molecular systems biology

Tristetraprolin-driven regulatory circuit controls quality and timing of mRNA decay in inflammation

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(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

1st Editorial Decision 22 July 2011

Thank you again for submitting your work to Molecular Systems Biology. We have now heard back from the three referees who agreed to evaluate your manuscript. As you will see from the reports below, the referees generally found your study of interest. They do, however, raise some important concerns on your work, which, I am afraid to say, preclude its publication in its present form.

Most importantly, two reviewers independently indicated that substantial additional experiments were needed, both supporting the generality of the temporal mRNA degradation model presented in Figure 6 and verifying some of the novel TTP targets predicted by the transcriptomic analysis (see Reviewer #2 point 1 and Reviewer #3 point 2). The reviewers also had a series of more specific concerns that will require some additional clarification and in some cases may require additional control experiments (e.g. Reviewer #2 point 2).

If you feel you can satisfactorily deal with the concerns raised by the referees, you may wish to submit a revised version of your manuscript. Please attach a covering letter giving details of the way in which you have handled each of the points raised by the referees. A revised manuscript will be once again subject to review and you probably understand that we can give you no guarantee at this stage that the eventual outcome will be favorable.

In addition, I would like to acknowledge that the peer review process took somewhat longer than usual in this case, because we experienced significant delays in receiving the reviewers' reports. I apologize for the delay.

Yours sincerely,
Editor - Molecular Systems Biology

Referee reports:

Reviewer #1 (Remarks to the Author):

This is an interesting paper that confirms and extends findings emerging from several laboratories and elucidates waves of RNA regulons (original concept should be cited) that initiate, maintain and then resolve an LPS-induced inflammatory response. At first take, I was concerned that this paper was repeating findings of other groups including Hao and Baltimore 2009 and other global mRNA decay regulon papers, but it is clearly unique. However, as a "systems biology" study for MSB, some similar pioneering studies in other immune models should be cited including perhaps: (A. Raghavan 2004, C. Cheadle 2005, PS Ray & PL Fox 2007, N. Mukherjee 2009, etc.) so that the generalized importance of systems approaches to problems in inflammation are put into a more comprehensive context without reviewing the entire field. Importantly, this study is in the "systems" category because it uses some uniquely integrated global approaches including RNA-RIP, SB203580 treatment to block p38 MAPK and actinomycin-type RNA stability measurements, and moreover, animal studies. Given the addition of multiple inhibitors over the time course of these experiments, I would have preferred to have seen the global decay data derived using the 4-thiouridine pulsing method rather than actinomycin D. However, recent studies suggest that 4-thiouridine results appear to correspond reasonably well with data using actinomycin to inhibit transcription. In aggregate, the data in this study are definitive and exciting. The model at the end is not unlike that of Baltimore as reviewed in Anderson, with a slight twist and based on different activators and responding components. But I am not sure that I agree with using the term "binary" in this case as these responses result from combinatorial regulation at the level of multiple trans-acting factors.
One of the dilemmas with global RNA studies of TTP and other RNA-binding proteins involved in mRNA decay is that because RNA binding is associated with degradation, the very transcript being identified is rapidly disappearing. This is not a problem with mRNA stabilizers such as HuR. Indeed, there are discrepant mRNA targeting results in the G. Stoecklin 2008 and J. Emmons 2008 publications. This is largely attributed by experts in the field to differences in the methods of immune activation used and the cell types (macrophage versus dendritic) as well as key differences in the RIP procedures used (e.g. antibody, tag, washing, etc.). Thus, rather than lumping the targets derived in the two studies together, it would be useful for the authors to discriminate which of the mRNA targets of TTP derived in each of those studies are being discussed in the text of this paper. This can be simple categorization without necessarily taking a side as to the validity of the two studies; but in the long-term development of the field this is likely to prove useful.

