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Adhesion shapes T cells for prompt and sustained T cell receptor signaling

Rita Lucia Contento, Silvia Campello, Anna Elisa Trovato, Elena Magrini, Fabio Anselmi and Antonella Viola

Corresponding authors: Antonella Viola and Rita Lucia Contento, Istituto Clinico Humanitas IRCCS

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

1st Editorial Decision 20 April 2010

Thank you for submitting your manuscript to the EMBO Journal. Your manuscript has now been seen by three referees and their comments to the authors are provided below. While the referees appreciate the potential interest of the findings reported, both referees # 2 and 3 also raise significant concerns with the analysis that I am afraid preclude its publication here at this stage.

As you can see, the referees appreciate the finding that chemokine/LFA-1 signaling modulates mitochondria dynamics. However, both referees #2 and 3 also find that the present analysis does not provide strong enough support for the physiological significance of this observation and that LFA-1 mediated effect on mitochondrial polarization is important for T cell signaling and for the "priming" of T cells. Also further work to uncouple this from TCR signaling is needed. With such serious reservation, I am afraid that I can unfortunately not offer to commit to a revised version at this stage and I see no other choice but to reject the present version. However, given the potential interest of the findings reported, I can offer that should be able to strengthen the findings significantly and add more data along the lines that the referees suggest, we are willing to look at a new submission. As a standard, we do not allow resubmission of rejected manuscripts but can do so in this case. For resubmissions we consider the novelty of data at the time of resubmission and may, if needed, bring in new referee(s).

I thank you in any case for the opportunity to consider this manuscript. I am sorry that we cannot be more positive on this occasion, but I hope that you will find our referees' comments helpful.

Yours sincerely,

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Editor
The EMBO Journal

REFEREE REPORTS

Referee #1 (Remarks to the Author):

In the present ms, Contento et al. very convincingly demonstrate that a major player of T cell adhesion, LFA1, can polarize mitochondria (an the MTOC) in the presence of chemokines independently of TCR triggering. They focus their attention mostly on mitochondria and find that LFA-1 function is required to establish cell polarity in T cells. They find that TCR was only required in case no chemokines were present. This is very interesting since most groups in the past have assumed that TCR activation was a necessary step for T cell polarization.

T cell polarization is a fundamental concept of T cell activation (and of course also very important in many other cell types). Thus the data by Contento et al. reveal not only an important new mechanism relevant for T cell polarization, but provide mechanistic insight into a process which is highly relevant for many other cell types.

I have only four minor suggestions that could improve an already very interesting ms further:

1. The most important point is to provide a more quantitative analysis of the "% of ISs with recruited mitochondria", because it is not clear how "recruited to the IS" is distinguished from "not recruited to the IS". The authors give percentage values but it is not clear to me how they arrived at these values.

It is not clear how the "% of ISs with recruited mitochondria" values in Fig. 1A and in several of the subsequent figures were calculated. Did the authors measure how much fluorescence was close (< xx nm) to the IS? Or did they measure how far away the closest mitochondrial structure with regard to the IS was? A solid quantification is required in this case because this parameter is the most important one in the whole paper. Also in Figure 1 (or Supple Fig 1) it would be good to show an example where mitochondria were not close following contact as this scenario is present in 40% of the cells (this would help to have a visual distinction between "recruited to IS" and "not recruited to IS" right at the beginning of the ms).

Fig 3C,D: I assume that lane 1,2 and 3,4 show different projections of the same cell as described for 3D. Again, it is however not clear to me how the localization of mitochondria was quantified. It would help to describe the algorithm and quantification. Otherwise it is very difficult for the reader to understand the differences in case of ICAM2 (C) or for the activating LFA1 antibody (D) from the pictures.

