

TRAM promotes Toll-like receptor-free myddosome signal transduction

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

Version 0:

Reviewer comments:

Referee #1

(Remarks to the Author)

The authors follow on from their previous Nature paper (2024) defining the central role of TLR-free Myddosomes (cytosolic myddosomes, 'CMs' here for short) in TLR signaling, by asking and addressing the question as to how plasma membrane (PM) based proto-myddosomes are transitioned to CMs for optimal signaling.

There are two main conceptual advances revealed (1) a key role for TRAM (TICAM2) in controlling the transition to CMs and (2) differential roles for proto-myddosomes and CMs in terms of the signaling outcomes and longevity of signal. Specifically, they show that (1) an unbiased screen reveals a role for TRAM in the release of proto-myddosomes and subsequent formation of CMs; (2) in the absence of TRAM, MyD88 is retained at the PM after either TLR2 or TLR4 stimulation, and signaling terminates early and MyD88 shows a different interactome compared to when TRAM is present; (3) in a broad analysis of TIR-TIR interactions, a MyD88-TRAM interaction ranks highly in terms of affinity; (4) TRAM requires localisation at PI(4,5)P2-rich PM subdomains for this function, where it competes with TIRAP for TLR4 binding; (5) the relationship between TLR4-TRAM and TIRAP can be recapitulated in vitro; (6) a chemical inhibitor of MyD88 self-association confirms the model by a distinct approach and also allows them to show that in vivo long-lived CMs are required for proper TLR signaling.

The techniques and approaches used are elegant and sophisticated, and allow a deeper analysis of MyD88 dynamics in intact cells than has previously been possible. The conclusions are justified by the data. My comments for further improvement of the manuscript before publication relate to the need for the authors to give more context to their findings both in terms of what was already known and what is now still unclear (in the narrative) and some important controls that are lacking to confirm the specificity of the TRAM role for CM signaling:

MAJOR:

1. There is a lack of controls showing that non-MyD88-dependent signaling is unaffected by absence of TRAM. Only Ext Fig 1C shows a lack of effect on TNF signaling for one readout. It would be straightforward in both the iBMDM and primary BMDMs to assess whether absence of TRAM affects TRIF-dependent signalling (TLR3), non-TLR signalling (e.g. via RIG-like receptors) and other pathways using MyD88 (IL-1 or IL-18). These are important experiments to perform to determine whether the role of TRAM is specific for TLR2 and TLR4 signaling or also true for other MyD88-dependent pathways, and crucially to confirm that absence of TRAM does not cause an impairment or delay in non-MyD88 signaling.

MINOR:

2. Although the discoveries with TRAM here are surprising and novel, the authors do brush over some previous work that hinted at a role for TRAM outside of TRIF signaling. TRAM had previously been shown to be a sorting adaptor for both TLR2 and TLR4, required to relocate TLR4 or TLR2 to endosomes. How does this other function of TRAM in TLR2 and TLR4 signaling relate to this? Are there TRAM-TLR complexes for TLR2&4 at the endosome as well as and distinct from CMs containing TRAM?

3. Related, although the authors show convincingly that TRAM and MyD88 interact, this is not entirely novel - previous

studies have shown that MyD88 and TRAM interact using different techniques and albeit in different contexts, which should be acknowledged (Ref 39 and Ohnishi 2012 PLoS ONE 7: e38423).

4. The TIRAP:TRAM ratio is shown to be important for optimal signaling via CMs. Thus, does the likelihood of signaling in individual cells depend on TIRAP:TRAM ratio, for example in responder vs bystander cells in Fig 1?
5. What exactly drives the displacement of TIRAP for TRAM during the transition from proto-myddosomes to CMs?
6. Please comment on the need for CMs to eventually dissociate to end potentially harmful signaling and how the cell might achieve this signal termination?

Referee #2

(Remarks to the Author)

In this submission Jon Kagan's group present evidence that the biogenesis of Myddosome structures in innate immune cells is regulated by the TIR adaptor protein TRAM (also known as TICAM2). This is surprising as TRAM has previously been understood to function as an adaptor protein in TLR4/TRIF mediated activation of interferon and other responses.

In the first experiments they develop a cell sorting method to separate a mouse macrophage cell line (IBMDM) in which the endogenous MyD88 gene has been replaced with a fluorescently tagged version. These cells are activated with TLR4 and 2 ligands LPS and LTA and cells that have formed Myddosomes are identified by high light side scattering. This revealed two populations of IBMDMs, with and without Myddosomes. Subsequent RNAseq analysis showed robust expression of primary and secondary response genes. These experiments provide a proof of principle for the method.

Next, they have used this approach for a CRISPR/Cas9 screen to identify genes involved in Myddosome assembly, that is mutant cells with low light scattering. This approach identified the expected TLR pathway components and in addition the TLR4/TRIF adaptor TRAM. Further analysis of the TRAM $-/-$ IBMDM cells provides some evidence that TRAM mutant cells have a partial defect in the release of early Myddosomes from the TLR, and a corresponding attenuation in downstream signalling for example to NF B and IL6. This finding is both surprising and unexpected because TRAM is well characterised as driving interferon response factor signaling and it is not very clear how to reconcile these two very different functions. Nevertheless, KO of TRAM was previously shown to interfere with later phase signalling to JNK consistent the defect described here¹. By contrast TRAM KO mice have normal LPS-induced early phase of NF B activation. A key question raised by this study is whether the observed effects are mechanistically important for signaling function or for example the consequence of using TRAM protein null cells. So an important control would be to determine whether these regulatory effects require TRAM signalling function. This could be tested using IBMDMs with the inactivating TRAM mutation in the BB-loop (C114H). It is also important to verify that TRAM/TRIF signalling in IBMDMs is normal with IRF3 and IFN assays.

Another general problem with the study is that in almost all experiments a single dose of LPS, 10ng/ml, is used which will only partially activate the TLR4 pathway. A consequence of that is shown in Extended Fig 3e which measures levels of cell surface TLR at different doses of LPS. At 10ng/ml surface levels of TLR are unaltered but at higher LPS about 80% is endocytosed. It is known that the timing of assembly and number of Myddosome formed is highly dependent on LPS dose and it is important to carry out a dose-response analysis with WT and null TRAM to determine whether TRAM effects are seen at higher doses, at least for most central experiments such as Fig 2e. Another issue is that activation of TLR4 by LPS is coupled to endocytosis and release of Myddosome with engagement of TRAM at the endosome which implies that endocytosis is a critical step in TRAM/TRIF signalling, or alternatively that a subpopulation of TLR4 is trafficked directly to the endosome. To address this, it would be useful to image fluorescently labelled TRAM and TRAM C114H during the signalling process. In this regard it is interesting that in Extended Fig 3D that TRAM is recruited to MyD88 complexes very transiently at about 1 hour which would be consistent with the idea that TRAM displaces nascent Myddosomes as TLR4 is trafficked to the endosome where the TLR4/TRAM complex engages with TRIF, ie there is a two-step process.

The next experiment uses split luciferase to measure interactions between TIR domains. These kinds of assay are prone to artefacts because the TIRs are being expressed from a very strong CMV promoter in HEK293 cells. Another problem is that the molecules of most interest, TRAM and Mal/TIRAP, are presumably lacking their respective PIP2 and myristoyl sequences, although the deletion boundaries used are not given. The membrane localisation of TRAM and Mal/TIRAP is critical for function because it increases greatly the effective concentration of the molecules at the membrane, enhancing interactions that would otherwise not be detected. Another problem is that no interactions are detected with either TLR2 or TLR4 except with Mal/TIRAP which gives a signal with everything, probably because it forms large filaments and aggregates when overexpressed. Many of these interactions have already been characterised and so overall I think this data adds very little to the central proposal about TRAM function and could be removed or replaced with a small table showing the TRAM/MyD88 interactions.

Next the authors created a series of TRAM chimeras with other membrane association motifs including the Mal/TIRAP PIP2 binding sequence. The main finding is that PIP2 motif could localise TRAM to the PIP2 rich domains and that it was functional in terms of releasing Myddosomes to the cytosol. While this is interesting the conclusion that TRAM requires PIP2 domains for release is not well founded as the TRAM native myristylation site is evenly distributed in the plasma membrane. The data in Figure 4 to support this work is not of great quality, for example the cells in Figure 4a should have a plasma membrane marker.

Following on, they reconstituted TRAM-HA with the IBMDM TRAM null cells and then manipulated the levels of Mal/TIRAP and TRAM finding that the ratio rather than the amount of the adaptors determined release of Myddosomes. While this conclusion is drawn using a high artificial overexpress system it does corroborate the central hypothesis. Using these same

cells they then did tandem immunoprecipitations to determine the temporal pattern of Mal/TRAM association with MyD88. This shows a switch from initial MyD88/Mal complexes by 40 minutes to a predominant complex with TRAM. A MyD88/Mal/TRAM ternary complex is also formed transiently. This provides further support that TRAM perhaps in tandem with trafficking displaces receptor bound Myddosomes. To validate these interactions they used turbidity measurements to probe the assembly of oligomeric TIR structures of purified TIR domains of MyD88, Tirap and Tram. The formation of these complexes in vitro with high concentrations of protein and the observed interference in assembly have been reported before (ref 26). In my view these structures are unlikely to represent physiological complexes and the experiments add little to the conclusions drawn in vivo unless they can show that the structures of the complexes in vitro and in vivo are cognate.

