The Bardet-Biedl syndrome complex component BBS1 controls T cell polarity during immune synapse assembly

Chiara Cassioli, Anna Onnis, Francesca Finetti, Nagaja Capitani, Jlenia Brunetti, Ewoud B. Compeer, Veronika Niederlova, Ondrej Stepanek, Michael L. Dustin and Cosima T. Baldari
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We have now reached a decision on the above manuscript.

To see the reviewers' reports and a copy of this decision letter, please go to: https://submit-jcs.biologists.org and click on the 'Manuscripts with Decisions' queue in the Author Area. (Corresponding author only has access to reviews.)

As you will see, the reviewers find the manuscript of interest, but raise a few criticisms that prevent me from accepting the paper at this stage. In particular, reviewers 2 and 3 are requesting a few additional experiments and textual clarifications that should be easy to perform. If you think that you can deal satisfactorily with the criticisms on revision, I would be pleased to see a revised manuscript. We would then return it to the reviewers.

We are aware that you may be experiencing disruption to the normal running of your lab that makes experimental revisions challenging. If it would be helpful, we encourage you to contact us to discuss your revision in greater detail. Please send us a point-by-point response indicating where you are able to address concerns raised (either experimentally or by changes to the text) and where you will not be able to do so within the normal timeframe of a revision. We will then provide further guidance. Please also note that we are happy to extend revision timeframes as necessary.

Please ensure that you clearly highlight all changes made in the revised manuscript. Please avoid using 'Tracked changes' in Word files as these are lost in PDF conversion.

I should be grateful if you would also provide a point-by-point response detailing how you have dealt with the points raised by the reviewers in the 'Response to Reviewers' box. Please attend to all of the reviewers' comments. If you do not agree with any of their criticisms or suggestions please explain clearly why this is so.

Reviewer 1

Advance summary and potential significance to field

In this manuscript Cassioli, Baldari and coworkers provide a novel insight into the role of ciliary transport proteins in a non-ciliated cell, the T lymphocyte. They nicely extend the work on ciliary transport in T lymphocytes and in immunological synapse formation, which the Baldari lab pioneered about ten years ago and further complemented with a series of interesting papers. Here they point out the Bardet-Biedl syndrome complex as an important machinery in T cells.
I found of particular interest the relationship with the proteasome complex, previously studied in T cells, but to my knowledge, not precisely localized before during the process of T cell activation. Furthermore, it nicely complements the molecular bases of actin polymerization and centrosomal dynamics in lymphocytes unveiled by other laboratories.
In summary, this work opens new and deeper insights into molecular transport in T cells and its important role in immunological synapse formation a key platform for T cell antigen receptor signaling. It opens new interesting questions, in particular on the influence of proteasome localization in building receptor signaling machinery, interesting to be address in the future.

Comments for the author

I found the work well performed and complete. The use both Jurkat and primary human T cells in parallel and the use of both shRNA knock down and CRISPR-Cas9 know out cell in various instances provides with solid complementary approaches. Microscopy is well performed and image quantification appropriate and statistically tested.

The work goes from unveiling BBS1 to the identification of its detailed molecular mechanism (at least one of them) describing it in a coherent manner.
Experimentally, I find the work sufficiently complete, by I have some minor requests on the text detailed below:
1. In the introduction: Sentence starting by: Many other ciliary proteins have since been demonstrated… (e.g. Unc-119, Rac-1 interacting protein FIP3, …)
Comment: FIP3 is more commonly known as Rab11 family interacting protein, Rab11 effector, rather than Rac-1-interacting protein.

2. Sup Fig S2 S3: Fig legends appear to be inversed

2. Figure 2 message is hard to follow on the fig and in the legend: I suggest to label on top of the panels what corresponds to Jurkat or primary T cells and on the left what is Crt and KO which appear to be missing in C and D

3. The understanding of data on Fig 4 (and other figures too) would be easier if the authors show with arrows and a frame the zoomed regions. The reader would better understand which is the “centrosomal structure/region” they are describing.

In Fig 4 they describe that F-actin clearance from the “centrosomal structure” requires BBS1. They say that F-actin in that area may be mainly associated with Rab5 endosomal compartment (Fig S5). However, the quantitative data appears contradictory, such as clearance from PCNT structures (Fig 4) but not from Rab5 ones. This deserves additional description and discussion in the text.

Reviewer 2

Advance summary and potential significance to field

In this work, the authors show that BBS1 is involved in the polarisation of the centrosome at the CD4 T cell immune synapse, using a cell line model (Jurkat T cells) and peripheral blood T cells. BBS1 localizes to the pericentrosomal area and is necessary for the recruitment of the proteasome element 19S to the centrosome upon TCR engagement. This relocalisation of the proteasome then allows the clearing of Arp2/3-dependent centrosomal actin, unlinking the centrosome and the nucleus, and allowing centrosome polarisation to the immune synapse. These results are very interesting as they highlight once again the close relationship between primary cilia and immune synapses, focusing here on the role of BBS1. The importance of centrosomal actin clearing for centrosome polarisation had been shown in mouse B lymphocytes cell lines as well as in Jurkat T cell lines, but the authors are the first to show this phenomenon in primary lymphocytes, which makes it particularly convincing.

Comments for the author

Specific comments
- In Fig1D, the colocalization coefficient between Gamma-Tubulin and BBS1 in Jurkat T cells appears relatively low compared with the colocalization coefficient with CD3. However, looking at illustrations it is clear that BBS1 is at the centrosome. Is the BBS1 signal outside the centrosomal area only background due to plasmid expression, or is endogenous BBS1 really also scattered in the cytoplasm?

Another way to quantify colocalization could be provided. Also, comparing with a random pattern in the cell (cytosplasmic staining, generation of random masks inside the cell area) could help highlight the specificity of colocalization in case BBS1 is indeed also present all around the cytosplasm.

- The authors do not see any effect of BBS1 deficiency on the synaptic polymerization of F-actin, but they cannot distinguish the F-actin pool from the Raji B cell or from the T cell. This statement would be more convincing if they could separate the two pools, by expressing a fluorescent F-actin probe in the Raji B cell.

- The authors suggest that BBS1 recruits 19S RP to the centrosome thanks to the dynein motor. What they show is that 1) dynein and 19S RP interact with BBS1, and 2) without BBS1, 19S RP and dynein do not co-immuno precipitate. This message would benefit from complementary experiments, such as dynein inhibition, or imaging of dynein/19S RP trajectories towards the centrosome.
As dynein is also recruited to the immune synapse, I think it raises additional questions:
How does this dynein pool compare with the synaptic dynein pool that drives TCR microcluster formation and immune synapse formation? Are they the same dyneins or is there a competition between the centrosome and the immune synapse?

