nature portfolio

Peer Review File

Development of conformation-selective antibodies targeting human SLC15A4

Corresponding Author: Professor Pu Gao

This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
SLC15A4 is an endolysosomal solute transporter implicated in TLR7/8/9 signaling and the ensuing immune response, making it a potential therapeutic target for diseases associated with these TLRs. In this study, the authors immunized mice to generate two novel anti-SLC15A4 antibodies, designated clone 107 and clone 235, using single-cell BCR technology. Both antibodies were shown to bind to the luminal side of SLC15A4 but exhibited opposing effects on TLR7/8-mediated THP1 activation by modulating TASL binding. The authors further illustrated the binding mechanisms of these antibodies to SLC15A4 through CryoEM, proposing a model for how they elicit contrasting activities. Although the structure of SLC15A4 in complex with TASL was previously resolved by Chen et al. in 2023, this study offers new insights into how the transporter can be conformationally altered using antibodies to modulate downstream functional activity. The experimental design is robust, and the data largely support the conclusions drawn. While the development of these anti-SLC15A4 antibodies serves as valuable research tools, the authors did not sufficiently demonstrate their potential therapeutic applications in vitro or in vivo.

Major concern:
The authors suggest that the generated anti-SLC15A4 antibodies may aid in developing selective therapies and research tools targeting human SLC15A4. However, as SLC15A4 is primarily localized intracellularly and not on the plasma membrane, and the authors did not perform experiments or discuss strategies to deliver the antibodies to the cytoplasm, they should clarify how these antibodies could have potential therapeutic applications.
More in vitro and in vivo assays could be performed to demonstrate the therapeutic utility of these antibodies.

Minor concern:
For Figure 1b FACS assay, it should be clarified if SLC15A4 is expressed on the plasma membrane or cytoplasm of HEK293T cells.
For Figure 1b-f, why was clone 235 used as a Fab while clone 107 was not? This is particularly important for internalization assay in which bivalent antibody is more likely to induce SLC15A4 clustering and more internalization.
For Figure 1c-f, can authors clarify how this method can be used to study internalization? Will AF647 be quenched efficiently once internalized?
For Supplementary Figure 2a, the change to an adherent morphology is difficult to spot, and Fab107 mediated block of R848-induced morphological switch is not convincing. Can the authors quantify the level of adherence and label the THP1 cells with fluorescent dye to make the adherence more easily observable?
For Figure 3b, could the authors provide SDS-PAGE data for all the peaks from size-exclusion chromatography? Also some experimental description is needed here; for example, is SLC15A4 mixed with TASL first and then antibody added or the other way around?

Reviewer #2

(Remarks to the Author)
Review of Zhu at al, NCOMMS-24-50481: Development of conformation-selective antibodies targeting SLC15A4 with opposite immunoregulatory activities

Zhu and colleagues report on the identification of two functional antibodies targeting the solute carrier SLC15A4. SLC15A4 plays a crucial role in endosomal TLR7,8 and 9 signalling and the two identified antibodies shows opposing effects by either blocking or sustaining the activation of this pathway by regulating the ability of SLC15A4 to form a complex with the recently identified immune adaptor TASL.
Overall, the findings are of interest for the field, the data are of high quality and the conclusions are well supported by the experimental evidence provided.
My main comments aim at further clarify some mechanistic aspects and providing additional information that will increase the impact of the study.

Main points:
- The authors rightfully state that their study and the two antibodies provide valuable research tools for the field, therefore they need to provide the full sequences of the two antibodies in the manuscript (as supplemental information) and facilitate the access of other labs to these tools by depositing the encoding plasmids in repositories such as Addgene.
- Accordingly to the previously reported specific role of SLC15A4-TASL complex in IRF5 activation (ref 20, 31, 34), it would be important to confirm that the two antibodies act through this mechanism by monitoring in THP1 cells R848-induced IRF5 activation, which should be affected (increased or decreased) by the two antibodies, as well as NFkB and MAPK, which should not be affected. Moreover, by locking SLC15A4 in the two opposing conformations, Fab107 and Fab235 should lead to TASL protein degradation or stabilisation, respectively (ref 20, 34). Is this observed?
- In the experiments of figure 2, THP1 cells were pre-treated O/N with the antibodies before R848 stimulation, while in fig 1c and 1e 4 hours were sufficient for significant antibody uptake. Which is the minimal time of pretreatment needed to observe a functional effect of the two antibodies on TLR7/8 responses? Does this correlate with the time required to observe TASL degradation/accumulation in cells?

Minor points:
- Some statements should be edited to better reflects previous findings. For examples the claim that the study “reveal the mechanism of TASL recruitment” (line 76,) or “our work elucidates how hSLC15A4 recruits TASL at a molecular level” (line 399) should be revised to mention that this has been previously described (refs 32, 33, 34). Similarly, “This feature holds promise for the development of drugs based on the SLC15A4-TASL adaptor complex for various diseases” (lines) should acknowledge the work in ref 34.
- Fig 4G: The figure illustrating the steric hindrance is not ideal. It would be useful to provide a clearer visualisation of the predicted clashes by zooming in these regions.
- Bar graphs should show individual values.

Reviewer #3

(Remarks to the Author)
The study by Zhu et al. employs a comprehensive screening and validation approach to identify two novel functional antibodies, clones 107 and 235. The data show that Clone 107 functions as an inhibitory antibody, binding selectively to hSLC15A4 in a luminal-open state that is incompatible with TASL binding. In contrast, Clone 235 acts as a stimulatory antibody, stabilizing hSLC15A4 in a cytoplasmic-open state that supports TASL binding. Despite recent reports on SLC15A4 structures, this study provides significant contributions to both therapeutic development and as a functional research tool for SLC15A4. Therefore, I recommend it for publication in Nature Communications. However, there are several points that require attention before publication:

Major concerns:
1. The structures of fab107-SLC15A4 and fab235-SLC15A4-TASL were solved in differing environments, with Fab107-SLC15A4 in a Nanodisc (Fig. 4a) and Fab235-SLC15A4-TASL in GDN detergent (Fig. 5a). Could the varying detergent environments, rather than the antibody itself, influence SLC15A4 conformation? Additionally, Chen et al. (2013, Nature Communications) suggested that apo SLC15A4 may form a dimeric state. Is it possible that the SLC15A4+Fab107 complex also forms a dimer?

