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 Mφ (BMMφ); interferon (IFN); Toll/IL-1 receptor domain-

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Abstract

Activation of TLRs by microbial molecules triggers intracellular signaling cascades and the expression of cytokines, as IL-10. *Il10* expression is tightly controlled to ensure effective immune responses, whilst preventing pathology. Maximal TLR-induction of *Il10* transcription in Mφ requires signaling through the MAPKs ERK and p38. Signals via p38 downstream of TLR4 activation also regulate IL-10 at the post-transcriptional level, but whether this mechanism operates downstream of other TLRs is not clear. We compared the regulation of IL-10 production in TLR2 and TLR4 stimulated BM-derived Mφ (BMMφ) and found different stability profiles for the *Il10* mRNA. TLR2 signals promoted a rapid induction and degradation of *Il10* mRNA, whereas TLR4 signals protected the *Il10* mRNA from rapid degradation, due to the activation of Toll/IL-1 receptor domain-containing adaptor inducing IFN-β (TRIF) and enhanced p38 signaling. Consequently, TLR4 is a stronger inducer of IL-10 secretion than TLR2. Our study provides a molecular mechanism for the differential IL-10 production by TLR2- or TLR4-stimulated BMMφ, showing that p38-induced stability is not common to all TLR signaling pathways. Furthermore, this mechanism is also observed upon bacterial activation of TLR2 or TLR4 in BMMφ, thus contributing to IL-10 modulation in these cells in an infection setting.

Introduction

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

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

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

post-transcriptional control [6]. Although these broad mechanisms likely operate in all IL-10-producing

cells, cell-specific factors have also been described [6]. For example, a specific NF-κB-binding enhancer

sequence at the *Il10* locus regulates *Il10* transcription in Mϕ and DCs stimulated via TLRs, but not in IL-

10-producing T cells [12]. In addition to NF-κB, other signaling cascades have been implicated in the

regulation of IL-10 induction by TLR-activated Mϕ and DCs, including the MAPKs ERK [3, 13-17] and.

p38 [13, 14, 18-22]. Indeed, ERK activation is required for IL-10 expression in different cells, from Mϕ

and DCs to Th cells [6]. ERK activation upregulates the transcription factor cFOS, which in turn

enhances IL-10 transcription [15, 17]. Additionally, ERK activation has been shown to lead to the

induction of IL-10 by increasing the *IL10* locus accessibility to the binding of transcription factors [13].

Regulation of IL-10 transcription by p38 has also been described, and shown to involve the transcription

factor Sp1 [18] and the activation of the downstream kinases MSK1 and MSK2 and CREB

phosphorylation [23]. Activation of p38 has been further implicated in the post-transcriptional regulation

of IL-10 by Mϕ in response to TLR4 ligation by helping to protect the *Il10* mRNA from rapid

degradation induced by the RNA binding protein tristetraprolin (TTP) [24]. Consistent with a role for

TTP in inducing the rapid degradation of the *Il10* mRNA, TTP deficient (-/-) Mϕ show elevated levels of

Il10 mRNA upon TLR4 stimulation [25]. Other post-transcriptional mechanisms for *Il10* gene regulation

have been described, including the participation of certain microRNAs [26, 27]. Targeting mRNA

stability is therefore an important mechanism for the regulation of IL-10 production, and indeed the

production of other cytokines [28]. However, this mechanism has been mainly studied downstream of

TLR4 and it is not clear how it operates downstream of other TLRs.

In this study, we compared the induction of IL-10 in Mϕ stimulated via the TLR2 and TLR4

ligands, Pam3CSK4 and LPS, respectively, as well as whole bacteria which predominantly signal through

either of these TLRs. We have found that independently of the stimuli, a peak of *Il10* mRNA was

observed as early as 1 h post-stimulation. However, whereas TLR2 signaling led to a rapid degradation of

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 Mϕ. Furthermore, we show that triggering of Mϕ

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.

