-well plates. After activating cells
456 with the concentrations and durations as described earlier, BMDMs were detached by scraping
457 in 450µL of Accutase. Cells were then washed with FACS buffer (PBS+ 2% (v/v) FBS with 1mM
458 EDTA) and incubated with a 1:500 dilution of TruStain FCX (Biolegend, 101320) for 5 minutes.
459 Cells were next stained for 30 mins on ice with a 1:300 dilution of antibodies raised against mouse
460 CD206 (Biolegend,141710), CD301(Biorad, MCA2392A647T), or CD71(Biolegend, 113812).
461 Cells were then washed and resuspended in FACS buffer containing 1 µg/mL DAPI (Invitrogen,
462 D1306) for viability analysis, and data was captured on an Attune NXT flow cytometer. Data were
463 analyzed using FlowJo X software.

464 *Endocytosis assay*

465 Following differentiation, BMDMs were seeded in black-walled 96-well plates at 3 x10^4
466 cells/well for high-content imaging to quantify uptake of FITC-dextran. 48 hr. after compound
467 treatment, medium was replaced with high-glucose DMEM containing 1mg/mL FITC-dextran
468 (Sigma, FD40) and 10ng/mL Hoechst 33342. Following a 1hr. incubation at 37°C, cells were
469 washed twice with PBS and fixed with 4% (v/v) paraformaldehyde (PFA) in PBS. Images were
470 captured with a PerkinElmer Operetta, and FITC-positive foci per cell was calculated using
471 Harmony software.

472

473 *Quantification of short-chain acyl CoAs*

474 Quantification of acyl CoAs was conducted according to previously established methods[18]
475 . Following differentiation, BMDMs were seeded in 10cm^2 dishes at 5 x10^6 cells/dish. Following
476 48hr. stimulation, cells were rinsed twice with ice-cold PBS, scraped into 1.5mL microfuge tubes
477 and pelleted via centrifugation at 4°C . 200µL of an ice-cold extraction solution [2.5% (w/v) 5-
478 sulfosalicylic acid (SSA) along with 1µM Crotonoyl CoA as an internal standard] was added to
479 each cell pellet and subsequently vortexed. Samples were centrifuged at 18,000*g* for 15 min at
480 4°C. Supernatants containing short-chain acyl CoAs were then removed and transferred to glass
481 LC-MS vials for analysis as is thoroughly described elsewhere[18] .

482

483 *Seahorse XF Analysis*

484 After 6 days of differentiation, BMDMs were plated at 3.0 x 10^4 cells/well in XF96 plates.
485 Following 48 hr. of treatment with compounds under investigation, respirometry assays were
486 conducted with an Agilent Seahorse XFe96 Analyzer. Oligomycin (2 µM), two injections of FCCP
487 (750 nM each), and rotenone (200 nM) with antimycin A (1 µM) were added acutely to the wells,
488 and respiratory parameters calculated according to best practices[77,78]. Measurements were
489 conducted in unbuffered DMEM (Sigma #5030) supplemented with 5 mM HEPES, 8 mM glucose,
490 2 mM glutamine, and 2 mM pyruvate. Lactate efflux was measured by correcting rates of
491 extracellular acidification for microplate sensor coverage and confounding respiratory
492 acidification[79].

493 Metabolomics and stable isotope tracing of polar metabolites

494 After 6 days of differentiation, BMDMs were plated at 1 x10^6 cells/well in 6-well dishes.
495 Cells were then stimulated in medium where either glucose or glutamine was replaced with
496 uniformly labeled $^{13}C_6$-glucose (CLM-1396) or uniformly labeled $^{13}C_5$-glutamine (CLM-1822). After
497 48 hr., cells were harvested and extracted for GC/MS using established methods, with all steps

498 conducted on ice[80]. Briefly, cell plates were washed twice with 0.9% (w/v) NaCl and samples were
499 extracted with 500 µL methanol, 200 µL water containing 1 µg of norvaline (internal standard),
500 and 500 µL chloroform. Samples were vortexed for 1 min and spun at 10,000 *g* for 5 min at 4°C,
501 and the aqueous layers containing the polar metabolites were transferred to GC/MS sample vials
502 and dried overnight using a refrigerated CentriVap. Once dry, the samples were resuspended in
503 20 µL of 2% (w/v) methoxyamine in pyridine and incubated at 37°C for 45 minutes. This was
504 followed by addition of 20 µL of MTBSTFA + 1% TBDMS (N-tert-Butyldimethylsilyl-N-
505 methyltrifluoroacetamide with 1% tertButyldimethylchlorosilane). Following a second 45-minute
506 incubation at 37°C, samples were run as previously described [80]. Analysis was conducted using
507 Agilent MassHunter software, and stable isotope tracing data was corrected for natural
508 abundance of heavy isotopes with FluxFix software using a reference set of unlabeled metabolite
509 standards [81].
510
511 *De novo lipogenesis*
512 Briefly, after 6 days of differentiation, BMDMs were plated at 1 x10^5 cells/ well in 24-well
513 dishes Cells were stimulated in medium in which unlabeled glucose was replaced with 10mM
514 uniformly labeled $^{13}C_6$-glucose. Extraction of fatty acids, quantification of *de novo* synthesis, and
515 normalization to cell number was conducted using an Agilent 5975C mass spectrometer coupled
516 to a 7890 gas chromatograph as previously described[76,82].
517
518 *HEK-Blue hTLR reporter assays*
519 HEK-Blue reporter cells expressing either human TLR2 (hkb-htlr2), hTLR4 (hkb-htlr4), or
520 hTLR7 (hkb-htlr7v2) were purchased from InvivoGen and maintained according to the
521 manufacturer's instructions. To establish concentration-response curves, 2.5 x 10^3 reporter cells
522 were resuspended in HEK-Blue detection medium (Invivogen, hb-det2) in 96-well plates and
523 stimulated with the appropriate agonist (Pam3CSK4: 0.6pg/mL to 1 µg/mL; LPS: 0.6pg/mL to 1
524 µg/mL; CL307: 0.6pg/mL to 1 µg/mL). Following a 24hr. incubation, secreted alkaline
525 phosphatase reporter activity was determined by assessing OD_{630} with a plate reader. The OD_{630}
526 of cells treated with 1 mM CoA was compared relative to positive controls. Calibration cuves to
527 fit empirical data were generated using GraphPad Prism software.
528
529
530