The final section analyses the effects of methyl-piperidino-pyrazole (MPP) a previously characterized MyD88 inhibitor. Consistent with the previous study they found inhibition of MyD88 signalling, destabilisation of Myddosomes and cytokine production. Similar inhibition of TLR signalling was also seen when MPP was injected into mice. This work largely confirms the previous study (ref 62) and therefore has limited novelty.

In conclusion the authors describe a novel role for TRAM in releasing receptor bound Myddosomes to the cytosol where they are able to mature or be degraded. Establishing a dual role for TRAM is important but what I find odd is the failure of the authors to consider how TRAM/TRIF signalling to, for example, IRF3 fits in with this model. An obvious possibility is that signal induced endocytosis traffics receptor associated TRAM to endosomes to initiate the TLR4/TRAM/TRIF signalling. There is abundant published evidence for this general model and if the authors believe it to be wrong they should explain why.

Referee #3

(Remarks to the Author)

In the manuscript by Fisch et al., the authors present several compelling and fundamental new discoveries in TLR signalling. Building on their previous significant study defining proto- and mature myddosome complexes (Fisch et al., Nature 2024), the authors make the surprising discovery that the adaptor, TRAM is a critical factor for TLR4 and TLR 2 myddosome release into the cytosol for maturation and sustained signalling for maximal cytokine secretion. Firstly, using an CRSIPR whole genome screen in combination with an innovative flow cytometry-based assay, TICAM-2, the gene for TRAM was identified as a key component for both TLR2 and TLR4 myddosome assembly. Using a variety of approaches the authors go on to demonstrate that TRAM is required for proto-myddosomes to be released from the receptor complex at the cell surface into the cytosol for more signalling competence and secondary response genes including ISGs. Mechanistically, the authors show that TRAM-TIR can bind directly to MyD88-TIR and further that TRAM outcompetes TIRAP for PIP2 binding at the cell membrane to bind MyD88 and release proto-myddosomes into the cytosol. The authors elegantly demonstrate that MyD88 interacts first with TIRAP then with both TIRAP and TRAM before exclusively with TRAM. If TIRAP is forced to endure, myddosomes are unable to mature in the cytosol. Finally, the authors utilise MyD88-TIR inhibitors and show both in vitro and in vivo that when MyD88-TIRs are inhibited after stimulation for 30-60 mins with LTA/LPS, early responses persist, sustained signalling and later cytokine responses are lost/reduced. This in vivo analysis included impressive endogenous MyD88 IPs of splenocytes from treated mice that showed both the formation of myddosomes in vivo and the importance of their maturation (longer lasting myddosomes) for normal cytokine responses.

Overall, this manuscript was extremely well executed building a clear and exciting narrative. The authors present a substantial body of thorough and well-executed data. Both the quality and innovation of the experiments performed in this manuscript were of the highest order making the findings highly convincing. Appropriate controls were presented in many experiments to provide further confidence in the results. The significant finding that TRAM is required for myddosome maturation, including in an in vivo setting makes the study highly novel and impactful. It provides a new model for the key roles of TIRAP and TRAM required for maximal TLR2/4 immune responses. Given the fundamental importance of TLR for innate immune responses this work will be of broad interest to immunologists, cell biologists, clinicians and the general readers of Nature.

The authors should address the following minor comments:

1. Main text— line 38: Author should also include DAMPs as activators of TLRs.
2. Figure 1a-c— line 74: What proportion of the LPS bulk cells are responders vs bystanders? Are the responders much higher??
3. Figure 1b— line 79: Can the authors suggest why some cells (SSC lo bystanders) don't form active myddosomes?
4. Figure 1c— line 82-84: Should this be reworded to state that both responders and bystanders exhibited ISG signature...?
5. Figure 1d— line 87: In the same experimental setup the authors should show bystanders don't respond to IFNs = no ISGs (e.g. surface ISGs like Siglec-1, Bst2).
6. Figure 1f— line 122: It appears that rather than lacking cytosolic myddosomes, TRAM KO cells have significantly reduced numbers. I know that numbers and cell surface vs cytosolic myddosomes are quantified at a later point but if these quantifications are presented here, it strengthens the conclusions drawn from this data.
7. Extended data Fig. 3: Rather than just using P-p38, inclusion of phosphorylated-IRAK4 in WCLs as readout for early/proximal myddosome activation would add value to this impressive data set.
8. Figure 2e: The progression of the times for each sample is not obvious (0, 0.2, 0.5, 1 ... 12 h). All the lanes should be labelled (as not stated in legend) and to accommodate they could be slightly reduced in size.
9. Extended data Fig. 3d: The authors show p62 (autophagy regulator) associates with myddosomes at post stimulation (3 h), which is accelerated in TRAM KO cells (2 h) leading to faster clearance of signalling molecule interactions. In the previous work published by this group, the interactions with p62 appeared more delayed (6-12 h). Please explain this

difference.

10. Figure 3: Using the primary BMDMs from WT and TRAM KO mice, although unlikely, it would be valuable to also examine the stimulation of other TLR pathways that use MyD88 (e.g. TLR5, TLR9, TLR7) to see if there is any impact on TNF and IL-6 with TRAM deficiency. Do other TLRs need TRAM for myddosome release/maturation and cytokine secretion?

11. Figure 4h: In the imaging data can the authors make the images larger – they are a little hard to make see without extreme zooming in. Also, what does the concentration indicate? PH dye? I don't think this was stated anywhere.

12. Extended data Fig. 9a– line 421: Authors state “LTA-triggered low-level IP-10 production was also reduced by MPP or TSI-13-57 pre-treatments.” Why is this the case when LPS IP-10 was unaffected?

13. Figure 6f: Is it possible to also examine TIRAP and TRAM in these IPs? If possible, it would be informative.

14. Discussion: Can the authors present a cartoon figure (supp) to summarise their new model of TRAM-mediated myddosome release and maturation.

15. Conclusion– line 510-513: The authors reframe “TLR signalling” as a 2-step process. However, is this true for all myddosome forming TLRs? or just TLR4 and TLR2? Other TLRs (see point 10 above), like endosomal TLR9, are not thought to use TRAM so how are the myddosomes from these TLRs released? Don't they require released into the cytosol for efficient cytokine production or is it only when TIRAP is involved? This is a fundamental part of the finding, and it is currently unclear if this mechanism is specific to TLR2 and TLR4 so it should be addressed. If it is specific, this should be made clear, including in the title perhaps.

Version 1:

Reviewer comments:

Referee #1

(Remarks to the Author)

The authors have significantly improved on their original submission by extensively and comprehensively addressing all the reviewers comments

Referee #2

(Remarks to the Author)

The authors have addressed adequately many of the issues I raised in the first round of review. The following points are outstanding.

- C114H mutation phenocopies KO, TRAM mutant cells support TLR3 TRIF signalling.
 - Requested dose response with LPS not done but analogous experiments with live bacteria have SRG defect. Not entirely convincing but goes some way to address point.
 - Imaging of C114H has been carried out and discussion of two different models of trafficking for TLR4 added.
 - I don't agree with the authors that the in vitro study be retained. As previously stated, it is likely to generate artefacts and distract from the main message of the study. 'Added language' does not address the point and I can't find that language anyway.
 - With regard to the in vitro assemblies, the notion that mega Dalton sized complexes produced by TIR constructs at high concentration accurately recapitulate the cellular signalling process is not supported by the data presented and is not consistent with in vivo studies. How can constructs that lack critical N-terminal and C-terminal regulatory motifs (death domains, myristoylation, PIP2 binding) possibly reflect the intricately regulated signalling process? Instead using single molecule fluorescence to measure the stoichiometry and ontogeny of the TLR signalosomes in live cells would provide information on the actual size of the homo- and hetero- complexes formed by the various TIR domains during signaling. In that regard also TRAMTIR, shown not to oligomerise in Fig. 5H, does form homo-oligomers which is why Enokizono et al 1 used the C117H mutant for NMR structure determination. A further problem is with Fig S7 – SEC profiles are shown but the column is not calibrated nor is the type of column used stated. To be meaningful the void volume needs to be indicated. To me it looks like all these preps are in the excluded volume so are already oligomeric to some extent prior to turbidity measurements.
 - Whilst the points made are taken about the differences between pretreatment with MPP and treatment after TLR stimulation any differences are likely due to pharmacokinetics. It seems also that the inhibitory action of MPP directed at the MyD88 TIR domains will implicitly affect Myddosome biogenesis, so this finding is incremental in my view. As Ippagunta et al say 'MPP analog to mice protected them from TLR4-dependent inflammation' as also shown here.
 - To conclude most of my points have been addressed satisfactorily but there are some outstanding issues.
1. Enokizono, Y. et al. Structures and interface mapping of the TIR domain-containing adaptor molecules involved in interferon signaling. *Proc. Natl. Acad. Sci. U. S. A.* 110, 19908-19913 (2013).

Referee #3

(Remarks to the Author)

I would like to thank the authors for addressing my comments so thoroughly. I am satisfied that they have adequately answered all of my concerns and I believe in doing so they have improved the manuscript. I would recommend the manuscript now be published! Well done.

