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Peer Review File

CD300ld on pathologically activated neutrophils promotes tumour immune suppression via binding phosphatidylserine on CD8+ T cells

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Parts of this Peer Review File have been redacted as indicated to remove third-party material.

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)
Wang and colleagues presented the results demonstrating that CD300ld mediates neutrophil-driven contact-dependent suppression of cytotoxic CD8+ 10 T cells via binding to phosphatidylserine (PS). Mice with mutant CD300ld, which lack PS-binding capacity, exhibit reduced immunosuppressive activity, phenocopying CD300ld knockout mice in multiple tumor models. Blockade of the CD300ld-PS interaction by neutralizing antibodies demonstrate therapeutic efficacy against established tumors and synergize with anti-PD-1 therapy. Clinical analyses reveal elevated CD300LD expression inversely associates with CD8+ T cell infiltration and predicts poor responses to checkpoint blockade therapy.
Authors presented comprehensive analysis of interaction of CD300ld with PS and implicate it in PMN-MDSC suppressive activity. They generated several new mouse models to support their conclusions. The study is based on recently published observation that CD300ld mediated suppressive activity of PMN-MDSC. Now, they are focused on identification of binding partner-ligand that would mediate the effect.
Although interesting, this study generates many biological questions, and it seems over interpreted. Biological significance of the findings is questionable.
Major concerns.
- Authors previously have previously demonstrated that genetic deletion of CD300dl greatly weakened the suppressive function of PMN-MDSC. This effect was previously mediated via signaling to PMN-MDSC through STAT3-S100A8/A9 axis. Now they report that PS does not mediate this effect and is responsible for interaction with PShigh CD8+ T cells. The question what role the original observations have in current concept of PS mediated effect? Does it suggests that there is another potent mechanism not associated with PS;
- PS is widely expressed on all apoptotic cells, but reported to be very low in non-apoptotic cells. However, authors reported that 35% of non-apoptotic T cells express PS. Was something special about their staining? Is there a clear difference in the level of CD8+PS high cells between tumor tissues and spleen or BM to justify the selectivity of targeting. These data are missing.
- Authors argue that activated T cells express PS. It is reasonable. The problem is that they used potent non-physiological activation of T cells. Potent T-cell activation is not a feature of TME. How these observations are linked to biology remained unclear. Authors demonstrated that the effect is very dependent on OT1 T cells expressing high level of PS. Strong activation of T cells is required to achieve rather modest proportion of PShigh T cells. What will be the biological significance of these results? What was the representation of PShigh T cells in tumors?
- The difference between WT and KO mice in CD8PShi cells is rather small. Would it create sufficient window to avoid signal;
- In many in vivo experiments it seems that tumor growth slopes in WT and mutant mice were similar. It could represent just a delay in tumor progression rather than true antitumor effect. It often happens in genetically modified mice. Partially supporting this concern are the data in Figure 2j in GEMM model, where difference in survival are minimal (albeit statistically significant;
- Similarly antitumor effect of antibody in Figure 6l is very small and have the same issues as mentioned above;
- The phagocytosis part of the story is questionable. Contrary to authors claim, spontaneous apoptosis of tumor cells is not prevalent and often is minimal. To appropriately assess the effect, authors would need to induce apoptosis in tumor cells

and then compare phagocytosis between WT and KO neutrophils;
- On the same subject, why authors did not use apoptosis induced therapy in combination with their therapy to support their conclusion since PS is the main focus of their study;

Specific questions/concerns.
- Figure 1. Since I am not an expert in the field of computational screening, I stipulate that all data obtained are 100% accurate. The question is for Figure 1g. Authors showed quite strong binding of PS liposome to neutrophils with deleted CD300ld suggesting independent binding. It would be useful if authors comment on possible functional consequences of this binding.
- Figure 2. Authors data and interpretations of Figure 2a and Extended Figure 3 raise questions. Authors observed the effect of PS targeting on tumor growth in WT mice. The effect of PS targeting in vivo could be the result of various activities of PS. In support of the specific role of Cd300ld they used KO mice. However, in KO mice tumor growth was substantially reduced. Addition of Annexin V did not further enhance it. Authors concluded that this is because CD300ld mediate effect of PS. In fact, tumor growth in that group was the same as treatment with Annexin V in WT mice. There is simply no room to enhance further the effect of treatment.
- The experiments with liposomes in Figure 3k demonstrated only partial decrease of PS liposome binding to mutant CD200ld.

Reviewer #2

(Remarks to the Author)
This is a well-executed study that builds upon the authors' previous work published in Nature (2023 Sep; 621(7980):830–839), which identified CD300ld on neutrophils as a critical immune suppressor contributing to tumor immune resistance. In the current study, the authors further elucidate a previously unrecognized mechanism by which neutrophils suppress cytotoxic $CD8^+$ T cell activity through direct physical contact mediated by CD300ld binding to phosphatidylserine (PS). Using a combination of structural modeling, in vitro lipid-binding assays, in vivo tumor models, and sortase A–mediated proximity labeling, the study demonstrates the functional importance of CD300ld–PS interactions in immune suppression. The authors also present a CD300ld-blocking antibody with therapeutic potential and show that CD300LD expression correlates with poor response to immune checkpoint blockade (ICB) across multiple human tumors. This work offers valuable mechanistic and translational insights into the role of the CD300ld–PS axis in cancer immune evasion.

1. The labeling for Figure 1c is unclear and potentially misleading. It appears that CD300ld-ECD-Fc and the Fc control were applied to separate blots, each probed with a different subset of lipids (e.g., one with TAG, PA, etc., and the other with cholesterol, PIP3, etc.). However, for proper comparison, both CD300ld-ECD-Fc and Fc control should be tested side-by-side on the same blot containing the full panel of lipids. If that was indeed the case, the figure labeling should be revised to clarify this experimental setup.

2. The apparent size of the 50% PS liposome pulldown bands vary considerably across multiple figures, including Figure 1d, 1j, Extended Data Figure 2b, and Figure 6a. Please explain and provide more detailed information regarding all the liposome pulldown assays in terms of experimental conditions and systems.

3. What is the KO efficacy of the CD300ld KO mice? Fig 1g shows that neutrophils from CD300ld KO mice still exhibit substantial PS-binding activity, approximately half that of WT neutrophils. It raises questions about the completeness of the knockout or the presence of compensatory PS-binding receptors. It seems that other receptors on neutrophils may contribute to PS recognition and complicate the interpretation of CD300ld-PS–specific effects.

4. While the authors convincingly demonstrate the immunosuppressive role of CD300ld using total knockout (KO) mice across several figures (e.g., Fig. 2, 5, and 6), it is important to note that CD300ld is not restricted to neutrophils. CD300ld is also expressed on other myeloid populations such as monocytes and macrophages. To more precisely attribute the observed effects to neutrophil-specific CD300ld activity, the use of conditional KO models (e.g., S100A8-Cre or Ly6G-Cre) would be more appropriate and mechanistically informative.

5. Additionally, CD300ld-ECD-Fc treatment and CD300ld blocking antibodies may exert effects beyond neutrophils. In Figures 2g, 5g, and 6g, the impact of CD300ld mutant or CD300ld antibodies on other tumor-infiltrating immune cells—particularly macrophages and monocytes—is not addressed. It would be valuable for the authors to provide flow cytometry or transcriptomic analyses to assess whether these other myeloid cells are also affected by CD300ld functional loss or blockade. This would clarify whether the therapeutic effects are neutrophil-specific or involve broader modulation of the myeloid compartment within the tumor microenvironment.

6. In Figures 6j, 6k, and 6l, the authors analyze CD300LD expression in human cancer datasets to establish its association with PShigh $CD8^+$ T cell infiltration and immune checkpoint blockade response. However, it is unclear whether the CD300LD expression analyzed reflects neutrophil-specific expression or total expression across all cell types. As indicated in Extended Data Figure 4e, CD300ld may also be expressed on cancer cells and $CD8^+$ T cells, raising the possibility that the bulk expression data may not be neutrophil-specific.

7. How abundant are neutrophils in the tumor models used in this study, and what proportion of them express CD300ld? More importantly, do $CD300ld^+$ neutrophils physically exclude or sequester $CD8^+$ T cells within spatially restricted regions

of the tumor microenvironment (TME)? Addressing this question would strengthen the mechanistic impact of CD300ld-mediated suppression. The authors are encouraged to include multiplex co-staining (e.g., Ly6G, CD8, CD300ld) or spatial transcriptomic data to support the spatial proximity and suppressive positioning of CD300ld$^+$ PMN-MDSCs relative to cytotoxic T cells.

8. Most therapeutic interventions in the study are assessed over relatively short timelines. For greater clinical relevance, it is important to evaluate whether CD300ld blockade induces durable tumor control or long-term immune memory. The inclusion of extended treatment models that allow for full tumor regression, as well as rechallenge experiments, would provide important insight into whether the antitumor immunity elicited by CD300ld blockade is sustained and therapeutically meaningful. For example, using well-characterized, ICB-responsive models such as AP1 or Renca could help assess the durability and synergy of CD300ld blockade with established immunotherapies in a more clinically relevant setting.

9. The study identifies CD300ld as an immunosuppressive receptor, but the signals that drive its upregulation in neutrophils within the TME remain unclear. Are tumor-derived cytokines responsible for CD300ld induction? Elucidating upstream regulators may help inform a better target.

Reviewer #3

(Remarks to the Author)
The study highlights that CD300ld on neutrophils interacts with phosphatidylserine (PS) on CD8+ T cells, contributing to T cell suppression. Mice harboring a mutated form of CD300ld incapable of PS binding exhibit reduced T cell suppression, comparable to CD300ld-deficient mice. Therapeutic blockade of the CD300ld-PS interaction using novel Llama-derived nanobodies demonstrates efficacy in tumor treatment and enhances the effectiveness of anti-PD-1 therapy. Elevated CD300LD expression correlates with decreased T cell activity within tumors and diminished therapeutic responses. These findings suggest that the CD300ld-PS interaction is a mechanism by which neutrophils promote tumor immune escape, and targeting this interaction may improve immunotherapeutic outcomes.

The manuscript provides important insights into the mechanisms regulating the suppressive activity of neutrophils on T lymphocytes in the context of cancer, highlighting a previously unrecognized interaction between CD300ld and PS. The data are coherent, and the conclusions are well supported by carefully designed and rigorously executed experiments. Overall, the study represents a valuable contribution to our understanding of tumor-associated immune regulation. The following comments are intended to further improve the clarity and readability of the manuscript and to help the Authors better convey the significance of their findings.

The contextualization of the experiments using the Tmem30a-deficient melanoma cell line is not immediately clear. The observed overproduction of PS in knockout cells does not appear directly related to the intrinsic biological properties of the tumor. It should be clarified whether Tmem30a deficiency or downregulation commonly occurs in tumors and, if so, whether it correlates with increased aggressiveness and immune dysregulation. At the end of the experimental description, the Authors include the following statement, which seems out of context and only loosely related to the Tmem30a-deficient melanoma model: “Moreover, since CD300ld also binds to CD8$^+$ T cells, our findings suggest that PMN-MDSCs may suppress antitumor immunity not only through passive accumulation, but also via CD300ld-mediated contact-dependent interactions with CD8$^+$ T cells, contributing to localized immune suppression.”

Regarding Figure 3, the description of the cellular transfer is unclear. The Authors refer to mice receiving WT PMN-MDSCs (lane 258), but it is not related to the CD8+ T cell transfer that was actually performed. Furthermore, the substantial biotin labeling observed in splenic PMNs suggests a CD300ld-dependent cellular interaction in this organ. Does this imply that suppression occurs within secondary lymphoid organs as well? This interpretation would be consistent with Figure 3L, which shows an immune synapse-like structure between CD8$^+$ T cells and putative PMN-MDSCs in the spleen; however, this conclusion requires direct experimental validation. Additional questions should also be addressed: Are there PS-high CD8$^+$ T cells present in the spleen of tumor-bearing mice? According to the Authors’ model, these cells should represent the primary targets of CD300ld-expressing PMNs. Can normal neutrophils compete with PMN-MDSCs for binding to PS-high CD8$^+$ T cells?

Figure 1b is difficult to interpret. Is the length of the spikes proportional to the predicted binding affinity? If so, why were some lipids with high predicted affinity—such as PPA—not tested in the immunoblot analysis shown in Figure 1c?

Supplementary Figure 1g lacks quantification and statistical analysis.

Supplementary Figure 6d-e: The differences between plots are minimal, raising concerns about their biological relevance once additional samples and statistics are included. This also questions the sensitivity of the SortA approach in distinguishing immune synapse formation from simple cell-to-cell contact.

Please also correct the sentence beginning on line 243.

Extended Data Figures 8a-b are presented and discussed before Extended Data Figure 7 in the text; the order should be revised for consistency.

Figure 4e: The graphical representation of the trajectories should be improved to make starting and ending points and the

intermediate steps along each trajectory more clearly visible. Moreover, the color schemes used to indicate subsets in the UMAP plots (panels c and f) do not correspond to the clusters identified, making direct comparison difficult.

