nature portfolio

Peer Review File

Structural and mechanistic insights into the constitutive Themis–Grb2 complex in T cell signalling

Corresponding Author: Professor Savvas Savvides

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)
This manuscript by Clancy et al reports a cryo-EM structures of Themis alone and in complex with Grb2, accompanied by extensive biophysical and cellular assays analyzing mutants at the Themis/Grb2 interaction interface. Despite its unique fold and emergence as an important regular of T cell development, the only structural data available for Themis is a preprint describing the crystal structure of the CABIT2 domain (Beyett et al, Biorxiv, 2024). The data in Clancy et al will therefore be of great interest to those studying molecular mechanisms of immune cell signaling. I believe this manuscript is suitable for publication in Nature Communications, once my comments below, mostly minor, are addressed.

Major points:
1. BLI experiments should be conducted to measure binding of full length Grb2 to Themis/Nb256 complex. An analogous experiment has been conducted in Extended Data Fig. 7c using Grb2SH3C and Themis/Nb256, which shows a similar binding affinity to Grb2FL and Themis1-563 alone. However, the authors have not shown whether Nb256 (or PMb256) impacts Grb2FL binding, which is important for validating the neutrality of Nb256 binding. Nb256 binds distal to the Grb2 binding site as shown in Fig. 2, but Class 3 in Fig. 5b appears to show the SH3N domain approaching or even contacting Nb256, raising the possibility that the nanobody influences binding of full-length Grb2.

Minor Points
1. Authors should adjust shading of “site 2” in Fig.2e (left panel) by shifting it away from CABIT1 and more toward CABIT2. Currently, the shading is misleading because appears to focus on the interaction between the Themis CABIT1 domain and Grb2, while the inset in Fig. 2f and mutagenesis data are centered around three CABIT2 helix residues (Y541, R544, R545) and one CABIT1 domain residue, I181. Similarly, the subsection title “Both Themis CABIT domains and the PRS synergize to stably bind Grb2” does not seem to accurately summarize the data described in the section. Only one CABIT1 residue mutation (I181A) is tested, which reduced but did not abrogate binding of Grb2, while four CABIT2 domain residues and two PRS residues were tested. Placing greater emphasis on the CABIT2 domain and the site 2 hotspot on this subsection title may be warranted.
2. Line 199: It is claimed that “biochemical behavior and stability of all purified mutant Themis variants were comparable to WT” but only SDS PAGE data are shown in Extended Data Fig. 7a, which are indicative of chemical purity but not biochemical behavior or stability. SEC profiles of each mutant should be included to support the claim that the mutations do not impact the biochemical behavior of Themis.
3. Fig. 5 or Extended Data Fig. 9 should include panels showing rigid body fit of models of the SH3N-SH2 domains into the lower resolution maps. It would be helpful to understand where the SH2 domain C-terminus is located in relation to the SH3C N-terminus in each case. The authors should also note in the text or figure caption whether the relative orientation of SH3N and SH2 domains are fixed in each of the resolved classes via interdomain interactions; the densities in Fig. 5b suggest this could be the case.
4. Line 233: “ThemisL411V displayed comparable behavior to ThemisWT in terms of protein expression, stability, and ability to bind Grb2.” Based on the data shown in Fig. 3e,f, the authors can claim that biochemical behavior and Grb2 binding are similar to WT, but the protein yield seems orders of magnitude lower than in Fig. 1a (maybe fewer cells were used for expression?) and stability data are not shown. The authors should adjust these claims accordingly or provide additional data.
5. Conclusions regarding the lack of phenotype for the L411V mice should be toned down (last sentence of paragraph

starting at line 230). While the blood samples showed no significant phenotypes at day 60, it should be mentioned that earlier time points could yield different data.
6. Line 83: "…an immunological nexus poised to connect signalling by the TCR and LAT to guide thymocyte selection." and Line 102: "...serve as a constitutive signalling hub that bridges TCR signalling and LAT to steer T lymphocyte maturation." These similar sentences read as if the authors are proposing that Themis/Grb2 acts as a bridge between TCR and LAT, but the statements are probably meant to suggest that Themis/Grb2 connects proximal TCR signaling to T lymphocyte maturation. The sentences should be edited for clarity.
7. Lines 272-274: The sentence "Grb2SH3C binding anchors… hot spot for the engagement of Grb2" is confusing and needs to be reworded or explained further; how binding of a Grb2 domain would create a hotspot for binding itself is not intuitive.
8. Lines 196-197: "…a similar structural mechanism may drive the Thems2-Grb2 interaction.": It should be noted here or in the discussion that I181A and the loop harboring it are not conserved in Themis2, perhaps suggesting a different structural role of CABIT1 domain in Themis2 binding of Grb2.
9. Additional details should be provided in the cryoEM image processing: how were soft masks generated for local refinements? What initialization mode was used for 3D classification (simple/PCA)? What class similarity settings were used for Ab initio model generation for the Themis1-563-PMb256 complex?
10. It is stated in methods line 680 that numerous Themis mutants (I181A, F327A, E483A, Y541A, R544A, R545A, P554A, P557A, I181A/R545A, P554A/P557A) were generated for Jurkat expression, but Fig. 6a only shows Shp-1 phosphorylation data for four of these mutants. Why weren't data collected or shown for the other mutants of interest?
11. Line 29 of Extended Data Figure 1 should have been labeled C.
12. Period is missing at end of line 259.
13. Line 640 in methods: "Y2909A" is a typo.

Reviewer #2

(Remarks to the Author)
I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

Reviewer #3

(Remarks to the Author)
Themis has been identified as a critical regulator of thymocyte development, where it promotes positive selection by coupling the TCR to the transmembrane adaptor LAT. Mechanistically, Themis has been shown to be recruited to tyrosine phosphorylated LAT by forming a complex with LAT-associated Grb2 which activates the Ras/MAPK pathway and fine-tunes TCR signaling by recruiting the phosphatase SHP1. The structural basis of the formation of the Grb2-Themis complex remains to be elucidated. In this report the authors bring a major contribution towards filling this gap through a cryo-EM analysis of Themis, both unbound and as a complex with Grb2. They show that Themis uses its tandem CABIT domains to engulf the C-terminal SH3 domain of Grb2, allowing its interaction with the Themis proline-rich sequence while leaving the N-terminal SH3 domain available for interaction with other molecular partners, as exemplified by Sos. Based on the structural data, they identify three putative interaction interfaces with Grb2, which they then test functionally for the ability to bind Grb2 by mutagenesis. They show that sites 1 and 2, but not site 3, are required for Grb2 binding as well as for TCR-dependent SHP-1 phosphorylation. Interestingly, based on its position in the cryo-EM structure of the Themis-Grb2 complex and its in vivo characterization in a mouse model, they rule out a role for a Themis mutation that had been proposed as possibly pathogenic in rheumathoid arthritis.

The structural characterization of the Themis-Grb2 complex, carried out using a sound experimental approach and cutting-edge methodologies, is very exhaustive and extremely informative, and is expected to fuel further studies on CABIT domain containing proteins known to interact with Grb2. The excellent writing of the report (including a detailed Materials and Methods section) and the quality of the figures match the quality of the data, which provide unprecedented insights into the molecular determinants that orchestrate the formation and stability of the complex, with potential important therapeutic implications. The functional validation of the mutants would however deserve a more in-depth characterization of the defects in TCR signaling in the Jurkat Themis KO T cells transfected with the individual mutants, as detailed below.

Point 1. In figure 6A the authors claim that Themis mutations in sites 1 and 2, but not in site 3, abrogate SHP-1 phosphorylation but not Erk phosphorylation in response to TCR signaling. First, from the immunoblot shown, no increase in TCR-dependent SHP-1 phosphorylation is evident in cells expressing wild-type Themis (this also applies to figure 1C) or the site 3 mutant, while a decrease in pSHP-1 levels at all times tested is clearly detectable in the KO cells and those expressing the site 1 and site 2 mutants. Second, at variance with the authors' statement, a (moderate) decrease in Erk phosphorylation is detectable in the KO cells, which would be consistent with previous reports (e.g. EMBO J 2014) and consistent with Themis as a positive regulator of TCR signaling. I think that these data need to be consolidated by looking at the phosphorylation/activation of other mediators of TCR signaling that have been previously shown to be modulated by Themis (e.g. EMBO J 2014, Sci Signal 2016), namely CD3 zeta, LAT and Vav. Also, it is essential that immunoblots be quantified over multiple experiments (with stats).

