THE EMBO JOURNAL

Rewired signaling network in T cells expressing the chimeric antigen receptor (CAR)

Xiaolei Su, Rui Dong, Kendra Libby, Franziska Blaeschke, Walker Fuchs, Alexander Marson, and Ronald Vale
DOI: 10.15252/embj.2020104730

Corresponding author(s): Xiaolei Su (xiaolei.su@yale.edu) , Ronald Vale (ron.vale@ucsf.edu)

Review Timeline:

Transfer from Review Commons:	17th Feb 20
Editorial Decision:	19th Feb 20
Revision Received:	25th Apr 20
Editorial Decision:	25th May 20
Revision Received:	1st Jun 20
Accepted:	5th Jun 20

Editor: Karin Dumstrei

Transaction Report: This manuscript was transferred to The EMBO Journal following peer review at Review Commons.

1st Editorial Decision 19th Feb 2020

Dear Profs. Su and Vale,

Thank you for submitting your manuscript to The EMBO Journal. This manuscript was transferred from Review Commons where the review process was carried out.

I have now had a chance to take a careful look at your manuscript, the referee comments and your point-by-point response with your detailed revision plan.

I appreciate the included data on the 1st generation of CAR and find that this is a really nice and important addition to the paper. I also agree with your proposed revision plan on the other points raises. The timeline looks good as well. I would therefore like to invite you to submit a revised manuscript to The EMBO Journal - I have provided the revision link below.

Just a few editorial comments:

- I think it would be good to separate the results and discussion section. The manuscript would allow for a proper discussion of the findings. The manuscript is also fairly short to begin with and I think the introduction could also be expanded

- Please upload individual figure files

- Take a look at author guidelines regarding reference formatting, supplement figures, author contributions etc

Let me know if we need to discuss anything further - happy to do so!

With best wishes

Karin

Karin Dumstrei, PhD
Senior Editor
The EMBO Journal

Instructions for preparing your revised manuscript:

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, please visit our website:
https://www.embopress.org/page/journal/14602075/authorguide#transparentprocess

Please make sure you upload a letter of response to the referees' comments together with the revised manuscript.

Please also check that the title and abstract of the manuscript are brief, yet explicit, even to non-specialists.

When assembling figures, please refer to our figure preparation guideline in order to ensure proper formatting and readability in print as well as on screen:
http://bit.ly/EMBOPressFigurePreparationGuideline

IMPORTANT: When you send the revision we will require
- a point-by-point response to the referees' comments, with a detailed description of the changes made (as a word file).
- a word file of the manuscript text.
- individual production quality figure files (one file per figure)
- a complete author checklist, which you can download from our author guidelines (https://www.embopress.org/page/journal/14602075/authorguide).
- Expanded View files (replacing Supplementary Information)
Please see out instructions to authors
https://www.embopress.org/page/journal/14602075/authorguide#expandedview

Please remember: Digital image enhancement is acceptable practice, as long as it accurately represents the original data and conforms to community standards. If a figure has been subjected to significant electronic manipulation, this must be noted in the figure legend or in the 'Materials and Methods' section. The editors reserve the right to request original versions of figures and the original images that were used to assemble the figure.

Further information is available in our Guide For Authors:
https://www.embopress.org/page/journal/14602075/authorguide

The revision must be submitted online within 90 days; please click on the link below to submit the revision online before 19th May 2020.

Link Not Available

Please do not share this URL as it will give anyone who clicks it access to your account.

Reviews transferred from Review Commons

REVIEWER #1

Evidence, reproducibility and clarity

The authors compare the TCR alone to a CAR that contains signaling modules from three receptors- TCR, CD28 and 41BB. The data quality if good and the experiments done are. The difference is quite clear, and I would even like to see a little more of the evidence related to failure of the TCR system.

More specifically:

Su and colleagues show that a third generation CAR with TCR zeta, CD28 and 41BB signal transduction pathways can activate a T cell for microcluster formation and Gads/SLP-76 recruitment, but not IL-2 production, without LAT. This is surprising because LAT is generally considered, as is up held here, as an essential adapter protein for T cell activation. However, this is not a "fair" experiment as the CAR has sequences from TCR, and two co-stimulatory receptor- CD28 and 41BB. It would be important and very straight-forward to test first and second generation CARs to determine if LAT independence is a function of the CAR architecture itself, or the additional costimulatory sequences. If it turns out that a first generation CAR with only TCR sequences can trigger LAT independent clustering and SLP-76 recruitment then the comparison would be fair and no additional experiment would be needed to make the point that the CAR architecture is intrinsically LAT independent. If the CD28 and/or 41BB sequences are needed for LAT independence then the fair comparison would be to co-crosslink TCR, CD28 and 41BB (an inducible costimulator such that anti-CD27 might be substituted to have a constitutively expressed receptor with this similar motifs) should be cross-linked with the TCR to make this a fair comparison between the two architectures.

The authors may want to cite work from Vignali and colleagues that even the TCR has two signaling modules- the classical ZAP-70/LAT module that is responsible to IL-2 and a Vav/Notch dependent module that controls proliferation. Its not clear to me that the issue raised about distinct signaling by CARs is completely parallel to this, but its interesting that Vignali also associated the classical TCR signaling pathway as responsible for IL-2 with an alterive pathways that uses the same ITAMs to control distinct functions. See Guy CS, Vignali KM, Temirov J, Bettini ML, Overacre AE, Smeltzer M, Zhang H, Huppa JB, Tsai YH, Lobry C, Xie J, Dempsey PJ, Crawford HC, Aifantis I, Davis MM, Vignali DA. Distinct TCR signaling pathways drive proliferation and cytokine production in T cells. Nat Immunol. 2013;14(3):262-70.

I would be very interested to see a movie of the LAT deficient T cells interacting with the anti-CD3 coated bilayers in Figure 2A. Since OKT3 has a high affinity for CD3 and is coated on the suface at a density that should engage anti-CD3 I'm surprised there is no clustering even simply based on mass action. The result looks almost like a dominant negative effect of LAT deficiency on a high affinity extracellular interaction. It would be interesting to see how this interface evolves or if there is anti-adhesive behavior that emerges.

Significance

While it interesting that the CAR is LAT independent, its obvious that the signalling networks are different as the CAR has two sets of motifs that are absent in the TCR, so the experiments as presented are not that insightful about the specific nature of the differences that lead to the different outcomes. At present its not a particularly well controlled experiment as the third gen CAR is changing too many things in relation to the TCR for the experiment to be interpreted. It would be easy to address this is a revised manuscript. To publish as is the discussion would need to acknowledge these limitations. The work is preliminary as science, but it might be useful to T cell engineering field to have this information as a preliminary report, which might be an argument for adding discussion of limitations, but going forward without more detailed analysis of mechanism.

REVIEWER #2

Evidence, reproducibility and clarity

__Summary:__

> Provide a short summary of the findings and key conclusions (including methodology and model system(s) where appropriate).

In this study, the authors have interrogated CAR signaling by imaging CD19-CAR microclusters as well as T cell signaling molecules recruited to CAR microclusters. They report differences spatial assembly between CAR and TCR microclusters that form on a lipid bilayer containing ligand. They also report that LAT is not required for CAR microcluster formation, recruitment of downstream signaling molecules or IL-2 production in Jurkat cells, while in primary T cells IL-2 production by CARs show more of a LAT dependence. From these observations, they conclude that CAR T cells have a rewired signaling pathway as compared to T cells that signal through the TCR.

__Major comments:__

> Are the key conclusions convincing?

The conclusions made by the authors about CAR microclusters are convincing. However, the conclusion that there is a "rewired signaling network" different from TCR microclusters needs to be more convincingly demonstrated in side-by-side comparisons of TCR and CAR microclusters and synapses.