2. On page 9 the authors conclude that LFA1-dependent mitochondria accumulation was not dependent on ORAI channel activity. The authors use a dominant negative ORAI1 channel subunit (the ORAI1-E106A mutant) to partially inhibit ORAI channel activity. In Suppl Fig. S3 the authors use a FACS analyzer with Fluo-4 to analyze calcium signals.TG has been shown in several publications to raise the cytosolic calcium concentration in Jurkat T-cells to levels of 300 - 600 nM (depending on publication). Thus even in the ORAI1-E106A mutant (which partially blocks the calcium signals), the calcium signal after 6.5 minutes would be expected to be in the range of 400 nM using the non-calibrated values form Suppl. Fig S3, a value which is still significantly higher than needed for maximal NFAT activation. Therefore I find it difficult to understand the conclusion that ORAI1-dep. calcium entry is not important for LFA1-dep. recruitment of mitochondria to the IS. Another problem is that FACS is difficult to use in case that calcium signals have to be quantified and that Fluo-4 is not a ratiometric dye (which makes the calcium quantification with FACS even more difficult). In Fig. 7, the authors have used a ratiometric method, why not in Suppl. Fig 3? Some groups have successfully used Indo-1 for quantification with the FACS (but that requires a 360 nm laser).

Thus, I am not convinced that the authors can make the conclusion that ORAI1-dependent calcium

influx is not at all involved in LFA-1 triggered translocation of mitochondria. To safely make this conclusion, one could take away external calcium or block the ORAI1 channels completely (with 50 μM 2-APB or maybe 1 uM BTP2). The still sufficiently high intracellular calcium after 7 minutes (as seen in Suppl. Fig. 3 of the ms) could explain that the authors still observe mitochondria translocation, if mitochondrial translocation would be calcium dependent. Schwindling et al. (2010) observed that very low intracellular calcium concentrations were sufficient for mitochondrial translocation as cited by the authors. These data are in agreement with the reports of groups using other cell types, who found that small amounts of calcium are required for mitochondrial calcium transport while high calcium concentrations (or no calcium at all!) make mitochondria rather stationary. I believe that either the authors should discuss this point very carefully or - if they want to make a strong conclusion - they need to block calcium entry much more quantitatively.

3. Statistics of the calcium data in Fig. 7 would be helpful.

4. Quintana et al, Cell Calcium 45, 109-122, 2009 could be an interesting reference to discuss, because it analyzes the impact of morphological changes on calcium signals in T cells.

Referee #2 (Remarks to the Author):

In this manuscript the authors find that the activated integrin LFA-1, rather than the TCR, mediates the early recruitment of mitochondria to the conjugate interface. Furthermore they observe that this recruitment occurs in response to PI3K-dependent activation of LFA-1 mediated by chemokines. In view of the role that the mitochondria plays in sequestering calcium and promoting CRAC channel function, the authors suggest that the rapid recruitment of mitochondria through adhesive interactions renders the T cell able to mount enhanced calcium responses following antigenic stimulation.

The authors have used an imaging approach together with a series of pharmacological inhibitors and blocking/activating antibodies to establish their findings. On the whole this is a nice study and the observation that LFA-1 can mediate mitochondria to the IS is convincing. However, in my view the potential functional significance of this relocalization represents the weakest aspect of this study. Perhaps the manuscript would benefit from a more detailed investigation of this, rather than the inclusion of results that seem unremarkable (such as the involvement of microtubules in mitochondria relocation, or the role of PI3K in activation of LFA-1).

Major issue:

The authors must provide a more robust demonstration of the function and importance of chemokine-mediated LFA-1 activation leading to the enhanced calcium signalling. Can this be established in cells lacking chemokines receptors or chemokine receptor signalling? Does this enhanced calcium signalling translate into enhanced/prolonged signalling downstream of the TCR, or offer competitive in terms of a functional outcome for cell signalling?

Minor issues:

Is LFA-1 dispensable after the initial recruitment of the mitochondria? Or does 'inside-out' activation through the TCR contribute to the stability of mitochondria at the IS?

The recruitment of mitochondria in the presence on the inhibitor Ru360 should be shown alongside Figure 7C.

