

1 **Differential post-transcriptional regulation of IL-10 by TLR2 and TLR4 activated**
2 **macrophages**

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16 **List of Abbreviations:** BM-derived M ϕ (BMM ϕ); interferon (IFN); Toll/IL-1 receptor domain-
17 containing adaptor inducing IFN- β (TRIF); tristetraprolin (TTP)

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24 **Abstract**

25 Activation of TLRs by microbial molecules triggers intracellular signaling cascades and the expression of
26 cytokines, as IL-10. *Ii10* expression is tightly controlled to ensure effective immune responses, whilst
27 preventing pathology. Maximal TLR-induction of *Ii10* transcription in M ϕ requires signaling through the
28 MAPKs ERK and p38. Signals via p38 downstream of TLR4 activation also regulate IL-10 at the post-
29 transcriptional level, but whether this mechanism operates downstream of other TLRs is not clear. We
30 compared the regulation of IL-10 production in TLR2 and TLR4 stimulated BM-derived M ϕ (BMM ϕ)
31 and found different stability profiles for the *Ii10* mRNA. TLR2 signals promoted a rapid induction and
32 degradation of *Ii10* mRNA, whereas TLR4 signals protected the *Ii10* mRNA from rapid degradation, due
33 to the activation of Toll/IL-1 receptor domain-containing adaptor inducing IFN- β (TRIF) and enhanced
34 p38 signaling. Consequently, TLR4 is a stronger inducer of IL-10 secretion than TLR2. Our study
35 provides a molecular mechanism for the differential IL-10 production by TLR2- or TLR4-stimulated
36 BMM ϕ , showing that p38-induced stability is not common to all TLR signaling pathways. Furthermore,
37 this mechanism is also observed upon bacterial activation of TLR2 or TLR4 in BMM ϕ , thus contributing
38 to IL-10 modulation in these cells in an infection setting.

39

40 Introduction

41 TLRs, a class of germ-line encoded receptors, play an important role in the recognition of
42 microorganisms and initiation of innate immune responses [1]. Members of the TLR family recognize
43 different molecular patterns in microbes, from peptidoglycan to polysaccharides, proteins and nucleic
44 acids [1]. TLR triggering leads to a specific cellular transcriptional program with the expression of
45 different immune mediators, such as cytokines [1]. The activation of intracellular signaling cascades upon
46 TLR stimulation by PAMPs relies on the presence of the signaling adaptors MyD88 and/or Toll/IL-1
47 receptor-domain-containing adaptor inducing interferon IFN- β (TRIF) [1]. MyD88 is a common signaling
48 adaptor used by all TLRs with the exception of TLR3, which only activates the TRIF dependent pathway.
49 Triggering of TLR4 by its ligand, LPS, is the only known example of recruitment of both MyD88 and
50 TRIF [1]. In this case, whereas both MyD88 and TRIF contribute to full pro-inflammatory cytokine gene
51 expression, only the TRIF pathway is involved in the induction of type I IFN downstream of TLR4 [2-5].

52 In addition to pro-inflammatory cytokine secretion, TLR signaling also leads to the production of
53 IL-10 by innate immune cells. IL-10 is a powerful anti-inflammatory cytokine produced by many cells of
54 the immune system, including innate immune cells such as M ϕ [6]. Strict regulation of the balance
55 between IL-10 production and the pro-inflammatory immune response during infection is essential to
56 achieve clearance of the pathogen in the absence of immunopathology, while at the same time avoiding
57 the establishment of chronic infection [7-9]. IL-10 production by TLR4-activated M ϕ or DCs requires
58 both MyD88 and TRIF signals [10]. Moreover, not only the TNF receptor-associated factor (TRAF) 6 has
59 been shown to be essential for the MyD88 pathway and involved in the signaling of TRIF [11], but also
60 TRAF3 recruitment by either adaptor is fundamental for IL-10 production [3]. Overall, whereas the
61 molecular pathways mediated by MyD88 and implicated in IL-10 regulation are well understood, less is
62 known of the specific contribution of TRIF.

63 Several molecular mechanisms for *Il10* gene regulation have been described, including epigenetic control,
64 the activation of specific intracellular signaling cascades, the action of certain transcription factors and

65 post-transcriptional control [6]. Although these broad mechanisms likely operate in all IL-10-producing
66 cells, cell-specific factors have also been described [6]. For example, a specific NF- κ B-binding enhancer
67 sequence at the *IL10* locus regulates *IL10* transcription in M ϕ and DCs stimulated via TLRs, but not in IL-
68 10-producing T cells [12]. In addition to NF- κ B, other signaling cascades have been implicated in the
69 regulation of IL-10 induction by TLR-activated M ϕ and DCs, including the MAPKs ERK [3, 13-17] and
70 p38 [13, 14, 18-22]. Indeed, ERK activation is required for IL-10 expression in different cells, from M ϕ
71 and DCs to Th cells [6]. ERK activation upregulates the transcription factor cFOS, which in turn
72 enhances IL-10 transcription [15, 17]. Additionally, ERK activation has been shown to lead to the
73 induction of IL-10 by increasing the *IL10* locus accessibility to the binding of transcription factors [13].
74 Regulation of IL-10 transcription by p38 has also been described, and shown to involve the transcription
75 factor Sp1 [18] and the activation of the downstream kinases MSK1 and MSK2 and CREB
76 phosphorylation [23]. Activation of p38 has been further implicated in the post-transcriptional regulation
77 of IL-10 by M ϕ in response to TLR4 ligation by helping to protect the *IL10* mRNA from rapid
78 degradation induced by the RNA binding protein tristetraprolin (TTP) [24]. Consistent with a role for
79 TTP in inducing the rapid degradation of the *IL10* mRNA, TTP deficient (-/-) M ϕ show elevated levels of
80 *IL10* mRNA upon TLR4 stimulation [25]. Other post-transcriptional mechanisms for *IL10* gene regulation
81 have been described, including the participation of certain microRNAs [26, 27]. Targeting mRNA
82 stability is therefore an important mechanism for the regulation of IL-10 production, and indeed the
83 production of other cytokines [28]. However, this mechanism has been mainly studied downstream of
84 TLR4 and it is not clear how it operates downstream of other TLRs.

85 In this study, we compared the induction of IL-10 in M ϕ stimulated via the TLR2 and TLR4
86 ligands, Pam3CSK4 and LPS, respectively, as well as whole bacteria which predominantly signal through
87 either of these TLRs. We have found that independently of the stimuli, a peak of *IL10* mRNA was
88 observed as early as 1 h post-stimulation. However, whereas TLR2 signaling led to a rapid degradation of
89 *IL10* mRNA, TLR4 signals contributed to increased stability of *IL10* mRNA. This increased *IL10* mRNA

90 stability was dependent on TRIF-mediated activation of the MAPK p38 signaling pathway. We thus
91 provide evidence that the TRIF pathway regulates IL-10 production at the post-transcriptional level, thus
92 discriminating between TLR2 and TLR4 activation of M ϕ . Furthermore, we show that triggering of M ϕ
93 by TLR2- or TLR4-activating bacteria impacts IL-10 secretion by these cells, suggesting a potential
94 relevance of the described mechanism for modulating the course of the immune response during
95 infection.