- The authors state that the expression of the BBSome core components is comparable or higher in T cells compared to ciliated cells. However, in Fig S1A BBSome expression levels are different between Jurkat T cells and primary T cells:
while primary T cells have comparable or much higher expression levels than ciliated cells, Jurkat T cells display lower ones. This is especially true for the expression levels of BBS1, that are really low in Jurkat T cells. This is not as striking in Fig 1A, where they measure BBS1 levels by Western Blot, but Jurkat T cells still express lower amounts of BBS1.

- Please always report on the panel the conditions and cell type (for example in Fig 2C, write Ctrl, J KD)

- Please draw the outline of the cells when showing single staining (especially for the centrosome, in Fig 1D for example), or put only the merged image otherwise it is not possible to interpret the figure.

Reviewer 3

Advance summary and potential significance to field

This manuscript presents several important T cell functions that are lost or diminished in cells where BBS1 is depleted. The results described in this study parallel the findings previously reported for disruption of proteins in the IFT system. As a consequence of reducing or eliminating synthesis of the BBS1 protein they found that the MTOC failed to translocated to the immunological synapse. They further show that in the absence of BBS1 “endosomal” CD3 does not accumulate at synapse in Jurkat and T cells and that pTyr is reduced or absent. They show punctate (endosomal?) actin around the MTOC and claim that more actin remains around the MTOC in the BBS1 JKD cells. They show that the proteasome inhibitors Epoxomycin and MG132 block MTOC translocation and that 19S subunit of the proteasome is recruited to the MTOC in control but not in KO cells. Finally they show that dynein is pulled down with the 19S proteasomal subunit in control but not KO cells.

Comments for the author

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Comments

General comments

1. There are general themes in this study where the reader is repeatedly directed or misdirected to conclusions that are not justified.

a) The term actin clearance (from the centrosome) is used throughout the paper as if it is inhibiting centrosome translocation. The idea of “clearance” needs to be defined. There is no visual evidence of actin filaments linking the nucleus to the MTOC yet that is where the discussion leads. Rather, actin appears to be associated with punctate organelles consistent with the location of WASH. In this context, clearance could mean movement of these organelles or depolymerization of actin off of the surface of these organelles. One of the general arguments of the paper appears to be that the proteasome is not recruited to the centrosome in BBS1 JKD cells and that proteasomal activity is what is reducing actin. Yet there was no particular demonstration that any particular components of the actin polymerization network were ubiquitylated and destroyed by the proteasome upon T cell activation. While actin around the centrosome may be reduced upon T cell activation, there is no data showing that actin itself is interfering with centrosome movement. The argument further loses its force by the observation that the centrosome disconnects from the nucleus in CD4 and B cells but not in CD8 T cells.

b) Other similar studies (as noted below) show there is a pTyr signaling defect. The authors never quite show that the failure of centrosome translocation is not tied to a signaling defect.

Specific Comments

Primary T cells

2. The statement is made
Of note, dissociation from the nucleus is not required for centrosome polarization to the IS formed by cytotoxic T cells (CTL) (Lui-Roberts et al., 2012). The results presented here, obtained on a CD4 T cell-derived line and on primary peripheral T cells, which have a minor proportion of CTLs….

a) In the materials and methods section it was noted that “T cells are isolated from healthy

human donors" but they do not appear to be enriched for CD4 cells. Given the stated difference between CD4 and CD8 cells on MTOC translocation and use of Jurkat cells for comparison why would you not specifically use CD4 cells for comparison?

b) Assuming the statement quoted above is true, we have to ask how do treatments such as proteasome inhibitors and BBS1 knockdown affect CD8 T cells. Would that not be a critical comparison for your hypothesis that actin linkage between the centrosome and nucleus needs to be broken for centrosome translocation?

3. **Failure of TcR movement to the centrosome**
The paper by Galgano, D., A. Onnis, E. Pappalardo, F. Galvagni, O. Acuto and C. T. Baldari (2017). "The T cell IFT20 interactome reveals new players in immune synapse assembly." J Cell Sci **130**(6): 1110-1121. Already showed that knocking down components of the IFT20 complex prevents movement of TcR containing vesicles to the IS. Shouldn't they be cited?

4. **Figure 4 Actin WASH and proteasome activity**
The authors use the term F actin clearance to describe the change in actin in the area of the MTOC. Presumably these are endosomes and not amorphous blobs of actin. If so, are these organelles normally associated with motor proteins and capable of moving towards or away from the MTOC? Is the failure to move associated with loss of linkage to motor proteins associated with organelles?

5. **Figure 3 pTyr (A, B and Left, Right)** Of particular important is the statement "This suggests that the signaling events triggered by the membrane-associated TCR:CD3 pool that eventually lead to centrosome translocation were initiated normally, but were not sustained in the absence of BBS1". In an earlier paper (Finetti et al, 2009) depletion of IFT20 also resulted in failure of the TcR to move to the synapse. In that study they wrote, "Consistent with this finding, both the extent and duration of tyrosine phosphorylation was reduced in *IFT20*-knockdown cells, … Moreover, signalling was not productive"

a) Given the similarities between the Finetti paper and this one with regard to the translocation of TcRs to the IS, how do you eliminate the possibility that failure of MTOC translocation is not simply a signaling defect.

b) This is an odd comparison. They are comparing the % of the pTyr at the synapse where (A) indicates there are grossly different levels of pTyr between the control and KO. Similarly in B they indicate there is 20% pTyr at the synapse where no staining is visible at all. Jurkat cells are different from primary T cells insofar as merely plating them on polylysine triggers calcium signaling on contact. Could this account for the difference between A and B? In any case while technically accurate one wonders what meaning there is to it.

c) For A - recruitment index what is the time point? This should be stated in the legend.

d) In B there is no pTyr at the IS. Was there ever pTyr at the IS? For the recruitment index of A there is some difference whereas in B there is no difference. What are we to make of that?

e) Why are similar time points to A not shown in B?

f) It would add confidence if other indicators of activation were shown for the control and the JKO to demonstrate that these were not impaired. These could be calcium, LAT recruitment etc.

6. **Figure 5C** This data is helpful and it seems the statistical significance holds for the 15 minute and 30 minute time points but what about the earlier time points? In particular does a comparison between the 0 minute and the 1 / 5 minute time points show that their differences are statistically significant?