Referee #3 (Remarks to the Author):

In this manuscript, the authors address two interesting questions: first, the potential role of chemokine-promoted LFA-1-mediated adhesive signals in priming T cells for antigen recognition and TCR signalling and, second, the polarization of mitochondria to the site of T cell-APC contact by chemokine/LFA-1 signalling as the molecular mechanism behind that effect. Regarding the first point, Friedman and colleagues already stressed the importance of initial chemokine-promoted LFA-

1-mediated T cell-APC contacts for shaping or preparing antigen recognition and signalling through the TCR (Nature Immunology 2006, Oct, 7(10):1101); the molecular mechanism/s behind are unknown. The idea of mitochondria dynamics modulation by chemokine/LFA-1 signalling proposed by the authors (second point of the manuscript) to explain the "T cell priming" effect is very attractive. However, this reviewer has major concerns regarding some of the showed data and, thus, differs in several of the stated conclusions.

Major concerns
The authors show the frequency of immune synapse (IS) with polarized mitochondria along the manuscript, but do not mention anything about the frequency of conjugate formation with unpulsed- versus antigen pulsed-APC. You would expect a higher efficiency in conjugate formation under the presence of cognate interactions; is the chemokine enough to promote cell contacts at high frequency, independently of antigen presence?? What happens in the absence of chemokine?? How the different treatments (colcemid, wortmanin,...) affect to the frequency of conjugate formation?? These should be specified, as may shed light on the biological relevance of the results discussed on the manuscript.

The LFA-1 blocking experiments showed in figure 2 indicate a reduction in the polarization of T cell mitochondria, but not "an inhibition" as the authors claimed. Similarly, in figure 7, LFA-1 blocking experiments and ruthenium treatment show just a reduction in the calcium influx, but no "an inhibition".

Knowing the relevance and requirement of LFA-1/ICAM-1 interactions for the establishment of the IS upon TCR engagement, are the effects showed in figures 2 and 7 just due to an impairment on the TCR-promoted LFA-1 adhesive function?? What happens in the absence of chemokine signalling?? It would be crucial try to isolate the effect of chemokine receptor signalling from TCR signalling, as both modulate LFA-1 activity.

Resubmission - authors' response 06 August 2010

Referee #1

We are very grateful to the reviewer for his/her positive and constructive comments. Although he/she had only minor suggestions, we think that they were very useful to improve the quality of our manuscript.

1. It is not clear how the "% of ISs with recruited mitochondria" values in Fig. 1A and in several of the subsequent figures were calculated. Did the authors measure how much fluorescence was close (< xx nm) to the IS? Or did they measure how far away the closest mitochondrial structure with regard to the IS was? A solid quantification is required in this case because this parameter is the most important one in the whole paper.
We apologize if the explanation of the analysis method was not clear. We modified the method section accordingly. For each experimental condition, images were randomly taken from different wells of the microscope slide, observed and classified by three different operators. Cells were scored polarized when (1) at least 66% of the mitochondria fluorescence was localized in the half part of the cell close to the contact area and (2) mitochondria were localized very close to the plasma membrane. We applied this method of analysis because we wanted to take in account not only the distance between mitochondria and the membrane but also the total amount of mitochondria localized near the IS. These two criteria correspond exactly to the two methods of analysis suggested by the referee.
In fact we scored as "not recruited" both the cells with the majority of mitochondria localized in the half part of the cell close to the IS but not in close contact with the plasma membrane, and, conversely, cells with some mitochondria very close to the IS membrane, but with the majority of mitochondria at the opposite site of the cell. Proportions were calculated on cumulative frequencies from different experiments for each condition and statistical analysis was performed.
The independent evaluation by different operators represents, to our knowledge, the easiest and fastest way to combine both the criteria, especially when large numbers of conjugates are analyzed. However, to validate our results, for each experiment we performed a mathematical analysis on a

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small sample of our images, using Image J software. In the z-projection of the stacks we measured the proportion of total red fluorescence localized in the half part of the cell close to the IS, applying the following formula:
(integrated density of red fluorescence in the half part of the cell close to the IS) / (integrated density of red fluorescence in the whole cell).
We obtain a ratio >0.66 for all the cells that were scored as recruited by operators.