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Referee #1

We thank this referee for their very helpful and insightful comments. The issues raised were helpful and we hope our replies have addressed the concerns raised. Our response to each comment is listed below in **Bold Font**.

The authors follow on from their previous Nature paper (2024) defining the central role of TLR-free Myddosomes (cytosolic myddosomes, 'CMs' here for short) in TLR signaling, by asking and addressing the question as to how plasma membrane (PM) based proto-myddosomes are transitioned to CMs for optimal signaling.

There are two main conceptual advances revealed (1) a key role for TRAM (TICAM2) in controlling the transition to CMs and (2) differential roles for proto-myddosomes and CMs in terms of the signaling outcomes and longevity of signal. Specifically, they show that (1) an unbiased screen reveals a role for TRAM in the release of proto-myddosomes and subsequent formation of CMs; (2) in the absence of TRAM, MyD88 is retained at the PM after either TLR2 or TLR4 stimulation, and signaling terminates early and MyD88 shows a different interactome compared to when TRAM is present; (3) in a broad analysis of TIR-TIR interactions, a MyD88-TRAM interaction ranks highly in terms of affinity; (4) TRAM requires localisation at PI(4,5)P₂-rich PM subdomains for this function, where it competes with TIRAP for TLR4 binding; (5) the relationship between TLR4-TRAM and TIRAP can be recapitulated in vitro; (6) a chemical inhibitor of MyD88 self-association confirms the model by a distinct approach and also allows them to show that in vivo long-lived CMs are required for proper TLR signaling.

The techniques and approaches used are elegant and sophisticated, and allow a deeper analysis of MyD88 dynamics in intact cells than has previously been possible. The conclusions are justified by the data. My comments for further improvement of the manuscript before publication relate to the need for the authors to give more context to their findings both in terms of what was already known and what is now still unclear (in the narrative) and some important controls that are lacking to confirm the specificity of the TRAM role for CM signaling:

MAJOR:

1. There is a lack of controls showing that non-MyD88-dependent signaling is unaffected by absence of TRAM. Only Ext Fig 1C shows a lack of effect on TNF signaling for one readout. It would be straightforward in both the iBMDM and primary BMDMs to assess whether absence of TRAM affects TRIF-dependent signalling (TLR3), non-TLR signalling (e.g. via RIG-like receptors) and other pathways using MyD88 (IL-1 or IL-18). These are important experiments to perform to determine whether the role of TRAM is specific for TLR2 and TLR4 signaling or also true for other MyD88-dependent pathways, and crucially to confirm that absence of TRAM does not cause an impairment or delay in non-MyD88 signaling.

We thank this referee for this important suggestion. We agree it is important to determine if the phenotype observed in TRAM-deficient cells reflects a specific role in TLR2/TLR4 signaling or a broader defect in innate immune signaling pathways. To address this point, we have expanded our analysis in primary BMDMs and iBMDMs using a panel of innate immune stimuli (Supplementary Fig. 4). In addition to TLR2 and TLR4 agonists, we examined cytokine production induced by endosomal TLRs (R848, CpG), TLR3 to assess TRIF-specific signaling (poly(I:C)), cytosolic PRRs engaging the STING and RIG-I/MAVS pathways (DMXAA and Sendai virus, respectively), and IL-33 as a MyD88-dependent, non-TLR member of the IL-1 receptor family.

These experiments confirmed the literature on the established role of TRAM in TLR4-TRIF signaling, as LPS-induced IFN β and IP-10 production were lost in the absence of TRAM (Fig. 4d+f & Supplementary Fig. 4). Important to this question of specificity, we found that TRAM was not required for STING, RIG-I or IL-33 induced cytokine secretion (Supplementary Fig. 4). TRAM was also not required for TLR3-induced IFN β secretion (Supplementary Fig. 4).

In examining MyD88-dependent TLRs, we found that the severity of the cytokine production defects associated with TRAM deficiency was highest for plasma membrane localized TLRs, as compared to the endosome-localized TLR7-9 (Supplementary Fig. 4). Similar results have been reported for the role of TIRAP in TLR7-9 signaling, where cytokine-specific (and ligand-specific) effects of TIRAP deficiency are known (Bonham *et al.*, *Cell* 2014). Interestingly, regardless of the receptor agonized, ligands that displayed a dependence on TRAM for signaling were most defective for production of the SRG products

IL-6 and IL-12p40. TNF α production was less consistently affected by TRAM deficiency, in line with its classification as a PRG that can be induced by transient signaling (Supplementary Fig. 4).

These collective findings demonstrate that TRAM is not required for non-TLR innate immune responses and support a model in which TRAM functions as a regulator of signaling downstream of TIRAP- and MyD88-dependent TLR pathways, in addition to its well-established role in the TLR4-TRIF-pathway.

To explain how TRAM could function in MyD88 and TRIF pathways downstream from TLR4, we include in this revision an analysis of TRIF-dependent IFN β secretion (Fig. 4d+f & Extended Data Fig. 7g). We altered the localization of TRAM in iBMDMs by expressing mutant TRAM alleles that were either localized to the plasma membrane, endosomes or cytosol. As expected from prior work, initially by Fitzgerald and colleagues (Rowe *et al.*, *PNAS* 2006), cytosolic TRAM mutants (e.g. TRAM G2A) were completely defective for cytokine and IFN secretion (Fig. 4d). Interestingly, we found that endosomal TRAM mutants (e.g. TRAM 3X) retained the ability to activate the TLR4-TRIF pathway and induce IFN secretion (Fig. 4d) but were defective for the induction of MyD88-dependent cytokines TNF α and IL-6 (Fig. 4d). Conversely, TRAM mutants that were specifically localized to PI(4,5)P₂-rich plasma membrane subdomains supported MyD88-dependent cytokine production but were poor inducers of TRIF-dependent IFNs (Fig. 4f). These signaling activities tracked with the ability of each TRAM mutant to assemble myddosomes and detach proto-myddosomes from the plasma membrane (Fig. 4b+e). These findings suggest an advanced biological model of the function of TRAM. Plasma membrane localized TRAM (and specifically TRAM within PI(4,5)P₂-rich subdomains) functions to drive myddosome assembly and cytokine expression whereas endosomal TRAM supports TRIF signaling and IFN production. These findings help explain prior observations that TRAM is localized to the plasma membrane and endosomes, as we can now conclude that localization to both compartments is important for signaling processes associated with activation of TLR4.

Importantly, we do not observe obvious changes in TRAM localization during signaling (Extended Data Fig. 7c), suggesting that TRAM is not globally redistributed upon receptor activation, as is the case for CD14 and TLR4. Instead, we propose that TRAM is pre-positioned at the sites of TLR signaling, akin to what is known for TIRAP.

MINOR:

2. Although the discoveries with TRAM here are surprising and novel, the authors do brush over some previous work that hinted at a role for TRAM outside of TRIF signaling. TRAM had previously been shown to be a sorting adaptor for both TLR2 and TLR4, required to relocate TLR4 or TLR2 to endosomes. How does this other function of TRAM in TLR2 and TLR4 signaling relate to this? Are there TRAM-TLR complexes for TLR2&4 at the endosome as well as and distinct from CMs containing TRAM?

This referee is correct in stating that TRAM has been implicated in TLR2 signaling, with a specific focus on the low-level IFN activities (relative to TLR4) induced by TLR2. Our finding of a role for TRAM in expression of secondary response genes, which are robustly induced by TLR2, is a central point of novelty for our study. On the other topics highlighted above, one point may need correction. TRAM has not been implicated as being required for the endocytosis of TLRs. TRAM functions after TLR4 endocytosis to activate TRIF-dependent IFN expression. Consistent with this idea, we find that TRAM deficiency does not impair TLR4 endocytosis (Extended Data Fig. 3c).

3. Related, although the authors show convincingly that TRAM and MyD88 interact, this is not entirely novel - previous studies have shown that MyD88 and TRAM interact using different techniques and albeit in different contexts, which should be acknowledged (Ref 39 and Ohnishi 2012 PLoS ONE 7: e38423).

We thank this referee for highlighting this prior work. We have now cited both studies. It may be helpful to also note that this prior work performed proof-of-concept studies of TRAM-MyD88 interactions in 293 cells. No studies prior to our work have examined TLR-induced TRAM-MyD88 interactions in macrophages.

4. The TIRAP:TRAM ratio is shown to be important for optimal signaling via CMs. Thus, does the likelihood of signaling in individual cells depend on TIRAP:TRAM ratio, for example in responder vs bystander cells in Fig 1?

We agree that the importance of the TIRAP:TRAM ratio in myddosome signaling raises the possibility that this stoichiometry contributes to cell-to-cell variability in signaling outcomes, including the emergence of responder and bystander populations. To address this point, we performed additional analyses comparing *Tirap* and *Ticam2* mRNA abundance in responder and bystander cells in our RNA-Seq experiments (Fig. 1c & Supplementary Fig. 1c). We also performed immunoblots for TRAM from responder and bystander cells (Fig. 1f). Interestingly, and consistent with our studies using CRISPRa to intentionally change the TIRAP:TRAM ratio (Fig. 5a-c & Extended Data Fig. 8), these experiments revealed that bystander cells display a reduction in TRAM levels (mRNA and protein), resulting in an increased TIRAP:TRAM ratio compared with responder cells. These findings suggest that variability in TRAM abundance, and consequently in the TIRAP:TRAM ratio, are indeed associated with the likelihood of productive signaling at the single-cell level. We thank this referee for promoting this additional analysis.