7. **Figure 5 and 6. Proteasome and actin**
The data in these figures apparently provide the basis for the statement, "We provide evidence that BBS1 promotes the proteasome-dependent F-actin clearance around the centrosome, on which its mobilization to the IS crucially depends".

a) The term “clearance” needs to be explicitly defined and clarified. The reader keeps being directed (or misdirected) to the idea that proteasomal degradation of actin or some related factor (WASH, ARP 2/3 etc) leads to actin “clearance” but no specific ubiquitylation of any of these related proteins was shown. A demonstration of some specific target would certainly strengthened the case. In the intro it was stated that there were two pools of vesicles and WASH is associated with endosomal vesicles. It seems likely that the patchy actin staining around the centrosome is vesicular. Why couldn’t their just have been a loss of WASH or depolymerization of actin or movement of vesicles that explains the change in actin staining? The data shows a loss of connection

b) *The authors write “F-actin clearance from the centrosomal area has been recently shown to occur during IS formation in lymphocytes, allowing for centrosome polarization (Bello-Gamboa et al., 2020; Obino et al., 2016)”. “Collectively, these results indicate that BBS1 deficiency results in the failure of T cells to clear F-actin from the centrosomal area, at least in part due to a higher stability of the centrosome-associated WASH pool, which likely accounts for the inability of the centrosome to translocate to the IS”.*

The article by Ibanzez-Vega (2019) investigated the reduction of actin around the centrosome during B cell activation and found no overall reduction of actin. The results reported here seem to show the same thing. While it may not be the authors intent, the statement that they show proteasome-dependent clearance of actin certainly lends itself to the idea they are claiming that the proteasome degrades actin. Given that the patches of actin are on organelles, isn’t it likely that either actin depolymerization or organelle movement is involved.

9. Figure 6B The same or different pools of ubiquitin?

a) The antibodies used in this study do not appear to be Ub chain specific. The actin and WASH that appears to remain around the centrosome probably are on endosomal vesicles. WASH can be ubiquitylated on K63 which activates it [see Hao, Y. H., J. M. Doyle, S. Ramanathan, T. S. Gomez, D. Jia, M. Xu, Z. J. Chen, D. D. Billadeau, M. K. Rosen and P. R. Potts (2013). "Regulation of WASH-dependent actin polymerization and protein trafficking by ubiquitination." Cell **152**(5): 1051-1064]. The question then is, “How do you know that there are not two pools of ubiquitin being stained by IF. Most of the cytoplasmic UB IF staining goes away after activation. Perhaps what remains after activation is Ub attached to proteins in other ways such as on K63 of WASH. As noted above there is a higher stability of the WASH pool that could be what is being detected by Ub IF staining.

b) Why should ubiquitinated proteins accumulate at the centrosome in JKD cells if the proteasome is not recruited to the centrosome? Visually there does not appear to be much difference other than generally brighter staining? These DIC images of these cells look abnormal

Minor issues

10. Introduction

This sentence is difficult to dissect “We identified as an unexpected new player in synaptic trafficking the intraflagellar transport (IFT) system”. It would be good to put a reference here to previous papers, or, if BBS4 is meant then that should be made clear.

11. This sentence seems to be missing a verb and included is used redundantly

Based on the basal body localization of the BBSome in ciliated cells (Nachury, 2018; Wingfield et al., 2018), these included markers of the centrosome (γ-tubulin) and of the pericentrosomal (PCM-1, CEP131/AZI1, CEP290) and endocytic recycling (Rab11) compartments. Markers of the Golgi apparatus (GM130) and late endosomes (Rab7) were also included.

12. IS assembly

“Of note BBS4, which is responsible for priming the assembly of a pre-BBSome complex in ciliated cells (Prasai et al., 2020), also polarises to the IS together with the centrosome (Fig.S1B), suggesting that other BBS proteins might participate in IS assembly”.

In what sense is BBS4 participating in IS assembly? What is deficient?

13. Supplemental Figure S4 is presumably mislabeled. It goes from A to E to C

14. Figure S8D Left, Middle What are we looking at. These should be labeled for clarity. Both left and middle figures look to be the same. Is the middle supposed to be total?

15. How does CRISPR gene editing transiently knock down expression?
"T cells transiently knocked out for BBS1 expression (T KO; ~70% depletion) by CRISPR-Cas9 gene editing (Fig.S3B). BBS1 depletion did not affect either the levels of surface CD3 or expression of the other BBS core components (Fig.S3C,D)."

16. Figure 2 C legend "Right" is misspelled.

17. Dynein-dynactin and centrosome
This observation could account, at least in part, for the defects in centrosome polarization to the IS observed in T cells with defective activity of the dynein-dynactin complex (Martin-Cofreces et al., 2008)

There has been a lot of work on the role of dynein in centrosome polarization including evidence showing that the NDE1/LIS1 complex with dynein rather than the dynactin dynein complex is responsible for centrosome polarization (Nath et al, 2016). Simply saying it is the dynein- dynactin complex seems inappropriate.

First revision

Author response to reviewers' comments

Point-by-point response

Reviewer 1

We thank the Reviewer for his/her positive feedback on our work. Please find below the answers to the specific questions.

Point 1. *"In the introduction: Sentence starting by: Many other ciliary proteins have since been demonstrated… (e.g. Unc-119, Rac-1 interacting protein FIP3, …). Comment: FIP3 is more commonly known as Rab11 family interacting protein, Rab11 effector, rather than Rac-1-interacting protein."*

We thank the Reviewer for pointing this out. We now have used the correct name, RAB11FIP3.

Point 2. *"Sup Fig S2 S3: Fig legends appear to be inversed"*

Thanks for pointing this out. The order of the legends has been rectified.

Point 3. *"Figure 2 message is hard to follow on the fig and in the legend: I suggest to label on top of the panels what corresponds to Jurkat or primary T cells and on the left what is Crt and KO which appear to be missing in C and D"*

We have modified the figure according to the Reviewer's suggestion, using different labels (i.e. J KD and T KO for BBS1KD Jurkat cells and BBS1KO primary T cells, respectively) and different symbols in the histograms.

Point 4. *"The understanding of data on Fig 4 (and other figures too) would be easier if the authors*

show with arrows and a frame the zoomed regions. The reader would better understand which is the "centrosomal structure/region" they are describing. In Fig 4 they describe that F-actin clearance from the "centrosomal structure" requires BBS1. They say that F-actin in that area may be mainly associated with Rab5 endosomal compartment (Fig S5). However, the quantitative data appears contradictory, such as clearance from PCNT structures (Fig 4) but not from Rab5 ones. This deserves additional description and discussion in the text."

We have modified figure 4 (and the other figures) according to the Reviewer's suggestion. Regarding F-actin clearance from the centrosomal structure/region, we agree that the description was not adequate. After reading this Reviewer's and Reviewer's 3 comments we decided to re-analyze the data generating a mask that only covers the area stained by PCNT1/ γ-tubulin to quantify the centrosomal F-actin pool. The results show that the centrosomal F-actin pool decreases in control SAg- specific conjugates, which is consistent with previous reports on both (Jurkat) T cells (Bello-Gamboa 2020) and B cells (Obino 2016), while no decrease was detectable in conjugates formed by BBS1-deficient T cells (Fig.4A,B,S5F). We interpret these results as i) confirming centrosomal F-actin clearance in Jurkat cells and showing that it also occurs in primary T cells (total and $CD4^+$) undergoing IS formation; and ii) indicating a role for BBS1 in this process. The new analyses are presented in figures 4 and S4C (showing the strategy for generating the mask, that is also described in the Results, p.12).

