THE EMBO JOURNAL

Manuscript EMBO-2015-92649

Neutralization of pro-inflammatory monocytes by targeting TLR2 dimerization ameliorates colitis

Liraz Shmuel-Galia, Tegest Aychek, Avner Fink, Ziv Porat, Batya Zarmi, Biana Bernshtein, Ori Brenner, Steffen Jung and Yechiel Shai

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Review timeline:

Submission date:	23 July 2015
Editorial Decision:	31 August 2015
Revision received:	29 November 2015
Editorial Decision:	23 December 2015
Revision received:	13 January 2016

Editor: Karin Dumstrei

Transaction Report:

(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 31 August 2015

Thank you for submitting your manuscript to The EMBO Journal. Your study has now been seen by three referees and their comments are provided below.

As you can see the referees find the data interesting, but they also find that significant revisions are needed to consider publication here. The concerns raised are clearly indicated below - let me know if we need to discuss any of them further.

Should you be able to address the raised concerns in full then we would like to consider a revised version. I should add that it is EMBO Journal policy to allow a single round of revision only and it is therefore important to address the major concerns at this stage.

When preparing your letter of response to the referees' comments, please bear in mind that this will form part of the Review Process File, and will therefore be available online to the community. For more details on our Transparent Editorial Process, please visit our website: http://emboj.embopress.org/about#Transparent_Process

We generally allow three months as standard revision time. As a matter of policy, competing manuscripts published during this period will not negatively impact on our assessment of the conceptual advance presented by your study. However, we request that you contact the editor as soon as possible upon publication of any related work, to discuss how to proceed. Should you foresee a problem in meeting this three-month deadline, please let us know in advance and we may be able to grant an extension.

Thank you for the opportunity to consider your work for publication. I look forward to your revision.

REFEREE REPORTS

Referee #1:

In this paper, Shmuel-Galia et al use a novel approach to inhibit TLR2 mediated signalling in DSS colitis, providing evidence that a short peptide that binds to the transmembrane domain of TLR2 may be therapeutic in vivo. The authors conclude that this reflects inhibition of TLR2-TLR6 heterodimerization in Ly6Chi monocytes in the inflamed mucosa and the data are interesting, if a little preliminary in places. As the authors themselves acknowledge, the experiments do not prove that monocytes or dimerization with TLR6 are the specific targets of the peptide. For instance, the possible role of TLR1 is dismissed without evidence and it is not shown whether the binding of the peptide to the TLR2 TM region might have additional effects on eg interaction with adapter/signalling partners. There are a number of other specific deficiencies:
1) The manuscript is rather long, especially the Introduction and Discussion.
2) The images shown in Figure 1A & B do not give a clear view of where the active peptide is expressed, as it is not easy to identify what part of the cell is involved. The staining with the scrambled peptide appears to be clearly cell surface, but this is not obvious with the active peptide. Together with the infrequent amount of staining, this makes the results difficult to interpret.
3) As the authors state, an obvious issue with using small peptides of this kind in vivo is their bioavailability over long periods of time and indeed this could explain why most of the effects of peptide in the first DSS experiment are partial. The authors address this aspect in the second set of DSS experiments, but this means that the study is somewhat repetitive in nature. It would be preferable if the work focused on this second protocol of peptide administration and carried out the more detailed investigation used in the first experiments.
4) The staining for leukocytes in the mucosa appears unusual, as it appears that virtually all CD11b+ cells are CD11c+, including those which are Ly6Chi. This is contrary to the established knowledge that murine monocytes of this kind are CD11c-.
5) It is also not clear if the numbers shown represent only the MHCII-ve fraction of Ly6Chi cells, or if they include the MHCII+ve fraction that is known to expand in intestinal inflammation.
6) The cells analyzed do not appear to have been subjected to live/dead gating as is considered standard for such work.