5. *What exactly drives the displacement of TIRAP for TRAM during the transition from proto-myddosomes to CMs?*

We thank the reviewer for this mechanistically important question. The precise molecular mechanism driving the apparent displacement of TIRAP and enrichment of TRAM during this transition is not directly addressed by our current study and would require additional biochemical and structural analyses beyond its scope. While our data support a model in which TRAM disrupts TIRAP from interacting with MyD88 to promote myddosome assembly, they do not yet allow us to resolve whether this reflects active displacement, differential recruitment kinetics, competitive binding, or changes in local membrane microenvironments. We therefore consider this an important and interesting open question. One possibility is that progressive changes in receptor clustering, lipid environment or post-translational modifications during myddosome maturation alter adaptor binding affinities, thereby favoring TRAM incorporation over TIRAP. Alternatively, temporal differences in adaptor recruitment dynamics may result in sequential engagement rather than direct displacement, albeit less likely based on our biochemistry characterization. At this stage, as these remain speculative models, we prefer not to expand on them extensively in the manuscript to avoid overinterpretation beyond the presented data.

6. *Please comment on the need for CMs to eventually dissociate to end potentially harmful signaling and how the cell might achieve this signal termination?*

We agree that efficient termination of myddosome signaling is likely to be as important as its assembly, because prolonged persistence of mature cytosolic signaling complexes could result in excessive or chronic inflammatory responses. While the present study focuses on the mechanism by which TRAM promotes myddosome maturation, our previous work demonstrated that mature myddosomes ultimately lose plasticity through continued incorporation of IRAK4 followed by signaling termination. Upon signaling termination, the inactive myddosome complexes are cleared from the cell via p62-mediated autophagy (Fisch *et al.*, *Nature* 2024).

Referee #2:

We thank this referee for their very helpful and insightful comments. The issues raised were helpful and we hope our replies have addressed the concerns raised. Our response to each comment is listed below in **Bold Font**.

In this submission Jon Kagan's group present evidence that the biogenesis of Myddosome structures in innate immune cells is regulated by the TIR adaptor protein TRAM (also known as TICAM2). This is surprising as TRAM has previously been understood to function as an adaptor protein in TLR4/TRIF mediated activation of interferon and other responses.

We thank this referee for their thoughtful commentary and suggestions on our manuscript. To ensure clarity at this first section of the critique, we apologize if we did not make it clear that we fully confirm all prior work on the role of TRAM in the TLR4-TRIF pathway. This prevailing view is not refuted. Rather, we find that in addition to its endosomal function in the TRIF pathway, TRAM, and specifically the pool that is localized to PI(4,5)P₂-rich plasma membrane subdomains, gains an additional role in the MyD88-dependent pathway. We have made several changes to the manuscript and generated additional data (see point-by-point response below) that provides additional texture to the model we propose.

In the first experiments they develop a cell sorting method to separate a mouse macrophage cell line (IBMDM) in which the endogenous MyD88 gene has been replaced with a fluorescently tagged version. These cells are activated with TLR4 and 2 ligands LPS and LTA and cells that have formed Myddosomes are identified by high light side scattering. This revealed two populations of IBMDMs, with and without Myddosomes. Subsequent RNAseq analysis showed robust expression of primary and secondary response genes. These experiments provide a proof of principle for the method.

The statement offered above needs a slight clarification. Our MyD88-AGF cells express tagged MyD88 from its endogenous locus under control from its endogenous promoter. The protein has not been “replaced” but endogenously tagged with CRISPR knock-in techniques. The cells were thoroughly validated in our prior study (Fisch et al., Nature 2024).

Next, they have used this approach for a CRISPR/Cas9 screen to identify genes involved in Myddosome assembly, that is mutant cells with low light scattering. This approach identified the expected TLR pathway components and in addition the TLR4/TRIF adaptor TRAM. Further analysis of the TRAM -/- IBMDM cells provides some evidence that TRAM mutant cells have a partial defect in the release of early Myddosomes from the TLR, and a corresponding attenuation in downstream signalling for example to NFkB and IL6. This finding is both surprising and unexpected because TRAM is well characterised as driving interferon response factor signaling and it is not very clear how to reconcile these two very different functions. Nevertheless, KO of TRAM was previously shown to interfere with later phase signalling to JNK consistent the defect described here¹. By contrast TRAM KO mice have normal LPS-induced early phase of NFkB activation. A key question raised by this study is whether the observed effects are mechanistically important for signaling function or for example the consequence of using TRAM protein null cells. So an important control would be to determine whether these regulatory effects require TRAM signalling function. This could be tested using IBMDMs with the inactivating TRAM mutation in the BB-loop (C114H). It is also important to verify that TRAM/TRIF signalling in IBMDMs is normal with IRF3 and IFN β assays.

We are grateful for the above comments and appreciate the point that our findings of a new function for TRAM are unexpected. Indeed, we were surprised to identify TRAM in our forward genetic screen as a regulator of myddosome assembly. While we have verified these initial findings through a variety of studies described in our initial submission, we appreciate the comment of this referee on whether these phenotypes are a consequence of using TRAM protein null cells. Below we describe several reasons why we do not believe this is the case, and rather support a revised cell biological model of TLR signaling.

First, we provide the compulsory evidence that all phenotypes associated with genetic deficiency of TRAM were complemented with TRAM cDNAs (Fig. 4a-f & Extended Data Fig. 7a-b). There must therefore be a mechanistic link to TRAM, as there is no evidence of a problem with TRAM null cells separate from TRAM function (Supplementary Fig. 4).

Second, perhaps the most direct evidence to bypass the concern that these phenotypes are unique to TRAM KO cells is our analysis of the ratio of TIRAP:TRAM. As we described in our first submission, we can phenocopy all TRAM KO phenotypes by increasing the ratio of TIRAP:TRAM (Fig. 5a-c & Extended Data Fig. 8). Increasing this ratio leads to an arrest of MyD88 at the plasma membrane (Fig. 5a & Extended Data Fig. 8c) and a defect in proto-myddosome to myddosome maturation and signaling (Fig. 5b-c & Extended Data Fig. 8c), as are observed in TRAM KO cells. Because these cells are TRAM sufficient, we can conclude a direct link to TRAM function rather than a caveat of a null deficiency.

Third, and to address the specific suggestion of this referee, we introduce additional analysis of the signaling-defective BB-loop mutant TRAM^{C114H}. We find that TRAM^{C114H} phenocopies the defects observed in TRAM KO cells (Fig. 4d), despite exhibiting a localization pattern comparable to WT TRAM (Extended Data Fig. 7c & Referee Fig. 1). These collective findings demonstrate that the observed phenotypes are not simply a consequence of TRAM protein loss but instead require an intact and functional TRAM TIR domain.

It is also helpful for us to highlight additional analysis performed for this revision, with the goal of better defining the specificity of TRAM activities. We have characterized TRIF-dependent signaling in the

revised manuscript. Consistent with the established role of TRAM in the TLR4–TRIF pathway, we found that TRAM deficiency abolishes LPS-induced IFN β and IP-10 production (Supplementary Fig. 4). In contrast, TLR3-induced IFN β responses remain intact, demonstrating that TRIF signaling *per se* is not impaired and confirming the specific requirement for TRAM in coupling TLR4 to TRIF (Supplementary Fig. 4). Additionally, we found that STING- and RIG-I induced IFN responses are unaffected by TRAM deficiency (Supplementary Fig. 4). Based on these data, we conclude that the two functions of TRAM—promotion of proto-myddosome release/maturation and coupling of TLR4 to the TRIF–IRF3–IFN pathway—are genetically and mechanistically separable. Our results do not challenge the established role of TRAM in TRIF signaling; rather, they reveal an additional function that operates in parallel. During TLR signaling, TRAM can therefore contribute to myddosome maturation and to TRIF-dependent IFN induction, depending on the signaling context and subcellular pool involved.

Another general problem with the study is that in almost all experiments a single dose of LPS, 10ng/ml, is used which will only partially activate the TLR4 pathway. A consequence of that is shown in Extended Fig 3e which measures levels of cell surface TLR at different doses of LPS. At 10ng/ml surface levels of TLR are unaltered but at higher LPS about 80% is endocytosed. It is known that the timing of assembly and number of Myddosome formed is highly dependent on LPS dose and it is important to carry out a dose-response analysis with WT and null TRAM to determine whether TRAM effects are seen at higher doses, at least for most central experiments such as Fig 2e.

We thank this referee for their comments. It is worth noting that 10 ng/mL LPS is within the range of doses commonly used for genetic analysis of innate immunity. Depending on the assay and lab, 1-100 ng/mL are typical. For example, the original report of TRAM KO mice published by Akira used 10-100 ng/mL of LPS in different experiments (Yamamoto *et al.*, *Nat Immunol.* 2003), and the first cell biological analysis of TRAM by Fitzgerald (Rowe *et al.*, *PNAS* 2006) showed that 15 ng/mL was a near maximal stimulus of cytokine secretion. Additionally, the original study describing the TIRAP KO mice by Akira used 1 ng/ml (Yamamoto *et al.*, *Nature* 2002) and the original TRIF KO mouse report used a dose range of 1-100 ng/mL (Yamamoto *et al.*, *Science* 2003).