96 **RESULTS**

97 **Distinct post-transcriptional regulation of IL-10 through TLR2 versus TLR4 signaling in**
98 **macrophages**

99 To dissect the molecular mechanisms regulating the initial steps of *Il10* gene expression in BMM ϕ
100 stimulated via TLR2 or TLR4, we compared the kinetics of mRNA expression in response to ligands
101 Pam3CSK4 and LPS. The dose of TLR2 and TLR4 agonists used corresponded to maximum IL-10
102 production by stimulated BMM ϕ , as measured by immunoassay (Supplemental Fig. 1). Both stimuli
103 induced a peak of *Il10* mRNA at 1 h post-stimulation (Fig. 1A). Strikingly, the *Il10* mRNA induced upon
104 TLR2 activation of BMM ϕ rapidly declined, with much lower levels detected at 3 h post-stimulation (Fig.
105 1A). This was in contrast to TLR4 triggering of BMM ϕ , where the amount of *Il10* mRNA remained
106 constant between 1 h and 3 h post-stimulation (Fig. 1A). The profile of *Il10* mRNA observed upon TLR2
107 stimulation (Fig. 1A) is compatible with a rapid degradation of *Il10* mRNA. To test this hypothesis, we
108 assessed the stability of the TLR2- or TLR4-induced *Il10* mRNA, by adding ActD to the BMM ϕ cultures
109 at 1 h post-stimulation. As shown in Fig. 1B, while the *Il10* mRNA induced by the TLR2 agonist was
110 rapidly degraded upon the addition of ActD, *Il10* mRNA induced by TLR4 showed a prolonged $t_{1/2}$. In
111 line with this, the IL-10 protein resulting of TLR2 versus TLR4 stimulation of BMM ϕ was different, with
112 higher quantities of IL-10 being secreted upon TLR4 activation (Fig. 1C). Of note, at 3 hours post-
113 stimulation with LPS the *Il10* mRNA was unstable (Supplemental Fig. 2A), suggesting that post-
114 transcriptional mechanisms operate with either TLR, albeit with a different kinetics. We next investigated
115 the *Il10* post-transcriptional regulation using another MyD88-dependent TLR stimulus. The *Il10*
116 transcription and mRNA stability profile induced in BMM ϕ upon TLR9 triggering with CpG, which like
117 TLR2 signals via MyD88 alone, was similar to that induced upon TLR2 activation (Fig. 1D, E and F).

TRIF signaling enhances the stability of *I110* mRNA via prolonged p38 activation

Next we investigated the molecular mechanism underlying the enhanced *I110* mRNA stability upon TLR4 signaling. Since a major difference between TLR2 (and TLR9) versus TLR4 signaling pathways is the recruitment of TRIF in the case of TLR4 but not TLR2 (or TLR9), and as TRIF-dependent signals have been implicated in IL-10 production by M ϕ [10], we hypothesized that TRIF may play a role in the observed differences. To test this, we generated BMM ϕ from WT or TRIF^{-/-} mice and stimulated the cultures with LPS for 1 h. At this time point, ActD was added and the amount of *I110* mRNA measured overtime by real-time PCR. We observed that the absence of TRIF significantly decreased the stability of *I110* mRNA upon TLR4 stimulation, leading to its degradation (Fig. 2A). The amount of IL-10 protein detected in TLR4-stimulated cultures of TRIF^{-/-} BMM ϕ was significantly lower than that observed for WT BMM ϕ (Fig. 2B). In the absence of TRIF the *I110* transcription after TLR4 stimulation of BMM ϕ was decreased (Supplemental Fig. 2B), suggesting that the TRIF pathway is also important to provide transcriptional enhancing signals.

Both p38 and ERK have been extensively implicated in the regulation of *I110* expression [6] and a role for p38 in the post-transcriptional regulation of *I110* has also been described [24]. We therefore investigated whether the differential activation of these MAPKs was involved in the observed post-transcriptional regulation of *I110* via TLR4/TRIF. We compared the levels of p38 and ERK phosphorylation upon TLR2 and TLR4 stimulation of WT BMM ϕ . Although over the first 60 min post-stimulation the activation of p38 was similar between TLR2 and TLR4, after that time point, a decrease in p38 activation was observed in Pam3CSK4-stimulated cells, which was less pronounced in the case of LPS-stimulated cells (Fig. 3A). TLR2 triggering led to a faster activation of ERK than TLR4 signaling, but the deactivation of this MAPK was similar to both signals (Fig. 3B). We next investigated in more detail the enhanced p38 activation and found that it was dependent on TRIF signals (Fig. 3C). ERK phosphorylation in the absence of TRIF was also decreased (Fig. 3D). We then tested the impact of inhibiting the activation of p38 or ERK signaling on the stability of *I110* mRNA, by stimulating WT

146 BMM ϕ with LPS and, at 50 min post-stimulation, adding specific inhibitors for these MAPKs. We chose
147 to add the chemical inhibitors at 50 min post-stimulation to minimize effects on the initial induction of
148 *I110* transcription. As shown in Fig. 3E and F, inhibition of p38 led to the degradation of the *I110* mRNA,
149 whereas inhibition of ERK did not. In line with this, inhibition of p38 activation had a stronger effect on
150 IL-10 protein than ERK blockade (Fig. 3G and H). Of note, other p38 (BIRB) and ERK (PD184352 and
151 U0126) inhibitors were used with similar results (data not shown).

152 In summary, our data show that TRIF signals contribute to regulate IL-10 at the post-transcriptional level
153 through a mechanism that involves enhanced p38 activation.

154

155 **TTP deficiency impacts *I110* mRNA stability early upon TLR2 stimulation of BMM ϕ**

156 The RNA binding protein TTP has been described to target *I110* mRNA and induce its rapid degradation
157 [24, 25]. p38 activation has been shown to inhibit TTP, thus promoting an increase of the $t_{1/2}$ of *I110*
158 mRNA [24, 25, 29, 30]. Considering these reports and our findings showing that in TLR2-stimulated
159 BMM ϕ a rapid degradation of the *I110* mRNA occurs, in parallel with a reduced activation of p38, we
160 next investigated the stability of TLR2-induced *I110* mRNA in the absence of TTP. WT or TTP^{-/-} BMM ϕ
161 were stimulated for 1 h with LPS or Pam3CSK4 and at that time ActD was added to the cultures and *I110*
162 mRNA measured by real-time PCR. As shown in Fig. 4A, LPS stimulation in the absence of TTP did not
163 result in increased *I110* mRNA stability at this time point. However, when the cells were stimulated with
164 Pam3CSK4 a slight but significant increase in *I110* mRNA $t_{1/2}$ was observed in the absence of TTP (Fig.
165 4B). For both LPS and Pam3CSK4 stimuli, an increase in IL-10 protein secretion was observed in TTP^{-/-}
166 cells at 6 h post-stimulation (Fig. 4C and D). This is in line with previous reports showing that at later
167 time points post-stimulation with LPS, the absence of TTP increases the stability of the *I110* mRNA with
168 consequent increase on IL-10 production [24, 25, 29, 30]. We herein show that in the case of TLR2
169 activation, the absence of TTP influences the *I110* mRNA stability at an earlier time point than with
170 TLR4. The transcriptional profile of *TTP* observed in macrophages upon TLR2 or TLR4 activation was

171 similar (Fig. 4E). We also addressed the contribution of TRIF signals for *TTP* expression and found that
172 in macrophages stimulated with LPS the absence of TRIF decreased the expression of *TTP* (Fig. 4F).
173 These data suggest that the differences observed for the stability of the *Il10* mRNA do not reflect or
174 correlate to specific changes in the expression of *TTP*.

175 176 **Differential post-transcriptional regulation of IL-10 by TLR2 versus TLR4 upon BMM ϕ** 177 **stimulation with bacteria**

178 Our findings unveil a novel link between TRIF and IL-10 post-transcriptional regulation, mediated by
179 p38 and partly by TTP. However, these findings were obtained using TLR stimulation with chemically
180 pure, single ligands. We sought to investigate if the described mechanism was also in place when BMM ϕ
181 were stimulated with bacteria. To test that, we stimulated WT BMM ϕ with bacteria described in the
182 literature to mainly require TLR2 ligation, such as *M. tuberculosis* strain H37Rv [31] and *L.*
183 *monocytogenes* [32], or TLR4 ligation, such as *E. coli* and *S. enteritidis* [33], for maximal BMM ϕ
184 activation. In agreement with the data from the respective TLR ligands, *M. tuberculosis* stimulation of
185 BMM ϕ , which requires TLR2 to induce IL-10, led to a rapid degradation of *Il10* mRNA, whereas *Il10*
186 mRNA induced upon stimulation of these cells with *E. coli*, which predominantly induces IL-10 via
187 TLR4, was stable over time (Fig. 5A). In further support of our data, *L. monocytogenes*, which signals
188 mainly through TLR2 showed rapid degradation of induced *Il10* mRNA, whereas *S. enteritidis*, which
189 mainly signals through TLR4 resulted in the induction *Il10* mRNA which was stable over time (Fig. 5B).

