THE EMBO JOURNAL

Manuscript EMBO-2010-76570

Calcium microdomains at the immunological synapse: how ORAI channels, mitochondria and calcium pumps generate local calcium signals for efficient T-cell activation

Mathias Pasche, Christian Junker, Dalia Al-Ansary, Carsten Kummerow, Lucia Nuñez, Carlos Villalobos, Ute Becherer, Jens Rettig, Barbara Niemeyer, Markus Hoth

Corresponding author: Ariel Quintana, La Jolla Institute for Allergy and Immunology

Review timeline:

Submission date:	19 November 2010
Editorial Decision:	27 December 2010
Revision received:	08 July 2011
Acceptance letter:	18 July 2011
Accepted:	19 July 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 27 December 2010

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

While referee #3 is not persuaded that the advance and insight provided is sufficient to consider publication in the EMBO Journal, both referees #1 and 2 are more supportive of the study. However as you can also see below, there are also many different issues that have to be resolved before further consideration here. Most of the experiments are based upon transient transfections and in particular referee #1 finds that analysis needs to be carried out on stable cell lines that show moderate expression of the different constructs. Some further insight into how the mitochondria is recruited to the IS is also needed. Questions are also raised regarding the nocodazole experiment. So as you can see quite a bit of work is needed for consideration here. Should you be able to address the raised concerns in full, then we would consider a revised manuscript. I would like to add that it is EMBO Journal policy to allow a single major round of revision only and it is therefore important to address the raised concerns at this stage if you wish the manuscript ultimately to be accepted. We normally allow 3 months for a revision, but I can extend the deadline should it be necessary.

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
The EMBO Journal

_____

REFEREE REPORTS:

Referee #1 (Remarks to the Author):

In this study, Hoth and coworkers provide further details about the role of mitochondria in enhancing TCR-induced calcium signaling at the immunological synapse.
In particular, the authors show/propose that:

1) ORAI1, STIM1 and PMCA4b localize at the immunological synapse;
2) Mitochondria at the immunological synapse reduce sub-plasma membrane Ca2+ signals through modulation of PMCA
3) Mitochondria co-localize with PMCA at the immunological synapse

While accumulation of mitochondria, ORAI1 and STIM1 at the immunological synapse have been already described, the mitochondria-mediated modulation of PMCA activity is novel and interesting. Nonetheless, authors should try and address the following concerns:

1. ORAI1, STIM1 and PMCA4b localization at the immunological synapse.
All experiments were performed after transient transfections of T cells. In these conditions, there are two main problems: i) unreal localization of proteins and receptors and ii) protein accumulation in the Golgi. This is particularly relevant in the case of TIRFM data. Experiments should be repeated using stable cell lines that can be selected for moderate expression of the constructs.
The authors have indicated the % of cells showing recruitment of proteins to the immunological synapse. They have also provided examples of what they mean with "accumulation". However, a systematic, quantitative approach has to be used to define "accumulation", such as analysis of fluorescence distribution.
How was the statistic analysis performed? If cells were scored on the basis of phenotypes (accumulation vs no accumulation), the analysis should be performed considering that the variables are categorical (non continuous). How was S.D. calculated?

2. Recruitment of mitochondria.
It has been recently published in EMBO Journal that accumulation of mitochondria at the immunological synapse is driven by LFA-1 (Contento et al., 2010). Consistent with their previous publications, Hoth and coworkers have used anti-CD3 antibodies to induce localization of mitochondria at the plasma membrane. Have the authors tried to perform TIRFM experiments using a combination of anti-CD3 and anti-LFA1 antibodies? Does LFA-1 stimulation change the results shown in this manuscript?

3. Actin and tubulin.
To provide support to their hypothesis that accumulation of mitochondria at the immunological synapse is responsible for PMCA modulation, the authors used drugs blocking actin or tubulin polymerization. The rationale was that actin is necessary for immunological synapse formation whereas tubulin is not. In general, this approach is quite "brutal", because of the many effects that these drugs may have . However, the authors have tried to perform the appropriate controls.
There are two aspect that require attention.
i) It is written in the text that "CRAC/ORAI channels were always present at the IS independently of actin requirement (Supplementary Fig. 9)". However, Fig. 1 shows that ORAI1 accumulates at the immunological synapse. Now, if in the absence of actin polymerization the immunological synapse cannot be established (as correctly stated by the author), how can ORAI1, STIM1 and PMCA be correctly recruited? How can this condition be comparable to the normal one?
ii) It is not true that nocodazole has no effects on synapse formation. Nocodazole inhibits TCR

signaling, which is required for synapse formation (Huby et al. JBC 1998). Thus, this cannot be used as control for a condition in which the immunological synapse is functional.
Considering these problems, the authors should either identify other experimental strategies to demonstrate their hypothesis, or they should be open about other possible interpretations of their data.

Although the manuscript is very well written, the authors should make an effort to simplify the text in a way that all experiments can be fully understood by scientist working in different fields.

Referee #2 (Remarks to the Author):

This manuscript reports on Ca2+ signaling in the immunological synapse (IS). The main finding is that Ca2+ is significantly lower in the IS due to Ca2+ uptake by mitochondria that also reduced the activity of PMCA, which is also found in the SI upon T cell stimulation and IS formation.
This was very difficult manuscript to follow and I had to read several paragraph and conclusions multiple times to figure out what the authors intend to convey and the reasoning for each conclusions. Hence, the manuscript needs significant simplifications, especially with the large number of Figs. in the manuscript (20 including in the supplement). Several of the Figs. can be easily removed or simplified so that there will not be the need to refer so much to the supplementary figures with the very long Fig. legends.
After sorting through the manuscript I think it has a good message that is supported by the data, but significant modifications and clarification and additional experiments are needed before further considerations.
1. Page 7, first paragraph- recruitment of mitochondria is observed on formation of the IS but not after treatment with thapsigargin. This is different from several observations, including by the authors, that mitochondria play important role in controlling the CRAC current activated by thapsigargin. The reason for the differences and how mitochondria can control Ca2+ influx in thapsigargin-treated cells if they are not recruited to the plasma membrane need to be explained.

2. It is not clear how the mitochondria are recruited to the IS. Is the Ca2+ increase or treatment with CD3 independent of Ca2+ influx is sufficient to recruit the mitochondria?

3. Page 8, second paragraph: an important conclusion offered by the authors is that the low Ca2+ at the IS is required for high global Ca2+. This is not tested rigorously. The authors are in a good position to carefully determine the relationship between Ca2+ at the IS and global Ca2+ using the different protocols used in the other figures to manipulate Ca2+ at the IS. The authors should simultaneously measure Ca2+ at the IS and global Ca2+, as in Fig. 3 under each of the conditions that changes IS.

4. In relation to 3, the authors inferred the PMCA activity at the IS from measuring global Ca2+. This underestimates PMCA activity. The authors should measure PMCA activity at the IS using their protocol of Ca2+ out removal while recording Ca2+ at the IS, as in Fig. 3.

5. Page 11, lines 13-16: The authors state that Ca2+ clearance is increased in thapsigargin-treated cells incubated at 20 mM Ca2+ even though the global Ca2+ is the same, "as expected". It is not clear to me how can this be expected. On the contrary, if Ca2+ was the same, so should PMCA activity. The authors should explain how Ca2+ clearance rates are so different with global Ca2+ being the same. This is even more puzzling with the findings that GLOBAL PMCA activity appear the same when IS is formed. The authors INTERPRATE this to suggest that recruitment of PMCA to the IS and buffering of Ca2+ by the mitochondria at the IS to be the same. This should be shown experimentally. The authors should measure Ca2+ at the IS in the presence of 1 and 20 Ca2+ out and show it to be the same!

6. Page 13, lines 5, 6: It is stated that Orai3 is less sensitive to Ca2+. In fact Orai3 shows the strongest fast-Ca2+ dependent inactivation. The authors should be more specific to refer to the slow inhibition. The paper by Lis et al is not listed in the references.

7. Page 14, end of first paragraph: The conclusion from the nocodazole experiment is not clear at all. How the authors can conclude anything is not clear. Actually, since nocodazole has the same effect

as the actin disruptors call into question the specificity of the effects rather than suggest the far reaching conclusion that PMCA modulation by local Ca2+ next to the open Orai channels is controlled by subplasmalemal mitochondria at the IS. Do you really think that the nocodazole experiment suggest all of that?

8. A relevant paper on the relationship between CRAC current and mitochondria that should be discussed in the context of the present findings is the recent work by Pozzan et at (Mol Cell. 2010 Apr 23;38(2):280-90.)