1. One of the key conclusions in this study is that CAR microclusters form in the absence of LAT, but TCR microclusters require LAT (in JCam2.5 cells in Fig. 2 and primary T cells in Fig. 4B). The requirement of LAT for formation of TCR microclusters is surprising, given multiple

reports (one of which the authors have cited) that TCR and ZAP70 clusters form normally in the absence of LAT (pZAP microclusters form normally in JCam2.5 cells Barda-Saad Nature Immunology 2005 Figure 1; TCR clusters form normally in LAT CRISPR KO Jurkat cells Yi et al., Nature Communications, 2019 Figure 5). The authors should carefully evaluate TCR and ZAP70 clusters (that form upstream of LAT) in their assays.

2. The authors make major conclusions about LAT dependence and independence of TCR and CAR microclusters respectively, by using JCam2.5 Jurkat cells and CRISPR/Cas9 edited primary cells. Of relevance to this conclusion, differences in the phosphorylation status of ZAP70 and SLP76 have been described between JCam2.5 cells lacking LAT (in which LAT was found to be deleted by gamma radiation) and J.LAT cells (in which LAT was specifically deleted by CRISPR/Cas9 in Lo et al Nature Immunology 2018). Of importance, pZAP and pSLP76 appeared fairly intact in J.LAT cells, but absent in JCam2.5 cells (Lo et al., Nat Immunol. 2018, Supp Fig 2). Therefore, the authors should evaluate TCR, ZAP70, Gads and SLP76 in TCR and CAR microclusters in J.LAT cells. This may partly explain the discrepancy in LAT requirement for IL-2 production in JCam2.5 cells and primary cells with LAT CRISPRed out.

3. Since the authors are reporting differences between CAR synapses and TCR synapses, the authors should show side by side comparison of CAR and TCR synapses in Figure 1F.

4. The authors should evaluate Gads microcluster formation in response to TCR stimulation via OKT3 (in Figure 4A). Given that it has been reported that TCR, Grb2 and c-Cbl are recruited to microclusters in Jurkat cells lacking LAT by CRISPR deletion (Yi et al., Nature Communications, 2019), it is important to establish the differences between TCR microclusters and CAR microclusters in side by side comparisons in their assay system.

5. Similar to the comment about Gads above, the authors should evaluate pSLP76 microcluster formation in response to TCR stimulation via OKT3 in primary T cells lacking LAT in Figure 4C, i.e. side by side comparisons of pSLP76 in TCR and CAR synapses (with and without LAT) should be shown.

> Should the authors qualify some of their claims as preliminary or speculative, or remove them altogether?

1. The data shown in Figure 3C shows a reduction in conjugate formation from 80% (WT) to 30% (LAT -). This is a severe reduction and does not support the authors' claim in the corresponding Figure legend that "LAT is dispensable for cell conjugate formation between Jurkat T cells expressing CAR and Raji B cells" and the Abstract that "LAT.....is not required for....immunological synapse formation". Statistical analysis for variance should be shown here.

2. In a similar vein, based on data from Movie S5 (where in a single cell, CAR microclusters translocate from cell periphery to center), and Figure 3C where (as described above in point 1) conjugate formation appears to be severely reduced, the authors conclude in the Results and Abstract that "LAT....is not required for actin remodeling following CAR activation". This conclusion is not supported by the data and the authors should remove this claim.

Alternatively, actin polymerization in CAR expressing cells (that are LAT sufficient and deficient) can be easily evaluated using phalloidin or F-Tractin.

> Would additional experiments be essential to support the claims of the paper? Request additional experiments only where necessary for the paper as it is, and do not ask authors to open new lines of experimentation.

Yes. Please see major comments above.

> Are the suggested experiments realistic in terms of time and resources? It would help if you could add an estimated cost and time investment for substantial experiments.

Yes. It should take 3 months to complete these experiments, since reagents and experimental systems to do these experiments already exist.

> Are the data and the methods presented in such a way that they can be reproduced?

Yes. Methods are clearly explained.

> Are the experiments adequately replicated and statistical analysis adequate?

There is no statistical analysis to evaluate differences between samples in Figures 3 and 4. These must be included.

__Minor comments:__

> Specific experimental issues that are easily addressable.

Please see Major Comments above. We believe that the recommended experiments are not difficult to execute since reagents exist and experimental systems are already set up.

> Are prior studies referenced appropriately?

Authors reference 13 and 14 for the following sentence in Results section 2: "Deletion or mutation of LAT impairs formation of T cell microclusters". However, in Reference 14 Barda-Saad et al., actually show that pZAP clusters are intact in JCam2.5 cells lacking LAT. Perhaps authors should clarify that LAT (and downstream signaling molecule) microclusters are impaired when LAT is deleted or mutated.

> Are the text and figures clear and accurate?

Yes. But would be helpful if authors specify what "control" is in Fig. 3B and C. In Figure 3B it is lipid bilayers without CD19, while in 3C it is K562 cells that do not express CD19.

> Do you have suggestions that would help the authors improve the presentation of their data and conclusions?

Would be helpful if authors specify in every Figure or at least Figure legend the experimental bilayer system/ligand used, since they use both OKT3 and CD19 as ligands in the paper.

Significance

> Describe the nature and significance of the advance (e.g. conceptual, technical, clinical) for the field.

If CAR microclusters and synapses are appropriately compared in a side by side comparison with TCR microclusters and synapses (as described in comments above), this study will be a conceptual advance in the field of CAR signaling. CAR microclusters have not been studied previously.

> Place the work in the context of the existing literature (provide references, where appropriate).

Very little imaging has been done on CAR synapses and to our knowledge this is the first live cell imaging study describing CAR microclusters.

> State what audience might be interested in and influenced by the reported findings.

This study will have a broad audience. Both scientists that study basic T cell signaling as well as clinicians that use CAR Ts will be interested in this study.

> Define your field of expertise with a few keywords to help the authors contextualize your point of view. Indicate if there are any parts of the paper that you do not have sufficient expertise to evaluate.

T cell signaling and imaging of proximal T cell signaling responses.

REVIEWER #3

Evidence, reproducibility and clarity

This manuscript by Dong and colleagues characterizes the molecular requirements and consequences of engaging a third-generation chimeric antigen receptor (CAR) directed to CD19. Utilizing a biological system of JCaM2.5, a Jurkat T cell mutant with dramatically low levels of LAT, expressing a CAR directed to CD19 fused to the cytoplasmic tails of CD28, 4-1BB and CD3□ that is activated by CD19/ICAM1 reconstituted lipid bilayers, the authors demonstrate LAT is not required for microcluster formation, immunologic synapse formation or recruitment of GADS and pSLP76 to the plasma membrane. In contrast, LAT was required for anti-CD3 mediated microcluster formation and pSLP76 recruitment to the

plasma membrane. However, LAT does appear to contribute to efficient synapse formation, PIP2 hydrolysis and IL-2 secretion when CAR+ JCaM2.5 or primary T cells are presented with Raji B cells, respectively. These data provide intriguing insights into the molecular requirements for third-generation CAR-T cell functions.

The authors have developed quite a nice system to understand the molecular contributions for CAR-T function. A few suggestions are provided here to further enhance the accuracy and significance of the findings:

1. The authors can address whether the LAT-independent effects are due to the attributes of third generation CAR-Ts with inclusion of CD28 and 4-1BB cytoplasmic domains or whether these differences are intrinsic to all CAR-Ts (e.g., first and second generation CARs).

2. Since a first-generation CAR-T forms non-conventional synapses (Davenport, et al., PNAS 2018), the authors should consider more detailed kinetic analysis to understand the formation and dissolution of the constituents of the synapse with their third generation CAR. This should include measurements of the duration of microcluster and synapse formation as well as further analysis of c- and p-SMAC constituents (e.g., LFA-1, TALIN, LCK and pSLP76) over time.

3. The authors utilize two different activation platforms. While using CD19/ICAM1 reconstituted bilayers, CAR+ JCaM2.5 or CAR+ primary T cells demonstrate no differences compared to wildtype JCaM2.5 cells in the parameters studied. However, when using Raji B cells, the CAR+ JCaM2.5 cells or CAR+ primary T cells demonstrate a more intermediate phenotype with respect to cell conjugate formation (Figure 3C) and IL-2 production (Figure 4D). The authors should analyze whether the differences attributed to the different outcomes may be due to the stimulation mode. For example, is c-SMAC assembly and GADS or pSLP76 recruitment to the plasma membrane still LAT-independent when activated with Raji B cells?

