Toll signalling promotes blastema cell proliferation during cricket leg regeneration via insect macrophages

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Original submission

First decision letter

MS ID#: DEVELOP/2021/199916

MS TITLE: Toll signalling promotes blastema cell proliferation during cricket leg regeneration via insect macrophages

AUTHORS: Tetsuya Bando, Misa Okumura, Yuki Bando, Marou Hagiwara, Yoshimasa Hamada, Yoshiyasu Ishimaru, Taro Mito, Eri Kawaguchi, Takeshi Inoue, Kiyokazu Agata, Sumihare Noji, and Hideyo Ohuchi

I have now received all the referees reports on the above manuscript, and have reached a decision. The referees' comments are appended below, or you can access them online: please go to BenchPress and click on the 'Manuscripts with Decisions' queue in the Author Area.

As you will see, the referees consider that your manuscript is much improved. However they do all still have minor criticisms and suggestions for improvement prior to publication. Please attend to all of the reviewers' comments in your revised manuscript and detail them in your point-by-point response. If you do not agree with any of their criticisms or suggestions explain clearly why this is so.

Reviewer 1

Advance summary and potential significance to field

The revised work from Bando and co-authors is much improved, and shows more convincingly a role for macrophages in regeneration, via Toll signalling. This is significant, as it expands our understanding of the broader role played by macrophages during regenerative processes, besides immune responses. In my opinion it would deserve publication, after addressing the minor comments listed below.

Comments for the author

- The introduction provides too many details (for example mentioning several genes that are not relevant for the study), which do not help the reader focus on the points of the paper. I suggest focusing on the relevant aspects, for example the section at lines 82-109 could be removed/reduced.
- Lines 59, 62: please, change “creatures” to “animals” or “metazoans”
- Lines 150-158: The sequencing and mapping approaches belong to the Methods sections; please, move this information there.
- Line 165: please, insert between brackets or in italic the GO term nomenclature (eg “cell differentiation”).
- Line 181: the expression of Gb’Toll8 appears downregulated (compared to t0), in regenerating legs, not upregulated. Perhaps Gb’Toll2-1 should have been cited instead?
- Lines 448-455 and 555-564: the Authors offer a hypothesis for the non-straightforward effects observed on regeneration when suppressing different Toll genes. The hypothesis is reasonable, but requires a better understanding of the experimental procedure to be understood. Please, explain more clearly the timing of RNAi injection and regeneration experiments in the Methods section, and refer to it in the Discussion.
- Lines 210-223: With regard to the Gb’Toll1-2 and Gb’Toll2-2 RNAi phenotypes, I suggest reporting in the main text also the numbers of the class 2 phenotypes, as together they show nearly a 70% effect.
- Lines 235-245: please, specify that suppression of Gb’Toll2-2 has no effect on Gb’Toll2-1, otherwise the rationale for focusing on Gb’Toll2-2 is unclear.
- Line 347: please, change “enumerated” to “counted” or “quantified”.
- Line 514: the information about the aseptic conditions should be mentioned in the Methods.
- Lines 539-544: the RNAseq analysis is not clearly explained, in particular please explain better the mapping and the differential expression analysis. Were the data normalized? How was the fold change calculated? Were replicates performed?
- Lines 556, 596, 599: Please, specify how the specific injection volume (207 nl) was determined.
- Line 862: please provide a legend for the colours of Figure 2B.
- The Response_letter_figure_for_reviewer1 is relevant and strengthens the analysis: I recommend adding it to the Supplementary Figures and referring to it in the Methods section.
- This is perhaps a matter of personal taste, but I recommend adding a Conclusion to the manuscript.

Reviewer 2

Advance summary and potential significance to field

Summary: In this study the Bando group investigates the role of Toll receptors, related signaling factors, and hemocytes in cricket leg regeneration. The survey of Toll receptor expression following amputation is useful, as is the characterization of hemocyte distributions in unwounded and regenerating legs. Inclusion of documentation of RNAi knockdowns is a strength of the paper though specificity of knockdown could still be demonstrated further. Performing the Toll knockdowns in the clodronate-ablated animals is a nice approach. Overall this is a nice study with some reasonably compelling functional data. It will be of interest to those in the field of appendage regeneration and to those in other fields such as fly and fish. Some relatively minor suggestions for improving the paper are noted.