190 To investigate whether TRIF and p38 activation also accounted for the increased stability of the
191 *Il10* mRNA observed in response to bacteria signaling through TLR4, we further dissected the
192 stimulation of BMM ϕ with *E. coli*. Again *Il10* mRNA induced by *E. coli* stimulation of BMM ϕ lost its
193 stability in the absence of TRIF or p38 activation (Fig. 5C and D). In addition, the amount of IL-10
194 protein secreted in the absence of TRIF or in the presence of the p38 inhibitor was lower than that
195 obtained for WT cells in response to *E. coli* (Fig. 5E and F). Additionally, the comparison of BMM ϕ

196 activation by *M. tuberculosis* H37Rv or *E. coli* showed that the relative amounts of *Il10* and *TTP* mRNA
197 and of p38 activation followed the pattern described for TLR2 versus TLR4 activation with chemical
198 agonists (Supplemental Fig. 3A-C). Also, in the absence of TRIF, the *Il10* and *TTP* mRNA and p38
199 activation were decreased in BMM ϕ stimulated with *E. coli*, recapitulating the findings with LPS
200 stimulation of these cells (Supplemental Fig. 3D-F).

201 Taken together, our study suggests that recognition of pathogens by distinct TLRs has an impact
202 on the amount of IL-10 produced by BMM ϕ . Our data demonstrate that this results from post-
203 transcriptional mechanisms of *Il10* mRNA stabilization involving TLR4/TRIF/p38 signaling when
204 BMM ϕ are stimulated with TLR4-activating microbes. This reveals an important mechanism in
205 modulating the course of the immune response.

DISCUSSION

IL-10 plays a fundamental role in regulating inflammation and the level of activation of adaptive immune responses [34]. Several immune cells produce IL-10 in response to various stimuli, for example ligands for PRRs in M ϕ [6]. Among the extensive family of PRRs, TLRs are amongst the best characterized members, with many studies contributing to the understanding of the intracellular signaling cascades, as well as of the changes in the cell transcriptome, triggered by individual TLRs [1]. The regulation of *Il10* gene transcription induced by TLR activation has also been an active area of research owing to the important immuno-regulatory roles of this cytokine [6]. Several studies demonstrate the existence of various layers for modulating IL-10 production, from epigenetic control, to transcriptional and post-transcriptional regulation [6].

By comparing TLR2- versus TLR4-stimulated BMM ϕ , we studied the molecular mechanisms leading to the post-transcriptional regulation of *Il10* mRNA induced by these stimuli. Our findings show that TLR4 signals increase the $t_{1/2}$ of the *Il10* mRNA, via enhanced p38 activation which was dependent on TRIF. Despite initial transcriptional induction of the *Il10* gene at 1 hr post stimulation, both TLR2 and TLR9 that lack the activation of the TRIF signaling cascade, failed to sustain the $t_{1/2}$ of *Il10* mRNA subsequent to this. Our study confirms the role of TRIF for maximal IL-10 production by TLR4-stimulated BMM ϕ [10]. TRIF signals promote both a stronger transcription of the *Il10* gene and an increased stability of the *Il10* mRNA, thus resulting in more IL-10 protein produced by TLR4- than TLR2-activated M ϕ . Therefore, our study suggests that different TLRs regulate IL-10 expression in different ways, perhaps allowing the fine-tuning of IL-10 production to suit infections with different pathogens and/or commensals. Further studies are required to address whether the role of TRIF in enhancing p38 activity and *Il10* mRNA stability is direct or indirect. A possible candidate for an indirect role of TRIF in this process is type I IFN. On one hand, TLR2 agonists are poor inducers of IFN- β , as opposed to TLR4 ligands that induce IFN- β mRNA independently of MyD88 signaling [35]. On the other

231 hand, IFN- β has been implicated in the induction and sustained expression of IL-10 by LPS-stimulated
232 BMM ϕ [35-38].

233 The mechanism proposed in our study was recapitulated upon stimulation of BMM ϕ with
234 microbes, such as *M. tuberculosis*, *L. monocytogenes*, *E. coli* and *S. enteritidis*. Therefore, it is possible
235 that the manipulation of specific TLR activation by bacteria will have implications on the amounts of IL-
236 10 secreted by M ϕ . In this sense, bacteria that preferentially trigger TLR4 may be manipulating the
237 immune system to increase the amounts of IL-10, thus compromising the full efficacy of the immune
238 response. It will be interesting to investigate if these differential pathways regulating IL-10 at the post-
239 transcriptional level also occur in cells other than M ϕ , or in M ϕ located at different anatomical sites.

240 One of the first pieces of evidence demonstrating post-transcriptional regulation of *Il10* mRNA
241 expression came from studies of the 3' untranslated regions (UTR) of the *Il10* mRNA, which showed the
242 existence of adenylate-uridylylate (AU) rich elements (AREs), capable of mediating mRNA decay [39].
243 These ARE sequences recruit several ARE-binding proteins that positively or negatively regulate mRNA
244 stability [28]. One such protein is TTP that was found to promote rapid mRNA decay of several
245 transcripts, including that of TNF [40], by initiating the assembly of RNA decay machinery [41, 42].
246 More recently, several cytokines, such as IL-10, were found to be targets of TTP [24, 25]. In line with
247 this, the $t_{1/2}$ of *Il10* mRNA induced upon 5 h of LPS stimulation of M ϕ was increased in the absence of
248 TTP [25]. As we now show, TTP also influences the $t_{1/2}$ of the *Il10* mRNA induced by TLR2 activation,
249 but in this case it targets the *Il10* mRNA earlier than observed for LPS. This difference between TLR2
250 and TLR4 is most likely related to differential TTP activation/inactivation, as the transcriptional pattern
251 of TTP in BMM ϕ activated through TLR2 or TLR4 is similar. Furthermore, TRIF deficiency led to less
252 TTP transcription in LPS-stimulated BMM ϕ than in WT cells. Altogether, our data suggest that a direct
253 relation between TTP transcription and function is not in place, in support of the importance of TTP
254 regulation by post-translational modifications. Specifically, the activity of TTP is negatively regulated by
255 the MAPK p38 [24, 25, 29, 30]. Importantly, the impact of p38 control of TTP-mediated mRNA

256 destabilization varies with target mRNAs. For example, whereas in the case of TNF a certain p38
257 independence was observed [43], for IL-1 α [43], CXCL1 [44] and IL-10 [24] the inhibition of p38
258 activation was important for the TTP-mediated decay. We observed a TRIF-mediated increase of p38
259 signaling, which is in line with previous studies [2, 45], and associated it with an increased $t_{1/2}$ of *Ii10*
260 mRNA. It is thus likely that the activation of TRIF and p38 upon TLR4 triggering are stabilizing the *Ii10*
261 mRNA in part through TTP inactivation. Of note, our data may look in apparent disagreement with
262 previous studies showing that IL-10 negatively regulate p38 [43, 46]. However, we observed stronger p38
263 activation during an initial phase of TLR4 stimulation (up to 120 minutes). During this initial period, the
264 amount of IL-10 protein secreted by TLR2- or TLR4-stimulated BMM ϕ is similar (data not shown), so, at
265 this stage, differences in p38 are likely not related to differential regulation by IL-10. Finally, we report
266 that the MAPK ERK does not regulate IL-10 expression post-transcriptionally, although inhibition of this
267 cascade reduced the amount of IL-10 secreted by TLR4-stimulated BMM ϕ . This observation is in line
268 with previous reports demonstrating that ERK is involved in the molecular mechanisms regulating *Ii10*
269 gene expression [6]. Thus, it is possible that ERK plays a major role in transcriptionally regulating IL-10,
270 with p38 additionally participating at the post-transcriptional level. This observation suggests that the
271 signaling cascades downstream of ERK and p38 diverge for example in what concerns TTP regulation.

272 TRIF signals appear therefore to enhance the activation of the MAPK p38, so that TTP-mediated
273 mRNA degradation is delayed. One possible mediator of this mechanism is the MAPK phosphatase
274 DUSP1. DUSP-1 was shown to impair p38 activity [47-49], to impair TTP expression and production by
275 inhibiting p38 activation [50] and to induce the reduction of *Ii10* mRNA stability [49]. Also, DUSP1-/-
276 mice challenged in vivo with LPS showed increased production of IL-10 [51]. Since in response to LPS
277 stimulation, TRIF-/- macrophages showed decreased levels of DUSP1 activation relatively to WT cells
278 [22], it is possible that the differences observed in terms of p38 activation are not directly related to
279 DUSP1. Further studies are however needed to unequivocally answer this question.