Also in Figure 1 (or Supple Fig 1) it would be good to show an example where mitochondria were not close following contact as this scenario is present in 40% of the cells (this would help to have a visual distinction between "recruited to IS" and "not recruited to IS" right at the beginning of the ms).
We agree that can be helpful to show at the beginning of the manuscript a figure representative of conditions where mitochondria are recruited or not. This figure has been included as new Supplementary Figure S1.

Fig 3C,D: I assume that lane 1,2 and 3,4 show different projections of the same cell as described for 3D.
Yes, we modified the legend to improve its clarity.

Again, it is however not clear to me how the localization of mitochondria was quantified. It would help to describe the algorithm and quantification. Otherwise it is very difficult for the reader to understand the differences in case of ICAM2 (C) or for the activating LFA1 antibody (D) from the pictures.
Again we apologize if the explanation of the analysis method was not clear. We applied the same methods described before for cells conjugated with APCs, but in this case cells were scored polarized when at least 66% of the mitochondria fluorescence was localized in the half part of the cell close to the area in contact with the slide. We modified the method section accordingly.
We validated our analysis using Image J on a sample of our images. First we identified the image slice that divides the cell into two halves along the Z axis, and then we created z-projections for the two halves (the inferior part closer to the slide and the superior part), where we measured the integrated density of red fluorescence. Finally we applied the following formula:
[(integrated density of red fluorescence in the inferior half of the cell)] / [(integrated density of red fluorescence in the superior half of the cell) + (integrated density of red fluorescence in the inferior half of the cell)].
We obtain a ratio >0.66 for all the cells that were scored as polarized by operators.

2. In Fig. 7, the authors have used a ratiometric method, why not in Suppl. Fig 3? Some groups have successfully used Indo-1 for quantification with the FACS (but that requires a 360 nm laser). Thus, I am not convinced that the authors can make the conclusion that ORAI1-dependent calcium influx is not at all involved in LFA-1 triggered translocation of mitochondria. To safely make this conclusion, one could take away external calcium or block the ORAI1 channels completely (with 50 uM 2-APB or maybe 1 uM BTP2).
I believe that either the authors should discuss this point very carefully or - if they want to make a strong conclusion - they need to block calcium entry much more quantitatively.
In Supplementary Figure S3 (now Supplementary Figure S4), we used only Fluo-4 instead of Fluo-4 plus Fura-Red because the CFP and the Fura-Red calcium-bound have the same excitation spectrum, thus it would be impossible to select CFP-ORAI1+ cells for the analysis. The spectrum of INDO-1 is compatible with CFP, but unfortunately our FACS do not have the required laser line. For these reasons, we performed the control experiment using only Fluo-4.
We are grateful to the referee for suggesting us the experiment that may better demonstrate our hypothesis.
We performed the experiment of mitochondria recruitment in presence of 50 μM 2-APB. We found that 2-APB did not inhibit the recruitment of mitochondria at the IS induced by LFA-1 (5 minutes and 15 minutes of interaction), confirming the previous data.
The data are shown in the new Supplementary Figure S5.

3. Statistics of the calcium data in Fig. 7 would be helpful.
We performed the statistical analysis on calcium data considering three representative time points of each curve (new Figure 7 A, 7B and 7C).

4. Quintana et al, Cell Calcium 45, 109-122, 2009 could be an interesting reference to discuss, because it analyzes the impact of morphological changes on calcium signals in T cells.
We thank the reviewer for this suggestion. We discussed this point in the revised version of the manuscript.

In conclusion, we apologize for the evident lack of clarity of the previous manuscript regarding the way we had performed the analyses, we thank the reviewer for his/her constructive suggestions and we are confident that he/she has now no major concerns regarding the study.