Referee #3 (Remarks to the Author):

This manuscript is reporting that formation of the immunological synapse in T helper cells results in the increased presence of Orai1 and mitochondria close to one another at the synapse, but exclusion of PMCa pump and SERCA pump. Local Ca buffering by the mitochondria is believed to explain the small local Ca signal (measured with TIRFM). According to authors, small local Ca produces less inactivation of Orai1 proteins, and this produces a big global Ca signal. This is an interesting study. I have following points to make.

It is not so clear what is very new here, other than exclusion of PMCA at the synapse. Cahalan group already show Orai1 in the immunological synapse and Hoth group previously showed that mitochondrial move to the synapse and, recently, this position of mitochondria is very important for local calcium entry. They report that 'if mitochondria are close to any given local calcium entry site, calcium influx is large; if they are not close, calcium influx is small' (Schindling et al., J. Immunol. 2010). Actually, this seems different to this paper, where authors suggest local calcium influx is smaller at the synapse because of mitochondria.

Authors hypothesis does not fit with recent works. Pozzan group showed in Cell (2010) that mitochondria are not close to the plasma membrane and not detect calcium influx signals. This was shown using new probe on mitochondrial surface. Also, Graier show mitochondria regulate Orai1 activity without need of moving to plasma membrane. How explain contradictory results and why not discussed?

How is calcium measured here with TIRFM? I) I do not think TIRFM spatial resolution is sufficient to detect calcium microdomains. 2) There is no calibration of the dye signal. How high is the calcium? Authors speculate >10 uM, but this is based on dye affinity. 3) Data in Figure 3C/D is normalized deltaF. What is this here? Is it F-F0/F0? Why is local signal so much smaller than global, if local causes global?

How long time is needed for formation of the immune synapse? No time course is shown. Figure 1 shows it is present around 1 hour later, but some stimuli are given in the paper for shorter time (supplementary 4A, anti-CD3 for 15 minutes). Synapse might not have formed, so how to conclude no SERCA is accumulated here? Also, please show no role for SERCA using labeling studies.

1st Revision - authors' response 08 July 2011

Referee #1 (Remarks to the Author):

1. *ORAI1, STIM1 and PMCA4b localization at the immunological synapse. All experiments were performed after transient transfections of T cells. In these conditions, there are two main problems: i) unreal localization of proteins and receptors and ii) protein accumulation in the Golgi. This is particularly relevant in the case of TIRFM data. Experiments should be repeated using stable cell lines that can be selected for moderate expression of the constructs. The authors have indicated the % of cells showing recruitment of proteins to the immunological synapse. They have also provided examples of what they mean with "accumulation". However, a systematic, quantitative approach has to be used to define "accumulation", such as analysis of fluorescence distribution. How was the statistic*

analysis performed? If cells were scored on the basis of phenotypes (accumulation vs. no accumulation), the analysis should be performed considering that the variables are categorical (non continuous). How was S.D. calculated?

We agree with the reviewer regarding all points.

1. Figure 1. The reviewer is right that our analysis is categorical. We now show in Figure 1B how we scored the cells. Lines were taken from the IS to the distal pole and fluorescence was quantified along these lines. The peaks at the respective positions are very clear and could be scored into the different categories. Since we have analyzed every condition with different transfections or in different samples, we can provide an S.D between different experiments (which all contain many different cells).

2. Figure 1. We have tried to generate stable T cell lines. We have only succeeded with ORAI1. Here we have generated several cell lines. As the reviewer suggested, we have used a Jurkat cell line for the analysis that expresses low amounts of ORAI1. The picture of one cell is shown in Figure 1B (right panel) and analyzed below (green bar). The analysis revealed that this condition reflects very well the condition with transient ORAI1 and STIM1 overexpression. As the reviewer probably had in his/her mind the STIM1 expression is very important for ORAI1 localization as our analysis also shows. Unreal/artificial location in the Golgi (as the reviewer pointed out), occurs in case we over express only ORAI1 without STIM1. We discuss this in the results section.

3. Since we could not generate the other stable cell lines, we also tried to analyze endogenous proteins with commercial antibodies. We have tested several commercially available antibodies and found one for ORAI1 that in our hand works reliable and specific. This means that it recognizes both the unglycosylated and two complex glycosylated bands of the expected molecular masses and these signals, although relatively weak, are strongly decreased after transfection of ORAI1 siRNA (data shown to referees). Treatment with PNGase F retains the unglycosylated while ablating the complex glycosylated bands (data not shown). Band intensities are quantified. STIM1 siRNA was also used as control. ORAI1 siRNA dependent downregulation was confirmed by qRT-PCR (not shown).

The analysis of the endogenous protein was similar as the one with the stable cell line (compare Fig.1 B (right) and quantitative analysis in Fig. 1B lower panel, black bar). For PMCA, we also quantified the cells with clear PMCA accumulation at the IS using the over expression system and in addition we have used a well established antibody to quantify endogenous protein (Fig. 1D and E). 4. Figure 2. We now quantify the TIRFM data. ORAI1 and PMCA are enhanced at the IS (total fluorescence is quantified in Fig. 2D). We have also analyzed the stable ORAI cell line and got similar results (Fig. 2C, D). In addition we have quantified the TIRFM data in Fig. 7.

2. *Recruitment of mitochondria. It has been recently published in EMBO Journal that accumulation of mitochondria at the immunological synapse is driven by LFA-1 (Contento et al., 2010). Consistent with their previous publications, Hoth and coworkers have used anti-CD3 antibodies to induce localization of mitochondria at the plasma membrane. Have the authors tried to perform TIRFM experiments using a combination of anti-CD3 and anti-LFA1 antibodies? Does LFA-1 stimulation change the results shown in this manuscript?*

We agree with the reviewer's suggestion to include LFA-1 stimulation. We have performed the requested experiments. The results are shown in Fig. 4A, C. LFA-1 stimulation alone had a small effect on the mitochondrial localization, whereas TCR stimulation through anti-CD3 antibodies induced mitochondria accumulation at the IS, which was further enhanced by additional LFA stimulation. LFA-1 stimulation alone induced mitochondrial accumulation, however somewhat weaker when compared to Contento et al (Contento et al., 2010). We are currently not sure why our effect with LFA-1 alone is smaller than reported by Contento et al, possible reasons could include the passage number or strain of Jurkats used, different cytokine environments or treatment protocol. More interestingly, TCR stimulation together with LFA-1 (a more physiological stimulus) showed stronger than additive mitochondrial accumulation at the IS. Our experiments, therefore, confirm a role for LFA-1 in mitochondrial translocation to the IS. Consistent with our finding that mitochondrial accumulation decreased the calcium concentration at the immune synapse, we measured smaller calcium concentrations at the immune synapse if LFA-1 was used together with TCR stimulation compared to TCR stimulation alone (Fig. 4C).

3. *Actin and tubulin. To provide support to their hypothesis that accumulation of mitochondria at the immunological synapse is responsible for PMCA modulation, the authors used drugs blocking actin or tubulin polymerization. The rationale was that actin is necessary for immunological synapse formation whereas tubulin is not. In general, this approach is quite "brutal", because of the many effects that these drugs may have. However, the authors have tried to perform the appropriate controls. There are two aspects that require attention. i) It is written in the text that "CRAC/ORAI channels were always present at the IS independently of actin requirement (Supplementary Fig. 9)". However, Fig. 1 shows that ORAI1 accumulates at the immunological synapse. Now, if in the absence of actin polymerization the immunological synapse cannot be established (as correctly stated by the author), how can ORAI1, STIM1 and PMCA be correctly recruited? How can this condition be comparable to the normal one?*

We thank the reviewer for pointing out this mistake that leads to such confusion about the interpretation of the data.