Referee #2:

In this paper by Shmuel-Galia and Aychek et al., the authors study the impact of a TLR-2 transmembrane peptide (TLR-2-p) on TLR-2-TLR-6 assembly induced by natural ligands and its capacity to limit DSS-induced colitis. Identifying factors that can limit gastrointestinal inflammation and the mechanisms by which they act is an area of significant research interest and of high therapeutic relevance. The authors begin by demonstrating that TLR-2-p interacts with both TLR-2 and TLR-6 and show by FRET that TLR-2-p inhibits the dimerization of TLR-2-TLR-6 leading to attenuation of downstream signaling. Subsequently, they go on to find that when administered intraperitoneally during DSS colitis, TLR-2-p (but not a scrambled control) can reduce severity of colitic pathology and concurrently inflammatory cytokine production is limited. Moreover, it is determined that Ly6Chi monocyte cytokine release in particular is reduced even though Ly6Chi monocyte frequency is unchanged. Overall this is a clearly structured manuscript that explores an intriguing new mechanism to modulate inflammatory cell function during gut damage. A number of points should be addressed in order to support the claims made.

Major points:

(1) In Fig. 5 it is claimed that the mechanism by which TLR-2-p operates is that Ly6Chi monocyte recruitment is unaltered but their cytokine production is changed. To make this claim it is important that the authors show absolute numbers of Ly6Chi monocytes isolated from the tissue.
(2) Additionally, in Fig. 5 it is not apparent to me what is meant in Fig. 5B as % out of CD11b+ Ly6Chi cells. CD11b+ Ly6Chi cells are monocytes so to look at % out of them makes no sense to

me. Do the authors mean % of Ly6Chi CD11b+ of CD45+ or % of Ly6Chi CD11b+ of CD11c&CD11b+. It is possible that I am misunderstanding this graph - please clarify.
(3) Clearly TLR-2-p affects downstream signaling in macrophages in vitro. Given that suppression of monocyte/macrophage production of cytokines by TLR-2-p is suggested as a major activity in DSS, it would be important to show that TLR-2-p can suppress pro-inflammatory cytokine production by monocytes or macrophages directly in vitro in response to activating ligands.
(4) TNF-alpha is an important cytokine in DSS colitis and targeted as a therapy in UC. Is TNF-alpha production impacted by TLR-2-p?

Minor points:

(1) Monocytes can be a major source of IL-1b during colitis. Is IL-1b message expression (or protein release) reduced in monocytes by TLR-2-p treatment? This would add further support to the idea that in Figure 4A it is inhibition of pro-inflammatory cytokine production by monocytes that is primarily impacted by TLR-2-p.
(2) The English should be thoroughly proof read throughout and corrections made. In particular, there is frequent inappropriate usage of plurals e.g. TLRs dimerization; anti-cyokines therapy etc. This sometimes makes the text a little confusing to understand.

Referee #3:

Shmuel-Galia et al. do a nice job demonstrating that the TLR2-peptide they previously described blocks interaction between TLR2 and TLR6. Further, they convincingly demonstrate that intraperitoneal injection of this peptide during DSS administration, a commonly used colitis model, reduces disease severity. I have the following comments/suggestions:

Figure 1
• The confocal images would benefit from a membrane co-stain or at least DAPI co-stain. It's difficult to ascertain which stains are on the membrane vs. cytoplasmic.
• Since TLR antibodies are notoriously challenging to generate and often have issues with specificity, the authors should demonstrate that these particular antibodies don't stain cells from the respective TLR knockouts or reference a paper that demonstrates this.
• Panel C needs an axis label.

Figure 2
• The authors should more directly address the possibility that the TLR2 peptide blocks TLR2/1 interaction in addition to TLR2/6 interaction. In their previous paper (Fink et al. 2013), the authors demonstrated that cytokine responses to both TLR2/6 and TLR2/1 ligands were diminished after addition of the TLR2-peptide, so an effect on both combinations of dimerization seems likely. If possible, the authors should perform the same assays in Figure 1 and 2 including TLR1. Although the authors state that the TLR2-TLR6 interaction has been shown to be more important in colitis, they can't rule out a role for blockade of TLR2-TLR1 in their DSS + peptide model. Therefore, claiming that their peptide is "targeting TLR2/6 dimerization" in the title and throughout the text is a bit misleading. The authors should either prove that the peptide selectively blocks only the TLR2/6 interaction, prove that the TLR2/6 interaction is more important in their model (for example, using their DSS + peptide protocol in TLR1-/- mice), or reword their paper to more accurately allow for this possibility.