With the above statements made, we acknowledge that it may be difficult to feel comfortable comparing data in studies performed by different labs across several years. As such, it is worth examining the data we have presented. We find that 10 ng/mL LPS robustly activates not only MyD88-dependent inflammatory cytokine production but also the TRIF-dependent arm of the pathway, which we have now examined in this revision (Fig. 4d+f).

This referee suggested that a dose-response analysis may be helpful. While we have performed dose analyses for myddosome activities in our recent study (Fisch *et al.*, *Nature* 2024), one problem with this suggestion is that different readers may consider different doses of a given PAMP appropriate. Stated differently, there is no accepted dose of PAMP that is universally considered to be “correct”. For these reasons, we have opted to address this concern in a different manner, which reflects the actual macrophage-microbe interaction: In this revision, we introduce a series of live bacterial infections across a range of multiplicities of infection (MOI). Specifically, we infected primary and immortalized cells with *E. coli*, *S. Typhimurium*, *S. aureus*, *L. monocytogenes* and *L. lactis* at MOI ranging from 1-50 (Fig. 3g-h & Extended Data Fig. 5). These experiments demonstrated two important points: First, we found that the doses of LPS and LTA used throughout our studies yielded comparable macrophage responses to those observed across multiple MOI (Extended Data Fig. 5). Second, the phenotypes we defined in TRAM KO cells with PAMPs were evident in these infection models. Specifically, we found that (like with PAMPs), TRAM KO cells were principally defective for the secretion of the secondary response gene product IL-6, whereas the primary response gene product TNF α was minimally impacted. Based on these new data, we hope that this referee agrees that the experimental conditions used with PAMPs are applicable to host-bacteria interactions.

Finally, the observation that higher LPS doses induce more extensive receptor endocytosis (Extended Data Fig. 3c) is interesting but perhaps suggests that the threshold of TLR4 endocytosis needed for TRIF signaling is lower than the maximal extent of possible TLR4 internalization. This suggestion is consistent with our prior work showing that each cell responds in an all-or-nothing fashion, regardless of LPS dose (Fisch *et al.*, *Nature* 2024). While LPS dose does not affect the extent of myddosome

assembly in individual cells, dose is a key determinant of how many cells in a population respond. Similar conclusions were made by several labs over the years, who examined transcription factor activities (e.g. Kellogg *et al.*, *Elife* 2015), where all-or-nothing responses are observed in individual cells in a population.

Another issue is that activation of TLR4 by LPS is coupled to endocytosis and release of Myddosome with engagement of TRAM at the endosome which implies that endocytosis is a critical step in TRAM/TRIF signalling, or alternatively that a subpopulation of TLR4 is trafficked directly to the endosome. To address this, it would be useful to image fluorescently labelled TRAM and TRAM C114H during the signalling process. In this regard it is interesting that in Extended Fig 3D that TRAM is recruited to MyD88 complexes very transiently at about 1 hour which would be consistent with the idea that TRAM displaces nascent Myddosomes as TLR4 is trafficked to the endosome where the TLR4/TRAM complex engages with TRIF, ie there is a two-step process.

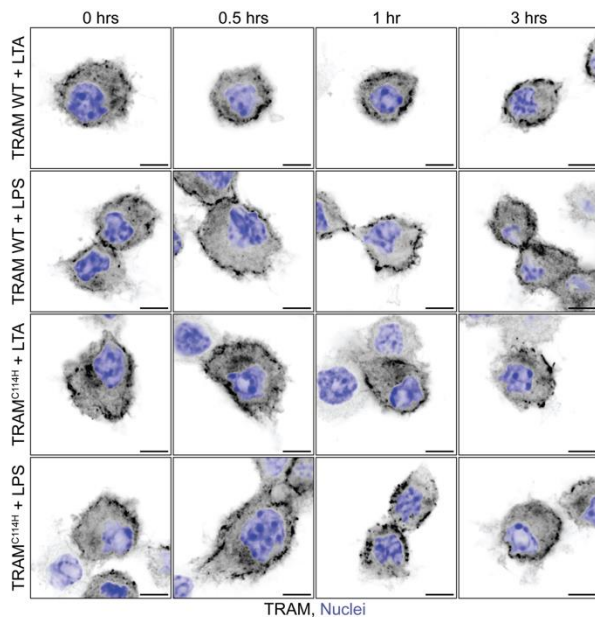
We thank this referee for this thoughtful comment. We agree that TLR4 endocytosis is a critical step in TRAM-dependent TRIF signaling and we do not challenge this well-established model. Indeed, the RNA-sequencing we performed (Fig. 3f) demonstrated complete loss of IFN/ISG expression in TRAM KO cells treated with LPS. We verified these findings at the protein level in this revision, by measuring IFN β secretion by ELISA (Supplementary Fig. 4).

However, the central question addressed in our study is distinct from the mechanism of TRIF activation on endosomal sites and instead concerns the role of TRAM in myddosome maturation. Several lines of evidence indicate that myddosome release is mechanistically separable from receptor endocytosis.

First, we find that TRAM deficiency does not impair TLR4 endocytosis (Extended Data Fig. 3c), as has been shown before (Zanoni *et al.*, *Cell* 2011). Second, we demonstrate that inhibition of receptor endocytosis does not prevent myddosome maturation (Extended Data Fig. 3b). Third, we observe analogous TRAM-dependent effects downstream of TLR2 stimulation, despite the fact that TLR2 does not undergo the same ligand-induced endocytic trafficking behavior that characterizes TLR4. Together, these findings argue that receptor internalization is not the driving force behind myddosome release and instead support a model in which these processes may occur independently.

With regard to the suggested imaging experiments, we have now examined the localization of WT TRAM and the C114H mutant and do not observe discernible differences in their spatial distribution (Referee Fig. 1, see below). This observation is consistent with the data on the localization of WT TRAM already presented in our initial submission (Extended Data Fig. 7c). While live-cell imaging of TRAM dynamics is an interesting question to address, our collective data suggest that there is a steady state distribution of TRAM between the plasma membrane and endosomes. In the absence of detectable stimulus-induced TRAM relocalization, despite clear changes in its biochemical interactions with MyD88, we propose that TRAM is pre-positioned at signaling sites prior to receptor activation.

We agree that both scenarios proposed by the referee remain possible: TRAM may act on receptor-associated complexes that subsequently traffic to endosomes, or alternatively a distinct receptor pool already within endosomes may engage the TRIF pathway. Our data do not distinguish between these possibilities, nor was that the objective of the present study. Rather, the key conclusion supported by our results is that TRAM has an additional role in myddosome maturation that is genetically and mechanistically separable from its established function in TLR4 endocytosis and TRIF signaling.



Referee Fig. 1: TRAM localization imaging.

Representative immunofluorescence images of TRAM WT- or TRAM^{C114H}-expressing cells stimulated with LTA or LPS for the indicated times. Cells were stained for TRAM (grey) and nuclei (blue). WT TRAM and TRAM^{C114H} displayed broadly similar spatial distributions throughout the time course, with no measurable changes in subcellular localization. Images are representative of n = 3 experiments. Scale bars, 5 µm.

The next experiment uses split luciferase to measure interactions between TIR domains. These kinds of assay are prone to artefacts because the TIRs are being expressed from a very strong CMV promoter in HEK293 cells. Another problem is that the molecules of most interest, TRAM and Mal/TIRAP, are presumably lacking their respective PIP2 and myristoyl sequences, although the deletion boundaries used are not given. The membrane localisation of TRAM and Mal/TIRAP is critical for function because it increases greatly the effective concentration of the molecules at the membrane, enhancing interactions that would otherwise not be detected. Another problem is that no interactions are detected with either TLR2 or TLR4 except with Mal/TIRAP which gives a signal with everything, probably because it forms large filaments and aggregates when overexpressed. Many of these interactions have already been characterised and so overall I think this data adds very little to the central proposal about TRAM function and could be removed or replaced with a small table showing the TRAM/MyD88 interactions.

We agree with this referee on the skepticism one must have for studies using overexpressed transgenes, especially in 293 cells. However, it is important to note that the interactions we have observed in this assay were validated by our biochemical studies of endogenous proteins in macrophages. In fact, with the exception of this single section of the study, which has value for the broad analysis of all TIRs in a single assay, all other work we presented examined proteins in macrophages (not 293 cells). For these reasons, we believe that retaining these data is appropriate and valuable for readers interested in the interaction landscape underlying the proposed model. To accommodate this concern, we have added language to this section of the manuscript highlighting caveats associated with 293 cell based analyses.

We thank this referee for pointing out that we did not provide the exact boundaries of the constructs used in these experiments. We apologize for this oversight and have added this information to the Supplementary Information (Supplementary Table 1).