531 *ATAC-Seq library prep*

532 ATAC-seq libraries were produced by the Applied Genomics, Computation and Translational Core
533 Facility at Cedars Sinai. Briefly 50,000 BMDMs per sample were lysed to collect nuclei and treated
534 with Tn5 transposase (Illumina) for 30 min at 37°C with gentle agitation. The DNA was isolated
535 with DNA Clean & Concentrator Kit (Zymo) and PCR amplified and barcoded with NEBNext High-
536 Fidelity PCR Mix (New England Biolabs) and unique dual indexes (Illumina). The ATAC-seq
537 library amplification was confirmed by real-time PCR, and additional barcoding PCR cycles were
538 added as necessary while avoiding overamplification. Amplified ATAC-seq libraries were purified
539 with DNA Clean & Concentrator Kit (Zymo). The purified libraries were quantified with Kapa
540 Library Quant Kit (KAPA Biosystems) and quality assessed on a 4200 TapeStation System
541 (Agilent). The libraries were pooled based on molar concentrations and sequenced on a HiSeq
542 4000 platform (paired end, 100 bp).

543

544 *ATAC-Seq analysis*

545 The peaks for all the ATAC-seq samples were used to generate a single reference peak file, and
546 the number of reads that fell into each peak was counted using deeptools multiBamSummary[83].
547 EdgeR[84] was used to determine the IL-4 significantly induced regions by applying a cutoff FDR
548 <0.01 and LFC > 1 of triplicate data upon IL-4 stimulation on WT BMDMs and to determine the
549 Pam3CSK4 co-treatment significant regions by applying a cutoff FDR <0.05 and LFC > 0.5.
550 Analysis of transcription factor motif enrichment was performed using findMotifsGenome function
551 in the HOMER suite[47], using all detected peaks as background. Reads were normalized by RPKM.
552 Data were visualized with ggplot2 or the pheatmap packages in R.

553

554 *RNA-Seq library prep and quantification*

555 RNA sequencing libraries were produced by the Technology Center for Genomics &
556 Bioinformatics at UCLA. Isolation of RNA was performed using Qiagen RNeasy Mini kit and RNA
557 libraries were prepared with KAPA stranded mRNA-Seq kit. High throughput sequencing was
558 performed on Illumina NovaSeq 6000 (paired end, 2x150bp) targeting 100 million reads per
559 sample. Demultiplexing was performed with Illumina Bcl2fastq v2.19.1. Gene expression
560 quantification from the resulting fastqs was performed using Salmon v1.21.1 in mapping-based
561 mode (Patro et al. 2017). Reads were selectively aligned to the GENCODE vM25 mouse
562 reference transcriptome with corrections for sequence-specific and GC content biases.

563

564 *RNA-Seq analysis*

565 Raw gene count data were analyzed using the R package DESeq2 v1.22.2[85] for library size
566 normalization and differential expression analysis. For differential expression results, genes with
567 adjusted p-values below 0.01 and log2 fold changes above 1 or below -1 were deemed significant.
568 For visualization in volcano plots, log2 fold changes above 10 or below -10 were set to 10 and -
569 10 respectively. Gene set enrichment analysis[86] was performed using the R package FGSEA
570 v1.15.0[87] based on gene lists ranked by the Wald statistic from differential expression results.
571 Genesets corresponding to the mouse transcriptome from KEGG, REACTOME, and BIOCARTA
572 within the Molecular Signatures Database[88] were accessed using the R package msigdbr v7.1.1[89]
573 Genesets with adjusted p-values below 0.05 were deemed significant.
574
575 *Statistical analysis*
576 All statistical parameters, including the number of biological replicates (n), can be found
577 in the figure legends. Statistical analyses were performed using Graph Pad Prism 5 software.
578 Data are presented as the mean ± standard deviation unless otherwise specified. Individual
579 pairwise comparisons were performed using two-tailed Student's t-test. For analysis involving
580 more than two groups, data were analyzed by repeated measures ANOVA followed by Dunnett's
581 post-hoc multiple comparisons tests (compared against vehicle controls unless otherwise
582 specified). Data were assumed to follow a normal distribution (no tests were performed). Values
583 denoted as follows were considered statistically significant: *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.
584

585 **AUTHOR CONTRIBUTIONS**

586 Conceptualization: AEJ, SJB, ASD; Data curation: AEJ, AR, NI, CC, NAB, ASD; Formal analysis:
587 AEJ, AR, NI, CC, NAB, ASD; Funding acquisition: TGG, AH, SJB, ASD; Investigation: AEJ, AR,
588 NI, CC, NAB, ABB, WYH, AS, ARB, AAC; Methodology: AEJ, CC, NAB, AS, ASD; Project
589 administration: SJB, ASD; Resources: TGG, AH, SJB, ASD; Supervision: AEJ, SJB, ASD ; Writing
590 - original draft: AEJ, ASD; Writing - review & editing: All authors
591

592 **ACKNOWLEDGEMENTS**

593 ASD is supported by National Institutes of Health (NIH) Grant R35GM138003, the W.M. Keck
594 Foundation (995337), and the Agilent Early Career Professor Award. SJB is supported by the NIH
595 Grants P01HL146358 and R01HL157710. AEJ was supported by the UCLA Tumor Cell Biology
596 Training Program T32 CA009056. ABB is supported by the UCLA Chemistry-Biology Interface
597 Training Grant T32GM136614. AH is supported by NIH Grant R01AI173214.