Point 2. While the structural data obtained on the complex assembled in vitro using purified Themis1-563 and Grb2 are clear, it cannot be excluded that the interaction of Grb2 with tyrosine phosphorylated LAT in activated T cells may impact on the stability and structure of the complex. The authors should consider looking at the recruitment of the Themis site 1-3

mutants to LAT in the reconstituted Themis KO T cells, for example in co-immunoprecipitation experiments. Similarly, the ability the Grb2-Themis complex to recruit SHP-1 in activated T cells when sites 1-3 are mutated should be assessed.

Point 3. The authors present very beautiful in vivo data that rule out a pathogenic role of the Themis L411V mutation in thymocyte development (and collagen-induced arthritis). In vivo validation of their structural data in Themis -/- transgenic mice expressing the Themis mutations that abrogate its interaction with Grb2 would be extremely valuable.

Reviewer #4

(Remarks to the Author)
Clancy et al. present cryo-EM structure of Themis1-Grb2 complex. Themis1 plays a critical role in T cell development and function of mature T cells, but the molecular and structural bases of Themis1 function have been elusive. The results of Clancy et al. are fascinating and important, as they help to resolve some Themis1-related discrepancies in the literature and provide solid basis for further interrogation of Themis1 mechanism of action. Themis1 (as well as the related proteins Themis2 and Themis3) has been predicted to consist of 2 CABIT domains, proline-rich region (PRR) and a disordered C-tail. Themis1 has been shown to bind constitutively to the adaptor Grb2 via PRR on Themis1 and C-terminal SH3 domain of Grb2 (Paster at al. 2013, GRB2-Mediated Recruitment of THEMIS to LAT Is Essential for Thymocyte Development). Themis-Grb2 complex is associated with the phosphatase Shp1, and can regulate its enzymatic activity as well as C-tail phosphorylation. Moreover, Themis1 has been shown to be recruited to LAT after TCR triggering.

However, it is still not known:
(a) how Themis1 regulates T cell development and activation (Themis1 was shown to be involved in signaling downstream of T cell receptor, cytokine receptors and the inhibitory receptor BLTA; the relevance of these different phenotypes for phenotype of Themis KO is currently unknown),
(b) if Themis1 is a positive or regulator of Shp1 activity (for example, Choi et al. 2017 THEMIS enhances TCR signaling and enables positive selection by selective inhibition of the phosphatase SHP-1 vs Zhang, et al. 2024 THEMIS is a substrate and allosteric activator of SHP1, playing dual 856 roles during T cell development.)
(c) how Themis interacts with Grb2 (and Shp1).

The cryo-EM work by Clancy et al. provide the first structure of Themis1-Grb2 complex, demonstrating that Themis uses three interaction interfaces on CABIT1, CABIT2 and PRR to bind Grb2 . Critically, the CABIT domains and the PRS synergise to bind Grb2, which explains inconclusive previous work on the role of the single CABIT domains in T cell development (Okada et al. 2014 Differential function of Themis CABIT domains during T cell development). Moreover, the Themis CABIT domains are flexible and become ordered upon Grb2 binding. Critically, Clancy et al. show that N-terminal SH3 domain and SH2 domain of Grb2 in complex in Grb2 are flexible and could interact with additional binding partners. This could explain the previous observation about Themis association with Vav1 (Lesourne et al. 2012 Interchangeability of Themis1 and Themis2 in thymocyte development reveals two related proteins with conserved molecular function). This work fills in important gaps in the current understanding of signal transduction in T cells.

This is an important paper, which will be of interest for researchers working in the fields of signal transduction, T cell development and structural biology. However, the current manuscript has few weaknesses that should be addressed by the authors:

1. pSHP1 analysis by Western blotting:
The authors discuss changes in “SHP-1 phosphorylation (pY564) after TCR stimulation”. However, it is clear from all the presented Western blot that p564 levels are comparable in stimulated and unstimulated samples. This suggests that Themis regulates Shp1 C-tail phosphorylation independently of TCR signal. The authors may want to revise their conclusions based on this.

2. Interpretation of pShp1 signal:
The authors interpret the increase in Shp1 Y564 phosphorylation as evidence for Shp1 activation. For example: “Themis variants that abrogate interactions with Grb2 also fail to activate the tyrosine phosphatase SHP-1 after TCR stimulation” However, it has been argued that Shp1 C-tail phosphorylation does not correlate with its phosphatase activity, and reduced pShp1 can actually result from high levels of enzymatic activity due to auto-dephosphorylation (for example: Choi et al. 2017 THEMIS enhances TCR signaling and enables positive selection by selective inhibition of the phosphatase SHP-1). Although the authors do not focus on the controversy of how Themis regulates Shp1, the paper will benefit from a more nuanced discussion of this topic.

3. TCR stimulation protocol:
Some information is missing from TCR stimulation protocol: were the cells stimulated in media with 10% serum (if yes, the authors may want to introduce the serum starvation step to reduce the baseline pShp1 signal), what was the cell number and volume used for stimulations? The experiments used plate-bound CD3 – were the cells centrifuged to facilitate contact with the plate-bound antibody? 30-60s seem to be very short time points, considering that the suspension cells need this time to settle on the plate.

4. Themis1-Grb2-Sos1 model:
The authors propose that Themis1-Grb2 complex can engage with additional partners such as Sos1, and show that Themis1-Grb2 complex binds Sos1-derived peptide. Without showing that full length Sos1 binds to Themis1-Grb2 complex

(for example: can Sos1 be detected in Themis1 IP?), this model remains speculative. Importantly, Lesourne at al. (Interchangeability of Themis1 and Themis2 in thymocyte development reveals two related proteins with conserved molecular function) detected Themis-Vav1, but not Sos1, interaction in thymocytes.

5. RA model:
What was the incidence of the disease? If all the mice developed arthritis, it could be that the double challenge model used was too strong to reveal any differences between WT and L411V. The authors may want to consider a model where not all WT mice develop CIA, for example either by using a single challenge or leaving out CFA from the second challenge. This is not necessary for the current paper, but could be of interest for the future.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
The authors have addressed my comments adequately. I believe the manuscript is suitable for publication. My only remaining comment is to fix the labeling error in Supplementary Figure 9b: in each of the docked models, one of the labels for Grb2 SH2 domains should be changed to Grb2 SH3N.

Reviewer #2

(Remarks to the Author)
I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

Reviewer #3

(Remarks to the Author)
The authors have addressed, with new experiments, my concerns regarding the immunoblots. The interpretation of the data is however not as clear-cut as they claim. They state "showed markedly reduced SHP-1 phosphorylation after TCR stimulation" and "Themis-deficient cells showed reduced phosphorylation of LAT, Vav1 and PLCgamma1 compared to control cells, which was restored upon expression of Themis WT (Fig. 6a). Reconstitution with Themis mutants restored phosphorylation of these proteins, although with delayed kinetics, suggesting temporal differences in this regulation".
I plotted the quantifications, and the only clear result is that SHP-1 phosphorylation is indeed reduced in Themis KO cells and that both WT Themis and its site 2, but not site 1 mutants restore activation-induced phosphorylation. However, neither Vav1 nor PLCgamma1 appears activated in response to TCR stimulation (in the case of Vav1, inducible phosphorylation can actually be detected in the KO cells, as opposed to control cells). Similar considerations apply to pLAT. Although in the presence of WT or mutant Themis the signals are in some cases higher, the statement that the stimulation-dependent signals are restored in these transfectants cannot be applied. Hence the authors should tune down their statements regarding these data and also discuss the results in the light of published reports linking Themis-Grb2 to TCR signaling. They should also briefly discuss their findings of a lack of effect of Themis deficiency or of expression of WT or mutant Themis on Erk phosphorylation as opposed to other studies, as they did in the rebuttal.