4. The authors should consider whether CAR expression level affects their observations. For example, do lower levels of CAR expression make the system LAT-dependent? Further, what is the level of the CAR relative to endogenous TCR expression on their primary T cells.

Minor comment:

1. Since JCaM2.5 has differences when compared to the parental Jurkat E6.1 T cell line, the authors should utilize JCaM2.5 reconstituted with wildtype LAT as a comparator.

Significance

The mechanism(s) by which CAR-Ts function is of high significance from both scientific and clinical viewpoints. From a scientific viewpoint, it provides important basic mechanistic information of how T cells are being activated to kill tumor cells. By understanding the molecular requirements, additional generations of CARs can be designed to provide greater efficacy, overcome resistance and possibly less toxicity.

This is an evolving field and little is known to date. Hence, this study could represent an insightful and important advance to the field.

Audience is to both basic immunologist and cancer biologists.

My expertise is in T cell signaling, T cell biology and immunotherapy.

Response to Referees: Review Commons Refereed Preprint #RC-2019-00134

General comments

We thank all three reviewers for providing their thoughtful and insightful review comments of our manuscript. We appreciate that the reviewers recognized the significance and impact of our work - "*Very little imaging has been done on CAR synapses and to our knowledge this is the first live cell imaging study describing CAR microclustsers*" (Reviewer 2); "*This is an evolving field and little is known to date. Hence, this study could represent an insightful and important advance to the field*" (Reviewer 3). A broad audience from both basic and clinical research sides will be interested in this work: "*This study will have a broad audience. Both scientists that study basic T cell signaling as well as clinicians that use CAR Ts will be interested in this study" (*Reviewer 2); "*Audience is to both basic immunologist and cancer biologists*" (Reviewer 3).

Meanwhile, we understand that the reviewers have raised a few major and minor issues, which we attempted to address. Most importantly, as suggested by both reviewer 1 and 3, we performed new experiments showing that LAT is not required for microcluster formation of the 1st generation of CAR (new Fig 4 and EV5). This finding suggests that the CAR-independent signaling is due to the intrinsic CAR architecture, and is not dependent on the co-signaling domains of CD28 and 4-1BB.

With the successful solutions to other issues, we believe the manuscript has been significantly improved and is ready for publication. Below we will provide point-to-point responses to each reviewer's comments.

Reviewer #1 (Evidence, reproducibility and clarity (Required)):

The authors compare the TCR alone to a CAR that contains signaling modules from three receptors- TCR, CD28 and 41BB. The data quality if good and the experiments done are. The difference is quite clear, and I would even like to see a little more of the evidence related to failure of the TCR system.

We appreciate the general positive comment of this reviewer.

More specifically:

Su and colleagues show that a third generation CAR with TCR zeta, CD28 and 41BB signal transduction pathways can activate a T cell for microcluster formation and Gads/SLP-76 recruitment, but not IL-2 production, without LAT. This is surprising because LAT is generally considered, as is up held here, as an essential adapter protein

for T cell activation. However, this is not a "fair" experiment as the CAR has sequences from TCR, and two co-stimulatory receptor- CD28 and 41BB. It would be important and very straight-forward to test first and second generation CARs to determine if LAT independence is a function of the CAR architecture itself, or the additional costimulatory sequences. If it turns out that a first generation CAR with only TCR sequences can trigger LAT independent clustering and SLP-76 recruitment then the comparison would be fair and no additional experiment would be needed to make the point that the CAR architecture is intrinsically LAT independent. If the CD28 and/or 41BB sequences are needed for LAT independence then the fair comparison would be to co-crosslink TCR, CD28 and 41BB (an inducible costimulator such that anti-CD27 might be substituted to have a constitutively expressed receptor with this similar motifs) should be cross-linked with the TCR to make this a fair comparison between the two architectures.

We agree with the reviewer that it is critical to make a “fair” comparison between TCR and CAR by testing the 1st generation CAR, which only contains the TCR/CD3ζ domain. Our new data showed that LAT is not required for microcluster and synapse formation of the 1st generation of CAR, in both Jurkat and primary T cells (new Fig 4 and EV5). This result is similar to our previously reported result from the 3rd generation CAR, although the 1st generation CAR induced less IL-2 production and CD69 expression in LAT null cells than the 3rd generation CAR did (new Fig 6). This suggests that the LAT-independent signaling is intrinsic to the CAR architecture, as the reviewer suggested. The co-signaling domains from CD28 and 4-1BB contribute to, but are not required for bypassing LAT to transduce the CAR signaling.

The authors may want to cite work from Vignali and colleagues that even the TCR has two signaling modules- the classical ZAP-70/LAT module that is responsible to IL-2 and a Vav/Notch dependent module that controls proliferation. Its not clear to me that the issue raised about distinct signaling by CARs is completely parallel to this, but its interesting that Vignali also associated the classical TCR signaling pathway as responsible for IL-2 with an alterive pathways that uses the same ITAMs to control distinct functions. See Guy CS, Vignali KM, Temirov J, Bettini ML, Overacre AE, Smeltzer M, Zhang H, Huppa JB, Tsai YH, Lobry C, Xie J, Dempsey PJ, Crawford HC, Aifantis I, Davis MM, Vignali DA. Distinct TCR signaling pathways drive proliferation and cytokine production in T cells. Nat Immunol. 2013;14(3):262-70.

We appreciate the reviewer’s mentioning this paper from Vignali’s group. It provides insights into understanding LAT-independent signaling in CAR T cells. We cited this paper and added a discussion about the mechanism of LAT-independent signaling.

I would be very interested to see a movie of the LAT deficient T cells interacting with the anti-CD3 coated bilayers in Figure 2A. Since OKT3 has a high affinity for CD3 and is coated on the surface at a density that should engage anti-CD3 I'm surprised there is no clustering even simply based on mass action. The result looks almost like a dominant negative effect of LAT deficiency on a high affinity extracellular interaction. It would be interesting to see how this interface evolves or if there is anti-adhesive behavior that emerges.

We now presented a movie showing the detailed process of LAT deficient GFP-CAR T cells landing on the bilayers coated with OKT3 (new Movie EV5), in which the bright field images delineate the locations of the cells, the OKT3 signal marks TCR, and the GFP signal marks CAR proteins on the plasma membranes. No TCR clusters (as indicated by OKT3) were formed during the landing process. We think the binding of bilayer-presented OKT3 to TCR is not sufficient to trigger TCR microclusters. However, TCR microclusters could form in LAT-deficient cells if OKT3 is presented by glass surface. This point is raised by reviewer 2. We added a discussion on the difference between bilayer and glass-presented OKT3 in inducing microcluster formation.

Reviewer #1 (Significance (Required)):

While it interesting that the CAR is LAT independent, its obvious that the signalling networks are different as the CAR has two sets of motifs that are absent in the TCR, so the experiments as presented are not that insightful about the specific nature of the differences that lead to the different outcomes. At present its not a particularly well controlled experiment as the third gen CAR is changing too many things in relation to the TCR for the experiment to be interpreted. It would be easy to address this is a revised manuscript. To publish as is the discussion would need to acknowledge these limitations. The work is preliminary as science, but it might be useful to T cell engineering field to have this information as a preliminary report, which might be an argument for adding discussion of limitations, but going forward without more detailed analysis of mechanism.

This is an excellent point and we have addressed it. See our response above on the new data of the 1st generation CAR.

Reviewer #2 (Evidence, reproducibility and clarity):

Summary:

Provide a short summary of the findings and key conclusions (including methodology and model system(s) where appropriate).