The mistake was made, as reviewer realized, by writing in the text that "CRAC/ORAI channels were always present "at the IS independently of actin requirement". So, if actin re-arrangement is blocked we of course do not have IS formation (Quintana et al., 2007) and it is absolutely wrong to state that CRAC/ORAI channels are at the IS (because there is no IS). What we really wanted to say is, that independently of actin accumulation to the cellcell, cell-bead or cell-Abs coated coverslips contact point (where the IS would build up as occurs under control condition), we still have CRAC/ORAI channels and PMCA at the plasma membrane and STIM1 in close contact with the plasma membrane. In addition STIM and ORAI proteins undergo normal ER and plasma membrane rearrangements following TG stimulation even in the presence of actin disrupting drugs. We and others have previously reported that both latrunculin and cytochalisin D do not have any effect in TG-induced Ca2+ signal (Mueller et al., 2007; Quintana et al., 2009; Quintana et al., 2007). In other words, actin is not required for STIM1- ORAI1 complex formation at the plasma membrane upon TG stimulation. In contrast, Ca2+ signals following IS stimulation are highly dependent on actin re-arrangement. This is explained by the dependence of TCR/CD3 on actin for accumulation at the IS (Campi et al., 2005; Dustin, 2008; Dustin and Cooper, 2000). Thus, one should expect that latrunculin-treated cells do not respond to anti-CD3 mAbs coated beads or coverslips. However, if one co-stimulates cells with TG as we did, then cells will respond the same as non treated cells (Quintana et al., 2007). Under these conditions, therefore, the mitochondrial movement driving force (Ca2+ influx through CRAC/ORAI channels, (Quintana et al., 2007)) is present as under control condition. However, disruption of actin polymerization blocks cell polarization and organelle distribution in the cytosol (Dustin, 2005; Dustin, 2008; Dustin and Cooper, 2000). Thus, in the absence of actin polymerization mitochondria are not able to accumulate beneath the plasma membrane (~ 200 nm), even though Ca2+ influx through CRAC/ORAI channels is ensured by TG stimulation ((Bakowski et al., 2001; Mueller et al., 2007), Supplementary Fig 2B-D). This was in general the rationale of those experiments using actin disrupting drugs. In case of nocodazole, a tubulin polymerization disrupting drug, the explanation is more straightforward. Again, for our experiments, it is most important that calcium signaling following IS formation works. This is clearly the case with nocodazole present because we have previously shown that nocodazole does not change calcium influx and also does not affect calcium release following formation of the IS (Quintana et al., 2006). Pump rates are also not changed (Quintana et al., 2006). This implies that the entire calcium influx and calcium release process is not grossly altered. As shown in the relevant Figure 8 of the paper, ISO cells show a comparable calcium plateau in 1 mM and 20 mM external calcium (compare Figure 6 or two papers from the Lewis lab, (Bautista et al., 2002; Bautista and Lewis, 2004), ref 16 and 23 in the original submission). These cells show significant calcium entry following IS formation and importantly PMCA modulation that is induced because mitochondrial accumulation is inhibited. This is the only relevant point of the drug experiments and we believe that we can safely conclude this. We have modified our text describing Figs. 7 and 8 to more clearly lay out the rationale and the conclusions of these experiments.

ii) It is not true that nocodazole has no effects on synapse formation. Nocodazole inhibits TCR signaling, which is required for synapse formation (Huby et al. JBC 1998). Thus, this cannot be used as control for a condition in which the immunological synapse is functional.

We agree with the reviewer that nocodazole can inhibit synapse formation. However, as shown in the study by Huby et al. (JBC 1998) as mentioned by the reviewer, the effect is greatly concentration dependent. Nocodazole has clear effects when used above 10 uM. It has very little effects below 5 uM. We have used 2 uM in our study to minimize effects on the synapse or other non-specific parameters. As explained above, we have also checked that calcium signaling is not changed by 2 uM nocodazole ((Quintana et al., 2007), Figure 4 C and D). For the reviewers convenience, we show (data shown to referees) that calcium release is not changed significantly by nocodazole and also Figure 4C and D (Quintana et al., 2007) that show that nocodazole does not interfere with IS-induced calcium signaling induced by the same beads that we have used for the nocodazole experiments. Thus, the IP3-dependent signaling cascade is not disrupted by nocodazole following TCR/CD3 complex stimulation because the calcium release from the endoplasmic reticulum was the same in control and in nocodazole treated cells. In other words, the abrogation of microtubule polymerization by nocodazole does not avoid the formation of signalosome beneath TCR/CD3 complex which induces the subsequent IP3 dependent signaling cascade.

We also proved as same as others (Bakowski et al., 2001) that at certain concentration and incubation time (previous to the stimulus) nocodazole does not have effect on CRAC/ORAI channels activity (Quintana et al., 2006; Quintana et al., 2007). The same is true for pump rates as shown in Figure b (also see Supplementary Fig 1 (Quintana et al., 2006)).

Thus, taken together we believe that under our conditions nocodazole treatment does not affect any triggering mechanisms of Ca2+ release activated Ca2+ channels following TCR stimulation other than microtubules dependent mitochondrial translocation to the plasma membrane and thereby the sustained activity of CRAC/ORAI channels.

Considering these problems, the authors should either identify other experimental strategies to demonstrate their hypothesis, or they should be open about other possible interpretations of their data.

1. We believe that the points that we discussed above now allow the interpretation the data in the way we did it. Nevertheless we followed the advice of the reviewer and tried other experimental strategies to prove our hypothesis. In addition, we also are now more critical and open about other interpretations (last paragraph of discussion). Considering the recent work by Baixauli et al (Baixauli et al., 2011), we have tried the mitochondrial fission inhibitor mdivi-1 to inhibit mitochondrial translocation. Mdivi-1 inhibited mitochondrial fission as reported but also killed the cells during the calcium readdition protocol. Thus, we could not find any conditions to use mdivi-1 to strengthen our conclusion that the mitochondrial distance regulates the IS calcium concentrations and thereby the pump rates.

2. Since we did not find other experimental ways to strengthen the findings related to the drugs we tested our hypothesis by mathematical modeling. To provide a third piece of independent evidence for our hypothesis (besides the direct TIRF measurements of local calcium signals and the dug based approach), we modeled calcium data as a function of the mitochondrial distance from the IS. We have used a one-dimensional mathematical model to predict the dependence of global Ca concentrations on local Ca concentrations at the IS as a function of the distance between IS and mitochondria. The model is described in the results section and in Fig. 9A-C. The mathematical details are summarized in the Supplementary Material. We believe that the model adds new evidence that mitochondrial localization is the key to determine local and global calcium, which would in turn modulate the pump rates.

Although the manuscript is very well written, the authors should make an effort to simplify the text in a way that all experiments can be fully understood by scientist working in different fields.

We fully agree with the reviewer that the MS is very complex and needs to be simplified and better explained such that most scientists from different fields can understand it. We have, therefore, re-written most of the MS in particular the whole results section has been completely reworked. Because the large number of supplementary figures hampered reading in the original ms, we have greatly reduced their number. The more important parts were moved into the regular figures 1-9, the less important ones are now only explained in the text.

Referee #2 (Remarks to the Author):

This manuscript reports on Ca2+ signaling in the immunological synapse (IS). The main finding is that Ca2+ is significantly lower in the IS due to Ca2+ uptake by mitochondria that also reduced the activity of PMCA, which is also found in the SI upon T cell stimulation and IS formation. This was very difficult manuscript to follow and I had to read several paragraph and conclusions multiple times to figure out what the authors intend to convey and the reasoning for each conclusions. Hence, the manuscript needs significant simplifications, especially with the large number of Figs. in the manuscript (20 including in the supplement). Several of the Figs. can be easily removed or simplified so that there will not be the need to refer so much to the supplementary figures with the very long Fig. legends.

We fully agree with the reviewer that the MS is very complex and needs to be simplified and better explained in a way that most scientists from different fields can understand it. We have, therefore, re-written most of the MS. We have also restructured the RESULTS section as evident from our new figure list. Since in the original ms, the many supplementary figures hampered the reading, we have greatly reduced their number. The more important parts were moved into the regular figures 1-9, the less important ones are now only explained in the text.

After sorting through the manuscript I think it has a good message that is supported by the data, but significant modifications and clarification and additional experiments are needed before further considerations. 1. Page 7, first paragraph- recruitment of mitochondria is observed on formation of the IS but not after treatment with thapsigargin. This is different from several observations, including by the authors, that mitochondria play important role in controlling the CRAC current activated by thapsigargin. The reason for the differences and how mitochondria can control Ca2+ influx in thapsigargin-treated cells if they are not recruited to the plasma membrane need to be explained.

