EMBO
reports

Manuscript EMBOR-2011-35121

Oxidative stress in the hematopoietic niche regulates the cellular immune response in Drosophila

Sergey A. Sinenko, Jiwon Shim and Utpal Banerjee

Corresponding author: Utpal Banerjee, University of California, Los Angeles

Review timeline:

Submission date:	07 June 2011
Editorial Decision:	28 June 2011
Revision received:	12 September 2011
Editorial Decision:	05 October 2011
Revision received:	12 October 2011
Accepted:	13 October 2011

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 28 June 2011

Thank you for the submission of your research manuscript to EMBO reports. We have now received the enclosed reports from the three referees that were asked to evaluate your study. As you will see, all the referees find the topic of interest and provide some suggestions for improvement that would render the study acceptable for publication in EMBO reports.

As you will see, the concerns of referees 1 and 2 are mainly of technical nature, aimed at strengthening your conclusions and rather straightforward to address. The analysis of lamellocyte differentiation in the presence of ROS scavengers, using a second method to measure ROS and providing more insight into the type of ROS and where it is generated would be important, as well as to analyze the plasmatocyte level and address other technical concerns of referee 2. Referee 3's concerns deal with the way in which EGFR signaling mediates the effects of ROS. To this end, it would be important to provide experimental data to address at which level ROS activates Spitz (as suggested in his/her point 1) and whether EGFR activation is occurring in the target tissue (CZ) or in the tissue where Spitz is activated (PSC). S/he has suggested several ways in which this can be done but not all of them have to be carried out, as long as the final results are conclusive. Given that addressing these issues relies on available tools and methodologies, and will strengthen the study, they should be addressed in a revised version.

© European Molecular Biology Organization

If the referee concerns can be adequately dealt with, we would be happy to accept your manuscript for publication. However, please note that it is EMBO reports policy to undergo one round of revision only and thus, acceptance of your study will depend on the outcome of the next, final round of peer-review.

I look forward to seeing a revised form of your manuscript when it is ready. In the meantime, do not hesitate to get in touch with me if I can be of any assistance.

Yours sincerely,

Editor
EMBO Reports

REFEREE REPORTS:

Referee #1:

This is a well written manuscript by Sinenko et al., 2011 (The oxidative status of a hematopoietic niche regulates cellular immune response in Drosophila) and is an important and novel study providing evidence that ROS are sensed in the hematopoietic niche with subsequent regulation of lamellocyte immune cells. This is based on data showing that in a Drosophila lymph gland model, the medullary zone (MZ; where hematopoietic cells reside and has moderate levels of ROS) populates the cortical zone (CZ) with differentiated cells (lamellocytes). The novel aspect is that this function is dependent on ROS levels within the posterior signalling center (PSC) based on studies which increased ROS artificially in the model using electron transport chain (ETC) inactivation (ND75 RNAi) or quenched ROS with Superoxide dismutase (SOD) overexpression. Finally, the production of ROS (during pathogen infection) causes the PSC to produce a cytokine signal that mediates the production of lamellocytes.

The authors interpret the data and suggest that metabolic dysfunction or parasitic infection induces an oxidatively stressed PSC that causes the activation of the PI3K pathway. What is not clear from experiments using wasp infection is how ROS are elevated or indeed what ROS are mediating this effect. In this 'parasitic' model, does inclusion of ROS scavengers (SOD/catalase) prevent lamellocyte differentiation as in the ND75 mutant? The clarity of the method used to measure ROS should be enhanced. Further given that ROS levels were assayed using a single probe it is prudent to measure ROS using a second assay system. This may provide information as to how ROS is mediating the effect or how ROS is produced. For example given that ROS can include superoxide, hydrogen peroxide (amongst others) is it superoxide defects mediating the phenotype or perhaps the more stable H_2O_2 which can diffuse between different cellular compartments. Could it be that the sensitivity/specificity of the probe cannot detect ROS in the PSC wt?