2. The ability of antibodies 107 and 235 to penetrate the plasma membrane and interact with the endolysosomal lumen side of SLC15A4 is intriguing, as observed via confocal fluorescence microscopy. Given that SLC15A4 is present in both endolysosomal and plasma membranes, could these antibodies initially bind to SLC15A4 at the plasma membrane and then be internalized via endocytosis to reach the endolysosomal membranes?

3. Previous studies have shown that the SLC15A4-TASL complex assumes an cytoplasmic-open conformation, while apo-SLC15A4 adopts an luminal-open conformation, which corresponds to the conformations of SLC15A4-TASL-Fab235 and SLC15A4-Fab107, respectively. It is noted that hSLC15A4 with Fab235 can also form a complex without TASL (Fig. 3b). What is the conformation of hSLC15A4+Fab235 in the absence of TASL? Can the cytoplasmic-open state be maintained without TASL, and does clone 235 stabilize SLC15A4 alone in this state?

Minor concerns:
1. The statement, “antibodies that target SLC15A4 with better biosafety and functional specificity have also not been discovered,” lacks supportive data on the biosafety profiles of antibodies.
2. Supplementary Fig. 3b contains formatting issues such as “PE Anti-M?”. Additionally, Supplementary Fig. 6d to j display “view” in a way that requires clarification.
3. Supplementary Fig.7 and Fig.8: The accession number of proteins from different species should be indicated at the figure

legends.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
The authors note in their revision that the functional immunomodulatory data for Fab 107 and Fab 235 reported in the original manuscript could not be reproduced upon testing with distinct antibody batches. They have addressed all other concerns raised. The identification of two novel SLC15A4-targeting antibodies in different states represents an advance; however, the lack of corroborating functional evidence raises concerns regarding whether the findings possess the broad significance and impact typically required for publication in Nature Communications.

Reviewer #2

(Remarks to the Author)
I understand and appreciate the decision of the authors to remove the functional data that they could not reproduce with the new batch of antibodies. Unfortunately, in my opinion this affects quite substantially the overall message and impact of the manuscript.
Indeed, without demonstration that these antibodies can have a direct functional effect on TLR7/9 responses and TASL protein stability, several claims concerning the impact of the antibodies on the conformation of SLC15A4 may be called into questions, especially regarding the idea that the antibody binding drives/induces the change in conformation (for example lines 252-253 “the conformational changes of hSLC15A4 upon binding to different antibodies” or lines 278-279“the distinct binding modes of Fab107 and Fab235 enable them to effectively target and stabilize unique conformations of hSLC15A4”).
It could be argued that the determinant factor for the conformation of recombinant SLC15A4 is the presence or not of TASL. It is clear that, at least in vitro, TASL binding to SLC15A4 drive the cytoplasmic open conformation (independently of the presence of the Fab235) and strong evidence suggest that apo SLC15A4 adopts a luminal open conformation in absence of TASL, independently of the presence of Fab107. Indeed in the structural studies by the Yang lab (cited Chen et al. Nat Commun 2023), as well as in the recent report by the Shimizu lab (PMID: 39719710) , the cryoEM structures of apo-SLC15A4 were both obtained in the luminal open conformation, while the presence of TASL was sufficient to induced the cytoplasmic open conformation.
The identification of antibodies that (preferentially) binds two different conformations of a proteins is different than showing that the binding of these antibodies can drive the conformational shift. Without functional evidence on the impact of the two antibodies on TLR7/9-induced responses, the current study falls in my opinion a bit short in formally demonstrating the latter. Indeed, as discussed in reviewer 3 point 3 and the reply from the authors, it appears that Fab235 binds both conformations and it is not sufficient to induce the conformational change in absence of TASL.
It is also unfortunate that the authors removed the data suggesting that the antibodies could be uptake by the cells (original first submission Fig 1c-f and SupFig 1e-f). Were these data also not reproducible with the new batch of antibodies?

Reviewer #3

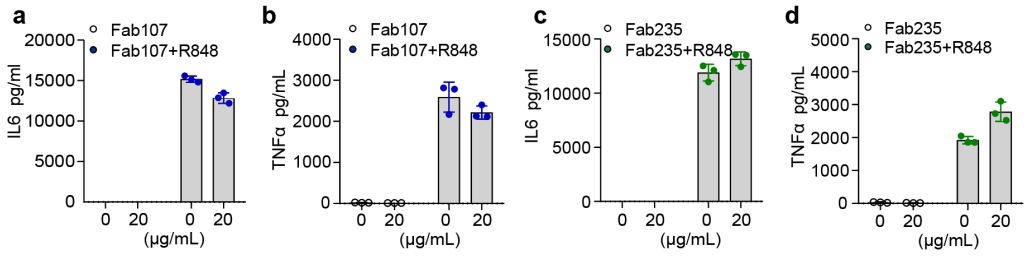
(Remarks to the Author)
Although the regulatory role of antibodies have been delete in this version, the structural analysis indeed provide important insights for the conformational change of SLC15A4. The related cellular function may require further investigation in the future. So I recommended it for publication.

Overall Response:

We are deeply grateful to the reviewers for their positive, constructive, and detailed feedback. These insightful comments have greatly helped improve our work. In response to the reviewers' suggestions, we have conducted additional experiments to address the raised concerns and strengthen our findings.

Through a systematic screening and validation approach, we identified two conformation-selective antibodies, clones 107 and 235, that target the endolysosomal luminal surface of hSLC15A4 with opposing conformational preferences. We further provide direct biochemical and structural evidence elucidating their conformation-specific recognition mechanisms and the molecular basis of SLC15A4's interaction with its downstream adaptor TASL. These data, consistently replicated across multiple experimental rounds in both the initial submission and the revision process, exhibit high reproducibility and reliability.