DISCLOSURES

None

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FIGURE LEGENDS

845 **Figure 1. Exogenous CoA provision enhances alternative macrophage activation**
846 **(a)** qPCR analysis of the IL-4-associated genes *Mgl2*, *Pdcd1gl2*, *Chil4*, *Ccl8,* and *Fizz1* in BMDMs
847 treated with CoA (1 mM), IL-4 (20 ng/mL), CoA + IL-4, or vehicle for 48 hr. (n≥9 independent
848 biological replicates). **(b-d)** Flow cytometric analysis of the IL-4-associated cell surface markers
849 CD206, CD301, and CD71 after treatments as in (a). (b) Contour plots with the percentage of
850 cells expressing both CD206 and CD301 is indicated in the upper right quadrant. Data shown are
851 from a single representative experiment. (c) Aggregate mean fluorescence intensity of CD206,
852 CD301, and CD71 (n=9 independent biological replicates). (d) Percentage of $CD206^+/CD301^+$
853 and $CD206^+/CD71^+$ populations (n=9 independent biological replicates). **(e)** Representative
854 images of BMDMs incubated for 1 hr. with FITC-Dextran (1mg/mL, green) and Hoechst 3342
855 (10ng/mL, blue) after stimulation with compounds as in (a). **(f)** Aggregate image analysis data for
856 experiments as in (e) (n=3 independent biological replicates). **(G)** Quantification of $CD206^+/CD71^+$
857 peritoneal macrophages from mice that were exposed to vehicle (PBS), CoA (40mg/kg), IL-4c (5
858 µg IL-4 and 25 µg anti-IL-4 monoclonal antibody), or the combination of IL-4c + CoA. (n≥3 mice
859 were used for each group). All data are presented as mean ± SEM. *p < 0.05; **p < 0.01; ***p <
860 0.001.
861
862 **Figure 2: CoA does not enhance alternative macrophage activation by boosting known**
863 **metabolic hallmarks of the IL-4 response.**
864 **(a)** Intracellular levels of CoA and acetyl CoA in BMDMs measured by LC-MS/MS. Cells were
865 treated with IL-4 (20 ng/mL), IL-4 + CoA (1 mM), or vehicle for 48 hr. as in Fig. 1 (n=3 independent
866 biological replicates). **(b)** Representative respirometry trace of BMDMs treated as in (a). (n=5
867 technical replicates from a single biological replicate). **(c)** Aggregate ATP-linked and FCCP-
868 stimulated respiration in intact BMDMs for treatments as in (a). Cells were offered 8 mM glucose,
869 2 mM pyruvate, and 2 mM glutamine in the experimental medium (n=8 independent biological
870 replicates). **(d)** Lactate efflux rate from respirometry experiments in (b & c) calculated using
871 Seahorse XF data and correcting for respiratory CO_2 (n=8 independent biological replicates). **(e)**
872 Metabolite abundances of citrate, α-ketoglutarate(α-KG), and malate in BMDMs treated as in (a)
873 (n=7 independent biological replicates). **(f)** Enrichment from uniformly labeled $^{13}C_6$-glucose into
874 the TCA cycle intermediates as in (e) (n=8 independent biological replicates). **(g)** Quantification
875 of newly synthesized palmitic acid (16:0) and palmitoleic acid (16:1) from BMDMs stimulated as
876 in (a). (data shown as n=8 technical replicates from n=2 independent biological replicates). **(h)**
877 Schematic depicting the mechanism of action of cyclopropane carboxylic acid (CPCA) and PZ-
878 2891**. (i)** Intracellular CoA levels of BMDMs stimulated with IL-4, IL-4 + CPCA (1 mM), or IL-4+PZ-
879 2891 (10 µM) for 48 h (n=5 independent biological replicates). **(j)** Flow cytometric quantification
880 of the $CD206^+/CD301^+$ population for BMDMs treated as in (i) (n=5 independent biological
881 replicates). All data are presented as mean ± SEM. *p < 0.05; **p < 0.01; ***p < 0.001.
882
883
884 **Figure 3. Exogenous CoA induces a pro-inflammatory response in BMDMs**
885 **(a)** Volcano plot from bulk RNA sequencing data from BMDMs treated IL-4 (20 ng/mL), IL-4 + 1
886 mM CoA, or vehicle control for 48 hr. comparing differential gene expression between IL-4 vs.
887 vehicle controls (left) and IL-4 + CoA vs. IL-4 (right). Genes associated with classical activation
888 are depicted in red, genes associated with alternative activation are shown in blue. **(b)** Gene Set
889 Enrichment Analysis of genes upregulated in BMDMs treated with IL-4+CoA vs. IL-4 alone. **(c)**
890 qPCR analysis of *Il1b*, *Tnf*, *Irg1*, and *Nos2* in BMDMs stimulated with CoA (1mM) or vehicle
891 control for 4 hr. (n=4 independent biological replicates). **(d)** qPCR analysis of the interferon-
892 stimulated gene *Mx1* in BMDMs stimulated with 1 mM CoA or vehicle control for 24 hr. (n=4
893 independent biological replicates). **(e)** Itaconate abundance after treatment with 1 mM CoA or
894 vehicle control for 48 hr. (n=6 independent biological replicates). **(f)** qPCR analysis of *Il1b* and
895 *Tnf* in the peritoneal exudate cells of mice treated with (40 mg/kg) CoA 6 hr. prior to collection

(n≥5 mice for each group). **(g)** Quantification of cytokines in the peritoneal lavage fluid (PLF) of mice treated as in (g) using Multiplexed ELISA (n=3 mice were used for each group). All data are presented as mean ± SEM. *p < 0.05; **p < 0.01.

Figure 4. CoA is a TLR4 agonist

(a) qPCR analysis of *Il1b*, *Tnf*, *Irg1*, *I12b,* and *Nos2* in WT and *Myd88*$^{-/-}$ BMDMs stimulated with 1 mM CoA or vehicle control for 4 hr. (n≥3 independent biological replicates). **(b)** Concentration-response curve of linked alkaline phosphatase activity in hTLR4 reporter cells with varying concentrations of LPS (black dots). The red dot and dashed lines represent the OD_{630} observed in response to 1 mM CoA treatment (n=4 independent biological replicates). **(c)** Aggregated response of 1 mM CoA compared to vehicle control in hTLR2, hTLR4, and hTLR7 relative to maximum TLR activation. (n≥3 independent biological replicates). **(d)** qPCR analysis of *Il1b*, *Tnf*, *I12b,* and *Nos2* in WT and *Tlr4*$^{-/-}$ BMDMs following treatment with 1 mM CoA for 4 hr. (n=3 independent biological replicates). **(e)** Abundance of itaconate in WT, *Myd88*$^{-/-}$, and *Tlr4*$^{-/-}$ BMDMs in response to 1 mM CoA treatment for 48 hr. or vehicle control (n≥5 independent biological replicates). All data are presented as mean ± SEM. *p < 0.05; ***p < 0.001.