Regarding the apparent contradiction highlighted by the Reviewer when comparing the results presented in new figure 5, we would like to point out that the quantifications of F-actin with Rab5 and Rab11 were carried out on individual endosomes and hence exclude the contribution of centrosomal F-actin. Additionally, at variance with the images used to quantify the centrosomal F-actin pool, which always include the PCNT1/γ-tubulin staining, the centrosomal F-actin pool may not have been included in the plane of the images used for the analysis of F-actin colocalization with Rab5/Rab11 endosomes, since in these experiments the centrosome was not labelled. We have explained this better in the figure legend and in the Materials and Methods.

To further strengthen the results we have quantified F-actin colocalization with early endosomes, which showed a higher colocalization compared to recycling endosomes, on cells triple-stained for PCNT1, F-actin and Rab5. For these images, where both the centrosomal and endosomal pools of F-actin can be clearly detected, we generated a mask that includes the endosomes and excludes the centrosome and measured the colocalization of F-actin with Rab5 within this mask. At variance with centrosomal F- actin, we found no alteration in endosomal F-actin in conjugates of control Jurkat T cells formed in the presence or absence of SEE (and no effect of BBS1 KD) (Fig.S6A,B), confirming the results obtained in the single-dot analysis on double- labelled cells (Fig.5E). We believe that this is due to the fact that this pool is highly dynamic, with continuous redistribution of WASH complexes that promote the Arp2/3- dependent polymerization of new actin filaments on endosomes and their coupling to microtubules for transport to the plasma membrane (Gomez & Billadeau, Dev Cell 2009). We have added this point to the Results (p.13). The new analyses are presented in figures S6A,B (the strategy for generating the masks is shown in figure S4C and also described in the Results, p.12).

Reviewer 2

We thank the Reviewer for his/her positive feedback on our work. Please find below the answers to the specific questions.

Specific comments

Point 1. *"In Fig1D, the colocalization coefficient between Gamma-Tubulin and BBS1 in Jurkat T cells appears relatively low compared with the colocalization coefficient with CD3. However, looking at illustrations it is clear that BBS1 is at the centrosome. Is the BBS1 signal outside the centrosomal area only background due to plasmid expression, or is endogenous BBS1 really also scattered in the cytoplasm? Another way to quantify colocalization could be provided. Also, comparing with a random pattern in the cell (cytosplasmic staining, generation of random masks inside the cell area) could help highlight the specificity of colocalization in case BBS1 is indeed also present all around the cytosplasm."*

The Reviewer is right. BBS1 localization is largely centrosomal, as in ciliated cells, with some colocalization with early and recycling endosomes that are clustered close to the centrosome (these include $CD3^+$ endosomes) (Fig.1B-E). To better illustrate these associations we have re-quantified the colocalizations and now show Manders' coefficients calculated as ratio of the compartment-specific pixels (e.g. γ-tubulin, Rab5 etc) to the BBS1-GFP pixels (Fig.1C).
Regarding the specificity of the cytoplamic staining, we had to carry out the colocalization analyses on BBS1-GFP-transfected cells, as there are no good anti- BBS1 antibodies suitable for immunofluorescence. We cannot therefore exclude that some cleavage might have occurred, leading to freely diffusible GFP, as supported by anti-GFP immunoblot of cell lysates showing a GFP immunoreactive band co- migrating with GFP in addition to the major immunoreactive band corresponding to BBS1-GFP (not shown). Some specific cytoplasmic staining may however be compatible with the existence of a cytosolic pool, since BBS proteins are produced as soluble gene products that are only subsequently assembled into the BBSome. We have modified the Materials and Methods to include these possibilities (p.25,26).

Point 2. *"The authors do not see any effect of BBS1 deficiency on the synaptic polymerization of F-actin, but they cannot distinguish the F-actin pool from the Raji B cell or from the T cell. This statement would be more convincing if they could separate the two pools, by expressing a fluorescent F-actin probe in the Raji B cell."*

We thank the Reviewer for the useful suggestion. We have transfected BBS1 ctr and KD T cells with the LifeAct reporter and quantified synaptic F-actin in conjugates formed with Raji B cells. The results confirm that BBS1 deficiency in T cells does not affect synaptic F-actin (Fig.5B,D).

Point 3. *"The authors suggest that BBS1 recruits 19S RP to the centrosome thanks to the dynein motor. What they show is that 1) dynein and 19S RP interact with BBS1, and 2) without BBS1, 19S RP and dynein do not co-immuno precipitate. This message would benefit from complementary experiments, such as dynein inhibition, or imaging of dynein/19S RP trajectories towards the centrosome. As dynein is also recruited to the immune synapse, I think it raises additional questions: How does this dynein pool compare with the synaptic dynein pool that drives TCR microcluster formation and immune synapse formation? Are they the same dyneins or is there a competition between the centrosome and the immune synapse?"*

We carried out new experiments to assess the outcome of dynein inhibition on 19S RP recruitment to the centrosome during IS assembly, as suggested by the Reviewer. Control Jurkat T cells were treated with the dynein inhibitor ciliobrevin and used to form SEE-specific conjugates with Raji B cells. The results show that, similar to BBS1 depletion (Fig.7A), ciliobrevin-treated cells had a defect in 19S recruitment to the centrosome compared to carrier-treated cells in SEE-specific conjugates (Fig.7G). The Results section has been modified accordingly (p.17). Together, these results support the notion that 19S RP recruitment to the centrosome is dynein-dependent and that BBS1 participates in this process by coupling 19S to dynein.
Regarding the centrosomal versus synaptic dynein pools, this is an interesting point. However, to our knowledge the exact composition of the dynein complexes that drive centrosome translocation and IS assembly (including TCR microcluster movement towards the c-SMAC) has not been characterized. In the report by Martin-Cofreces et al (JCB 2008) showing that dynein is required for centrosome translocation the authors refer to an antibody against dynein heavy chain, while in the report by Hashimoto- Tane et al (Immunity 2011) the authors refer to antibodies against dynein light chain 1 and intermediate chain 2. The latter is the one that we have used for our study. We believe that the current information is not sufficient to answer the Reviewer's question. For future reference we have indicated the specificity of the antibody used (dynein intermediate chain 2, clone 74.1) in the legend to figure 7 (see Table S2 with details about its source).