RESULTS

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

macrophages

To dissect the molecular mechanisms regulating the initial steps of *Il10* gene expression in BMMϕ

stimulated via TLR2 or TLR4, we compared the kinetics of mRNA expression in response to ligands

Pam3CSK4 and LPS. The dose of TLR2 and TLR4 agonists used corresponded to maximum IL-10

production by stimulated BMMϕ, as measured by immunoassay (Supplemental Fig. 1). Both stimuli

induced a peak of *Il10* mRNA at 1 h post-stimulation (Fig. 1A). Strikingly, the *Il10* mRNA induced upon

TLR2 activation of BMMϕ rapidly declined, with much lower levels detected at 3 h post-stimulation (Fig.

1A). This was in contrast to TLR4 triggering of BMMϕ, where the amount of *Il10* mRNA remained

constant between 1 h and 3 h post-stimulation(Fig. 1A). The profile of *Il10* mRNA observed upon TLR2

stimulation (Fig. 1A) is compatible with a rapid degradation of *Il10* mRNA. To test this hypothesis, we

assessed the stability of the TLR2- or TLR4-induced *Il10* mRNA, by adding ActD to the BMMϕ cultures

at 1 h post-stimulation. As shown in Fig. 1B, while the *Il10* mRNA induced by the TLR2 agonist was

rapidly degraded upon the addition of ActD, *Il10* mRNA induced by TLR4 showed a prolonged $t_{1/2}$. In

line with this, the IL-10 protein resulting of TLR2 versus TLR4 stimulation of BMMϕ was different, with

higher quantities of IL-10 being secreted upon TLR4 activation (Fig. 1C). Of note, at 3 hours post-

stimulation with LPS the *Il10* mRNA was unstable (Supplemental Fig. 2A), suggesting that post-

transcriptional mechanisms operate with either TLR, albeit with a different kinetics. We next investigated

the *Il10* post-transcriptional regulation using another MyD88-dependentTLR stimulus. The *Il10*

transcription and mRNA stability profile induced in BMMϕ upon TLR9 triggering with CpG, which like

TLR2 signals via MyD88 alone, was similar to that induced upon TLR2 activation (Fig. 1D, E and F).

TRIF signaling enhances the stability of *Il10* mRNA via prolonged p38 activation

Next we investigated the molecular mechanism underlying the enhanced *Il10* 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 Mφ [10], we hypothesized that TRIF may play a role in the observed differences. To test this, we generated BMMφ 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 *Il10* mRNA measured overtime by real-time PCR. We observed that the absence of TRIF significantly decreased the stability of *Il10* mRNA upon TLR4 stimulation, leading to its degradation (Fig. 2A). The amount of IL-10 protein detected in TLR4-stimulated cultures of TRIF-/- BMMφ was significantly lower than that observed for WT BMMφ (Fig. 2B). In the absence of TRIF the *Il10* transcription after TLR4 stimulation of BMMφ 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 *Il10* expression [6] and a role for p38 in the post-transcriptional regulation of *Il10* has also been described [24]. We therefore investigated whether the differential activation of these MAPKs was involved in the observed post-transcriptional regulation of *Il10* via TLR4/TRIF. We compared the levels of p38 and ERK phosphorylation upon TLR2 and TLR4 stimulation of WT BMMφ. 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 *Il10* mRNA, by stimulating WT

146   BMMϕ 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   *Il10* transcription. As shown in Fig. 3E and F, inhibition of p38 led to the degradation of the *Il10* 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 *Il10* mRNA stability early upon TLR2 stimulation of BMMϕ**

156   The RNA binding protein TTP has been described to target *Il10* 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 *Il10*

158   mRNA [24, 25, 29, 30]. Considering these reports and our findings showing that in TLR2-stimulated

159   BMMϕ a rapid degradation of the *Il10* mRNA occurs, in parallel with a reduced activation of p38, we

160   next investigated the stability of TLR2-induced *Il10* mRNA in the absence of TTP. WT or TTP-/- BMMϕ

161   were stimulated for 1 h with LPS or Pam3CSK4 and at that time ActD was added to the cultures and *Il10*

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 *Il10* mRNA stability at this time point. However, when the cells were stimulated with

164   Pam3CSK4 a slight but significant increase in *Il10* 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 *Il10* 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 *Il10* 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

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

Differential post-transcriptional regulation of IL-10 by TLR2 versus TLR4 upon BMMφ stimulation with bacteria

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

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

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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 BMMϕ 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 BMMϕ. Our data demonstrate that this results from post-

203 transcriptional mechanisms of *Il10* mRNA stabilization involving TLR4/TRIF/p38 signaling when

204 BMMϕ are stimulated with TLR4-activating microbes. This reveals an important mechanism in

205 modulating the course of the immune response.