280 The mechanism proposed in this study is likely to affect pro-inflammatory cytokines in addition to
281 IL-10. Activation of the TRIF pathway with subsequent enhanced p38 activation and an increase in the
282 $t_{1/2}$ of cytokine mRNAs, promotes a specific post-transcriptional control that may shift the immune
283 response towards a more inflammatory type. In this scenario, ensuring that IL-10 is also enhanced might
284 be of importance for the achievement of a balanced response. IL-10 has been implicated in inducing its
285 own transcription via STAT3 activation in human monocyte-derived M ϕ [52], which would constitute an
286 autocrine loop for IL-10 induction. IL-10 induces the destabilization of its own mRNA possibly via a
287 secondary factor [53]. More recently, it has been shown that in addition to being a target of TTP, IL-10 is
288 itself an activator of TTP, by reducing late p38 activity [43, 54]. Thus, in situations when IL-10 is being
289 produced, TTP is activated. This mechanism ensures a shut-down of pro-inflammatory cytokines, such as
290 TNF and IL-1 α [55], IL-6 and IL23p19 [55] or chemokines [56], mediated by IL-10 induced mRNA
291 decay. It is also possible that this mechanism subsequently limits IL-10 translation, thus guaranteeing the
292 appropriate balance of the immune response. For both TLR2 and TLR4 stimulation of BMM ϕ , early IL-
293 10 protein was detected in similar amounts or was higher for TLR4 signals, thus suggesting that in our
294 system, the differences in the stability of the *Ii10* mRNA observed are likely not due to distinct IL-10
295 autocrine signaling.

296 The broad array of regulatory mechanisms in place to modulate IL-10 expression might be a
297 consequence of IL-10 induction by a variety of stimuli in many different cell types. Uncovering the
298 extensive network underlying these mechanisms will be of interest to targeted modulation of IL-10
299 production. As we show here, this extensive network of molecular mechanisms differs with the type of
300 stimuli and involves many layers of regulation, including at the post-transcriptional level. We show that
301 although the early induction of *Ii10* transcription in BMM ϕ by TLR2 and TLR4 is similar, the cell then
302 fine tunes the amount of IL-10 via a mechanism of post-transcriptional regulation mediated by
303 TRIF/p38/TTP, which discriminates between these two TLRs. This mechanism operates in BMM ϕ
304 sensing whole bacteria and allows for distinct IL-10 induction by TLR2- versus TLR4-activating

305 microbes. As a consequence, TLR4 stimulation of BMM ϕ leads to higher levels of IL-10 production than
306 TLR2 activation, which may be beneficial to inhibit inflammatory pathologies or on the other hand
307 manipulated to the advantage of the pathogen.

308

309 **Materials and Methods**

310 **Animals**

311 Females of eight to twelve weeks of age on the C57BL/6 background were used. Wild type (WT) mice
312 were ordered from Charles River (Barcelona, Spain). TLR2^{-/-} [57] and TLR4^{-/-} [58] animals were bred
313 and maintained at ICVS. TRIF^{-/-} [5] mice were from MRC-NIMR. All mouse protocols followed the
314 European Union Directive 86/609/EEC and were previously approved by the national authority *Direcção*
315 *Geral de Veterinária*.

316 **Cell culture**

317 Complete (c) DMEM was prepared by supplementing DMEM (GIBCO) with 10% FBS, 1% sodium
318 pyruvate, 1% HEPES and 1% L-glutamine (all from GIBCO). BMM ϕ were generated in cDMEM
319 supplemented with 20% of L929-cell conditioned media (LCCM). On day 0, 4x10⁶ cells in 8 mL were
320 plated per petri dish (Sterilin) and kept at 37°C and 5% CO₂. Cell cultures were fed on day 4 with 10 mL
321 of cDMEM-20% LCCM. BMM ϕ were recovered on day 7 of culture, counted and stimulated for different
322 time-points as appropriate. TTP^{-/-} and control (WT) cells were from littermate male mice of 6-16 weeks
323 of age that had been backcrossed 28 generations into C57Bl/6NTac mice and have been described
324 previously [59]; cells were used to derive, as above, BMM ϕ , except that 30% of LCCM was used.

325 **Bacteria**

326 *Mycobacterium tuberculosis* H37Rv strain Pasteur, a kind gift from P. J. Cardona (Barcelona), was grown
327 in Proskauer Beck (PB) medium containing 0.05% Tween 80 to mid-log phase and frozen in PB medium
328 and 30% glycerol at -80°C. The DH5X strain of *E. coli* (ICVS) and *S. enteritidis* (a gift from J. Azeredo,
329 INL) were grown in Lysogeny broth (LB), to mid-log phase and frozen in PBS 1X and 30% glycerol at -
330 80°C. *L. monocytogenes* (ICVS) was grown in Antibiotic Medium 3. *E. coli* was heat inactivated for 60
331 min at 95°C, *S. enteritidis* was heat inactivated for 30 min at 72°C and *L. monocytogenes* for 60 min at
332 62°C prior to cell stimulation. All bacteria were added to the cell cultures at a multiplicity of infection of
333 two.

334 **Reagents**

335 LPS (*S. Minnesota*, Sigma) and Pam3CSK4 (InvivoGen) were used at 25 ng/mL and 2 µg/mL,
336 respectively. Actinomycin D (ActD) (Sigma) was used at 10 µg/mL and added to the cell cultures 1 h
337 post-stimulation, when indicated. p38 (SB 203580) and ERK (PD0325901) inhibitors, kind gifts of Prof.
338 Sir P. Cohen, were used at 2.5 µM and 0.1 µM [60], respectively, and added to the cell cultures 50 min
339 post-stimulation. Cell culture grade DMSO (Sigma) was used as a vehicle control, when appropriate.

340 **ELISA**

341 IL-10 quantification in the cell culture supernatants was performed by ELISA following the
342 manufacturer's instructions (eBioscience).

343 **RNA extraction, cDNA and quantitative real time PCR**

344 Total RNA from stimulated and non-stimulated cell culture samples was extracted at different time-points
345 with TRIzol® 143 Reagent (Invitrogen, San Diego, CA) and converted to cDNA according to the
346 manufacturer's instructions (Fermentas). *Il10* and *TTP* gene expression were assessed by real-time PCR
347 using SYBR Green (Fermentas) and TaqMan MasterMix (Applied Biosystems) and normalized against
348 ubiquitin or Hypoxanthine Phosphoribosyltransferase 1 (*Hprt1*) expression, respectively, as previously
349 described [12].

350 **Western Blot (WB)**

351 BMMφ were rested for 5 h in 1% FBS – cDMEM prior to stimulation. At the indicated time points post
352 stimulation, cell culture supernatants were discarded and cells gently washed with apyrogenic 1X PBS
353 (GIBCO). Protein extracts were obtained with a lysis buffer solution (100 mM Tris-HCl pH8; 10%
354 glycerol; 1 mM EDTA pH8; 5 mM MgCl₂; 50 mM NaCl; 1% NP-40; 1x protease inhibitor cocktail
355 (Roche); 1x phosphatase inhibitor cocktails II and III (Sigma); dH₂O) for 20 minutes on ice. Extracts
356 were kept at -80°C until further use. Samples were then centrifuged at 13000 rpm for 15 min and protein
357 extracts recovered. Immediately before use, protein extracts were heated 5 min at 95°C and 20 µg of each
358 sample resolved in a 12% SDS-PAGE and then transferred to nitrocellulose membranes. Total and

359 phospho p38 (Threonine 180 and Tyrosine 182) and total and phospho ERK (Threonine 202/185 and
360 Tyrosine 204/187) were detected by using specific antibodies (all from Cell Signaling). The secondary
361 antibody was anti-rabbit-HRP (Cell Signaling). The membranes were developed with SuperSignal Femto
362 substrate (Thermo Scientific) and read by a Universal Hood II (Bio-Rad). Quantity one (Bio-Rad)
363 software was used to analyze the results.