We agree and are now trying to explain this part better. As we explained in the third paragraph of our discussion, this apparent contradiction about the mitochondrial control of CRAC/ORAI channel activity between TG and IS stimulation based on the mitochondrial position is explained by the Ca2+ diffusion and Ca2+ microdomain theories of Erwin Neher (Neher, 1998). Basically, Neher demonstrated that only Ca2+ microdomains generated between 100-200 nm or further away from the plasma membrane can be modulated by endogenous or exogenous buffer. The latter feature results from the fact that binding of Ca2+ to buffers is simply not fast enough for Ca2+ to be intercepted by buffers (even those buffers with a fast Ca2+ binding kinetics like BAPTA) at shorter distances (i.e. 20-80 nm). So, it is clear those mitochondria as a Ca2+ buffer can not be faster or more efficient than BAPTA or EGTA. This is more critical if mitochondria are placed closer to the channel. We know from electrophysiological data (Gilabert et al., 2001; Gilabert and Parekh, 2000; Glitsch et al., 2002; Hoth et al., 2000; Montalvo et al., 2006; Quintana et al., 2006) that mitochondrial control of CRAC/ORAI channel is important under physiological conditions of weak intracellular Ca2+ buffering (1.2 mM EGTA) but not in the presence of high concentration of Ca2+ chelator (10 mM EGTA). From these results the size of Ca2+ microdomains that induces the slow Ca2+- dependent inactivation of ORAI channels can be predicted. The mean path length that a Ca2+ ion travels before being intercepted by a buffer molecule is given by (Dca/konB). It depends on the chelator's on-rate kon, the free buffer concentration B and the Ca2+ diffusion co-efficient Dca. Both Dca and kon are constant but buffer concentrations may change. While 10 mM of EGTA starts to intercept Ca2+ at 136 nm, 1.2 mM starts to buffer Ca2+ at 396 nm away from the pore of the channel. Thus, under physiological conditions, the slow Ca2+-dependent inactivation of ORAI channels is controlled by a Ca2+ microdomain whose size ranges between ~140 and 400 nm. This covers the distance range (~150-200 nm) between mitochondria and ORAI channels at the IS. Hence, the positioning of mitochondria relative to ORAI1/3 channels can explain the mitochondrial control of CRAC/ORAI channel activity as previously described. In addition, active ORAI channels form clusters (1600ñ9000 nm2) (Park et al., 2009) upon TG stimulation and cap-like structures after IS formation, which generate larger and higher Ca2+ microdomains than that predicted beneath a single channel (Fig. 9D). The latter explains why mitochondrial Ca2+ uptake and mitochondrial translocation towards the PM (once they reach the area < 900 nm from the PM)

upon TG stimulation also control CRAC/ORAI channel activity, although mitochondria never localize within 200 nm of the PM under these conditions. However, the mitochondrial control of ORAI channel activity is much less efficient in the absence of IS formation, since sustained global Ca2+ signals following TG stimulation are significantly lower than global Ca2+ signals in the presence of the IS (Quintana et al., 2007). We have polished this part a bit in the ms and hope it is now easier to understand.

2. It is not clear how the mitochondria are recruited to the IS. Is the Ca2+ increase or treatment with CD3 independent of Ca2+ influx is sufficient to recruit the mitochondria?

We have previously shown that mitochondria are recruited to the IS (Quintana et al., 2007) but not to the calcium source, however calcium influx through CRAC/ORAI channels but not Ca2+ release from stores is required to move mitochondria (Schwindling et al., 2010). We have also shown that mitochondrial movement is along microtubules in a kinesin/dynein dependent manner (Quintana et al., 2009; Quintana et al., 2006). Recently, Contento et al (Contento et al., 2010) have demonstrated that LFA-1 is also important for mitochondrial recruitment. We confirmed this in the revised version (Fig. 4A, C). Next to LFA-1, Baixauli et al (Baixauli et al., 2011) have reported that the fission factor DRP-1 may be involved in recruiting mitochondria as well. We have incorporated a sentence summarizing these findings in the ms where we introduce mitochondrial accumulation at the IS: "Mitochondrial accumulation at the IS depends on several factors including TCR activation and Ca2+ signaling (Quintana et al., 2007; Schwindling et al., 2010), LFA- 1 activation (Contento et al., 2010) and the fission factor DRP-1 (Baixauli et al., 2011)."

4. *Page 8, second paragraph: an important conclusion offered by the authors is that the low Ca2+ at the IS is required for high global Ca2+. This is not tested rigorously. The authors are in a good position to carefully determine the relationship between Ca2+ at the IS and global Ca2+ using the different protocols used in the other figures to manipulate Ca2+ at the IS. The authors should simultaneously measure Ca2+ at the IS and global Ca2+, as in Fig. 3 under each of the conditions that changes IS.*

We agree with the reviewer and have performed the suggested experiment. It is a difficult experiment because the TIRF angle has to be constantly controlled and readjusted. Fig. 4C and D now show simultaneous calcium signals at the IS (with TIRF) and global calcium signals with epifluorescence in the same cells (for no IS and IS with CD3 conditions, blue and red curves). The outcome is the same as before. IS formation induced a higher global Ca2+ signal while it significantly reduced the local Ca2+ signal compared to No IS stimulation. This is translated into a larger and more sustained translocation of NFAT in the nucleus (Fig. 4F) and thereby into a more efficient activation and larger proliferation of T-cells. We have also calibrated the fluorescence data as recommended by the third reviewer. This is rarely done in TIRF. However the values have to be interpreted with caution since we had to use some values from an in vitro calibration. We explain this in the results section. The individual measurements of the old local measurements are now shown in Fig. 4E. They are supplemented by a condition + CCCP and a condition without TG. + CCCP eliminates the differences between IS and NoIS, which makes sense if mitochondria control local ca signals.

5. *In relation to 3, the authors inferred the PMCA activity at the IS from measuring global Ca2+. This underestimates PMCA activity. The authors should measure PMCA activity at the IS using their protocol of Ca2+ out removal while recording Ca2+ at the IS, as in Fig. 3.*

We thank the reviewer for this suggestion. This is a very good and straight forward experiment that we had overlooked. Figure 6C shows the data. In this case we have overlayed the 1 and 20 mM calcium data (because the reviewer suggested below that "the authors should measure Ca2+ at the IS in the presence of 1 and 20 Ca2+ out and show it to be the same"). We have analyzed the local pump rates by TIRF microscopy. Fig. 6D shows that the local PMCA Ca2+ pump rate in the presence of IS the Ca2+ clearance rate of PMCA was not significantly different between 1

and 20 mM external Ca2+. From our epifluorescence analysis (Fig. 6 A-B), this was expected. However, the pump rate was increased by about twofold (Fig. 6D) in case no IS was formed and the Ca2+ microdomain near the channels increased (Fig. 4C) by enhancing the Ca2+ driving force through CRAC/ORAI channel by augmenting the extracellular Ca2+ concentration from 1 to 20 mM. Overall, we confirmed our previous results that PMCA activity is locally down regulated at the IS because the Ca2+ microdomain beneath CRAC/ORAI channels is significantly reduced by mitochondrial Ca2+ uptake following the large accumulation of mitochondria at the IS. Furthermore PMCA co-localizes more with mitochondria than with ORAI at the IS due to a specific redistribution of both PMCA and ORAI1 into discrete areas (Fig. 3D). However, in NO IS stimulation PMCA and ORAI1 overlapped strongly because of the absence of PMCA redistribution at the PM (Fig. 3D and F).

5. Page 11, lines 13-16: The authors state that Ca2+ clearance is increased in thapsigargin-treated cells incubated at 20 mM Ca2+ even though the global Ca2+ is the same, "as expected". It is not clear to me how can this be expected. On the contrary, if Ca2+ was the same, so should PMCA activity. The authors should explain how Ca2+ clearance rates are so different with global Ca2+ being the same. This is even more puzzling with the findings that GLOBAL PMCA activity appear the same when IS is formed. The authors INTERPRATE this to suggest that recruitment of PMCA to the IS and buffering of Ca2+ by the mitochondria at the IS to be the same. This should be shown experimentally. The authors should measure Ca2+ at the IS in the presence of 1 and 20 Ca2+ out and show it to be the same!

1. We explain the sentence "It is expected that Ca2+ clearance is increased in thapsigargin-treated cells incubated at 20 mM Ca2+ even though the global Ca2+ is the same" now better in the results section. The experiment rationale follows the findings from the Lewis lab ((Bautista et al., 2002; Bautista and Lewis, 2004), ref 16 and 23 in the original submission). In 20 mM external Ca there is more Ca entry through ORAI channels. If a "dynamic" steady state exists (meaning that global calcium does not change) calcium export rates have to be increased also, otherwise global calcium would increase. The Lewis lab showed that PMCA was up-regulated under these conditions. We have rewritten this part to explain it better.

2. We fully agree with the suggestions by the reviewer to measure Ca2+ at the IS in the presence of 1 and 20 Ca2+ out and show it to be the same". We have performed the suggested experiment. We show that local calcium signals following IS formation are indeed the same in the presence of 1 mM and 20 mM (Fig. 6C). These results confirm our previous analysis of the local Ca2+ microdomain dependent PMCA activity as explained above (see please response to point 4). Therefore, our conclusion based on the analysis of iso-cells is reliable, though it probably underestimated the Ca2+ microdomain mediated modulation of PMCA activity.