Figure 5. Myd88-linked TLR-ligands enhance IL-4 the response

(a-b) Flow cytometric analysis of CD206 and CD301 in BMDMs stimulated for 48 hr. with vehicle control, IL-4 (20 ng/mL), or IL-4 in combination with one of the following: LPS (0.1 ng/mL), Pam3CSK4 (5ng/mL), Imiquimod (10 μM), Flagellin (100 ng/mL), or Poly (I:C) (1 μg/mL) (a) Contour plots showing the percentage of cells expressing both CD206 and CD301 in the upper right quadrant. Data are from a single representative experiment. (b) Percentage of $CD206^{+}/CD301^{+}$ populations for treatments as in (a) (n=8 independent biological replicates). **(c)** qPCR analysis of *Mgl2* and *Ym1* in BMDMs stimulated with vehicle control, IL-4, or IL-4 in combination with LPS, Pam3CSK4, or Poly (I:C) for 48h. Concentrations as in (a) (n≥3 independent biological replicates). **(d)** Aggregate $FITC^{+}$ foci per cell for BMDMs stained with FITC-Dextran and treated as with vehicle control, IL-4, or IL-4 with either LPS or Pam3CSK4 as in (a). (n=4 independent biological replicates). **(e)** Percentage of $CD206^{+}/CD301^{+}$ populations in WT and *Myd88*$^{-/-}$BMDMs stimulated with vehicle control, IL-4, or IL-4 with either LPS or Pam3CSK4 as in (a) (n=8 independent biological replicates. **(f)** qPCR analysis of *Mgl2* and *Ym1* for BMDMs as in (e) (n=4 independent biological replicates). **(g)** Quantification of $CD206^{+}/CD71^{+}$ peritoneal macrophages from mice that were injected with vehicle control, IL-4c (5 μg IL-4 and 25 μg anti-IL-4 monoclonal antibody), IL-4c + LPS (125 μg), IL-4c +Pam3CSK4 (50 μg), or IL-4c + Poly (I:C) (200 μg). (n≥9 mice were used for each group). **(h)** Weights of subcutaneous B16-F10 melanoma tumors that were derived from the co-implantation of B16-F10 tumor cells and BMDMs that were stimulated with either vehicle control, IL-4, or IL-4 in combination with Pam3CSK4. All data are presented as mean ± SEM. *p < 0.05; **p < 0.01; ***p < 0.001; ns, not significant.

Figure 6. MyD88-linked signaling increases chromatin accessibility in IL-4-stumulated macrophages

(a) Heatmap of z-scored ATAC-seq signal for the 10,878 IL-4-inducible regions. The heatmap is arranged by increasing values for IL-4 with Pam3CSK4 co-treatment group and is divided into 10 equal bins. Side bar indicates distance to closest transcription start site (TSS). **(b)** Heatmap showing p-values of the most highly enriched motifs for each of the 10 bins that were generated in (a). **(c)** Top 3 hits from de novo transcription factor motif analysis on the significantly induced regions by Pam3CSK4 co-treatment (log_2 fold change >0.5, false discovery rate < 0.05). **(d)** Representative tracks of Chil4 and Ccl8 promoter regions with nearby c-Jun/AP-1 motifs**.**