In summary, this is an interesting, novel and well written manuscript but ROS measurement and interpretation needs to be discussed/analysed further given that the conclusion is centred around these reactive molecules

Referee #2:

Sinenko et al describe a function for reactive oxygen species (ROS) in the development of blood cells in the fruitfly Drosophila melanogaster. More specifically, the authors show that genetic induction of ROS in the posterior signaling center (PSC), a part of the haematopoetic lymph glands that has been likened to haematopoetic niches in vertebrates leads to differentiation of lamellocytes, a specific type of blood cell in a non cell autonomous way. An increase in ROS in the same organ is also shown to occur upon infection with parasitic wasps. The pathway involved is further characterized and shown to include Akt1/Foxo and Spitz, a ligand for epidermal growth factor receptor. The results are an extension to previous work from the same group where ROS had been

identified as major regulators of blood cell development in general. Here the relevance of the PSC is more specifically addressed and ROS shown to act in a non cell autonomous way.

This is an important contribution the body of the manuscript focuses on the main aspects of the work and presents them clearly I have only a few questions and comments:

Regarding the induction of ROS it seems as if they were also induced in parts of the lymph gland other than the PSC (Fig 1 A and B and Fig. 2 C and D). While this is less surprising for the wasp infestation (Fig. 2), ROS induction in Fig. 1 using PSC-specific drivers is expected to be more restricted. Is this due to some expression of the driver outside the PSC (perhaps unlikely since there is no green signal outside the PSC) or could it be that secondarily, ROS are also induced outside the PSC, relayed by cells or cytonemes? (see also comment below on the dot driver)

In Fig. S3, the point is made that JAK/STAT and TNF pathways don't play a role in lamellocyte differentiation via ROS. Here I wonder how well-documented the knockdowns are in this and/or previous work.

Page 3 Line 9 "in the absence of PSC": we wonder whether it would be more appropriate to say "in the absence of collier expression in the PSC."

Page 4 line 7. To our knowledge, the expression of dot-gal4 driver maybe not exclusively PSC restricted. It may be expressed in the primary and the secondary lobes; however its expression is much lower than in the PSC, therefore it could have some cell autonomous effects on blood cell differentiation. Maybe the authors have some comments on this.

Page 4: bottom the argument is made that that oxidative stress elicits a specific response and the similarity in crystal cell counts are give as an example. This point would be much strengthened if plasmatocytes had been included in the comparison.

Page 5 line 6. "Apoptosis induced in the PSC does not affect the lamellocyte differentiation" maybe this should be expressed more specifically such as by saying: "apoptosis in the PSC alone did not induce lamellocyte proliferation."

Page 5 the section between the lines 7-11 we wonder whether this sentence would not fit better at the end of the previous paragraph (bottom of page 4).

Bottom of page 6: the last sentence on that page did not make sense to me (formatting problem perhaps?)

p-7 line 11: I would not talk about melanotic tumors not even "pseudotumors" since in this case they are likely all melanotic capsules as a genuine response towards parasitic eggs (similarly in the figure legend).

Page 7 line 20 the authors refer to "a cytokine" we suggest to name Spitz again in brackets for clarity.

Methods section:
Page 9 Line 10, instead of 30ml 30µl
What fixative was used?
For ROS staining the authors refer to Owushu-Ansah&Banerjee 2009, but wasn't the actual protocol published in the 2008 paper

Finally I wonder whether the authors would have any comments whether polycomb group genes might also be involved in the PCS to promote lamellocyte differentiation.

Referee #3:

The paper by Sinenko et al. examines the role of pathogen-induced oxidative stress, in triggering