Figure 3
• The authors should plot mouse weights over a longer DSS timecourse, rather than just focusing on one time point. I understand that they sacrificed mice at the peak of inflammation to do histology and scoring, but weight loss will continue after day 7 and it would also be interesting to see if the peptide affects the rate of recovery.
• This may be beyond the scope of the paper, but it would be interesting to determine whether administration of the peptide after the initiation of inflammation has an effect (which would make this approach more relevant therapeutically).

Figure 5
• Percentages need to be listed for the flow plots.

• The authors should think about including cell numbers (although this is admittedly sometimes difficult for lamina propria).

Figure 6
• Panel B is unnecessary (and somewhat confusing) in this figure and could be moved to supplemental.
• The monocyte sorting is a good experiment and provides insight into potential mechanisms of DSS amelioration, but the authors did not demonstrate that the effect of TLR2/6 on monocyte activation is cell intrinsic. Perhaps they could try to use their rhodamine-labeled peptide and determine whether it is bound to monocytes during DSS in vivo. Or they could look at downstream TLR signaling pathways to show they are reduced within monocytes after administration of the peptide. If they don't show this, then they should be clearer about the fact that this effect might be indirect (not just mention this quickly in the discussion).

1st Revision - authors' response 29 November 2015

Referee #1

In this paper, Shmuel-Galia et al use a novel approach to inhibit TLR2 mediated signalling in DSS colitis, providing evidence that a short peptide that binds to the transmembrane domain of TLR2 may be therapeutic in vivo. The authors conclude that this reflects inhibition of TLR2-TLR6 heterodimerization in Ly6Chi monocytes in the inflamed mucosa and the data are interesting, if a little preliminary in places. As the authors themselves acknowledge, the experiments do not prove that monocytes or dimerization with TLR6 are the specific targets of the peptide. For instance, the possible role of TLR1 is dismissed without evidence and it is not shown whether the binding of the peptide to the TLR2 TM region might have additional effects on eg interaction with adapter/signalling partners. There are a number of other specific deficiencies:

We thank the reviewer for his constructive comment. We have now added data that substantiate our notion that TLR2p interferes with the formation of both TLR2/TLR6 and TLR2/TLR1 dimers.
In the below we will address the specific points of critique

1) The manuscript is rather long, especially the Introduction and Discussion.
We have considerably trimmed the manuscript to be more concise.

2) The images shown in Figure 1A & B do not give a clear view of where the active peptide is expressed, as it is not easy to identify what part of the cell is involved. The staining with the scrambled peptide appears to be clearly cell surface, but this is not obvious with the active peptide. Together with the infrequent amount of staining, this makes the results difficult to interpret.
We agree with the reviewer that the data presented in Figure 1 of our original paper lacked clarity. We therefore have redone the whole experiment and include the new improved data set in the revised draft. We now include DAPI staining for the nuclei and an improved membrane staining with DiD.
Of note, we include the staining with TLR1 that substantiates the notion that TLR2-p interferes with the formation of TLR2/TLR1 dimers.

3) As the authors state, an obvious issue with using small peptides of this kind in vivo is their bioavailability over long periods of time and indeed this could explain why most of the effects of peptide in the first DSS experiment are partial. The authors address this aspect in the second set of DSS experiments, but this means that the study is somewhat repetitive in nature. It would be preferable if the work focused on this second protocol of peptide administration and carried out the more detailed investigation used in the first experiments.
We agree with the reviewer that the pharmaco-kinetics of the peptide will have to be addressed if this approach is taken into the clinic. Indeed we are undertaking such approaches, such as the testing of more stable enantiomers. However, we believe that these efforts are beyond the scope of this study, which establishes to general potency of the interference with TLR dimerization as colitis treatment.

4) The staining for leukocytes in the mucosa appears unusual, as it appears that virtually all CD11b+

cells are CD11c+, including those, which are Ly6Chi. This is contrary to the established knowledge that murine monocytes of this kind are CD11c-.
We have modified the figure according to the reviewer's request and include the revised figure. The main aim is to highlight that the Ly6Chi monocyte infiltrates and we found the present display most clear.
The data are first gated on CD11c- CD11b+ cells, and then plotted as Ly6C vs. MHC II. This best demonstrates the monocyte infiltrates.