In this study, the authors have interrogated CAR signaling by imaging CD19-CAR microclusters as well as T cell signaling molecules recruited to CAR microclusters. They report differences spatial assembly between CAR and TCR microclusters that form on a lipid bilayer containing ligand. They also report that LAT is not required for CAR microcluster formation, recruitment of downstream signaling molecules or IL-2 production in Jurkat cells, while in primary T cells IL-2 production by CARs show more of a LAT dependence. From these observations, they conclude that CAR T cells have a rewired signaling pathway as compared to T cells that signal through the TCR.

Major comments:

- Are the key conclusions convincing?

The conclusions made by the authors about CAR microclusters are convincing. However, the conclusion that there is a "rewired signaling network" different from TCR microclusters needs to be more convincingly demonstrated in side-by-side comparisons of TCR and CAR microclusters and synapses.

1. One of the key conclusions in this study is that CAR microclusters form in the absence of LAT, but TCR microclusters require LAT (in JCam2.5 cells in Fig. 2 and primary T cells in Fig. 4B). The requirement of LAT for formation of TCR microclusters is surprising, given multiple reports (one of which the authors have cited) that TCRζ and ZAP70 clusters form normally in the absence of LAT (pZAP microclusters form normally in JCam2.5 cells Barda-Saad Nature Immunology 2005 Figure 1; TCRζ clusters form normally in LAT CRISPR KO Jurkat cells Yi et al., Nature Communications, 2019 Figure 5). The authors should carefully evaluate TCRζ and ZAP70 clusters (that form upstream of LAT) in their assays.

We thank the reviewer for raising this excellent point. LAT-independent TCR clusters were reported in the two papers mentioned by the reviewer, which we think is convincing. However, there is a key difference in the experimental settings between these two papers and ours. We use supported lipid bilayer to present MOBILE TCR-activating antibody to activate T cells, whereas these two papers used IMMOBILE TCR-activating antibody attached to the cover glass. We reasoned that the mobile surface of supported lipid bilayer more closely mimics the antigen-presenting cell surface where antigens are mobile on the membrane. We added a new discussion about the difference between supported lipid bilayer and cover glass-based activation.

We agree with the reviewer on the careful evaluation of TCR and ZAP70 clusters. We had showed the data of TCR clusters as marked by TCR-interacting OKT3 (Fig 3A). We performed new experiments on ZAP70 clusters (new Fig EV3). Our data suggest that, similar to TCR clusters, ZAP70 clusters are not formed in LAT-deficient T cells, if activated by OKT3, but are formed if activated by CD19.

2. The authors make major conclusions about LAT dependence and independence of TCR and CAR microclusters respectively, by using JCam2.5 Jurkat cells and CRISPR/Cas9 edited primary cells. Of relevance to this conclusion, differences in the phosphorylation status of ZAP70 and SLP76 have been described between JCam2.5 cells lacking LAT (in which LAT was found to be deleted by gamma radiation) and J.LAT cells (in which LAT was specifically deleted by CRISPR/Cas9 in Lo et al Nature Immunology 2018). Of importance, pZAP and pSLP76 appeared fairly intact in J.LAT cells, but absent in JCam2.5 cells (Lo et al., Nat Immunol. 2018, Supp Fig 2). Therefore, the authors should evaluate TCRζ, ZAP70, Gads and SLP76 in TCR and CAR microclusters in J.LAT cells. This may partly explain the discrepancy in LAT requirement for IL-2 production in JCam2.5 cells and primary cells with LAT CRISPRed out.

Jcam2.5 is a classical well-characterized LAT-deficient cell line that has been continuously used in the T cell signaling field (Barda-Saad Nature Immunology 2005, Rouquette-Jazdanian A, Mol. Cell, 2012; Balagopalan L, J Imm. 2013; Carpier J, J Exp Med, 2018; Zucchetti A, Nat. Comm. 2019). We agreed with the concern that the reviewer raised on the absence of pZAP70 and pSLP76 in JCam2.5 cells. As the reviewer suggested, we obtained J.LAT, which is LAT null but has intact pZAP70 and pSLP76. We introduced CAR into J.LAT and the wild-type control and performed the clustering assay as we did for Jcam2.5. Our results showed that, similar to Jcam2.5, CAR forms robust microclusters in J.LAT cells (new Fig EV2). More importantly, we presented data confirming the LAT-independent CAR clustering, SLP76 phosphorylation, and IL-2 production in human primary T cells (Fig 7). Therefore, the data from three independent cell sources support our conclusion on LAT-independent CAR signal transduction.

3. Since the authors are reporting differences between CAR synapses and TCR synapses, the authors should show side by side comparison of CAR and TCR synapses in Figure 1F.

We focused on characterizing CAR synapse in this manuscript and did not make any conclusion on the difference between TCR and CAR synapse. We are cautious about comparing CAR synapse to TCR synapse for technical reasons: it is critical to use antigen-specific TCRs (e.g. mouse OTI as a common model) to study the TCR synapse pattern so that the study will be physiologically relevant. However, we use human T cell line and human primary T cells for the CAR study. The technical barrier to introduce an

antigen-specific TCR complex into these cells, and to activate these cells by purified peptide-MHC complex, is very high. And the result is interesting, but beyond the scope of the current work.

4. The authors should evaluate Gads microcluster formation in response to TCR stimulation via OKT3 (in Figure 4A). Given that it has been reported that TCRζ, Grb2 and c-Cbl are recruited to microclusters in Jurkat cells lacking LAT by CRISPR deletion (Yi et al., Nature Communications, 2019), it is important to establish the differences between TCR microclusters and CAR microclusters in side by side comparisons in their assay system.

As the reviewer suggested, we evaluated Gads microcluster formation with TCR stimulation and found that Gads did not form microclusters in LAT-deficient cells (new Fig 5A). Because we only made conclusions on the Gads-SLP76 pathway, we think investigating Grb2 and c-Cbl microcluster, though interesting, is beyond the scope of this manuscript.

5. Similar to the comment about Gads above, the authors should evaluate pSLP76 microcluster formation in response to TCR stimulation via OKT3 in primary T cells lacking LAT in Figure 4C, i.e. side by side comparisons of pSLP76 in TCR and CAR synapses (with and without LAT) should be shown.

We totally agree and performed new experiment on pSLP76 in human primary T cells. Our data suggested that, similar to Jurkat, pSLP76 microclusters remain intact in LAT null primary cells (new Fig 7D and 7E).

- Should the authors qualify some of their claims as preliminary or speculative, or remove them altogether?

1. The data shown in Figure 3C shows a reduction in conjugate formation from 80% (WT) to 30% (LAT -). This is a severe reduction and does not support the authors' claim in the corresponding Figure legend that "LAT is dispensable for cell conjugate formation between Jurkat T cells expressing CAR and Raji B cells" and the Abstract that "LAT.....is not required for....immunological synapse formation". Statistical analysis for variance should be shown here.

We agree with the reviewer’s judgement. This cell conjugation analysis was performed using Jcam2.5 cells. As pointed by the reviewer, Jcam2.5 has additional defects in ZAP70 and SLP76 in addition to the lack of LAT. Therefore, we performed the same analysis again using J.LAT cells, which was recommended by the reviewer. Our new data showed that J.LAT cells form conjugates with Raji B cells in a similar rate as the wild-type cells do, as evaluated by statistical analysis (new Fig 6A). Therefore, we think these new data support the claim that LAT is dispensable for cell conjugate formation.

2. In a similar vein, based on data from Movie S5 (where in a single cell, CAR microclusters translocate from cell periphery to center), and Figure 3C where (as described above in point 1) conjugate formation appears to be severely reduced, the authors conclude in the Results and Abstract that "LAT....is not required for actin remodeling following CAR activation". This conclusion is not supported by the data and the authors should remove this claim. Alternatively, actin polymerization in CAR expressing cells (that are LAT sufficient and deficient) can be easily evaluated using phalloidin or F-Tractin.

As suggested by the reviewer, we evaluated actin polymerization in TCR or CAR stimulated cells using a filamentous actin reporter F-tractin. Our data showed that LAT is required for TCR-induced but not CAR-induced actin polymerization (new Fig 5C). Therefore, our results support the claim that LAT is not required for actin remodeling following CAR activation.