Referee #2

We thank the reviewer for his/her comments and for suggesting to include more experiments to strengthen the results of our study. The major issue raised by the referee was the functional significance of mitochondria accumulation at the IS. In the previous version of the manuscript we had shown that mitochondria relocation was pivotal to increase calcium signaling in stimulated T cells. In this new version, we have extended the initial finding and shown that the calcium buffering operated by mitochondria at the IS is essential to allow nuclear translocation of the transcription factor NFAT as well as synthesis and membrane expression of the IL-2 receptor CD25.
We think that these data, in addition to many other publications demonstrating a critical role for calcium signaling in T lymphocytes, indicate the functional significance of our data. We admit that the new data have strongly improved the quality of the study.

Major issue:

The authors must provide a more robust demonstration of the function and importance of chemokine-mediated LFA-1 activation leading to the enhanced calcium signalling. Can this be established in cells lacking chemokines receptors or chemokine receptor signalling?
T cells express several chemokine receptors and it would be impossible to knock-down all of them. However, chemokine receptors are GPCRs and most of them signal through Gαi, a Gα protein that is selectively inhibited by the Pertussis toxin (PTx) (J Exp Med 1985, Jul 1; 162(1):145-56). Thus, we analyzed TCR-induced calcium influx in cells pre-treated with pertussis toxin, in order to block chemokine receptor signaling (new Figure 7C). We found that TCR-induced calcium influx is significantly reduced in T cells lacking chemokine receptor signaling and stimulated by APCs, and that - in contrast to what observed in control cells - anti-LFA-1 blocking antibodies did not further reduce it, suggesting that the inhibition of chemokine receptor signaling has already blocked the ability of LFA-1 to enhance TCR-induced calcium influx. Interestingly, at later time points, pertussis toxin treated cells showed a significantly higher calcium influx than cells treated with pertussis toxin plus anti-LFA-1, suggesting that TCR signaling may replace the chemokine signaling and partially restore LFA-1 functions, even if not immediately. This experiment demonstrates that the amplification of calcium signaling by LFA-1 requires chemokine-mediated LFA-1 activation.

Does this enhanced calcium signalling translate into enhanced/prolonged signalling downstream of the TCR, or offer competitive in terms of a functional outcome for cell signalling?
To address the functional outcome of mitochondria translocation to the IS and mitochondrial calcium buffering, we analyzed both the localization of NFATc2, the main transcription factor controlled by intracellular calcium, and the expression of CD25, the main marker of T cell activation.
First, we measured the ratio between the nuclear and the cytoplasmic localization of HA-tagged NFATc2 in Jurkat cells treated or not with Ru360 and/or anti-LFA-1 blocking antibodies and conjugated with SEE-pulsed B cells. We found a strong correlation between the NFAT nuclear translocation and the recruitment of mitochondria to the IS. In fact, the NFAT nuclear translocation index was significantly higher in T cells with mitochondria recruited to the IS than in cells with mitochondria not recruited. Importantly, in presence of anti-LFA-1 blocking antibodies this correlation was maintained (because mitochondria were functional) but the overall percentage of IS with mitochondria recruited was reduced. Most interestingly, NFAT nuclear translocation was reduced - and equal to that of cells with evenly distributed mitochondria - when the organelles could not buffer calcium. This last experiment clearly indicates that NFAT translocation depends on mitochondria recruitment to the IS and on their ability to buffer calcium at that specific location.

Data are included in the revised version of the manuscript as new Figure 8A.