The statement that “myeloid-lymphocyte doublets had higher total gene counts” (Extended Data Figures 7e-f) requires clarification: higher than what? What is the biological significance of this observation?

Reviewer #4

(Remarks to the Author)
The authors identified phosphatidylserine (PS) as a ligand for CD300ld on myeloid-derived suppressor cells (PMN-MDSCs) and showed that PMN-MDSCs contact with cytotoxic PShighCD8+ T cells through the CD300ld-mediated recognition of PS and suppress PShighCD8+ T cell functions via ROS release, which impairs antitumor immunity during cancer progression. They extensively performed in vitro and in vivo experiments, appropriately analyzed statistically processed data, and drew a solid conclusion. The anti-tumor mechanisms described in this paper are novel, unique, interesting, important, and helpful for the researchers in the relevant field. However, several concerns need to be addressed.

1. In abstract, neutrophils to PMN-MDSCs are not distinguished. The similarities and differences between them in the expression and functions of CD300ld may be discussed.

2. There exist many receptors for PS, including CD300 family molecules, TIM family molecules, and so on. Other PS receptors on neutrophils/PMN-MDSCs may exhibit the same functions as CD300ld. Expression profiles of other PS receptors on neutrophils and PMN-MDSCs should be shown. This point should be discussed.

3. Do PShighCD4+ T cells and PShighNK cells exist? If so, can PMN-MDSCs interact with these cells to exert specific functions in the context of anti-tumor immunity?

4. It was previously reported by Comas-Casellas et al. (JBC, 2012) that human CD300d is not expressed on the surface of human myeloid cells. Did the authors examine the surface expression of human CD300ld on human neutrophils and OMN-MDSC?

5. Line 63: The references (regarding Extended Data Figure 1) are lacking.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
Authors provided extensive answers to all my questions and concerns. I am thankful for clarification of several points I raised and additional experiments performed to address the others. However, I did not find some responses clear or helpful.

"The experiments with liposomes in Figure 3k demonstrated only partial decrease of PS
liposome binding to mutant CD300ld." - I did not find the answer helpful since it suggests only partial specificity of the event.

"Authors data and interpretations of Figure 2a and Extended Figure 3 raise questions. Authors
observed the effect of PS targeting on tumor growth in WT mice. ... In support of the specific role of Cd300ld they used KO mice. However, in KO mice tumor growth was substantially reduced. Addition of Annexin V did not further enhance it" - Authors provided some general explanation that did not clarify the nature of concerns. Lack of the effect is lack of the effect. It may require different experimental set up or change of interpretation but explanation of the authors were not convincing.

I raised several comments regarding very small antitumor effect of the different treatment. I did not find clear answer or interpretation of the data to this concern. In contrast, by answering different question by using higher affinity antibody authors obtained much more convincing data. it suggests that their system is amendable for improvement in theory.

Finally, the main question I had is a link between a recent study published by this group and current data. Authors wrote in response "Previously, we reported that CD300ld genetic deletion diminishes PMN-MDSC suppressive function via the STAT3-S100A8/A9 axis, reflecting a cell-intrinsic regulatory mechanism. In contrast, our current study demonstrates that CD300ld on PMN-MDSCs mediates intercellular crosstalk with PS-high $CD8^+$ T cells through a direct physical interaction, representing a distinct extrinsic mechanism. Notably, our data show that CD300lddependent immunosuppression strongly requires cell-cell contact (Figure to reviewer 1a), and importantly, pharmacological inhibition of STAT3 or S100A9 does not disrupt the physical interaction between PMN-MDSCs and PS-high $CD8^+$ T cells (Figure to reviewer 1b). This indicates that the STAT3-S100A8/A9 axis and PS-mediated cell crosstalk operate through distinct pathways: the former drives PMN-MDSC intrinsic functionality, while the latter facilitates their targeting of effector T cells". Authors proposed existence of different ligand driving S100A8/A9-STAT3 distinct from PS: "we propose that CD300ld likely also binds additional as-yet-uncharacterized ligands that drive the STAT3-S100A8/A9-dependent intrinsic regulation described previously".

Now it is getting really complicated. In a nutshell, there are several totally independent mechanisms regulated by the same receptor. PS is responsible for binding, bit not CD300ld downstream signaling? What would prevent CD300ld to signal in that situation? Other ligands (not yet identified) target CD300ld and provide for signaling? Is it possible, yes. Is it likely, probably not since there very few examples of such phenomenon and it usually required formation of different receptor-ligand clusters. The concern is how much observed phenomenon is the result of artificial experimental conditions?

Reviewer #2

(Remarks to the Author)
The authors have added a substantial amount of new experiments, which largely addressed my previous comments. I do not have further questions or comments.

Reviewer #3

(Remarks to the Author)
No additional comments

Reviewer #4

(Remarks to the Author)
This reviewer's concerns were satisfactorily addressed.

Version 2:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
Authors addressed my questions. I have no more questions or concerns.

Referee #1 (Remarks to the Author):

Wang and colleagues presented the results demonstrating that CD300ld mediates neutrophil-driven contact-dependent suppression of cytotoxic CD8+ 10 T cells via binding to phosphatidylserine (PS). Mice with mutant CD300ld, which lack PS-binding capacity, exhibit reduced immunosuppressive activity, phenocopying CD300ld knockout mice in multiple tumor models. Blockade of the CD300ld-PS interaction by neutralizing antibodies demonstrate therapeutic efficacy against established tumors and synergize with anti-PD-1 therapy. Clinical analyses reveal elevated CD300LD expression inversely associates with CD8+ T cell infiltration and predicts poor responses to checkpoint blockade therapy.

Authors presented comprehensive analysis of interaction of CD300ld with PS and implicate it in PMN-MDSC suppressive activity. They generated several new mouse models to support their conclusions. The study is based on recently published observation that CD300ld mediated suppressive activity of PMN-MDSC. Now, they are focused on identification of binding partner-ligand that would mediate the effect.

Although interesting, this study generates many biological questions, and it seems over interpreted. Biological significance of the findings is questionable.

We thank Reviewer #1 for the time, care, and attention s/he has taken to review our study. We appreciate his/her positive feedback and insightful comments, to which we respond in detail, below.

1. Authors previously have previously demonstrated that genetic deletion of CD300dl greatly weakened the suppressive function of PMN-MDSC. This effect was previously mediated via signaling to PMN-MDSC through STAT3-S100A8/A9 axis. Now they report that PS does not mediate this effect and is responsible for interaction with PShigh CD8+ T cells. The question what role the original observations have in current concept of PS mediated effect? Does it suggests that there is another potent mechanism not associated with PS;

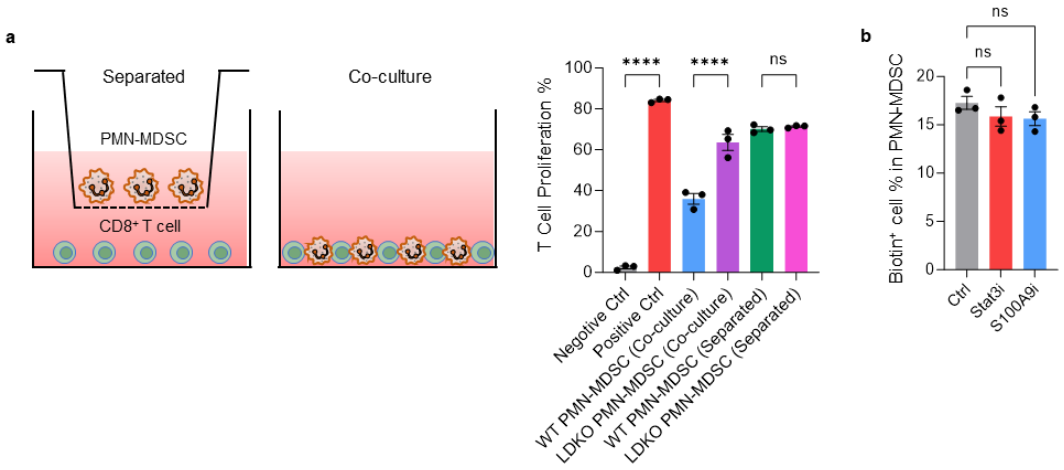
We sincerely appreciate the reviewer's insightful question, which leads us to clarify the mechanistic interplay between our current findings and prior observations.

A key clarification is that our recent work does not contradict the earlier characterization of the STAT3-S100A8/A9 axis instead, it expands the functional repertoire of CD300ld in immune regulation. Previously, we reported that CD300ld genetic deletion diminishes PMN-MDSC suppressive function via the STAT3-S100A8/A9 axis, reflecting a cell-intrinsic regulatory mechanism: CD300ld serves as a signaling receptor that modulates PMN-MDSC activation through intracellular pathways (e.g., STAT3 phosphorylation). In contrast, our current study demonstrates that CD300ld on PMN-MDSCs mediates intercellular crosstalk with PS-high $CD8^+$ T cells through a direct physical interaction, representing a distinct extrinsic mechanism. Notably, our data show that CD300ld-dependent immunosuppression strongly requires cell-cell contact (**Figure to reviewer 1a**), and importantly, pharmacological inhibition of STAT3 or S100A9 does not disrupt the physical interaction between PMN-MDSCs and PS-high $CD8^+$ T cells (**Figure to reviewer 1b**). This indicates that the STAT3-S100A8/A9 axis and PS-mediated cell crosstalk operate through distinct pathways: the former drives PMN-MDSC intrinsic functionality, while the latter facilitates their targeting of effector T cells.

While our current data highlight PS as a key ligand mediating $CD8^+$ T cell interactions, we propose that CD300ld likely also binds additional as-yet-uncharacterized ligands that drive the STAT3-S100A8/A9-dependent intrinsic regulation described previously. This concept aligns with the well-

documented multifunctionality of classic membrane proteins. For instance, TIM-3 engages in diverse interactions[1]: the interaction between Gal-9 and TIM-3 induces phosphorylation of the tyrosine motif within its intracellular domain, recruits the downstream tyrosine kinase Fyn, and ultimately leads to T cell functional exhaustion[2]; meanwhile, $CD8^+$ T cell-derived PS mediates cellular interactions between T cells and TIM-3-positive dendritic cells, thereby promoting antigen cross-presentation by DCs[3]. Identifying CD300ld's novel ligands and their downstream signaling pathways will be a critical focus of our future work, as it will further resolve the complexity of CD300ld's multifaceted role in immune suppression .

We have expanded the discussion section (line 587 to line 599) of our manuscript to explicitly address the potential for CD300ld to bind additional ligands beyond PS, integrating this perspective into our broader framework of CD300ld's functional versatility. We deeply appreciate the reviewer's critical insight, which has significantly enhanced the depth and clarity of our mechanistic interpretation.



For Reviewer Fig.1: a, WT or LDKO PMN-MDSCs were either co-cultured with or separated from T cells, and the suppression of $CD8^+$ T cell proliferation was quantified by flow cytometry. **b**, Flow cytometric analysis of $Biotin^+$ labeling in PMN-MDSCs pre-treated with Stat3i (WP1066, 5 μM) or S100A9i (Tasquinimod, 50 μM) after co-incubation with $SrtA^+$ $CD8^+$ T cells.

2. PS is widely expressed on all apoptotic cells, but reported to be very low in non-apoptotic cells. However, authors reported that 35% of non-apoptotic T cells express PS. Was something special about their staining? Is there a clear difference in the level of CD8+PS high cells between tumor tissues and spleen or BM to justify the selectivity of targeting. These data are missing.

We sincerely appreciate the reviewer's critical questions, which have helped us clarify key aspects of our study. Below, we address each point systematically:

Regarding PS expression in non-apoptotic $CD8^+$ T cells

The observation of PS expression on non-apoptotic T cells—including the 35% of non-apoptotic $CD8^+$ T cells reported in our study-does not stem from specialized staining protocols. Our PS staining protocol follows established methods widely used in the field. We utilized Annexin V combined with viability dyes (7-AAD) to distinguish PS exposure. Importantly, this phenomenon of PS upregulation on activated, non-apoptotic $CD8^+$ T cells is supported by a growing body of literature. Here are some studies (**For Reviewer Fig.2**) that have similarly documented physiological PS externalization on viable, activated T cells during immune responses, both *in vitro* (e.g., after

TCR stimulation[4,5]) and *in vivo* (e.g., within tumor microenvironments[6]). To directly address whether PS-high CD8⁺ T cells exhibit apoptotic propensity, we performed functional validation experiments. Tumor-infiltrating CD8⁺ T cells were sorted into PS-high and PS-low subsets. Sorted cells were cultured *ex vivo* for 48 hours, and cell viability was monitored. PS-high CD8⁺ T cells maintained comparable viability to PS-low counterparts throughout the culture period (**Extended Data Figure 6a**). These results confirm that PS-high CD8⁺ T cells in our model are functionally viable and distinct from apoptotic populations.