In our initial submission, we also presented cellular data showing that these two antibodies, when extracellularly added, could markedly enhance or suppress TLR7/8-mediated immune responses. While these cellular effects were consistently observed with the original antibody batch, newly purified batches during revision exhibited a significant decline in immunoregulatory activity at the cellular level (please see the figure below showing the diminished cellular immunoregulatory activities with the newly purified antibodies). Despite exhaustive efforts over several months, we have not yet identified a convincing explanation for this discrepancy between different antibody batches. A plausible hypothesis is that, although these antibodies exhibit clear conformation selectivity at the biochemical and structural levels, their efficiency in cellular internalization and delivery to the endolysosomal lumen may be insufficient to achieve robust immunomodulation in cells.



Cytokine production (TNFα and IL6) of THP1 cells by using newly purified batches of antibodies, unstimulated or stimulated with R848, pretreated with Fab107 (a and b) or Fab235 (c and d). Mean ± s.d. (n = 3 biological replicates).

Given these findings, and in the interest of scientific rigor and responsible reporting to the community, we have decided to retain only the robust antibody screening, biochemical, structural, and mutagenesis data in the revised manuscript and to remove the cellular immunoregulatory data for the time being. We sincerely hope the reviewers will understand and find this change acceptable, we also hope that they will agree that these conformation-selective antibodies still hold promise for future development and engineering as potent modulators of cellular and *in vivo* immune responses.

Point-by-Point Response:

In our point-by-point response below, we have specifically addressed the reviewers' concerns. The reviewers' comments are in red and our responses are in black. As the manuscript has undergone substantial revisions, we have not marked individual additions and deletions in the revised version.

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Reviewer #1:

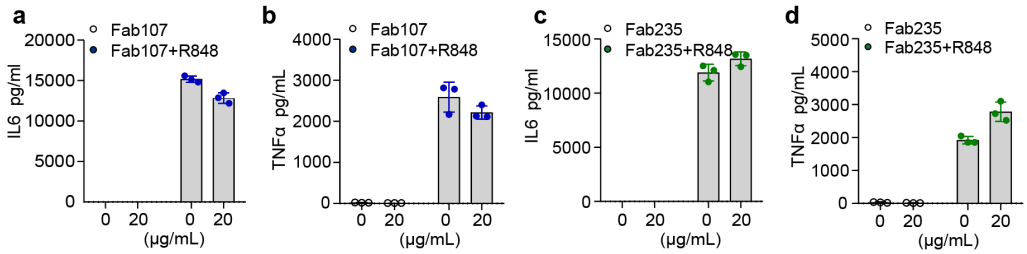
SLC15A4 is an endolysosomal solute transporter implicated in TLR7/8/9 signaling and the ensuing immune response, making it a potential therapeutic target for diseases associated with these TLRs. In this study, the authors immunized mice to generate two novel anti-SLC15A4 antibodies, designated clone 107 and clone 235, using single-cell BCR technology. Both antibodies were shown to bind to the luminal side of SLC15A4 but exhibited opposing effects on TLR7/8-mediated THP1 activation by modulating TASL binding. The authors further illustrated the binding mechanisms of these antibodies to SLC15A4 through CryoEM, proposing a model for how they elicit contrasting activities. Although the structure of SLC15A4 in complex with TASL was previously resolved by Chen et al. in 2023, this study offers new insights into how the transporter can be conformationally altered using antibodies to modulate downstream functional activity. The experimental design is robust, and the data largely support the conclusions drawn. While the development of these anti-SLC15A4 antibodies serves as valuable research tools, the authors did not sufficiently demonstrate their potential therapeutic applications in vitro or in vivo.

We sincerely thank the reviewer for summarizing the main contributions of this study and for recognizing that it "*offers new insights into how the transporter can be conformationally altered using antibodies to modulate downstream functional activity*" and that "*the experimental design is robust, and the data largely support the conclusions drawn*". In response to the reviewer's valuable suggestions, we have conducted extensive experiments. However, due to the reduced cellular immunoregulatory activities observed in newly purified batches of antibodies (as noted in our Overall Response), we have decided to remove the cellular functional data to ensure responsible reporting. Nevertheless, our biochemical and structural data robustly demonstrate that these two antibodies target distinct conformations of SLC15A4. To our knowledge, there have been no prior reports of using antibodies to regulate different conformations of the same SLC transporter. Thus, we believe our study still represents a potential strategy for harnessing antibodies to modulate the inhibitory or stimulatory states of hSLC15A4.

1. The authors suggest that the generated anti-SLC15A4 antibodies may aid in developing selective therapies and research tools targeting human SLC15A4. However, as SLC15A4 is primarily localized

intracellularly and not on the plasma membrane, and the authors did not perform experiments or discuss strategies to deliver the antibodies to the cytoplasm, they should clarify how these antibodies could have potential therapeutic applications.

We thank the reviewer for raising this important point. Indeed, SLC15A4 is primarily localized to the endolysosomal membrane rather than the plasma membrane, and direct application of antibodies may result in low immunoregulatory activity, which could account for the reduced efficacy observed with newly purified antibody batches (see figure below). To address this limitation, we plan to develop a bispecific antibody that combines Fab107 or Fab235 with a rapidly internalizing antibody targeting pDCs, B cells, or tumor cells. By leveraging the efficient internalization properties of these additional antibodies, we aim to ensure effective delivery of our SLC15A4-targeting Fab fragments to the endolysosomal compartment. Additionally, conjugating the antibodies with cell-penetrating peptides (CPPs) may further enhance cellular uptake. These strategies represent important directions for our future research, which we have now discussed in the revised manuscript (lines 351-359).



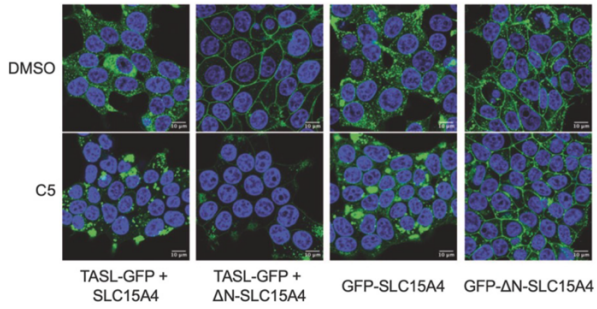
Cytokine production (TNFα and IL6) of THP1 cells by using newly purified batches of antibodies, unstimulated or stimulated with R848, pretreated with Fab107 (a and b) or Fab235 (c and d). Mean ± s.d. (n = 3 biological replicates).