6. *Page 13, lines 5, 6: It is stated that Orai3 is less sensitive to Ca2+. In fact Orai3 shows the strongest fast-Ca2+ dependent inactivation. The authors should be more specific to refer to the slow inhibition. The paper by Lis et al is not listed in the references*.

The reviewer is completely right that we must be more specific about which Ca2+ dependent modulation we talk about since ORAI3 channels, as reviewer pointed out, showed a strong fast inactivation but almost no slow inactivation compared to ORAI1 channels. Now we are clearer in turn of what kind of Ca2+ dependent inactivation of CRAC/ORAI channels we are talking about. We have incorporated the following part in the ms: "However, mitochondria by itself cannot regulate channel activity since they localize around 200 nm away from channel clusters. The local Ca2+ domain is likely to influence the activity of Ca2+ binding proteins such as calmodulin, a well known Ca2+ binding protein. Calmodulin interacts with and induces fast Ca2+-dependent inactivation of ORAI1 and ORAI3 (Frischauf et al., 2011; Mullins et al., 2009). Differences in the degree of Ca2+-dependent inactivation have been reported for ORAI1, ORAI2 and ORAI3, with ORAI3 showing the strongest fast inactivation but little slow inactivation (Lis et al., 2007). Whether calmodulin also influences the slow mitochondrial-dependent inactivation is not known. Calmodulin has also been reported to bind to the polybasic domain of STIM (Bauer et al., 2008), where it may affect association of this domain with the

PM and subsequently the Ca2+ influx through CRAC/ORAI channels. Recently a new Ca2+ binding protein, CRACR2A, has been described to participate in the formation and stability of STIM-ORAI1 complex (Srikanth et al., 2010). Although we do not yet know the threshold affinity of CRACR2A for Ca2+, one can speculate that mitochondrial-dependent Ca2+ microdomains around channels may regulate the function of CRACR2A and subsequently CRAC/ORAI channels. By preventing large accumulation of Ca2+ near the sites that govern slow inactivation of ORAI channels, mitochondria also reduce the Ca2+ microdomain-dependent modulation of PMCA. In this case, even though mitochondria and PMCA co-localize strongly at the IS, we have not found so far any evidence of organelle-protein interaction (data not shown). Nevertheless, since calmodulin has been described to modulate PMCA activity (Brini et al., 2003; Caride et al., 2007), one can again speculate that calmodulin dependent PMCA activity would be highly sensitive to the subplasmalemmal Ca2+ signal."

7. *Page 14, end of first paragraph: The conclusion from the nocodazole experiment is not clear at all. How the authors can conclude anything is not clear. Actually, since nocodazole has the same effect as the actin disruptors call into question the specificity of the effects rather than suggest the far reaching conclusion that PMCA modulation by local Ca2+ next to the open Orai channels is controlled by subplasmalemal mitochondria at the IS. Do you really think that the nocodazole experiment suggest all of that?*

We agree with the reviewer #1 (see above) and also reviewer #2 that nocodazole can inhibit synapse formation. However, we want also to point out that the disruption of microtubules polymerization by nocodazole is drug concentration and incubation time dependent (Huby et al 1998, Campello et al 2006, Yoder et al 2011, Takesono et al 2010, Quintana et al 2006). As shown in the study by Huby et al. (JBC 1998) mentioned by the reviewer #1, the effect is greatly concentration dependent. Nocodazole has clear effects when used above 10 uM. It has very little effects below 5 uM. We have used 2 uM in our study to minimize effects on the synapse or other non-specific parameters. For the reviewers convenience, we show (data shown to referees) that calcium release is not changed significantly by nocodazole and also Figure 4C and D (from PNAS 2007) that show that nocodazole does not interfere with ISinduced calcium signaling activated by the same beads that we have used for the nocodazole experiments. Thus, the IP3-dependent signaling cascade is not disrupted by nocodazole following TCR/CD3 complex stimulation because the calcium release from the endoplasmic reticulum was the same in control and in nocodazole treated cells. In other words, the abrogation of microtubule polymerization by nocodazole does not avoid the formation of signalosome beneath TCR/CD3 complex which induces the subsequent IP3 dependent signaling cascade.

CRAC/ORAI1 activity, pump rates and Ca2+ dependent K+ channel activity (not shown) are also not changed. This shows that the whole calcium influx and calcium release processes are not altered. We have also confirmed that nococdazole does not change ORAI1 accumulation in the TIRF experiments (quantification in Fig. 7). Consistent with the literature, actin is also accumulating at the IS. This means that IS stimulation activates normal calcium signaling with 2 uM nocodazole present. Indeed, the ISO cells analyzed in the relevant Figure 8 (with nocodazole) of the paper show a calcium plateau in 1 mM and 20 mM external calcium comparable to control (compare Figure 8C to Fig. 6A). These cells show significant calcium entry following IS formation and importantly local modulation of PMCA is now induced because mitochondrial accumulation is inhibited. That means we do have IS-stimulated calcium entry in the absence of mitochondrial accumulation, the condition we wanted to test. While nocodazole clearly allows IS formation, the actin poison drugs do not. Nocodazole is thus the stronger experiment. Nevertheless, without IS (as in the case of actin disrupters) and without mitochondrial accumulation, we expect pump modulation despite focal stimulation of the cells with beads in the presence of TG. This is what we found. We thus believe, these experiments make sense. We have clarified this in the text which reads now: "In case of actin poison drugs the situation was slightly different because the abrogation of actin polymerization disrupt of course IS formation (Fig. 7A) and thereby the accumulation of mitochondria into the vicinity of IS. But the final result was the same as that obtained with nocodazole treatment because CRAC/ORAI channels, STIM1 and PMCA undergo a normal ER and PM re-arrangement following TG stimulation as previously reported (Bakowski et al., 2001; Quintana et al., 2009; Quintana et al., 2006; Quintana et al., 2007). The ORAI fluorescence at the TIRF layer and the

ORAI fluorescence measured with epifluorescence were not significantly altered. In other words actin is not required for STIM1-ORAI1 dependent Ca2+ influx upon TG stimulation (Bakowski et al., 2001; Mueller et al., 2007), Supplementary Fig. 2). Therefore, if one co-stimulates latrunculin or cytochalasin D-treated cells with antibodies coated coverslips and TG, then cells should respond the same as No IS stimulated cells (Quintana et al., 2007). Thus, in the absence of actin polymerization mitochondria are not able to accumulate beneath the IS (Fig. 8A-B), even though Ca2+ influx through CRAC/ORAI channels is ensured by TG stimulation (Bakowski et al., 2001; Mueller et al., 2007), Supplementary Fig 2)." Nevertheless, we tried to come up with other experimental conditions to test pump modulations. Considering the recent work by Baixauli et al (Baixauli et al., 2011), we have tried the mitochondrial fission inhibitor mdivi-1 to inhibit mitochondrial translocation. Mdivi-1 inhibited mitochondrial fission as reported by Baixauli et al but also killed the cells during the calcium readdition protocol. Thus, we could not find any conditions to use mdivi-1 to strengthen our conclusion that the mitochondrial distance regulates the IS calcium concentrations and thereby the pump rates. Since we did not find other experimental ways to strengthen the findings related to the drugs we tested our hypothesis by mathematical modeling. To provide a third piece of independent evidence for our hypothesis (besides the direct TIRF measurements of local calcium signals and the dug based approach), we modeled calcium data as a function of the mitochondrial distance from the IS. We have used a one-dimensional mathematical model to predict the dependence of global Ca concentrations on local Ca concentrations at the IS as a function of the distance between IS and mitochondria. The model is described in the results section and in Fig. 9A-C. The mathematical details are summarized in the Supplementary Material. We believe that the model adds new evidence that mitochondrial localization is the key to determine local and global calcium, which would in turn modulate the pump rates.

8. *A relevant paper on the relationship between CRAC current and mitochondria that should be discussed in the context of the present findings is the recent work by Pozzan et at (Mol Cell. 2010 Apr 23;38(2):280-90.)*

We agree with the reviewer. We discuss the paper by the Pozzan group on HeLa cells and in addition we have performed experiments with TIRF on HeLa cells. Our experiments completely strengthen the point by Pozzan et al that mitochondria do not play a significant role in controlling SOC in HeLa cells. TIRF signals are very small and most importantly very little of the local signal is transported into a global signal (Figure 6 E and F). Thus we fully agree with Pozzan et al. when it comes to HeLa cells, however the immune synapse in T cells is significantly different. Here mitochondria are polarized to the IS where they come very close to the cell-cell contact site at the IS and can control the local calcium influx signals whereas in HeLa they control calcium release.

