THE EMBO JOURNAL

Manuscript EMBO-2010-75989

The mitochondrial fission factor Drp1 modulates T Cell Receptor signaling at the immune synapse

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Review timeline:

Submission date:	13 September 2010
Editorial Decision:	06 October 2010
Revision received:	20 December 2010
Additional Correspondence:	10 January 2011
Accepted:	12 January 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 06 October 2010

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 three referees find the analysis interesting, insightful and support publication the EMBO Journal, pending satisfactory revisions. Revisions are needed and in particular referees #2 and 3 raise a number of issues that have to be resolved in order for further consideration here. Should you be able to address the concerns raised then we would be happy to consider a revised version. I should remind you that it is EMBO Journal policy to allow a single major round of revision and that it is therefore important to address the raised 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 initiative, please visit our website: http://www.nature.com/emboj/about/process.html

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

Yours sincerely,

Editor
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REFEREE REPORTS

Referee #1 (Remarks to the Author):

The authors present data that suggests the recent studies on mitochondria in the immunological synapse and the role of myosin IIA in immunological synapse formation are related. It has been shown that mitochondrial localization to the immunological synapse plays an important role in buffering cytoplasmic Calcium ion increases. Another study showed that myosin IIA is required for immunological synapse formation and normal signaling. This study confirms that mitochondria are localized at the immunological synapse and that this requires activity to Drp1. A novel observation is that mitochondrial localation to the immunological synapse also influences myosin IIA activation and movement of TCR microclusters to form an immunological synapse. This energetics component emphasizes the need to have a very local source of ATP since lymphocytes are small cells and its unexpected that displacement of mitochondria by a few microns impairs function of the system. The mitochondria are concentrated directly in the region in which actin flow generates the pSMAC structure.
The title might include a reference to mitochondia.

Referee #2 (Remarks to the Author):

The immunological synapse (IS) is a large junctional structure formed between a T cell and an antigen-presenting cell (APC) consisting of a central cluster of T cell receptors (cSMAC) surrounded by a ring of adhesion and signaling molecules (pSMAC), and plays an essential role in controlling T cell activation. In early step during T cell activation, dynamic reorientation of MTOC and Golgi apparatus towards nascent IS occurs. It has also been reported that mitochondria translocate towards nascent IS to become an essential component of the signaling complex.

Baixauli et al. demonstrated in this manuscript that mitochondrial fission GTPase Drp1 plays an important role in mitochondrial translocation toward IS; Drp1 knockdown inhibited the mitochondrial translocation, which led to decreased $\Delta\Psi$ and ATP production to compromise ATP-dependent myosin activation, and overall affected cSMAC assembly and T cell signaling.

The authors' finding of the involvement of Drp1 in the mitochondrial translocation toward IS is very interesting (Figures 1-3). However, they did not address most important issues how Drp1 is involved in the translocation and whether mitochondrial division activity of Drp1 is essential for the function. All other data (Figures 4-6) are the overall phenotype of compromised mitochondrial translocation expected from previous reports.

Comments:
1. Figure 1 & 2: mitochondrial morphology and localization changes in the activated T cells should be analyzed by EM.
2. Figure 2C and D: fractionation data for minus SEE or minus HA control cells should be provided to show that mitochondrial recruitment of Drp1 is primed indeed by T cell activation.
3. Figure 2F: endogenous Drp1 signal seems to be too high. Why?
4. Figure 2: the effect of Drp1 re-expression should be analyzed to rule out off-target effects of siRNAs.
5. Figure 4A: this figure does not mean anything in the absence of control experiments.
6. Figure 5: this reviewer do not understand the logic why these marginal effects of Drp1 knockdown on ATP and $\Delta\Psi$ compromised ATP-dependent myosin activation, and why this marginal inhibition of myosin phosphorylation affected cSMAC assembly. The authors should analyze myosin (or F-actin) morphology changes by microscopy in Drp1 knockdown cells.
7. The authors should analyze the effect of dominant-negative Drp1 mutants and Fis1-knockdown on the mitochondrial translocation. The effect of Drp1 GTPase inhibitor mdivi-1 on the transport would be informative.