5) It is also not clear if the numbers shown represent only the MHCII-ve fraction of Ly6Chi cells, or if they include the MHCII+ve fraction that is known to expand in intestinal inflammation.
We apologize for having been unclear. What the bar diagram shows is '% of Ly6Chi MHCII- monocytes out of CD11b+ cells.

6) The cells analyzed do not appear to have been subjected to live/dead gating as is considered standard for such work.
The cells are gated according to FSC/SSC and on CD45 live cells. These gates are now included for clarity in supplementary Fig 1 (A).

Referee #2:

In this paper by Shmuel-Galia and Aychek et al., the authors study the impact of a TLR-2 transmembrane peptide (TLR-2-p) on TLR-2-TLR-6 assembly induced by natural ligands and its capacity to limit DSS-induced colitis. Identifying factors that can limit gastrointestinal inflammation and the mechanisms by which they act is an area of significant research interest and of high therapeutic relevance. The authors begin by demonstrating that TLR-2-p interacts with both TLR-2 and TLR-6 and show by FRET that TLR-2-p inhibits the dimerization of TLR-2-TLR-6 leading to attenuation of downstream signaling. Subsequently, they go on to find that when administered intraperitoneally during DSS colitis, TLR-2-p (but not a scrambled control) can reduce severity of colitic pathology and concurrently inflammatory cytokine production is limited. Moreover, it is determined that Ly6Chi monocyte cytokine release in particular is reduced even though Ly6Chi monocyte frequency is unchanged. Overall this is a clearly structured manuscript that explores an intriguing new mechanism to modulate inflammatory cell function during gut damage. A number of points should be addressed in order to support the claims made.

Major points:

(1) In Fig. 5 it is claimed that the mechanism by which TLR-2-p operates is that Ly6Chi monocyte recruitment is unaltered but their cytokine production is changed. To make this claim it is important that the authors show absolute numbers of Ly6Chi monocytes isolated from the tissue.
We have learned from our past experience with the study of mononuclear phagocytes in gut tissue that due to variations in the isolation procedure it very difficult to impossible to obtain reliable absolute numbers. To evaluate a monocyte infiltration we therefore prefer to show the accumulation of the cells in reference to a tissue resident population, such as CD11b+ cells.
Our conclusion is built on our more detailed analysis of monocyte infiltration and its kinetics in the Zigmond et al study, Immunity 2012 : Ly6Chi Monocytes in the Inflamed Colon Give Rise to Proinflammatory Effector Cells and Migratory Antigen-Presenting Cells.

(2) Additionally, in Fig. 5 it is not apparent to me what is meant in Fig. 5B as % out of CD11b+ Ly6Chi cells. CD11b+ Ly6Chi cells are monocytes so to look at % out of them makes no sense to me. Do the authors mean % of Ly6Chi CD11b+ of CD45+ or % of Ly6Chi CD11b+ of CD11c&CD11b+. It is possible that I am misunderstanding this graph - please clarify.
We apologize for the confusion. What the bar diagram shows is % of Ly6Chi monocytes out of CD11b+ cells.

(3) Clearly TLR-2-p affects downstream signaling in macrophages in vitro. Given that suppression of monocyte/macrophage production of cytokines by TLR-2-p is suggested as a major activity in DSS, it would be important to show that TLR-2-p can suppress pro-inflammatory cytokine production by monocytes or macrophages directly in vitro in response to activating ligands
We thank the reviewer for his suggestions. In the revised manuscript we include now the analysis of

primary BM-derived CD115+ cells, i.e. BM monocytes, in response to various ligands and the potency of TLR2-p to inhibit the response. The results confirm our previous data

(4) TNF-alpha is an important cytokine in DSS colitis and targeted as a therapy in UC. Is TNF-alpha production impacted by TLR-2-p?
We agree that TNF-alpha is an important cytokine but the factor can have both pro-inflammatory and healing activity. Moreover, TNF-alpha is produced as a latent factor that is membrane anchored. TNF-alpha measurements by RT-PCR are hence less informative. This is why we focused in our study on IL6 and IL-1. However, we added to the revised paper a new set of experiments on BM-derived monocytes/macrophages and show there that TLR2-p does block TNF alpha production by these cells.