Referee #3 (Remarks to the Author):

This manuscript is reporting that formation of the immunological synapse in T helper cells results in the increased presence of Orai1 and mitochondria close to one another at the synapse, but exclusion of PMCa pump and SERCA pump. Local Ca buffering by the mitochondria is believed to explain the small local Ca signal (measured with TIRFM). According to authors, small local Ca produces less inactivation of Orai1 proteins, and this produces a big global Ca signal. This is an interesting study. I have following points to make. It is not so clear what is very new here, other than exclusion of PMCA at the synapse. Cahalan group already show Orai1 in the immunological synapse and Hoth group previously showed that mitochondrial move to the synapse and, recently, this position of mitochondria is very important for local calcium entry. They report that 'if mitochondria are close to any given local calcium entry site, calcium influx is large; if they are not close, calcium influx is small' (Schindling et al., J. Immunol. 2010). Actually, this seems different to this paper, where authors suggest local calcium influx is smaller at the synapse because of mitochondria.

The reviewer is right that our data support the prediction of the mitochondria model of controlling calcium channels like CRAC/ORAI or VOCs. There is no contradiction with the

paper by Schwindling et al (Schwindling et al., 2010), which shows that calcium influx is large if mitochondria are present at the IS or plasma membrane. In the present paper, calcium influx is also large if mitochondria are close to the IS. The large calcium influx generates the large global calcium concentration (Figure 4D). However, local calcium domains are low at the IS, because the calcium is rapidly taken up by the mitochondria, which later slowly export it back deep in the cytosol. The net calcium influx rate into the local domain is large, however the efflux out of the local domain (mostly into mitochondria) is also large. Exactly this finding is predicted by the model that mitochondria prevent slow CRAC channel inactivation. To do this they have to decrease the calcium between 100-400 nm away from the calcium entry sites. Through detailed and novel measurements of local calcium at the IS with TIRF we show that the subplasmalemmal Ca2+ signal beneath the IS is much lower than predicted (when taking into consideration the large accumulation of CRAC/ORAI channels at the cell-cell contact point). The low local calcium signal at the IS is required for T cell activation. Such measurements were not available up to now. In addition, our analysis of the interplay between mitochondria, ORAI and PMCA is also completely novel including the dependence of pump modulation on mitochondria. All these results are in our opinion very accurate and allow a very good understanding of calcium dependent T cell activation at the immune synapse. ORAI localization at the IS was also controversial because Cahalan's data did not fit completely with Samelson's data. Our localization analysis now clearly shows that ORAI1 is enriched at the IS in most cells and that the correct localization depends on a sufficient amount of STIM1 (we have analyzed ORAI1 and ORAI1/STIM1 transient over expression, as well as a low ORAI1 expressing stable cells line and endogenous protein). We believe that our novel results obtained by TIRFM are unique and shed light about those very important nanoscale events at the IS.

Authors hypothesis does not fit with recent works. Pozzan group showed in Cell (2010) that mitochondria are not close to the plasma membrane and not detect calcium influx signals. This was shown using new probe on mitochondrial surface. Also, Graier show mitochondria regulate Orai1 activity without need of moving to plasma membrane. How explain contradictory results and why not discussed?

The reviewer is of course right that Pozzan's group (Giacomello et al., 2007) and Graier's group (Naghdi et al., 2010) did not find that mitochondria moved to the plasma membrane. Pozzan's group used HeLa cells and Graier's group an endothelial cell line. Pozzan's group reported that mitochondria did not regulate CRAC/ORAI in Hela cells, while Graier's group in contrast showed that mitochondria did regulate CRAC/ORAI channels in the endothelial cells line but without moving. We have used the same conditions as we have used for the T cells to repeat the data in HeLa cells. We essentially find the same as the Pozzan group, namely that mitochondria do not regulate SOC in HeLa. In line with Pozzans group there are very few mitochondria at the plasma membrane (data not shown). We analyzed TIRF signals at the plasma membrane and the corresponding global calcium signals (Figure 6 E,F). Very little of the local calcium gets transported deeper into the cytosol. This means that mitochondria do not control plasma membrane signals in HeLa cells (exactly in line with Pozzans group). HeLa cells have little store-dependent CRAC/ORAI entry and compared to their size relatively few mitochondria at the plasma membrane. Consequently they have very small global calcium signals. Interestingly, the ratio between the local and the global calcium is very low (much lower as for the T cells, Figure 6E, F). This means that HeLa cells are not able to efficiently get the calcium away from the plasma membrane into the cytosol (which fits well with Pozzan's data). HeLa are much more calcium-release dependent while T cells depend a lot more on calcium influx. In our hands, HeLa and T cells are very different regarding the regulation of calcium by mitochondria and thus cannot be directly compared. The fact that cell type matters when it comes to specific differences, of which kind of Ca2+ microdomains mitochondria sense, has been previously also documented by others (Hoth et al., 1997; Montero et al., 2000; Varadi et al., 2004). Recently, Munos et al 2011 showed that mitochondria in Vascular Smooth Muscles cells take up Ca2+ from CRAC/ORAI channels but not from VOCE. This allows mitochondria to control the Ca2+ influx dependent proliferation of those cells. Although we do not know exactly the cell type specific mechanism that control the mitochondrial decision to take up Ca2+ from one or other Ca2+ source, one can speculate based on all information we have today. In case of T-cells the major, if not sole, Ca2+ influx pathway is the STIM1-ORAI1 mediated capacitative Ca2+ entry (Feske et al., 2006; Feske et al., 2005). T-cell activation and subsequent proliferation requires a sustained Ca2+ signal for hours (APC-T cell contact has been reported to take until 16-18 hours

(Friedman et al., 2010; Miller et al., 2002)). In this case, one would expect T-cells to control somewhat the slow Ca2+ dependent inactivation of CRAC/ORAI channels in order to keep a long lasting Ca2+ influx. Since mitochondria is a very powerful intracellular Ca2+ buffer (Rizzuto and Pozzan, 2006) but also a highly mobile organelle (Saotome et al., 2008; Yi et al., 2004), it is not surprising that T-cell decides to accumulate them (as also does with other organelles like Golgi or centrosome) right beneath the IS where all decision about cell activation takes place. This is now more striking with the enrichment of ORA1 and STIM1 molecules at the IS. In other words, the strategic localization of mitochondria within T-cells guarantees an efficient immune response. The movement and localization of mitochondria to the IS has been recently confirmed by two other groups (Baixauli et al., 2011; Contento et al., 2010). However, we would like to point out again the importance to understand the Ca2+ microdomain theory in the context of Ca2+ diffusion. Intracellular Ca2+ buffer (like calmodulin, ER, mitochondria) under physiological conditions would be able to sequester Ca2+ from channels if their position relative to those channels is the right one. The right position does not have to be the closer one. The latter is explained by the Ca2+ diffusion coefficient and the Ca2+ binding kinetic (Kon) of each buffer. Ca2+ diffusion and Ca2+ microdomain theories developed by Erwin Neher predicted that only Ca2+ microdomains generated in the range of about 100-200 nm or further away the mouth of a Ca2+ channel can be modulated by endogenous buffers. The same is true for exogenous buffers when used at concentrations that mimic physiological cell buffering capacity (1.2 mM EGTA). The latter features result from the fact that binding of Ca2+ to buffers is simply not fast enough for Ca2+ to be intercepted by buffers (Kon) at shorter distance (i.e. 20-80 nm).Therefore, it is not reasonable to expect that a mitochondrion, which is much less efficient and slower in sequestering Ca2+ than BAPTA or EGTA, immobilize at the plasma membrane within 5-10 nm (the mAKAP-RFP-CAAX plasma membrane linker is not longer than 5 nm linearly (Csordas et al., 2006)), would buffer Ca2+ more efficiently than one localize within 150-300 nm away of the plasma membrane. Then, from this analysis it is easy to explain the results of Graier's group. We actually obtain similar results by measuring local Ca2+ signals at the IS. With mitochondria anchored to the plasma membrane, local Ca2+ signal at the IS was as high as that observed in TG stimulated cells (data not shown). The only way mitochondria could take up more Ca2+ once attached to the membrane is if mitochondria co-localized with ORAI1. However that is not the case as shown here and by others (Korzeniowski et al., 2009). Such a close contact between mitochondria and Ca2+ channels has been shown for IP3 receptors in the ER. Recently Hajnoszky's group (Csordas et al., 2010) very elegantly demonstrated that both the area and gap width of the ER-mitochondria interface are important determinants of ER-derivate mitochondrial Ca2+ homeostasis. They found by changing the length of an artificial linker that anchored mitochondria to the ER that the closest contact is not the most effective to deliver the local Ca2+ signal into mitochondria. We hope that our arguments (and the HeLa cell data) can convince the reviewer that mitochondria play a very different role in T cells. They do influence ORAI/CRAC activity and they do move to the IS.