Reviewer #2 (Remarks to the Author):

The manuscript by Kratochvil et al. expands upon previous knowledge on the role of TTP in cytokine mRNA regulation. By incubating with the p38 MAPK inhibitor SB203580 while measuring genome wide mRNA decay rates in TTP-/- and wt cells, the authors were able to identify novel TTP targets. Cxcl2, one verified target, demonstrated varying TTP-dependent decay activity with different duration of LPS exposure. Highly stable at 3 hours of LPS treatment, Cxcl2 is more rapidly degraded at 6 and 8 hours, in correlation to the change in p38 MAPK activity. TNF on the other hand, is readily affected by TTP at 3 hours. Mice with myeloid-specific TTP deficiency appeared fully healthy under normal conditions, only experiencing elevated cytokine expression when immunologically challenged.

This study proposes an intriguing model of TTP activity where the destabilizing effect, after LPS stimulation, is restricted to certain mRNAs at first and then spread to more transcripts. Another important contribution is the generation of TTP conditional knockout mice. This will be an important tool for the field, enabling more detailed analysis of TTP function in cell and tissue types. The manuscript should be publishable after addressing the following specific comments.

Specific comments:
1. Very limited data is presented to back up the proposed model in Figure 6. Only 2 genes, TNF and Cxcl2 were analyzed as examples, which can be discredited as merely exceptions instead of representing a general phenomenon. Therefore more targets should be characterized, and TNF should be analyzed at all time points.

2. When verifying Il1a and Cxcl2 as true TTP targets, reasonable experiments were carried out yet poorly described in the results section (Figures 1b and 1c). Although the methods and figure legends offer more detail, it is confusing for readers outside the RNA decay field. The experiment should be described better in the text, and the figures should be better labeled. Also, a better negative control that does not show up in all lanes, should be used in Figure 1b.

3. The strong correlation of TTP-mediated decay with the motifs AUUUA or WWAUUUAWW in the 3'UTR is already well established. This analysis can be supporting evidence for the validity of TTP targets uncovered, instead of being a separate major point.

Reviewer #3 (Remarks to the Author):

Kratochvill et al. probe the mRNA-destabilizing effects of TTP in mouse macrophages using a global mRNA stability assay and show that the decay of as many as 1/3 of "unstable" genes stimulated by LPS require TTP. They further demonstrate that conditional silencing of TTP in mouse macrophages leads to an exaggerated inflammatory response to LPS, without changes in baseline phenotype in the absence of inflammatory stimulus. They also propose that the activity of TTP inversely correlates with p38 MAPK activity.

Main concerns are as follows:

1. Novelty: p38MAPK has already been known to inhibit TTP function by phosphorylation and the concept of integrated regulation of p38/TTP is quite well established.
2. The validation of the array study needs to be significantly expanded and more rigorous - authors need to confirm direct TTP effects and transcript binding of more than just two genes (Cxcl2 and IL1a). Also, both of these genes are already known to regulate TTP. They should characterize novel genes, identified by their array study and not previously known to regulate TTP. Both these approaches - expanding the number of validated genes and characterize previously unknown TTP targets - would increase the novelty and value of the study.

Specific comments:

1. It is not completely clear from the Results section how the Authors determine which genes are unstable. It appears they used the 90 min time point and just compared relative levels in +/- treatment and +/- TTP rather than half-life to generate the p-values. Was this the case? Also, they mention that these p-values, not half-life, was used as the main criteria for classification of an mRNA as stable. The rationale of this choice should be addressed in the manuscript.
2. Half-lives were determined using only the 0 and 90 minute time points. Why did the authors not use all three time points to generate a slope to determine half-life, as this would be the most accurate?
3. The Authors state that: "In untreated cells Cxcl2 mRNA was unstable in WT but stable in TTP-/- cells showing that without inflammatory signaling the basal by means of Western blotting undetectable TTP is fully functional due to the low p38 MAPK activity (Supplementary Figure 3)." If TTP is undetectable in the untreated WT cells, it seems implausible that it is responsible for the observed changes. This effect could rather be due to intrinsic changes between the WT and TTP-/- cells (possibly differential expression of other RBPs). This issue needs to be either experimentally addressed or at least discussed with more alternative hypotheses.
4. The data presented in figure 2C would be enhanced by calculating and plotting each half-life at different time points and presenting this as an inset or another panel.
5. The Authors describe a difference in the stability profile in CXD2 and TNF as a function of time. The authors use the findings on these two genes to make a general argument about the mechanism of action of p38 MAPK and TTP activity. In order to generalize these data, several additional transcripts need to be characterized within this system. Furthermore, it is not clear why, If TTP binds to both transcripts, would the temporal activity of p38 MAPK have differential effects on these transcripts.