206

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 Mϕ [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 BMMϕ, 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 BMMϕ [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 Mϕ. 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   BMMϕ [35-38].

233   The mechanism proposed in our study was recapitulated upon stimulation of BMMϕ with

234   microbes, such as *M. tuberculosis*, *L. monocytogenes*, *E. coli* and *S. enteriditis*. 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 Mϕ. 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 Mϕ, or in Mϕ 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-uridylate (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 Mϕ 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 BMMϕ activated through TLR2 or TLR4 is similar. Furthermore, TRIF deficiency led to less

252   TTP transcription in LPS-stimulated BMMϕ 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-1α [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 *Il10*

260 mRNA. It is thus likely that the activation of TRIF and p38 upon TLR4 triggering are stabilizing the *Il10*

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 BMMϕ 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 BMMϕ. This observation is in line

268 with previous reports demonstrating that ERK is involved in the molecular mechanisms regulating *Il10*

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 *Il10* 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 Mφ [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-1α [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 BMMφ, 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 *Il10* 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 *Il10* transcription in BMMφ 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 BMMφ

304   sensing whole bacteria and allows for distinct IL-10 induction by TLR2- versus TLR4-activating

305 microbes. As a consequence, TLR4 stimulation of BMMφ 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

Materials and Methods

Animals

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

Cell culture

Complete (c) DMEM was prepared by supplementing DMEM (GIBCO) with 10% FBS, 1% sodium pyruvate, 1% HEPES and 1% L-glutamine (all from GIBCO). BMMϕ were generated in cDMEM supplemented with 20% of L929-cell conditioned media (LCCM). On day 0, 4×10^6 cells in 8 mL were plated per petri dish (Sterilin) and kept at 37ºC and 5% CO_2. Cell cultures were fed on day 4 with 10 mL of cDMEM-20% LCCM. BMMϕ were recovered on day 7 of culture, counted and stimulated for different time-points as appropriate. TTP$^{-/-}$ and control (WT) cells were from littermate male mice of 6-16 weeks of age that had been backcrossed 28 generations into C57Bl/6NTac mice and have been described previously [59]; cells were used to derive, as above, BMMϕ, except that 30% of LCCM was used.

Bacteria

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

Reagents

LPS (*S. Minnesota*, Sigma) and Pam3CSK4 (InvivoGen) were used at 25 ng/mL and 2 µg/mL,

respectively. Actinomycin D (ActD) (Sigma) was used at 10 µg/mL and added to the cell cultures 1 h

post-stimulation, when indicated. p38 (SB 203580) and ERK (PD0325901) inhibitors, kind gifts of Prof.

Sir P. Cohen, were used at 2.5 µM and 0.1 µM [60], respectively, and added to the cell cultures 50 min

post-stimulation. Cell culture grade DMSO (Sigma) was used as a vehicle control, when appropriate.

ELISA

IL-10 quantification in the cell culture supernatants was performed by ELISA following the

manufacturer's instructions (eBioscience).

RNA extraction, cDNA and quantitative real time PCR

Total RNA from stimulated and non-stimulated cell culture samples was extracted at different time-points

with TRIzol® 143 Reagent (Invitrogen, San Diego, CA) and converted to cDNA according to the

manufacturer's instructions (Fermentas). *Il10* and *TTP* gene expression were assessed by real-time PCR

using SYBR Green (Fermentas) and TaqMan MasterMix (Applied Byosistems) and normalized against

ubiquitin or Hypoxanthine Phosphoribosyltransferase 1 (*Hprt1*) expression, respectively, as previously

described [12].