Comments for the author

Major concerns:
1. Documentation of on-target RNAi knockdowns (Figure S4) is good and essential for this study. What is the y-axis for this graph? Normalized transcript level? But the actual transcript is different for each set of bars? It seems the authors could also address specificity of the RNAi here as well- if they measured a TollR different than the one targeted for each pair. This would reassure that they see both knockdown and specificity.

2. With respect to the depletion of the plasmatocytes/macrophages by clodronate- the documentation of the depletion would be stronger with an independent marker- like a surface marker. Using BODIPY-lipo it seems possible that the cells could still be present but just not capable of taking up the marker/label. Has this been examined?

3. The word “blastema” here seems to be used a bit loosely. It is not a synonym for “where proliferation is occurring”. But that appears to be how it is mostly used. For instance there is proliferation throughout the leg in Fig. 6D but presumably only those cells near the amputation plane are physically within the blastemal structure. Definitely less proliferation with clodronate but this effect does not appear specific to the cells near the amputation. Some clarification as to what the authors mean by the word “blastema” both structurally and functionally, would be helpful.

Minor Concerns:
1. Please label Y-axis in Figure 1B. there are numerous other graphs with no Y-axis labels as well- please label all of them so the reader can understand what is being measured.
2. Figure 1 C probably best as supplemental.
3. Figure 3- unamputated control for comparison to class 3 would be helpful. This is the first place the reader sees a cricket leg.
4. Lines 213/214- To further analyse… First, there should be a paragraph break there. Second spz are not intracellular components… Please rewrite.
5. Line 557- spelling of section header.
6. For this reviewer’s tastes Fig. S6 should be in the main paper- this is the most precise documentation of the Toll2-2 RNAi phenotype. Why bury it?

Reviewer 3

Advance summary and potential significance to field

Both the text and the data have been greatly improved. However, the authors should polish the points below prior to publication:

Comments for the author

The authors have still not provided the RNAseq data in a readily accessible format. As requested previously the entire RNAseq dataset must be provided with the paper, as a supplementary excel file.
It would be good to state explicitly that Gb Toll-2 is phylogenetically related to Drosophila Toll-9 and not Toll-2, to avoid confusions.
Authors should cite work of others in Drosophila: Toll-6 promotes cell survival, cell-cell interactions and cell shape changes in neurons (McIlroy et al 2013 Nature Neuroscience; McLaughlin et al 2014 J Cell Biol; Ward et al 2015 Neuron); Toll-2 promotes cell proliferation in Drosophila (Li et al 2020 eLife) and planar cell polarity via PI3K (Tamada et al 2021 Dev Cell).

Lines 75-77: “In insects,… and by PGRPLC and IMd signalling for gram-positive bacteria”. Mistake. The Toll pathway responds to gram-positive bacteria and the Imd pathway is activated by gram-negative bacteria.
Line 80-81: “These insect immunity signalling pathways…”. This sentence is not grounded on evidence.
Figure 1: check the nomenclature: TLR or Toll correct for all?
Line 214-215: “We found two paralogous genes, Gb spz and Gb spz2..”. It would be interesting if the authors could clarify whether Gb spz and spz-2 are orthologues of Drosophila spz-1 and spz-2 (also known as Drosophila neurotrophin 1 , see Zhu et al 2008 PLoS Biol).
Lane 216-222: authors should check Li et al 2020 eLife e52743 DOI: 10.7554/eLife.52743 which shows that: 1) Toll-2 induces cell proliferation in Drosophila; 2) however, this does not require the MyD88 pathway, and in fact, MyD88 inhibits proliferation.
Line 269:”These results suggest that Toll-2-2 upregulates the expression of its own ligands…”. Or that the cells that normally produce the ligands are no longer there.
Line 384-385: “The cricket genome lacks a Persephone homologue (data not shown)”. Either show the data or remove the claim.