Differential PS expression on CD8⁺ T cells across tissues

To address the selectivity of PS-high CD8⁺ T cell targeting, we expanded our analysis to compare PS levels on CD8⁺ T cells across multiple organs (spleen, blood, bone marrow, lymph nodes and tumor tissues) in tumor-bearing mice. Tumor-infiltrating CD8⁺ T cells exhibited significantly higher PS positivity (**Extended Data Figure 6b**). This tissue-specific disparity, restricted to the tumor microenvironment, strongly supports the selectivity of PMN-MDSC-CD8⁺ T cell interactions. Given that PMN-MDSCs are enriched in tumor tissues and exert their immunosuppressive functions via cell-cell contact, the localized elevation of PS-high CD8⁺ T cells in tumors underscores the spatial specificity of this regulatory axis.

Figure Redacted

For Reviewer Fig.2: Key figures reported in previous studies showing the presence of PS signaling on activated CD8⁺ cells.

3. Authors argue that activated T cells express PS. It is reasonable. The problem is that they used potent non-physiological activation of T cells. Potent T-cell activation is not a feature of TME. How these observations are linked to biology remained unclear. Authors demonstrated that the effect is very dependent on OT1 T cells expressing high level of PS. Strong activation of T cells is required to achieve rather modest proportion of PShigh T cells. What will be the biological significance of these results? What was the representation of PShigh T cells in tumors?

We sincerely appreciate the reviewer's critical inquiry regarding the physiological relevance of PS expression in T cells and its connection to TME biology. Below, we address concerns and experimental validations to clarify the biological significance of our observations.

First, despite the immunosuppressive state in TME, we and others have confirmed that tumor-infiltrating CD8⁺ T cells exhibit elevated PS signals physiologically (**For Reviewer Fig.3a[6]**). Additionally, we observed that PS levels on CD8⁺ T cells serve as an indicator of the TME's immunosuppressive degree: CD8⁺ T cells display higher PS signals when the microenvironment is less immunosuppressive (**Revised Extended Data Figure 7k**). Mechanistically, PS upregulation on CD8⁺ T cells correlate with their cytotoxic activity: during target cell killing, perforin release transiently increases surface PS, which acts as a "self-protective shield" to limit autocrine perforin-mediated damage (**For Reviewer Fig.3b[7]**). This is functionally supported by our data showing that PS-high CD8⁺ T cells in TILs express significantly higher CD107a (a degranulation marker) than PS-low counterparts, directly linking PS to their effector cytotoxic state (**Revised Extended Data Figure**

6e).

Second, to address concerns about artificial activation, we extended our experiments to physiological conditions: we co-cultured SrtA immobilized PMN-MDSCs with tumor-infiltrating $CD8^+$ T cells (not artificially activated OT-1 cells) and Flow cytometric analysis revealed that CD300ld WT PMN-MDSCs preferentially bound to PS-high $CD8^+$ T cells, whereas CD300ld KO PMN-MDSCs showed minimal binding (**Revised Figure 3h, i**).

These data demonstrate that PS-high $CD8^+$ T cells are not artifacts of artificial activation but a physiologically relevant subset in the TME, where their PS expression reflects **cytotoxic activity** and mediates selective interactions with PMN-MDSCs. This bridges our experimental observations to TME biology, reinforcing the biological significance of our findings.

We deeply appreciate reviewer's critical insight, which has significantly strengthened the rigor of our mechanistic interpretation.

Figure Redacted

For Reviewer Fig.3: a, The correlation between CD107a and PS expression in PD-1^+ Tim-3^{hi} $CD8^+$ TILs. **b**, Perforin binding to phosphatidylserine on the CTL plasma membrane.

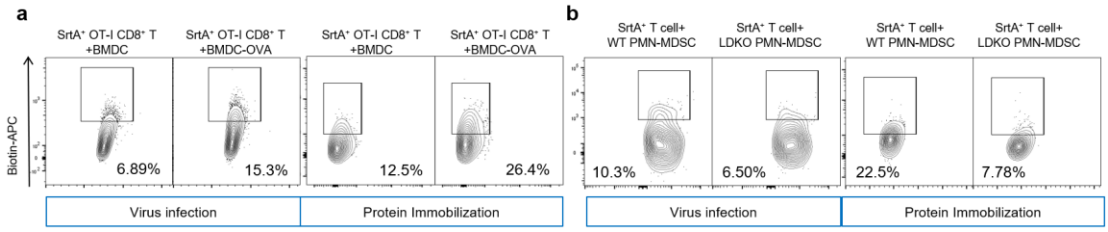
4. The difference between WT and KO mice in CD8PShi cells is rather small. Would it create sufficient window to avoid signal;

We sincerely appreciate the reviewer's critical observation regarding the relatively small difference in $CD8^+PS^+$ cells between WT and KO mice. This concern stems from our initial experimental approach, where we used viral infection to express SrtA on $CD8^+$ T cells. Viral infection efficiency is inherently variable, often resulting in suboptimal labeling of target cells and diluting the signal difference between genotypes. To address this limitation, we refined our methodology by employing chemical conjugation to covalently attach SrtA to the surface of $CD8^+$ T cells. This approach significantly improved labeling efficiency-whether assessing interactions between $CD8^+$ T cells and DCs (**For Reviewer Fig.4a**) or $CD8^+$ T cells and neutrophils (**For Reviewer Fig.4b**). Importantly, this optimized labeling system also reduced T cell proportion within the co-cultured cell mixture. By lowering the T cell density during $CD8^+$ T cell-neutrophil co-incubation, we effectively minimized interference from other potential receptor-ligand pairs (e.g., non-specific adhesion molecules or low-affinity interactions) that could mask the specific CD300ld-PS binding signal. With this reduced background noise, the difference in **interacting** $CD8^+$ T cells between WT and KO PMN-MDSCs became more pronounced under the improved conditions. (**Revised Figure 3d, e, f, Extended Data Figure 6i, j, k, Extended Data Figure 10i**).

Notably, while chemical conjugation demonstrated superior labeling efficiency *in vitro*, we observed that the SrtA enzyme signal diminishes over time, with detectable activity fading by approximately 24 hours post-modification. This limitation precludes its use in *in vivo* labeling experiments. Nevertheless, we believe the enhanced signal clarity and reproducibility achieved

with this optimized *in vitro* system-coupled with the consistent biological trend observed-provide compelling evidence to support our conclusion regarding the role of CD300ld-PS interactions in driving PMN-MDSC-mediated contact and suppression of PS-high $CD8^+$ T cells.

We thank the reviewer for prompting this methodological refinement, which has not only clarified the experimental results but also underscored the importance of rigorous tool optimization in capturing subtle but biologically meaningful differences.



For Reviewer Fig.4 a, Flow cytometry analysis of the Ratio of labeled bone marrow-derived DC (BMDC; Biotin+) after co-culture with virus infected or protein immobilized $SrtA^+$ $CD8^+$ T cells. **b**, Flow cytometry analysis of the Ratio of labeled PMN-MDSCs after co-culture with virus infected or protein immobilized SrtA+ CD8+ T cells.

5. In many in vivo experiments it seems that tumor growth slopes in WT and mutant mice were similar. It could represent just a delay in tumor progression rather than true antitumor effect. It often happens in genetically modified mice. Partially supporting this concern are the data in Figure 2j in GEMM model, where difference in survival are minimal (albeit statistically significant;

We sincerely appreciate the reviewer's critical observation regarding the tumor growth kinetics in our WT and CD300ld mutant mice, and we fully acknowledge your concern that delayed tumor progression may not equate to true antitumor efficacy-a nuanced challenge common in genetically modified models. We would like to clarify that CD300ld's antitumor role is not mediated by direct cytotoxicity toward tumor cells but rather by regulating the immune-suppressive function of PMN-MDSCs, key regulators of T cell immunity. By inhibiting PMN-MDSC-mediated suppression, CD300ld deficiency or mutant enhances the effector function of $CD8^+$ T cells, indirectly restricting tumor progression; this immune-modulatory mechanism, rather than direct tumor cell killing, underlies its effects.

Notably, the evaluated tumor models (B16-F10 melanoma, TC-1 lung carcinoma, and primary hepatocellular carcinoma, **Figure 2e, h, j, k**) are inherently immunologically "cold"-marked by low $CD8^+$ T cell infiltration, high immunosuppressive myeloid cell burdens, and poor immune activation responsiveness. In these stubbornly resistant microenvironments, where conventional immunotherapies rarely induce meaningful regression, suppressive signals dominate the immune landscape, offering little therapeutic opportunity. Against this backdrop, CD300ld's partial reversal of immune suppression (via PMN-MDSC regulation)-though manifesting only as a measurable delay in tumor progression-carries profound implications for tumor immunotherapy, underscores CD300ld's potential to prime "cold" tumors for future success.

In contrast, in the "hot" tumor model MC38 (colorectal adenocarcinoma, **Revised Figure 2i**) or Renca (renal cell carcinoma, **Revised Figure 5h**), which exhibits higher baseline $CD8^+$ T cell infiltration and greater susceptibility to immune modulation, CD300ld deficiency or blockade resulted in more pronounced tumor growth inhibition, aligning with our mechanism: relieving PMN-MDSC-mediated suppression in immunologically active microenvironments unmasks tumors to enhanced T cell attack, amplifying the antitumor effect. Furthermore, combination therapy with

anti-PD-1 antibodies significantly potentiated CD300ld's efficacy across multiple models (e.g., MC38, B16-F10), strongly supporting its role as an immune modulator.

We deeply thank the reviewer for prompting this detailed clarification.

6. Similarly antitumor effect of antibody in Figure 6I is very small and have the same issues as mentioned above;

We sincerely appreciate reviewer's observation regarding the limited antitumor effect of the humanized antibody shown in **initial Figure 6i**, we think the reduced efficacy of the humanized antibody compared to its murine counterpart primarily stems from its relatively lower affinity and blocking capacity for human CD300LD (3.323E-08 *vs* 7.184E-08), To address this, we adjusted the dosing from 10 mg/kg to 20 mg/kg (Method line 678 to line 682), which resulted in a marked enhancement of the antibody's antitumor activity (**Revised Figure 6i and Extended Data Figure 10k**).

Notably, while the humanized antibody demonstrated only modest efficacy at the initial dose, this result still serves a critical purpose: it validates the conceptual feasibility of targeting human CD300LD for antitumor therapy. That is, even with suboptimal affinity/blocking capacity, the antibody retains the ability to modulate CD300LD-dependent pathways when administered at higher doses, confirming that CD300LD is a viable therapeutic target in humans. We recognize that optimizing the affinity and blocking capacity of the humanized antibody is essential to maximize its clinical potential. Our subsequent efforts will focus on engineering next-generation variants with enhanced binding kinetics and potent CD300LD neutralization, which we anticipate will further improve therapeutic outcomes in both preclinical and clinical settings.

7. The phagocytosis part of the story is questionable. Contrary to authors claim, spontaneous apoptosis of tumor cells is not prevalent and often is minimal. To appropriately assess the effect, authors would need to induce apoptosis in tumor cells and then compare phagocytosis between WT and KO neutrophils;

We sincerely appreciate the reviewer's critical observation regarding the phagocytosis experiment, and we acknowledge that our initial description was insufficiently detailed, leading to potential misunderstanding. In fact, our phagocytosis assay utilized tumor cells induced to undergo apoptosis via camptothecin treatment-a standard approach to induce cell apoptosis. Details of this protocol and confirmation of apoptosis were explicitly included (**Revised Extended Data Figure 4f**). We regret that this critical detail was not emphasized sufficiently in the main text. We thank the reviewer for highlighting this oversight; it has prompted us to improve the clarity of our methods description.

8. On the same subject, why authors did not use apoptosis induced therapy in combination with their therapy to support their conclusion since PS is the main focus of their study;

We sincerely appreciate the reviewer's thoughtful question regarding the absence of apoptosis-induced therapy in our study, particularly given the central focus on PS in our work. Below, we clarify our rationale and connect it to the broader context of PS biology:

In our study, PS is characterized as a key immunosuppressive signal: tumor-derived PS recruits PMN-MDSCs, and PS exposed on activated $CD8^+$ T cells further enhances PMN-MDSC-mediated T cell suppression-a phenomenon consistent with prior reports that PS functions as an immunosuppressive signal in cancer[8]. Given this role, apoptosis-inducing therapies (e.g.,

chemotherapy or radiotherapy), which typically increase PS externalization on dying cells, might theoretically amplify tumor immune suppression by enriching PS signals. This could potentially render tumor-bearing hosts less responsive to immunotherapy.

Our study focused on elucidating the mechanism by which CD300ld on PMN-MDSCs binds PS to drive immune suppression, while apoptosis induction is a relevant clinical strategy, integrating it into our current experimental framework could divert focus from our primary research objective. We acknowledge the value of exploring this angle in future work, as it could directly inform clinical strategies to mitigate PS-driven immune suppression during apoptosis-inducing treatments. We thank the reviewer for raising this critical point-it has deepened our consideration of how PS biology intersects with standard therapies and highlighted potential avenues for extending our findings into translational contexts.