© European Molecular Biology Organization

lamellocyte differentiation, to initiate an innate immune response in the Drosophila larva. The beauty of this system is that it is possible to induce the oxidative stress specifically within each of the three subpopulations of the lymph gland, and pinpoint the ROS response to the PSC region, which subsequently induces lamellocyte differentiation in the CZ. They have shown that the response induced by wasp infestation is mediated by the same mechanism, and that the signal transmitted between the activated PSC and the CZ is the EGFR ligand Spitz.
The work is compelling in that it identifies in a context of the whole organism, the detailed molecular responses to pathogen infestation, and maps them to discrete tissues. In that perspective it is novel and of broad interest, and certainly belongs to the caliber of papers that should appear in EMBO Reports.
I found the paper lacking, however, in the analysis of the effects of ROS (in the PSC) on the induction of the EGFR ligand Spitz, and subsequent triggering of EGFR (presumably in the CZ). This is a very central point for the paper, and should be characterized mechanistically in more detail. The following experiments would significantly advance the paper:
1. Since the Spitz precursor is usually broadly expressed, it is probably the intra-membrane protease Rhomboid which is induced by ROS, to facilitate Spitz processing. Recombineered lines where GFP has been fused to the endogenous Rho1 and Rho3 loci are available (Yogev et al., 2010), and can be used to demonstrate induction of Rhomboid expression following ROS. In the future (certainly beyond the scope of this paper), identification of the regulatory elements within the Rhomboid promoter that respond to ROS would be important.
2. Spitz is known to require the chaperone Star (which was in fact originally cloned by the Banerjee lab) for its processing. Expression of RNAi for Star in the PSC should give rise to effects similar to Spitz RNAi.
3. Do we know if Spitz induces lamellocyte differentiation in the CZ directly, or may operate in the PSC through a relay pathway? Staining for dpERK in the CZ following ROS would be informative.
4. In addition, expression of an EGFR dominant-negative construct in the CZ following ROS, should demonstrate loss of both lamellocyte induction and of dpERK induction. Alternatively, the same effect may be achieved by expression of EGFR RNAi specifically in the CZ (but not in the PSC).
5. Conversely, expression of activated EGFR or activated Ras in the CZ and monitoring its effects on lamellocyte induction in the absence of ROS is essential, in order to know if ROS activates a linear pathway through Spitz, or if additional effectors of ROS function in parallel to Spitz. The reported experiment with secreted Spitz expressed in the PSC indicates that a linear pathway may indeed be the case. However, since secreted Spitz works over a limited range because of its retention in the ER, expression of activated downstream constructs specifically in the CZ may provide a more dramatic effect, and again rule out the option of a relay signal functioning as a mediator between Spitz/EGFR in the PSC, and lamellocyte induction in the CZ.

1st Revision - authors' response — 12 September 2011

Reviewer 1

<Description>
This is a well written manuscript by Sinenko et al., 2011 (The oxidative status of a hematopoietic niche regulates cellular immune response in Drosophila) and is an important and novel study providing evidence that ROS are sensed in the hematopoietic niche with subsequent regulation of lamellocyte immune cells. This is based on data showing that in a Drosophila lymph gland model, the medullary zone (MZ; where hematopoietic cells reside and has moderate levels of ROS) populates the cortical zone (CZ) with differentiated cells (lamellocytes). The novel aspect is that this function is dependent on ROS levels within the posterior signalling center (PSC) based on studies which increased ROS artificially in the model using electron transport chain (ETC) inactivation (ND75 RNAi) or quenched ROS with Superoxide dismutase (SOD) overexpression. Finally, the production of ROS (during pathogen infection) causes the PSC to produce a cytokine signal that mediates the production of lamellocytes.
…

© European Molecular Biology Organization

In summary, this is an interesting, novel and well written manuscript but ROS measurement and interpretation needs to be discussed/analysed further given that the conclusion is centred around these reactive molecules.

<Critique>
1. How ROS are elevated or indeed what ROS are mediating this effect. In this 'parasitic' model, does inclusion of ROS scavengers (SOD/catalase) prevent lamellocyte differentiation as in the ND75 mutant?

As suggested by the reviewer, we have now clearly shown that expression in the PSC of either SOD2 or catalase, which scavenge superoxide and hydrogen peroxide respectively (Finkel & Holbrook, 2000), but not GTPx, which causes reduction of thioredoxin mediated effects (Missirlis et al, 2003), can reduce the number of lamellocytes as well as their maturation upon wasp parasitization (Supplementary Figure 4C). Moreover, these animals exhibit reduced melanotic capsule formation as seen with expression of *Spitz* RNAi in the PSC (Figure 2J, M). Thus, specific types of ROS including superoxide and hydrogen peroxide mediate this effect. We have now said this on page 7.