Reviewer #4

(Remarks to the Author)
The authors have fully addressed my questions/concerns, and I fully endorse publication of the manuscript.

NCOMMS-25-29649-A, Clancy *et al.*

Point-by-point response to reviewer comments

We thank all reviewers for their expert assessment of our work and constructive remarks, which have collectively helped us to improve our manuscript in several ways. We address all remarks below in point-by-point fashion.

Reviewer #1

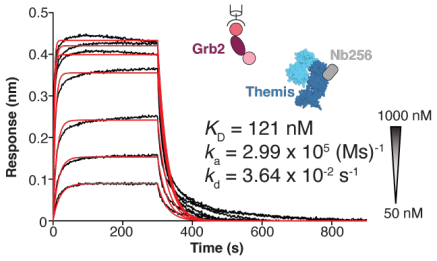
This manuscript by Clancy et al reports a cryo-EM structures of Themis alone and in complex with Grb2, accompanied by extensive biophysical and cellular assays analyzing mutants at the Themis/Grb2 interaction interface. Despite its unique fold and emergence as an important regular of T cell development, the only structural data available for Themis is a preprint describing the crystal structure of the CABIT2 domain (Beyett et al, Biorxiv, 2024). The data in Clancy et al will therefore be of great interest to those studying molecular mechanisms of immune cell signaling. I believe this manuscript is suitable for publication in Nature Communications, once my comments below, mostly minor, are addressed.

We thank the reviewer for the positive assessment of our work and for endorsing our manuscript for publication in Nature Communications. We address the comments raised by the reviewer in a point-by-point response.

Major points:

1. BLI experiments should be conducted to measure binding of full length Grb2 to Themis/Nb256 complex. An analogous experiment has been conducted in Extended Data Fig. 7c using Grb2SH3C and Themis/Nb256, which shows a similar binding affinity to Grb2FL and Themis1-563 alone. However, the authors have not shown whether Nb256 (or PMb256) impacts Grb2FL binding, which is important for validating the neutrality of Nb256 binding.

We thank the reviewer for raising this potential issue and for the experimental suggestion. We agree that it is essential to confirm that Nb256 binding does not influence the interaction between Themis and full-length Grb2.To address this, we have performed additional BLI experiments to measure the binding of full-length Grb2 to Themis in complex with Nb256. These experiments show that Grb2 binds to the Themis-Nb256 complex with a similar affinity to Themis alone, confirming that Nb256 does not affect Themis binding to Grb2. These new data are now included in Extended Data Figure 2d (also provided here).



Extended Data Figure 2d. BLI response curve measuring the interaction of immobilised full-length Grb2 with Themis$^{1\text{-}563}$ in complex with Nb256. Start and end concentrations of the dilution series is shown inset. Response curves were fitted with a 1:1 model (red) to quantify the kinetics (k_a, k_d) and binding affinity (K_D).

Nb256 binds distal to the Grb2 binding site as shown in Fig. 2, but Class 3 in Fig. 5b appears to show the SH3N domain approaching or even contacting Nb256, raising the possibility that the nanobody influences binding of full-length Grb2.

As shown in Figure 2, Nb256 binds to the CABIT2 domain of Themis, distal to the Grb2 binding site. The apparent proximity of Grb2^{SH3N} domain to Nb256 in Class 3 likely reflects a snapshot of the conformational heterogeneity and flexibility of Grb2, rather than a stable or functionally relevant

contact. The BLI experiments support this by confirming that binding of Themis to Grb2 is unaffected by Nb256 binding.

Minor Points

1. Authors should adjust shading of “site 2” in Fig.2e (left panel) by shifting it away from CABIT1 and more toward CABIT2. Currently, the shading is misleading because appears to focus on the interaction between the Themis CABIT1 domain and Grb2, while the inset in Fig. 2f and mutagenesis data are centered around three CABIT2 helix residues (Y541, R544, R545) and one CABIT1 domain residue, I181. Similarly, the subsection title “Both Themis CABIT domains and the PRS synergize to stably bind Grb2” does not seem to accurately summarize the data described in the section. Only one CABIT1 residue mutation (I181A) is tested, which reduced but did not abrogate binding of Grb2, while four CABIT2 domain residues and two PRS residues were tested. Placing greater emphasis on the CABIT2 domain and the site 2 hotspot on this subsection title may be warranted.

We thank the reviewer for this helpful suggestion. We have revised Figure 2 to more accurately reflect the location of the site 2 interaction interface defined by our structural and mutagenesis data. Specifically, we adjusted the visualisation of site 2 to include the central CABIT2 helix and replaced the previous shading with boxes highlighting the interfaces. We also added complementary surface views of the complex alongside the cartoon representations. The updated figure is now included in the revised manuscript and also provided here.

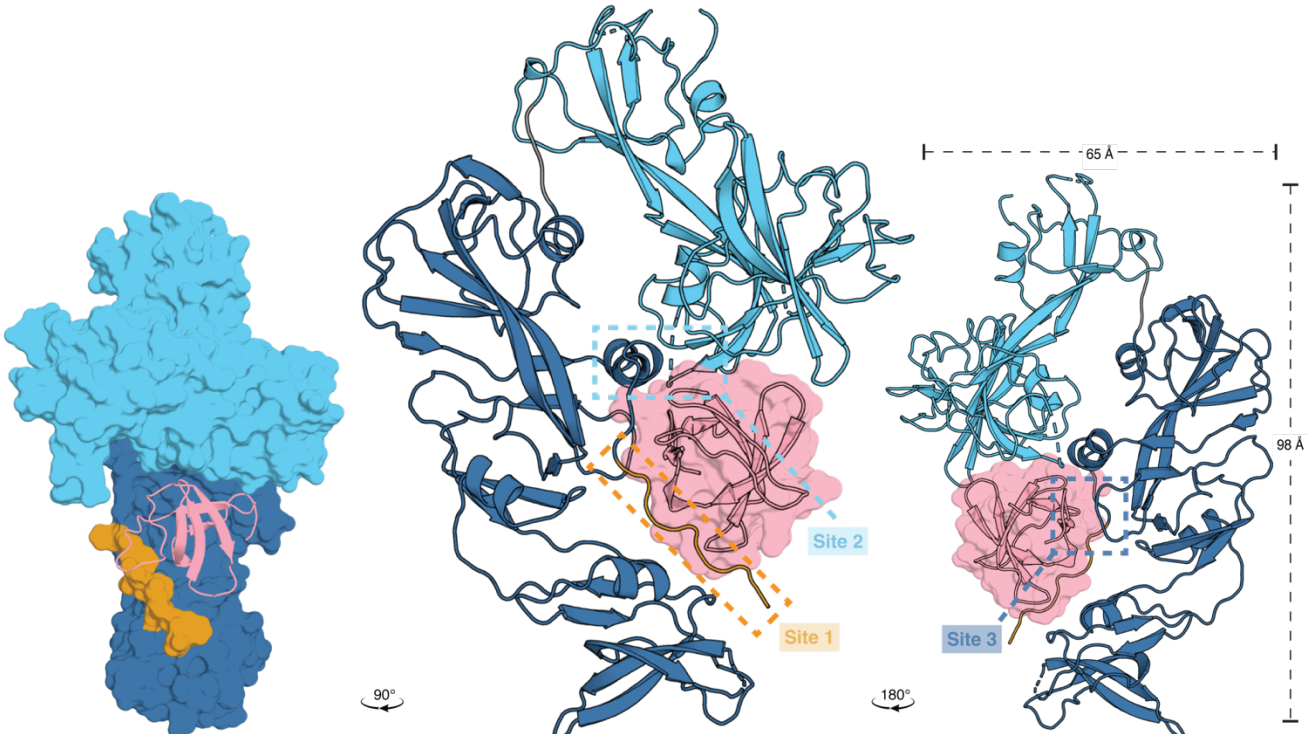


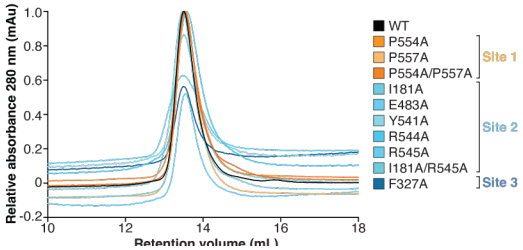
Figure 2d. Real-space refined model of the Themis1-563-Grb2SH3C complex. Cartoon representations of the front and back views are shown, with Grb2SH3C depicted in surface view. Side view shown as a surface representation of Themis, illustrating how Grb2SH3C is engulfed by the CABIT domains. Site 1, site 2 and site 3 interaction interfaces are highlighted in dashed boxes, with the site 2 centred around the central helix bridging the CABIT1 and CABIT2 domains.