9. Since I am not an expert in the field of computational screening, I stipulate that all data obtained are 100% accurate. The question is for Figure 1g. Authors showed quite strong binding of PS liposome to neutrophils with deleted CD300ld suggesting independent binding. It would be useful if authors comment on possible functional consequences of this binding.

We sincerely appreciate the reviewer's critical question regarding the robust binding of PS liposomes to CD300LD-deficient neutrophils observed in Figure 1g. In our initial experiments, to achieve better staining, we incubated liposomes with neutrophils at room temperature under dark conditions. However, this approach inadvertently led to significant liposome internalization by the cells, resulting in elevated background signals. In revised experiments, all staining steps were performed at ice to suppress phagocytic activity (Method line 803 to line 805), ensuring observed PS binding reflected direct surface interactions rather than internalization. Under these conditions, we observed a significant reduction in PS liposome binding to CD300ld-KO neutrophils compared to WT controls, confirming CD300ld as a major mediator of PS binding in neutrophils (**Revised Figure 1g and Extended Data Figure 1f, g**). Notably, even with phagocytosis inhibited, CD300ld-KO neutrophils retained ~30% of WT-level binding, indicating residual PS recognition via CD300ld-independent mechanisms. To explore this, we analyzed transcriptomic sequencing[9] to compare expression of reported PS-binding proteins in neutrophils and PMN-MDSCs. While CD300ld is the most abundantly expressed PS-binding protein in PMN-MDSCs and the third in neutrophils, cells also expressed other PS-binding proteins, which may compensate for CD300ld loss to mediate the remaining binding (**Revised Extended Data Figure 1h**).

We thank the reviewer for raising this critical point, which strengthens our understanding of PS binding dynamics in the tumor microenvironment.

10. Authors data and interpretations of Figure 2a and Extended Figure 3 raise questions. Authors observed the effect of PS targeting on tumor growth in WT mice. The effect of PS targeting in vivo could be the result of various activities of PS. In support of the specific role of Cd300ld they used KO mice. However, in KO mice tumor growth was substantially reduced. Addition of Annexin V did not further enhance it. Authors concluded that this is because CD300ld mediate effect of PS. In fact, tumor growth in that group was the same as treatment with Annexin V in WT mice. There is simply no room to enhance further the effect of treatment.

We appreciate the reviewer's concern regarding the lack of additive effects between Annexin V and PS-targeting agents in CD300LD-KO mice. This experiment confirms that CD300LD primarily exerts its pro-tumor effects through its PS-binding capacity. In WT mice, PS-targeting agents

inhibited tumor growth, consistent with PS's immunomodulatory role. In CD300ld-KO mice, this signaling axis is disrupted: PS cannot engage its main receptor, severely impairing its ability to influence tumor-immune crosstalk. When we treated these mice with Annexin V, no further tumor growth reduction occurred. This lack of synergy confirms CD300ld's irreplaceability in PS-dependent tumor regulation. Another concern is why Annexin V does not exert its effects by blocking these other PS-binding proteins in CD300ld-KO mice. Two factors explain: First, CD300ld is the most abundantly expressed PS-binding protein on PMN-MDSCs. Second, other PS-binding proteins may be functionally irrelevant to maintaining tumor immune suppression; instead, they might primarily mediate phagocytic processes (e.g., clearing apoptotic cells or debris) rather than directly regulating immune activation or suppression. These data confirm CD300ld on neutrophils as the central mediator of PS-dependent tumor regulation, with alternative PS-binding proteins unable to compensate for its loss. We thank the reviewer for highlighting this, which strengthens our mechanistic interpretation.

11. The experiments with liposomes in Figure 3k demonstrated only partial decrease of PS liposome binding to mutant CD200ld.

We sincerely appreciate the reviewer's question regarding the partial reduction in PS liposome binding observed in CD300ld mutant neutrophils in **revised Extended Data Figure 3j**. We wish to emphasize that our binding assays directly validate the loss of PS-binding activity in CD300ld mutants (**Figure 1i, j**): both CD300ld-KO neutrophils and CD300ld mutant neutrophils (with disrupted PS-binding residues) exhibit identical phenotypes, including failure to recruit PMN-MDSCs to the tumor microenvironment and inability to suppress $CD8^+$ T cell activation. The partial PS liposome binding observed in CD300ld mutants aligns with our prior analysis of PS-binding protein expression, as neutrophils (and PMN-MDSCs) express low levels of other PS-binding proteins (e.g., CD300a, TIM-3) that, while less abundant than CD300ld, can mediate PS interactions in CD300ld-KO or mutant neutrophils. We thank the reviewer for raising this critical point.

Referee #2 (Remarks to the Author):

This is a well-executed study that builds upon the authors' previous work published in Nature (2023 Sep; 621(7980):830–839), which identified CD300ld on neutrophils as a critical immune suppressor contributing to tumor immune resistance. In the current study, the authors further elucidate a previously unrecognized mechanism by which neutrophils suppress cytotoxic $CD8^+$ T cell activity through direct physical contact mediated by CD300ld binding to phosphatidylserine (PS). Using a combination of structural modeling, in vitro lipid-binding assays, in vivo tumor models, and sortase A–mediated proximity labeling, the study demonstrates the functional importance of CD300ld–PS interactions in immune suppression. The authors also present a CD300ld-blocking antibody with therapeutic potential and show that CD300LD expression correlates with poor response to immune checkpoint blockade (ICB) across multiple human tumors. This work offers valuable mechanistic and translational insights into the role of the CD300ld–PS axis in cancer immune evasion.

We thank Reviewer #2 for the time, care, and attention s/he has taken to review our study. We appreciate his/her positive feedback and insightful comments, to which we respond in detail, below.

1. The labeling for Figure 1c is unclear and potentially misleading. It appears that CD300ld-ECD-Fc and the Fc control were applied to separate blots, each probed with a different subset of lipids (e.g., one with TAG, PA, etc., and the other with cholesterol, PIP3, etc.). However, for proper comparison, both CD300ld-ECD-Fc and Fc control should be tested side-by-side on the same blot containing the full panel of lipids. If that was indeed the case, the figure labeling should be revised to clarify this experimental setup.

We sincerely appreciate reviewer's careful review and constructive feedback regarding the labeling clarity of **initial Figure 1c**. We acknowledge that the initial figure annotation could be misleading, and we apologize for any confusion caused. To clarify, CD300ld-ECD-Fc and the Fc control were indeed incubated with the same lipid We coated the membrane with a full panel of lipids at consistent positions across replicate experiments. Both CD300ld-ECD-Fc and Fc control were then applied to the membrane in parallel, followed by synchronized protein incubation, antibody detection, and development steps to ensure strict comparability. We have revised the figure legend (**Revised Figure 1c**). We confirm that the data remain robust and the conclusions-regarding CD300ld-ECD-Fc's specific binding to PS over neutral lipids-are fully supported. We thank the reviewer for highlighting this critical point; it has helped us improve the clarity and rigor of our presentation.

2. The apparent size of the 50% PS liposome pulldown bands vary considerably across multiple figures, including Figure 1d, 1j, Extended Data Figure 2b, and Figure 6a. Please explain and provide more detailed information regarding all the liposome pulldown assays in terms of experimental conditions and systems.

We sincerely appreciate reviewer's careful observation regarding the variability in band sizes across the PS liposome pulldown assays in **initial Figures 1d**, **1j**, **Extended Data Figure 2b**, and **Figure 6a**. We apologize for the initial lack of detailed explanation, which may have caused confusion, and clarify the experimental context and conditions below. The primary reason for the

differences in band sizes is the use of full-length *vs* truncated forms of CD300ld-ECD-Fc across experiments: we employed the full-length mouse CD300ld-ECD-Fc fusion protein (~60 kDa) in **Figure 1d**, and a truncated form of mouse CD300ld-ECD-Fc (~50 kDa, generated by removing a C-terminal linker region) in **Figures 1j** and **Extended Data Figure 2b**. All pulldown assays, regardless of protein form, were performed under identical conditions to ensure comparability. To confirm truncation did not affect specificity, we repeated the assay with full-length mutant proteins and obtained consistent results (**Revised Figure 1j**). We have also added specific protein information to the figure legends. For **Figure 6a**, we used the full-length form of human CD300LD-ECD-Fc (~53 kDa). We deeply regret the initial lack of clarity and have revised the manuscript to explicitly note the protein forms used in each assay and their rationale, ensuring readers can better interpret the band size differences. We thank the reviewer for raising this critical point-it has strengthened the transparency of our methods.

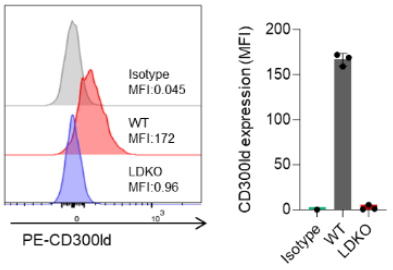
3. What is the KO efficacy of the CD300ld KO mice? Fig 1g shows that neutrophils from CD300ld KO mice still exhibit substantial PS-binding activity, approximately half that of WT neutrophils. It raises questions about the completeness of the knockout or the presence of compensatory PS-binding receptors. It seems that other receptors on neutrophils may contribute to PS recognition and complicate the interpretation of CD300ld-PS–specific effects.

We sincerely appreciate reviewer's critical inquiry regarding the residual PS-binding activity observed in CD300ld-KO neutrophils (**Initial Figure 1g**) and the implications for knockout completeness. We are pleased to clarify both the rigor of our CD300ld KO and the nuanced interpretation of these data. First, we confirm that the CD300ld KO was generated with complete efficiency (**For Reviewer Fig.5**) and this eliminates concerns about incomplete knockout as a confounding factor.

To clarify the residual PS-binding activity observed in CD300ld-KO neutrophils, we first refined our staining protocol. In initial experiments, to improve signal clarity, we incubated liposomes with neutrophils at room temperature. However, this approach inadvertently promoted significant liposome internalization by the cells, elevating background signals. In revised experiments, we performed all staining steps on ice to suppress phagocytic activity. Under these optimized conditions, we observed a marked reduction in PS liposome binding to CD300ld-KO neutrophils compared to WT controls (**Revised Figure 1g and Extended Data Figure 1g**). We attribute the remaining residual binding (~30%) to the presence of other PS-binding proteins on neutrophils. Transcriptomic analysis comparing PS-binding protein expression in neutrophils and PMN-MDSCs revealed that while CD300ld is the most abundant PS-binding protein in PMN-MDSCs (and the third most abundant in neutrophils), other reported PS-binding proteins (e.g., CD300a, TIM-3) are also expressed in both cell types (**Extended Data Figure 1h**). Though these proteins are less abundant than CD300ld, their collective contribution likely explains the residual PS binding in CD300ld-KO neutrophils-particularly under conditions of high PS availability.

Critically, even with this residual binding, our earlier functional data remain robust: the partial PS-binding activity in CD300ld-KO neutrophils is insufficient to rescue the pro-tumor phenotype driven by CD300ld loss (**Initial Figure 2a**). This aligns with our conclusion that CD300ld on neutrophil is the dominant effector of PS-dependent tumor-immune crosstalk. We thank the

reviewer for raising this important point, as it has strengthened our interpretation of PS-binding mechanisms.



For Reviewer Fig.5 Flow cytometric analysis (***left***) and quantification (***right***) of CD300ld expression on CD300ld WT or KO neutrophils.

4. While the authors convincingly demonstrate the immunosuppressive role of CD300ld using total knockout (KO) mice across several figures (e.g., Fig. 2, 5, and 6), it is important to note that CD300ld is not restricted to neutrophils. CD300ld is also expressed on other myeloid populations such as monocytes and macrophages. To more precisely attribute the observed effects to neutrophil-specific CD300ld activity, the use of conditional KO models (e.g., S100A8-Cre or Ly6G-Cre) would be more appropriate and mechanistically informative.

We sincerely appreciate reviewer's suggestion to use conditional KO models to more precisely attribute the observed effects to neutrophil-specific CD300ld activity. In fact, our prior published work has already addressed this by leveraging the S100A8-Cre model to selectively delete CD300ld in neutrophils. Notably, this study revealed that even with neutrophil-specific CD300ld deletion, tumor progression remained significantly slowed, mirroring the phenotype of global CD300ld-KO mice (**For Reviewer Fig.6a**). To further validate this, we repeated these experiments using S100A8-Cre to generate neutrophil-specific CD300ld conditional KO (cKO) mice and conducted tumor growth assays, which yielded phenotypes nearly identical to those observed in global CD300ld-KO mice (**Revised Extended Data Figure 3a, b**). Besides that, a neutrophil depletion experiments using Anti Ly6G antibodies also showed that eliminating neutrophils (regardless of CD300ld status) abrogated the differential tumor growth between WT and CD300ld-KO mice (**For Reviewer Fig.6b**). These results collectively indicate that CD300ld's immunosuppressive effects are primarily mediated by neutrophils/PMN-MDSCs, rather than other myeloid populations. Reviewer's suggestion reinforces the need to highlight this specificity, and we have updated the manuscript to more explicitly connect our current findings to the neutrophil-specific CD300ld activity established in our earlier work. We thank the reviewer for raising this critical point-it has helped us strengthen the mechanistic narrative and clarify the cellular basis of CD300ld's effects.