Minor points:

(1) Monocytes can be a major source of IL-1b during colitis. Is IL-1b message expression (or protein release) reduced in monocytes by TLR-2-p treatment? This would add further support to the idea that in Figure 4A it is inhibition of pro-inflammatory cytokine production by monocytes that is primarily impacted by TLR-2-p.
We thank the reviewer for these suggestions. However due to the scarcity of the material we can obtain from the inflamed tissue, it is impossible to perform a protein analysis on the monocyte infiltrates.

(2) The English should be thoroughly proof read throughout and corrections made. In particular, there is frequent inappropriate usage of plurals e.g. TLRs dimerization; anti-cyokines therapy etc. This sometimes makes the text a little confusing to understand.
We apologize and have made efforts to correct

Referee #3:

Shmuel-Galia et al. do a nice job demonstrating that the TLR2-peptide they previously described blocks interaction between TLR2 and TLR6. Further, they convincingly demonstrate that intraperitoneal injection of this peptide during DSS administration, a commonly used colitis model, reduces disease severity. I have the following comments/suggestions:

Figure 1
• The confocal images would benefit from a membrane co-stain or at least DAPI co-stain. It's difficult to ascertain which stains are on the membrane vs. cytoplasmic.
In response to the reviewer's request we have replaced Figure 1 with a new data set. We now include DAPI staining for the nuclei and an improved membrane staining with DiD. While our conclusions have not changed, the data are we believe of much better quality. We thank the reviewer for his suggestion.

• Since TLR antibodies are notoriously challenging to generate and often have issues with specificity, the authors should demonstrate that these particular antibodies don't stain cells from the respective TLR knockouts or reference a paper that demonstrates this.
This is indeed a good point and was also a concern for us. The TLR2 antibody we use in our study is well established and respective controls have been reported (Hoffmann, O. et al., JI 2007: TLR2 Mediates Neuroinflammation and Neuronal Damage)
With respect to the anti-TLR6 reagent we have had an extensive correspondence with the vendor. Unfortunately though they have been unable to provide us with convincing evidence that this reagent is specific in immuno-staining. We therefore decided not to include the data obtained with the anti-TLR6 reagent in our co-localization study (Figure 1). Rather, in the new revised experiment we resort to an anti-TLR1 antibody for which specificity controls have been published (Alexopoulou L. et al., Nat Med 2002: Hyporesponsiveness to vaccination with Borrelia burgdorferi OspA in humans and in TLR1- and TLR2-deficient mice)

• Panel C needs an axis label.
Corrected

Figure 2

• The authors should more directly address the possibility that the TLR2 peptide blocks TLR2/1 interaction in addition to TLR2/6 interaction. In their previous paper (Fink et al. 2013), the authors demonstrated that cytokine responses to both TLR2/6 and TLR2/1 ligands were diminished after addition of the TLR2-peptide, so an effect on both combinations of dimerization seems likely. If possible, the authors should perform the same assays in Figure 1 and 2 including TLR1. Although the authors state that the TLR2-TLR6 interaction has been shown to be more important in colitis, they can't rule out a role for blockade of TLR2-TLR1 in their DSS + peptide model. Therefore, claiming that their peptide is "targeting TLR2/6 dimerization" in the title and throughout the text is a bit misleading. The authors should either prove that the peptide selectively blocks only the TLR2/6 interaction, prove that the TLR2/6 interaction is more important in their model (for example, using their DSS + peptide protocol in TLR1-/- mice), or reword their paper to more accurately allow for this possibility.

We thank the reviewer for his comment. Indeed, TLR2-p targets dimerization of TLR2 with TLR1 and TLR6, as highlighted in the functional assay (Figure 2A). To further strengthen this notion we include in the new Figure 1 of the revised manuscript data (under B) demonstrating co-localization of the peptide with TLR1.

We have modified the manuscript throughout to accommodate interference with both TLR2/TLR6 and TLR2/TLR1 dimerization.

Figure 3

• The authors should plot mouse weights over a longer DSS time course, rather than just focusing on one time point. I understand that they sacrificed mice at the peak of inflammation to do histology and scoring, but weight loss will continue after day 7 and it would also be interesting to see if the peptide affects the rate of recovery.