2. More in vitro and in vivo assays could be performed to demonstrate the therapeutic utility of these antibodies.

We thank the reviewer for the valuable suggestion. To demonstrate the therapeutic utility of our antibodies, we systematically conducted a series of additional functional experiments during the revision process. However, in replicating these experiments, we observed a significant reduction in potency in the newly prepared antibody batches, and despite several months of efforts, we have not yet identified a convincing explanation for this discrepancy. To ensure the reliability of our conclusions, we have removed the cellular functional data in the revised manuscript. The current core findings are now supported by reproducible structural and biochemical validation. We hope that the reviewer will agree that these conformation-selective antibodies still hold promise for future development and engineering as potent modulators of cellular and *in vivo* immune responses.

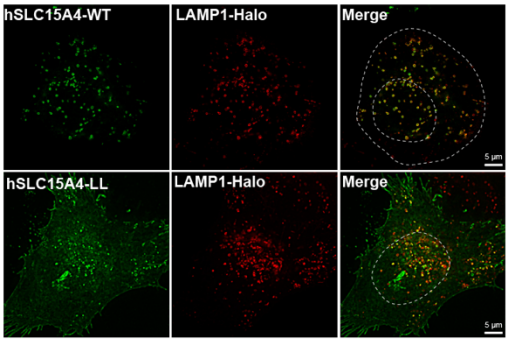
3. For Figure 1b FACS assay, it should be clarified if SLC15A4 is expressed on the plasma membrane or cytoplasm of HEK293T cells.

We thank the reviewer for raising this issue. The subcellular distribution of SLC15A4 in HEK293T cells has been characterized in a recent peer-reviewed study (*Nat Commun 14, 6626, 2023*). As demonstrated by confocal microscopy in this paper (figure below), full-length SLC15A4 predominantly localizes to endolysosomal compartments with minimal plasma membrane association in HEK293T cells. In contrast, the N-terminal truncated variant (ΔN-SLC15A4) exhibits complete redistribution to the plasma membrane. These observations are consistent with our FACS data obtained through indirect surface staining. Specifically, compared to the prominent plasma membrane localization of the N-terminal mutations (L14A/L15A) or deletions (Δ1-31), full-length SLC15A4 only exhibits weak surface staining in HEK293T cells that highly overexpress SLC15A4 (Fig. 1b).



Data from Nat Commun 14, 6626, 2023

Furthermore, we also examined the subcellular distribution of SLC15A4 in HeLa cells, which express it at relatively lower levels than HEK293T cells. As shown in the confocal microscopy data (figure below), wild-type SLC15A4 primarily localizes to endolysosomal compartments, while the LL mutant (L14A/L15A) displays a marked redistribution to the plasma membrane. We speculate that only under conditions of high overexpression (e.g., in HEK293T cells) might a small portion of SLC15A4 localize to the plasma membrane.



Live cell fluorescence microscopy of Hela cells expressing the indicated constructs.

4. For Figure 1b-f, why was clone 235 used as a Fab while clone 107 was not? This is particularly important for internalization assay in which bivalent antibody is more likely to induce SLC15A4 clustering and more internalization.

We thank the reviewer for pointing out this issue and for the helpful suggestion. In the revised manuscript, we have conducted the assays using the bivalent antibody form of clone 235 instead of its Fab form (Fig. 1b).

5. For Figure 1c-f, can authors clarify how this method can be used to study internalization? Will AF647 be quenched efficiently once internalized?

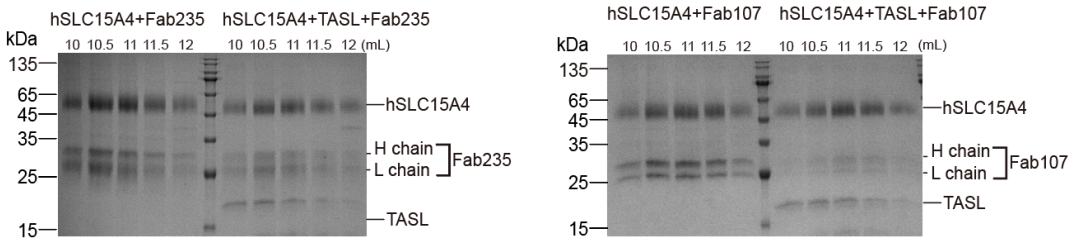
We thank the reviewer for raising these questions. AF647 is a pH-insensitive dye and will not be quenched once internalized. However, due to the reduced cellular immunoregulatory efficacy observed with newly purified antibody batches (as noted in our Overall Response), we have decided to exclude these data from the revised manuscript.

6. For Supplementary Figure 2a, the change to an adherent morphology is difficult to spot, and Fab107 mediated block of R848-induced morphological switch is not convincing. Can the authors quantify the level of adherence and label the THP1 cells with fluorescent dye to make the adherence more easily observable?

We thank the reviewer for pointing out these issues. Due to the reduced cellular immunoregulatory efficacy observed with newly purified antibody batches (as noted in our Overall Response), we have decided to exclude these data from the revised manuscript.

7. For Figure 3b, could the authors provide SDS-PAGE data for all the peaks from size-exclusion chromatography?

By following the reviewer's suggestion, we have performed SDS-PAGE analysis for all the peaks from size-exclusion chromatography (figure below). We have included these data in the revised manuscript (Supplementary Fig. 2g, h).



Also some experimental description is needed here; for example, is SLC15A4 mixed with TASL first and then antibody added or the other way around?

Yes, we first purified the hSLC15A4-TASL complex and then added the antibody. We have included the experimental description in the Methods section (lines 555-557).