We appreciate the reviewer’s point regarding the relative emphasis on the CABIT2 domain in this subsection. While our mutational analysis focuses primarily on CABIT2 and the PRS, mutation of the CABIT1 residue I181A substantially reduced Grb2 binding, with only minimal association detected by BLI. In line with this, and as noted in the Discussion, previous studies have reported that Themis constructs lacking the CABIT1 domain fail to bind Grb2 (Paster et al., 2013; Okada et al., 2014; Choi et al., 2017; Beyett et al., 2024), further supporting a requirement for both CABIT domains in stable complex formation. Taken together, we interpret our data as consistent with a model in which both

CABIT domains and the PRS synergise to stabilise Themis and its complex with Grb2, even though only a subset of CABIT1 residues was directly tested in this study.

2. Line 199: It is claimed that "biochemical behavior and stability of all purified mutant Themis variants were comparable to WT" but only SDS PAGE data are shown in Extended Data Fig. 7a, which are indicative of chemical purity but not biochemical behavior or stability. SEC profiles of each mutant should be included to support the claim that the mutations do not impact the biochemical behavior of Themis.

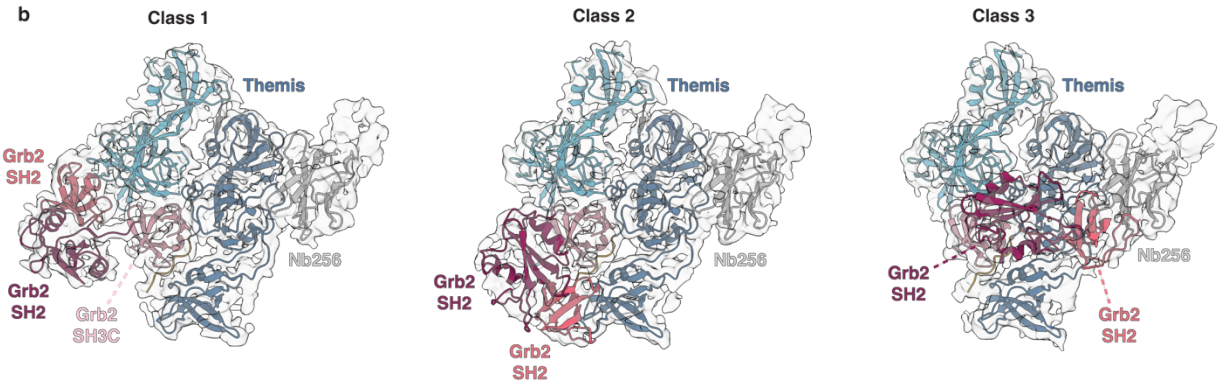
We thank the reviewer for pointing this out. To directly address the experimental suggestion, we have now included size-exclusion chromatography (SEC) profiles for all purified Themis mutant variants. As shown in the revised Extended Data Figure 7a, the chromatographic elution profiles of all mutants are indistinguishable from Themis WT, indicating their biochemical equivalence in terms of monodispersity, oligomeric state, and hydrodynamic radius.



Extended Data Figure 7a. Representative SEC chromatograms of purified Themis$^{1-563}$ mutants. All variants display similar biochemical behaviour to wild-type (WT) Themis$^{1-563}$.

3. Fig. 5 or Extended Data Fig. 9 should include panels showing rigid body fit of models of the SH3N-SH2 domains into the lower resolution maps. It would be helpful to understand where the SH2 domain C-terminus is located in relation to the SH3C N-terminus in each case. The authors should also note in the text or figure caption whether the relative orientation of SH3N and SH2 domains are fixed in each of the resolved classes via interdomain interactions; the densities in Fig. 5b suggest this could be the case.

As suggested by the reviewer, we now provide a new panel to Extended Data Figure 9 showing the rigid body fit of the SH3N and SH2 domains of Grb2 into the low resolution cryo-EM maps obtained after 3D classification in cryoSPARC. Models of Grb2 SH3N and SH2 domains were predicted by AlphaFold3 and independently rigid body fitted into the corresponding cryo-EM maps in ChimeraX. The flexible loops connecting each domain of Grb2 were subsequently connected and adjusted using local real-space refinement in Coot, specifically restricted to regions supported by density.



Extended Data Figure 9b. Rigid body fitting of Grb2^{SH3N} (dark pink) and Grb2^{SH2} (wine) domains in cryo-EM maps from Class 1-3 identified from 3D classification.

While the SH3N and SH2 domains can be placed with reasonable confidence using this approach, the resolution of these maps, approximately 6-10 Å after refinement, does not allow unambiguous

determination of the relative orientation of the domains or definitive assessment of specific interdomain interactions.

4. Line 233: “ThemisL411V displayed comparable behavior to ThemisWT in terms of protein expression, stability, and ability to bind Grb2.” Based on the data shown in Fig. 3e,f, the authors can claim that biochemical behavior and Grb2 binding are similar to WT, but the protein yield seems orders of magnitude lower than in Fig. 1a (maybe fewer cells were used for expression?) and stability data are not shown. The authors should adjust these claims accordingly or provide additional data.
We thank the reviewer for this comment. The yield of Themis^{L411V} is indeed comparable to that of Themis^{WT} under the same expression conditions. We note that the apparent difference in protein yield in Fig. 3e was due to a typo in the y-axis of the chromatogram, which was incorrectly labelled as 0-0.8 mAu, instead of the correct 0-80 mAu. This has now been corrected in the revised figure.

5. Conclusions regarding the lack of phenotype for the L411V mice should be toned down (last sentence of paragraph starting at line 230). While the blood samples showed no significant phenotypes at day 60, it should be mentioned that earlier time points could yield different data.
We thank the reviewer for alerting us about this. To address this, we have explicitly specified the experimental end point as 60 days after CIA induction and revised the concluding statement accordingly. We have added the following to the main text: “While these results indicate that the L411V mutation does not significantly alter susceptibility to CIA at this stage of disease, we cannot exclude that some phenotypic differences may emerge at earlier timepoints. Accordingly, Themis^{L411V} may require co-segregating pathogenic variants in immune-related genes, such as CD36, to contribute to autoimmune disease.”

6. Line 83: “…an immunological nexus poised to connect signalling by the TCR and LAT to guide thymocyte selection.” and Line 102: “...serve as a constitutive signalling hub that bridges TCR signalling and LAT to steer T lymphocyte maturation.” These similar sentences read as if the authors are proposing that Themis/Grb2 acts as a bridge between TCR and LAT, but the statements are probably meant to suggest that Themis/Grb2 connects proximal TCR signaling to T lymphocyte maturation. The sentences should be edited for clarity.
We thank the reviewer for highlighting this potential ambiguity. We agree that the original phrasing could be read as proposing a direct bridging role of the Themis-Grb2 complex between the TCR and LAT. To clarify our intended meaning, we have revised both sentences to emphasise the integration of proximal TCR signalling with downstream pathways that regulate thymocyte selection and T cell maturation. The revised text now reads: “Thus, the Themis–Grb2 complex has emerged as an immunological nexus poised to connect proximal TCR signalling to downstream pathways that guide thymocyte selection and maturation,” (Line 82-84) and “…serve as a constitutive signalling hub that integrates TCR signalling to steer T lymphocyte maturation.” (Line 101-102).