- Would additional experiments be essential to support the claims of the paper? Request additional experiments only where necessary for the paper as it is, and do not ask authors to open new lines of experimentation.

Yes. Please see major comments above.

- Are the suggested experiments realistic in terms of time and resources? It would help if you could add an estimated cost and time investment for substantial experiments. Yes. It should take 3 months to complete these experiments, since reagents and experimental systems to do these experiments already exist.

- Are the data and the methods presented in such a way that they can be reproduced? Yes. Methods are clearly explained.

We appreciate the reviewer’s recognition of the clarity of the methods part.

- Are the experiments adequately replicated and statistical analysis adequate? There is no statistical analysis to evaluate differences between samples in Figures 3 and 4. These must be included.

We now added statistical analysis in Fig 5B and 6A (old figure 3 and 4).

Minor comments:

- Specific experimental issues that are easily addressable.

Please see Major Comments above. We believe that the recommended experiments are not difficult to execute since reagents exist and experimental systems are already set up.

- Are prior studies referenced appropriately?

Authors reference 13 and 14 for the following sentence in Results section 2: "Deletion or mutation of LAT impairs formation of T cell microclusters". However, in Reference 14 Barda-Saad et al., actually show that pZAP clusters are intact in JCam2.5 cells lacking LAT. Perhaps authors should clarify that LAT (and downstream signaling molecule) microclusters are impaired when LAT is deleted or mutated.

As the reviewer suggested, we now clarified that clustering of LAT downstream binding partners is impaired when citing reference (Barda-Saad et al).

- Are the text and figures clear and accurate?

Yes. But would be helpful if authors specify what "control" is in Fig. 3B and C. In Figure 3B it is lipid bilayers without CD19, while in 3C it is K562 cells that do not express CD19.

We now specified “control” in the figure.

- Do you have suggestions that would help the authors improve the presentation of their data and conclusions?

Would be helpful if authors specify in every Figure or at least Figure legend the experimental bilayer system/ligand used, since they use both OKT3 and CD19 as ligands in the paper.

We now specified the ligand in the figure or legend.

Reviewer #2 (Significance):

- Describe the nature and significance of the advance (e.g. conceptual, technical, clinical) for the field.

If CAR microclusters and synapses are appropriately compared in a side by side comparison with TCR microclusters and synapses (as described in comments above), this study will be a conceptual advance in the field of CAR signaling. CAR microclusters have not been studied previously.

- Place the work in the context of the existing literature (provide references, where appropriate).

Very little imaging has been done on CAR synapses and to our knowledge this is the first live cell imaging study describing CAR microclusters.

We appreciate this reviewer's comment on our work as a conceptual advance in understanding CAR signaling.

- State what audience might be interested in and influenced by the reported findings. This study will have a broad audience. Both scientists that study basic T cell signaling as well as clinicians that use CAR Ts will be interested in this study.

We appreciate this reviewer's recognition of the broad audience of this manuscript.

- Define your field of expertise with a few keywords to help the authors contextualize your point of view. Indicate if there are any parts of the paper that you do not have sufficient expertise to evaluate.

T cell signaling and imaging of proximal T cell signaling responses.

Reviewer #3 (Evidence, reproducibility and clarity):

This manuscript by Dong and colleagues characterizes the molecular requirements and consequences of engaging a third-generation chimeric antigen receptor (CAR) directed to CD19. Utilizing a biological system of JCaM2.5, a Jurkat T cell mutant with dramatically low levels of LAT, expressing a CAR directed to CD19 fused to the cytoplasmic tails of CD28, 4-1BB and CD3ζ that is activated by CD19/ICAM1 reconstituted lipid bilayers, the authors demonstrate LAT is not required for microcluster formation, immunologic synapse formation or recruitment of GADS and pSLP76 to the plasma membrane. In contrast, LAT was required for anti-CD3 mediated microcluster formation and pSLP76 recruitment to the plasma membrane. However, LAT does appear to contribute to efficient synapse formation, PIP2 hydrolysis and IL-2 secretion when CAR+ JCaM2.5 or primary T cells are presented with Raji B cells, respectively. These data provide intriguing insights into the molecular requirements for third-generation CAR-T cell functions.

The authors have developed quite a nice system to understand the molecular contributions for CAR-T function. A few suggestions are provided here to further enhance the accuracy and significance of the findings:

1. The authors can address whether the LAT-independent effects are due to the attributes of third generation CAR-Ts with inclusion of CD28 and 4-1BB cytoplasmic domains or whether these differences are intrinsic to all CAR-Ts (e.g., first and second generation CARs).

This is an excellent point. We have included new data showing LAT-independent cluster formation of the 1st generation CAR in both Jurkat and primary T cells (new Fig 4 and EV5). Therefore, we favor the second possibility as pointed by the reviewer that LAT-independent effects are intrinsic to CAR architecture.

2. Since a first-generation CAR-T forms non-conventional synapses (Davenport, et al., PNAS 2018), the authors should consider more detailed kinetic analysis to understand the formation and dissolution of the constituents of the synapse with their third generation CAR. This should include measurements of the duration of microcluster and synapse formation as well as further analysis of c- and p-SMAC constituents (e.g., LFA-1, TALIN, LCK and pSLP76) over time.

We agree with the reviewer on a more detailed characterization of the CAR synapse. We measured the duration of the unstable CAR synapse and time from cell landing to the start of retrograde flow (new Fig 2C). We also determined the localization of CD45, a marker for d-SMAC (new Fig 2D). We found that the formation of dSMAC is also not common in CAR T synapse, strengthening our conclusion that CAR forms non-typical immunological synapse.

3. The authors utilize two different activation platforms. While using CD19/ICAM1 reconstituted bilayers, CAR+ JCaM2.5 or CAR+ primary T cells demonstrate no differences compared to wildtype JCaM2.5 cells in the parameters studied. However, when using Raji B cells, the CAR+ JCaM2.5 cells or CAR+ primary T cells demonstrate a more intermediate phenotype with respect to cell conjugate formation (Figure 3C) and IL-2 production (Figure 4D). The authors should analyze whether the differences attributed to the different outcomes may be due to the stimulation mode. For example, is c-SMAC assembly and GADS or pSLP76 recruitment to the plasma membrane still LAT-independent when activated with Raji B cells?

As the reviewer suggested, we examined c-SMAC assembly in Raji B cells conjugated with CAR T cells. We found that the majority of CAR do not form cSMAC (new Fig EV4), which is consistent with the result from the bilayer activation system. Since both Gads and SLP76 are cytosolic proteins, they keep largely in the cytosolic pool which obscures their recruitment and clustering on the plasma membrane when imaged by confocal microscopy at the cross-section of cell-cell synapse.

4. The authors should consider whether CAR expression level affects their observations. For example, do lower levels of CAR expression make the system LAT-dependent? Further, what is the level of the CAR relative to endogenous TCR expression on their primary T cells.

We agree with the reviewer that it is informative to determine if LAT-independent signaling is dose dependent. We tried to measure the CAR concentration relative to the endogenous TCR/CD3ζ. By western blot using two different antibodies against CD3ζ, we detected TCR/CD3ζ expression, but found no bands corresponding to CAR. We believe this reflects a low expression of CAR in our system, which is confirmed by FACS. The general low expression of CAR makes it challenging to sort an even lower CAR-expressing population. Therefore, we sought alternative ways to determine the dose-dependence; we titrated the CD19 concentrations on the bilayer. As shown in the new Figure EV1, CAR formed microclusters similarly in the wild-type versus LAT-deficient cells in a wide range of CD19 concentration. Therefore, we conclude that the LAT-independent cluster formation is robust at low antigen density as well.

Minor comment:

1. Since JCaM2.5 has differences when compared to the parental Jurkat E6.1 T cell line, the authors should utilize JCaM2.5 reconstituted with wildtype LAT as a comparator.