We agree with the reviewer that it would have been preferable to have data from a longer course, even after withdrawal of the peptide. However, we would like to ask the reviewer not to insist on these data since they would involve major new experimentation. Also, we believe that we make the main point that the TLR2-p has therapeutic potential with the presented data. The analysis of the extend of the effect belongs arguably to a follow-up study that will optimize peptide application, the testing of variants, such as enantiomers.

This may be beyond the scope of the paper, but it would be interesting to determine whether administration of the peptide after the initiation of inflammation has an effect (which would make this approach more relevant therapeutically).

Again this is of course a very interesting point, but as we believe indeed beyond the scope of the present paper.

Figure 5

Percentages need to be listed for the flow plots.

We apologize for the omission and have added the percentages.

• The authors should think about including cell numbers (although this is admittedly sometimes difficult for lamina propria).

We have learned from past experience with the study of mononuclear phagocytes in gut tissue that due to variations in the isolation procedure it is very difficult to impossible to obtain reliable absolute numbers. To evaluate a monocyte infiltration we therefore prefer to show the accumulation of the cells in reference to a tissue resident population, such as CD11b+ cells.

Our conclusion is built on our more detailed analysis of monocyte infiltration and its kinetics in the Zigmond et al study, Immunity 2012 : Ly6Chi Monocytes in the Inflamed Colon Give Rise to Proinflammatory Effector Cells and Migratory Antigen-Presenting Cells.

Figure 6

• Panel B is unnecessary (and somewhat confusing) in this figure and could be moved to supplemental.

We would prefer to include panel B in Fig 6 (now Fig. 7) in the text. Even though repetitive, it substantiates the anti-colitis effect of TLR2-p and it also refers to the same animals that where used to retrieve the monocytes analyzed in Fig7C.

• The monocyte sorting is a good experiment and provides insight into potential mechanisms of DSS

amelioration, but the authors did not demonstrate that the effect of TLR2/6 on monocyte activation is cell intrinsic. Perhaps they could try to use their rhodamine-labeled peptide and determine whether it is bound to monocytes during DSS in vivo. Or they could look at downstream TLR signaling pathways to show they are reduced within monocytes after administration of the peptide. If they don't show this, then they should be clearer about the fact that this effect might be indirect (not just mention this quickly in the discussion).

We thank the reviewer for appreciating the experiment.

Indeed we believe that this is the key figure. We show in Fig3B of the revised manuscript that the monocyte/ macrophage response to TLR ligands is blocked by TLR2-p in vitro. We have previously established that engagement of TLR2 on monocytes is critical for them to acquire the pro-inflammatory signature (Zigmond et al 2012). In light of these findings we consider that it is highly likely that the TLR2-p acts intrinsically on the monocytes. However we of course cannot rule out more complex scenarios that we sketch in the discussion.

2nd Editorial Decision 23 December 2015

Thanks for submitting your revised manuscript to The EMBO Journal. Your revision has now been re-reviewed by the referees and their comments are provided below.

As you can see the referees appreciate the introduced changes and support publication here. They raise a few minor concerns that I would like to ask you to address in a final round of revision. Once we get the revised version back I will accept it for publication here.

REFEREE REPORTS

Referee #1:

The authors have addressed the original points and the manuscript is much improved. One or two minor comments:

1) The authors mention that they have been unable to verify the specificity of the anti-TLR6, which is the reagent used in the FRET studies. Perhaps this warrants comment.

2) The effects of TLR2p on IL6 production in the DSS mice shown in Figure 5 are very small. In the same figure, the control scrambled peptide has had almost as much effect on IL23 mRNA as the TLR2p.

Referee #2:

In reference to my first major point, I agree with the authors that it may be difficult for many reasons to absolutely quantify numbers of monocytes in an inflamed colon. I would therefore prefer if the authors reworded their results section to reflect this uncertainty. Perhaps they could use a phrase like: When challenged with DSS, "frequency" of Ly6Chi cells within the total CD11b+ pool increased in the colon, corroborating earlier studies...

Other than this minor issue the authors have satisfactorily addressed my concerns.