Western Blot (WB)

BMMϕ were rested for 5 h in 1% FBS – cDMEM prior to stimulation. At the indicated time points post

stimulation, cell culture supernatants were discarded and cells gently washed with apyrogenic 1X PBS

(GIBCO). Protein extracts were obtained with a lysis buffer solution (100 mM Tris-HCl pH8; 10%

glycerol; 1 mM EDTA pH8; 5 mM MgCl2; 50 mM NaCl; 1% NP-40; 1x protease inhibitor cocktail

(Roche); 1x phosphatase inhibitor cocktails II and III (Sigma); dH2O) for 20 minutes on ice. Extracts

were kept at -80ºC until further use. Samples were then centrifuged at 13000 rpm for 15 min and protein

extracts recovered. Immediately before use, protein extracts were heated 5 min at 95ºC and 20 µg of each

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 ± 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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Figure legends

Figure 1. Distinct post-transcriptional regulation of IL-10 through TLR2 versus TLR4 signaling in BMMϕ. WT BMMϕ were stimulated with Pam3CSK4 (2 µg/mL) or LPS (25 ng/mL) (A, B and C) or with CpG (1 µM) (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 BMMϕ with Pam3CSK4 or LPS, cell culture supernatants were collected and the amount of IL-10 measured by ELISA. (D, E and F) BMMϕ were stimulated with CpG (diamonds) and treated as in (A), (B) and (C), respectively. Results are Mean ± 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) BMMϕ 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) BMMϕ with LPS the cell culture supernatants were collected and the amount of IL-10 measured by ELISA. Results are Mean ± 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); ***$p < 0.001$.

Figure 3. p38 mediates the TRIF-induced stability of the *Il10* mRNA. (A and B) WT BMMϕ were stimulated with Pam3CSK4 (2 µg/mL; crosses) or LPS (25 ng/mL; open squares) and, at the indicated time points post-stimulation, total cell extracts were prepared, separated in SDS-PAGE and the ratio of phospho p38/total p38 (A) or phospho ERK/total ERK (B) assessed by WB. (C and D) WT (solid line) or TRIF$^{-/-}$ (dashed line) BMMϕ were stimulated with LPS (25 ng/mL) and the ratio of phospho p38/total p38 (C) or phospho ERK/total ERK (D) assessed by WB as indicated in (A) and (B). Data represent pooled protein extracts from each three replicates per condition. Shown is a representative Western Blot from the 3 independent experiments performed and the resulting quantification. Results are Mean ± SEM of 3 independent experiments. Each time point represented was performed in triplicate for each experiment. (E and F) WT BMMϕ were stimulated with LPS for 50 min. At this time point, DMSO (as a control, open squares), SB203580 (p38 inhibitor, at 2.5 µM; close squares) or PD0325901 (ERK inhibitor, at 0.1 µM; close circles) were added to the cultures. Ten min later, ActD was added to all wells and the $t_{1/2}$ of the *Il10* mRNA determined as in Figure 1B. (G and H) Cells were treated as before, but with no addition of ActD, and at 6 h post-stimulation the supernatants were collected and the amount of IL-10 measured by ELISA. Results are Mean ± 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-F) or by the Student´s t test (G and H); * *p*<0.05; ** *p*<0.01; ****p*<0.001.

Figure 4. Absence of TTP delays the early degradation of the *Il10* mRNA induced upon TLR2-signaling. (A and B) WT (solid lines) or TTP$^{-/-}$ (dashed lines) BMMϕ (generated from frozen cells) were stimulated with LPS (25 ng/mL, open squares) or Pam3CSK4 (2 µg/mL, crosses) for 1h and the *Il10* mRNA $t_{1/2}$ was determined upon addition of ActD as described in Figure 1B. (C and D) WT (black bars) or TTP$^{-/-}$ (white bars) BMMϕ were generated and stimulated as above, except that ActD was not added, and the amount of IL-10 in the cell culture supernatants was measured by ELISA 6 h post-stimulation. (E) WT BMMϕ were stimulated with LPS (25 ng/mL, open squares) or Pam3CSK4 (2 µg/mL, crosses) and the *TTP* mRNA