Line 456-461:"…..Therefore, Toll signalling may interfere with Hippo signalling….". In Drosophila, Toll-2 may function together with Yki to promote cell proliferation, rather than interfering with it (Li et al 2020 eLife).

First revision

Author response to reviewers' comments

Replies to Reviewer #1
We appreciate Reviewer #1's comments to our manuscript. We have agreed all the points raised by the reviewer, and revised the text and figures accordingly.

Reviewer 1 Comments for the author...
The introduction provides too many details (for example mentioning several genes that are not relevant for the study), which do not help the reader focus on the points of the paper. I suggest focusing on the relevant aspects, for example the section at lines 82-109 could be removed/reduced.
Response: We have reduced the section to avoid irrelevant details (lines 81-100).

- Lines 59, 62: please, change "creatures" to "animals" or "metazoans"
Response: We corrected the term from "creatures" to "animals" as you suggested in lines 59 and 62.

- Lines 150-158: The sequencing and mapping approaches belong to the Methods sections; please, move this information there.
Response: We moved the sentences related to the methods of RNA-seq analysis from the Result section to the Materials and Methods section in lines 550-554. We also revised the paragraph about RNA-seq analysis in the Results section in lines 139-147.

- Line 165: please, insert between brackets or in italic the GO term nomenclature (eg "cell differentiation").
Response: As you suggested, we added double-quotation to each GO term as "such as "cell differentiation" and "cell death" in biological processes" in line 153.

- Line 181: the expression of Gb'Toll8 appears downregulated (compared to t0), in regenerating legs, not upregulated. Perhaps Gb'Toll2-1 should have been cited instead?
Response: In this sentence, we summarized the expression changes based on the RNA-seq analysis. Gb'Toll8 was upregulated in the RNA-seq analysis (Fig. S2B), but we could not confirm by qPCR (Fig. 2B). In contrast to the Gb'Toll8, we confirmed the upregulation of Gb'Toll2-5 and Gb'Toll2-2 revealed by RNA-seq analysis (Fig. S2B) by qPCR (Fig. 2B). In this RNA-seq analysis, we used a GS FLX Titanium (Roche 454) sequencer, which reads longer transcripts but a fewer number of them than a short read NGS, in general. We sometimes faced the issue that expression changes obtained by NGS were not confirmed by qPCR, especially when we used the long read NGS, because numbers of reads were few.

- Lines 448-455 and 555-564: the Authors offer a hypothesis for the non-straightforward effects observed on regeneration when suppressing different Toll genes. The hypothesis is reasonable, but requires a better understanding of the experimental procedure to be understood. Please, explain more clearly the timing of RNAi injection and regeneration experiments in the Methods section, and refer to it in the Discussion.
Response: We added the detailed explanation about RNAi, leg amputation and regeneration, and nymphal growth in lines 574-579, as "Wound regions are usually covered with scar within a day in third instar. The third instar nymphs moult to fourth instar within four days. In the fourth instar, newly formed cuticles cover the wound region instead of the scar. The fourth instar nymphs moult to fifth instar within five days and the lost leg tissues are reconstructed in miniature in the fifth instar. After RNAi and leg amputation, we observed RNAi phenotypes of regenerating legs on the tenth day, which corresponds to the late fifth instar."

- Lines 210-223: With regard to the Gb'Toll1-2 and Gb'Toll2-2 RNAi phenotypes, I suggest reporting in the main text also the numbers of the class 2 phenotypes, as together they show nearly a 70% effect.
Response: We added the description about class 2 phenotype as "In addition, 18.8% and 32% of Gb'Toll2-1RNAi and Gb'Toll2-2RNAi crickets showed class 2 phenotype, respectively, thus, > 70% of these RNAi crickets showed abnormalities during leg regeneration." in lines 201-203.