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Reviewer #2:

Review of Zhu at al, NCOMMS-24-50481: Development of conformation-selective antibodies targeting SLC15A4 with opposite immunoregulatory activities. Zhu and colleagues report on the identification of two functional antibodies targeting the solute carrier SLC15A4. SLC15A4 plays a crucial role in endosomal TLR7,8 and 9 signaling and the two identified antibodies shows opposing effects by either blocking or sustaining the activation of this pathway by regulating the ability of SLC15A4 to form a complex with the recently identified immune adaptor TASL. Overall, the findings are of interest for the field, the data are of high quality and the conclusions are well supported by the experimental evidence provided. My main comments aim at further clarify some mechanistic aspects and providing additional information that will increase the impact of the study.

We thank the reviewer for summarizing the pivotal role of SLC15A4 in endosomal TLR7/8/9 signaling and the key contributions of our work. We also appreciate the reviewer's recognition that our study is "*of interest for the field, the data are of high quality and the conclusions are well supported by the experimental evidence provided*". We fully agree with the reviewer's suggestion to "*further clarify some mechanistic aspects and provide additional information to enhance the study's impact*", and have carefully addressed these points in our revised manuscript.

As mentioned in our above Overall Response, the newly purified antibody batches unfortunately did not replicate the pronounced cellular immunoregulatory activity observed with the original batches. Despite several months of dedicated efforts, we have not identified a convincing explanation for this discrepancy between different antibody batches. In the interest of scientific rigor and responsible communication to the research community, we have therefore decided to retain only the robust antibody screening, biochemical, structural, and mutagenesis data in the revised manuscript, while temporarily removing the cellular immunoregulatory data. We sincerely hope the reviewer will understand and find this change acceptable, we also hope that the reviewer will agree that these conformation-selective antibodies still hold promise for future development and engineering as potent immune modulators.

1. The authors rightfully state that their study and the two antibodies provide valuable research tools for the field, therefore they need to provide the full sequences of the two antibodies in the manuscript (as supplemental information) and facilitate the access of other labs to these tools by depositing the encoding plasmids in repositories such as Addgene.

We thank the reviewer for this valuable suggestion. In our initial submission, we did not include the full sequence information because the structural data we deposited in the PDB database already contains these sequences. In response to the reviewer's comment, we have now also incorporated the complete antibody sequence information in the revised manuscript (Supplementary Table 2). Additionally, we will

ensure that the plasmids are made available to the community, either by depositing them in repositories such as Addgene, as the reviewer suggested, or by directly sharing the plasmids upon request.

2. Accordingly to the previously reported specific role of SLC15A4-TASL complex in IRF5 activation (ref 20, 31, 34), it would be important to confirm that the two antibodies act through this mechanism by monitoring in THP1 cells R848-induced IRF5 activation, which should be affected (increased or decreased) by the two antibodies, as well as NFkB and MAPK, which should not be affected. Moreover, by locking SLC15A4 in the two opposing conformations, Fab107 and Fab235 should lead to TASL protein degradation or stabilisation, respectively (ref 20, 34). Is this observed?

3. In the experiments of figure 2, THP1 cells were pre-treated O/N with the antibodies before R848 stimulation, while in fig 1c and 1e 4 hours were sufficient for significant antibody uptake. Which is the minimal time of pretreatment needed to observe a functional effect of the two antibodies on TLR7/8 responses? Does this correlate with the time required to observe TASL degradation/accumulation in cells?

We thank the reviewer for the questions and suggestions. We regret that reproducibility issues with the newly purified antibody batches have prevented us from conclusively addressing the reviewer's concerns (as noted in our Overall Response). To ensure scientific rigor, we have de-emphasized the immunoregulatory analyses in the revised manuscript and instead focused on the robust structural and biochemical data. We believe these data still demonstrate that the two antibodies target distinct conformations of SLC15A4, offering prospects for their future application.

We plan to develop a bispecific antibody that combines Fab107 or Fab235 with a rapidly internalizing antibody. Additionally, conjugating these antibodies with cell-penetrating peptides (CPPs) may further enhance cellular uptake. These strategies represent important directions for our future research, which we have now discussed in the revised manuscript (lines 351–359). At that time, we will follow the reviewer's suggestion and assess the effects of the two antibodies on the NFκB and MAPK pathways, their impact on IRF5 activation, as well as the optimal time for antibody uptake and associated changes in TASL, as proposed by the reviewer in this feedback.

4. Some statements should be edited to better reflects previous findings. For examples the claim that the study "reveal the mechanism of TASL recruitment" (line 76,) or "our work elucidates how hSLC15A4 recruits TASL at a molecular level" (line 399) should be revised to mention that this has been previously described (refs 32, 33, 34). Similarly, "This feature holds promise for the development of drugs based on the SLC15A4-TASL adaptor complex for various diseases" (lines) should acknowledge the work in ref 34.

We thank the reviewer for the suggestion. We have added these references in the revised manuscript (lines 73, 361, and 368).

5. Fig 4G: The figure illustrating the steric hindrance is not ideal. It would be useful to provide a clearer visualisation of the predicted clashes by zooming in these regions.

We thank the reviewer for the suggestion. In the revised manuscript, we have provided a clearer visualization of the predicted clashes by zooming in on these regions (Fig. 2g).

6. Bar graphs should show individual values.

We thank the reviewer for the suggestion. We have added the individual value in bar graphs.

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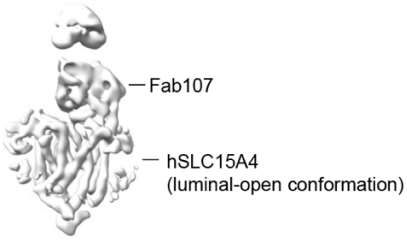
Reviewer #3:

The study by Zhu et al. employs a comprehensive screening and validation approach to identify two novel functional antibodies, clones 107 and 235. The data show that Clone 107 functions as an inhibitory antibody, binding selectively to hSLC15A4 in a luminal-open state that is incompatible with TASL binding. In contrast, Clone 235 acts as a stimulatory antibody, stabilizing hSLC15A4 in a cytoplasmic-open state that supports TASL binding. Despite recent reports on SLC15A4 structures, this study provides significant contributions to both therapeutic development and as a functional research tool for SLC15A4. Therefore, I recommend it for publication in Nature Communications. However, there are several points that require attention before publication:

We thank the reviewer for summarizing the key insights of our work and recognizing this paper as "*significant contributions to both therapeutic development and as a functional research tool for SLC15A4*", as well as for recommending it "*for publication in Nature Communications*". In response, we have provided detailed explanations to address the reviewer's concerns.