Referee #3:

The authors have addressed my concerns. I recommend that the paper be accepted.

2nd Revision - authors' response 13 January 2016

Referee #1

The authors mention that they have been unable to verify the specificity of the anti-TLR6, which is the reagent, used in the FRET studies. Perhaps this warrants comment.

We agree with the reviewer that antibody specificity is critical. However, we believe that in the specific case of FRET, the specificity of the antibody is controlled for in the experiment, itself. For FRET to occur a specific interaction between TLR2 and TLR6 is needed as the energy transfer is between the TLR6-PEand TLR2-Cy5 antibodies. In this experiment, upon stimulation with specific TLR2-TLR6 ligand, LTA, FRET was observed. This indicates that the TLR6 antibody is specific as it is crucial for FRET.

The effects of TLR2p on IL6 production in the DSS mice shown in Figure 5 are very small. In the same figure, the control scrambled peptide has had almost as much effect on IL23 mRNA as the TLR2p.

We thank the reviewer for his comment. Although the scrambled peptide does seem to have some effect in part of the results in Fig5B, this did not reach significance. To clarify this point, we indicated in the revised manuscript significant and non-significant results in the figure.

Referee #2

We have accepted the reviewer's comment and changed the text as he suggested.

EMBO PRESS

YOU MUST COMPLETE ALL CELLS WITH A PINK BACKGROUND ↓

Corresponding Author Name: Yechiel Shai
Journal Submitted to: EMBO Journal
Manuscript Number: 92649

Reporting Checklist For Life Sciences Articles (Rev. July 2015)

This checklist is used to ensure good reporting standards and to improve the reproducibility of published results. These guidelines are consistent with the Principles and Guidelines for Reporting Preclinical Research issued by the NIH in 2014. Please follow the journal's authorship guidelines in preparing your manuscript.

A- Figures

1. Data

The data shown in figures should satisfy the following conditions:

- the data were obtained and processed according to the field's best practice and are presented to reflect the results of the experiments in an accurate and unbiased manner.
- figure panels include only data points, measurements or observations that can be compared to each other in a scientifically meaningful way.
- graphs include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical replicates.
- if n< 5, the individual data points from each experiment should be plotted and any statistical test employed should be justified
- Source Data should be included to report the data underlying graphs. Please follow the guidelines set out in the author ship guidelines on Data Presentation.

2. Captions

Each figure caption should contain the following information, for each panel where they are relevant:

- a specification of the experimental system investigated (eg cell line, species name).
- the assay(s) and method(s) used to carry out the reported observations and measurements
- an explicit mention of the biological and chemical entity(ies) that are being measured.
- an explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.
- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- a statement of how many times the experiment shown was independently replicated in the laboratory.
- definitions of statistical methods and measures:
  - common tests, such as t-test (please specify whether paired vs. unpaired), simple χ^2 tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
  - are tests one-sided or two-sided?
  - are there adjustments for multiple comparisons?
  - exact statistical test results, e.g., P values = x but not P values < x;
  - definition of 'center values' as median or average;
  - definition of error bars as s.d. or s.e.m.

Any descriptions too long for the figure legend should be included in the methods section and/or with the source data.

Please ensure that the answers to the following questions are reported in the manuscript itself. We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.

In the pink boxes below, provide the page number(s) of the manuscript draft or figure legend(s) where the information can be located. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable).

B- Statistics and general methods

Please fill out these boxes ↓ (Do not worry if you cannot see all your text once you press return)

1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	Material and Method/ statistical analysis - page 14
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	
3. Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. randomization procedure)? If yes, please describe.	
For animal studies, include a statement about randomization even if no randomization was used.	
4.a. Were any steps taken to minimize the effects of subjective bias during group allocation or/and when assessing results (e.g. blinding of the investigator)? If yes please describe.	
4.b. For animal studies, include a statement about blinding even if no blinding was done	
5. For every figure, are statistical tests justified as appropriate?	Material and Method/ statistical analysis - page 14
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	Material and Method/ statistical analysis - page 14
Is there an estimate of variation within each group of data?	Material and Method/ statistical analysis - page 14
Is the variance similar between the groups that are being statistically compared?	Material and Method/ statistical analysis - page 14