Figure Redacted

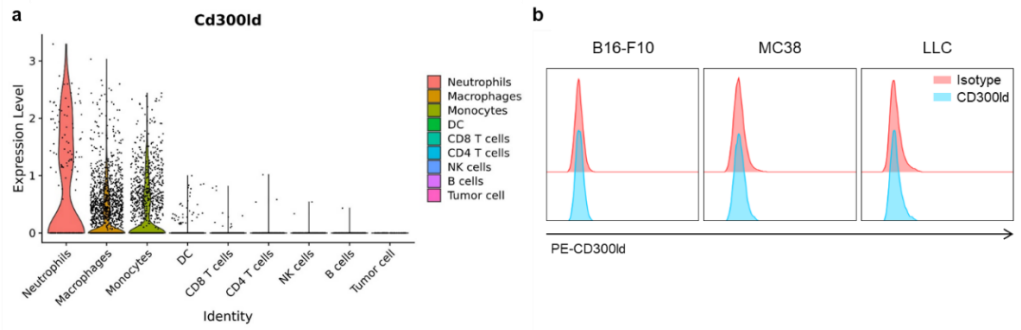
For Reviewer Fig.6 a, B16-F10 tumour growth curves in CD300ld$^{fl/fl}$S100a8^{cre} mice. **b**, CD300ld WT or KO B16-F10 tumor baering mice were treatment with anti-Ly6G or isotype control every other day. Tumour growth curves are shown.

5. Additionally, CD300ld-ECD-Fc treatment and CD300ld blocking antibodies may exert effects beyond neutrophils. In Figures 2g, 5g, and 6g, the impact of CD300ld mutant or CD300ld antibodies on other tumor-infiltrating immune cells—particularly macrophages and monocytes—is not addressed. It would be valuable for the authors to provide flow cytometry or transcriptomic analyses to assess whether these other myeloid cells are also affected by CD300ld functional loss or blockade. This would clarify whether the therapeutic effects are neutrophil-specific or involve broader modulation of the myeloid compartment within the tumor microenvironment.

We sincerely appreciate the reviewer's thoughtful suggestion to assess the impact of CD300ld functional loss or blockade on other tumor-infiltrating myeloid cells beyond neutrophils-an important consideration for clarifying the specificity of its therapeutic effects. In response, we have updated our manuscript to include flow cytometric analyses of key myeloid populations (monocytes, macrophages) and lymphoid populations (CD4$^+$ T cells) in the tumor microenvironment across relevant figures (**Initial Figures 2g, 5g, 6g**). These supplementary analyses reveal no significant differences in the frequencies of macrophages and monocytes between WT and CD300ld-KO/mutant mice (**Revised Extended Data Figure 3k and 10f**), or between CD300ld-ECD-Fc-treated and control groups (**Revised Extended Data Figure 3g**). The blocking antibodies used are consistent (**Revised Extended Data Figure 8g**). This aligns with our prior conclusion that CD300ld's immunomodulatory effects are primarily mediated by neutrophils/PMN-MDSCs, as other myeloid subsets show no detectable changes in response to CD300ld loss or blockade. We thank the reviewer for highlighting this critical point; it has strengthened our mechanistic narrative by confirming the neutrophil-specificity of CD300ld's role in tumor immunity. These updates are now included in the revised manuscript to provide a more comprehensive view of the cellular dynamics involved.

6. In Figures 6j, 6k, and 6l, the authors analyze CD300LD expression in human cancer datasets to establish its association with PShigh CD8$^+$ T cell infiltration and immune checkpoint blockade response. However, it is unclear whether the CD300LD expression analyzed reflects neutrophil-specific expression or total expression across all cell types. As indicated in Extended Data Figure 4e, CD300ld may also be expressed on cancer cells and CD8$^+$ T cells, raising the possibility that the bulk expression data may not be neutrophil-specific.

We sincerely appreciate reviewer's critical question regarding the specificity of CD300ld expression data in human cancer datasets (**Initial Figures 6j, l**) and the potential confounding factor of CD300LD expression in non-neutrophil cells. We hope the following elaboration on context and methodology will help clarify our approach and address your point: First, we note that **initial Extended Data Figure 4e** illustrates the binding of CD300ld-ECD (extracellular domain) to various cell types in the tumor microenvironment, not the endogenous expression of CD300ld. This distinction is critical: the figure demonstrates CD300ld's capacity to interact with multiple immune populations but does not reflect where CD300ld is expressed endogenously. Importantly, our scRNA-sequence (**For Reviewer Fig.7a**) and Flow cytometric analysis (**For Reviewer Fig.7b**) confirmed that CD300ld is not significantly expressed in tumor cells. Thus, the CD300ld expression levels reported in **Figures 6j, 6l** primarily reflect neutrophil-specific expression, with minimal contribution from non-neutrophil populations. We have revised the manuscript to clarify these methodological details in the figure legends, ensuring readers can confidently interpret the specificity of our dataset analyses.



For Reviewer Fig.7 a, Violin plots showing the expression of CD300ld in indiciated cluster**. b,** Flow cytometric analysis of CD300ld expression on indiciated tumor cells.

7. How abundant are neutrophils in the tumor models used in this study, and what proportion of them express CD300ld? More importantly, do CD300ld$^+$ neutrophils physically exclude or sequester CD8$^+$ T cells within spatially restricted regions of the tumor microenvironment (TME)? Addressing this question would strengthen the mechanistic impact of CD300ld-mediated suppression. The authors are encouraged to include multiplex co-staining (e.g., Ly6G, CD8, CD300ld) or spatial transcriptomic data to support the spatial proximity and suppressive positioning of CD300ld$^+$ PMN-MDSCs relative to cytotoxic T cells.

We sincerely appreciate reviewer's thoughtful questions regarding the abundance of neutrophils, CD300ld expression, and their spatial relationship with CD8$^+$ T cells in the tumor microenvironment-critical aspects for strengthening the mechanistic basis of CD300ld-mediated suppression. In the tumor models mainly used mainly (B16-F10 melanoma and MC38 colon carcinoma), neutrophils account for ~5-10% of total immune cells in B16-F10 tumors and ~30% in MC38 tumors, consistent with prior reports of neutrophil infiltration in solid tumors. Using scRNA-seq and Flow, we confirmed CD300ld is broadly expressed across neutrophil subsets[10] (**For Reviewer Fig.8a**), with higher expression in neutrophils enriched for immunosuppressive molecules (CD14)[9] (**For Reviewer Fig.8b**).

To investigate spatial proximity, we performed immunofluorescence staining on tumor sections (WT B16-F10 models) using antibodies against MPO (neutrophil marker), CD8 (T cell marker), and

CD300ld. We observed spatial overlap between CD300ld$^+$ PMN-MDSCs and CD8$^+$ T cells in regions (**Revised Figures 3n**). Critically, we further leveraged published spatial transcriptomic data to correlate CD300ld expression on neutrophils with the expression of CD8$^+$ T cell effector molecules (Gzmb, Prf1) in the tumor microenvironments. This analysis revealed a spatial negative correlation: regions with higher CD300ld$^+$ neutrophil density showed reduced Gzmb/Prf1 expression in adjacent CD8$^+$ T cells (**For Reviewer Fig.8c**). Although direct physical interactions between CD300ld$^+$ neutrophils and CD8$^+$ T cells were detected, we posit that such contacts are transient. As tumors advance, the sustained immunosuppressive activity of CD300ld$^+$ neutrophils likely contributes to the progressive exclusion of CD8$^+$ T cells from inflamed tumor regions. Notably, while we currently lack tools to continuously monitor this dynamic process, we are confident that future investigations into this area will yield significant insights. We extend our gratitude to the reviewer for raising these critical questions, which have greatly enhanced the rigor and clarity of our work.

Figure Redacted

For Reviewer Fig.8 a, UMAP shows CD300ld expression in most neutrophils; **b,** Representative flow cytometry histograms (*left*) and statistics (*right*) showing the expression of CD300ld in CD14$^-$, CD14^{mid} and CD14^{high} PMN-MDSCs. **c,** Spatial transcriptome analysis shows the correlation between CD300ld and Gzmb/Prf1.

8. Most therapeutic interventions in the study are assessed over relatively short timelines. For greater clinical relevance, it is important to evaluate whether CD300ld blockade induces durable tumor control or long-term immune memory. The inclusion of extended treatment models that allow for full tumor regression, as well as rechallenge experiments, would provide important insight into whether the antitumor immunity elicited by CD300ld blockade is sustained and therapeutically meaningful. For example, using well-characterized, ICB-responsive models such as AP1 or Renca could help assess the durability and synergy of CD300ld blockade with established immunotherapies in a more clinically relevant setting.

We sincerely appreciate the reviewer's thoughtful suggestion to evaluate the durability of CD300LD blockade's antitumor effects and assess long-term immune memory, which is critical for enhancing the clinical relevance of our findings. In response, we have expanded our analysis to include two well-characterized ICB-responsive models: Renca (renal cell carcinoma) and MC38 (colon adenocarcinoma)-the latter being particularly valuable for evaluating synergy with

established immunotherapies.

A key consideration in our experimental design was the genetic background of tumor models: Renca was originally isolated from a kidney tumor in a Balb/c mouse, whereas our KO and point mutation mice are on a C57BL/6 background. To address this, we instead used CD300ld-blocking antibodies in Renca-bearing Balb/c mice. Notably, in this setting, 4 out of 15 mice (27%) treated with CD300ld antibodies achieved complete tumor regression. When these tumor-free mice were rechallenged with fresh Renca cells, none developed new tumors, demonstrating robust and durable immune memory (**Revised Figures 5h, i**).

We further validated these findings in MC38-bearing mice, although CD300ld blockade alone exerted efficacy, combining it with anti-PD-1 antibodies led to striking results: 9 out of 15 mice (60%) achieved complete tumor regression. Notably, when these tumor-free mice were rechallenged with fresh MC38 cells post-treatment, no new tumors developed, demonstrating robust immune memory (**Revised Figures 5k, l**).

Regrettably, we were unable to locate detailed characterization of the AP1 cell line referenced in reviewer's suggestion. However, our data in MC38 and Renca-two widely used models-strengthen the conclusion that CD300ld blockade, particularly in combination with PD-1, elicits durable antitumor immunity and functional memory. These updates are now integrated into the manuscript to explicitly address reviewer's concern, providing critical insights into the long-term efficacy of CD300ld-targeted therapy.

9. The study identifies CD300ld as an immunosuppressive receptor, but the signals that drive its upregulation in neutrophils within the TME remain unclear. Are tumor-derived cytokines responsible for CD300ld induction? Elucidating upstream regulators may help inform a better target.

We sincerely appreciate the reviewer's insightful question regarding the upstream signals driving CD300ld upregulation in tumor-infiltrating neutrophils - a critical mechanistic gap that deserves further exploration. We fully agree that identifying the drivers of CD300ld expression (e.g., tumor-derived cytokines) would enhance our understanding of its context-dependent regulation and potentially reveal novel therapeutic targets. In our current study, we focused primarily on characterizing CD300ld's functional role in neutrophil-mediated immunosuppression rather than dissecting its transcriptional or post-transcriptional regulation. That said, we recognize the importance of elucidating upstream regulators, and our team is actively exploring this direction in follow-up studies, including cell factor screening, transcriptomic analysis, and genetic validation to identify and characterize the regulatory mechanisms driving CD300ld expression.

While these explorations are beyond the scope of our current manuscript, we view them as essential to completing the mechanistic puzzle of CD300ld's role in tumor immunity. We plan to share progress on these studies as they mature. We thank the reviewer for highlighting this key question. It has sharpened our focus on the broader regulatory landscape of CD300ld and reinforced our commitment to a multi-dimensional understanding of its biology. We believe these future investigations will not only complement our existing findings but also provide critical insights to guide the development of more effective CD300ld-targeted therapies.

Referee #3 (Remarks to the Author):

The study highlights that CD300ld on neutrophils interacts with phosphatidylserine (PS) on CD8+ T cells, contributing to T cell suppression. Mice harboring a mutated form of CD300ld incapable of PS binding exhibit reduced T cell suppression, comparable to CD300ld-deficient mice. Therapeutic blockade of the CD300ld-PS interaction using novel Llama-derived nanobodies demonstrates efficacy in tumor treatment and enhances the effectiveness of anti-PD-1 therapy. Elevated CD300LD expression correlates with decreased T cell activity within tumors and diminished therapeutic responses. These findings suggest that the CD300ld-PS interaction is a mechanism by which neutrophils promote tumor immune escape, and targeting this interaction may improve immunotherapeutic outcomes.