1st Revision - authors' response 12 October 2011

Point-by-point reply (cited parts are in *italic*):

Reviewer #1:

1) "*However, as a "systems biology" study for MSB, some similar pioneering studies in other immune models should be cited including perhaps: (A. Raghavan 2004, C. Cheadle 2005, PS Ray & PL Fox 2007, N. Mukherjee 2009, etc.) so that the generalized importance of systems approaches to problems in inflammation are put into a more comprehensive context without reviewing the entire field.*"

We have included the suggested papers Raghavan & Bohjanen, 2004, Cheadle et al, 2005 and Mukherjee et al, 2009 on page 3 (Introduction section). In addition, to account for the recent advances in understanding of the effect of translation and miRNAs on RNA stability & gene expression we have included the reviews by Dahan et al, 2011 and Chekulaeva & Filipowicz, 2009.

2) "*But I am not sure that I agree with using the term "binary" in this case as these responses result from combinatorial regulation at the level of multiple trans-acting factors.*"

We completely agree with the reviewer that besides TTP and p38 MAPK additional factors contribute to the removal of inflammatory mRNAs. With the term "binary" we wanted to stress the dominance of TTP and p38 MAPK in the overall process since to our knowledge no other proteins individually exhibit such a decisive impact on the overall stability profile of inflammatory mRNAs. However, since the reviewer feels that this term is misleading, for reasons of semantics we completely removed the term "binary" and replaced it in few cases with the term "TTP- and p38 MAPK-dominated".

3) "*Indeed, there are discrepant mRNA targeting results in the G. Stoecklin 2008 and J. Emmons 2008 publications. This is largely attributed by experts in the field to differences in the methods of immune activation used and the cell types…… Thus, rather than lumping the targets derived in the two studies together, it would be useful for the authors to discriminate which of the mRNA targets of TTP derived in each of those studies are being discussed in the text of this paper. This can be simple categorization without necessarily taking a side as to the validity of the two studies; but in the long-term development of the field this is likely to prove useful.*"

In fact, we intend to provide the scientific community with the information as to what target was found in what screen, as suggested by the reviewer. However, with feel that such information should be available in a most reader-friendly way possible. For this reason we will provide this information in the Internet via the searchable AREsite (http://rna.tbi.univie.ac.at/cgi-bin/AREsite.cgi) that we recently released (Gruber et al, 2011, *Nucleic Acids Res*, Database issue: D66-9). Such feature was implemented in the database prior to its launch: AREsite displays references for all listed targets of TTP, AUF1 and HuR. Upon publication of our current study, AREsite will be amended by our RNA decay dataset allowing direct comparison with targets found in other screen. This is now more explicitly stated in the manuscript (page 16, Materials and Methods section, last sentence in Microarray analysis).

In addition, on page 6 we suggest e.g. cell type specific expression of targets as one possible explanation for different results obtained in other studies.

Reviewer #2:

1) "*Very limited data is presented to back up the proposed model in Figure 6. Only 2 genes, TNF and Cxcl2 were analyzed as examples, which can be discredited as merely exceptions instead of representing a general phenomenon. Therefore more targets should be characterized, and TNF should be analyzed at all time points.*"

This major concern is shared with the reviewer #3 (point 2).

We have performed additional experiments to support the proposed model:

(i) Stability profile of *Tnf* mRNA is now shown for all time points (new **Figure 4**), as requested by the reviewer.