Point 3. *"The authors state that the expression of the BBSome core components is comparable or higher in T cells compared to ciliated cells. However, in Fig S1A, BBSome expression levels are different between Jurkat T cells and primary T cells: while primary T cells have comparable or much higher expression levels than ciliated cells, Jurkat T cells display lower ones. This is especially true for the expression levels of BBS1, that are really low in Jurkat T cells. This is not as striking in Fig 1A, where they measure BBS1 levels by Western Blot, but Jurkat T cells still*

express lower amounts of BBS1."

We agree with the Reviewer's considerations and have changed the Results to better describe these findings (p.6). We believe that the apparent discrepancy in BBS1 expression at the mRNA and protein levels in Jurkat cells might reflect a higher stability of the protein compared to primary T cells. We have also commented on this point (p.6).

Point 4. *"Please always report on the panel the conditions and cell type (for example in Fig 2C, write Ctrl, J KD)"*

We have edited all figures according to the Reviewer's suggestion.

Point 5. *"Please draw the outline of the cells when showing single staining (especially for the centrosome, in Fig 1D for example), or put only the merged image, otherwise it is not possible to interpret the figure."*

We have drawn the outlines of cells showing single staining as requested.

Reviewer 3

We thank the Reviewer for the critical assessment of our manuscript and the useful suggestions. We have carried out several additional experiments to address the issues raised by the Reviewer and extensively edited the manuscript to better clarify several points that were obviously not sufficiently clear in the previous version. Since the major points in the "General comments" are reiterated and expanded in the "Specific comments" we have provided a point-by-point response to the latter.

Point 1." *In the materials and methods section it was noted that "T cells are isolated from healthy human donors" but they do not appear to be enriched for CD4 cells. Given the stated difference between CD4 and CD8 cells on MTOC translocation and use of Jurkat cells for comparison why would you not specifically use CD4 cells for comparison?"*
"Assuming the statement quoted above is true, we have to ask how do treatments such as proteasome inhibitors and BBS1 knockdown affect CD8 T cells. Would that not be a critical comparison for your hypothesis that actin linkage between the centrosome and nucleus needs to be broken for centrosome translocation?

We agree with the Reviewer and repeated the experiment on purified peripheral $CD4^+$ T cells co-stained for PCNT-1 and nesprin-2. The results show that the distance of the centrosome from the nucleus increases in $CD4^+$ T cells that have formed an IS, concomitant with centrosome translocation (Fig.S5D,E). Due to the restrictions caused by the COVID-19 pandemic, which include the supply of buffy coats, we had to use some frozen left-over aliquots of purified $CD4^+$ T cells that we had previously prepared for other experiments. We could therefore carry out only selected experiments, namely the measurement of the distance of the centrosome from the nucleus (Fig.S5E), as requested by the Reviewer, as well as centrosomal F-actin density in 15-min conjugates (mature synapses) (Fig.S5F) and 19S RP recruitment to the centrosome early during IS assembly (1 min) (Fig.S5G). These results recapitulate the results obtained on Jurkat (Fig.4B;7A) and primary T cells (Fig.4B,7C).

Unfortunately, we did not have stored $CD8^+$ T cells. However, since $CD8^+$ T cells had been previously shown not to require centrosome detachment from the nucleus to translocate to the IS (Lui-Roberts et al., 2012), we believe that, in the present circumstances, we have addressed to our best the major concern raised by the Reviewer. We have edited accordingly the part of the Discussion on the differences between $CD4^+$ T cells (this manuscript) and $CD8^+$ T cells (Lui-Roberts et al., 2012) in the dissociation of the centrosome from the nucleus during IS assembly (p.20).

Point 2. "*The paper by Galgano, D., A. Onnis, E. Pappalardo, F. Galvagni, O. Acuto and C. T. Baldari (2017). "The T cell IFT20 interactome reveals new players in immune synapse assembly." J Cell Sci 130(6): 1110-1121. Already showed that knocking down components of the IFT20 complex prevents movement of TcR containing vesicles to the IS. Shouldn't they be cited?*"

We have cited the paper as requested. Please note however that knockdown of BBS1 gives a different IS phenotype compared to IFT20 knockdown, with a defect of the centrosome in the former (this manuscript) and a defect in polarized TCR recycling with normal centrosome translocation in the latter (Finetti et al, 2009; Finetti et al, 2014), indicating that BBS1 and IFT20 act at different steps of IS assembly. We commented on this in the Discussion (p.20).

Point 3. *"The authors use the term F actin clearance to describe the change in actin in the area of the MTOC. Presumably these are endosomes and not amorphous blobs of actin. If so, are these organelles normally associated with motor proteins and capable of moving towards or away from the MTOC? Is the failure to move associated with loss of linkage to motor proteins associated with organelles?"*

As discussed in the response to Reviewer 1 (point 4), our description of "F-actin clearance" in the area of the MTOC was obviously not adequate. After reading this Reviewer's and Reviewer's 1 comments we decided to re-analyze the data generating a mask that only covers the area stained by PCNT1/ γ-tubulin to quantify the centrosomal F-actin pool. Regarding the endosomal pool, we complemented the colocalization analyses carried out on cells double-stained for F-actin and Rab5 or Rab11 with new experiments on conjugates triple-stained for PCNT1, Rab5 and F- actin (we selected Rab5 because of its higher colocalization with F-actin compared to Rab11). For these images we generated a mask that includes the endosomes and excludes the centrosome and measured the colocalization of F-actin with Rab5 within this mask on the entire Rab5 compartment. The new analyses are presented in figure Fig.S6A,B (the strategy for generating the mask is shown in figure S4C and it is also described in the Results, p.12).
Our results show that the centrosomal F-actin pool decreases in control antigen- specific conjugates, which is consistent with previous reports on both (Jurkat) T cells (Bello-Gamboa 2020) and B cells (Obino 2016), while no decrease was detectable in conjugates formed using BBS1-deficient T cells (Fig.4A,B,S5F). We interpret these results as i) confirming centrosomal F-actin clearance in Jurkat cells and showing that it also occurs in primary T cells (total and $CD4^+$) undergoing IS formation; and ii) indicating a role for BBS1 in this process. At variance with centrosomal F-actin, we found no alteration in endosomal F-actin in conjugates of control T cells formed in the presence or absence of SEE (and no effect of BBS1 KD), as assessed in conjugates co-stained for Rab5 (Fig.S6A,B). This is in agreement with the colocalization analysis carried out on individual endosomes (Fig.5E), which excludes the contribution of centrosomal F-actin. We believe that this is due to the fact that this pool is highly dynamic, with continuous redistribution of WASH complexes that promote the Arp2/3- dependent polymerization of new actin filaments on endosomes and their coupling to microtubules for their transport to the plasma membrane (Gomez&Billadeau, Dev Cell 2009). We have edited the Results section accordingly (p.13).
Regarding the movement of endosomes towards and away from the MTOC, this process is indeed mediated by microtubule motor proteins. During IS formation recycling endosomes remain clustered around the centrosome and co-translocate with it to the IS. This process is dependent on centrosome repositioning to the IS (Bustos- Moran 2016). We believe that F-actin-positive endosomes do not polarize to the IS in BBS1 KD/KO T cells because the centrosome itself does not translocate. In this context, T cells deficient for IFT20 or other ciliogenesis proteins show a different behaviour of recycling endosomes, which fail to polarize to the IS despite a normal translocation of the centrosome due to an interruption of the pathway at different steps, from sorting at early endosomes to coupling to microtubule motors (Finetti 2014, Onnis 2015, Capitani 2020). We have clarified this point in the Discussion (p.20).