The manuscript provides important insights into the mechanisms regulating the suppressive activity of neutrophils on T lymphocytes in the context of cancer, highlighting a previously unrecognized interaction between CD300ld and PS. The data are coherent, and the conclusions are well supported by carefully designed and rigorously executed experiments. Overall, the study represents a valuable contribution to our understanding of tumor-associated immune regulation. The following comments are intended to further improve the clarity and readability of the manuscript and to help the Authors better convey the significance of their findings..

We thank Reviewer #3 for the time, care, and attention s/he has taken to review our study. We appreciate his/her positive feedback and insightful comments, to which we respond in detail, below.

1. The contextualization of the experiments using the Tmem30a-deficient melanoma cell line is not immediately clear. The observed overproduction of PS in knockout cells does not appear directly related to the intrinsic biological properties of the tumor. It should be clarified whether Tmem30a deficiency or downregulation commonly occurs in tumors and, if so, whether it correlates with increased aggressiveness and immune dysregulation. At the end of the experimental description, the Authors include the following statement, which seems out of context and only loosely related to the Tmem30a-deficient melanoma model: “Moreover, since CD300ld also binds to $CD8^+$ T cells, our findings suggest that PMN-MDSCs may suppress antitumor immunity not only through passive accumulation, but also via CD300ld-mediated contact-dependent interactions with $CD8^+$ T cells, contributing to localized immune suppression.”

We sincerely appreciate the reviewer’s thoughtful question regarding the experimental design using the Tmem30a-deficient melanoma cell line and the relevance of the concluding statement. We clarify our logic below to address your concerns: Our goal was to investigate whether tumor cell-expressed PS could modulate CD300ld function, given our prior findings that CD300ld-ECD binds both PS and tumor cells. However, PS is a lipid molecule, making direct manipulation (e.g., knockout/overexpression) in tumor cell lines technically challenging. To indirectly regulate tumor cell PS levels, we focused on Tmem30a-a key regulator of PS flipping. Prior studies (**For Reviewer Fig.9a**[11]) and our new analysis of melanoma patient datasets from TCGA demonstrate that Tmem30a expression is tightly linked to tumor biology and clinical outcomes: high Tmem30a

expression correlates with improved patient survival (**For Reviewer Fig.9b**), while its loss is associated with aggressive, immunosuppressive tumor phenotypes. This provided a rationale to use Tmem30a-deficient melanoma cells: they model a scenario where tumor cell PS levels are elevated, allowing us to test whether such PS enrichment impacts CD300ld-dependent tumor-immune interactions.

Regarding the final statement ("Moreover, since CD300ld also binds to $CD8^+$ T cells…"), we acknowledge its apparent disconnect from the Tmem30a-deficient model. This was intended to logically bridge our tumor cell-centric findings (PS-CD300ld interactions) to the broader immune context ($CD8^+$ T cell suppression), emphasizing that CD300ld's role extends beyond tumor cell signaling to include direct engagement with cytotoxic T cells. We agree that clarifying the link between Tmem30a deficiency and tumor intrinsic properties would strengthen the rationale. We have revised the manuscript to explicitly note that Tmem30a loss recapitulates features of aggressive, immunosuppressive tumors (line 201 to line 204) and revised the transition description (line 221 to line 225). We thank the reviewer for raising this point-it has helped us refine the narrative to better highlight the logical flow and biological relevance of our model choice.

Figure Redacted

For Reviewer Fig.9 a, Flow cytometry analysis of surface PS levels in WT and Tmem30a-KO cells. **b,** Kaplan-Meier analysis showing the overall survival of TMEM30A expression in SKCM patients.

2. Regarding Figure 3, the description of the cellular transfer is unclear. The Authors refer to mice receiving WT PMN-MDSCs (lane 258), but it is not related to the CD8+ T cell transfer that was actually performed. Furthermore, the substantial biotin labeling observed in splenic PMNs suggests a CD300ld-dependent cellular interaction in this organ. Does this imply that suppression occurs within secondary lymphoid organs as well? This interpretation would be consistent with Figure 3L, which shows an immune synapse-like structure between $CD8^+$ T cells and putative PMN-MDSCs in the spleen; however, this conclusion requires direct experimental validation. Additional questions should also be addressed: Are there PS-high $CD8^+$ T cells present in the spleen of tumor-bearing mice? According to the Authors' model, these cells should represent the primary targets of CD300ld-expressing PMNs. Can normal neutrophils compete with PMN-MDSCs for binding to PS-high $CD8^+$ T cells?

We sincerely appreciate reviewer's careful review of Figure 3 and the thoughtful questions raised. Below, we address each point to clarify our experimental design and findings:

First, we apologize for the initial ambiguity in describing the cellular transfer in **Figure 3j** and **3k**. The reference to "WT PMN-MDSCs (lane 258)" was an error in labeling, we have now clarified in the revised manuscript (line 299). This mislabeling did not affect the core interpretation of the data.

Regarding immune suppression in secondary lymphoid organs, we agree that such interactions may occur, and our data support this. In tumor-bearing mice, we observed that splenic $CD8^+$ T cells exhibited PS exposure (**Revised Extended Data Figure 6b**). Splenic PMN-MDSCs from these mice also displayed robust immunosuppressive activity, comparable to their tumor-infiltrating counterparts (**For Reviewer Fig.10a**[12]). Additionally, splenic $CD8^+$ T cells from tumor-bearing mice showed lower expression of activation markers than those from healthy controls (**For Reviewer Fig.10b**), indicating impaired functionality even in secondary lymphoid tissues-though the degree of suppression was milder than in the tumor microenvironment. These findings collectively suggest that immune suppression mediated by PMN-MDSCs and CD300ld-PS interactions extends to secondary lymphoid organs, albeit with reduced potency relative to the tumor bed.

To address whether normal neutrophils can compete with PMN-MDSCs for binding to PS-high $CD8^+$ T cells, we performed a competitive binding assay: $SrtA^+$ $CD8^+$ T cells were co-incubated with splenic neutrophils from tumor-free mice or PMN-MDSCs from tumor-bearing mice. We found that $CD8^+$ T cells binding to PMN-MDSC were much higher than neutrophils, primarily due to their lower CD300ld expression (**Revised Extended Data Figure 6l**). This suggests that while normal neutrophils can weakly binding for $CD8^+$ T cells, PMN-MDSCs-owing to their higher CD300ld levels-remain the dominant mediators of this interaction in the TME.

We have corrected the description error and we thank the reviewer for highlighting these critical points, which have strengthened the rigor and clarity of our analysis.

Figure Redacted

For Reviewer Fig.10 a, Suppression of $CD8^+$ T cell proliferation by different neutrophils. **b,** Flow cytometry detection of Gzmb positive CD8+ T cells in spleen under the indicated condition.

3. Figure 1b is difficult to interpret. Is the length of the spikes proportional to the predicted binding affinity? If so, why were some lipids with high predicted affinity—such as PPA—not tested in the immunoblot analysis shown in Figure 1c?

We sincerely appreciate reviewer's thoughtful question regarding the interpretation of Figure 1b and the rationale behind the selection of lipids for testing in Figure 1c. We clarify the experimental design and considerations below to address your concerns:

The length of the "spikes" in Figure 1b does indeed correspond to the predicted binding affinity of each lipid for CD300ld-ECD, with longer spikes indicating higher predicted affinity. To prioritize candidates for experimental validation, we selected the top 30 lipids with the highest predicted affinity scores from our in-silico screening.

Regarding the absence of phosphatidic acid (PPA) in the immunoblot analysis (**Figure 1c**), two key factors influenced this decision: First, a critical limitation was the lack of access to purified PPA in a commercially available, free lipid form suitable for our assay. Despite efforts to source or synthesize this lipid, we were unable to obtain a validated, biochemically pure preparation for testing. Second, PPA is relatively under characterized in biological contexts compared to other lipids. Actually, to date, not only PPA (18:1/18:1) but this class of lipid has not been identified in mammalian cells[13]. This lack of prior data has reduced its priority for initial experimental testing.

We regret that the initial explanation did not sufficiently emphasize these practical and contextual constraints. We have revised the figure legend to explicitly clarify the relationship between spike length and predicted affinity, as well as the specific reasons for excluding PPA (line 82-83, line 1230-1231). We thank the reviewer for raising this critical point.

4. Supplementary Figure 1g lacks quantification and statistical analysis.

We sincerely appreciate the reviewer's observation regarding **Supplementary Figure 1g**. To clarify, we intentionally placed the key quantitative data and statistical analysis in the main figure (**Figure 1g**) to emphasize the critical impact of CD300ld deletion on PS binding.

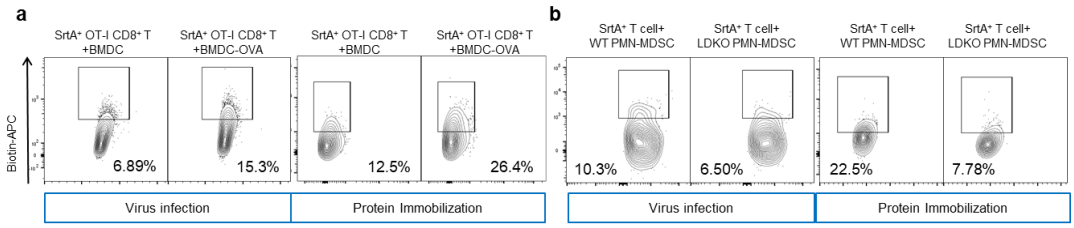
5. Supplementary Figure 6d-e: The differences between plots are minimal, raising concerns about their biological relevance once additional samples and statistics are included. This also questions the sensitivity of the SortA approach in distinguishing immune synapse formation from simple cell-to-cell contact.

We sincerely appreciate the reviewer's critical observation regarding the subtle differences observed in **Supplementary Figure 6d-e** and their concerns about biological relevance and the sensitivity of the SrtA approach. We acknowledge that the lower labeling efficiency in our initial experiments likely arose from the inherent variability of viral transduction, which led to inconsistent SrtA expression on T cells. To resolve this, we refined our methodology by replacing viral transduction with chemical conjugation to covalently attach SrtA to the surface of $CD8^+$ T cells. This approach drastically improved labeling efficiency by minimizing variability and ensuring consistent, high-density SrtA expression on the cell membrane, this approach significantly improved labeling efficiency-whether assessing interactions between $CD8^+$ T cells and DCs (**For Reviewer Fig.11a**) or $CD8^+$ T cells and neutrophils (**For Reviewer Fig.11b**). Crucially, this optimized system also reduced the proportion of T cells in co-culture experiments, effectively lowering background noise from non-specific interactions that could obscure the specific SrtA-mediated immune synapse signals. With these improvements, the differences in SrtA-dependent interactions between WT and KO conditions became more pronounced, as evidenced by clearer separation of

clusters in the revised plots (**Revised Extended Data Figure 6i**). These adjustments not only enhance the visibility of biologically meaningful differences but also validate the SrtA approach's sensitivity: by reducing non-specific background.

Notably, while our imaging data suggest that T cells and neutrophils form synapse-like structures via CD300ld-PS interactions, we emphasize that current evidence is insufficient to definitively confirm the formation of functional immune synapses-this remains a critical area for future investigation. Our present work primarily focuses on characterizing the cell-cell interactions driven by CD300ld-PS binding, and we aim to delve deeper into the mechanistic details of synapse formation in subsequent studies. So we omitted "synapse-like morphology" from the manuscript to avoid misinterpretation.

We thank the reviewer for raising these important points, as they have prompted us to refine our interpretations and highlight the forward directions of our research.



For Reviewer Fig.11 a, Flow cytometry analysis of the Ratio of labeled bone marrow-derived DC (BMDC; Biotin$^+$) after co-culture with virus infected or protein immobilized SrtA$^+$ CD8$^+$ T cells. **b**, Flow cytometry analysis of the Ratio of labeled PMN-MDSCs after co-culture with virus infected or protein immobilized SrtA$^+$ CD8$^+$ T cells.

6. Please also correct the sentence beginning on line 243.

We sincerely thank the reviewer for pointing out the need to refine the sentence starting at Line 243. We agree that the initial phrasing was unclear and prone to misinterpretation. We have revised it as: "We next asked whether CD300ld-PS adhesive interactions contribute to T cell-PMN-MDSC cell-cell contact. To test this, SrtA-immobilized PS^{high} and PS^{low} OT-I T cells were sorted and co-cultured with WT or CD300ld KO PMN-MDSCs." (line 275-278) This clarification eliminates confusion and ensures the logic between the research question and methodology is transparent..

7. Extended Data Figures 8a-b are presented and discussed before Extended Data Figure 7 in the text; the order should be revised for consistency.

We sincerely appreciate the reviewer's observation regarding the order of **initial Extended Data Figures 8a-b** and **7** in the text. We have revised the manuscript to reorder the extended data figures. This adjustment aligns the figure sequence with the narrative progression of our results, enhancing clarity and consistency throughout the text. We thank the reviewer for highlighting this critical detail.