(ii) Stability profile of *Il1a* and *Il6* is shown for 3, 6 and 9 h of LPS treatment (new **Supplementary Figure 6**). We did not obtain consistent data for untreated cells because the low expression of *Il1a* and *Il6* prior to LPS treatment did not allow reliable decay measurements.

(iii) We compared mRNA stability of 9 novel and 7 known targets after 3 and 9 h of LPS treatment using a new microarray-based experiment (new **Table 3** and **Supplementary Figure 3**). We show the decay rates only for those mRNAs for which the TTP-dependent degradation is confirmed by an independent approach (i.e. RNA immunoprecipitation shown in new **Figure 2D**) or has been demonstrated by others in the past.

All these new experiments are in good agreement with the proposed model of gradually unfolding extent of TTP-mediated mRNA decay, both in terms of quality and quantity. In addition, the outcome of the *Tnf* stability profile revealed that prior to LPS treatment a significant part of *Tnf* mRNA degradation does not depend on TTP. The TTP-independent degradation was reduced by LPS treatment. This observation prompted us to examine the expression of the TTP homologues Tis11b and Tis11c (new **Supplementary Figure 7**) since these proteins have been previously implicated in *Tnf* mRNA degradation. The expression of both TTP homologues was high prior to LPS treatment but dropped rapidly upon LPS addition suggesting that Tis11b and/or Tis11c might account for the TTP-independent *Tnf* mRNA degradation in unstimulated cells. Since the targets of Tis11b/Tis11c *in vivo* and the regulation of mRNA degradation by these proteins are largely unexplored, more studies are needed in the future to establish the connectivity map integrating the TTP homologues in the global mRNA decay.

2) "*When verifying Il1a and Cxcl2 as true TTP targets, reasonable experiments were carried out yet poorly described in the results section (Figures 1b and 1c). Although the methods and figure legends offer more detail, it is confusing for readers outside the RNA decay field. The experiment should be described better in the text, and the figures should be better labeled. Also, a better negative control that does not show up in all lanes, should be used in Figure 1b*"

We added the detailed procedure of RNA-EMSA to Materials and methods. In addition, the labeling of the RNA-EMSA experiment (now **Figure 1B**) was made simpler and additional information was included in the figure legend. The RNA immunoprecipitation experiments (originally Figure 1B) were redone and more targets included (new **Figure 4D**). In case of *Junb* the unused PCR primers appear as a band in the negative control lanes. This primer band runs faster than the specific *Junb* band as seen in the lane containing the TTP antibody-precipitated sample (lane labeled as AB).

3) "*The strong correlation of TTP-mediated decay with the motifs AUUUA or WWAUUUAWW in the 3'UTR is already well established. This analysis can be supporting evidence for the validity of TTP targets uncovered, instead of being a separate major point.*"
We agree with this view: we deleted this part and incorporated it in a shortened version into the second part of the results sections (last paragraph, page 8).

Reviewer #3:
1) "*Novelty: p38MAPK has already been known to inhibit TTP function by phosphorylation and the concept of integrated regulation of p38/TTP is quite well established.*"
In the manuscript, we appreciate in several occasions the reported effects of p38 MAPK on TTP activity. However, we believe that the functional consequences of this coupled system on the global control of specificity and timing of inflammatory mRNA decay, as revealed in our current study, establish a novel concept in the field of systems biology of negative regulation of inflammation. This concept is now supported by additional data, as requested by the reviewer (see points 2 and 7 below). We also believe that by employing mice with myeloid cell-specific TTP deletion, a new tool generated in the course of the study, we substantiate the functional importance of the p38 MAPK/TTP-driven regulatory circuit for the first time *in vivo*. Further important finding (concluded from the good health of mice with myeloid-specific TTP ablation) is that myeloid TTP is critically involved in the re-installment of immune homeostasis after inflammatory stimulus rather than in the previously suggested maintenance of steady state immune homeostasis.