Exactly how ROS is elevated upon parasitization needs further investigation and is not fully understood in any host-pathogen system. However, our work is consistent with previous studies (Arsenijevic et al, 2000) in mammals that have shown that mitochondrial ROS can trigger systemic signals that reinforce innate immune responses (discussed on page 9).

2. The clarity of the method used to measure ROS should be enhanced. Further given that ROS levels were assayed using a single probe it is prudent to measure ROS using a second assay system. This may provide information as to how ROS is mediating the effect or how ROS is produced. For example given that ROS can include superoxide, hydrogen peroxide (amongst others) is it superoxide defects mediating the phenotype or perhaps the more stable H2O2 which can diffuse between different cellular compartments. Could it be that the sensitivity/specificity of the probe cannot detect ROS in the PSC wt? In summary, this is an interesting, novel and well written manuscript but ROS measurement and interpretation needs to be discussed/analysed further given that the conclusion is centred around these reactive molecules.

Following the reviewer's suggestion, we measured H_2O_2 levels using the Redoxsenser Red (Molecular probe, R14060) dye. Wild type PSC does not express Redoxsensor Red (Supplementary Figure 4A-A'). However, subset of PSC cells express Redoxsensor Red upon wasp infestation (Supplementary Figure 4B-B') but at a lower level compared to that seen by DHE staining to detect superoxide. We conclude that superoxide is the major ROS that is elevated upon infestaion, although it can convert to low levels of hydrogen peroxide (now discussed on page 7). The PSC and MZ cells are in the same tissue and not separated by any barriers although they express different developmental genes. There is no obvious reason that we can think of why two neighboring set of cells should behave differently upon exposure to DHE dye or redoxsensor dyes. It is of course very reasonable that the PSC also has some ROS level, just not detectable in comparison with the MZ. We now say this on page 3.

Reviewer 2

<Description>
Sinenko et al describe a function for reactive oxygen species (ROS) in the development of blood cells in the fruitfly Drosophila melanogaster. More specifically, the authors show that genetic induction of ROS in the posterior signaling center (PSC), a part of the haematopoetic lymph glands that has been likened to haematopoetic niches in vertebrates leads to differentiation of lamellocytes, a specific type of blood cell in a non cell autonomous way. An increase in ROS in the same organ is also shown to occur upon infection with parasitic wasps. The pathway involved is further characterized and shown to include Akt1/Foxo and Spitz, a ligand for epidermal growth factor receptor. The results are an extension to previous work from the same group where ROS had been identified as major regulators of blood cell development in general. Here the relevance of the PSC is more specifically addressed and ROS shown to act in a non cell autonomous way.

This is an important contribution the body of the manuscript focuses on the main aspects of the work

and presents them clearly I have only a few questions and comments:

<Critique>
1. Regarding the induction of ROS it seems as if they were also induced in parts of the lymph gland other than the PSC (Fig 1 A and B and Fig. 2 C and D). While this is less surprising for the wasp infestation (Fig. 2), ROS induction in Fig. 1 using PSC-specific drivers is expected to be more restricted. Is this due to some expression of the driver outside the PSC (perhaps unlikely since there is no green signal outside the PSC) or could it be that secondarily, ROS are also induced outside the PSC, relayed by cells or cytonemes? (see also comment below on the dot driver)

We apologize if this was not properly explained in the manuscript. This is now made clearer in the text and legend. Indeed, as the reviewer points out, the wild type lymph gland, without wasp infection has regions of high ROS. We have published this work (Owusu-Ansah & Banerjee, 2009) and shown that the progenitor population of the medullary zone (MZ) utilizes ROS as a developmental signal. Importantly for this paper however, without wasp infection, the ROS level in the PSC is low compared to the MZ cells (Fig. 1A-B' and Fig 2C-D', now made clear on page 3 and 4). ND75RNAi expression or wasp infestation induces ROS in the PSC independent of the medullary zone ROS.

2. In Fig. S3, the point is made that JAK/STAT and TNF pathways don't play a role in lamellocyte differentiation via ROS. Here I wonder how well-documented the knockdowns are in this and/or previous work.