Referee #3 (Remarks to the Author):

The present paper shows that down regulation of dynamin-related protein 1 (DRP1), which has previously been shown to regulate mitochondrial fusion/fisson, interferes with several aspects that

are important for T cell activation: These include mitochondrial positioning and IS formation but also ATP production and mitochondrial membrane potential. The authors use mostly imaging technology to understand DRP1 function in T cells but apply also biochemistry and FACS analysis. Up to now, DRP1 has not been reported to modulate mitochondrial positioning or IS formation. In fact, the molecular mechanism of mitochondrial relocalization to the IS is not understood very well at present. The novel results about DRP1 function presented here allow a better understanding of T cell polarization and IS formation. However, there are several major problems that need to be addressed:

1. The two most important findings of the paper are in my opinion that mitochondrial positioning relative to the IS is changed by DRP1 down regulation and that IS formation is impaired (reduction of CD3 assembling at the cSMAC, yet proximal signaling at the IS is enhanced!). Mitochondrial function is also impaired (i.e. ATP production is reduced). It is not clear to me what is cause here and what is effect. Are mitochondria not localized to the IS in DRP1 knock-down cells because CD3 assembling is reduced or is CD3 assembling reduced because for instance mitochondrial ATP production is impaired or another signaling parameter is changed? Alternatively, are there several different targets for DRP1 which cause parallel but unrelated problems during T cell polarization? These are important questions and need to be addressed more rigorously.

2. It is not clear to me how the reduced CD3 assembly at the IS is related to an enhanced proximal TCR signaling. The authors speculate about this finding. One problem that I see is that while CD3 assembly at the IS is analyzed by target cell activation on short time scales (20 minutes etc.), some of the functional assays (i.e. calcium measurements) are carried out following anti-CD3 antibody activation (antibodies are applied in solution) or are analyzed on long time scales (like the IL-2 assay):

2a. The IL-2 assay was performed with T cells activated following IS formation (with SEE-pulsed Raji), however IL-2 was measured after 16 hours while most other assays (mitochondrial positioning, IS formation, calcium measurements) presented in the paper are more short term (20 minutes etc.). It is difficult to analyze IL-2 secretion at very short (30-120 minutes) time scales but it is possible. It would strengthen the ms if the T-cell polarization data and IL-2 production could be measured on similar time scales.

2b. It has been shown in the past that calcium signals but also other T-cell activation parameters differ greatly when T cells are stimulated through an IS compared to antibodies in solution. For instance, almost all cells show calcium increases following IS formation but many (solution) antibody-treated cells only show calcium signals after long delays and others never show calcium signals depending on the antibodies used. It would be important to carry out the calcium experiments in SEE-pulsed Raji stimulated T cells (or at least following IS formation). This is of course not possible with FACS analysis but can be done with imaging technology (which is available to the authors). Such experiments would allow a much better qualitative and quantitative statement about proximal TCR signaling following T cell polarization. (It would also be great but potentially very difficult to interfere with CD3 assembly but not change mitochondrial localization and analyze proximal TCR signaling under these conditions).

3. Loading conditions are not well described for the calcium experiments. The authors use a rather high Indo-1 concentration for loading but other details like temperature, loading time, etc. are not mentioned. These details are important to judge whether or not Indo-1 already significantly buffers cytosolic calcium or not. Usually 1 ug/ml of calcium dye loaded for not more than 30 minutes at room temperature is more than enough to measure cytosolic calcium with a very good signal to noise ratio in the cytosol.

4. In many cases a more quantitative statistical analysis is required. This is for instance the case in Fig. 1 (how much mitochondria fluorescence is close to the IS in how many cells? Statistical significance?) but also in other Figures: Fig.2, ...

5. Maybe I have overlooked it: Did the authors show or analyze, how DRP1 down-regulation changed the fusion/fission state of mitochondria? Wouldn't one expect that mitochondria are more fused under these conditions?

6. Why are calcium signals and IL-2 secretion changed in the DRP1 knock down cells: because mitochondria are mis-localized or because CD3 assembly is impaired?

minor points:

7. Quantification of the blots in Fig. 2 C, D would be required.

8. Fig. 2G, y axis: please define this axis in the figure legend. It is explained in the methods but not easy to understand.

1st Revision - authors' response 20 December 2010

Point by Point Reply

Reviewer 1
We very much appreciate the reviewer's positive comments. As requested, we have revised the title to directly mention mitochondria. The new title is: "*The mitochondrial fission factor dynamin-related protein 1 modulates T cell receptor signaling at the immune synapse*".