- Lines 235-245: please, specify that suppression of Gb'Toll2-2 has no effect on Gb'Toll2-1, otherwise the rationale for focusing on Gb'Toll2-2 is unclear.
Response: We added the description about Gb'Toll2-1 expression in Gb'Toll2-2(RNAi) regenerating legs as "but expression levels of other cricket Toll genes, including Gb'Toll2-1, were not significantly changed." in lines 234-235.

- Line 347: please, change "enumerated" to "counted" or "quantified".
Response: We revised "counted" as you suggested in line 351.

- Line 514: the information about the aseptic conditions should be mentioned in the Methods.
Response: We conducted RNAi and leg amputation using sterilized glass needles, scissors and tweezers, and kept these crickets in clean insect cages, as usual. We did not inoculate pathogens such as E. coli in our experiments. That strategy is used in some references to analyze the immunity function of Toll signalling. We revised the sentence as "Cricket leg amputation experiments here were conducted without artificial pathogen inoculation." in lines 523-524.

- Lines 539-544: the RNAseq analysis is not clearly explained, in particular please explain better the mapping and the differential expression analysis. Were the data normalized? How was the fold change calculated? Were replicates performed?
Response: We added the explanation about the mapping methods and software in the Materials and Methods section. The expression level of each assembled contig in the regenerating legs and non-regenerating legs was normalized by calculating the RPKM value. We just divided the RPKM values of RL by corresponding RPKM value of NL to estimate the relative expression changes (listed in Table S1). Blast results to the contigs upregulated in RLs (3 hpa) compared with NLs (0 hpa) are listed in Table S2 and selected signalling pathway genes were shown in Fig. S2B. Since the Roche 454 sequencer had been discontinued, we could not replicate the RNA-seq analysis. We therefore confirmed the expression changes as revealed by RNA-seq using qPCR.

- Lines 556, 596, 599: Please, specify how the specific injection volume (207 nl) was determined.
Response: We used an auto-nanoliter injector, Nanoject II, for liquid injection to the crickets. The maximum volume of this instrument is 69 nL, and we conducted three injections of dsRNA, liposome or EdU solution. Thus, the total injection volume of each solution was 69 nL x 3 =207 nL. We added this information in the Materials and Methods section in lines 572-573.

- Line 862: please provide a legend for the colours of Figure 2B.
Response: We separated the colours of graph bars by patterns of gene expression changes in Fig 2B. Genes upregulated during regeneration were shown in red bars and down regulated genes were shown in blue. Genes whose expression changes were less than 2-fold or more than half were shown in yellow. We added this explanation in lines 898 and 899.

- The Response_letter_figure_for_reviewer1 is relevant and strengthens the analysis: I recommend adding it to the Supplementary Figures and referring to it in the Methods section.
Response: We added the Response_letter_figure_for_reviewer1 figures in Fig. S6, and added brief explanation in Results section in lines 240-245.

- This is perhaps a matter of personal taste, but I recommend adding a Conclusion to the manuscript.
Response: We added a concluding summary to the final paragraph of the Discussion section as "In conclusion, this study provides new insights into the function of Toll-related signalling for leg regeneration via plasmatocytes, cooperatively with JAK/STAT signalling. Recognition of apoptotic cells via the scavenger receptor Crq on plasmatocytes also promotes leg regeneration." in lines

532-534, since there is no paper that includes the Conclusion section in the latest issue of Development.

Replies to Reviewer #2
We are grateful for Reviewer #2's review comments. We carefully revised our manuscript following the Reviewer #2's comments.

Reviewer 2 Comments for the author...
Major concerns:
1. Documentation of on-target RNAi knockdowns (Figure S4) is good and essential for this study. What is the y-axis for this graph? Normalized transcript level? But the actual transcript is different for each set of bars? It seems the authors could also address specificity of the RNAi here as well- if they measured a TollR different than the one targeted for each pair. This would reassure that they see both knockdown and specificity.
Response: The graph bars for the qPCR experiments indicate relative expression level and we added the explanation about that in the figure legends as "The y-axis indicates normalized expression of Gryllus Toll genes in the DsRedRNAi samples and relative expression levels in the RNAi samples against the corresponding Toll genes." in lines 42-44 in Supplementary materials.
We calculated the actual expression level when we performed the qPCR experiments. We found that actual expression of Gb'Toll was much higher than Gb'Toll2-1 and Gb'Toll2-2, and it seemed that it was difficult to show the graph using actual expression level, thus we prepared the graphs using normalized expression level.