Next the authors created a series of TRAM chimeras with other membrane association motifs including the Mal/TIRAP PIP2 binding sequence. The main finding is that PIP2 motif could localise TRAM to the PIP2 rich domains and that it was functional in terms of releasing Myddosomes to the cytosol. While this is interesting the conclusion that TRAM requires PIP2 domains for release is not well founded as the TRAM native myristylation site is evenly distributed in the plasma membrane. The data in Figure 4 to support this work is not of great quality, for example the cells in Figure 4a should have a plasma membrane marker.

We thank this referee for raising the importance of protein localization, as this has been a centerpiece of work in our lab for many years. As this referee correctly indicated, and consistent with the original

work by Fitzgerald on this topic (Rowe *et al.*, *PNAS* 2006), TRAM localizes to a wide stretch of the plasma membrane. As we report here, included in TRAM's plasma membrane localization is enrichment in PI(4,5)P₂ rich plasma membrane subdomains, which we mark with the PH domain from PLCδ1 (Fig. 4g).

Our chimeric approach, where we swapped the localization domain of TRAM with several others, was necessary to delineate the importance of endosomal and plasma membrane subdomain localization (Fig. 4d+f). As we describe below, our revision was expanded to include the study of TRIF-induced IFNs, in order to better link TRAM to MyD88 and TRIF pathways.

As this referee described, we altered the localization of TRAM in iBMDMs by expressing mutant TRAM alleles that were either localized to the plasma membrane, endosomes or cytosol (Extended Data Fig. 7a-e). Within the plasma membrane, we targeted TRAM specifically to PI(4,5)P₂-rich subdomains or more generally to the entire cell surface. This work revealed that TRAM mutants that were specifically localized to PI(4,5)P₂-rich plasma membrane subdomains supported MyD88-dependent cytokine production but were poor inducers of TRIF-dependent IFNs (Fig. 4f). General plasma membrane localization, using the SH4 domain from Fyn, did not support MyD88 signaling (Fig. 4f), thus our conclusion that localization to PI(4,5)P₂-rich domain is central for TRAM function. Interestingly, we found that endosomal TRAM mutants (e.g. TRAM 3X) retained the ability to activate the TLR4-TRIF pathway and induce IFN secretion but were defective for the induction of MyD88-dependent cytokines (Fig. 4d). As expected from prior work, cytosolic TRAM mutants (e.g. TRAM G2A) were completely defective for cytokine and IFN secretion (Fig. 4d). These signaling activities tracked with the ability of each TRAM mutant to assemble myddosomes and detach proto-myddosomes from the plasma membrane (Fig. 4b+e).

Based on the new data presented, we now offer a more advanced biological model of the function of TRAM. Plasma membrane (specifically PI(4,5)P₂ localized) TRAM functions to drive myddosome assembly and cytokine expression whereas endosomal TRAM supports TRIF signaling. These findings help explain prior reports that TRAM is localized to the plasma membrane and to endosomes, as we can now conclude that localization to both compartments is important for TLR4.

Finally, we respectfully disagree with the assertion that the microscopy data in our study is of poor quality. In fact, the imaging and quantitative analyses presented in this study were viewed differently by the other referees. Reviewer #1 stated that “the techniques and approaches used are elegant and sophisticated” and that “the conclusions are justified by the data” while Reviewer #3 described the work as experimental quality “of the highest order”.

Following on, they reconstituted TRAM-HA with the IBMDM TRAM null cells and then manipulated the levels of Mal/TIRAP and TRAM finding that the ratio rather than the amount of the adaptors determined release of Myddosomes. While this conclusion is drawn using a high artificial overexpress system it does corroborate the central hypothesis.

We would like to clarify that these experiments were not performed using an ectopic overexpression system. Rather, we employed CRISPR activation (CRISPRa; Konermann *et al.*, *Nature* 2014) to selectively modulate the expression of the endogenous *Tirap* and *Ticam2* genes. This approach allowed us to achieve controlled changes in a spectrum of protein abundance (depending on sgRNA used), while preserving endogenous contexts. Based on this method, we found that overexpression is not the determinant of function. Rather, it is the ratio of TIRAP:TRAM that determines myddosome assembly (Fig. 5a-c). Even under conditions of high level of overexpression using a subset of the CRISPRa vectors, WT myddosomes activities could be maintained if the ratio of TIRAP:TRAM was preserved (Fig. 5c).

Using these same cells they then did tandem immunoprecipitations to determine the temporal patten of Mal/TRAM association with MyD88. This shows a switch from initial MyD88/Mal complexesby 40 minutes to a predominant complex with TRAM. A MyD88/Mal/TRAM ternary complex is also formed transiently. This provides further support that TRAM perhaps in tandem with trafficking displaces receptor bound Myddosomes. To validate these interactions they used turbidity measurements to probe the assembly of oligomeric TIR structures of purified TIR domains of MyD88, Tirap and Tram. The formation of these complexes in vitro with high concentrations of protein and the observed interference in assembly have been reported before (ref 26). In my view these structures are

unlikely to represent physiological complexes and the experiments add little to the conclusions drawn in vivo unless they can show that the structures of the complexes in vitro and in vivo are cognate.

We are pleased that this referee agreed on the insight generated from our analysis in macrophages of the transition of complexes of TIRAP-MyD88 → TIRAP-MyD88-TRAM → MyD88 alone. This finding drove much of our thinking of how myddosome maturation occurs. We also agree that there is an assumption with all recombinant protein studies that the observed behaviors reflect natural biochemical activities. This assumption is not unique to this project and creates a mandate to correlate observations made with recombinant proteins to activities in cells. With this concept in hand, we would like to highlight that our study has offered this corroboration, including an important new addition prompted by this comment.

The turbidity assay we used has been described by others to study TIR domains, including the work cited by this referee (Ve et al., NSMB 2017). However, our work advances beyond what was published, as we investigated how TRAM and TIRAP influence MyD88 assembly dynamics. The phenotypes we observed using recombinant TIR domains include 1) TIRAP-MyD88 complexes are small, 2) MyD88 complexes are large and 3) TRAM frees MyD88 from the size restriction imposed by TIRAP. While these phenotypes match our findings in macrophage, we recognize the value of additional evidence supporting the relationship between *in vitro* and *in cellulo* studies. Towards this end, and in response to this query, we performed studies using the signaling-defective TIRAP^{P125H} mutant (Extended Data Fig. 9f). We find that unlike the WT TIR domain of TIRAP, this mutant not only lacks activity in myddosome assembly in macrophages but also has no impact on MyD88 oligomerization *in vitro* using the turbidity assay. The concordance between the cellular phenotype and *in vitro* behavior provides evidence that the turbidity measurements report on biologically relevant TIR-domain interactions. The addition of the TIRAP^{P125H} mutant to our recombinant protein studies was an excellent one and we thank this referee for prompting us to provide additional data.

We have revised the manuscript to more clearly explain the purpose, limitations and interpretation of these experiments and have incorporated the new TIRAP^{P125H} data in Extended Data Fig. 9f.

The final section analyses the effects of methyl-piperidino-pyrazole (MPP) a previously characterized MyD88 inhibitor. Consistent with the previous study they found inhibition of MyD88 signalling, destabilisation of Myddosomes and cytokine production. Similar inhibition of TLR signalling was also seen when MPP was injected into mice. This work largely confirms the previous study (ref 62) and therefore has limited novelty.

It is important to note that while MPP and its derivative TSI-13-57 were described previously, the work done by others focused exclusively on prophylactic treatments prior to TLR ligand exposure *in vitro* and *in vivo*. No study has examined the impact of these inhibitors on pre-existing inflammatory states of cells or animals, which is more physiological if one wants to consider impacts on disease. We also note that these inhibitors had not been used to assess direct impacts on myddosome activities, which was our focus. For these reasons, we would respectfully ask this referee to reconsider the position that our work largely confirms prior work.

To summarize, our studies provided the first biochemical evidence for myddosome formation *in vivo*. We were excited by this finding, as the composition matched what we observe *in vitro*, thereby solidifying the myddosome as the site of multiple signaling enzymes, including IKKs, MAPKs, TBK1 (Fig. 6b). Additionally, we demonstrate that MPP and its derivative TSI-13-57 disrupts pre-existing myddosomes, revealing an unanticipated capacity to reverse established signaling assemblies. Finally, by combining our *in vivo* myddosome assembly assay with chemical treatments, we found that TSI-13-57 administered after TLR activation disrupts myddosomes and halts inflammatory gene expression and cytokine detection in the serum. None of these studies were performed in prior publications.

In response to this query, we have moved all *in vitro* data to Extended Data Fig. 10 and only retained data from our *in vivo* experiments in the main figure (Fig. 6).

In conclusion the authors describe a novel role for TRAM in releasing receptor bound Myddosomes to the cytosol where they are able to mature or be degraded. Establishing a dual role for TRAM is important but what I find odd is the failure of the authors to consider how TRAM/TRIF signalling to, for example, IRF3 fits in with this model. An obvious possibility is that signal induced endocytosis traffics receptor associated TRAM to endosomes to

initiate the TLR4/TRAM/TRIF signalling. There is abundant published evidence for this general model and if the authors believe it to be wrong they should explain why.