364 **Statistical analysis**

365 Data are expressed as Mean \pm SEM and analyzed by the two-way ANOVA test with a Bonferroni post-
366 test or by the two-tailed Student's t test, as indicated. The p values considered as having statistical
367 significance were *, $p \leq 0.1$; **, $p \leq 0.01$ and *** $p \leq 0.001$.

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369

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381

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Figure legends

Figure 1. Distinct post-transcriptional regulation of IL-10 through TLR2 versus TLR4 signaling in BMM ϕ . WT BMM ϕ were stimulated with Pam3CSK4 (2 μ g/mL) or LPS (25 ng/mL) (A, B and C) or with CpG (1 μ M) (D, E and F). (A) At the indicated time points post-stimulation with Pam3CSK4 (crosses) or LPS (open squares) total RNA was harvested, converted to cDNA and the *Il10* mRNA expression determined by real-time PCR and normalized to ubiquitin. (B) At 1 h post-stimulation with Pam3CSK4 (crosses) or LPS (open squares), ActD was added to the cultures and 30, 60 or 90 min later the *Il10* mRNA expression determined as in (A). The percentage of *Il10* mRNA present at each time point is relative to the amount of *Il10* mRNA measured at 1 h post-stimulation in the absence of ActD. The *Il10* mRNA half-life ($t_{1/2}$) was calculated as the time post ActD addition where 50% of *Il10* mRNA was still present. (C) 6 h post-stimulation of BMM ϕ with Pam3CSK4 or LPS, cell culture supernatants were collected and the amount of IL-10 measured by ELISA. (D, E and F) BMM ϕ were stimulated with CpG (diamonds) and treated as in (A), (B) and (C), respectively. Results are Mean \pm SEM of 3 (A, B and C) or 2 (D, E and F) independent experiments. Each time point represented was performed in triplicate for each experiment. *p* values were determined by the two-way ANOVA with a Bonferroni post-test (A and B) or by the Student's *t* test (C); ****p*<0.001.

Figure 2. TRIF signaling enhances the stability of *Il10* mRNA. (A) BMM ϕ generated from WT (solid line) or TRIF^{-/-} (dashed line) mice were stimulated with LPS (25 ng/mL) and the *Il10* mRNA half-life ($t_{1/2}$) was determined at 1h post-stimulation, as described in Figure 1B. (B) 6 h post-stimulation of WT (black bar) or TRIF^{-/-} (white bar) BMM ϕ with LPS the cell culture supernatants were collected and the amount of IL-10 measured by ELISA. Results are Mean \pm SEM of 2 independent experiments. Each time point represented was performed in triplicate for each experiment. *p* values were determined by the two-way ANOVA with a Bonferroni post-test (A) or by the Student's *t* test (B); ****p*<0.001.

584 **Figure 3.** p38 mediates the TRIF-induced stability of the *Ii10* mRNA. (A and B) WT BMM ϕ were
585 stimulated with Pam3CSK4 (2 μ g/mL; crosses) or LPS (25 ng/mL; open squares) and, at the indicated
586 time points post-stimulation, total cell extracts were prepared, separated in SDS-PAGE and the ratio of
587 phospho p38/total p38 (A) or phospho ERK/total ERK (B) assessed by WB. (C and D) WT (solid line) or
588 TRIF^{-/-} (dashed line) BMM ϕ were stimulated with LPS (25 ng/mL) and the ratio of phospho p38/total
589 p38 (C) or phospho ERK/total ERK (D) assessed by WB as indicated in (A) and (B). Data represent
590 pooled protein extracts from each three replicates per condition. Shown is a representative Western Blot
591 from the 3 independent experiments performed and the resulting quantification. Results are Mean \pm SEM
592 of 3 independent experiments. Each time point represented was performed in triplicate for each
593 experiment. (E and F) WT BMM ϕ were stimulated with LPS for 50 min. At this time point, DMSO (as a
594 control, open squares), SB203580 (p38 inhibitor, at 2.5 μ M; close squares) or PD0325901 (ERK
595 inhibitor, at 0.1 μ M; close circles) were added to the cultures. Ten min later, ActD was added to all wells
596 and the $t_{1/2}$ of the *Ii10* mRNA determined as in Figure 1B. (G and H) Cells were treated as before, but
597 with no addition of ActD, and at 6 h post-stimulation the supernatants were collected and the amount of
598 IL-10 measured by ELISA. Results are Mean \pm SEM of 2 independent experiments. Each time point
599 represented was performed in triplicate for each experiment. *p* values were determined by the two-way
600 ANOVA with a Bonferroni post-test (A-F) or by the Student's *t* test (G and H); * *p*<0.05; ** *p*<0.01;
601 ****p*<0.001.

602 **Figure 4.** Absence of TTP delays the early degradation of the *Ii10* mRNA induced upon TLR2-signaling.
603 (A and B) WT (solid lines) or TTP^{-/-} (dashed lines) BMM ϕ (generated from frozen cells) were stimulated
604 with LPS (25 ng/mL, open squares) or Pam3CSK4 (2 μ g/mL, crosses) for 1h and the *Ii10* mRNA $t_{1/2}$ was
605 determined upon addition of ActD as described in Figure 1B. (C and D) WT (black bars) or TTP^{-/-} (white
606 bars) BMM ϕ were generated and stimulated as above, except that ActD was not added, and the amount of
607 IL-10 in the cell culture supernatants was measured by ELISA 6 h post-stimulation. (E) WT BMM ϕ were
608 stimulated with LPS (25 ng/mL, open squares) or Pam3CSK4 (2 μ g/mL, crosses) and the *TTP* mRNA

609 measured over time as indicated in Figure 1A. (F) WT (solid lines) or TRIF^{-/-} (dashed lines) BMM ϕ were
610 stimulated with LPS and the *TTP* mRNA measured over time as indicated in Figure 1A. Results are Mean
611 \pm SEM of 2 (A-D) or 3 (E and F) independent experiments. Each time point represented was performed in
612 triplicate for each experiment. *p* values were determined by the two-way ANOVA with a Bonferroni post-
613 test (A, B, E and F) or by the Student's *t* test (C and D); * *p*<0.05; ** *p*<0.01; ****p*<0.001.

614 **Figure 5.** Differential post-transcriptional regulation of IL-10 by TLR2 versus TLR4 upon BMM ϕ
615 stimulation with intact bacteria. (A and B) WT BMM ϕ were stimulated with heat-killed *E. coli* (crosses),
616 *S. enteritidis* (diamonds), *L. monocytogenes* (inverted triangles) or with live *M. tuberculosis* H37Rv (open
617 circles) at a moi of 2. The *Il10* mRNA *t*_{1/2} was determined at 1h post-stimulation, as indicated in Figure
618 1B. (C) WT (solid lines) or TRIF^{-/-} (dashed lines) BMM ϕ were stimulated with heat-killed *E. coli* (moi of
619 2) and the *Il10* mRNA *t*_{1/2} determined at 1h post-stimulation, as indicated in Figure 1B. (D) WT BMM ϕ
620 were stimulated with heat-killed *E. coli* (moi of 2) in the presence of DMSO (as a control, solid lines) or
621 of SB203580 (p38 inhibitor, at 2.5 μ M; dashed lines) and the *Il10* mRNA *t*_{1/2} determined at 1h post-
622 stimulation, as indicated in Figure 1B. (E) 6 h post-stimulation of WT (black bar) or TRIF^{-/-} (white bar)
623 BMM ϕ with *E. coli* the cell culture supernatants were collected and the amount of IL-10 measured by
624 ELISA. (F) WT BMM ϕ were stimulated with *E. coli* in the presence of DMSO (as a control, black bar) or
625 of SB203580 (p38 inhibitor, white bar) for 6 h and the IL-10 protein present in the supernatants of the
626 stimulated cultures determined by ELISA. Results are Mean \pm SEM of 3 (A and B) or 2 (C-F)
627 independent experiments. Each time point represented was performed in triplicate for each experiment. *p*
628 values were determined by the two-way ANOVA with a Bonferroni post-test (A-D) or by the Student's *t*
629 test (E and F); * *p*<0.05; ** *p*<0.01; ****p*<0.001.