7. Lines 272-274: The sentence “Grb2SH3C binding anchors… hot spot for the engagement of Grb2” is confusing and needs to be reworded or explained further; how binding of a Grb2 domain would create a hotspot for binding itself is not intuitive.
We thank the reviewer for highlighting this point. We agree that the original wording was not clear and we have now revised the text in the main Results section to clarify this (Line 277-280): “The cryo-EM structure of unbound Themis$^{1-563}$ highlights the conformational plasticity and flexibility of the CABIT domains. Grb2^{SH3C} binding to the central helix between the CABIT1 and CABIT2 domains (site 2) appears to serve as a conformational switch poised to prime the formation of a stable Themis-Grb2 complex via site 1.”

8. Lines 196-197: “…a similar structural mechanism may drive the Thems2-Grb2 interaction.”: It should be noted here or in the discussion that I181A and the loop harboring it are not conserved in

Themis2, perhaps suggesting a different structural role of CABIT1 domain in Themis2 binding of Grb2.
We agree that the lack of conservation of I181 and the surrounding loop in Themis2 is an important point to note. To address this, we have added text in the main Results section to explicitly highlight the lack of conservation of I181 in Themis2 (Line 195-199). In addition, we have expanded the Discussion to further emphasise differences in the CABIT1 domains of Themis and Themis2, and to raise the possibility that the Themis2-Grb2 may adopt a distinct architecture to the canonical complex (Line 384-389). These additions clarify the limitations of extrapolating the Themis-Grb2 interaction mechanism to Themis2 and highlight the need for future dedicated investigation of Themis2-Grb2 and related protein complexes.

9. Additional details should be provided in the cryoEM image processing: how were soft masks generated for local refinements? What initialization mode was used for 3D classification (simple/PCA)? What class similarity settings were used for Ab initio model generation for the Themis1-563-PMb256 complex?
We thank the reviewer for requesting additional details regarding the cryo-EM image processing. We have now revised and expanded the Methods section to provide a more complete description of the workflow.

Default class similarity settings were used for all Ab-initio reconstructions, for both the Themis$^{1-563}$-PMb256 and Themis$^{1-563}$-Grb2-PMb256 complexes. 3D classification was performed using the simple initialisation mode in cryoSPARC.

Soft masks for local refinement were generated from a predicted AlphaFold-Multimer model of the Themis$^{1-563}$-Grb2^{SH3C} complex to focus on obtaining higher resolution information for the Themis-Grb2 interaction interfaces. This predicted model was converted to a volume using the molmap command in ChimeraX, blurred to 20 Å, resampled on a map grid from a map generated by non-uniform refinement. This initial volume was imported to cryoSPARC to generate a soft mask with a dilation radius of 5 pixels and soft padding width of 15 pixels for use in local refinement.

These details have been added to the Materials and Methods section (Lines 597-608).

10. It is stated in methods line 680 that numerous Themis mutants (I181A, F327A, E483A, Y541A, R544A, R545A, P554A, P557A, I181A/R545A, P554A/P557A) were generated for Jurkat expression, but Fig. 6a only shows Shp-1 phosphorylation data for four of these mutants. Why weren't data collected or shown for the other mutants of interest?
We thank the reviewer for pointing out this inconsistency. This statement was an error in the original Materials and Methods, where all generated Themis mutants were listed rather than only those expressed in Jurkat cells for activation assays. We have corrected the Methods section to specify only the mutants that were transfected and analysed for these experiments, resolving the discrepancy between the Methods and figure.

11. Line 29 of Extended Data Figure 1 should have been labeled C.
12. Period is missing at end of line 259.
13. Line 640 in methods: "Y2909A" is a typo.
We thank the reviewer for their careful reading of the manuscript and for pointing out these errors. All issues have been corrected in the revised text and figures.

Reviewer #2

I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

We thank the reviewer for their engagement and time taken in reviewing our manuscript. We also gratefully acknowledge the co-review conducted through this initiative to support training and recognition of Early Career Researchers.

Reviewer #3

Themis has been identified as a critical regulator of thymocyte development, where it promotes positive selection by coupling the TCR to the transmembrane adaptor LAT. Mechanistically, Themis has been shown to be recruited to tyrosine phosphorylated LAT by forming a complex with LAT-associated Grb2 which activates the Ras/MAPK pathway and fine-tunes TCR signaling by recruiting the phosphatase SHP1. The structural basis of the formation of the Grb2-Themis complex remains to be elucidated. In this report the authors bring a major contribution towards filling this gap through a cryo-EM analysis of Themis, both unbound and as a complex with Grb2. They show that Themis uses its tandem CABIT domains to engulf the C-terminal SH3 domain of Grb2, allowing its interaction with the Themis proline-rich sequence while leaving the N-terminal SH3 domain available for interaction with other molecular partners, as exemplified by Sos. Based on the structural data, they identify three putative interaction interfaces with Grb2, which they then test functionally for the ability to bind Grb2 by mutagenesis. They show that sites 1 and 2, but not site 3, are required for Grb2 binding as well as for TCR-dependent SHP-1 phosphorylation. Interestingly, based on its position in the cryo-EM structure of the Themis-Grb2 complex and its in vivo characterization in a mouse model, they rule out a role for a Themis mutation that had been proposed as possibly pathogenic in rheumathoid arthritis.

The structural characterization of the Themis-Grb2 complex, carried out using a sound experimental approach and cutting-edge methodologies, is very exhaustive and extremely informative, and is expected to fuel further studies on CABIT domain containing proteins known to interact with Grb2. The excellent writing of the report (including a detailed Materials and Methods section) and the quality of the figures match the quality of the data, which provide unprecedented insights into the molecular determinants that orchestrate the formation and stability of the complex, with potential important therapeutic implications. The functional validation of the mutants would however deserve a more in-depth characterization of the defects in TCR signaling in the Jurkat Themis KO T cells transfected with the individual mutants, as detailed below.

We thank the reviewer for their thorough evaluation of our study and for their expressed appreciation of the rigor and technical details of the manuscript. We gratefully acknowledge the reviewer's recognition of the significance of elucidating the structural basis of the Themis-Grb2 interaction and for highlighting the value of these analyses in advancing our understanding of the role of Theis in T cell signalling. We appreciate the constructive feedback regarding the functional characterisation of the Themis mutants introduced in this study and have addressed these points below.

Point 1. In figure 6A the authors claim that Themis mutations in sites 1 and 2, but not in site 3, abrogate SHP-1 phosphorylation but not Erk phosphorylation in response to TCR signaling. First, from the immunoblot shown, no increase in TCR-dependent SHP-1 phosphorylation is evident in cells expressing wild-type Themis (this also applies to figure 1C) or the site 3 mutant, while a decrease in pSHP-1 levels at all times tested is clearly detectable in the KO cells and those expressing the site 1 and site 2 mutants. Second, at variance with the authors' statement, a (moderate) decrease in Erk phosphorylation is detectable in the KO cells, which would be consistent with previous reports (e.g. EMBO J 2014) and consistent with Themis as a positive regulator of TCR signaling. I think that these data need to be consolidated by looking at the phosphorylation/activation of other mediators of TCR signaling that have been previously shown to be modulated by Themis (e.g. EMBO J 2014, Sci Signal 2016), namely CD3 zeta, LAT and Vav. Also, it is essential that immunoblots be quantified over multiple experiments (with stats).

We thank the reviewer for this detailed and constructive comment. We have substantially revised the data presented in Fig. 6a using newly generated immunoblots, which more clearly illustrate the effects of Themis mutations on SHP-1 phosphorylation.