1. The structures of fab107-SLC15A4 and fab235-SLC15A4-TASL were solved in differing environments, with Fab107-SLC15A4 in a Nanodisc (Fig. 4a) and Fab235-SLC15A4-TASL in GDN detergent (Fig. 5a). Could the varying detergent environments, rather than the antibody itself, influence SLC15A4 conformation?

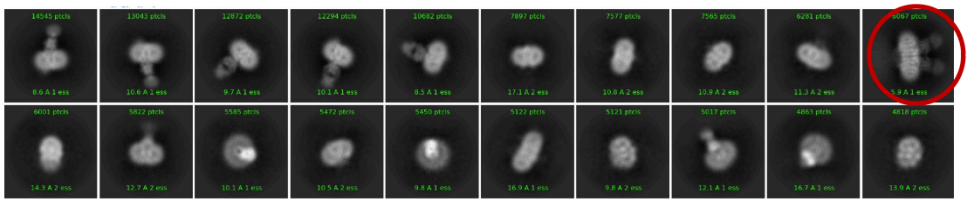
We thank the reviewer for raising this concern. To address it, we collected additional cryo-EM data of Fab107-SLC15A4 complex in the GDN detergent environment and reconstructed a density map at a resolution of 4.85 Å (bellow figure). The density map demonstrates that SLC15A4 adopts a luminal-open conformation in the GDN environment, consistent with the conformation observed in the nanodisc condition. This confirms that the conformational differences in SLC15A4 are not attributable to the detergent environment.



hSLC15A4-Fab107 (4.85 Å, GDN detergent environment)

Additionally, Chen et al. (2013, Nature Communications) suggested that apo SLC15A4 may form a dimeric state. Is it possible that the SLC15A4+Fab107 complex also forms a dimer?

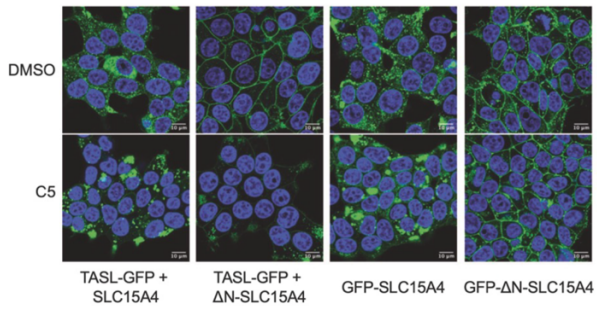
Thank you. Although we did not observe dimeric particles in the Nanodisc environment, 2D classification of the cryo-EM data under GDN conditions revealed a small population of dimeric particles, in which Fab107 remains bound (bellow figure). This demonstrates that the SLC15A4-Fab107 complex is capable of forming dimers.



hSLC15A4-Fab107 (2D classification, GDN detergent environment)
Representative dimer particles are circled in red circles.

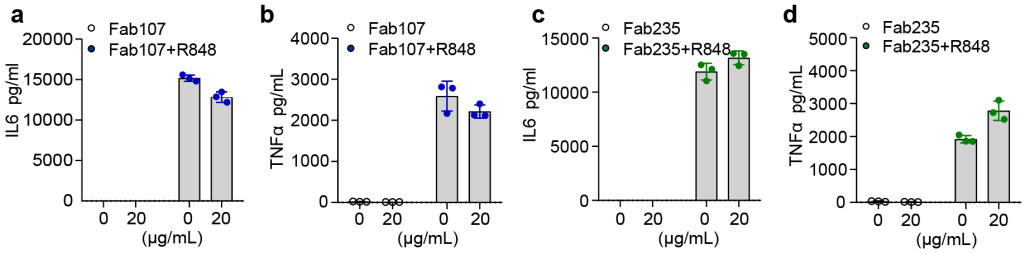
2. The ability of antibodies 107 and 235 to penetrate the plasma membrane and interact with the endolysosomal lumen side of SLC15A4 is intriguing, as observed via confocal fluorescence microscopy. Given that SLC15A4 is present in both endolysosomal and plasma membranes, could these antibodies initially bind to SLC15A4 at the plasma membrane and then be internalized via endocytosis to reach the endolysosomal membranes?

We thank the reviewer for raising this question. The subcellular distribution of SLC15A4 in HEK293T cells has been characterized in a recent peer-reviewed study (*Nat Commun 14, 6626, 2023*). As demonstrated by confocal microscopy in this paper (figure below), full-length SLC15A4 predominantly localizes to endolysosomal compartments with minimal plasma membrane association in HEK293T cells. In contrast, the N-terminal truncated variant (ΔN-SLC15A4) exhibits complete redistribution to the plasma membrane. These observations are consistent with our FACS data obtained through indirect surface staining. Specifically, compared to the prominent plasma membrane localization of the N-terminal mutations (L14A/L15A) or deletions (Δ1-31), full-length SLC15A4 only exhibits weak surface staining in HEK293T cells that highly overexpress SLC15A4 (Fig. 1b in the revised manuscript).



Data from Nat Commun 14, 6626, 2023

We agree with the reviewer that the antibodies may initially bind to SLC15A4 at the plasma membrane and then be internalized via endocytosis to reach the endolysosomal compartments. However, given the minimal plasma membrane localization of SLC15A4, the direct extracellular application of antibodies may lead to low internalization efficiency. As mentioned in our Overall Response above, the newly purified antibody batches unfortunately did not replicate the pronounced cellular immunoregulatory activity observed with the original batch (figure below). Despite several months of dedicated efforts, we have not yet identified a convincing explanation for this discrepancy between different antibody batches. In the interest of scientific rigor and responsible communication to the research community, we have therefore decided to retain only the robust antibody screening, biochemical, structural, and mutagenesis data in the revised manuscript, while temporarily removing the cellular immunoregulatory data.