C- Reagents

USEFUL LINKS FOR COMPLETING THIS FORM

http://www.antibodypedia.com
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http://www.consort-statement.org/checklists/view/32-consort/66-title

http://www.equator-network.org/reporting-guidelines/reporting-recommendations-for-tun

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http://oba.od.nih.gov/biosecurity/biosecurity_documents.html
http://www.selectagents.gov/

6. To show that antibodies were profiled for use in the system under study (assay and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile. e.g., Antibodypedia (see link list at top right), 1DegreeBio (see link list at top right).	Material and method- pages 9-14
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	Material and method- page 10

* for all hyperlinks, please see the table at the top right of the document

D- Animal Models

8. Report species, strain, gender, age of animals and genetic modification status where applicable. Please detail housing and husbandry conditions and the source of animals.	Material and method- page 12
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	Material and method- page 12
10. We recommend consulting the ARRIVE guidelines (see link list at top right) (PLoS Biol. 8(6), e1000412, 2010) to ensure that other relevant aspects of animal studies are adequately reported. See author guidelines, under 'Reporting Guidelines'. See also: NIH (see link list at top right) and MRC (see link list at top right) recommendations. Please confirm compliance.	

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	NA
12. Include a statement confirming that informed consent was obtained from all subjects and that the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.	
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	
14. Report any restrictions on the availability (and/or on the use) of human data or samples.	
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	
16. For phase II and III randomized controlled trials, please refer to the CONSORT flow diagram (see link list at top right) and submit the CONSORT checklist (see link list at top right) with your submission. See author guidelines, under 'Reporting Guidelines'. Please confirm you have submitted this list.	
17. For tumor marker prognostic studies, we recommend that you follow the REMARK reporting guidelines (see link list at top right). See author guidelines, under 'Reporting Guidelines'. Please confirm you have followed these guidelines.	

F- Data Accessibility

18. Provide accession codes for deposited data. See author guidelines, under 'Data Deposition'. Data deposition in a public repository is mandatory for: a. Protein, DNA and RNA sequences b. Macromolecular structures c. Crystallographic data for small molecules d. Functional genomics data e. Proteomics and molecular interactions	NA
19. Deposition is strongly recommended for any datasets that are central and integral to the study; please consider the journal's data policy. If no structured public repository exists for a given data type, we encourage the provision of datasets in the manuscript as a Supplementary Document (see author guidelines under 'Expanded View' or in unstructured repositories such as Dryad (see link list at top right) or Figshare (see link list at top right).	NA
20. Access to human clinical and genomic datasets should be provided with as few restrictions as possible while respecting ethical obligations to the patients and relevant medical and legal issues. If practically possible and compatible with the individual consent agreement used in the study, such data should be deposited in one of the major public access-controlled repositories such as dbGAP (see link list at top right) or EGA (see link list at top right).	NA
21. As far as possible, primary and referenced data should be formally cited in a Data Availability section. Please state whether you have included this section. Examples: **Primary Data** Wetmore KM, Deutschbauer AM, Price MN, Arkin AP (2012). Comparison of gene expression and mutant fitness in Shewanella oneidensis MR-1. Gene Expression Omnibus GSE39462 **Referenced Data** Huang J, Brown AF, Lei M (2012). Crystal structure of the TRBD domain of TERT and the CR4/5 of TR. Protein Data Bank 4O26 AP-MS analysis of human histone deacetylase interactions in CEM-T cells (2013). PRIDE PXD000208	NA
22. Computational models that are central and integral to a study should be shared without restrictions and provided in a machine-readable form. The relevant accession numbers or links should be provided. When possible, standardized format (SBML, CellML) should be used instead of scripts (e.g. MATLAB). Authors are strongly encouraged to follow the MIRIAM guidelines (see link list at top right) and deposit their model in a public database such as Biomodels (see link list at top right) or JWS Online (see link list at top right). If computer source code is provided with the paper, it should be deposited in a public repository or included in supplementary information.	NA

G- Dual use research of concern

23. Could your study fall under dual use research restrictions? Please check biosecurity documents (see link list at top right) and list of select agents and toxins (APHIS/CDC) (see link list at top right). According to our biosecurity guidelines, provide a statement only if it could.	NA