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Supplemental Figure legends

Figure S1. IL-10 production by BMM ϕ in response to increasing doses of Pam3CSK4 (A) or LPS (B). WT BMM ϕ were stimulated with the indicated doses of Pam3CSK (A) or LPS (B) and 6 h post-stimulation the amount of IL-10 in the culture supernatants measured by ELISA. Represented is the Mean \pm SEM of 2 independent experiments. Each time point represented was performed in triplicate for each experiment.

Figure S2. (A) WT BMM ϕ were stimulated with LPS (25 ng/mL) and 1h (open squares) or 3h (inverted triangles) later the *Il10* mRNA $t_{1/2}$ determined as indicated in Figure 1B. (B) WT (solid lines) or TRIF $^{-/-}$ (dashed lines) BMM ϕ were stimulated with LPS (25 ng/mL) and at the indicated time points the *Il10* mRNA determined as in Figure 1A. Represented is the Mean \pm SEM of 2 independent experiments. Each time point represented was performed in triplicate for each experiment. *p* values were determined by the two-way ANOVA with a Bonferroni post-test; ****p*<0.001.

Figure S3. (A, B and C) WT BMM ϕ were stimulated with live *M. tuberculosis* H37Rv (open circles) or with heat-killed *E. coli* (crosses) at a moi of 2. At the indicated time points post-stimulation the expression of *Il10* (A) or *TTP* (B) mRNA was determined as detailed in Figure 1A. The ratio of phospho p38/total p38 (C) was also determined as detailed in Figure 3A. (D-F) WT (solid lines) or TRIF $^{-/-}$ (dashed lines) BMM ϕ were stimulated with heat-killed *E. coli* (moi of 2) and the expression of *Il10*, *TTP* or the ratio phospho p38/total p38 were determined as before. Represented is the Mean \pm SEM of 2 independent experiments. Each time point represented was performed in triplicate for each experiment. *p* values were determined by the two-way ANOVA with a Bonferroni post-test; * *p*<0.05; ** *p*<0.01; ****p*<0.001.

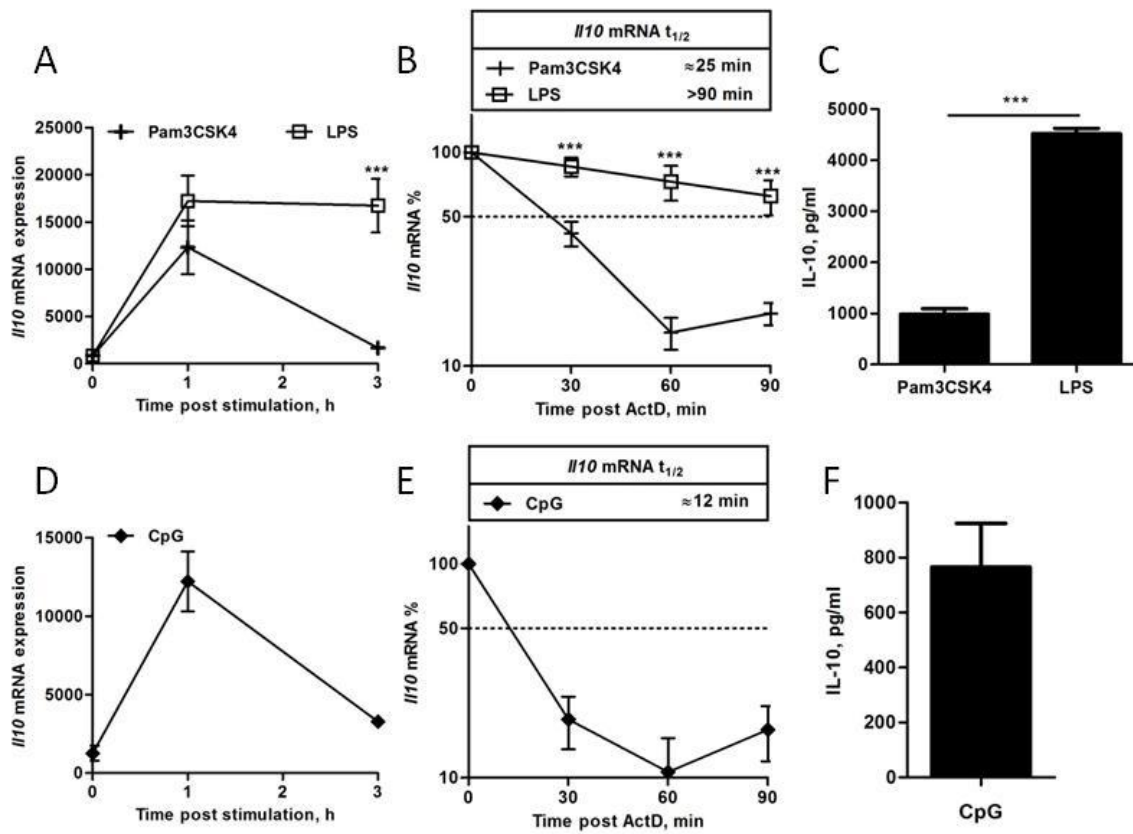


Figure 1

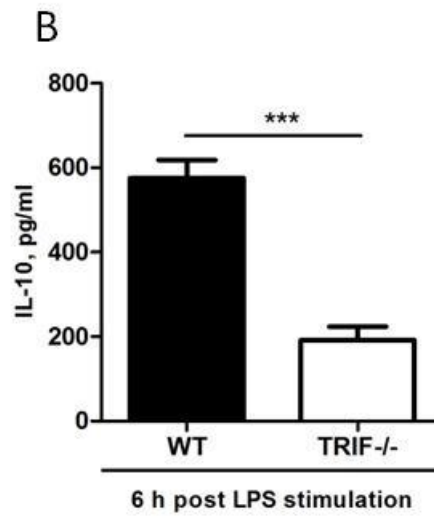
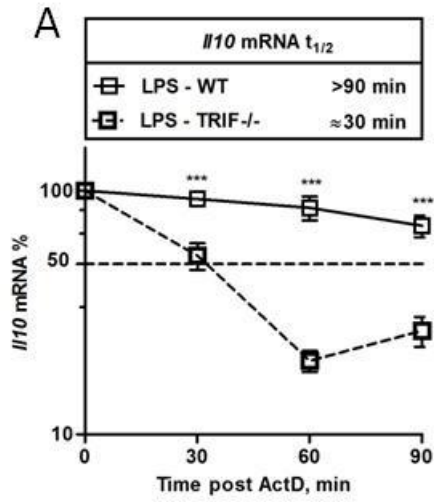