We now discuss this in some more detail on page 6. We have added additional data that show that ROS is upstream of Spi activation (Supplementary Figure 4D-F'). The experiments that suggest that JAK/STAT and JNK are not involved downstream of ROS in this process are similar in nature to those that provide the data that to that Spi is involved. However, we are not claiming that these other pathways are not involved in lamellocyte differentiation in different contexts (for example, Makki et al, 2010) nor are we saying that ROS function is limited to this one context (for example, (Owusu-Ansah & Banerjee, 2009). However, as far as the PSC is concerned, the receptor for JAK/STAT (*Domeless*) is not expressed there (Makki et al, 2010), loss of ligands *upd1-3* does not give the phenotype (Supplementary figure 3A) that is seen with loss of *Spi*. Loss of *eiger* does not give the phenotype (Supplementary figure 3A) seen with loss of Spi. Overexpression of these ligands in the PSC will not cause lamellocyte formation in wild-type as overexpression of Spi does (Figure 2B). And downstream target genes such as Tep4 (Krzemien et al, 2007) are not expressed in the PSC. We have now discussed this more clearly in the text (page 6).

3. Page 3 Line 9 "in the absence of PSC": we wonder whether it would be more appropriate to say "in the absence of collier expression in the PSC."

Thank you. We changed this phrase to "in the absence of *collier* expression in the PSC".

4. Page 4 line 7. To our knowledge, the expression of dot-gal4 driver maybe not exclusively PSC restricted. It may be expressed in the primary and the secondary lobes; however its expression is much lower than in the PSC, therefore it could have some cell autonomous effects on blood cell differentiation. Maybe the authors have some comments on this.

The reviewer is of course correct. Our primary driver for this purpose is *Antp-Gal4* which is not expressed anywhere in the lymph gland except in the PSC. However, as *Antp* is expressed in tissues outside of the lymph gland, we wanted to use a second driver that is largely in the blood system and expressed highly in the PSC. There is no driver that is fully blood specific and only in the PSC, so we are using the two together to make our argument. We say this now on page 4.

5. Page 4: bottom the argument is made that that oxidative stress elicits a specific response and the similarity in crystal cell counts are give as an example. This point would be much strengthened if plasmatocytes had been included in the comparison.

This is a good point. Following the reviewer's suggestion, we have now counted the total

© European Molecular Biology Organization

number of hemocytes (which are largely plasmatocytes), in the *HHLT-Gal4 UAS-ND75RNAi* background. We detect no change in hemocyte count compared with wild type (now presented as Supplementary Figure 1D). This was a valuable suggestion, and it strengthens our conclusion that ROS specifically elicits a lamellocyte-specific response.

6. Page 5 line 6. "Apoptosis induced in the PSC does not affect the lamellocyte differentiation" maybe this should be expressed more specifically such as by saying: "apoptosis in the PSC alone did not induce lamellocyte proliferation."

We have changed the line as suggested.

7. Page 5 the section between the lines 7-11 we wonder whether this sentence would not fit better at the end of the previous paragraph (bottom of page 4).

We have moved the lines to the previous paragraph.

8. Bottom of page 6: the last sentence on that page did not make sense to me (formatting problem perhaps?)

We removed this sentence.

9. p-7 line 11: I would not talk about melanotic tumors not even "pseudotumors" since in this case they are likely all melanotic capsules as a genuine response towards parasitic eggs (similarly in the figure legend).

We changed "melanotic tumors" to "melanotic capsules".

10. Page 7 line 20 the authors refer to "a cytokine" we suggest to name Spitz again in brackets for clarity.

We put Spitz in brackets for clarity as suggested.

Methods section:
11. Page 9 Line 10, instead of 30ml 30µl
What fixative was used?
For ROS staining the authors refer to Owushu-Ansah&Banerjee 2009, but wasn't the actual protocol published in the 2008 paper

We have used 4% formaldehyde as a fixative (indicated in text page 11). Detailed description of ROS staining protocol is published in *Nature Protocol Exchange*, 2008 (Owusu-Ansah et al., doi:10.1038/nprot.2008.23, Protocol Exchange 2008) as a companion to the *Nature* manuscript. It does not show up on pubmed independently of the 2009 paper except as a doi.