measured over time as indicated in Figure 1A. (F) WT (solid lines) or TRIF$^{-/-}$ (dashed lines) BMMφ were stimulated with LPS and the *TTP* mRNA measured over time as indicated in Figure 1A. Results are Mean ± SEM of 2 (A-D) or 3 (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, B, E and F) or by the Student´s t test (C and D); * $p<0.05$; ** $p<0.01$; ***$p<0.001$.

Figure 5. Differential post-transcriptional regulation of IL-10 by TLR2 versus TLR4 upon BMMφ stimulation with intact bacteria. (A and B) WT BMMφ were stimulated with heat-killed *E. coli* (crosses), *S. enteriditis* (diamonds), *L. monocytogenes* (inverted triangles) or with live *M. tuberculosis* H37Rv (open circles) at a moi of 2. The *Il10* mRNA $t_{1/2}$ was determined at 1h post-stimulation, as indicated in Figure 1B. (C) WT (solid lines) or TRIF$^{-/-}$ (dashed lines) BMMφ were stimulated with heat-killed *E. coli* (moi of 2) and the *Il10* mRNA $t_{1/2}$ determined at 1h post-stimulation, as indicated in Figure 1B. (D) WT BMMφ were stimulated with heat-killed *E. coli* (moi of 2) in the presence of DMSO (as a control, solid lines) or of SB203580 (p38 inhibitor, at 2.5 μM; dashed lines) and the *Il10* mRNA $t_{1/2}$ determined at 1h post-stimulation, as indicated in Figure 1B. (E) 6 h post-stimulation of WT (black bar) or TRIF$^{-/-}$ (white bar) BMMφ with *E. coli* the cell culture supernatants were collected and the amount of IL-10 measured by ELISA. (F) WT BMMφ were stimulated with *E. coli* in the presence of DMSO (as a control, black bar) or of SB203580 (p38 inhibitor, white bar) for 6 h and the IL-10 protein present in the supernatants of the stimulated cultures determined by ELISA. Results are Mean ± SEM of 3 (A and B) or 2 (C-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-D) or by the Student´s t test (E and F); * $p<0.05$; ** $p<0.01$; ***$p<0.001$.

Supplemental Figure legends

Figure S1. IL-10 production by BMMϕ in response to increasing doses of Pam3CSK4 (A) or LPS (B). WT BMMϕ 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 ± SEM of 2 independent experiments. Each time point represented was performed in triplicate for each experiment.