We are most grateful that this referee appreciates the value of the dual function of TRAM in MyD88 and TRIF signaling. The focus on our first submission was on the link to myddosomes, to ensure focus on the most novel concepts. However, we now recognize that we should have elaborated further on the relationship between these new activities and those that were already established. In this revision, we have added new experiments to compare the MyD88 and TRIF pathway regulation by TRAM and have modified the text in several instances to ensure clarity on the model that we propose. Although indicated earlier in our response to this referee, it is worth highlighting again here. Our data support a model whereby the unique localization pattern of TRAM enables it to be pre-positioned at the plasma membrane and on endosomes (Extended Data Fig. 7b-c). In PI(4,5)P₂-rich regions of the plasma membrane, TRAM functions to displace TIRAP from MyD88, thereby facilitating the proto-myddosome to myddosome transition. If TRAM is localized exclusively to endosomes, its functions in myddosome regulation are lost, but its functions in promoting TRIF signaling are retained (Fig. 4d). Conversely, if TRAM is localized exclusively to PI(4,5)P₂-rich regions of the plasma membrane, its functions in TRIF signaling are lost but its functions in myddosome regulation are retained (Fig. 4f). Thus, we propose that TRAM is the most receptor proximal factor that coordinates the two pathways activated by TLR4. These functions also apply to macrophage encounters with the diverse bacterial infections we have performed in this revision (Extended Data Fig. 5). We now offer a graphic model describing these activities (Fig. 6f), to ensure a clear view of these mechanisms by the community.

Referee #3:

We thank this referee for their very helpful and insightful comments. The issues raised were helpful and we hope our replies have addressed the concerns raised. Our response to each comment is listed below in **Bold Font**.

In the manuscript by Fisch et al., the authors present several compelling and fundamental new discoveries in TLR signalling. Building on their previous significant study defining proto- and mature myddosome complexes (Fisch et al., Nature 2024), the authors make the surprising discovery that the adaptor, TRAM is a critical factor for TLR4 and TLR 2 myddosome release into the cytosol for maturation and sustained signalling for maximal cytokine secretion. Firstly, using an CRSIPR whole genome screen in combination with an innovative flow cytometry-based assay, TICAM-2, the gene for TRAM was identified as a key component for both TLR2 and TLR4 myddosome assembly. Using a variety of approaches the authors go on to demonstrate that TRAM is required for proto-myddosomes to be released from the receptor complex at the cell surface into the cytosol for more signalling competence and secondary response genes including ISGs. Mechanistically, the authors show that TRAM-TIR can bind to directly to MyD88-TIR and further that TRAM outcompetes TIRAP for PIP2 binding at the cell membrane to bind MyD88 and release proto-myddosomes into the cytosol. The authors elegantly demonstrate that MyD88 interacts first with TIRAP then with both TIRAP and TRAM before exclusively with TRAM. If TIRAP is forced to endure, myddosomes are unable to mature in the cytosol. Finally, the authors utilise MyD88-TIR inhibitors and show both in vitro and in vivo that when MyD88-TIRs are inhibited after stimulation for 30-60 mins with LTA/LPS, early responses persist, sustained signalling and later cytokine responses are lost/reduced. This in vivo analysis included impressive endogenous MyD88 IPs of splenocytes from treated mice that showed a both the formation of myddosomes in vivo and the importance of their maturation (longer lasting myddosomes) for normal cytokine responses.

Overall, this manuscript was extremely well executed building a clear and exciting narrative. The authors present a substantial body of thorough and well-executed data. Both the quality and innovation of the experiments performed in this manuscript were of the highest order making the findings highly convincing. Appropriate controls were presented in many experiments to provide further confidence in the results. The significant finding that TRAM is required for myddosome maturation, including in an in vivo setting makes the study highly novel and impactful. It provides a new model for the key roles of TIRAP and TRAM required for maximal TLR2/4 immune responses. Given the fundamental importance of TLR for innate immune responses this work will be of broad interest to immunologists, cell biologists, clinicians and the general readers of Nature.

The authors should address the following minor comments:

1. Main text– line 38: Author should also include DAMPs as activators of TLRs.

We have updated the text as requested.

2. Figure 1a-c– line 74: What proportion of the LPS bulk cells are responders vs bystanders? Are the responders much higher??

Yes, the LPS responsive cells are much higher proportion than non-responders. LPS is such a strong TLR agonist that concentrations as low as 1 ng/mL induce myddosome formation in the majority of cells (Fisch et al., *Nature* 2024; Kellogg et al., *Elife* 2015). At the LPS concentration used in these experiments (10 ng/mL) we typically observe ~70% of cells containing myddosomes. We have updated the text with this information.

3. Figure 1b– line 79: Can the authors suggest why some cells (SSC^{lo} bystanders) don't form active myddosomes?

This is a very interesting question. We are intrigued to report in this revision that the SSC^{lo} bystanders express lower *Ticam2* mRNA (by RNA-Seq) than SSC^{hi} responders (Fig. 1c and Supplementary Fig. 1c). Immunoblot analysis after cell sorting of these populations demonstrated that this trend translates to the protein level (Fig. 1f). The low level of TRAM expression results in a higher TIRAP:TRAM ratio in bystander cells, which do not assemble myddosomes efficiently. This conclusion correlates with the data reported in our first submission of this manuscript, where we found that intentionally (through CRISPRa) increasing the TIRAP:TRAM ratio diminished myddosome assembly (Fig. 5a-c & Extended Data Fig. 8). Based on these data, and our prior work showing that MyD88 expression can vary at the single cell level (with functional consequences; Fisch et al., *Nature* 2024), we propose that heterogeneous TRAM expression levels within a cell population is a means by which signaling efficiency can be controlled.

4. Figure 1c– line 82-84: Should this be reworded to state that both responders and bystanders exhibited ISG signature...?

We thank this referee for suggesting this adjustment. We have updated the text to incorporate this suggestion.

5. Figure 1d– line 87: In the same experimental setup the authors should show bystanders don't respond to IFNs = no ISGs (e.g. surface ISGs like Siglec-1, *Bst2*).

We thank the referee for this great suggestion. We have repeated the experiments to analyze cell surface expression of Tetherin (*Bst2*). As predicted by this referee, when treated with Brefeldin A, the bystanders did not display an increase in Tetherin surface levels (Fig. 1d). In the absence of Brefeldin A, we observed robust increases in Tetherin surface levels in responders and bystanders alike, indicating an intact IFN-response in all cells and corroborating our RNA-Seq analysis.

6. Figure 1f– line 122: It appears that rather than lacking cytosolic myddosomes, TRAM KO cells have significantly reduced numbers. I know that numbers and cell surface vs cytosolic myddosomes are quantified at a later point but if these quantifications are presented here, it strengthens the conclusions drawn from this data.

We thank this referee for this helpful observation. We agree that the images in Fig. 1f (now Fig. 1g) show that TRAM-deficient cells are not *completely* devoid of cytosolic myddosomes, but rather exhibit a marked reduction in their abundance, with the vast majority of assemblies remaining associated with the plasma membrane (>90%). To better support this interpretation and as suggested here, we have moved the relevant quantification of MyD88 localization from Fig. 2 to Fig. 1h-i, where these imaging data were first presented. This change should allow the reader to directly assess the relative distribution of MyD88 and strengthens the conclusion that TRAM deficiency nearly abolishes the process of myddosome maturation.

7. Extended data Fig. 3: Rather than just using P-p38, inclusion of phosphorylated-IRAK4 in WCLs as readout for early/proximal myddosome activation would add value to this impressive data set.

We thank the reviewer for this suggestion. We agree that p-IRAK4 is a useful proximal readout of myddosome activation and could, in principle, complement the p-p38 measurements shown in Extended Data Fig. 3a-b. However, we respectfully note that our current dataset in this manuscript already includes multiple orthogonal readouts spanning proximal signaling events and downstream functional outputs,

which together support the conclusions drawn from our experiments. In this context, adding p-IRAK4 immunoblotting would not provide substantive new mechanistic insight that would change interpretations of the data. It may also be helpful to note that our previous study explored the mechanistic importance of p-IRAK4 in myddosome activities (Fisch *et al.*, *Nature* 2024). While IRAK4 protein was absolutely required for myddosome assembly and activities, IRAK4 phosphorylation was of minimal importance. Given this, and in light of the other experimental additions included in the revision, we have not incorporated p-IRAK4 measurements in the revised manuscript.

8. Figure 2e: The progression of the times for each sample is not obvious (0, 0.2, 0.5, 1...12 h). All the lanes should be labelled (as not stated in legend) and to accommodate they could be slightly reduced in size.

We have updated the labels in Fig. 2e (now Fig. 2b) as requested and also made the same improvement in Extended Data Fig. 10f.

9. Extended data Fig. 3d: The authors show p62 (autophagy regulator) associates with myddosomes at post stimulation (3 h), which is accelerated in TRAM KO cells (2 h) leading to faster clearance of signalling molecule interactions. In the previous work published by this group, the interactions with p62 appeared more delayed (6-12 h). Please explain this difference.