12. Finally I wonder whether the authors would have any comments whether polycomb group genes might also be involved in the PCS to promote lamellocyte differentiation.

We have not looked into this. This is a good suggestion for future studies but does not fit into the results here. The PSC, of course is not differentiating but sending out a signal to the precursors to differentiate. It is possible that this signal that leads to lamellocyte formation functions through Polycomb. Indeed it is also possible that the ROS signal in the PSC is interpreted through Polycomb as the reviewer implies, but we have no particular reason to believe this is the case. It is a great idea, though, worth following up for the future.

Reviewer 3

<Description>
The paper by Sinenko et al. examines the role of pathogen-induced oxidative stress, in triggering lamellocyte differentiation, to initiate an innate immune response in the Drosophila larva. The beauty of this system is that it is possible to induce the oxidative stress specifically within each of the

three subpopulations of the lymph gland, and pinpoint the ROS response to the PSC region, which subsequently induces lamellocyte differentiation in the CZ. They have shown that the response induced by wasp infestation is mediated by the same mechanism, and that the signal transmitted between the activated PSC and the CZ is the EGFR ligand Spitz.
The work is compelling in that it identifies in a context of the whole organism, the detailed molecular responses to pathogen infestation, and maps them to discrete tissues. In that perspective it is novel and of broad interest, and certainly belongs to the caliber of papers that should appear in EMBO Reports.
I found the paper lacking, however, in the analysis of the effects of ROS (in the PSC) on the induction of the EGFR ligand Spitz, and subsequent triggering of EGFR (presumably in the CZ). This is a very central point for the paper, and should be characterized mechanistically in more detail. The following experiments would significantly advance the paper:

<Critique>
1. Since the Spitz precursor is usually broadly expressed, it is probably the intra-membrane protease Rhomboid which is induced by ROS, to facilitate Spitz processing. Recombineered lines where GFP has been fused to the endogenous Rho1 and Rho3 loci are available (Yogev et al., 2010), and can be used to demonstrate induction of Rhomboid expression following ROS. In the future (certainly beyond the scope of this paper), identification of the regulatory elements within the Rhomboid promoter that respond to ROS would be important.

This was a very fine suggestion. We did this experiment and found that Rhomboid 1 is up-regulated specifically in the PSC upon wasp infestation, whereas wild-type PSC does not express detectable Rhomboid 1 (Figure 2F-G', and text on page 7-8). This result strongly supports our model that Spitz is secreted from the PSC upon wasp infestation. How ROS might activate Rhomboid expression will need to be followed up in the future.
Within 1.5 kb of Rho1, binding sites for EcR, GATA1, AP1 and CREB are found, all of which could directly or indirectly be affected by ROS activation. But figuring this logic out will take a lot of analysis.

2. Spitz is known to require the chaperone Star (which was in fact originally cloned by the Banerjee lab) for its processing. Expression of RNAi for Star in the PSC should give rise to effects similar to Spitz RNAi.

To our pleasant surprize, we found that a single copy loss of Star in either a *$Star^{1}$*/+ or *$Star^{X}$*/+ genetic background gives significant suppression of lamellocytes formation upon wasp infestation (Figure 2E, and text on page 7-8), and inhibits melanotic capsule formation in a manner similar to that seen upon Spitz inactivation (Figure 2K, N, and text on page 8). These results are consistent with, and add to, our model.

3. Do we know if Spitz induces lamellocyte differentiation in the CZ directly, or may operate in the PSC through a relay pathway? Staining for dpERK in the CZ following ROS would be informative.

To further define whether Spitz induces lamellocyte differentiation directly in the circulating hemocytes (where we have presented most of the analysis, not in the CZ), we analyzed expressions of dpERK in the presence or absence of ROS induction. In normal growth conditions, hemocytes express basal levels of dpERK, however, in agreement with release of Spitz, parasitizing animals significantly increases dpERK levels in the hemocytes (Figure 3B,D). Similarly, ROS induction in the PSC by inactivation of ND75 causes phosphorylation of ERK in the hemocytes (Figure 3C). These results strongly suggest that ROS induction in the PSC activates the EGFR/ERK pathway in the hemocytes (we describe this on page 8). A similar effect is seen in the CZ (data not shown).