8. Figure 4e: The graphical representation of the trajectories should be improved to make starting and ending points and the intermediate steps along each trajectory more clearly visible. Moreover, the color schemes used to indicate subsets in the UMAP plots (panels c and f) do not correspond

to the clusters identified, making direct comparison difficult.

We sincerely appreciate the reviewer's constructive feedback on the clarity of our UMAP visualizations and trajectory plots. We agree that improving the clarity of trajectory plots and aligning color schemes with identified clusters is critical for enhancing readability and interpretability. we first unified the color scheme between **Figure 4c** (UMAP) and **Figure 4f** (trajectory analysis) by ensuring each cluster is represented by a distinct, standardized color that directly corresponds to the cluster labels in the figure legend, eliminating ambiguity and enabling direct comparison of cell subsets across panels. To improve visibility of starting/ending points and intermediate steps in the trajectories, we displayed pseudotime values for each cluster along the trajectory, using a color gradient where darker hues represent earlier (starting) stages of differentiation and lighter hues denote later (terminal) stages (**Revised Extended Data Figure 7f**). We thank the reviewer's invaluable guidance, as it has driven us to refine our visual presentation to better align with best practices in data communication, and the updated figures now more effectively support our conclusions, enhancing accessibility for readers.

9. The statement that "myeloid-lymphocyte doublets had higher total gene counts" (Extended Data Figures 7e-f) requires clarification: higher than what? What is the biological significance of this observation?

We sincerely appreciate reviewer's question regarding the statement that "myeloid-lymphocyte doublets had higher total gene counts" in **initial Extended Data Figures 7e-f**. We clarify that this comparison refers to single myeloid cells and single lymphocytes-the gene counts of doublets were higher than those of either individual cell type.

Biologically, this observation aligns with the definition of cellular doublets: a doublet consists of two physically associated cells (e.g., a myeloid and a lymphocyte) that are co-encapsulated during single-cell dissociation or sequencing. Because each doublet contains two distinct cells, it inherently has a higher total gene count than a single cell (either myeloid or lymphocyte alone) (**Revised Extended Data Figure 7i**). This is a well-documented feature of doublets in single-cell omics data, where aggregated gene counts from two cells are reflected in the total count. Thus, the higher gene counts in myeloid-lymphocyte doublets directly support their identification as bona fide doublet populations. We have revised the manuscript (line 343) to explicitly state this comparison to clarify the basis of this observation. We thank the reviewer raising this point-it has helped us strengthen the interpretation of our single-cell data and ensure clarity in reporting.

Referee #4 (Remarks to the Author):

The authors identified phosphatidylserine (PS) as a ligand for CD300ld on myeloid-derived suppressor cells (PMN-MDSCs) and showed that PMN-MDSCs contact with cytotoxic PShighCD8+ T cells through the CD300ld-mediated recognition of PS and suppress PShighCD8+ T cell functions via ROS release, which impairs antitumor immunity during cancer progression. They extensively performed in vitro and in vivo experiments, appropriately analyzed statistically processed data, and drew a solid conclusion. The anti-tumor mechanisms described in this paper are novel, unique, interesting, important, and helpful for the researchers in the relevant field. However, several concerns need to be addressed.

We thank Reviewer #4 for the time, care, and attention s/he has taken to review our study. We appreciate his/her positive feedback and insightful comments, to which we respond in detail, below.

1. In abstract, neutrophils to PMN-MDSCs are not distinguished. The similarities and differences between them in the expression and functions of CD300ld may be discussed.

We sincerely appreciate the reviewer's insightful suggestion to clarify the distinctions and similarities between neutrophils and PMN-MDSCs, particularly regarding CD300ld expression and function. We concur that explicitly addressing these points will significantly enhance the manuscript's clarity.

In the original Abstract, these cell populations (neutrophil and PMN-MDSC) were not adequately differentiated. To resolve this, we have revised the text to directly contrast their phenotypic and functional profiles (lines 27–36). We further refined the description to underscore their shared dependence on CD300ld while emphasizing divergent functional outcomes: CD300ld is specifically expressed in neutrophils (**For Reviewer Fig. 12a**) and upregulated in PMN-MDSCs (**For Reviewer Fig. 12b**)-a key distinction highlighted in our prior work. In the revised manuscript, we have expanded this discussion to explicitly delineate both shared and divergent roles of CD300ld in these cells: whereas neutrophils constitutively express CD300ld, PMN-MDSCs display elevated CD300ld levels (lines 44–45), which enhance their immunosuppressive capacity. This clarification enables readers to more readily appreciate the nuanced contributions of CD300ld to neutrophil and PMN-MDSC biology. We thank the reviewer for raising this important point-it has helped refine our presentation of these critical cell populations.

Figure Redacted

For Reviewer Fig.12 a, Flow cytometry histograms showing the expression of CD300ld in immune cell subpopulations. **b ,** Flow cytometry histograms statistics showing expression of CD300ld in splenic neutrophils from tumour-free mice or B16-F10 tumour-bearing mice.

2. There exist many receptors for PS, including CD300 family molecules, TIM family molecules, and so on. Other PS receptors on neutrophils/PMN-MDSCs may exhibit the same functions as CD300ld. Expression profiles of other PS receptors on neutrophils and PMN-MDSCs should be shown. This point should be discussed.

We sincerely appreciate the reviewer's insightful question regarding the potential contribution of other PS receptors on neutrophils/PMN-MDSCs and the need to address their expression profiles. Below, we clarify our analysis and interpretation to address your concerns:

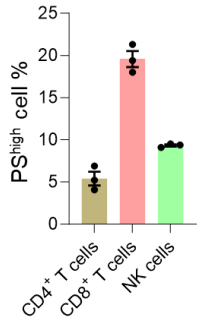
Regarding the expression of other PS receptors, we agree that a comprehensive profile of PS-binding proteins on these cells is critical for contextualizing our findings. We analyzed transcriptomic data comparing PS-binding protein expression in neutrophils and PMN-MDSCs, and while CD300ld was the most abundantly expressed PS-binding protein in PMN-MDSCs (and the second most in neutrophils) (**Revised Extended Data Figure 1h**), we also identified other reported PS-binding proteins (e.g., CD300a, TIM-3) in both cell types. This observation explains the residual PS-binding activity (~30%) observed in CD300ld KO neutrophils. We posit that this residual activity primarily stems from the collective contribution of these other PS-binding proteins. Although their individual expression levels are lower than that of CD300ld, their combined expression may synergistically mediate PS binding-particularly under conditions where PS availability is high. Notably, even with this residual activity, our functional data remain robust: the partial PS-binding capacity of CD300ld KO neutrophils is insufficient to rescue the pro-tumor phenotype driven by CD300ld loss. This aligns with our conclusion that CD300ld on neutrophils is the dominant effector of PS-dependent tumor-immune crosstalk, whereas other PS-binding proteins play non-essential, auxiliary roles. We thank the reviewer for raising this important point, as it has strengthened our discussion of PS-binding mechanisms and underscored the specificity of CD300ld in mediating critical tumor-immune interactions.

3. Do PShigh CD4+ T cells and PShigh NK cells exist? If so, can PMN-MDSCs interact with these cells to exert specific functions in the context of anti-tumor immunity?

We sincerely appreciate reviewer's thoughtful questions regarding the presence of PS-high $CD4^+$ T cells and NK cells and their potential interactions with PMN-MDSCs in anti-tumor immunity. Our data indicate that low levels of non-apoptotic PS signals are detectable on both $CD4^+$ T cells and NK cells in the tumor microenvironment, though these signals are much weaker compared to those on $CD8^+$ T cells (flow cytometry quantification revealed approximately 5% PS^{high} $CD4^+$ T cells and 8% PS^{high} NK cells, **For Reviewer Fig.13**). Consistent with this reduced PS expression, we did not observe significant binding of CD300LD-ECD to $CD4^+$ T cells or NK cells (**Extended Data Figures 4e**),

suggesting CD300ld does not directly mediate PMN-MDSC interactions with these cell types at the PS-dependent level.

To assess functional impacts, we performed depletion experiments: depleting $CD8^+$ T cells (using anti-CD8 antibodies) abrogated the tumor-growth-inhibitory effect of CD300ld-KO mice, with no difference in tumor progression between WT and KO mice, aligning with our prior conclusion that $CD8^+$ T cells are the primary targets of CD300ld-dependent PMN-MDSC suppression. In contrast, depleting $CD4^+$ T cells (anti-CD4 antibodies) or NK cells (anti-NK1.1 antibodies) did not rescue the tumor-growth-inhibitory phenotype of KO mice-even in the absence of these cells, KO mice still exhibited slower tumor growth compared to WT controls (**Revised Extended Data Figures 6f**). These results suggest that while PMN-MDSCs may suppress $CD4^+$ T cells and NK cells, this suppression is not mediated by CD300ld. We thank the reviewer for raising these critical points-they have deepened our understanding of the heterogeneity of PMN-MDSC interactions and strengthened the precision of our mechanistic conclusions.

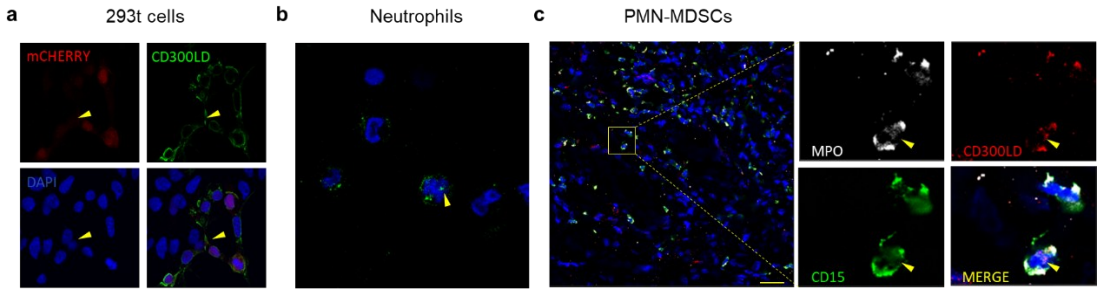


For Reviewer Fig.13 PS^{high} cell proportion in in indicated cells.

4. It was previously reported by Comas-Casellas et al. (JBC, 2012) that human CD300d is not expressed on the surface of human myeloid cells. Did the authors examine the surface expression of human CD300ld on human neutrophils and OMN-MDSC?

We sincerely appreciate the reviewer's insightful question regarding the surface expression of human CD300LD and its consistency with the previously reported data. To address these concerns, we performed ectopic overexpression of human CD300LD in 293T cells, where confocal microscopy revealed that the majority of CD300LD localized to the cell membrane, with only a minor fraction in the cytoplasm (**For Reviewer Fig.14a**). We further validated this using endogenous CD300LD antibody staining in primary human cells: immunofluorescence analysis of neutrophils from healthy donor peripheral blood (**For Reviewer Fig.14b**) and PMN-MDSCs from melanoma patients showed a similar pattern-CD300LD predominant membrane localization with faint cytoplasmic staining (**For Reviewer Fig.14c**). We attribute the apparent discrepancy with the 2012 study[14] to potential differences in experimental contexts, including cell type and different protein tag, which can collectively influence protein subcellular targeting. Collectively, our data-supported by both overexpression and endogenous staining-confirm that human CD300LD is primarily membrane-localized in neutrophils and PMN-MDSCs. We thank the reviewer for raising this critical point,

which has prompted rigorous validation of CD300LD localization across systems and enhanced the robustness of our mechanistic conclusions.



For Reviewer Fig.14 a, human CD300LD with Flag were infectd to 293T cells and than IF staining were performed. **b ,** IF staining with endogenous CD300LD antibody to detect peripheral blood neutrophils from healthy individuals. **c ,** IF staining with endogenous CD300LD antibody to detect tumor slices of melanoma patients. The yellow arrow represents the localization of CD300LD in the cytoplasm.

5. Line 63: The references (regarding Extended Data Figure 1) are lacking.

We sincerely thank the reviewer for carefully noting the missing references associated with **Extended Data Figure 1**. This oversight stemmed from our incomplete final checks, and we deeply appreciate the reviewer's attention to detail in highlighting it. In the revised manuscript, we have now added the appropriate citations to ensure Extended Data Figure 1 is fully referenced (line 67-69).

References

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6 Pagliano, O. *et al.* Tim-3 mediates T cell trogocytosis to limit antitumor immunity. *J Clin Invest* **132**, doi:10.1172/jci152864 (2022).

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13 Graber, Z. *et al.* The Electrostatic Basis of Diacylglycerol Pyrophosphate-Protein Interaction. *Cells* **11**, doi:10.3390/cells11020290 (2022).

14 Comas-Casellas, E. *et al.* Cloning and characterization of CD300d, a novel member of the human CD300 family of immune receptors. *J Biol Chem* **287**, 9682-9693, doi:10.1074/jbc.M111.279224 (2012).

Reviewer #1 (Remarks to the Author):

Authors provided extensive answers to all my questions and concerns. I am thankful for clarification of several points I raised and additional experiments performed to address the others. However, I did not find some responses clear or helpful.