How is calcium measured here with TIRFM? I) I do not think TIRFM spatial resolution is sufficient to detect calcium microdomains.

We have used Fluo derivatives to measure calcium with TIRFM as has been routinely done by many other groups (Becherer et al. Nat Neuroscience 2003; Shgetomi Nature Neurosci 2010). Almost of what we know today about Ca2+ microdomain dependent exocytosis mechanisms in living cells has been possible by employing TIRFM. TIRFM has a z-direction resolution of about 150-200 nm which is given by the penetration depth of the evanescent wave. In xy direction TIRF has a resolution of approximately half the wavelength used for the measurement. This is usually in the range of about 200-300 nm. That means that one can clearly measure microdomains with a TIRF system, because the resolution is clearly below 1 uM. On the other hand, Ca2+ diffusion and Ca2+ microdomain theories developed by Erwin Neher (Neher, 1998) predicted that only Ca2+ microdomains generated in the range of about 100- 200 nm or further away the mouth of a Ca2+ channel can be modulated by endogenous or exogenous buffer. The latter feature results from the fact that binding of Ca2+ to buffers is simply not fast enough for Ca2+ to be intercepted by buffers (even those buffers with a fast Ca2+ binding kinetics like BAPTA) at shorter distance (i.e. 20-80 nm). So, it is easy to understand that from the technical

point of view TIRFM is maybe the best technique to allow an approximation of Ca2+ microdomains in living cells today.

2) There is no calibration of the dye signal. How high is the calcium? Authors speculate >10 uM, but this is based on dye affinity. 3) Data in Figure 3C/D is normalized deltaF. What is this here? Is it F-F0/F0? Why is local signal so much smaller than global, if local causes global?

The reviewer is completely right. And of course the local signal can not be lower than the global signal. We did not intend to make this statement. We were sloppy to speculate about the calcium concentration in the way we did. We have changed this. Usually, Fluo is not calibrated in TIRF measurements. We however completely agree with the reviewer that it should be tried in particular if we compare local and global calcium. We have performed new experiments and calibrated the data (see new Figure 4C and D). The global Ca2+ concentrations measured with Fluo-5F are by a factor of 2 lower than the Ca2+ concentrations we have measured previously with Fura-2 using an epifluorescence video-imaging system (Quintana and Hoth, 2004; Quintana et al., 2009; Quintana et al., 2006; Quintana et al., 2007). This may be a problem of the in-vitro calibration we had to perform for Fluo-5F with the TIRFM setup because we could technically not perform a calibration for each cell, which is in our opinion required for a reliable in-vivo calibration with a single wavelength dye. Our fluorescence were analyzed in the way that the reviewer mentioned, only that the values were multiplied by 100 (F = 100*((F-F0)/F0)) We have clarified this in the paper.

How long time is needed for formation of the immune synapse? No time course is shown. Figure 1 shows it is present around 1 hour later, but some stimuli are given in the paper for shorter time (supplementary 4A, anti-CD3 for 15 minutes). Synapse might not have formed, so how to conclude no SERCA is accumulated here? Also, please show no role for SERCA using labeling studies.

The reviewer is right to criticize this part. We should have been more explicit. Under our conditions an IS is initiated after very few minutes (compare also (Quintana et al., 2007), Fig. 4C) and it is stable for more than an hour (compare to (Schwindling et al., 2010), Fig. 1). In Figure 1 and 2 it is shown that IS formation starts after few minutes. The actin ring which is a hallmark of IS formation is fully established at 8 minutes (Supplementary Figure 1a), ORAI 1 is also already there but its content at the IS can vary during time. All of our functional analysis are performed at a time where an IS has been for sure formed. SERCA should be present at the IS (because STIM1 and the ER have to be there). Since in many experiments we have used Thapsigargin to inhibit SERCA, it does not play a functional role under our conditions. However, we have done one experiment that the reviewer had possibly in mind: We found that local Ca2+ signals without TG were similar to the ones obtained with TG at the IS (see Figure 4E), indicating that SERCA activity has no significant influence on local Ca2+ signals if an IS is formed. We were first very surprised by this result. However, it can be explained by the mitochondrial calcium uptake capability and kinetics when mitochondria are exposed to a high Ca2+ microdomain generated close to Ca2+ channels (Giacomello et al., 2010; Montero et al., 2000). Within this nanoscale environment mitochondrial Ca2+ uptake capacity and/or efficiency is much higher than the SERCA one (Rizzuto and Pozzan, 2003; Rizzuto and Pozzan, 2006). Therefore, this highlights the dominant role of mitochondria as intracellular Ca2+ buffer at the IS. In addition we have analyzed the distribution of SERCA in Fig. 1F. The figure shows that there is no preferential accumulation of SERCA at the IS formed with a Raji cell (left). In TIRF we have found the same (not shown but mentioned in the ms). This was seen in all analyzed cells. SERCA is not enriched at the IS and also from this point of view not a major player.

References 1. Baixauli, F., Martin-Cofreces, N. B., Morlino, G., Carrasco, Y. R., Calabia-Linares, C., Veiga, E., Serrador, J. M., and Sanchez-Madrid, F. (2011). The mitochondrial fission factor dynamic-related protein 1 modulates T-cell receptor signalling at the immunological synapse. Embo J 30, 1238- 1250.

2. Bakowski, D., Glitsch, M. D., and Parekh, A. B. (2001). An examination of the secretion-like coupling model for the activation of the Ca2+ release-activated Ca2+ current I(CRAC) in RBL-1 cells. J Physiol 532, 55-71.

3. Bauer, M. C., O'Connell, D., Cahill, D. J., and Linse, S. (2008). Calmodulin binding to the polybasic C-termini of STIM proteins involved in store-operated calcium entry. Biochemistry 47, 6089-6091.

4. Bautista, D. M., Hoth, M., and Lewis, R. S. (2002). Enhancement of calcium signalling dynamics and stability by delayed modulation of the plasma-membrane calcium-ATPase in human T cells. J Physiol 541, 877-894.

5. Bautista, D. M., and Lewis, R. S. (2004). Modulation of plasma membrane calcium-ATPase activity by local calcium microdomains near CRAC channels in human T cells. J Physiol 556, 805-817.

6. Brini, M., Colleto, L., Pierobon, N., Kraev, N., Guerini, D., and Carafoli, E. (2003). A comparative functional analysis of plasma membrane Ca2+ pump isoforms in intact cells. J Biol Chem 278, 24500-24508.

7. Campi, G., Varma, R., and Dustin, M. L. (2005). Actin and agonist MHC-peptide complexdependent T cell receptor microclusters as scaffolds for signaling. J Exp Med 202, 1031-1036.

8. Caride, A. J., Filoteo, A. G., Penniston, J. T., and Strehler, E. E. (2007). The plasma membrane Ca2+ pump isoform 4a differs from isoform 4b in the mechanism of calmodulin binding and activation kinetics: implications for Ca2+ signaling. J Biol Chem 282, 25640-25648.

9. Contento, L. R., Campello, S., Trovato, E. A., Magrini, E., Anselmi, F., and Viola, A. (2010). Adhesion shapes T cells for prompt and sustained T-cell receptor signalling. Embo J Oct 15, 1-13.

10. Csordas, G., Renken, C., Varnai, P., Walter, P., Weaver, D., Buttle, K. F., Balla, T., Mannella, C. A., and Hajnoczky, G. (2006). Structural and functional features and significance of the physical linkage between ER and mitochondria. J Cell Biol 174, 915-921.

11. Csordas, G., Varnai, P., Golenar, T., Roy, S., Purkins, G., Schneider, T. G., Balla, T., and Hajnoczky, G. (2010). Imaging interorganelle contacts and local calcium dynamics at the ERmitochondrial interface. Mol Cell 39, 121-132.

12. Dustin, M. L. (2005). A dynamic view of the immunological synapse. Semin Immunol 17, 400- 410.

13. Dustin, M. L. (2008). T-cell activation through immunological synapses and kinapses. Immunol Rev 221, 77-89.

14. Dustin, M. L., and Cooper, J. A. (2000). The immunological synapse and the actin cytoskeleton: molecular hardware for T cell signaling. Nat Immunol 1, 23-29.