2) "*The validation of the array study needs to be significantly expanded and more rigorous - authors need to confirm direct TTP effects and transcript binding of more than just two genes (Cxcl2 and IL1a). Also, both of these genes are already known to regulate TTP. They should characterize novel genes, identified by their array study and not previously known to regulate TTP. Both these approaches - expanding the number of validated genes and characterize previously unknown TTP targets - would increase the novelty and value of the study.*"
As requested by the reviewer, we performed additional experiments to increase the novelty and importance of the study. We would like to refer to our reply to the reviewer #2 (point 1) who requested similar experiments.
Briefly:
(i) We show TTP binding to *Ccl2, Ccl4, Ccl7, Cxcl2, Ets2, Il1a, Junb, Plaur, Tnfsf9* using RNA immunoprecipitation (new **Figure 1B**). These are to the best of our knowledge novel targets. We are not aware of any other reports directly demonstrating these mRNAs to be destabilized by TTP. We feel that this is indeed true also for *Cxcl2* and *Il1a*, that are for the first time shown by us to directly bind to TTP *in vivo*, and to be destabilized via AREs in their 3´ UTR.
(ii) We determined mRNA stability and confirmed TTP-dependent decay of the novel targets *Ccl4, Ccl7, Cxcl2, Ets2, Il1a, Junb, Plaur, Tnfsf9* as well as several known targets (*Ccl3, Cxcl1, Ier3, Il6, Il10, Il12b, Tnf*) at different times of LPS treatment also in the absence of the p38 MAPK inhibitor (new **Table 3**). This

experiment further proves the validity of our array study and confirms that our approach comes close to the identification of the upper limit of TTP targets in the chosen experimental system.

3) "*It is not completely clear from the Results section how the Authors determine which genes are unstable. It appears they used the 90 min time point and just compared relative levels in +/- treatment and +/- TTP rather than half-life to generate the p-values. Was this the case? Also, they mention that these p-values, not half-life, was used as the main criteria for classification of an mRNA as stable. The rationale of this choice should be addressed in the manuscript.*"

Our rationale for this is that the variance differs among genes. The half-life does not incorporate this difference in variance, whereas the p-value does. If a gene has a big difference in half-life, but also a big variance among replicates, it may not be significantly differentially regulated at all and thus should not be considered. With the p-value, the gene-specific variance is accounted for. This is now explained in the manuscript (page 5, bottom, and page 6, top).

4) "*Half-lives were determined using only the 0 and 90 minute time points. Why did the authors not use all three time points to generate a slope to determine half-life, as this would be the most accurate?*"

We agree with the reviewer that by including all three time points an accurate determination of half-lives should be possible. However, we could not consider the 45 min time point for half-life calculations since the difference in means was low compared to the variation at this time point. This is now mentioned on page 5 (bottom) and page 6 (top) and in the Materials & methods section (Microarray analysis, page 18). The low difference in means was most likely caused by the too short time (45 min) of the decay measurement compared to the mean half-life of all unstable mRNA (approx. 200 min).

5) "*The Authors state that: "In untreated cells Cxcl2 mRNA was unstable in WT but stable in TTP-/- cells showing that without inflammatory signaling the basal by means of Western blotting undetectable TTP is fully functional due to the low p38 MAPK activity (Supplementary Figure 3)." If TTP is undetectable in the untreated WT cells, it seems implausible that it is responsible for the observed changes. This effect could rather be due to intrinsic changes between the WT and TTP-/- cells (possibly differential expression of other RBPs). This issue needs to be either experimentally addressed or at least discussed with more alternative hypotheses.* "

We now show that both *Tnf* (new experiment, shown in new **Figure 4A**) and *Cxcl2* (original **Figure 2C**) mRNAs are destabilized by TTP also prior to LPS treatment. Although TTP expression is indeed low in unstimulated cells, both the TTP mRNA as well as TTP protein can be detected (new **Supplementary Figure 7**). To prove the specificity of the TTP signal on Western blot, and to demonstrate the presence of TTP mRNA and protein in untreated cells we employed cells completely lacking TTP as a negative control. The TTP protein band in extracts from unstimulated cells was detectable only after longer exposure of the Western blot. We however believe that by employing two different methods (qRT-PCR and Western blot) together with an appropriate negative control we convincingly demonstrate TTP expression prior to LPS treatment. We of course cannot entirely exclude that the decreased degradation of *Cxcl2* and *Tnf* mRNAs in untreated