Figure 1

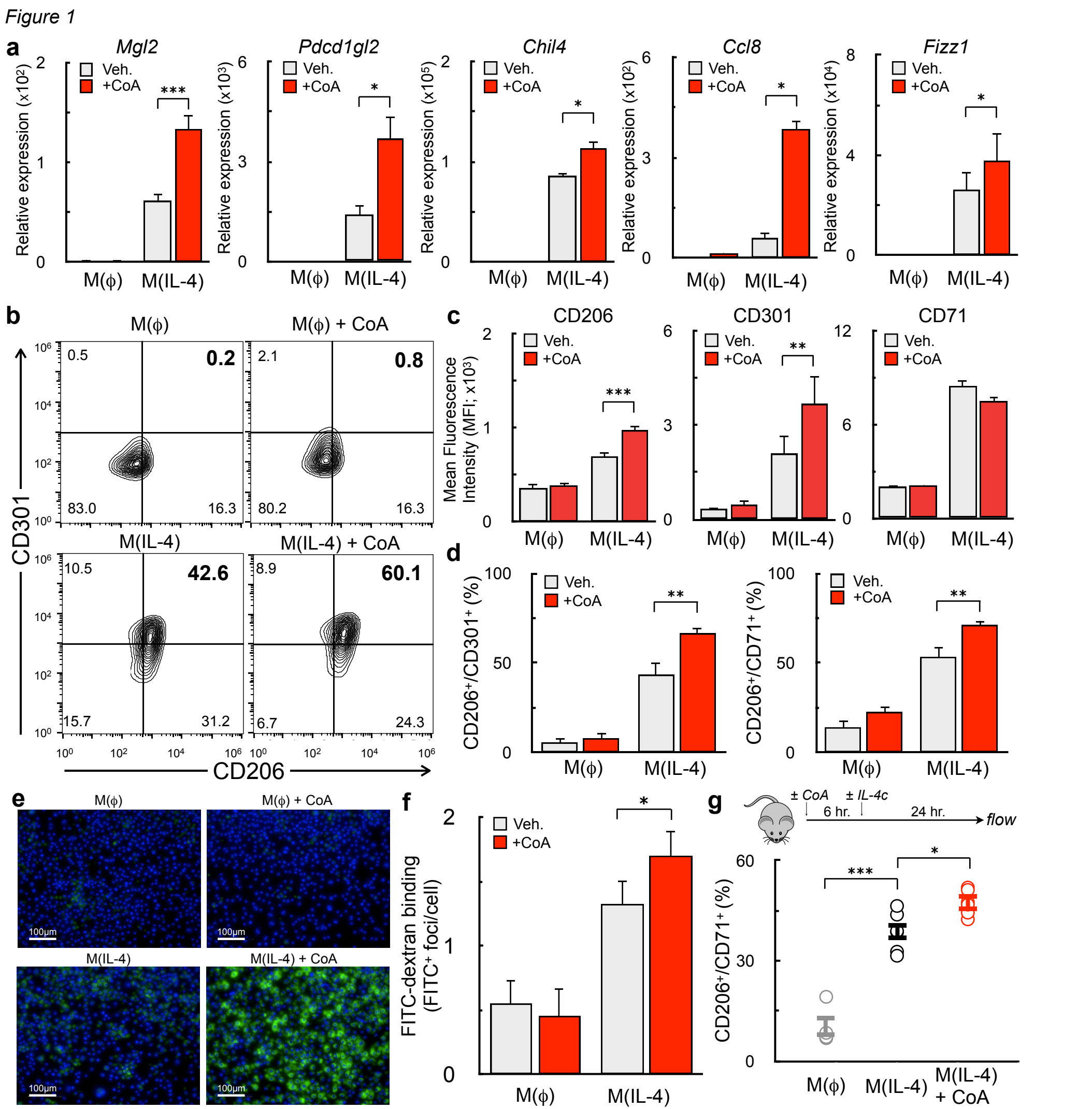

a
Mgl2
Pdcd1gl2
Chil4
Ccl8
Fizz1
Veh.
+CoA
Relative expression (x10²)
Relative expression (x10³)
Relative expression (x10⁵)
Relative expression (x10²)
Relative expression (x10⁴)
M(ϕ)
M(IL-4)
b
M(ϕ)
M(ϕ) + CoA
M(IL-4)
M(IL-4) + CoA
0.5
0.2
83.0
16.3
2.1
0.8
80.2
16.3
10.5
42.6
15.7
31.2
8.9
60.1
6.7
24.3
CD301
CD206
c
CD206
CD301
CD71
Mean Fluorescence Intensity (MFI; x10³)
d
CD206⁺/CD301⁺ (%)
CD206⁺/CD71⁺ (%)
e
M(ϕ)
M(ϕ) + CoA
M(IL-4)
M(IL-4) + CoA
100μm
f
FITC-dextran binding (FITC⁺ foci/cell)
g
± CoA
± IL-4c
6 hr.
24 hr.
flow
CD206⁺/CD71⁺ (%)
M(IL-4) + CoA

Figure 2

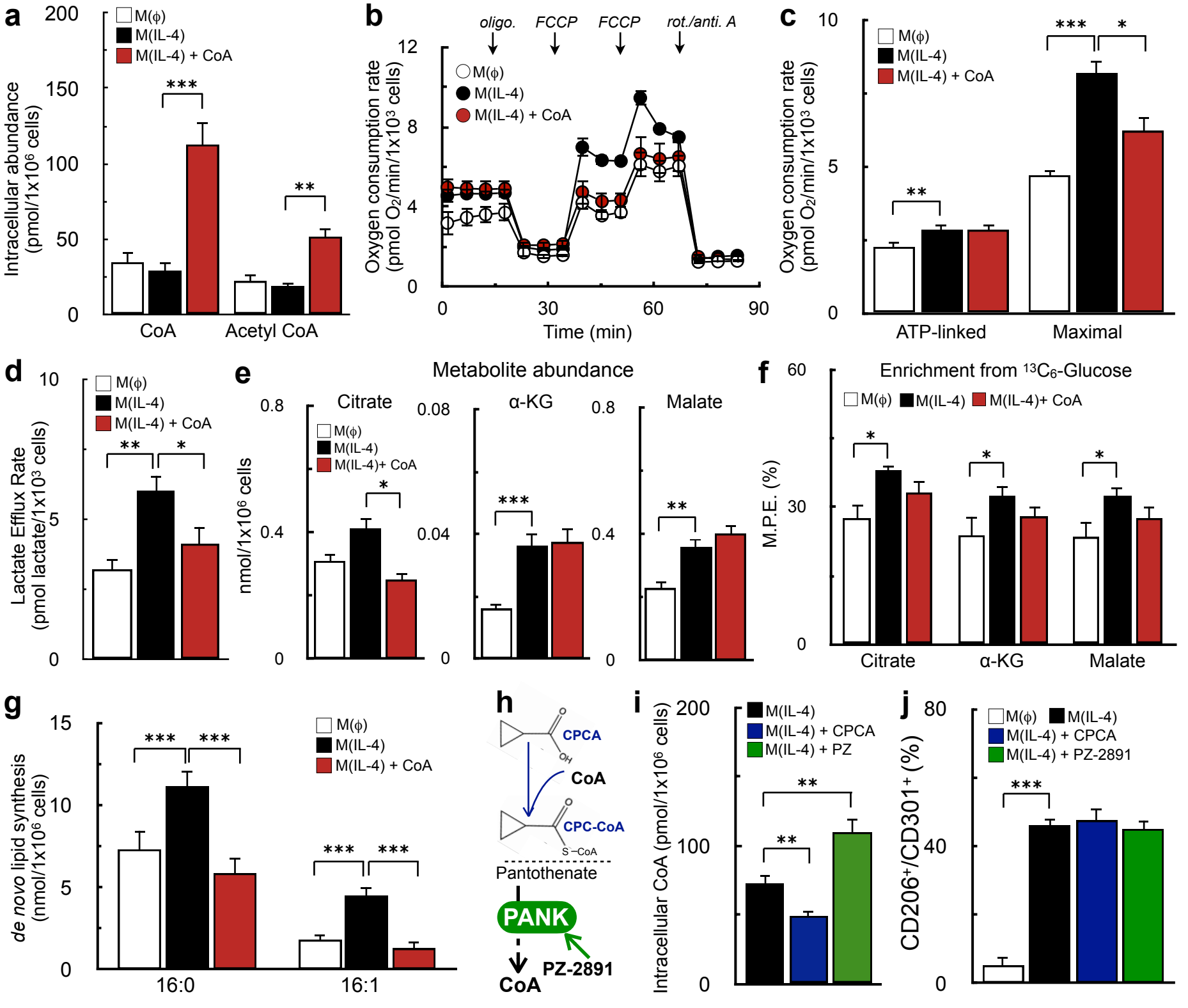

a
Intracellular abundance (pmol/1x10⁶ cells)
M(ϕ)
M(IL-4)
M(IL-4) + CoA
CoA
Acetyl CoA
b
oligo.
FCCP
FCCP
rot./anti. A
Oxygen consumption rate (pmol O2/min/1x10³ cells)
Time (min)
c
ATP-linked
Maximal
d
Lactate Efflux Rate (pmol lactate/1x10³ cells)
e
Metabolite abundance
Citrate
α-KG
Malate
nmol/1x10⁶ cells
f
Enrichment from 13C6-Glucose
M.P.E. (%)
g
de novo lipid synthesis (nmol/1x10⁶ cells)
16:0
16:1
h
CPCA
CoA
CPC-CoA
Pantothenate
PANK
PZ-2891
i
Intracellular CoA (pmol/1x10⁶ cells)
M(IL-4) + CPCA
M(IL-4) + PZ
j
CD206+/CD301+ (%)
M(IL-4) + PZ-2891