2. With respect to the depletion of the plasmatocytes/macrophages by clodronate- the documentation of the depletion would be stronger with an independent marker- like a surface marker. Using BODIPY-lipo it seems possible that the cells could still be present but just not capable of taking up the marker/label. Has this been examined?
Response: In some reports on Drosophila hemocytes, croquemort (crq), which is a CD36 homologue, is one of the surface markers for insect macrophages (plasmatocytes). In our study, crq expression was decreased in plasmatocyte-depleted regenerating legs, and crq(RNAi) affects leg regeneration, as demonstrated by the fact that plasmatocyte-depleted crickets showed a regeneration-defective phenotype. These results indicate that crq could be used as a plasmatocyte marker in crickets as well, however, we could not prepare an anti-cricket Crq antibody. Although there are some anti-CD36 antibodies commercially-available, the epitope of the antibodies did not show homology to cricket Crq. Thus, we have not tried to detect plasmatocytes using a surface marker. We will try such experiments in the next study.

3. The word "blastema" here seems to be used a bit loosely. It is not a synonym for "where proliferation is occurring". But that appears to be how it is mostly used. For instance there is proliferation throughout the leg in Fig. 6D but presumably only those cells near the amputation plane are physically within the blastemal structure. Definitely less proliferation with clodronate but this effect does not appear specific to the cells near the amputation. Some clarification as to what the authors mean by the word "blastema" both structurally and functionally, would be helpful.
Response: A blastema is a mass of proliferating cells, and blastema cells are the source of regenerating tissues in several regenerative animals, however, the specific definition, cell types, and marker genes/proteins of the blastema cells are varied depending on animal species, such as prrx1 in amphibians, PIWI, pERK, mkpA in planarians. In the cricket, the marker genes/proteins to determine the blastema have not been identified. We define the blastema as a whitish tissue beneath the wound site (Fig. 7C).

As you mentioned, proliferating cells were distributed not only in the blastema structure but also in the tibial stump region in regenerating cricket legs. The proliferating cells in the blastema are for leg regeneration and the proliferation in the stump region are for nymphal growth. In Fig. 5A and Fig. 7D, all cells were localized in the blastema and more proximal region was not included in these figures. We therefore conclude that less proliferation was caused by Clo-lipo treatment or Gb'Toll2-2(RNAi) in the blastema at least.
According to your suggestions, we added some clarification regarding cricket blastemas as, "In cricket regenerating legs, the blastema appears as a whitish tissue localised in the distal region (shown by brackets in Fig. 7C)." in lines 315-316.

Minor Concerns:

1. Please label Y-axis in Figure 1B. there are numerous other graphs with no Y-axis labels as well- please label all of them so the reader can understand what is being measured.
Response: In Figure 1B, graph bars indicate the number of contigs that annotated the GO terms. We added the explanation of y-axis as “The y-axis indicates numbers of contigs.” in line 888. There was no explanation in other qPCR graphs, so we also added the explanation in figure legends.

2. Figure 1 C probably best as supplemental.
Response: We moved the Fig. 1C to Fig. S2B.

3. Figure 3- unamputated control for comparison to class 3 would be helpful. This is the first place the reader sees a cricket leg.
Response: We added the contralateral unamputated leg photo in Fig. 3A to compare with class 3 regenerating legs.

4. Lines 213/214- To further analyse… First, there should be a paragraph break there. Second spz are not intracellular components… Please rewrite.
Response: We broke the paragraph as you suggested and rewrote the sentence as “To further analyse the role of Toll signalling, RNAi targeting ligands and intracellular component genes of the signalling was performed” in lines 206-207.

5. Line 557- spelling of section header.
Response: We showed full spell and abbreviation of RNAi as “RNA interference (RNAi)” in line 569.