TTP-deficient cells is only indirectly caused by the lack of TTP. As pointed out by the reviewer, this decreased degradation could be caused by e.g. increased expression of mRNA-stabilizing proteins such as HuR. However, HuR expression was comparable in WT and TTP-deficient cells, as stated on page 16 (first paragraph). Furthermore, we observed that *Tnf* mRNA is destabilized in untreated cells in part in TTP-dependent and in part in TTP-independent way (new **Figure 4A**). The TTP-independent *Tnf* mRNA degradation might be caused by the TTP homologues Tis11b and Tis11c that are expressed in untreated cells but inhibited upon treatment with LPS (new **Supplementary Figure 6**). This issue is discussed (Discussion section).

6) "*The data presented in figure 2C would be enhanced by calculating and plotting each half-life at different time points and presenting this as an inset or another panel.*"
We present the half-lives in all figures next to the respective decay curves.

7) "*The Authors describe a difference in the stability profile in CXD2 and TNF as a function of time. The authors use the findings on these two genes to make a general argument about the mechanism of action of p38 MAPK and TTP activity. In order to generalize these data, several additional transcripts need to be characterized within this system. Furthermore, it is not clear why, If TTP binds to both transcripts, would the temporal activity of p38 MAPK have differential effects on these transcripts.*"
We would like to refer to our reply to the reviewer #2 (point 1). Briefly, we added the stability profiles of *Tnf* (new **Figure 4**), *Il1a* and *Il6* (new **Supplementary Figure 5**) and stability data of 16 mRNAs for 3 and 9 h of LPS treatment (new **Table 3**). The new data support the proposed model.
To the mechanism of differential regulation of *Tnf* and *Cxcl2* mRNA stability by TTP: TTP mRNA-destabilizing activity is regulated by p38 MAPK both at the level RNA binding but also by other means such as binding to 14-3-3 proteins (reviewed by Sandler & Stoecklin, Biochem Soc Trans. 2008 Jun;36(Pt 3):491-6.). Thus, besides TTP binding to *Tnf* and *Cxcl2* mRNAs other regulatory mechanism can contribute to the differential regulation of mRNA stability. These mechanisms might also include cis-acting stabilizing or destabilizing elements present in the 3´ UTRs and the trans-acting microRNAs, as discussed in the Discussion section.

We believe that the revised manuscript addresses all issues pointed out by the reviewers, and hope that our study has been sufficiently improved to meet the standards required for publication in *Mol Syst Biol*.
Thank you for considering the revised manuscript for publication.

Acceptance letter 07 November 2011

Thank you again for sending us your revised manuscript. We are now satisfied with the modifications made and I am pleased to inform you that your paper has been accepted for publication.

Please note, that in addition to our capacity to host datasets in our supplementary information section, we provide a new functionality that allows readers to directly download the 'source data' associated with selected figure panels (e.g. <http://tinyurl.com/365zpej>). This sort of figure-associated data may be particularly appropriate for some of the figures in this work. Please see our Instructions of Authors for more details on preparation and formatting of figure source data (<http://www.nature.com/msb/authors/index.html#a3.4.3>).

Thank you very much for submitting your work to Molecular Systems Biology.

Sincerely,

Editor - Molecular Systems Biology

Referee reports:

Reviewer #1 (Remarks to the Author):

I am essentially satisfied with the manuscript and the authors' response to my questions.

Reviewer #2 (Remarks to the Author):

In this revised manuscript, the authors have addressed the main concerns of the reviewers. The manuscript describes a nice list of TTP targets in LPS-treated BMDMs and confirms a subset of those targets as directly interacting with TTP. Moreover, they describe a conditional TTP knockout mouse, which will be an invaluable tool for future research into TTP function. I therefore highly recommend publication in MSB.