Reviewer 2

We appreciate the constructive comments of the reviewer and agree that our original manuscript did not fully address how Drp1 drives mitochondrial translocation. As suggested by the reviewer, we have investigated whether the mitochondrial division activity of Drp1 is essential for mitochondrial translocation. We used two approaches: i) Transmission electron microscopy-based morphometric analysis of mitochondria in Drp1-inactivated or control T cells forming superantigen-specific conjugates with APCs; ii) Confocal microscopy-based analysis of mitochondria localization upon uncoupling from Drp1. In these experiments, T cells were either transfected with the mitochondria binding-deficient Drp1 mutant S637D or treated with the Drp1 GTPase inhibitor mdivi-1; both of these experimental tools increase mitochondrial fusion by inhibiting fission (Chang and Blackstone, 2010; Cerenghetti et al., 2008; and Cassidy-Stone et al., 2008; Tanaka and Joule, 2008). Using these approaches, we found that inhibition of Drp1 docking at the mitochondrial outer membrane disturbs mitochondrial translocation toward the T cell IS and that mitochondrial de-localization in mdivi-1-treated cells was concomitant with enlargement of mitochondrial area and perimeter. These new results strongly suggest that Drp1 binding to mitochondria and mitochondrial fission are essential for redistribution of mitochondria toward the IS. These new data are presented in the revised manuscript in the new Figure 3, and under Results, page 8 .

Response to comments

1.- We have performed localization studies and morphometric analyses of mitochondria in superantigen-specific conjugates formed between APCs and mdivi-1-treated or control T cells. The results indicate that mitochondria in mdivi-1-treated T cells do not localize to the IS, and are larger in area and perimeter than those observed in control T cells. These results are presented in new panels E and F of Fig 3, and are commented on in the Results section (page 8).

2.- Figure 2C and D: Fractionation experiments with antigen-independent T cell-APC conjugates are now shown. The results indicate that Drp1 translocation toward the mitochondrial subcellular fraction was insignificant in the absence of antigen. These data have been included as new Supplemental Figure S2.

3.- Figure 2F: We have replaced the corresponding panel in Figure 2F with a more representative one (new Figure 2G), in which the interference in Drp1 expression is more clearly observed.
4.- Figure 2: New experiments have been performed to address this issue. The results indicate that mitochondrial de-localization in Drp1-interfered T cells was specific, since Drp1 re-expression reverted mitochondrial translocation defects. These data have been added to Figure 2 (new panels J-

L), and Results, page 7, last paragraph.

5.- As controls for the results shown in Figure 4A. we investigated mitochondrial depolarization in cells plated on poly-L-Lys plus control IgG. The results, added as new Supplemental Figure S4, show that there is no mitochondrial depolarization under this experimental condition.

6.- Figure 5: Regarding the reviewer's question about why the effects of Drp1 knockdown on ATP and compromised ATP-dependent myosin phosphorylation affected cSMAC assembly, myosin-IIA and phosphorylated myosin light chain (MLC) have been shown to concentrate at the pSMAC, controlling the actin flux that directs TCR/CD3 microclusters toward the cSMAC (Illani et al., 2009). Other studies have shown that TCR activation is initiated in the early-assembled peripheral microclusters of TCR/CD3 complexes, and is down-modulated in the cSMAC, where activated TCR/CD3 signaling complexes are switched off after ubiquitination, internalization and degradation (Lee et al., 2003; Malissen 2003; Vardhana et al., 2010; and Varma et al., 2006). As requested by the reviewer, we have analyzed the localization of phosphorylated MLC in Drp1 knockdown T cells. We found that Drp1 silencing reduced MLC phosphorylation at the immune synapse. Our findings thus provide a plausible explanation of how de-localization of mitochondria from the IS may disturb cSMAC formation and subsequent signaling. We propose that, by reducing actomyosin fueling at the IS, mitochondrial de-localization in Drp1-interfered T cells reduces TCR/CD3 centripetal flux, and thus sustains signaling by maintaining TCR/CD3 complexes away from cSMAC-localized degradation pathways. These results are shown in new Figure 6F and commented on under Results, page 10, last paragraph in the Discussion , page 12-13.

7.- As requested, we have analyzed the effects of overexpressing mitochondria binding-deficient Drp1 S637D mutant and of treating T cells with the Drp1 GTPase inhibitor mdvi-1, experimental tools that increase mitochondrial fusion by inhibiting fission (Chang and Blackstone, 2010; Cerenghetti et al., 2008; Cassidy-Stone et al., 2008; and Tanaka and Youle, 2008). These approaches disrupted mitochondria redistribution to the IS in a similar way to Drp1 silencing. These data are shown in new Figure 3A-D, and commented on in the Results section (page 8, first paragraph).