Point 4.1. *"Given the similarities between the Finetti paper and this one with regard to the translocation of TcRs to the IS, how do you eliminate the possibility that failure of MTOC translocation is not simply a signaling defect."*
As mentioned above, IFT20 controls TCR sorting at early endosomes and their transit to recycling endosomes (Finetti et al, 2014), while BBS1 acts at the previous step, i.e. centrosome polarization to the IS (this manuscript). TCR signaling is kept operational by two receptor pools. The plasma membrane-associated pool triggers the mobilization of surface TCRs, integrins and co-stimulatory receptors to the T cell contact with the APC and their redistribution therein to generate the mature IS architecture. It also triggers centrosome polarization followed by re-routing of recycling endosomes that follow the centrosome to the IS. These allow for sustained signaling by delivering

fresh supplies of endosome-associated TCRs to the synaptic membrane as engaged, exhausted TCRs are internalized (Soares et al., 2013; Dustin 2014). As pointed out by the Reviewer, the fact that the centrosome does not polarize to the IS in BBS1-deficient T cells could indeed suggest a signaling defect. To rule out this possibility, we looked at the accumulation of tyrosine phosphoproteins (signaling readout) at the nascent IS (5-min conjugates), when receptor recruitment and redistribution are still in progress. The working hypothesis was that, if the failure of the centrosome to polarize towards the T cell-APC contact in BBS1 KD/KO cells was due to defective signaling, then we would observe an impairment in local tyrosine phosphoprotein accumulation also at an earlier time point, when the IS architecture has not consolidated yet to its mature configuration. As shown in figure 3A for BBS1 KD Jurkat cells and in figure 3B that includes new data obtained on primary BBS1 KO T cells, p-Tyr accumulation at the IS was comparable to control cells at 5 min. These data indicate that early signaling, on which centrosome translocation depends, occurs normally in BBS1 KD/KO T cells, while later signaling is impaired, likely due to the failure of endosomal TCRs to be delivered to the synaptic membrane in the absence of centrosome translocation. This is further supported by the flow cytometric analysis of p-Tyr$^+$ conjugates shown in figure 3E, where a defect in BBS1 KD cells could only be observed at 15- and 30-min conjugates, but not at earlier time points. This led us to hypothesize alternative explanations for the defect in centrosome translocation in BBS1 T cells, centrosomal F-actin clearance appearing a strong possibility based on the reports by Obino et al (2016) on B cells and Bello-Gamboa et al (2020) on T cells. We now confirmed the lack of an early signaling defect in BBS1-deficient T cells using an alternative readout, namely the synaptic accumulation of the active forms of the tyrosine kinase ZAP-70 and and of the transmembrane adaptor LAT in SEE-specific conjugates, as suggested by the Reviewer (Fig.3C,D; please see also the response to point 4.5). We have expanded the Results accordingly (p.10-11).

Point 4.2. *"This is an odd comparison. They are comparing the % of the pTyr at the synapse where (A) indicates there are grossly different levels of pTyr between the control and KO. Similarly in B they indicate there is 20% pTyr at the synapse where no staining is visible at all. Jurkat cells are different from primary T cells insofar as merely plating them on polylysine triggers calcium signaling on contact. Could this account for the difference between A and B? In any case while technically accurate one wonders what meaning there is to it."*

We obviously have not explained clearly what has been measured for the histograms. The percentages indicate the proportion of antigen-specific conjugates showing a strong and selective synaptic p-Tyr staining among the conjugates scored. The recruitment index indicates the ratio of pTyr fluorescence at the IS membrane to the p-Tyr fluorescence in the whole cell. We have described this more in detail in the Materials and Methods (p.31).
The histogram in figure 3A (left panel) shows that the percentage of SEE-specific conjugates harboring strong p-Tyr positivity of the synaptic membrane is comparable between control and BBS1 KD Jurkat cells at 5 min (early signaling). This percentage increases at 15 min (post-centrosome translocation sustained signaling) in control cells, but decreases in BBS1 KD cells. As stated in the response to point 4.1, this supports the notion that early signaling, on which centrosome translocation depends, is triggered normally in BBS1 KD cells, but that signaling is not sustained due to the failure of endosomal TCRs to be delivered to the synaptic membrane in the absence of centrosome translocation.

To measure the outcome of the centrosome translocation defect on p-Tyr signaling at the mature IS in BBS1 KD cells at the single cell level, we quantified the p-Tyr signal at the IS membrane in 15-min conjugates and expressed it as a ratio to the p-Tyr signal in the whole cell (recruitment index). We observed an increase in the synaptic accumulation of p-Tyr in SEE-specific conjugates of control Jurkat cells and a lesser accumulation in SEE-specific conjugates of BBS1 KD cells (Fig.3A, right panel), which indicates that, as predicted, the defect in centrosome translocation leads to defective signaling in these cells.

Regarding primary T cells, in the original submission we had validated only the results obtained on 15-min conjugates of Jurkat cells, with overall similar results. We have now integrated the data on primary T cells with new experiments on 5-min conjugates as well as 15-min conjugates. The results, presented in the new histogram in figure 3B (left panel), confirm that p-Tyr accumulation at the IS in 5-min conjugates is comparable between control and BBS1 KO T cells and hence that

the defect is in sustained, post-centrosome translocation signaling. Of note, based on the new analyses carried out on all experiments, the basal synaptic p-Tyr staining is not significantly different in BBS1 KO cells compared to control cells (Fig.3B, right panel). We have re-written this part of the Results to better explain the rationale, the experimental setting and the conclusions drawn based on the results (p.10-11).

Point 4.3. *"For A - recruitment index what is the time point? This should be stated in the legend."*

We thank the Reviewer for pointing this out. This information is now stated in the legend.

Point 4.4 *"In B there is no pTyr at the IS. Was there ever pTyr at the IS? For the recruitment index of A there is some difference whereas in B there is no difference. What are we to make of that?" "Why are similar time points to A not shown in B?"*

As detailed above (response to point 4.2) we have now added the 5-min time point which shows a normal initial p-Tyr accumulation at the IS formed by BBS1 KO T cells (Fig.3B), similar to Jurkat cells (Fig.3A). Representative images are shown in the figure below.