In these revised data, cells reconstituted with Themis^{WT} reproducibly display higher levels of SHP-1 phosphorylation following TCR stimulation compared with control cells, whereas Themis^{KO} cells and cells expressing site 1 and 2 mutants consistently displayed reduced SHP-1 phosphorylation across the timepoints examined.

We agree that some previous studies have reported reduced p-ERK levels in Themis-deficient primary thymocytes, while other reports have observed no differences in ERK activation (Johnson et al., 2009; Lesourne et al., 2009). Prior studies were performed using primary thymocytes stimulated with antigens of varying affinity, which may account for differences in proximal TCR signalling relative to the Jurkat cell system and antibody stimulation used here. In the revised immunoblots presented in this manuscript, and consistent across multiple independent replicates, we did not observe reproducible differences in ERK phosphorylation between Themis^{KO} cells or cells reconstituted with WT or mutant Themis variants.

To further consolidate this analysis, as suggested by the reviewer, we extended our experiments to additional proximal TCR signalling mediators previously reported to be regulated by Themis, including LAT, Vav1 and PLCγ1. Consistent with published studies, Themis knockout resulted in reduced phosphorylation of these proteins after TCR stimulation, which was restored upon expression of Themis^{WT}. Reconstitution with Themis mutants also restored phosphorylation of these mediators, albeit with altered kinetics, indicating that disruption of the Themis-Grb2 complex does not broadly impair proximal TCR signalling. However, we note that larger differences may be more apparent in primary cells or under conditions of lower affinity stimulation.

Phosphorylation of SHP-1, LAT, Vav1 and PLCγ1 was quantified within each biological replicate following normalisation to ERK. Effects in phosphorylation were consistent across replicates, however, we observed variability in signal intensity between experiments, consistent with the use of very short stimulation periods required to detect phosphorylation events after TCR stimulation. Therefore, we have based our conclusions on reproducible observations across independent experiments and present representative immunoblots along with corresponding quantifications. The figure legend has been revised to reflect this.

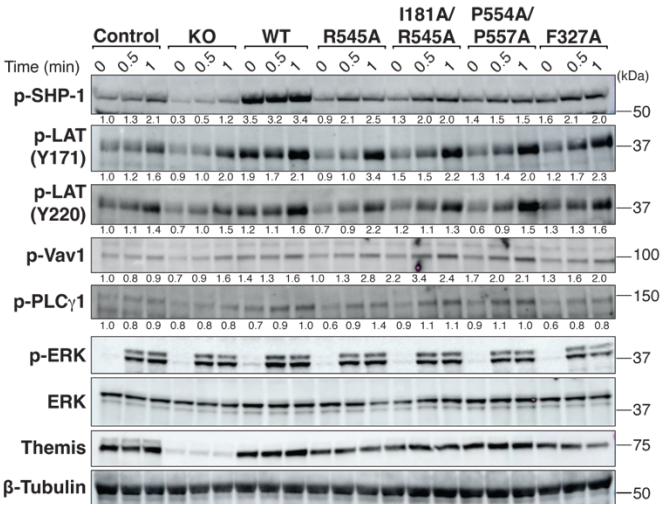


Figure 6a. Western blot analysis of SHP-1 phosphorylation (pY564) after TCR activation. Cells were stimulated with plate-bound anti-CD3 (2 μg/mL) and soluble anti-CD28 (1 μg/mL). The indicated proteins were analysed by western blot. Band intensities for p-SHP-1, p-LAT, p-Vav1 and p-PLCγ1 were quantified and normalized to total ERK. Values below the blots indicate quantified fold changes

Point 2. While the structural data obtained on the complex assembled in vitro using purified Themis1-563 and Grb2 are clear, it cannot be excluded that the interaction of Grb2 with tyrosine phosphorylated LAT in activated T cells may impact on the stability and structure of the complex. The authors should consider looking at the recruitment of the Themis site 1-3 mutants to LAT in the reconstituted Themis KO T cells, for example in co-immunoprecipitation experiments. Similarly, the ability the Grb2-Themis complex to recruit SHP-1 in activated T cells when sites 1-3 are mutated should be assessed.

We thank the reviewer for this comment. Previous work had established that Grb2 binding is critically required for the recruitment of Themis to LAT after TCR stimulation. In particular, Paster et al, (2013) demonstrated that a Grb2-binding-deficient Themis mutant (P555A/P558A), analogous to the site 1 mutants characterised in our study, fails to associate with LAT after TCR engagement. On this basis, we predict that the site 1 and site 2 mutants generated based on our structural analysis would likewise be impaired in LAT recruitment.

The role of the Themis-Grb2 complex in SHP-1 recruitment has been debated. Choi et al. (2017) reported that while both Grb2 and SHP-1 immunoprecipitated with full-length Themis, a Themis variant lacking the PRS failed to recruit Grb2, as expected, but retained SHP-1 binding, suggesting a direct interaction between Themis and SHP-1. In addition, a truncated Themis construct containing only the CABIT1 domain also immunoprecipitated SHP-1. In line with this, a recent study from Zhang et al. (2024) showed that phosphorylation of the highly conserved residue Y35 within the Theims CABIT1 domain is required for SHP-1 binding, distal to the Grb2 binding site (Extended Data Figure 10). In contrast, Paster et al. (2014) showed that the Themis PRS mutant (P555A/P558A) failed to immunoprecipitate SHP-1 following TCR stimulation, suggesting a dependency on Grb2 to facilitate the interaction with SHP-1.

In this study, we have shown that unbound Themis displays extensive interdomain flexibility, which is stabilised by Grb2 binding. While the predicted SHP-1 binding site is distal to the Grb2 interface, the increased stability of the CABIT1 domain induced by Grb2 binding likely stabilises Themis to facilitate the interaction with SHP-1.

Given the scope of the current study, we have focused on integrating structural, biochemical and functional readouts to define how distinct Grb2 binding interfaces within Themis regulate complex formation and downstream signalling, particularly SHP-1 phosphorylation. We believe that this approach provides a mechanistic framework that will inform future functional studies to investigate higher order Themis-Grb2-SHP-1 complexes in T cell signalling.

Point 3. The authors present very beautiful in vivo data that rule out a pathogenic role of the Themis L411V mutation in thymocyte development (and collagen-induced arthritis). In vivo validation of their structural data in Themis -/- transgenic mice expressing the Themis mutations that abrogate its interaction with Grb2 would be extremely valuable.

We thank the reviewer for this valuable suggestion and for their positive assessment of the in vivo data presented in this manuscript. We agree that in vivo validation of additional Themis mutants would be highly informative. However, the generation and characterisation of these transgenic mice represents a substantial undertaking that is beyond the scope of the current study. We have therefore limited our in vivo analysis to the previously reported L411V variant, while using complementary biochemical and cellular approaches to characterise structure-guided mutants important for Themis-Grb2 complex formation. We believe these combined approaches provide a strong framework for future in vivo studies, and hope to inspire readers of our work to undertake experimental work in this direction.

Reviewer #4

Clancy et al. present cryo-EM structure of Themis1-Grb2 complex. Themis1 plays a critical role in T cell development and function of mature T cells, but the molecular and structural bases of Themis1 function have been elusive. The results of Clancy et al. are fascinating and important, as they help to resolve some Themis1-related discrepancies in the literature and provide solid basis for further interrogation of Themis1 mechanism of action. Themis1 (as well as the related proteins Themis2 and Themis3) has been predicted to consist of 2 CABIT domains, proline-rich region (PRR) and a disordered C-tail. Themis1 has been shown to bind constitutively to the adaptor Grb2 via PRR on Themis1 and C-terminal SH3 domain of Grb2 (Paster at al. 2013, GRB2-Mediated Recruitment of THEMIS to LAT Is Essential for Thymocyte Development). Themis-Grb2 complex is associated with the phosphatase Shp1, and can regulate its enzymatic activity as well as C-tail phosphorylation. Moreover, Themis1 has been shown to be recruited to LAT after TCR triggering.