Reviewer 3

We appreciate the reviewer's positive and constructive comments, all of which have been taken into account in the preparation of the revised manuscript.

1.- We have addressed the issue of cause and effect in relation to mitochondrial translocation and CD3 clustering at the IS cSMAC. For this we used the Drp1 inhibitor mdivi-1, which blocks Drp1 assembly and binding at the mitochondrial outer membrane, thus inhibiting mitochondrial fission by reducing the constriction activity of Drp1. Inhibition of Drp1 with mdivi-1 disrupted TCR/CD3 clustering at the T cell IS cSMAC, confirming our previous experiments in which Drp1-mitochondria functional interactions were disrupted by Drp1 silencing or targeting of mitochondria with the uncoupling agent FCCP or the mitochondrial F1F0ATPase inhibitor oligomycin. These results indicate that CD3 assembly at the cSMAC of Drp1 knockdown cells was reduced because mitochondrial redistribution, positioning and function near the pSMAC is impaired. The revised manuscript provides a more clear-cut message than the former version on this issue. The new set of experiments is shown in the new panels C and D of Figure 6.

2.- Previous studies have shown that activation of the TCR is initiated throughout the cell-cell contact area with antigen-pulsed APC in the early-assembled peripheral microclusters of TCR/CD3 complexes. Once microclusters are concentrated at the cSMAC, TCR activity would be down-regulated as the TCR/CD3 signaling complexes are switched off by ubiquitination, internalization and degradation (Lee et al., 2003; Malissen 2003; Vardhana et al., 2010; and Varma et al., 2006). We have commented on this rationale in the Discussion (page 13) and have cited the most important publications supporting this point (page 13 and corresponding cites under References).

2a.- We agree with that IL-2 secretion was analyzed long after most the assays aimed at characterizing the role of Drp1 in mitochondrial redistribution and immune synapse organization. We studied IL-2 protein production as a global readout of T cell activation by ELISA; this standard procedure requires at least 16 h of stimulation to yield sufficient accumulation of the cytokine in

culture supernatants. As the reviewer suggested, we have now studied the production of IL-2 at very short times. Unfortunately, despite all our efforts to detect IL-2 at 60 and 120 min in SEE-stimulated J77 T cells, we could not detect any significant amount. This does not seem to us to be a problem, since the IL-2 assay at 16 h gives a valid downstream readout of the earlier effect of mitochondria mislocalization in Drp1 interfered cells.

2b.- We agree that the kinetics of calcium release may differ between T cells stimulated with CD3 Abs or antigen-pulsed APCs. Thus, as suggested by the reviewer, we have now performed calcium imaging experiments in J77 T cells stimulated with SEE-pulsed Raji B cells. The results basically confirm our previous findings with CD3 Abs in solution. Drp1-silenced T cells stimulated with SEE-pulsed Raji APCs show a more sustained release of $[Ca^{2+}]_i$ than control SEE-stimulated T cell-APC conjugates. These results are included as new Figures 7E. Because the restrictions on figure sizes, former panel E in Figure 6 is presented in the revised manuscript as new Supplemental Figure S5. Regarding the possibility of interfering in CD3 assembly at the cSMAC to analyze proximal TCR signaling without affecting mitochondrial localization, while this could be very interesting we do not think it is an appropriate approach for the current study.

3.- A detailed description of how intracellular calcium levels were detected in flow cytometry experiments, including Indo-1 loading conditions, is now provided in Material and Methods (pages 21-22).

4.- The quantitative statistical analyses corresponding to Figures 1A-B and Figures 2A-B have been now included as new Supplemental Figure S1 and Figure 2C, respectively.

5.- We have evaluated this issue in the new version of the manuscript. Localization studies and morphometric analysis of mitochondria in mdivi-1-treated or control APC-activated T cells indicate that mitochondria do not localize near the contact area between mdivi-1-treated T cells and APCs. Mitochondria are also larger in area and perimeter than those in control T cells. As result of these studies, we have added new panels E and F to Figure 3 and commentary in the Results section (page 8, first paragraph). The detection of larger mitochondria in mdivi-1-treated cells strongly suggests that mitochondria are more fused under these conditions. These results are supported by overexpression of the mitochondria binding-deficient mutant Drp1-S637D, a Drp1 phosphomimetic mutant that increases mitochondrial fusion by reducing fission (Chang and Blackstone, 2010). Drp1-S637D overexpression also disrupted mitochondria redistribution toward the IS of T cells. This result is shown in new panels A and B in Figure 3.