This referee is correct in the statement that we did not observe robust p62 incorporation into myddosomes at 3 hrs post LPS stimulation in our previous work (Fisch *et al.*, *Nature* 2024; Extended Data Fig. 7a). We believe this is due to exposure times of immunoblot membranes, as we previously included samples from a 12 hr time point when the p62 band intensity was much higher (leading to shortened exposure times and no detection of fainter signals). We would like to note that the association of p62 at 3 hrs post LTA or LPS stimulation of WT cells in this work (Extended data Fig. 3d) was barely detectable and only became robust at ≥ 6 hrs.

10. Figure 3: Using the primary BMDMs from WT and TRAM KO mice, although unlikely, it would be valuable to also examine the stimulation of other TLR pathways that use MyD88 (e.g. TLR5, TLR9, TLR7) to see if there is any impact on TNF and IL-6 with TRAM deficiency. Do other TLRs need TRAM for myddosome release/maturation and cytokine secretion?

We thank this referee for this suggestion. We agree it is important to determine if the phenotype observed in TRAM-deficient cells reflects a specific role in TLR2/TLR4 signaling or a broader defect in innate immune signaling pathways. To address this point, we have expanded our analysis in primary BMDMs and iBMDMs using a panel of innate immune stimuli (Supplementary Fig. 4). In addition to TLR2 and TLR4 agonists, we examined cytokine production induced by endosomal TLRs (R848, CpG), TLR3 to assess TRIF-specific signaling (poly(I:C)), cytosolic PRRs engaging the STING and RIG-I/MAVS pathways (DMXAA and Sendai virus, respectively), and IL-33 as a MyD88-dependent, non-TLR member of the IL-1 receptor family.

These experiments confirmed the literature on the established role of TRAM in TLR4-TRIF signaling, as LPS-induced IFN β and IP-10 production were lost in the absence of TRAM (Fig. 4d+f & Supplementary Fig. 4). Important to this question of specificity, we found that TRAM was not required for STING, RIG-I or IL-33 induced cytokine secretion (Supplementary Fig. 4). TRAM was also not required for TLR3-induced IFN β secretion (Supplementary Fig. 4).

In examining MyD88-dependent TLRs, we found that the severity of the cytokine production defects associated with TRAM deficiency was highest for plasma membrane localized TLRs, as compared to the endosome-localized TLR7-9 (Supplementary Fig. 4). Similar results have been reported for the role of TIRAP in TLR7-9 signaling, where cytokine-specific (and ligand-specific) effects of TIRAP deficiency are known (Bonham *et al.*, *Cell* 2014). Interestingly, regardless of the receptor agonized, ligands that displayed a dependence on TRAM for signaling were most defective for production of the SRG products IL-6 and IL-12p40. TNF α production was less consistently affected by TRAM deficiency, in line with its classification as a primary response gene that can be induced by transient signaling (Supplementary Fig. 4).

These collective findings demonstrate that TRAM is not required for non-TLR innate immune responses and support a model in which TRAM functions as a regulator of signaling downstream of TIRAP- and MyD88-dependent TLR pathways, in addition to its established role in the TLR4-TRIF-pathway.

To explain how TRAM could function in MyD88 and TRIF pathways downstream from TLR4, we include in this revision an analysis of TRIF-dependent IFN β secretion (Fig. 4d+f & Extended Data Fig. 7g). We altered the localization of TRAM in iBMDMs by expressing mutant TRAM alleles that were either localized to the plasma membrane, endosomes or cytosol. As expected from prior work, initially by Fitzgerald and colleagues, cytosolic TRAM mutants were completely defective for cytokine and IFN secretion (Fig. 4d). Interestingly, we found that endosomal TRAM mutants (e.g. TRAM 3X) retained the ability to activate the TLR4-TRIF pathway and induce IFN secretion (Fig. 4d) but were defective for the induction of MyD88-dependent cytokines TNF α and IL-6 (Fig. 4d). Conversely, TRAM mutants that were specifically localized to PI(4,5)P₂-rich plasma membrane subdomains supported MyD88-dependent cytokine production but were poor inducers of TRIF-dependent IFNs (Fig. 4f). These signaling activities tracked with the ability of each TRAM mutant to assemble myddosomes and detach proto-myddosomes from the plasma membrane (Fig. 4b+e). These findings suggest an advanced biological model of the function of TRAM. Plasma membrane localized TRAM (and specifically TRAM within PI(4,5)P₂-rich subdomains) functions to drive myddosome assembly and cytokine expression whereas endosomal TRAM supports TRIF signaling and IFN production. These findings help explain prior observations that TRAM is localized to the plasma membrane and endosomes, as we can now conclude that localization to both compartments is important for signaling processes associated with activation of TLR4.

Importantly, we do not observe obvious changes in TRAM localization during signaling (Extended Data Fig. 7c), suggesting that TRAM is not globally redistributed upon receptor activation, as is the case for CD14 and TLR4. Instead, we propose that TRAM is pre-positioned at the sites of TLR signaling, akin to what is known for TIRAP.

11. Figure 4h: In the imaging data can the authors make the images larger – they are a little hard to make see without extreme zooming in. Also, what does the concentration indicate? PH dye? I don't think this was stated anywhere.

We apologize that these images were too small to see comfortably. We have re-organized the figure and enlarged the images as requested. The labels did not indicate concentrations but z-positions from 3D-imaging data. We have clarified this point in the figure legend.

12. Extended data Fig. 9a– line 421: Authors state “LTA-triggered low-level IP-10 production was also reduced by MPP or TSI-13-57 pre-treatments.” Why is this the case when LPS IP-10 was unaffected?

This is an interesting point, as the question of how TLR2 induces IP-10 has been ambiguous in the literature. It has been reported in different studies that MyD88 (Stack *et al.*, *J Immunol.* 2014) or TRIF (Nilsen *et al.*, *J Biol Chem* 2015) control IP-10 expression in the TLR2 pathway. Regardless of mechanism of signaling, there is consensus that the amount produced is far less than what is observed for TLR4. For example, the amount of IP-10 produced by TLR4 exceeds that induced by TLR2 by >15-fold (Extended Data Fig. 10a). This finding confirms the original work by Vogel and colleagues on the selective ability of TLR4 (compared to TLR2) to induce IP-10 and other IFN associated factors (Toshchakov *et al.*, *Nat Immunol.* 2002).

In the case of TLR4, IP-10 is well-verified as a TRIF-dependent ISG. In considering this prior work with ours, one could expect that the MyD88 inhibitors should not affect LPS-induced IP-10 production, which is what we report here (Extended Data Fig. 10a). In the case of LTA induced low-level IP-10 production, our finding that this process is prevented by MPP and TSI-13-57 supports the central role of MyD88 in TLR2 signaling. We have added this information to the manuscript.

13. Figure 6f: Is it possible to also examine TIRAP and TRAM in these IPs? If possible, it would be informative. This is a great suggestion, and we attempted to detect TIRAP and TRAM. However, due to the complexity of the *in vivo* system (minimum of 30 minutes is required to process samples collected from these mice) we were not able to record an informative immunoblot for TIRAP and TRAM following immunoprecipitation of MyD88. As TIRAP and TRAM act very early in the myddosome pathway, and are

lost rapidly from the complex, we expect that new methods will need to be developed to track these very early times of TLR signaling where TIRAP and TRAM participate. At present, it appears that our *in vivo* assay is best-suited to track stable long lasting myddosome components.

14. Discussion: Can the authors present a cartoon figure (supp) to summarise their new model of TRAM-mediated myddosome release and maturation.

We have generated a cartoon summarizing our findings which has been added as Fig. 6f.

15. Conclusion— line 510-513: The authors reframe “TLR signalling” as a 2-step process. However, is this true for all myddosome forming TLRs? or just TLR4 and TLR2? Other TLRs (see point 10 above), like endosomal TLR9, are not thought to use TRAM so how are the myddosomes from these TLRs released? Don't they require released into the cytosol for efficient cytokine production or is it only when TIRAP is involved? This is a fundamental part of the finding, and it is currently unclear if this mechanism is specific to TLR2 and TLR4 so it should be addressed. If it is specific, this should be made clear, including in the title perhaps.

We thank the reviewer for this important point and agree that the scope of our proposed model should be more clearly defined. The additional experiments performed in response to referee comments were quite informative. In addition to being dispensable for STING-, RIG-I/MAVS-, TLR3-, and IL-33-signaling (see reply to query 10 above), our data identify a broader role for TRAM in a subset of MyD88-dependent pathways. Specifically, we found that TLR2-, TLR4-, TLR7/8-, and TLR9-induced production of SRG products, such as IL-6, was more dependent on TRAM than the production of PRG products, such as TNF α . Yet, we also note that endosomal MyD88-dependent TLRs displayed a less severe dependence on TRAM in general. Similar findings were reported previously for TIRAP (Bonham *et al*, *Cell*, 2014).

Based on these data, we have revised the text to clarify the two-step myddosome maturation model. We highlight that the TIRAP-TRAM competition controlling MyD88 is particularly important for plasma membrane TLRs, and to a lesser extent for endosomal TLRs.

Below is our reply to the instructions by Dr. Weiss to address the last query from referee 2.

Referee 2 comment: *I don't agree with the authors that the in vitro study be retained. As previously stated, it is likely to generate artefacts and distract from the main message of the study. 'Added language' does not address the point and I can't find that language anyway.*

In response, we have removed the specific experiments in question, which used luciferase-based assays to screen for TIR domain interactions *in vitro*.