However, it is still not known:
(a) how Themis1 regulates T cell development and activation (Themis1 was shown to be involved in signaling downstream of T cell receptor, cytokine receptors and the inhibitory receptor BLTA; the relevance of these different phenotypes for phenotype of Themis KO is currently unknown),
(b) if Themis1 is a positive or regulator of Shp1 activity (for example, Choi et al. 2017 THEMIS enhances TCR signaling and enables positive selection by selective inhibition of the phosphatase SHP-1 vs Zhang, et al. 2024 THEMIS is a substrate and allosteric activator of SHP1, playing dual 856 roles during T cell development.)
(c) how Themis interacts with Grb2 (and Shp1).

The cryo-EM work by Clancy et al. provide the first structure of Themis1-Grb2 complex, demonstrating that Themis uses three interaction interfaces on CABIT1, CABIT2 and PRR to bind Grb2 . Critically, the CABIT domains and the PRS synergise to bind Grb2, which explains inconclusive previous work on the role of the single CABIT domains in T cell development (Okada et al. 2014 Differential function of Themis CABIT domains during T cell development). Moreover, the Themis CABIT domains are flexible and become ordered upon Grb2 binding. Critically, Clancy et al. show that N-terminal SH3 domain and SH2 domain of Grb2 in complex in Grb2 are flexible and could interact with additional binding partners. This could explain the previous observation about Themis association with Vav1 (Lesourne et al. 2012 Interchangeability of Themis1 and Themis2 in thymocyte development reveals two related proteins with conserved molecular function). This work fills in important gaps in the current understanding of signal transduction in T cells.

This is an important paper, which will be of interest for researchers working in the fields of signal transduction, T cell development and structural biology. However, the current manuscript has few weaknesses that should be addressed by the authors:

We thank the reviewer for the strong endorsement of the importance of our work. We address the comments raised in a point-by-point response below.

1. pSHP1 analysis by Western blotting:
The authors discuss changes in “SHP-1 phosphorylation (pY564) after TCR stimulation”. However, it is clear from all the presented Western blot that p564 levels are comparable in stimulated and unstimulated samples. This suggests that Themis regulates Shp1 C-tail phosphorylation independently of TCR signal. The authors may want to revise their conclusions based on this.
We thank the reviewer for this careful observation. We agree that SHP-1 pY564 is detectable in unstimulated cells, indicating a basal level of phosphorylation. However, we consistently observe a clear increase in SHP-1 Y564 phosphorylation following TCR engagement, consistent with previously published reports. This increase is reduced in Themis-deficient cells and cells expressing site 1 or site 2 mutants. Notably, reconstitution with WT Themis or the truncated Themis$^{1-563}$ variant enhances

SHP-1 phosphorylation even in the absence of TCR stimulation. Under these conditions, TCR engagement does not lead to a pronounced further increase in pSHP-1.

Previous work has suggested that Themis and SHP-1 also form a constitutive complex in T cells in a Grb2 dependent manner (Paster et al. 2014), raising the possibility that Themis regulates SHP-1 independent of TCR activation. Further structural, biochemical and functional studies will be required to confirm this and further characterise this higher order complex.

2. Interpretation of pShp1 signal:
The authors interpret the increase in Shp1 Y564 phosphorylation as evidence for Shp1 activation. For example: “Themis variants that abrogate interactions with Grb2 also fail to activate the tyrosine phosphatase SHP-1 after TCR stimulation”
However, it has been argued that Shp1 C-tail phosphorylation does not correlate with its phosphatase activity, and reduced pShp1 can actually result from high levels of enzymatic activity due to auto-dephosphorylation (for example: Choi et al. 2017 THEMIS enhances TCR signaling and enables positive selection by selective inhibition of the phosphatase SHP-1). Although the authors do not focus on the controversy of how Themis regulates Shp1, the paper will benefit from a more nuanced discussion of this topic.
We thank the reviewer for raising this point and agree that the relationship between SHP-1 C-terminal phosphorylation and its activity is complex and has been interpreted differently across studies. While it has been shown that tyrosine phosphorylation of Y536 and Y564 in the C-terminus of SHP-1 increases its PTP activity, reduced SHP-1 phosphorylation may also reflect increased catalytic activity due to auto-dephosphorylation, as noted by the reviewer. Recent evidence shows that Themis binds to and stabilises SHP-1 in an open, active state and increases its phosphatase activity (Zhang et al., 2024), however how this correlates to SHP-1 Y564 phosphorylation was not investigated.

We used p-Y564 as a biochemical readout of SHP-1 regulation in the context of TCR activation. We agree that phosphorylation at this site should be interpreted as indicative changes in SHP-1 regulation, rather than as a definitive measurement of enzymatic activity. To address this, further functional characterisation of SHP-1 phosphatase activity in the presence of Themis or the Themis-Grb2 complex is required. In light of this, we have revised the text to remove language implying direct activation of SHP-1 and instead focus on describing the modulation of SHP-1 Y564 phosphorylation downstream of TCR activation. Our results demonstrate that disruption of the Themis-Grb2 complex alters SHP-1 Y564 phosphorylation, supporting a central role for this complex in regulating SHP-1 in T cell signalling

We have added the following text to the Discussion to address this ambiguity and the larger unresolved controversy of how Themis regulates SHP-1 (Lines 396-407):
“A long unresolved question is whether Themis functions as a positive or negative regulator of SHP-1 phosphatase activity. Previous reports have proposed that Themis enhances SHP-1 activity, with increased SHP-1 C-terminal phosphorylation often used as an indicator of elevated PTP function. However, it has been argued that SHP-1 phosphorylation does not correlate with catalytic activity, as reduced pSHP-1 can reflect increased enzymatic activity due to auto-dephosphorylation. Conversely, others have proposed that Themis acts as a negative regulator of SHP-1 via reactive oxygen species (ROS)-dependent regulation of its catalytic cysteine. In this study, we interpret changes in SHP-1 Y564 phosphorylation as indicative of altered regulation of SHP-1 within TCR signalling complexes, rather than as a direct measure of enzymatic activity. Dissecting how Themis integrates and regulates SHP-1 catalytic activity will require future studies combining structural, biochemical and enzymatic approaches.”

3. TCR stimulation protocol:
Some information is missing from TCR stimulation protocol: were the cells stimulated in media with 10% serum (if yes, the authors may want to introduce the serum starvation step to reduce the baseline pShp1 signal), what was the cell number and volume used for stimulations? The experiments used plate-bound CD3 – were the cells centrifuged to facilitate contact with the plate-bound antibody? 30-60s seem to be very short time points, considering that the suspension cells need this time to settle on the plate.
We thank the reviewer for these helpful comments and for highlighting areas where additional methodological detail is necessary. All TCR stimulation assays were carried in media supplemented with 10 % FCS. We tested lower serum concentrations, including serum starvation, 1 %, 2% and 5 % FCS,. While serum-starvation and lower serum concentrations reduced non-specific antibody binding on western blots, the signal-to-noise ratio for specific SHP-1 phosphorylation was consistently highest at 10 % FCS. We therefore performed all experiments under these conditions.

We apologise for the ambiguity in our original description of the experimental setup. Jurkat cells were incubated with plate-bound anti-CD3 for 2 h at 37 ^{o}C to allow sufficient contact with antibody-coated surface prior to stimuliaton. Co-stimulatory anti-CD28 antibody was added in soluble form, with the 30 – 60s timepoints referring to the duration of stimulation following addition of anti-CD28. These timepoints are consistent with previously published studies measuring early TCR signalling events (Fu et al., 2013).

To address these points, we have added the following details to the Materials and Methods section (Lines 739-748): Jurkat cells (1 x 10^{6} cells per well) were stimulated with 2 mg/mL plate-bound anti-CD3 antibody (BD Biosciences) and 1 mg/mL soluble anti-CD28 antibody (BD Biosciences). In brief, anti-CD3 antibody (2 mg/mL) was diluted in phosphate-buffered saline (PBS) and 100 µL per well was added to 96 well plates. Plates were incubated for 1 h at 37 ^{o}C to allow antibody binding. Cells were counted using a Luna Automated cell counter and diluted in RPMI media supplemented with 10% FCS to the desired cell density. After incubation, unbound antibody solution was removed. Jurkat cells were added at 1 x 10^{6} cells/well in 50 µL total volume and incubated for 2 h at 37 ^{o}C. Soluble anti-CD28 antibody was diluted in RPMI supplemented with 10% FCS and 50 µL was added to the wells for 30–60 s. Timepoints refer to the duration of stimulation following addition of anti-CD28 antibody.