4. In addition, expression of an EGFR dominant-negative construct in the CZ following ROS, should demonstrate loss of both lamellocyte induction and of dpERK induction. Alternatively, the

© European Molecular Biology Organization

same effect may be achieved by expression of EGFR RNAi specifically in the CZ (but not in the PSC).

We are grateful to the reviewer for pointing this out. We have further examined involvement of EGFR followed by ROS/Spitz induction.
We performed extensive genetic analysis of EGFR with the use of blood-specific drivers to show that blood system indeed requires EGFR in lamellocyte formation. Expression of dominant-negative EGFR ($EGFR^{DN}$) in all the blood system including the lymph gland and circulating hemocytes (using *HHLT-Gal4 UAS-$EGFR^{DN}$*) leads to dramatic suppression of lamellocyte formation (Figure 3A and text on page 8). This phenotype is virtually identical to Spitz RNAi driven by *Antp-Gal4*, indicating that Spitz secreted from the PSC acts through EGFR in the blood system. Please note, however, that most of our analysis in this paper is regarding circulating hemocytes (and not CZ) and so that is where we report most of these data for consistency with the rest of the manuscript.
As *HHLT-Gal4* is pan-hemocyte driver, we utilize compartment specific drivers to further prove requirement of EGFR in the CZ or in circulating hemocytes. The suppression of EGFR specifically in both CZ and circulating hemocytes (using lineage-traced *Hemloectin-Gal4*) prevents *Hml^+* cells from becoming lamellocytes upon wasp infestation (Supplementary Figure 4G, and text on page 8). This is consistent with our data. Additionally, as an internal control, we have found that a small subset of lamellocytes do not express Hml in the wild-type background. Consequently these do not express EGFR-DN and these lamellocytes are still present when *Hml* is used as a driver. All L1 positive cells are missing when HHLT (which is expressed in all hemocytes) is used as a driver. These results greatly enhance the quality of the presented data and we thank the reviewer for suggesting the experiments.

5. Conversely, expression of activated EGFR or activated Ras in the CZ and monitoring its effects on lamellocyte induction in the absence of ROS is essential, in order to know if ROS activates a linear pathway through Spitz, or if additional effectors of ROS function in parallel to Spitz. The reported experiment with secreted Spitz expressed in the PSC indicates that a linear pathway may indeed be the case. However, since secreted Spitz works over a limited range because of its retention in the ER, expression of activated downstream constructs specifically in the CZ may provide a more dramatic effect, and again rule out the option of a relay signal functioning as a mediator between Spitz/EGFR in the PSC, and lamellocyte induction in the CZ.

We have now done this experiment, and expressed activated EGFR by using lineage-traced *Hemolectin-Gal4*, and show robust induction of lamellocytes in the hemocytes (Figure 3A). This is consistent with the notion that secreted Spitz directly activates lamellocyte differentiation in the hemocyte. It should be noted (and we say this on page 8) that the fact that activated Ras increases total number of hemocytes, including lamellocytes, was reported by Dearolf's group several years ago (Asha et al, 2003).

Reference

Arsenijevic D, Onuma H, Pecqueur C, Raimbault S, Manning BS, Miroux B, Couplan E, Alves-Guerra MC, Goubern M, Surwit R, Bouillaud F, Richard D, Collins S, Ricquier D (2000) Disruption of the uncoupling protein-2 gene in mice reveals a role in immunity and reactive oxygen species production. Nat Genet **26:** 435-439

Asha H, Nagy I, Kovacs G, Stetson D, Ando I, Dearolf CR (2003) Analysis of Ras-induced overproliferation in Drosophila hemocytes. Genetics **163:** 203-215
Finkel T, Holbrook NJ (2000) Oxidants, oxidative stress and the biology of ageing. Nature **408:** 239-247
Krzemien J, Dubois L, Makki R, Meister M, Vincent A, Crozatier M (2007) Control of blood cell homeostasis in Drosophila larvae by the posterior signalling centre. Nature **446:** 325-328