15. Feske, S., Gwack, Y., Prakriya, M., Srikanth, S., Puppel, S. H., Tanasa, B., Hogan, P. G., Lewis, R. S., Daly, M., and Rao, A. (2006). A mutation in Orai1 causes immune deficiency by abrogating CRAC channel function. Nature 441, 179-185.

16. Feske, S., Prakriya, M., Rao, A., and Lewis, R. S. (2005). A severe defect in CRAC Ca2+ channel activation and altered K+ channel gating in T cells from immunodeficient patients. J Exp Med 202, 651-662.

17. Friedman, R. S., Beemiller, P., Sorensen, C. M., Jacobelli, J., and Krummel, M. F. (2010). Realtime analysis of T cell receptors in naive cells in vitro and in vivo reveals flexibility in synapse and signaling dynamics. J Exp Med 207, 2733-2749.

18. Frischauf, I., Schindl, R., Bergsmann, J., Derler, I., Fahrner, M., Muik, M., Fritsch, R., Lackner, B., Groschner, K., and Romanin, C. (2011). Cooperativeness of Orai cytosolic domains tunes subtype-specific gating. J Biol Chem 286, 8577-8584.

19. Giacomello, M., Drago, I., Bortolozzi, M., Scorzeto, M., Gianelle, A., Pizzo, P., and Pozzan, T. (2010). Ca2+ hot spots on the mitochondrial surface are generated by Ca2+ mobilization from stores, but not by activation of store-operated Ca2+ channels. Mol Cell 38, 280-290.

20. Giacomello, M., Drago, I., Pizzo, P., and Pozzan, T. (2007). Mitochondrial Ca2+ as a key regulator of cell life and death. Cell Death Differ 14, 1267-1274.

21. Gilabert, J. A., Bakowski, D., and Parekh, A. B. (2001). Energized mitochondria increase the dynamic range over which inositol 1,4,5-trisphosphate activates store-operated calcium influx. Embo J 20, 2672-2679.

22. Gilabert, J. A., and Parekh, A. B. (2000). Respiring mitochondria determine the pattern of activation and inactivation of the store-operated Ca(2+)

current I(CRAC). Embo J 19, 6401-6407.

23. Glitsch, M. D., Bakowski, D., and Parekh, A. B. (2002). Store-operated Ca2+ entry depends on mitochondrial Ca2+ uptake. Embo J 21, 6744-6754.

24. Hoth, M., Button, D. C., and Lewis, R. S. (2000). Mitochondrial control of calcium-channel gating: a mechanism for sustained signaling and transcriptional activation in T lymphocytes. Proc Natl Acad Sci U S A 97, 10607-10612.

25. Hoth, M., Fanger, C. M., and Lewis, R. S. (1997). Mitochondrial regulation of store-operated calcium signaling in T lymphocytes. J Cell Biol 137, 633-648.

26. Korzeniowski, M. K., Szanda, G., Balla, T., and Spat, A. (2009). Store-operated Ca(2+) influx and subplasmalemmal mitochondria. Cell Calcium 46, 49-55.

27. Lis, A., Peinelt, C., Beck, A., Parvez, S., Monteilh-Zoller, M. K., Fleig, A., and Penner, R. (2007). CRACM1, CRACM2, and CRACM3 are store-operated Ca2+ channels with distinct functional properties. Curr Biol 17, 794-800.

28. Miller, M. J., Wei, S. H., Parker, I., and Cahalan, M. D. (2002). Two-photon imaging of lymphocyte motility and antigen response in intact lymph node. Science 296, 1869-1873.

29. Montalvo, G. B., Artalejo, A. R., and Gilabert, J. A. (2006). ATP from subplasmalemmal mitochondria controls Ca2+ dependent inactivation of CRAC channels. J Biol Chem 281, 35616- 35623.

30. Montero, M., Alonso, M. T., Carnicero, E., Cuchillo-Ibanez, I., Albillos, A., Garcia, A. G., Garcia-Sancho, J., and Alvarez, J. (2000). Chromaffin-cell stimulation triggers fast millimolar mitochondrial Ca2+ transients that modulate secretion. Nat Cell Biol 2, 57-61.

31. Mueller, P., Quintana, A., Griesemer, D., Hoth, M., and Pieters, J. (2007). Disruption of the cortical actin cytoskeleton does not affect store operated Ca2+ channels in human T-cells. FEBS Lett 581, 3557-3562.

32. Mullins, F. M., Park, C. Y., Dolmetsch, R. E., and Lewis, R. S. (2009). STIM1 and calmodulin interact with Orai1 to induce Ca2+ dependent inactivation of CRAC channels. Proc Natl Acad Sci U S A 106, 15495-15500.

33. Naghdi, S., Waldeck-Weiermair, M., Fertschai, I., Poteser, M., Graier, W. F., and Malli, R. (2010). Mitochondrial Ca2+ uptake and not mitochondrial motility is required for STIM1-ORAI1 dependent store-operated Ca2+ entry. J Cell Sci 123, 2553-2564.

34. Neher, E. (1998). Vesicle pools and Ca2+ microdomains: new tools for understanding their roles in neurotransmitter release. Neuron 20, 389-399.

35. Park, C. Y., Hoover, P. J., Mullins, F. M., Bachhawat, P., Convington, E. D., Rausenr, S., Walz, T., Garcia, K. C., Dolmetsch, R., and Lewis, R. S. (2009). STIM1 clusters and activates CRAC channels via direct binding of a cytosolic domain to Orai1. Cell 136, 876-890.

36. Quintana, A., and Hoth, M. (2004). Apparent cytosolic calcium gradients in T-lymphocytes due to fura-2 accumulation in mitochondria. Cell Calcium 36, 99-109.

37. Quintana, A., Kummerow, C., Junker, C., Becherer, U., and Hoth, M. (2009). Morphological changes of T cells following formation of the immunological synapse modulate intracellular calcium signals. Cell Calcium 45, 109-122.

38. Quintana, A., Schwarz, E. C., Schwindling, C., Lipp, P., Kaestner, L., and Hoth, M. (2006). Sustained activity of CRAC channels requires translocation of mitochondria to the plasma membrane. J Biol Chem.

39. Quintana, A., Schwindling, C., Wenning, A. S., Becherer, U., Rettig, J., Schwarz, E. C., and Hoth, M. (2007). T cell activation requires mitochondrial translocation to the immunological synapse. Proc Natl Acad Sci U S A 104, 14418-14423.

40. Rizzuto, R., and Pozzan, T. (2003). When calcium goes wrong: genetic alterations of a ubiquitous signaling route. Nat Genet 34, 135-141.

41. Rizzuto, R., and Pozzan, T. (2006). Microdomains of intracellular Ca2+: molecular determinants and functional consequences. Physiol Rev 86, 369-408.

42. Saotome, M., Safiulina, D., Szabadkai, G., Das, S., Fransson, A., Aspenstrom, P., Rizzuto, R., and Hajnoczky, G. (2008). Bidirectional Ca2+-dependent control of mitochondrial dynamics by the Miro GTPase. Proc Natl Acad Sci U S A 105, 20728-20733.

43. Schwindling, C., Quintana, A., Krause, E., and Hoth, M. (2010). Mitochondria positioning controls local calcium influx in T cells. J Immunol 184, 184-190.

44. Srikanth, S., Jung, H.-J., Kim, K.-D., Souda, P., Whitelegge, J., and Gwack, Y. (2010). A novel EF-hand protein, CRACR2A, is a cytosolic Ca2+ sensor that stabilizes CRAC channels in T cells. Nat Cell Biol 12, 436-446.

45. Varadi, A., Cirulli, V., and Rutter, G. A. (2004). Mitochondrial localization as a determinant of capacitative Ca2+ entry in HeLa cells. Cell Calcium 36, 499-508. 46. Yi, M., Weaver, D., and Hajnoczky, G. (2004). Control of mitochondrial motility and distribution by the calcium signal: a homeostatic circuit. J Cell Biol 167, 661-672.

Acceptance letter 18 July 2011

Thank you for submitting your manuscript to the EMBO Journal.

I asked the original referees #1 and 2 to review the revised version and I have now received their comments back. As you can see below both referees appreciate the added changes and support publication in the EMBO Journal. I am therefore pleased to proceed with the acceptance of the paper for publication here. You will receive the formal acceptance letter shortly.

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

Editor

The EMBO Journal

Referee #1 I feel that the authors have done an excellent job responding to the criticisms. The new data significantly strengthen the paper and the editorial changes have considerably improved the readability of the manuscript.I appreciate the attention the authors have made to address all of the points I have brought up and feel that the paper should be accepted for publication.

Referee #2 The authors thoroughly address all concerns and this excellent study is now suitable for publication in the EMBO Journal.