6. For this reviewer’s tastes Fig. S6 should be in the main paper- this is the most precise documentation of the Toll2-2 RNAi phenotype. Why bury it?
Response: According to your suggestion, we moved the SEM figure to Fig. 4.

Replies to Reviewer #3
We are thankful for Reviewer #3's comments. We have included new data related to the RNA-seq analysis and revised the text according to the suggestions.

Reviewer 3 Comments for the author...
The authors have still not provided the RNAseq data in a readily accessible format. As requested previously, the entire RNAseq dataset must be provided with the paper, as a supplementary excel file.
Response: The RNA-seq raw data is available through the DNA DataBank of Japan (DDBJ). In addition, we also deposited an assembled transcriptome data to DDBJ, and we provided a Supplementary Tables that includes expression change data between regenerating legs and non-regenerating legs, based on the RPKM values, and Blast results of selected contigs in Table S1 and S2.

It would be good to state explicitly that Gb Toll-2 is phylogenetically related to Drosophila Toll-9 and not Toll-2, to avoid confusions.
Response: We added the explanation about Gryllus Toll2 paralogues as “Five paralogues Gb’Toll2-1 to Gb’Toll2-5 were phylogenetically closed to termite Zootermopsis nevadensis Toll like receptor 2, but not closed to Drosophila Toll-2, since Drosophila Toll-2 was closed to Toll-7 (Fig. 2A)” in lines 167-169.

Authors should cite work of others in Drosophila: Toll-6 promotes cell survival, cell-cell interactions and cell shape changes in neurons (McIlroy et al 2013 Nature Neuroscience; McLaughlin et al 2014 J Cell Biol; Ward et al 2015 Neuron); Toll-2 promotes cell proliferation in Drosophila (Li et al 2020 eLife) and planar cell polarity via PI3K (Tamada et al 2021 Dev Cell).
Response: Thank you for your recommendation of these references. We cited them in the Discussion section as “Drosophila Toll-2 regulates cell proliferation and planar cell polarity (Li et al., 2020; Tamada et al., 2021) and Toll-6 promotes neuronal cell shape, survival and interactions (McIlroy et al., 2013; McLaughlin et al., 2016; Ward et al., 2015)” in lines 441-444.

Lines 75-77: “In insects,… and by PGRPLC and IMd signalling for gram-positive bacteria”. Mistake. The Toll pathway responds to gram-positive bacteria and the Imd pathway is activated by gram-negative bacteria.
Response: Thank you for pointing out the misdescription. We rewrote the sentences “In insects, infectious microbes such as gram-positive bacteria, yeasts and fungi are mostly detected by Toll via proteoglycan recognition proteins (PGRPs) and clip-domain serine proteinases (clip-SPs), and gram-negative bacteria are detected by PGRP-LC and immune deficiency (Imd) signalling” in lines 75-78.

Line 80-81: “These insect immunity signalling pathways…”. This sentence is not grounded on evidence.
Response: This sentence included the starting hypothesis for our research and is partially overlapped with lines 98-100 and 108-109. Thus, we deleted the sentence.

Figure 1: check the nomenclature: TLR or Toll correct for all?
Response: Thank you very much for pointing out the nomenclature. We changed from “Toll-like receptor signaling” to “Toll signalling” in Fig. S2B.

Line 214-215: “We found two paralogous genes, Gb spz and Gb spz2..”. It would be interesting if the authors could clarify whether Gb spz and spz-2 are orthologues of Drosophila spz-1 and spz-2 (also known as Drosophila neurotrophin 1 , see Zhu et al 2008 PLoS Biol).
Response: We analysed the amino acid homologies between six Drosophila Spz ligands and two Gryllus Spz ligands, and found that Gb’Spz and Gb’Spz2 were homologous to Drosophila Spz and Spz5, respectively. We added the explanation and rewrote the sentence as “We found two spz paralogues, which encode Toll ligands, in the Gryllus genome (Fig. 3B), whereas Drosophila has six spz paralogues (Viljakainen, 2015). Gb'Spz and Gb'Spz2 were paralogous to Drosophila Spz and Spz5, respectively.” in lines 207-209.