Figure 2

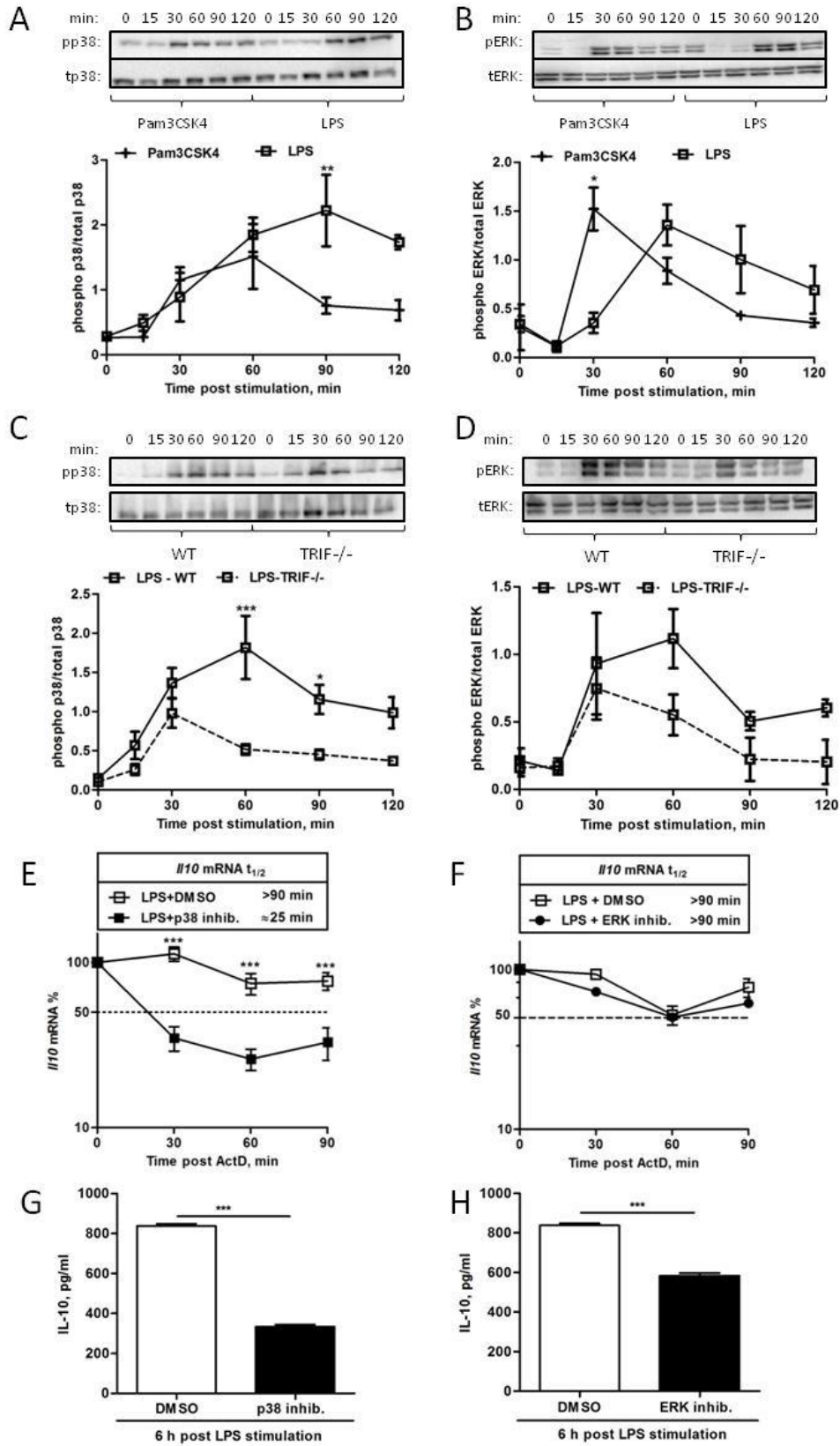


Figure 3

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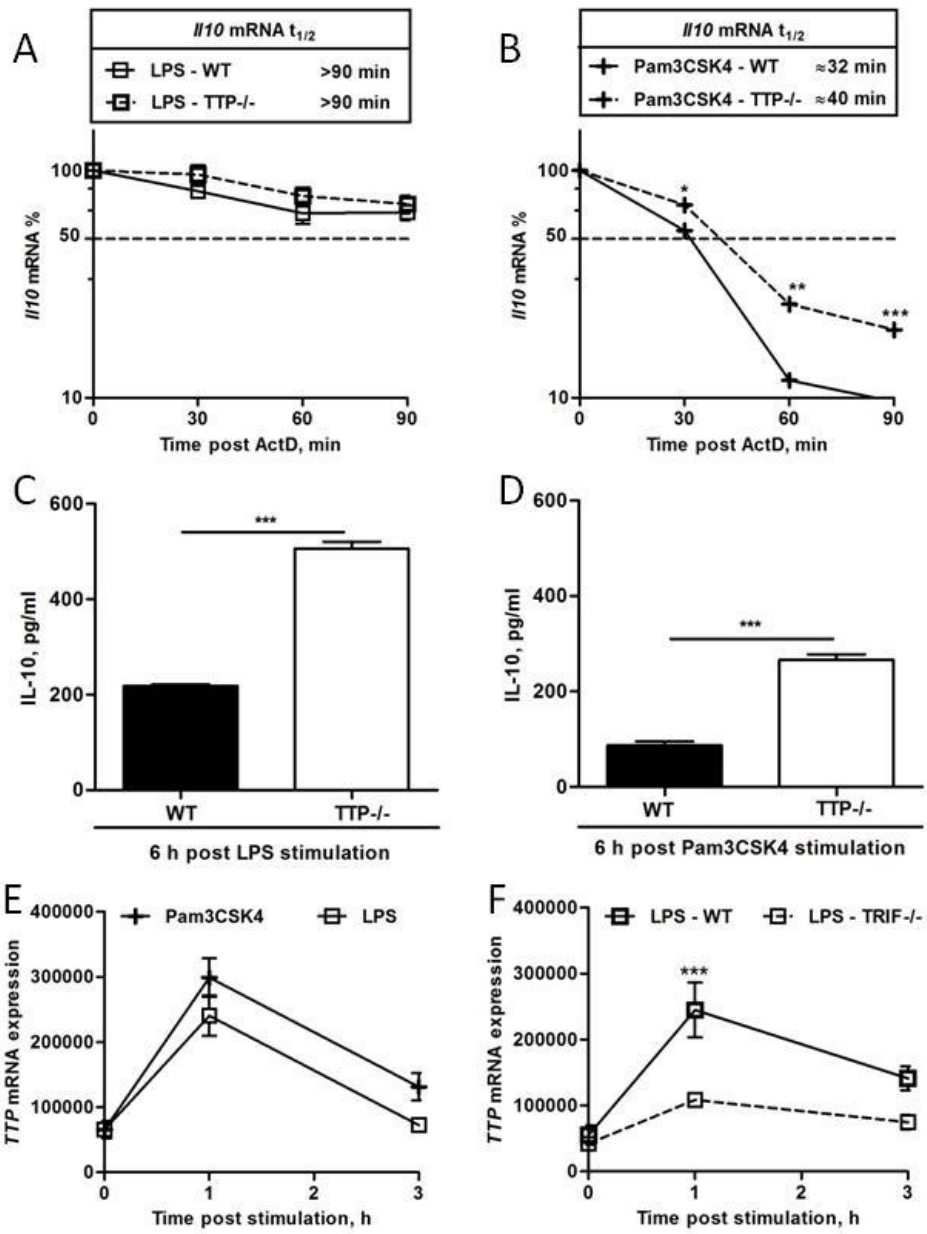