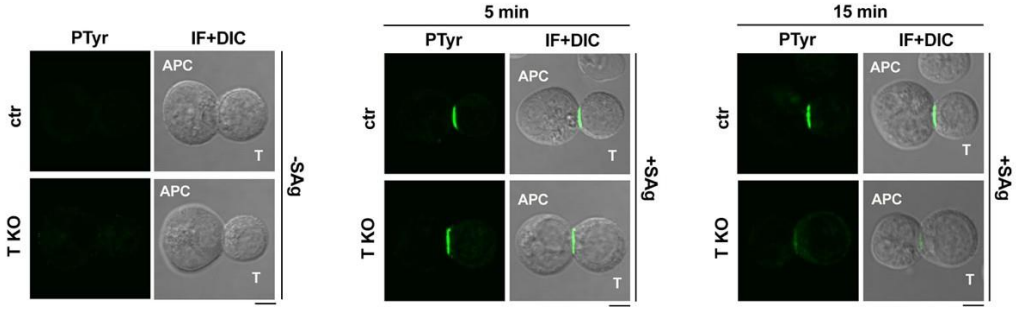

Figure 1. BBS1 is required for sustained TCR signaling at the IS. Immunofluorescence analysis of tyrosine phosphoproteins (p-Tyr) in 5-min and 15-min conjugates of control (ctr) and BBS1KO (T KO) primary T cells and SAg-pulsed Raji B cells. Conjugates of control (ctr) and BBS1KO (T KO) primary T cells formed in the absence of SAg were used as negative controls.

Point 4.5. *"It would add confidence if other indicators of activation were shown for the control and the JKO to demonstrate that these were not impaired. These could be calcium, LAT recruitment etc."*

As mentioned above (response to point 4.1) we carried out new experiments using the synaptic accumulation of the active forms of the tyrosine kinase ZAP-70 and the adaptor LAT as different indicators of activation. The results, shown in figure 3C,D, confirm that signaling is initiated normally in BBS1 KD T cells but is not sustained at later time points.

Point 5. *"Figure 5C This data is helpful and it seems the statistical significance holds for the 15 minute and 30 minute time points but what about the earlier time points? In particular does a comparison between the 0 minute and the 1 / 5 minute time points show that their differences are statistically significant?"*

The differences between control and BBS1 KD cells at early time points of activation (1 and 5 min) are not statistically significant, supporting the notion that BBS1 regulates later (post-centrosomal translocation) signaling. The differences between activated and non-activated samples are statistically significant for all time points in control cells but only for the early time points (1 and 5 min) in BBS1 KD cells, consistent with a defect in sustained TCR signaling. We have added this information to the figure (now Fig.3E).

Point 6.1. *"The term "clearance" needs to be explicitly defined and clarified. The reader keeps being directed (or misdirected) to the idea that proteasomal degradation of actin or some related factor (WASH, ARP 2/3 etc) leads to actin "clearance" but no specific ubiquitylation of any of these related proteins was shown. A demonstration of some specific target would certainly strengthened the case. In the intro it was stated that there were two pools of vesicles and WASH*

is associated with endosomal vesicles. It seems likely that the patchy actin staining around the centrosome is vesicular. Why couldn't their just have been a loss of WASH or depolymerization of actin or movement of vesicles that explains the change in actin staining? The data shows a loss of connection"

We do not want to propose that actin is being degraded. Since the F-actin pool at any given time is the result of a balance between polymerization and depolymerization of actin filaments, we think that one or more of the many regulators of the polymerization/depolymerization process might be the target of the centrosomal proteasome pool. In the discussion we have hypothesized WASH as one possible indirect target, since the centrosomal pool of WASH decreases during IS formation (Fig.4C,D and Obino 2016 for B cells) and this would tilt the balance towards F-actin depolymerization, leading to its clearance from the centrosome. The hypothesis is plausible, since it has been demonstrated that TRIM27, one of the main regulators of WASH, undergoes proteasome-mediated degradation (Hao 2013). We have further elaborated this point in the Discussion, also hypothesizing alternative targets of the proteasome among known actin regulators (p.21).
Regarding the distinction of centrosomal versus vesicular WASH in our measurements, we carried out new analyses using a mask that only includes the centrosome, as described in the response to point 3 for F-actin. The results confirm the presence of a dynamic WASH pool at the centrosome that decreases in activated control cells (which would result in a loss of F-actin due to a shift in the balance towards depolymerization) but not in BBS1 KD cells (Fig.4C,D). We re-analyzed and carried out further experiments on conjugates stained for WASH and Rab5 to assess the impact of BBS1 deficiency on the vesicular WASH pool (colocalization of WASH analyzed on single Rab5$^+$ dots). The results did not reveal any significant changes in either control or BBS1-deficient cells (Fig.S6C; please see also figure S6D for new analysis of WASH-Rab11 colocalization), consistent with the fact that only specific receptors and signaling mediators undergo polarized recycling to the IS, such that WASH-dependent actin polymerization is limited to these endosomes. Additionally, polarized recycling occurs over an extended timeframe, such that changes in endosomal WASH at any given time might go undetected. This point has been mentioned in the Results (p.13).

Point 6.2. *"The article by Ibanzez-Vega (2019) investigated the reduction of actin around the centrosome during B cell activation and found no overall reduction of actin. The results reported here seem to show the same thing. While it may not be the authors intent, the statement that they show proteasome-dependent clearance of actin certainly lends itself to the idea they are claiming that the proteasome degrades actin. Given that the patches of actin are on organelles, isn't it likely that either actin depolymerization or organelle movement is involved."*

As mentioned in the response to point 6.1, we do not claim that the proteasome degrades centrosomal actin but rather that it affects local F-actin polymerization (possibly indirectly by inhibiting WASH), such that the balance is tilted towards depolymerization, as hypothesized by the Reviewer and further addressed in the Discussion (p.21). Regarding the possibility that F-actin clearance from the centrosome is caused by the movement of F-actin$^+$ endosomes, this appears unlikely since we did not observe activation-dependent changes in endosomal F-actin, as detailed in the response to point 3 and shown in figure 5E,F.

Point 7.1 *"The antibodies used in this study do not appear to be Ub chain specific. The actin and WASH that appears to remain around the centrosome probably are on endosomal vesicles. WASH can be ubiquitylated on K63 which activates it [see Hao,*
Y. H., J. M. Doyle, S. Ramanathan, T. S. Gomez, D. Jia, M. Xu, Z. J. Chen, D. D. Billadeau, M. K. Rosen and P. R. Potts (2013). "Regulation of WASH-dependent actin polymerization and protein trafficking by ubiquitination." Cell 152(5): 1051-1064]. The question then is, "How do you know that there are not two pools of ubiquitin being stained by IF. Most of the cytoplasmic UB IF staining goes away after activation. Perhaps what remains after activation is Ub attached to proteins in other ways such as on K63 of WASH. As noted above there is a higher stability of the WASH pool that could be what is being detected by Ub IF staining."