Lane 216-222: authors should check Li et al 2020 eLife e52743 DOI: 10.7554/eLife.52743 which shows that: 1) Toll-2 induces cell proliferation in Drosophila; 2) however, this does not require the MyD88 pathway, and in fact, MyD88 inhibits proliferation.
Response: Thank you for the comment about the functional differences of Toll-2 and MyD88 between Drosophila and Gryllus. We added the description about this difference in the Discussion section as “although Drosophila Toll-2 does not require MyD88 for cell proliferation (Li et al., 2020)” in lines 404-405.

Line 269: ”These results suggest that Toll-2-2 upregulates the expression of its own ligands…”. Or that the cells that normally produce the ligands are no longer there.
Response: Thank you for the comments. In fact, Gb’Toll2-2 promotes the accumulation of plasmatocytes in the regenerating legs (Fig. 8B) and the expression of Gb’spz and Gb’spz2 was decreased (Fig. 8E) in the plasmatocytes-depleted regenerating legs. Thus, we added the assumption as “or Gb’Toll2-2 affects localisation of the ligands expressing cells during Gryllus leg regeneration.” in lines 270-271.

Line 384-385: “The cricket genome lacks a Persephone homologue (data not shown)”. Either show the data or remove the claim.
Response: We analysed the amino acid sequences homologies of serine proteinases between Gryllus and Drosophila and showed a phylogenetic tree in Fig. S11. We cited the Fig. S11 as “We could not find a persephone (psh) homologue in the Gryllus genome (Fig. S11).” in line 388-389.

Line 456-461:”…..Therefore, Toll signalling may interfere with Hippo signalling….”. In Drosophila, Toll-2 may function together with Yki to promote cell proliferation, rather than interfering with it (Li et al 2020 eLife).
Response: We appreciate your comment. We added the explanation about the functional difference of Toll-2 between Gryllus and Drosophila, as “although Drosophila Toll-2 cooperatively promotes cell proliferation with Yki (Li et al., 2020)” in lines 469-470.

Second decision letter

MS ID#: DEVELOP/2021/199916

MS TITLE: Toll signalling promotes blastema cell proliferation during cricket leg regeneration via insect macrophages

AUTHORS: Tetsuya Bando, Misa Okumura, Yuki Bando, Marou Hagiwara, Yoshimasa Hamada, Yoshiyasu Ishimaru, Taro Mito, Eri Kawaguchi, Takeshi Inoue, Kiyokazu Agata, Sumihare Noji, and Hideyo Ohuchi

You will be pleased to hear that the referees are happy with your revisions and there are just a few minor comments for you to consider before we proceed to publication. The referees' comments are appended below, or you can access them online: please go to BenchPress and click on the 'Manuscripts with Decisions' queue in the Author Area.

Reviewer 1

Advance summary and potential significance to field

The present manuscript investigates the regulation of blastema in regenerating cricket legs, finding that Toll-related signalling in macrophages promotes cell proliferation. It is a relevant finding, that sheds lights on a shared mechanism for the regulation of regeneration.

Comments for the author

The Authors have made great efforts in addressing all the reviewer's comments.
The RNAseq study is now clearly explained, and all the data is made available. I have one additional concern, regarding the RNAseq analysis, where there are no replicates and the differentially expressed genes are found by calculating the ratio between the two conditions. This approach is problematic for the lowly expressed genes, as the read counts might just be due to noise. For example, the gene isotig04104 has RPKM values of 0.17 and 0.59 (so fundamentally they are not expressed), but as the ratio gives 3.47, the gene would be considered as upregulated at 3 hpa. There is no "best" way of dealing with this issue, but some strategies can be applied. I recommend either setting a threshold for the analysis (e.g. considering only genes with RPKM >5), or adding a +1 to all RPKM values, before calculating the ratio (the ratio for isotig04104 would then become 1.35). Further log2-tranforming the ratio would help comparing the results. Hopefully, this correction would also generally help with the discrepancies that the Authors observe between RNAseq and qPCR results. Please note, this correction of low counts will not change the key findings of the present manuscript (I checked), but might affect the total numbers of up/down-regulated contigs.
Additional points:
- please explain how the number of annotated contigs was chosen (32 and 561 contigs).
- Please correct "closed" to "close" (lines 168 and 169).