Figure S2. (A) WT BMMϕ were stimulated with LPS (25 ng/mL) and 1h (open suares) 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) BMMϕ 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 ± 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 BMMϕ 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 phospho p38/total p38 (C) was also determined as detailed in Figure 3A. (D-F) WT (solid lines) or TRIF^{-/-} (dashed lines) BMMϕ 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 ± 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.
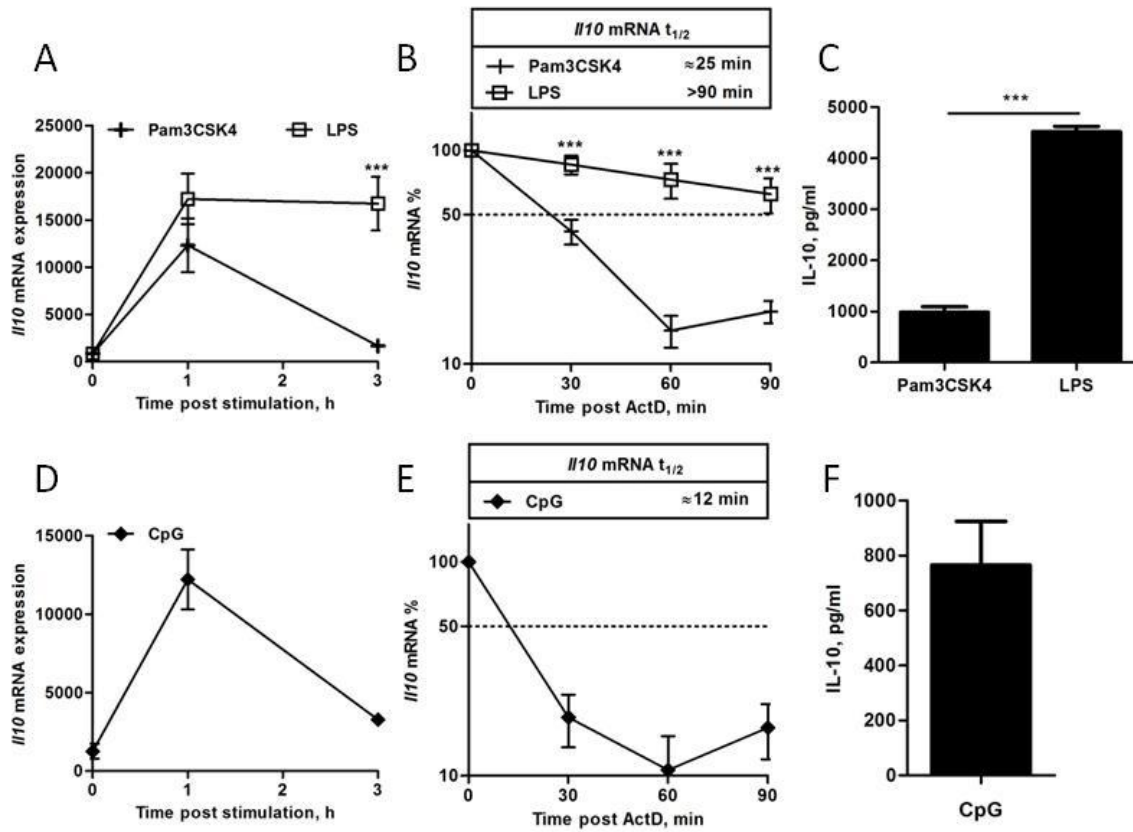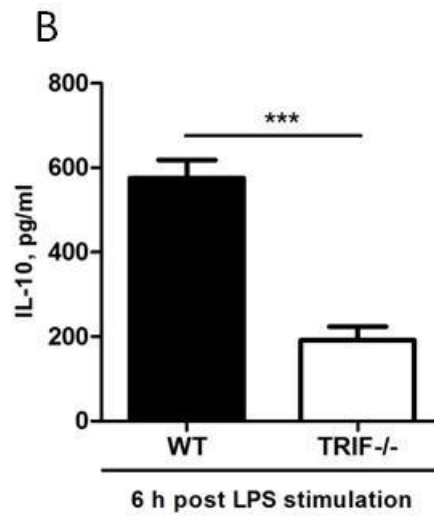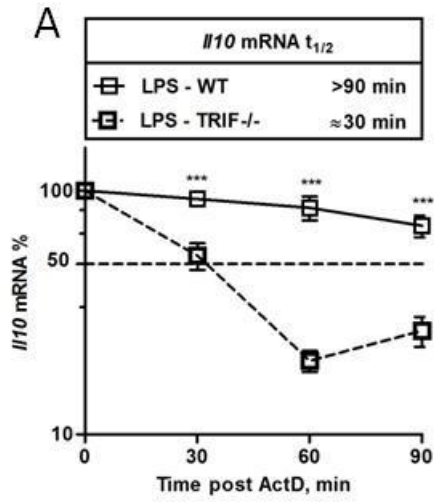
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653 **Figure 1**

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Figure 2

Figure 3

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662 **Figure 4**

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Figure 5

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A

B

Supp. Fig. 1

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A

Il10 mRNA t$_{1/2}$

LPS - 1h   >90 min
LPS - 3h   ≈18 min

Il10 mRNA %

Time post ActD, min

B

LPS - WT   LPS - TRIF-/-

Il10 mRNA expression

Time post stimulation, h

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672   **Supp. Fig. 2**

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675   **Supp. Fig. 3**