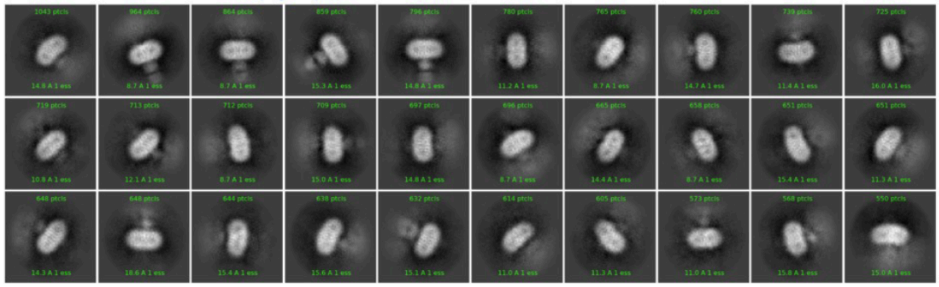
Cytokine production (TNFα and IL6) of THP1 cells by using newly purified batches of antibodies, unstimulated or stimulated with R848, pretreated with Fab107 (a and b) or Fab235 (c and d). Mean ± s.d. (n = 3 biological replicates).

3. Previous studies have shown that the SLC15A4-TASL complex assumes an cytoplasmic-open conformation, while apo-SLC15A4 adopts an luminal-open conformation, which corresponds to the conformations of SLC15A4-TASL-Fab235 and SLC15A4-Fab107, respectively. It is noted that hSLC15A4 with Fab235 can also form a complex without TASL (Fig. 3b). What is the conformation of hSLC15A4+Fab235 in the absence of TASL? Can the cytoplasmic-open state be maintained without TASL, and does clone 235 stabilize SLC15A4 alone in this state?

We thank the reviewer for these insightful questions. The cryo-EM structure of the SLC15A4-TASL-Fab235 complex shows that the binding interface between Fab235 and SLC15A4 in the cytoplasmic-open conformation (1086 Å²) is larger than the predicted interface in the luminal-open conformation (764

Å²). This suggests that Fab235 preferentially binds to the cytoplasmic-open conformation of SLC15A4 but can also interact with the luminal-open conformation.

To clarify whether Fab235 can push and stabilize SLC15A4 in the cytoplasmic-open conformation without TASL, we collected cryo-EM data for the SLC15A4-Fab235 complex. Unlike the SLC15A4-TASL-Fab235 complex, for which a high-resolution 3D reconstruction was obtained, the data for the SLC15A4-Fab235 complex failed to yield a reliable 3D reconstruction (figure below). These indirect results suggest that Fab235 may facilitate the cytoplasmic-open conformation when TASL is present, while in the absence of TASL, the SLC15A4-Fab235 complex may remain in a dynamic equilibrium between conformations. Based on these analyses, we believe that Fab235 can bind to both conformational states of SLC15A4 but exhibits a higher binding preference for the TASL-bound cytoplasmic-open conformation.



hSLC15A4-Fab235 (2D classification, GDN detergent environment)

4. The statement, “antibodies that target SLC15A4 with better biosafety and functional specificity have also not been discovered,” lacks supportive data on the biosafety profiles of antibodies.

We agree with the reviewer that SLC15A4 antibodies may not necessarily have better biosafety and functional specificity, and we have deleted this sentence in the revised manuscript.

5. Supplementary Fig. 3b contains formatting issues such as “PE Anti-M?”. Additionally, Supplementary Fig. 6d to j display “view” in a way that requires clarification.

Thank you. We have corrected these formatting mistakes in the revised manuscript.

6. Supplementary Fig.7 and Fig.8: The accession number of proteins from different species should be indicated at the figure legends.

Thank you. We have added the accession number of proteins in the revised manuscript.

We thank the reviewers for their valuable comments. In response to their suggestions and the editorial requests, we have made necessary revisions to the manuscript. Our detailed responses to reviewers' concerns are presented below. The reviewers' comments are in red and our responses are in black.

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Reviewer #1:

The authors note in their revision that the functional immunomodulatory data for Fab 107 and Fab 235 reported in the original manuscript could not be reproduced upon testing with distinct antibody batches. They have addressed all other concerns raised. The identification of two novel SLC15A4-targeting antibodies in different states represents an advance; however, the lack of corroborating functional evidence raises concerns regarding whether the findings possess the broad significance and impact typically required for publication in Nature Communications.

We thank the reviewer for recognizing the advance of our work and appreciate the thoughtful concerns. In the revised manuscript, we have emphasized the conformation-specific properties of these antibodies, which—as the reviewer noted—represent a key advance. Regarding immunomodulatory functions, although the effects observed with the new antibody batches were relatively modest, we consistently detected inhibitory and stimulatory trends on the TLR7/8 pathway. To address this point more thoroughly, we have expanded the Discussion to explore potential reasons for the limited functional potency, and to outline future directions for antibody optimization and engineering. While this remains a proof-of-concept study, we believe it provides a critical structural and mechanistic foundation for future developing these antibodies toward robust immunomodulatory activity.

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Reviewer #2:

I understand and appreciate the decision of the authors to remove the functional data that they could not reproduce with the new batch of antibodies. Unfortunately, in my opinion this affects quite substantially the overall message and impact of the manuscript.

Indeed, without demonstration that these antibodies can have a direct functional effect on TLR7/9 responses and TASL protein stability, several claims concerning the impact of the antibodies on the conformation of SLC15A4 may be called into questions, especially regarding the idea that the antibody

binding drives/induces the change in conformation (for example lines 252-253 “the conformational changes of hSLC15A4 upon binding to different antibodies” or lines 278-279“the distinct binding modes of Fab107 and Fab235 enable them to effectively target and stabilize unique conformations of hSLC15A4”).

We thank the reviewer for the questions and suggestions. In the revised manuscript, we have clarified that these two antibodies selectively bind to and stabilize distinct states of SLC15A4, rather than actively inducing or enforcing conformational transitions. This interpretation is supported by our structural and biochemical data, which consistently demonstrate that each antibody preferentially recognizes a specific conformation of SLC15A4. We also acknowledge the imprecise wording in the original text (Lines 252–253: “*the conformational changes of hSLC15A4 upon binding to different antibodies*”) and have revised this sentence to “*the conformational differences of hSLC15A4 upon binding to different antibodies*” (now Line 251) to more accurately reflect our findings.