Makki R, Meister M, Pennetier D, Ubeda JM, Braun A, Daburon V, Krzemien J, Bourbon HM, Zhou R, Vincent A, Crozatier M (2010) A short receptor downregulates JAK/STAT signalling to control the Drosophila cellular immune response. PLoS Biol **8:** e1000441

© European Molecular Biology Organization

Missirlis F, Rahlfs S, Dimopoulos N, Bauer H, Becker K, Hilliker A, Phillips JP, Jackle H (2003) A putative glutathione peroxidase of Drosophila encodes a thioredoxin peroxidase that provides resistance against oxidative stress but fails to complement a lack of catalase activity. Biol Chem **384:** 463-472

Owusu-Ansah E, Yavari A, Banerjee U (2008) A protocol for in vivo detection of reactive oxygen species. *Nat Protocols* **doi::** 10.1038/nprot.2008.1023

Owusu-Ansah E, Banerjee U (2009) Reactive oxygen species prime Drosophila haematopoietic progenitors for differentiation. Nature **461:** 537-541

2nd Editorial Decision 05 October 2011

Thank you for your patience while we have reviewed your revised manuscript. As you will see from the reports below, the referees are now all positive about its publication in EMBO reports. I am therefore happy to write an 'accept in principle' decision, which means that we will accept your manuscript for publication once a few minor issues/corrections have been addressed, as follows.

In going through the file in detail prior to acceptance, I have noted several problems with the description of the statistics performed in most of the figures. Please go through your manuscript carefully once more and ensure that all relevant figures and supplementary figures have been generated according to proper statistical analysis procedures (for guidance, please refer to for guidance: Cumming et al. JCB 2007) and all figure legends legends include information on the number of independent experiments measured, the type of error bars used and statistical test applied to the data.

In addition, I have noted that -at over 38,000 characters- your manuscript text is considerably longer than our 28,000 characters maximum. As you have only three main figures, I think we can be flexible in this case. However, please go through the text once more and try to more succinctly convey the same information wherever possible. It would be ideal to decrease the length by 2,000-4,000 characters if doable.

Once all these remaining issues have been attended to, you will receive an official decision letter from the journal accepting your manuscript for publication in the next available issue of EMBO reports. This letter will also include details of the further steps you need to take for the prompt publication of your study.

Thank you for your contribution to EMBO reports!

Yours sincerely,

Editor
EMBO Reports

REFEREE REPORTS:

Referee #1:

The authors have addressed the previous concerns adequately.

© European Molecular Biology Organization

Referee #2:

Sorry about the late response, we were quite positive about the first draft and all the points we raised have been adequately addressed. Due to time constraints I am not able to review in detail all comments raised by the other reviewers but from a first glance they appear to have been addressed as well, in some case adding even further to the quality of the manuscript.

Referee #3:

I was highly impressed by the amount of additional work that was put in, which significantly improved the paper, its claims and rigorousness. It was also rewarding to see that the experiments I suggested worked out according to the model. I recommend publication of the paper in its current form.

2nd Revision - authors' response 12 October 2011

Thank you for your help with our manuscript submitted to EMBO Reports and for your suggestions about changes to the text. Please find attached a revised version of our manuscript entitled, "Oxidative stress in the hematopoietic niche regulates the cellular immune response in *Drosophila*". We incorporated the suggested changes in the title and abstract. The length of the paper is now significantly decreased to 32,760 characters with only three figures. We have eliminated over 5000 characters from the main text as per your suggestion. We have also included the number of samples used and the description of the error bars in the figure legends and methods.
We hope that with these corrections made, this manuscript is now ready for publication in EMBO reports. Once again, I really appreciate your help through the review process.

3rd Editorial Decision 13 October 2011

I am very pleased to accept your manuscript for publication in the next available issue of EMBO reports.

Thank you for your contribution to EMBO reports and congratulations on a successful publication. Please consider us again in the future for your most exciting work.

Yours sincerely,

Editorial Staff
EMBO Reports

© European Molecular Biology Organization