Agreeing with this reviewer, we recognized that Jcam2.5 was generated by mutagenesis which may result in protein expression difference for genes besides Lat. As suggested by reviewer1, we used J.LAT, a genuine LAT knockout cell line that is generated by CRISPR-mediated gene targeting, to perform the clustering assay (new Fig EV2). Our results showed that, similar to Jcam2.5, CAR but not the TCR formed microclusters in J.LAT cells.

Reviewer #3 (Significance):

The mechanism(s) by which CAR-Ts function is of high significance from both scientific and clinical viewpoints. From a scientific viewpoint, it provides important basic mechanistic information of how T cells are being activated to kill tumor cells. By understanding the molecular requirements, additional generations of CARs can be designed to provide greater efficacy, overcome resistance and possibly less toxicity.

This is an evolving field and little is known to date. Hence, this study could represent an insightful and important advance to the field.

Audience is to both basic immunologist and cancer biologists.

We appreciate this reviewer's comments on the high significance of our work to the field of both basic immunology and clinical application.

My expertise is in T cell signaling, T cell biology and immunotherapy.

1st Revision - Editorial Decision 25th May 2020

Dear Xiaolei,

Thank you for submitting your revised manuscript to The EMBO Journal. Your study has now been re-reviewed by three referees and their comments are provided below.

As you can see from the comments, the referees appreciate the introduced changes and support publication here. Ref #2 and 3 has two additional requests based upon the newly added data. the experiments should be technical feasible and not to time intensive. I think the addition of such experiments would indeed complete the analysis - let me know if there are any unforeseen problems with doing the experiments.

When you re-submit your revised manuscript will you please take care of the following issues as well.

- Please make sure that the funding information is correctly listed for all authors (discrepancy between the funding info listed for the Care-for-Rare Foundation between the online system and the MS file). Should HHMI funding be added for Ron Vale?

- Fig 4 panels need to be called out. The same goes for EV figure panels.

- Each movie needs to be zipped with its legend and the legends should be removed from the article.

- please double check that the figures have scale bars

- we also require a Data Availability Section - if there is no data deposited in external databases please state: This study includes no data deposited in external repositories

- we include a synopsis of the paper (see http://emboj.embopress.org/). Please provide me with a general summary statement and 3-5 bullet points that capture the key findings of the paper.

- we also need a summary figure for the synopsis. The size should be 550 wide by 400 high (pixels).

- I have asked our publisher to do their pre-publication checks on the paper. They will send me the file within the next few days.

Let me know if we need to discuss anything further

With best wishes

Karin

Karin Dumstrei, PhD
Senior Editor
The EMBO Journal

Instructions for preparing your revised manuscript:

Please make sure you upload a letter of response to the referees' comments together with the revised manuscript.

Please also check that the title and abstract of the manuscript are brief, yet explicit, even to non-specialists.

When assembling figures, please refer to our figure preparation guideline in order to ensure proper formatting and readability in print as well as on screen:
http://bit.ly/EMBOPressFigurePreparationGuideline

IMPORTANT: When you send the revision we will require
- a point-by-point response to the referees' comments, with a detailed description of the changes made (as a word file).
- a word file of the manuscript text.
- individual production quality figure files (one file per figure)
- a complete author checklist, which you can download from our author guidelines (https://www.embopress.org/page/journal/14602075/authorguide).
- Expanded View files (replacing Supplementary Information)
Please see out instructions to authors
https://www.embopress.org/page/journal/14602075/authorguide#expandedview

Please remember: Digital image enhancement is acceptable practice, as long as it accurately represents the original data and conforms to community standards. If a figure has been subjected to significant electronic manipulation, this must be noted in the figure legend or in the 'Materials and Methods' section. The editors reserve the right to request original versions of figures and the original images that were used to assemble the figure.

Further information is available in our Guide For Authors:
https://www.embopress.org/page/journal/14602075/authorguide

The revision must be submitted online within 90 days; please click on the link below to submit the revision online before 23rd Aug 2020.

https://emboj.msubmit.net/cgi-bin/main.plex

Referee #1:

The authors have addressed by concerns by looking at 1st generate CARs and looking at down-stream signaling in different cellular contexts. The paper is much more complete and they make a clear case that LAT is optional for some kind of organised synapse formation, but more important for cytokine production. I have not further concerns and endorse publication.

Referee #2:

In the revised version of the manuscript, the authors have addressed the major concerns we previously brought up with new data and analyses. The inclusion of JLAT CRISPER KO cells as well as inclusion of a 1st generation CAR strengthens the observation that a subset of signaling microclusters form downstream of CAR engagement in a LAT independent manner. However, downstream signaling such as CD69 expression and cytokine production is LAT dependent, which brings up the following concern, which should be addressed before acceptance:

The authors need to show that the surface levels of CAR molecules on WT and lat-/- cells (both Jurkats and PBMCs) are equivalent. Especially concerning is the new data shown in Figure 4C that clustering of CD19 induced by both 1st and 3rd generation CARs is significantly higher in lat-/- cells. This data is supported by Figure 3B showing higher CD19 clustering in lat-/- cells expressing 3rd gen CARs. These observations bring to light the need to consider that higher CAR expression levels on the lat-/- cells compensate for the lack of LAT. The surface levels of CARs can easily be determined by FACS staining and should not be a technically challenging experiment.

Minor comments:
1. Figure 5C: the authors need to quantify actin polymerization in several cells and include the statistical analysis of this quantification.
2. In the 2nd paragraph of the Discussion, the authors state that their data showing that CARs may bypass LAT could explain the faster proximal signaling previously shown in CAR expressing cells (Davenport et al., 2018). However, the Davenport paper reported faster signaling in CAR expressing cells at the level of Lck (upstream of LAT). The authors should discuss this reference appropriately.

Referee #3:

This is an interesting study by Dong and colleagues to suggest a differential requirement of LAT in TCR and CAR-T cell mediated functions. The results are intriguing and will be of interest to the community. There is one experiment needed to fully support the authors' conclusions.

Major concern:
1. As the authors have elegantly translated their findings from Jurkat into primary human T cells in figure 7 and since the primary function of CAR-Ts is to engage and kill CD19 tumor cells, the authors should analyze killing of target cells by primary WT vs lat- CAR Ts cells to support the biochemical and cellular analysis performed in Jurkat T cells.

Minor concerns:
1. Figure 3B. Is there any statistical difference between OKT3 vs CD19 in the WT conditions?
2. In their comparisons of 1st vs 3rd generation CARs (figure 4), the authors should show data that the expression levels of these two generations are similar.
3. Page 7, Figure 5A- since the authors just discussed in figure 4 and page 6 1st vs 3rd generation CARs, they should clarify that experiments in figure 5A were performed presumably with 3rd generation CARs to avoid confusion.
4. Page 7, line 17: Wardenburg et al., Immunity 1998 was the first description of NCK-SLP76 and actin assembly in T cells.

5. Figure 5A: Is the absolute GADS level in wt vs lat- cells statistically different? If so, this would seem to indicate that there is a significantly higher level of CAR expressed in the J.lat- than in WT.
Figure 5A: Is the absolute GADS level in wt vs lat- cells statistically different? If so, this would seem to indicate that there is a significantly higher level of CAR expressed in the J.lat- than in WT. If so, how might this alter the authors' conclusions?
6. Figure 5C: There needs to be quantitation of F-actin to determine if there are any differences in F-actin levels between WT vs lat-. In the single cell shown, it appears that there might be more peripheral F-actin intensity in lat-. Together with the data shown in figure 5A, is it possible that there is an enhanced GADs and F-actin in the absence of lat?
7. EV1. Is the [CD19] concentration in figure incorrect? The figure states 0.4 nM, but text reads 0.2 nM.

Response to Referees: EMBO J-2020-104730R1

We thank all three reviewers for providing their second round of comments. Reviewer 1 is completely satisfied and endorses publication. Reviewer 2 requested data showing the expression level of CAR, which we have included in the resubmission. Reviewer 3 asked if LAT is required for the killing activity of CAR T. We agree that this is an interesting question but it is beyond the major conclusion of the paper: LAT is not required for signaling cluster and synapse formation.