Finally, we analyzed the ability of LFA-1 to enhance CD25 expression in T cells (please see new Figure 8B). We activated human CD4+ cells with superantigens and murine fibroblasts that selectively express human ICAM1 and/or MHCII (as described in Figures 3B and 5C). We found that TCR-LFA-1 co-engagement was able to increase the expression of CD25. Most importantly, this costimulatory function of LFA-1 depended on the ability of mitochondria to buffer calcium: in presence of Ru360 the LFA-1 induced up-regulation of CD25 was significantly reduced. Altogether, these data demonstrate that the recruitment of mitochondria to the IS and their role in sustaining TCR-induced calcium influx are pivotal to support the nuclear translocation of NFAT and thus facilitate T cell activation.

Minor issues:

Is LFA-1 dispensable after the initial recruitment of the mitochondria? Or does 'inside-out' activation through the TCR contribute to the stability of mitochondria at the IS?
To understand if TCR-induced stabilization of mitochondria at the IS requires LFA-1, we blocked (or not) LFA-1 only after the initial 15 minutes of T-APC interaction and we allowed these conjugates to interact for 3 more hours, after which we analyzed the mitochondria recruitment to the IS. During the initial phase of this experimental setting (first 15 minutes), LFA-1 could therefore induce mitochondria relocation to the IS, as demonstrated in Figures 2 and 3. During the rest of the incubation period, as already shown in Figure 6B and 6C, TCR should be able to stabilize and maintain mitochondria positioning at the IS. The new experiment demonstrates that the effects of TCR signaling on mitochondria localization depend on LFA-1, because TCR signaling could not stabilize the organelles at the IS in the presence of anti-LFA-1 blocking antibodies (please see new Figure 6D).
Interestingly, the fact that TCR signaling is required to stabilize mitochondria at the IS indicates that during long-lasting cellular interactions the chemokine receptor signaling is not sufficient to control LFA-1-induced mitochondria stabilization at the IS (Figure 6B, 6C and 6D). In contrast, during the initial T-APC interactions, both chemokine receptors and TCR can trigger LFA-1 activation that in turn induces mitochondria recruitment to the IS (Supplementary Figure S3 and Figure 6A). Therefore we can conclude that the requirements for LFA-1 activation that controls mitochondria positioning are finely tuned over time.

The recruitment of mitochondria in the presence on the inhibitor Ru360 should be shown alongside Figure 7C.
We included this control. In presence of Ru360 mitochondria were normally recruited at the IS. Data about the recruitment of mitochondria after 5 minutes or 15 minutes of interaction with APCs are shown in Supplementary Figure S6 and in Figure 8A, respectively.

In conclusion, we thank the reviewer for his/her constructive suggestions. We included all the requested data and we are confident that he/she has now no major concerns regarding the study.

Referee #3

We thank the reviewer for his/her positive and constructive comments and for suggesting experiments to improve the quality of our work. The main concern of the referee was related to the effects of the different treatments on the frequency of conjugate formation. For each experiment shown in the manuscript, we had already analyzed the conjugate numbers but we had not shown the results. In the new version, we have included a table indicating the frequency of conjugates for each experimental condition.

Major concerns

The authors show the frequency of immune synapse (IS) with polarized mitochondria along the manuscript, but do not mention anything about the frequency of conjugate formation with unpulsedversus
antigen pulsed-APC. You would expect a higher efficiency in conjugate formation under the presence of cognate interactions; is the chemokine enough to promote cell contacts at high

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frequency, independently of antigen presence?? What happens in the absence of chemokine?? How the different treatments (colcemid, wortmanin,...) affect to the frequency of conjugate formation?? These should be specified, as may shed light on the biological relevance of the results discussed on the manuscript.
We agree with the referee that if in any of our experimental condition the number of conjugates between T cells and APCs was dramatically reduced, the analysis of mitochondria recruitment would be completely meaningless. In fact we had evaluated by FACS the percentage of T cells coupled with B cells for each treatment. We apologize if in the previous version of the manuscript we omitted to mention these important controls. Data are now shown in Supplementary Table I.
On one hand, not surprisingly, TCR stimulation by SEE superantigen induced a significant increase in conjugate number. Anyhow, even without SEE, the number of conjugates was high enough to perform our analyses. Moreover, the "unpulsed" condition clearly resembles in vitro the first phase of in vivo interactions between T cells and APCs before antigen recognition and it is therefore physiologically relevant.
On the other hand, none of the used drugs reduced the number of conjugates, with the exception of PP2, an inhibitor of src kinases downstream TCR triggering (J Biol Chem 1996, Jan 12; 271(2):695-701), that, as expected, blocked the increase in conjugate number induced by TCR. Nevertheless, we performed all our analyses on conjugated cells only and we expressed all values as percentage of conjugates, thus small differences in conjugate number among conditions due to SEE addition and normal experimental variability are irrelevant.