As detailed in point 3, we have carried out new analyses to better discriminate the changes in F-actin, WASH, 19S and Ub at the centrosome. We believe that we do not see two Ub pools because the centrosomal one is tightly confined to a small area while the cytosolic pool is diffuse and hence

undetectable as a specific entity. We fully agree with the Reviewer regarding the regulation of WASH stability by K63 ubiquitylation, however the presence of a dynamic proteasome pool at the centrosome that locally degrades a variety of ubiquitylated proteins has been well established (Vora&Phillips 2016), as also shown in a recent report for T cells (Martin-Cofreces et al, Sci Adv 2020; now cited). Our prediction was that, if the proteasome was not effectively recruited to the centrosome in BBS1 KD cells (as shown in figure 7A), then we would see a local accumulation of ubiquitylated proteins compared to control cells, where an accumulation of 19S RP can instead be detected at the centrosome. Our results (Fig.7B) are consistent with this hypothesis.
The Reviewer is right about the fact that, among the ubiquitylated proteins at the centrosome, some might be modified by K63-linked rather than K48-linked ubiquitin chains, which tag for proteasome-mediated degradation. To address this issue experimentally we carried out additional experiments using an antibody specific for K48 polyubiquitin (we also used an antibody specific for K63 polyubiquitin, but it gave a too weak staining, making it unsuitable for this type of analysis). The results provide evidence of a centrosomal pool of K48 polyubiquitin that decreases during IS formation in control cells, but not in BBS1 KD cells. The results are presented in figure 7D and have been added to the Results (p.16).

Point 7.2. *"Why should ubiquitinated proteins accumulate at the centrosome in JKD cells if the proteasome is not recruited to the centrosome? Visually there does not appear to be much difference other than generally brighter staining? These DIC images of these cells look abnormal."*

Since a proteasome pool is present at the centrosome also under basal conditions one would expect the presence of ubiquitylated proteins that is likely to change during cell activation to allow for the regulation of centrosome movement or function, as shown for other cell types (Vora&Phillips 2016; Ibanez-Vega 2019). We show that in control cells the basal centrosomal Ub pool increases early during IS formation followed by a decrease, concomitant with 19S RP recruitment (Fig.7A,B). We think that BBS1 KD cells have a higher basal centrosomal Ub pool, because the local basal proteasome pool might be also regulated by BBS1. At variance with control cells, the centrosomal Ub pool does not undergo significant changes in BBS1 KD cells compared to control cells following activation with SEE-loaded Raji cells (Fig.7B). This is likely accounted for by the fact that the proteasome is not recruited to or activated at the centrosome in SEE-specific conjugates formed by BBS1 KD T cells and hence ubiquitylated proteins are not degraded. We have commented on this point in the Results (p.16). Please note that the proteasome connection is supported by the results of the new experiments, suggested by the Reviewer, providing evidence that the dynamic Ub pool is, at least in part, K48-linked (Fig.7D).
Regarding the differences between ctr and KD cells, the Ub staining was normalized to the staining of the centrosome within the same cell (using a mask that only includes the PCNT1 staining). We detailed this better in the Materials and Methods (p.31). We also used images with better DIC morphology.

Point 8. *"This sentence is difficult to dissect "We identified as an unexpected new player in synaptic trafficking the intraflagellar transport (IFT) system". It would be good to put a reference here to previous papers, or, if BBS4 is meant then that should be made clear."*

The sentence was modified and references added as requested.

Point 9. *"Based on the basal body localization of the BBSome in ciliated cells (Nachury, 2018; Wingfield et al., 2018), these included markers of the centrosome (γ- tubulin) and of the pericentrosomal (PCM-1, CEP131/AZI1, CEP290) and endocytic recycling (Rab11) compartments."*

The sentence was edited as requested.

Point 10. *"Of note BBS4, which is responsible for priming the assembly of a pre- BBSome complex in ciliated cells (Prasai et al., 2020), also polarises to the IS together with the centrosome (Fig.S1B), suggesting that other BBS proteins might participate in IS assembly". In what sense is BBS4 participating in IS assembly? What is deficient?"*

In this manuscript we addressed the function of BBS1. The data presented in figure S1A showing that the other core BBSome components are expressed and that one of these, BBS4, polarizes to the IS (Fig.S1B), suggest the possibility that other BBSome components may participate in IS

assembly. This is just a hypothesis, but their functional characterization will be the topic of future work. We have toned own our statement in the Results (p.7).

Point 11. *"Supplemental Figure S4 is presumably mislabeled. It goes from A to E to C"*

We apologize for the odd lettering. Actually the "E" was a truncated "B". We checked lettering in new figure S4.

Point 12. *"Figure S8D Left, Middle What are we looking at. These should be labeled for clarity. Both left and middle figures look to be the same. Is the middle supposed to be total?"*

We labelled the panels as requested. The middle panel shows CD3□ MFI measured on permeabilized cells (total). We have rectified the Y-axis label in the figure (now Fig.S7C) and clarified the contents in the figure legend.

Point 13. *"How does CRISPR gene editing transiently knock down expression?"*

We apologize for the misstatement. We have removed the word “transiently”. Point 14.
"Figure 2 C legend “Right” is misspelled."
The spelling has been rectified.

Point 15. *"There has been a lot of work on the role of dynein in centrosome polarization including evidence showing that the NDE1/LIS1 complex with dynein rather than the dynactin dynein complex is responsible for centrosome polarization (Nath et al, 2016). Simply saying it is the dyneindynactin complex seems inappropriate."*

We have modified the statement and included the indicated reference (Discussion, p.22).

Second decision letter

MS ID#: JOCES/2021/258462

MS TITLE: The Bardet-Biedl Syndrome complex component BBS1 controls T cell polarity during immune synapse assembly

AUTHORS: Chiara Cassioli, Anna Onnis, Francesca Finetti, Nagaja Capitani, Jlenia Brunetti, Ewoud B Compeer, Veronika Niederlova, Ondrej Stepanek, Michael L Dustin, and Cosima Baldari

ARTICLE TYPE: Research Article

I am happy to tell you that your manuscript has been accepted for publication in Journal of Cell Science, pending standard ethics checks.

Reviewer 1

Advance summary and potential significance to field

I believe this is an original and well performed work that provides key insights into the mechnaisms of immunological synapse formation.

Comments for the author

I am satisfied with the responses the authors provided to my comments on the first review and I have no further requests.
I recomment publication

Reviewer 2

Advance summary and potential significance to field

The revisions have addressed the questions we raised in our first review, the paper is now ready for publication.

Comments for the author

The revisions have addressed the questions we raised in our first review, the paper is now ready for publication.