With the successful addressing to all other issues, we believe the manuscript is ready for publication. Below we will provide point-to-point responses to each reviewer's comments.

Referee #1

The authors have addressed by concerns by looking at 1st generate CARs and looking at down-stream signaling in different cellular contexts. The paper is much more complete and they make a clear case that LAT is optional for some kind of organised synapse formation, but more important for cytokine production. I have not further concerns and endorse publication.

Thank you!

Referee #2

In the revised version of the manuscript, the authors have addressed the major concerns we previously brought up with new data and analyses. The inclusion of JLAT CRISPER KO cells as well as inclusion of a 1st generation CAR strengthens the observation that a subset of signaling microclusters form downstream of CAR engagement in a LAT independent manner. However, downstream signaling such as CD69 expression and cytokine production is LAT dependent, which brings up the following concern, which should be addressed before acceptance:

The authors need to show that the surface levels of CAR molecules on WT and lat-/- cells (both Jurkats and PBMCs) are equivalent. Especially concerning is the new data shown in Figure 4C that clustering of CD19 induced by both 1st and 3rd generation CARs is significantly higher in lat-/- cells. This data is supported by Figure 3B showing higher CD19 clustering in lat-/- cells expressing 3rd gen CARs. These observations bring

to light the need to consider that higher CAR expression levels on the lat-/- cells compensate for the lack of LAT. The surface levels of CARs can easily be determined by FACS staining and should not be a technically challenging experiment.

We agree with the reviewer that it is important to determine the expression level of CAR in the WT and LAT null cells. The CAR T cells used in this study were sorted with a similar CAR expression level when they were made by lentiviral transduction. However, it is possible that the CAR expression level changed during the amplification of sorted cells. Therefore, we determined the CAR expression level by FACS when the functional assays were performed. Because we noticed that LAT null cells are larger than the WT cells, we quantified the CAR expression level by normalizing the total fluorescence intensity to cell size. We think this normalized expression represents the intracellular concentration of LAT better than the total intensity. We found that the normalized expressions between the wild-type and LAT null cells are similar (Appendix Figure S1). Unfortunately, we have not found any commercially available antibodies recognizing the extracellular part of CAR. There are homemade antibodies (Jena B, PLoS one 2013) but we were not able to obtain them at a reasonable time frame. This made it challenging to determine the cell surface level of CAR. Meanwhile, we think determining the total CAR expression level should address the reviewer's concern on the compensation of CAR expression in LAT null cells.

Minor comments:

1. Figure 5C: the authors need to quantify actin polymerization in several cells and include the statistical analysis of this quantification.

We now included the quantification and statistical analysis.

2. In the 2nd paragraph of the Discussion, the authors state that their data showing that CARs may bypass LAT could explain the faster proximal signaling previously shown in CAR expressing cells (Davenport et al., 2018). However, the Davenport paper reported faster signaling in CAR expressing cells at the level of Lck (upstream of LAT). The authors should discuss this reference appropriately.

We carefully reviewed the Lck data in the Davenport 2018 paper. Figure 1E showed a lower Lck accumulation in the synapse after CAR activation than TCR. Figure 2A showed a higher phosphorylated Lck in CAR-activated than TCR-activated cells. We did not find any kinetic data showing Lck phosphorylation is faster, which should be best revealed by $t_{1/2}$, in CAR-activated than TCR-activated cells. We appreciate that the reviewer pointed out that our original statement on "faster proximal signaling" is not so

appropriate. On the hand, Figure 4A in the Davenport paper showed that CAR triggers faster lytic granule recruitment to the synapse than TCR. We now changed our discussion to match what the data exactly demonstrated.

Referee #3

This is an interesting study by Dong and colleagues to suggest a differential requirement of LAT in TCR and CAR-T cell mediated functions. The results are intriguing and will be of interest to the community. There is one experiment needed to fully support the authors' conclusions.

Major concern:

1. As the authors have elegantly translated their findings from Jurkat into primary human T cells in figure 7 and since the primary function of CAR-Ts is to engage and kill CD19 tumor cells, the authors should analyze killing of target cells by primary WT vs lat- CAR Ts cells to support the biochemical and cellular analysis performed in Jurkat T cells.

We agree with the reviewer that it will be interesting to compare the killing activity of WT vs lat- CAR Ts. However, our major conclusion for this work is that LAT is not required for signaling cluster and synapse formation. The result of the killing ability is relevant but beyond the focus of the manuscript.

Minor concerns:

1. Figure 3B. Is there any statistical difference between OKT3 vs CD19 in the WT conditions?

Yes, OKT3 is higher than CD19 in the WT condition ($P<0.0001$ by two-tailed unpaired t test). However, we do not think this statistical analysis accurately reflects the clustering difference between OKT3 and CD19 because these two different ligands have different dye-labeling efficiencies.

2. In their comparisons of 1st vs 3rd generation CARs (figure 4), the authors should show data that the expression levels of these two generations are similar.

We now show the data in Appendix Figure S1. Indeed, the CAR expression of the two generations are similar.

3. Page 7, Figure 5A- since the authors just discussed in figure 4 and page 6 1st vs 3rd generation CARs, they should clarify that experiments in figure 5A were performed presumably with 3rd generation CARs to avoid confusion.

That is a good suggestion. We now clarified it's the 3rd generation CAR in the figure legend.

4. Page 7, line 17: Wardenburg et al., Immunity 1998 was the first description of NCK-SLP76 and actin assembly in T cells.

Thanks for suggesting this reference. It has been included.

5. Figure 5A: Is the absolute GADS level in wt vs lat- cells statistically different? If so, this would seem to indicate that there is a significantly higher level of CAR expressed in the J.lat- than in WT. Figure 5A: Is the absolute GADS level in wt vs lat- cells statistically different? If so, this would seem to indicate that there is a significantly higher level of CAR expressed in the J.lat- than in WT. If so, how might this alter the authors' conclusions?

The reviewer raises an interesting point. The absolute GADS level recruited to membrane is statistically higher in lat- than in WT cells ($P<0.005$ by two-tailed unpaired t test). We determined the total CAR expression level in WT and lat- cells and found they are comparable (Appendix Figure S1). We suspect that LAT could recruit additional factors to the membrane that inhibits the recruitment of Gads but the exact mechanism needs further investigation.

6. Figure 5C: There needs to be quantitation of F-actin to determine if there are any differences in F-actin levels between WT vs lat-. In the single cell shown, it appears that there might be more peripheral F-actin intensity in lat-. Together with the data shown in figure 5A, is it possible that there is an enhanced GADs and F-actin in the absence of lat?

We determined the F-actin level in both central and peripheral regions in the CAR T synapse and there is no significant difference between WT vs lat- (new figure 5C).

7. EV1. Is the [CD19] concentration in figure incorrect? The figure states 0.4 nM, but text reads 0.2 nM.

Thanks for catching this discrepancy. It was a typo in the figure legend. We have corrected it.

2nd Revision - Editorial Decision 5th Jun 2020

Dear Xiaolei,

Thank you for submitting your revised manuscript to The EMBO Journal. I have now had a chance to take a careful look at everything and all looks good.

I am therefore very pleased to accept the manuscript for publication here.

Congratulations on a nice manuscript

with best wishes

Karin

Karin Dumstrei, PhD
Senior Editor
The EMBO Journal

Please note that it is EMBO Journal policy for the transcript of the editorial process (containing referee reports and your response letter) to be published as an online supplement to each paper. If you do NOT want this, you will need to inform the Editorial Office via email immediately. More information is available here: http://emboj.embopress.org/about#Transparent_Process

Your manuscript will be processed for publication in the journal by EMBO Press. Manuscripts in the PDF and electronic editions of The EMBO Journal will be copy edited, and you will be provided with page proofs prior to publication. Please note that supplementary information is not included in the proofs.

Should you be planning a Press Release on your article, please get in contact with embojournal@wiley.com as early as possible, in order to coordinate publication and release dates.