The LFA-1 blocking experiments showed in figure 2 indicate a reduction in the polarization of T cell mitochondria, but not "an inhibition" as the authors claimed. Similarly, in figure 7, LFA-1 blocking experiments and ruthenium treatment show just a reduction in the calcium influx, but no "an inhibition".
Whit the word "inhibition" we intended "partial inhibition". Of course, as already specified at page 5 of the previous version of the manuscript, we believe that the complete absence of LFA-1 binding, and thus the total inhibition of LFA-1 effects, is a condition that is unlikely achieved with blocking antibodies. For this reason, when possible, we used several different approach and/or experimental conditions to confirm the effect of anti-LFA-1 blocking antibodies. Anyway, we appreciate that "inhibition" may be misleading for the readers. Thus, we thank the referee for the observation and we substituted "reduction" for "inhibition" in the revised version of the manuscript.

Knowing the relevance and requirement of LFA-1/ICAM-1 interactions for the establishment of the IS upon TCR engagement, are the effects showed in figures 2 and 7 just due to an impairment on the TCR-promoted LFA-1 adhesive function?? What happens in the absence of chemokine signalling?? It would be crucial try to isolate the effect of chemokine receptor signalling from TCR signalling, as both modulate LFA-1 activity.
As shown in the new Supplementary Table I, in our experimental conditions used for immunofluorescence experiments of Figure 2 (15 minutes of interaction at 37°C and then adhesion onto microscope slides or analysis by FACS), the addition of anti-LFA-1 blocking antibodies did not modify the number of conjugates, irrespectively of TCR triggering.
The same is true for the experimental conditions used for FACS analysis of Figure 7 (centrifugation al low speed to promote and synchronize interactions, 5 minutes of recording and then SEE addition). Anti-LFA-1 blocking did not reduce the number of conjugates before (control (mean_SD): 40,07_1,71% ; anti-LFA-1: 40,29_4,46%; t-test: p=0,91) or after TCR triggering (control (mean_SD): 38,47+/-2,36% ; anti-LFA-1: 39,16+/-4,47%; t-test: p=0,82).
Thus, we concluded that the results were not influenced by the LFA-1 adhesive functions.
We thank the referee for the very interesting suggestion of analyzing the different roles of chemokine receptors and TCR in LFA-1 activation. With this aim, we pre-treated T cells with pertussis toxin, in order to block the signaling of any chemokine receptor *(J Exp Med 1985, Jul 1; 162(1):145-56)*, and/or with PP2, that specifically inhibits the early TCR signaling *(J Biol Chem 1996, Jan 12; 271(2):695-701)*. As shown in Supplementary Table I, the treatment with pertussis toxin did not significantly reduce the number of conjugated T cells. We found that chemokine receptor signaling alone is able to induce mitochondria recruitment through LFA-1 activation. In the presence of chemokine receptor signaling, the contribution of TCR was irrelevant. On the contrary, in the absence of chemokine receptor signaling, the contribution of TCR was essential to induce mitochondria recruitment (new Figure 6A). These data demonstrate that during the initial TAPC interactions both chemokine receptors and TCR may activate LFA-1 and thus trigger mitochondria recruitment, without additive effect when both the receptors are engaged.
Moreover, we performed a new experiment to understand if the TCR contribution to the stability of