Reviewer 2

Advance summary and potential significance to field

I believe the authors have done their best to satisfactorily address the majority of the comments raised by the reviewers. The paper will be interesting to the field.

Comments for the author

The majority of the textual/data layout concerns have been addressed. The experimental concerns that were addressable in a reasonable timeframe have been addressed.

Reviewer 3

Advance summary and potential significance to field

The involvement of Tolls in cell proliferation and regeneration is a very interesting and important finding.
Tolls were originally discovered for their functions in development, but are best known for their functions in innate immunity. This manuscript highlights the relevance of Tolls in a wide variety of cellular contexts and independently of infection.

Comments for the author

The authors have addressed all my previous criticisms. Thus, the manuscript is ready for publication in Development.

Second revision

Author response to reviewers' comments

Reviewer 1 Comments for the author...
The Authors have made great efforts in addressing all the reviewer's comments.
The RNAseq study is now clearly explained, and all the data is made available. I have one additional concern, regarding the RNAseq analysis, where there are no replicates and the differentially expressed genes are found by calculating the ratio between the two conditions. This approach is problematic for the lowly expressed genes, as the read counts might just be due to noise. For example, the gene isotig04104 has RPKM values of 0.17 and 0.59 (so fundamentally they are not expressed), but as the ratio gives 3.47, the gene would be considered as upregulated at 3 hpa. There is no "best" way of dealing with this issue, but some strategies can be applied. I recommend either setting a threshold for the analysis (e.g. considering only genes with RPKM > 5), or adding a +1 to all RPKM values, before calculating the ratio (the ratio for isotig04104 would then become 1.35). Further log2-tranforming the ratio would help comparing the results. Hopefully, this correction would also generally help with the discrepancies that the Authors observe between RNAseq and qPCR results. Please note, this correction of low counts will not change the key findings of the present manuscript (I checked), but might affect the total numbers of up/down-regulated contigs.

Response: We appreciate Reviewer #1's suggestions to our RNAseq analyses. In our RNAseq analysis, we analyzed only contigs that the read counts were more than 10, to exclude the lowly expressed genes. Following with your recommendation, we additionally excluded the contigs whose RPKM values were less than 5. The RPKM values of almost all contigs that the read counts were more than 10 were more than 5, therefore, annotated contigs were slightly reduced. We revised the number of analyzed contigs in Fig. 1A (from 561 to 549). GO terms annotated to the contigs were also slightly changed, thus we revised Fig. 1B and Fig. S2A. We revised the explanation about contig selections based on the read numbers and RPKM values in the Results section in lines 146-152.

Additional points:
- please explain how the number of annotated contigs was chosen (32 and 561 contigs).
Response: We have revised the explanation about annotated contigs in lines 146-152.

- Please correct "closed" to "close" (lines 168 and 169).
Response: Thank you so much for pointing out the typos. We have corrected these typos in lines 171 and 172.

Reviewer 2 Comments for the author...
The majority of the textual/data layout concerns have been addressed. The experimental concerns that were addressable in a reasonable timeframe have been addressed.
Response: We appreciate Reviewer 2's critical reading of the manuscript.

Reviewer 3 Comments for the author...
The authors have addressed all my previous criticisms. Thus, the manuscript is ready for publication in Development.
Response: We appreciate Reviewer 3's critical reading of the manuscript.

Third decision letter

MS ID#: DEVELOP/2021/199916

MS TITLE: Toll signalling promotes blastema cell proliferation during cricket leg regeneration via insect macrophages

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ARTICLE TYPE: Research Article

I am happy to tell you that your manuscript has been accepted for publication in Development, pending our standard ethics checks.