It could be argued that the determinant factor for the conformation of recombinant SLC15A4 is the presence or not of TASL. It is clear that, at least in vitro, TASL binding to SLC15A4 drive the cytoplasmic open conformation (independently of the presence of the Fab235) and strong evidence suggest that apo SLC15A4 adopts a luminal open conformation in absence of TASL, independently of the presence of Fab107. Indeed in the structural studies by the Yang lab (cited Chen et al. Nat Commun 2023), as well as in the recent report by the Shimizu lab (PMID: 39719710), the cryoEM structures of apo-SLC15A4 were both obtained in the luminal open conformation, while the presence of TASL was sufficient to induced the cytoplasmic open conformation.

We thank the reviewer for these comments. As established in prior studies, cryo-EM structures have consistently shown that apo-SLC15A4 adopts a luminal-open conformation, whereas TASL binding induced a transition to the cytoplasmic-open conformation. Building on this framework, we conducted biochemical analyses to further probe antibody-conformation preferences. Our results demonstrate that Fab107 binds preferentially to apo-SLC15A4 and exhibits markedly reduced affinity for the SLC15A4–TASL complex (Fig. 1d; Supplementary Fig. 2e, f, h), strongly indicating that Fab107 selectively recognizes the luminal-open conformation. This conclusion is further supported by our cryo-EM structure of the SLC15A4–Fab107 complex (Fig. 2a, b, g). While Fab235 exhibits detectable binding to both apo- and TASL-bound SLC15A4 in biochemical assays, structural analysis indicates that it preferentially stabilizes the cytoplasmic-open conformation (Fig. 3a, b, i, j). Collectively, these findings support the conclusion that both antibodies function as conformation-specific stabilizers, with Fab107 and Fab235 selectively recognizing and stabilizing distinct structural states of SLC15A4.

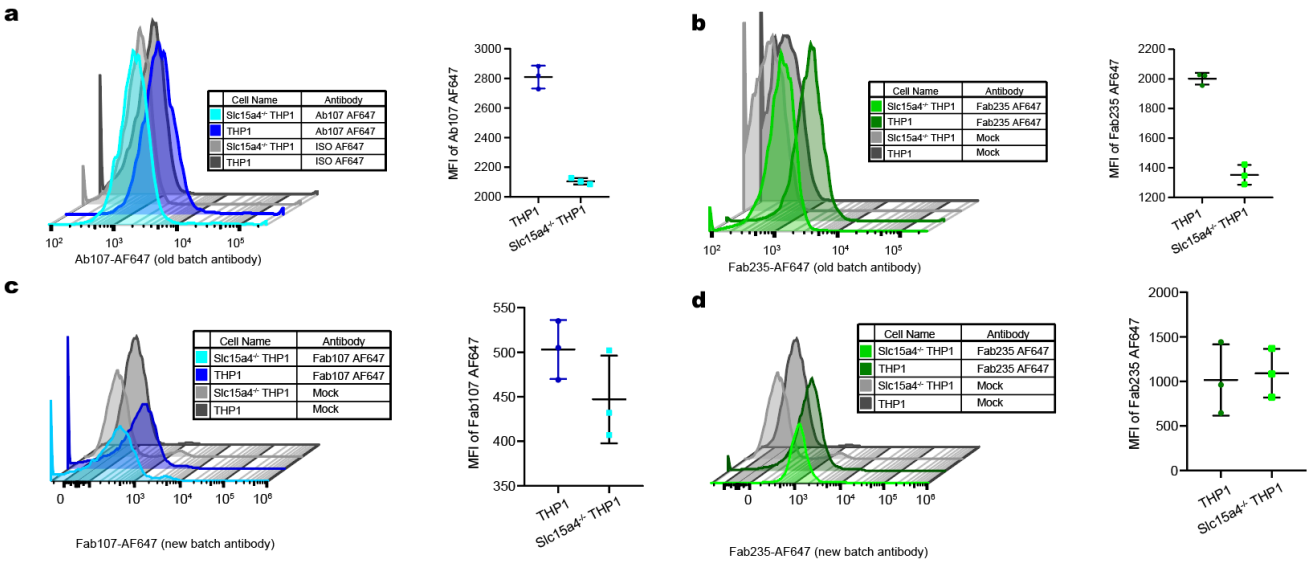
The identification of antibodies that (preferentially) binds two different conformations of a proteins is different than showing that the binding of these antibodies can drive the conformational shift. Without functional evidence on the impact of the two antibodies on TLR7/9-induced responses, the current study falls in my opinion a bit short in formally demonstrating the latter. Indeed, as discussed in reviewer 3

point 3 and the reply from the authors, it appears that Fab235 binds both conformations and it is not sufficient to induce the conformational change in absence of TASL.

We fully agree with the reviewer that "*antibodies preferentially binding to different protein conformations are distinct from antibodies capable of driving conformational shifts.*" In the revised manuscript, we have explicitly clarified that these two antibodies function by selectively binding to and stabilizing distinct conformational states of SLC15A4, rather than inducing or driving conformational transitions.

It is also unfortunate that the authors removed the data suggesting that the antibodies could be uptake by the cells (original first submission Fig 1c-f and SupFig 1e-f). Were these data also not reproducible with the new batch of antibodies?

We thank the reviewer for raising this point. Upon repeating the antibody internalization assay using a newly purified antibody batch, we observed a markedly reduced internalization efficiency compared to the original batch (see figure below). We cannot exclude the possibility that impurities present in the original preparation may have contributed to the apparent uptake. In light of this uncertainty, we have removed these data from the revised manuscript to ensure the accuracy and reproducibility of the results we report.



Flow cytometry analyses of THP1 or *Slc15a4*$^{-/-}$THP1 cells stained with antibodies labeled with AF647. a, b, old batch antibodies. c, d, new batch antibodies.

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Reviewer #3:

Although the regulatory role of antibodies have been delete in this version, the structural analysis indeed provide important insights for the conformational change of SLC15A4. The related cellular function may require further investigation in the future. So I recommended it for publication.

We thank the reviewer for summarizing the key insights of our work and the positive comments.