mitochondria at the IS involves the inside-out activation of LFA-1. We blocked (or not) LFA-1 only after the initial 15 minutes of T-APC interaction and we allowed these conjugates to interact for 3 more hours, after which we analyzed the mitochondria recruitment to the IS. During the initial phase of this experimental setting (first 15 minutes), LFA-1 could therefore induce mitochondria relocation to the IS, as demonstrated in Figures 2 and 3. During the rest of the incubation period, as already shown in Figure 6B and 6C, TCR should be able to stabilize and maintain mitochondria positioning at the IS. The new experiment demonstrates that the effects of TCR signaling on mitochondria localization depend on LFA-1, because TCR signaling could not stabilize the organelles at the IS in the presence of anti-LFA-1 blocking antibodies (please see new Figure 6D). Interestingly, the fact that TCR signaling is required to stabilize mitochondria at the IS indicates that during long-lasting cellular interactions the chemokine receptor signaling is not sufficient to control LFA-1-induced mitochondria stabilization at the IS (Figure 6B, 6C and 6D). In contrast, during the initial T-APC interactions, both chemokine receptors and TCR can trigger LFA-1 activation that in turn induces mitochondria recruitment to the IS (Supplementary Figure S3 and Figure 6A). Therefore we can conclude that the requirements for LFA-1 activation that controls mitochondria positioning are finely tuned over time.

In conclusion, we thank the reviewer for his/her positive and constructive comments and for suggesting to include more experiments to strengthen the results of our study. We addressed all the refereeís questions and we are confident that he/she has now no major concerns regarding the study. We have also cited and discussed the paper suggested by the referee (Nat Immunol 2006, Oct, 7(10):1101-8).

Additional Correspondence 14 September 2010

Thank you for submitting your manuscript to the EMBO Journal. This is a resubmission of MS # 74343 that reject post review earlier this year. In this case I offered that should you be able to extend the analysis along the lines suggested by the referees that we would be willing to consider a new submission. I asked the original three referees to review the paper for us and their comments are provided below. The referees are listed in the same order as on the original submission. As you can see below all three referees appreciate the introduced changes. While referees #1 and 3 are very supportive of the study, referee #2 is undecided if the advance provided is sufficient to consider publication in the EMBO Journal. Given all the available input, I would like to go with the recommendations of referee #1 and 3 and I am therefore very pleased to proceed with the acceptance of the paper for publication here - no further revisions are needed. You will receive the formal acceptance letter shortly

Best wishes

Editor
The EMBO Journal

REFEREE REPORTS

Referee #1:

Again, this is a very interesting manuscript with exciting data. The authors have satisfactorily responded to my minor suggestions. The mechanistic insight of this ms will certainly help to understand signalling during T cell polarization better.

Referee #2:

In the revised manuscript the authors have gone some way to address our original

concerns in terms of the functional impact of mitochondrial calcium signalling and chemokine-mediated LFA-1 activation. The authors show that blocking chemokine receptor signalling through pertussis toxin reduces calcium signalling. In addition they use the relocalization of NFAT as a functional readout for mitochondrial calcium signalling and observe reduced relocalization in the presence of the Ru360 inhibitor. These additional experiments are in line with the conclusions derived from their original studies. This paper does not provide a clear-cut demonstration of enhanced TCR signalling as a result of mitochondrial relocalization, but such a demonstration is by no means trivial and potentially beyond the scope of the current manuscript. Thus the paper in its current form would contribute some new observations to the field, but it is likely that these observations will be moderate in their impact.

Referee 3:

In the revised version of the manuscript entitled "Adhesion shapes T cells for prompt and sustained T cell receptor signalling" by Rita L. Contento and colleagues, the authors have made a considerable effort to address the major concerns pointed out by this referee on the previous version. The stated conclusions have gained in clarity and robustness with the new set of added data, together with the clarifications in the text. This referee has no other concerns to make regarding the revised version of the manuscript, and would like to congratulate the authors for the effort made and final result.

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