Figure 3

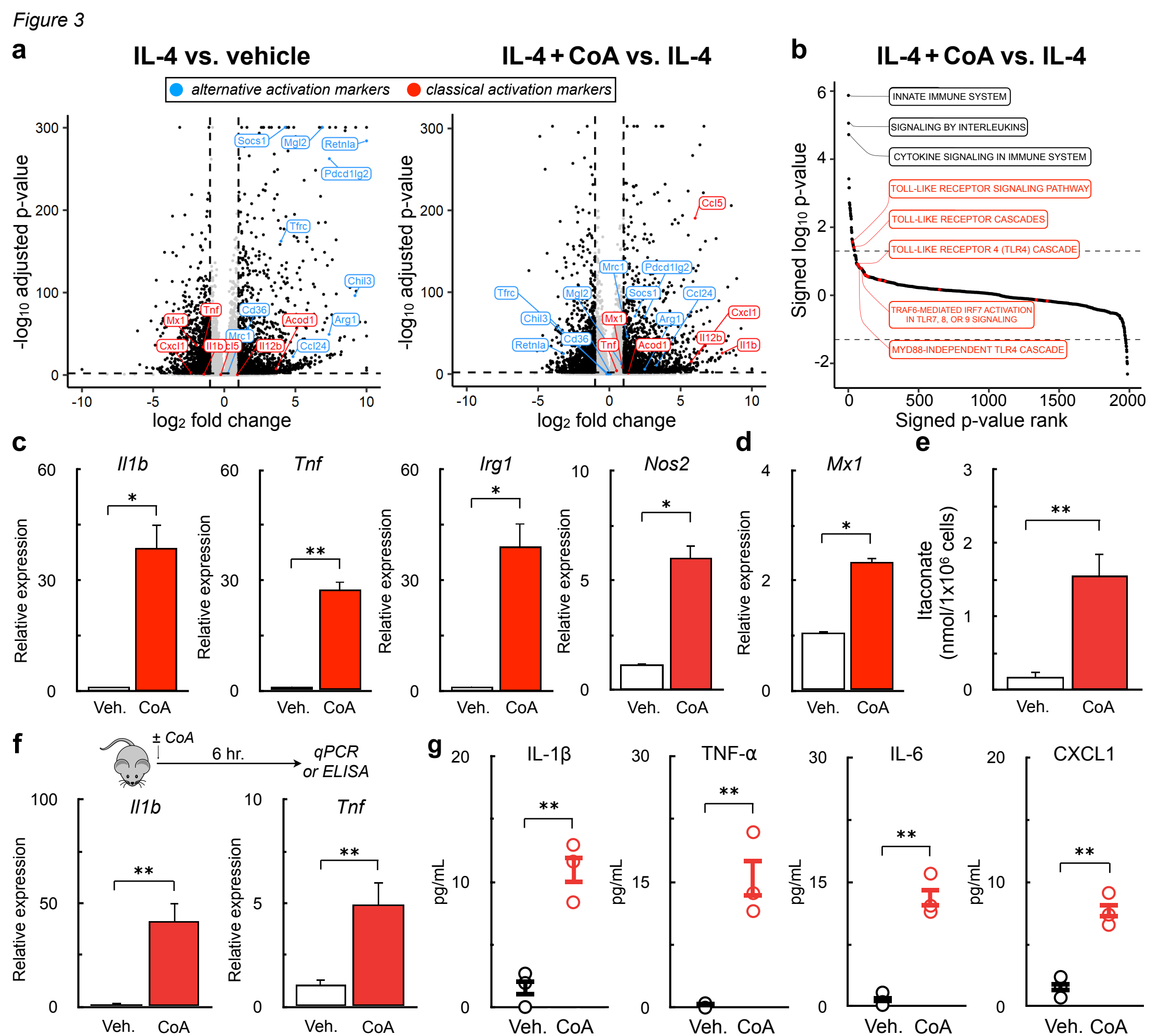

a
IL-4 vs. vehicle
IL-4 + CoA vs. IL-4
alternative activation markers
classical activation markers
-log10 adjusted p-value
log2 fold change
Socs1
Mgl2
Retnla
Pdcd1lg2
Tfrc
Chil3
Cd36
Acod1
Arg1
Mx1
Tnf
Mrc1
Cxcl1
Il1b
Ccl5
Il12b
Ccl24
b
IL-4 + CoA vs. IL-4
Signed log10 p-value
Signed p-value rank
INNATE IMMUNE SYSTEM
SIGNALING BY INTERLEUKINS
CYTOKINE SIGNALING IN IMMUNE SYSTEM
TOLL-LIKE RECEPTOR SIGNALING PATHWAY
TOLL-LIKE RECEPTOR CASCADES
TOLL-LIKE RECEPTOR 4 (TLR4) CASCADE
TRAF6-MEDIATED IRF7 ACTIVATION IN TLR7, 8, OR 9 SIGNALING
MYD88-INDEPENDENT TLR4 CASCADE
c
Il1b
Tnf
Irg1
Nos2
Relative expression
Veh.
CoA
d
Mx1
e
Itaconate (nmol/1x10^6 cells)
f
± CoA
6 hr.
qPCR or ELISA
Il1b
Tnf
g
IL-1β
TNF-α
IL-6
CXCL1
pg/mL