If you have any questions, please do not hesitate to call or email the Editorial Office. Thank you for your contribution to The EMBO Journal.

** Click here to be directed to your login page: http://emboj.msubmit.net

EMBO PRESS

YOU MUST COMPLETE ALL CELLS WITH A PINK BACKGROUND ↓

PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

Corresponding Author Name: Xiaolei Su, Ronald Vale
Journal Submitted to: EMBO Journal
Manuscript Number: 2020-104730

Reporting Checklist For Life Sciences Articles (Rev. June 2017)

This checklist is used to ensure good reporting standards and to improve the reproducibility of published results. These guidelines are consistent with the Principles and Guidelines for Reporting Preclinical Research issued by the NIH in 2014. Please follow the journal's authorship guidelines in preparing your manuscript.

A- Figures

1. Data

The data shown in figures should satisfy the following conditions:

- ➔ the data were obtained and processed according to the field's best practice and are presented to reflect the results of the experiments in an accurate and unbiased manner.
- ➔ figure panels include only data points, measurements or observations that can be compared to each other in a scientifically meaningful way.
- ➔ graphs include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical replicates.
- ➔ if n< 5, the individual data points from each experiment should be plotted and any statistical test employed should be iustified
- ➔ Source Data should be included to report the data underlying graphs. Please follow the guidelines set out in the author ship guidelines on Data Presentation.

2. Captions

Each figure caption should contain the following information, for each panel where they are relevant:

- ➔ a specification of the experimental system investigated (eg cell line, species name).
- ➔ the assay(s) and method(s) used to carry out the reported observations and measurements
- ➔ an explicit mention of the biological and chemical entity(ies) that are being measured.
- ➔ an explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.

- ➔ the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- ➔ a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- ➔ a statement of how many times the experiment shown was independently replicated in the laboratory.
- ➔ definitions of statistical methods and measures:
  - common tests, such as t-test (please specify whether paired vs. unpaired), simple $\chi 2$ tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
  - are tests one-sided or two-sided?
  - are there adjustments for multiple comparisons?
  - exact statistical test results, e.g., P values = x but not P values < x;
  - definition of 'center values' as median or average;
  - definition of error bars as s.d. or s.e.m.

Any descriptions too long for the figure legend should be included in the methods section and/or with the source data.

In the pink boxes below, please ensure that the answers to the following questions are reported in the manuscript itself. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable). We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.

B- Statistics and general methods

Please fill out these boxes ↓ (Do not worry if you cannot see all your text once you press return)

1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	Sample size (number of cells/data set) was determined based on the sample size utilized routinely in the T cell biology studies.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	NA
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	No exclusions were made in the data.
3. Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. randomization procedure)? If yes, please describe.	Image acuiqisition were done randomly within one sample group using an automated acquisition plug-ins in Micro-Manager. The sample names were anonymous to the investigators during data collection by the use of a numeric code for each sample.
For animal studies, include a statement about randomization even if no randomization was used.	NA
4.a. Were any steps taken to minimize the effects of subjective bias during group allocation or/and when assessing results (e.g. blinding of the investigator)? If yes please describe.	Key experiments were repeated independently in two collaborating labs.
4.b. For animal studies, include a statement about blinding even if no blinding was done	NA
5. For every figure, are statistical tests justified as appropriate?	Yes.
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	Yes.
Is there an estimate of variation within each group of data?	Results were represented in dot plots when possible, so the variation within data can be appreciated. SD is also shown.

USEFUL LINKS FOR COMPLETING THIS FORM

http://www.antibodypedia.com
http://1degreebio.org
http://www.equator-network.org/reporting-guidelines/improving-bioscience-research-repor

http://grants.nih.gov/grants/olaw/olaw.htm
http://www.mrc.ac.uk/Ourresearch/Ethicsresearchguidance/Useofanimals/index.htm
http://ClinicalTrials.gov
http://www.consort-statement.org
http://www.consort-statement.org/checklists/view/32-consort/66-title

http://www.equator-network.org/reporting-guidelines/reporting-recommendations-for-tumo

http://datadryad.org

http://figshare.com

http://www.ncbi.nlm.nih.gov/gap

http://www.ebi.ac.uk/ega

http://biomodels.net/

http://biomodels.net/miriam/
http://jjj.biochem.sun.ac.za
http://oba.od.nih.gov/biosecurity/biosecurity_documents.html
http://www.selectagents.gov/

Is the variance similar between the groups that are being statistically compared?	Yes.

C- Reagents

6. To show that antibodies were profiled for use in the system under study (assay and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile. e.g., Antibodypedia (see link list at top right), 1DegreeBio (see link list at top right).	All used antibodies in this study were described in the Materials and Methods, with the vendor and catalog number specified.
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	Sources of cell lines were described in the Materials and Methods. Cells lines were regularly checked for mycoplasma contamination.

* for all hyperlinks, please see the table at the top right of the document

D- Animal Models

8. Report species, strain, gender, age of animals and genetic modification status where applicable. Please detail housing and husbandry conditions and the source of animals.	NA
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	NA
10. We recommend consulting the ARRIVE guidelines (see link list at top right) (PLoS Biol. 8(6), e1000412, 2010) to ensure that other relevant aspects of animal studies are adequately reported. See author guidelines, under 'Reporting Guidelines'. See also: NIH (see link list at top right) and MRC (see link list at top right) recommendations. Please confirm compliance.	NA

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	NA
12. Include a statement confirming that informed consent was obtained from all subjects and that the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.	NA
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	NA
14. Report any restrictions on the availability (and/or on the use) of human data or samples.	NA
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	NA
16. For phase II and III randomized controlled trials, please refer to the CONSORT flow diagram (see link list at top right) and submit the CONSORT checklist (see link list at top right) with your submission. See author guidelines, under 'Reporting Guidelines'. Please confirm you have submitted this list.	NA
17. For tumor marker prognostic studies, we recommend that you follow the REMARK reporting guidelines (see link list at top right). See author guidelines, under 'Reporting Guidelines'. Please confirm you have followed these guidelines.	NA

F- Data Accessibility

18: Provide a "Data Availability" section at the end of the Materials & Methods, listing the accession codes for data generated in this study and deposited in a public database (e.g. RNA-Seq data: Gene Expression Omnibus GSE39462, Proteomics data: PRIDE PXD000208 etc.) Please refer to our author guidelines for 'Data Deposition'. Data deposition in a public repository is mandatory for: a. Protein, DNA and RNA sequences b. Macromolecular structures c. Crystallographic data for small molecules d. Functional genomics data e. Proteomics and molecular interactions	NA
19. Deposition is strongly recommended for any datasets that are central and integral to the study; please consider the journal's data policy. If no structured public repository exists for a given data type, we encourage the provision of datasets in the manuscript as a Supplementary Document (see author guidelines under 'Expanded View' or in unstructured repositories such as Dryad (see link list at top right) or Figshare (see link list at top right).	NA
20. Access to human clinical and genomic datasets should be provided with as few restrictions as possible while respecting ethical obligations to the patients and relevant medical and legal issues. If practically possible and compatible with the individual consent agreement used in the study, such data should be deposited in one of the major public access-controlled repositories such as dbGAP (see link list at top right) or EGA (see link list at top right).	NA
21. Computational models that are central and integral to a study should be shared without restrictions and provided in a machine-readable form. The relevant accession numbers or links should be provided. When possible, standardized format (SBML, CellML) should be used instead of scripts (e.g. MATLAB). Authors are strongly encouraged to follow the MIRIAM guidelines (see link list at top right) and deposit their model in a public database such as Biomodels (see link list at top right) or JWS Online (see link list at top right). If computer source code is provided with the paper, it should be deposited in a public repository or included in supplementary information.	NA

G- Dual use research of concern

22. Could your study fall under dual use research restrictions? Please check biosecurity documents (see link list at top right) and list of select agents and toxins (APHIS/CDC) (see link list at top right). According to our biosecurity guidelines, provide a statement only if it could.	No.