Figure 4

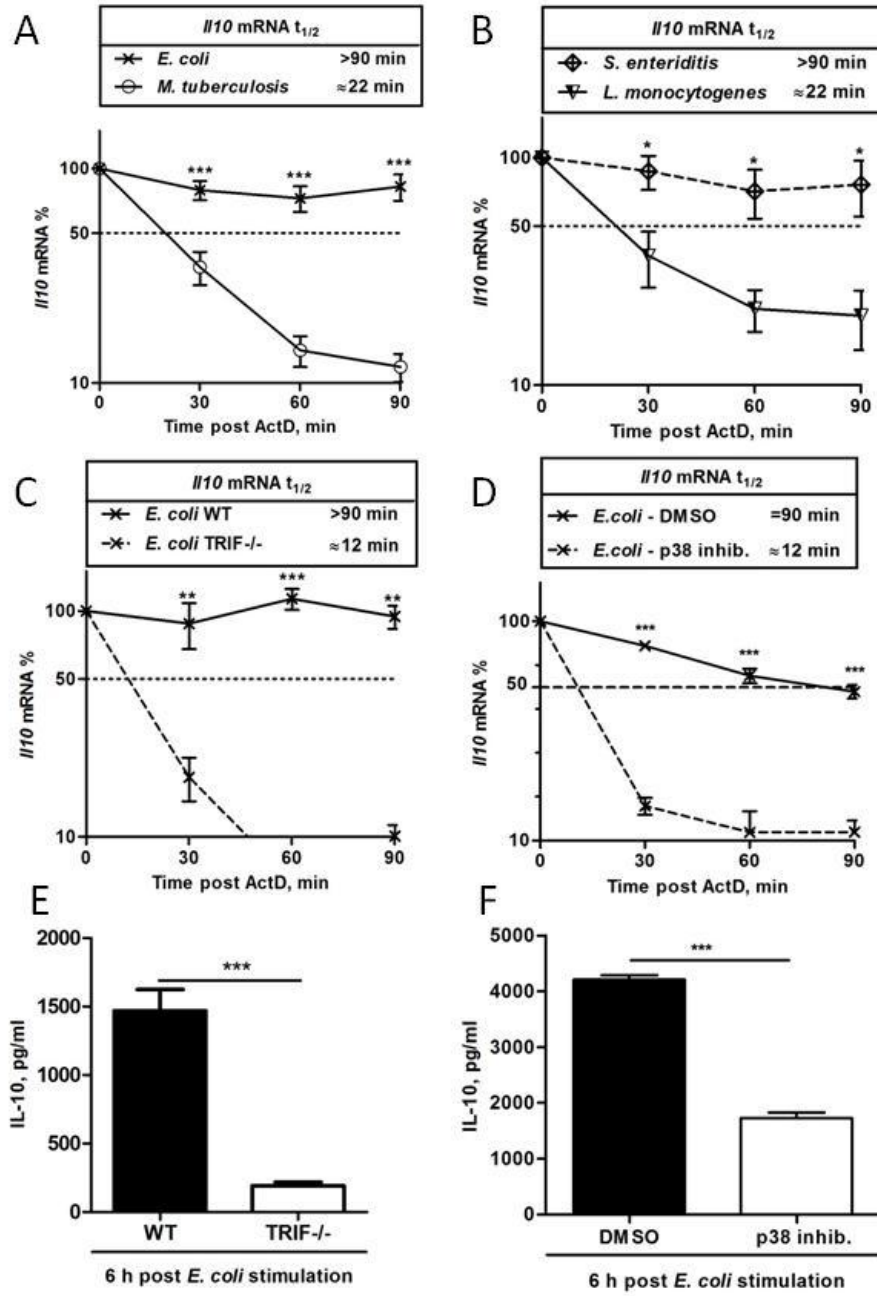
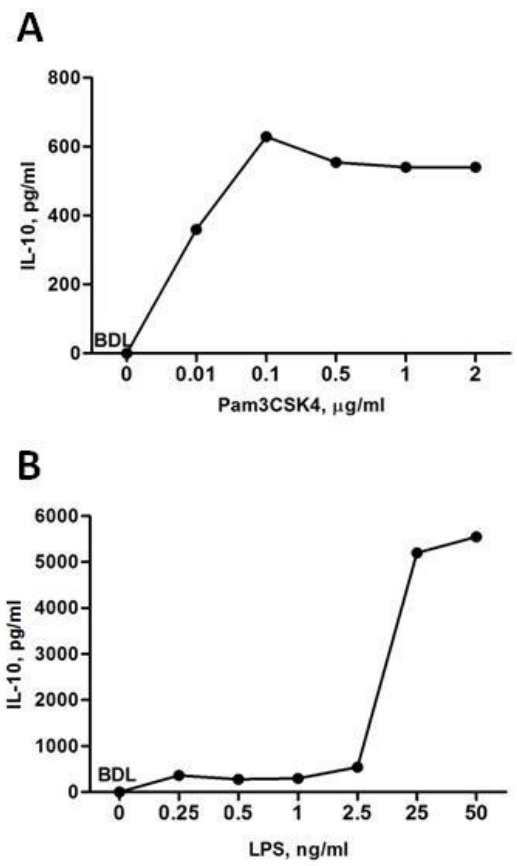


Figure 5

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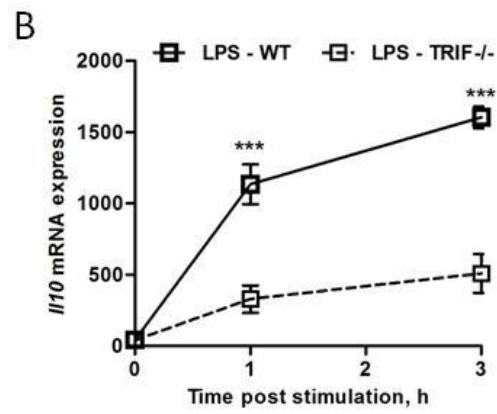
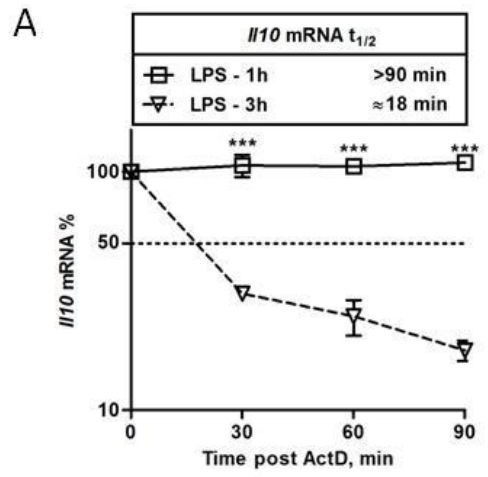


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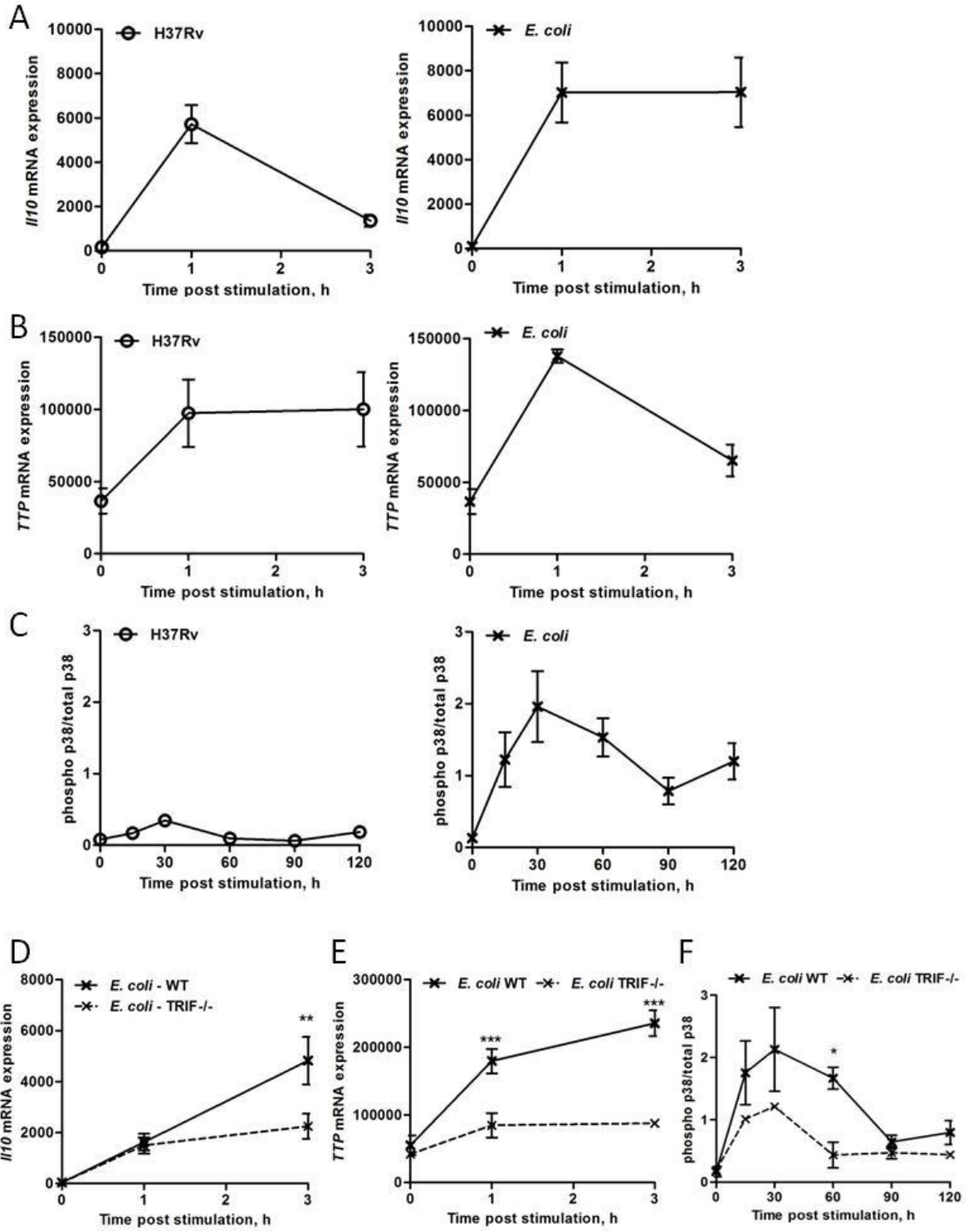
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Supp. Fig. 1

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Supp. Fig. 2



Supp. Fig. 3