Figure 4

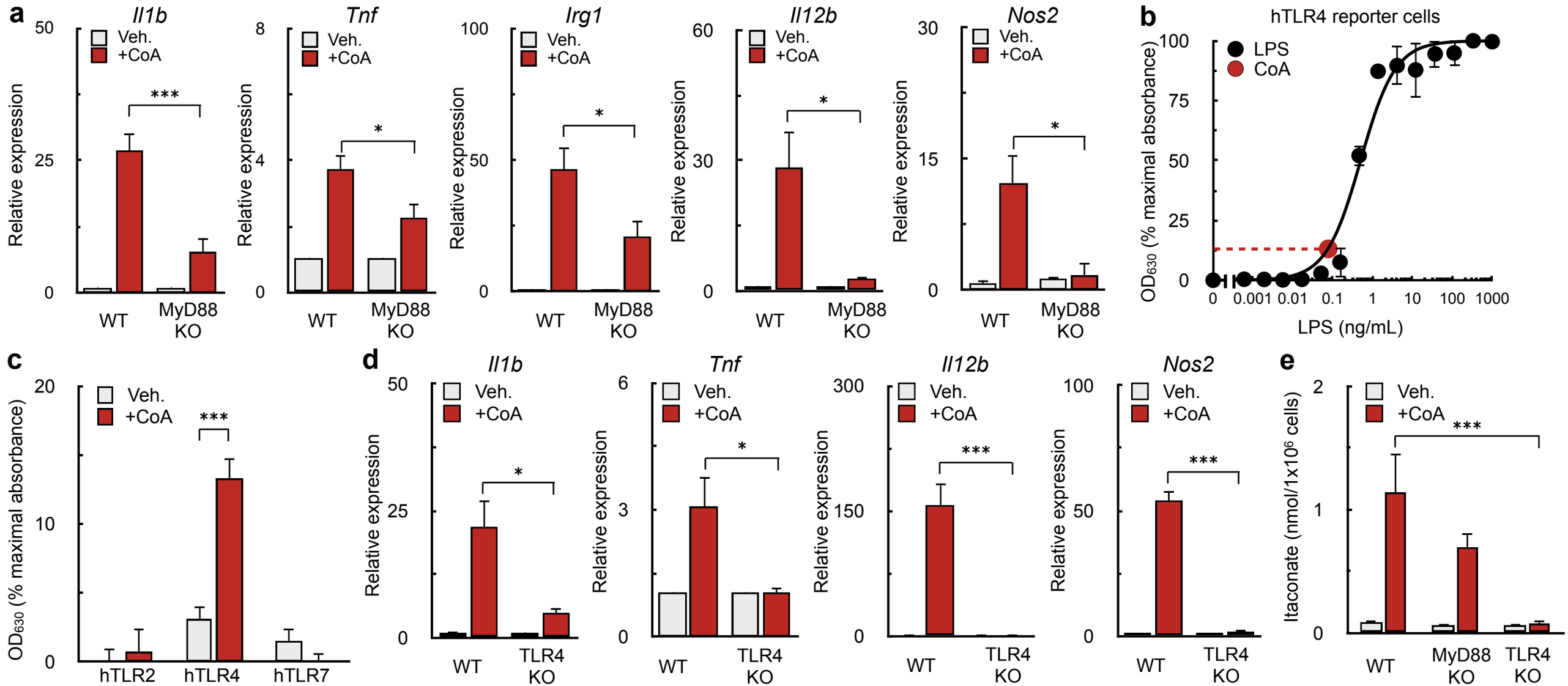

a
Il1b
Tnf
Irg1
Il12b
Nos2
Veh.
+CoA
Relative expression
***
*
WT
MyD88 KO
b
hTLR4 reporter cells
LPS
CoA
OD630 (% maximal absorbance)
LPS (ng/mL)
c
OD630 (% maximal absorbance)
hTLR2
hTLR4
hTLR7
d
TLR4 KO
e
Itaconate (nmol/1x10^6 cells)