6.- In the case of the sustained $[Ca^{2+}]_i$ signals observed in Drp1-silenced T cells upon APC stimulation, we think that both dispersion of CD3 at the IS and mitochondrial mislocalization may play a role, favoring $[Ca^{2+}]_i$ entry and reducing buffering, respectively (see Discussion, pages13--14). In the case of IL-2, since $[Ca^{2+}]_i$ signaling is essential for the production of IL-2 and we consider that $[Ca^{2+}]_i$ levels can be affected by mitochondrial positioning, we cannot rule out the possibility that IL-2 production may also be affected by mitochondrial mislocalization directly, in addition to the increased signaling due to impaired CD3 assembly.

Minor points:

7.- Normalized densitometric band intensities for the blots presented in the former Figures 2C and D are now presented beneath each lane.

8.- Figure 2G y-axis has been defined in the corresponding Figure Legend.

Additional Correspondence 10 January 2011

Thank you for submitting your revised manuscript to the EMBO Journal. I asked the original referee # 2 and 3 to review the revised manuscript. I have now head back from the referees and their comments are provided below. As you can see below, both referees appreciate the introduced changes and support publication in the EMBO Journal. I am therefore very pleased to proceed with its acceptance here.

There is one remaining point to clarify and that is the concentration of Flou-4 AM used - Referee # 3 point #3. Could you please incorporate this into the manuscript and send me a modified word document by email. Please also send along a point-by-point response. Once we receive the revised version we will proceed with its acceptance here.

Thank you for submitting your interesting study to the EMBO Journal.

Best wishes

Editor
The EMBO Journal

REFEREE REPORTS

Referee #2

The paper is well revised and answers satisfactorily the points that reviewers have raised. I believe this is an important report and will bring new concept into the regulation of T-cell receptor signaling.

Referee #3

Baixauli et al. have responded satisfactorily to all my suggestions/criticisms. They have included new experiments to address the following points:

Point 1 and 2: The authors now show that DRP1-induced fission and mislocalization of mitochondria reduce CD3 assembly at the cSMAC (Fig. 6 C,D). This will ultimately lead to a drecreased down-regulation of TCR activity (as discussed by the authors). Thus, calcium signaling and IL2 production should be enhanced in DRP1 treated T cells (as shown by the authors (Figures 7 E, F)-

Point 2a: The authors have tried to measure IL2 at shorter times but have not succeeded. I am a little sceptical that IL2 production at 16 hours can always be correlated with rather early TCR signaling events but I am willing to follow the authors' argumentation.

Point2b: The authors now show calcium responses following IS formation (Figure 7E), which clearly strengthens their point. They did not try to interfere with CD3 assembly and not change mitochondrial localization at the same time, and subsequently analyze proximal TCR signaling under these conditions. In my original review I suggested this experiment but made it clear that I would not insist on this experiment (even if I believe that it is a very important experiment and should be performed in a future publication by the authors).

Point 3: Details of calcium measurements are now provided, however the concentration of Fluo-4 AM is lacking. In their original ms, they used a rather high Indo-1 concentration. How high was the concentration of Fluo-4 used in Figure 7E? I am a little surprised to see that the authors load at 37 C because such high temperatures can induce dye accumulation in the ER and mitochondria of T cells.

Point 4: The authors have provided statistics for Fig. 1 (in S1) and Fig. 2.

Point 5: The authors have analyzed the fusion/fission state under some conditions (Figure 3).

Points 6: The authors discuss this point.

Minor points have been addressed.

Additional Correspondence 12 January 2011

Point-by-point reply to the reviewer's comments.

Reviewer 2:

We appreciate the positive comments about the significance of this study.

Reviewer 3:
We appreciate the positive comments of this reviewer.
With regard to the remaining minor point 3, the concentration used for Fluo-4 AM has been added to the revised manuscript and it was 2 μM, which is in the range of concentrations recommended by the manufacturer (Invitrogen). Although compartmentalization of the dye may occur (which is an inherent problem of the ester-based cell loading technique), it was negligible in the experiments conducted during the imaging of SEE-induced immunological synapse formation.