4. Themis1-Grb2-Sos1 model:
The authors propose that Themis1-Grb2 complex can engage with additional partners such as Sos1, and show that Themis1-Grb2 complex binds Sos1-derived peptide. Without showing that full length Sos1 binds to Themis1-Grb2 complex (for example: can Sos1 be detected in Themis1 IP?), this model remains speculative. Importantly, Lesourne at al. (Interchangeability of Themis1 and Themis2 in thymocyte development reveals two related proteins with conserved molecular function) detected Themis-Vav1, but not Sos1, interaction in thymocytes.
We thank the reviewer for this comment. We agree that our data do not directly demonstrate binding of Sos1 to the Themis-Grb2 complex, and we did not intend to present this model as definitive. Sos1 was used as a well-established and extensively characterised binding partner of $Grb2^{SH3N}$ domain. It is known that $Grb2^{SH3N}$ preferentially interacts with PxxPxR motifs, as found in Sos1, which allowed us to test if engagement of $Grb2^{SH3C}$ by Themis constrains the ability of Grb2 to interact with an isolated Sos1 peptide via its N-terminal domain.

Our data using a Sos1-derived PRS peptide demonstrates that Grb2 remains competent to engage additional ligands using its SH3N domain, opening the possibility that Sos1 may associate with Themis-Grb2 complexes under certain cellular or developmental contexts, while not excluding the involvement of other Grb2 effectors.

Lesourne et al. indeed detected interaction between Themis and Vav1, but not Sos1 in thymocytes. Sos1 deletion in mice leads to a partial block at the double negative to double positive (DP) transition during thymocyte development, suggesting that Sos1 is required for pre-TCR but not TCR-dependent developmental signals at the DP stage (Kortum et al., 2011). These observations suggest that Themis-Grb2 signalling complexes may be context dependent and vary with developmental stage or signalling conditions.

Accordingly, we have revised the Results section and accompanying schematic figure to clarify the use of Sos1 as a test case to strengthen and deepen our structural insights on the flexibility and availability of unbound Grb2 domains, primarily the SH3N domain while engaged with Themis. Characterisation of a putative Themis-Grb2-Sos1 complex and identification of physiologically relevant $Grb2^{SH3N}$ binding partners within Themis-Grb2 signalling assemblies will require further investigation.

5. RA model:
What was the incidence of the disease? If all the mice developed arthritis, it could be that the double challenge model used was too strong to reveal any differences between WT and L411V. The authors may want to consider a model where not all WT mice develop CIA, for example either by using a single challenge or leaving out CFA from the second challenge. This is not necessary for the current paper, but could be of interest for the future.
We thank the reviewer for this input. The incidence of CIA in our study was 65% in wild-type mice and 61% in Themis L411V transgenic mice, indicating that not all animals developed disease under the conditions used. As C57BL/6 mice are not very susceptible to CIA, co-administration of CFA during both the immunisation and challenge steps is commonly used to achieve disease induction in this background. Given the comparable disease incidence observed in both genotypes, we consider it unlikely that the model was overly strong or masked potential differences between WT and Themis L411V mice at the time point analysed. We agree, however, that alternative protocols with earlier experimental end points may be informative and represent an interesting direction for future studies.

NCOMMS-25-29649-T, Clancy *et al.*

Point-by-point response to reviewer comments

We thank all reviewers for their expert assessment of our work and constructive remarks, which have collectively helped us to improve our manuscript in several ways. We address all remarks below in point-by-point fashion.

Reviewer #1

The authors have addressed my comments adequately. I believe the manuscript is suitable for publication. My only remaining comment is to fix the labeling error in Supplementary Figure 9b: in each of the docked models, one of the labels for Grb2 SH2 domains should be changed to Grb2 SH3N.

We thank the reviewer for the positive assessment of our work and for endorsing our manuscript for publication in Nature Communications. We thank the reviewer for noticing this error, which we have now corrected in the manuscript.

Reviewer #2

I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

We thank the reviewer for their engagement and positive evaluation our manuscript.

Reviewer #3

The authors have addressed, with new experiments, my concerns regarding the immunoblots. The interpretation of the data is however not as clear-cut as they claim. They state "showed markedly reduced SHP-1 phosphorylation after TCR stimulation" and "Themis-deficient cells showed reduced phosphorylation of LAT, Vav1 and PLCgamma1 compared to control cells, which was restored upon expression of Themis WT (Fig. 6a). Reconstitution with Themis mutants restored phosphorylation of these proteins, although with delayed kinetics, suggesting temporal differences in this regulation". I plotted the quantifications, and the only clear result is that SHP-1 phosphorylation is indeed reduced in Themis KO cells and that both WT Themis and its site 2, but not site 1 mutants restore activation-induced phosphorylation. However, neither Vav1 nor PLCgamma1 appears activated in response to TCR stimulation (in the case of Vav1, inducible phosphorylation can actually be detected in the KO cells, as opposed to control cells). Similar considerations apply to pLAT. Although in the presence of WT or mutant Themis the signals are in some cases higher, the statement that the stimulation-dependent signals are restored in these transfectants cannot be applied. Hence the authors should tune down their statements regarding these data and also discuss the results in the light of published reports linking Themis-Grb2 to TCR signaling. They should also briefly discuss their findings of a lack of effect of Themis deficiency or of expression of WT or mutant Themis on Erk phosphorylation as opposed to other studies, as they did in the rebuttal.

We thank the reviewer for this careful analysis of our work. We agree that the most robust and reproducible result is the effect on SHP-1 phosphorylation, while the effects on Vav1 and PLCg1 are modest and variable under the experimental conditions used here. LAT phosphorylation is reduced in ThemisKO cells, and increased in cells reconstituted with wildtype Themis. Reconstitution with Themis variants results in lower pLAT levels in comparison to wildtype Themis, with similar phosphorylation levels only reached at later timepoints, suggesting delayed kinetics after TCR stimulation.

We have revised the text accordingly to reflect this. In particular, we now highlight that the limited effects on Vav1 and PLCg1 phosphorylation, as well as the differences in ERK phosphorylation, may reflect differences in cellular context and stimuation conditions, as suggested by the reviewer.

We have added the following text to the results section (Lines 427-442):

"Themis has been reported to modulate other proximal TCR signalling events, including phosphorylation of LAT, Vav1 and PLCg[15,20]. In line with this, Themis-deficient cells exhibited reduced phosphorylation of LAT at residues Y171 and Y220, which was restored upon expression of Themis^{WT} (**Fig. 6a**). Reconstitution with Themis mutants also restored LAT phosphorylation, although with delayed kinetics, suggesting temporal differences in this regulation. Under these conditions, Vav1 and PLCg1 were not robustly activated in response to TCR activation. While a slight reduction in basal phosphorylation was observed in Themis^{KO} cells, reconstitution with Themis variants did not consistently alter phosphorylation. In addition, no differences in TCR-induced ERK activation was observed between Themis^{KO} and empty vector-transduced control cells, or upon reconstitution of any Themis mutants (**Fig. 6a**). Previous studies reporting altered ERK phosphorylation in Themis-deficient cells were performed using primary thymocytes stimulated with antigens of varying affinity[36], which may account for differences in proximal TCR signalling relative to the Jurkat cell system and antibody stimulation used here. Together, these data underscore the central role of the Themis-Grb2 complex as a critical regulatory node controlling SHP-1 phosphorylation after TCR stimulation."

Reviewer #4

The authors have fully addressed my questions/concerns, and I fully endorse publication of the manuscript.

We thank the reviewer for their positive endorsement of our work and for supporting its publication.