Figure 5

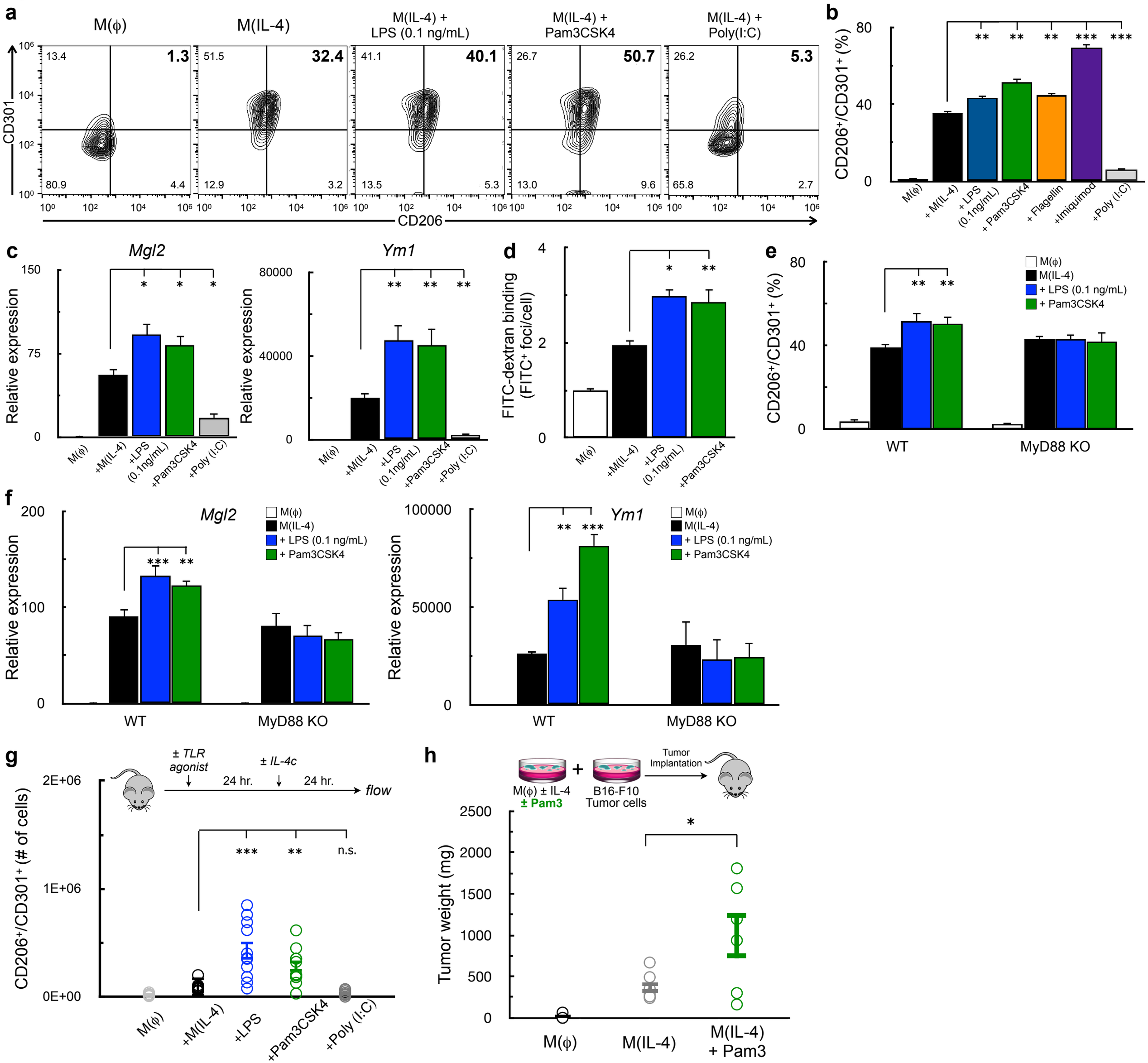
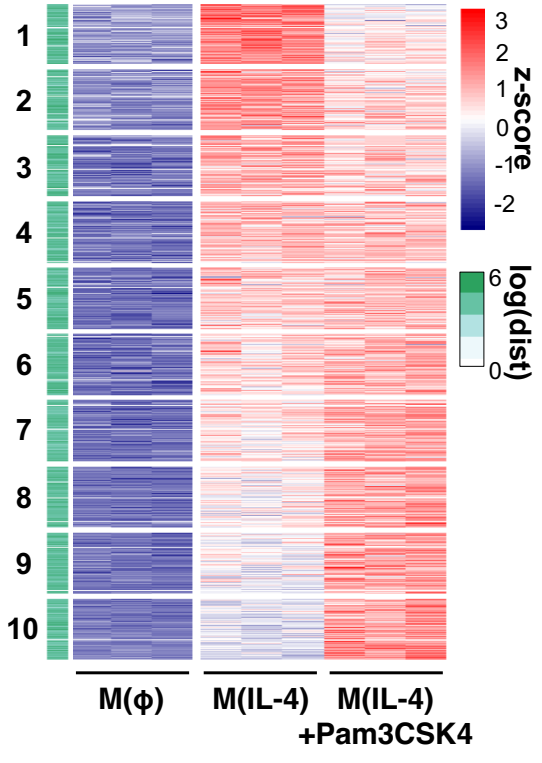
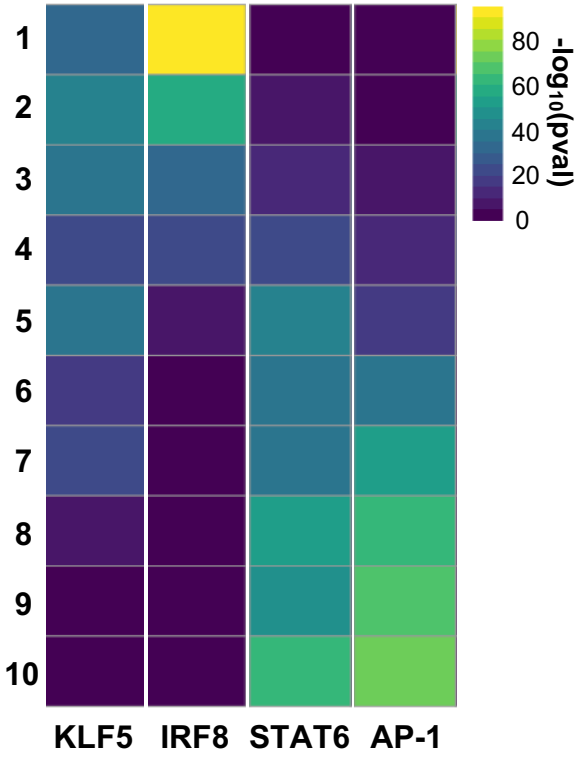

a
M(ϕ)
M(IL-4)
M(IL-4) + LPS (0.1 ng/mL)
M(IL-4) + Pam3CSK4
M(IL-4) + Poly(I:C)
CD301
CD206
13.4
1.3
80.9
4.4
51.5
32.4
12.9
3.2
41.1
40.1
13.5
5.3
26.7
50.7
13.0
9.6
26.2
5.3
65.8
2.7
b
CD206+/CD301+ (%)
M(ϕ)
+ M(IL-4)
+ LPS (0.1ng/mL)
+ Pam3CSK4
+ Flagellin
+Imiquimod
+Poly (I:C)
c
Mgl2
Ym1
Relative expression
M(ϕ)
+M(IL-4)
+LPS (0.1ng/mL)
+Pam3CSK4
+Poly (I:C)
d
FITC-dextran binding (FITC+ foci/cell)
e
CD206+/CD301+ (%)
M(ϕ)
M(IL-4)
+ LPS (0.1 ng/mL)
+ Pam3CSK4
WT
MyD88 KO
f
Mgl2
Ym1
Relative expression
WT
MyD88 KO
g
± TLR agonist
± IL-4c
24 hr.
24 hr.
flow
CD206+/CD301+ (# of cells)
n.s.
M(ϕ)
+M(IL-4)
+LPS
+Pam3CSK4
+Poly (I:C)
h
M(ϕ) ± IL-4
± Pam3
B16-F10 Tumor cells
Tumor Implantation
Tumor weight (mg)
M(ϕ)
M(IL-4)
M(IL-4) + Pam3

Figure 6

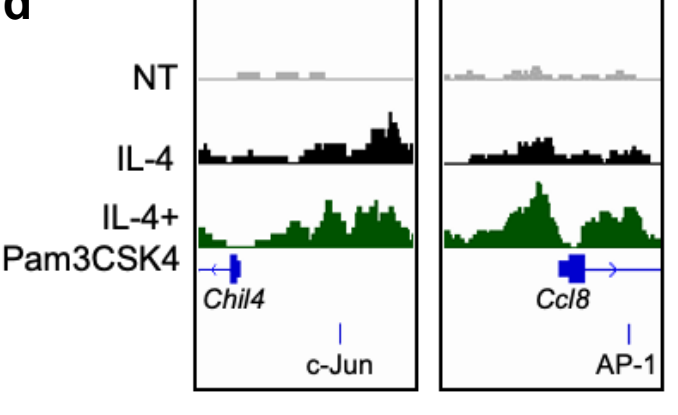
a



b



c

De novo motif	Best Match	% Targets	$-\log_{10}$(pval)
	Fra1 (bZIP)	61	144
	Stat6	31	108
	Egr2	21	47

d