"The experiments with liposomes in Figure 3k demonstrated only partial decrease of PS liposome binding to mutant CD300ld." - I did not find the answer helpful since it suggests only partial specificity of the event.

"Authors data and interpretations of Figure 2a and Extended Figure 3 raise questions. Authors observed the effect of PS targeting on tumor growth in WT mice. ... In support of the specific role of Cd300ld they used KO mice. However, in KO mice tumor growth was substantially reduced. Addition of Annexin V did not further enhance it" - Authors provided some general explanation that did not clarify the nature of concerns. Lack of the effect is lack of the effect. It may require different experimental set up or change of interpretation but explanation of the authors were not convincing.

I raised several comments regarding very small antitumor effect of the different treatment. I did not find clear answer or interpretation of the data to this concern. In contrast, by answering different question by using higher affinity antibody authors obtained much more convincing data. it suggests that their system is amendable for improvement in theory.

Finally, the main question I had is a link between a recent study published by this group and current data. Authors wrote in response "Previously, we reported that CD300ld genetic deletion diminishes PMN-MDSC suppressive function via the STAT3-S100A8/A9 axis, reflecting a cell-intrinsic regulatory mechanism. In contrast, our current study demonstrates that CD300ld on PMN-MDSCs mediates intercellular crosstalk with PS-high $CD8^+$ T cells through a direct physical interaction, representing a distinct extrinsic mechanism. Notably, our data show that CD300lddependent immunosuppression strongly requires cell-cell contact (Figure to reviewer 1a), and importantly, pharmacological inhibition of STAT3 or S100A9 does not disrupt the physical interaction between PMN-MDSCs and PS-high $CD8^+$ T cells (Figure to reviewer 1b). This indicates that the STAT3-S100A8/A9 axis and PS-mediated cell crosstalk operate through distinct pathways: the former drives PMN-MDSC intrinsic functionality, while the latter facilitates their targeting of effector T cells". Authors proposed existence of different ligand driving S100A8/A9-STAT3 distinct from PS: "we propose that CD300ld likely also binds additional as-yet-uncharacterized ligands that drive the STAT3-S100A8/A9-dependent intrinsic regulation described previously".

Now it is getting really complicated. In a nutshell, there are several totally independent mechanisms regulated by the same receptor. PS is responsible for binding, bit not CD300ld downstream signaling? What would prevent CD300ld to signal in that situation? Other ligands (not yet identified) target CD300ld and provide for signaling? Is it possible, yes. Is it likely, probably not since there very few examples of such phenomenon and it usually required formation of different receptor-ligand clusters. The concern is how much observed phenomenon is the result of artificial

experimental conditions?

Reviewer #2 (Remarks to the Author):

The authors have added a substantial amount of new experiments, which largely addressed my previous comments. I do not have further questions or comments.

Reviewer #3 (Remarks to the Author):

No additional comments

Reviewer #4 (Remarks to the Author):

This reviewer's concerns were satisfactorily addressed.

Reviewer #1:

Authors provided extensive answers to all my questions and concerns. I am thankful for clarification of several points I raised and additional experiments performed to address the others. However, I did not find some responses clear or helpful.

We thank Reviewer #1 for the time, care, and attention s/he has taken to review our study. We appreciate his/her feedback and insightful comments, to which we respond in detail, below.

1. "The experiments with liposomes in Figure 3k demonstrated only partial decrease of PS liposome binding to mutant CD300ld." - I did not find the answer helpful since it suggests only partial specificity of the event.

We sincerely appreciate the reviewer's critical question regarding the partial reduction in PS liposome binding observed in CD300ld mutant neutrophils. We wish to emphasize that our "assay of liposome binding with neutrophil" serves as one of multiple orthogonal approaches to validate CD300ld's PS-binding activity. Indeed, we have employed extensive molecular experiments, including liposome pulldown and SPR binding assays, all of which converge to confirm CD300ld as a PS binding protein (**Figure 1d, e**) and mutant CD300ld lose this capacity entirely (**Figure 1i, j**). These complementary lines of evidence leave us highly confident in the specific role of CD300ld in mediating PS recognition.

Regarding the reviewer's concern about assay specificity, we acknowledge that residual binding in CD300ld mutants may reflect contributions from other low-abundance PS-binding proteins, as previously noted, "while CD300ld is the most abundantly expressed PS-binding protein in PMN-MDSCs and the third in neutrophils, cells also expressed other PS-binding proteins (e.g., CD300a, TIM-3), which may compensate for CD300ld loss to mediate the remaining binding." This possibility is consistent with transcriptomic data (**Extended Data Figure 1h**), which reflects the broader expression profile of PS-binding receptor in neutrophils.

To definitively address the reviewer's concern about binding specificity, we performed antibody-blocking experiments with L14, a CD300ld-blocking antibody (**Figure 5c**). Incubation of WT neutrophils with L14 dose-dependently reduced PS-liposome binding, while no effect was seen in CD300ld-KO neutrophils (**Revised Extended Data Figure 8e**). Notably, even at saturating L14 concentrations, binding in WT neutrophils did not drop to zero-consistent with the residual binding in **Figure 1g and Extended Data Figure 3j** and underscoring that other PS receptors contribute to this baseline interaction (**line 411-416**). This experiment directly confirms CD300ld's role in mediating the majority of PS binding, while the incomplete blockade (and partial reduction in KO and mutant) reflects the presence of auxiliary receptors.

In summary, while minor residual binding in mutants may arise from compensatory mechanisms, our multi-method validation and proposed antibody blocking experiment provide compelling evidence for CD300ld's specific role in PS recognition.

2. "Authors data and interpretations of Figure 2a and Extended Figure 3 raise questions. Authors observed the effect of PS targeting on tumor growth in WT mice. ... In support of the specific role of Cd300ld they used KO mice. However, in KO mice tumor growth was substantially reduced. Addition of Annexin V did not further enhance it" - Authors provided some general explanation that did not clarify the nature of concerns. Lack of the effect is lack of the effect. It may require different experimental set up or change of interpretation but explanation of the authors were not

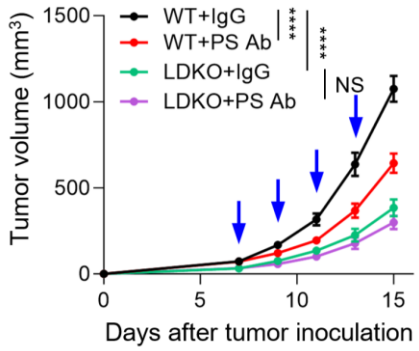
convincing.

We sincerely thank the reviewer for raising this critical concern, which has prompted us to re-examine our data, repeat key experiments, and refine our interpretations to better address the nuances of PS-targeting effects in CD300ld KO mice. We acknowledge that our initial description of the effects of PS-targeting agents (e.g., Annexin V or anti-PS antibodies) in CD300ld KO mice may have been slightly overly emphasized, and we appreciate the opportunity to clarify with new experimental evidence.

Specifically, a closer analysis of the raw data reveals that while the anti-PS antibody treatment in CD300ld KO mice did not show statistically significant inhibition compared to control IgG under our current experimental conditions, there was a subtle but consistent trend of slightly reduced tumor growth relative to controls, an observation we may have previously understated (**Initial Extended Data Figure 3e, which was shown below**). We then repeated this key experiment and increased the number of PS-targeting agent administrations from 4 to 6 doses (extending the treatment duration). The revised data reveal a clearer picture: in KO mice treated with Annexin V, tumor sizes in the measurement focus were significantly smaller than those in control IgG-treated KO mice (p=0.0459; **Revised Figure 2a**). For the anti-PS antibody group, while a consistent downward trend in tumor growth was observed, the difference did not reach statistical significance (p=0.1701; **Revised Extended Data Figure 3e**), likely due to higher biological variability in this cohort (**line 144-148**). Importantly, these results demonstrate that PS-targeting agents do exert a measurable inhibitory effect in KO mice under optimized conditions-though this effect is modest compared to the robust tumor suppression seen in WT mice.

This pattern aligns with our core hypothesis: CD300ld drives tumor progression primarily via its PS-binding activity, so its genetic ablation (KO) already attenuates tumor growth substantially. Consequently, PS-targeting agents in KO mice act on a "baseline-lowered" tumor microenvironment, yielding only subtle additional inhibition (potentially mediated by other functions like anti-angiogenesis) rather than the dramatic enhancement seen in WT mice (where CD300ld is fully functional).

Moreover, we want to emphasize that Annexin V or PS-blocking antibodies are not the sole evidence supporting this conclusion: point-mutated CD300ld ECDs (**Figure 2b**) and CD300ld mutant mice (**Figure 2e, h, i, j**), which directly disrupt PS binding, provide more definitive data demonstrating that the PS-binding activity of CD300ld is tightly correlated with its pro-tumor function, reinforcing the validity of our main findings.



For Reviewer Fig.1: Tumor growth curves of WT or CD300ld KO mice treated with Isotype or PS Ab starting from day 7 post B16-F10 tumor inoculation

3. I raised several comments regarding very small antitumor effect of the different treatment. I did not find clear answer or interpretation of the data to this concern. In contrast, by answering different question by using higher affinity antibody authors obtained much more convincing data. it suggests that their system is amendable for improvement in theory.

We sincerely thank the reviewer for acknowledging the improvement of our experimental system, and we appreciate the constructive feedback. Regarding his or her concern about the perceived "very small antitumor effect" of CD300ld deletion or mutation, we aim to provide a systematic response and clarification based on our complete dataset. For instance, in the B16-F10 tumor model, CD300ld KO or mutant mice exhibited over 50% reduction in tumor burden compared to controls (**Figure 2e**), while in the immunologically more responsive MC-38 model, tumor load was reduced by more than 65% (**Figure 2i**). Even in the TC-1 and HCC models, which the reviewer might consider to have weaker effects, we observed clearly significant antitumor activity (TC-1 ~40% reduction, (**Figure 2h**) and HCC models ~50% reduction, (**Figure 2j**)). Furthermore, when combining CD300ld-blocking antibodies with anti-PD-1 therapy, the synergistic effect was even more pronounced: tumor burden decreased by approximately 70% in B16-F10 (**Extended Data Figure 9i**) and over 90% in MC-38 models (**Extended Data Figure 9l**). These data collectively demonstrate that the antitumor impact of CD300ld deficiency or mutation is far from negligible. While we respect the reviewer's perspective, we believe labeling these effects as "very small" may underestimate their biological significance.

Importantly, our use of CD300ld KO or mutant mice was not intended to highlight dramatic therapeutic efficacy per se, but rather to establish causality-specifically, that CD300ld drives tumorigenesis primarily through its PS-binding capacity. Even if the antitumor effects in certain models appear modest, the consistent and statistically significant phenotypes across multiple models, combined with the mechanistic validation from PS-binding-disrupted mutants, strongly support our core conclusion. Thus, we maintain that these data robustly validate CD300ld's PS-dependent pro-tumor function.

We sincerely appreciate the reviewer's acknowledgment of improvements in the therapeutic efficacy of our CD300ld humanized antibodies (**Figure 6i**); While optimizing the antibody is relatively straightforward with established protocols, however, we regret that further optimization of CD300ld KO or mutant mouse models is not feasible for us at this stage.

4. Finally, the main question I had is a link between a recent study published by this group and current data. Authors wrote in response "Previously, we reported that CD300ld genetic deletion diminishes PMN-MDSC suppressive function via the STAT3-S100A8/A9 axis, reflecting a cell-intrinsic regulatory mechanism. In contrast, our current study demonstrates that CD300ld on PMN-MDSCs mediates intercellular crosstalk with PS-high $CD8^+$ T cells through a direct physical interaction, representing a distinct extrinsic mechanism. Notably, our data show that CD300lddependent immunosuppression strongly requires cell-cell contact (Figure to reviewer 1a), and importantly, pharmacological inhibition of STAT3 or S100A9 does not disrupt the physical interaction between PMN-MDSCs and PS-high $CD8^+$ T cells (Figure to reviewer 1b). This indicates that the STAT3-S100A8/A9 axis and PS-mediated cell crosstalk operate through distinct pathways: the former drives PMN-MDSC intrinsic functionality, while the latter facilitates their targeting of effector T cells". Authors proposed existence of different ligand driving S100A8/A9-STAT3 distinct from PS: "we propose that CD300ld likely also binds additional as-yet-uncharacterized ligands that drive the STAT3-S100A8/A9-dependent intrinsic regulation described previously".

Now it is getting really complicated. In a nutshell, there are several totally independent mechanisms regulated by the same receptor. PS is responsible for binding, bit not CD300ld downstream signaling? What would prevent CD300ld to signal in that situation? Other ligands (not yet identified) target CD300ld and provide for signaling? Is it possible, yes. Is it likely, probably not since there very few examples of such phenomenon and it usually required formation of different receptor-ligand clusters. The concern is how much observed phenomenon is the result of artificial experimental conditions?

We sincerely thank the reviewer for their thoughtful and persistent questioning regarding the apparent complexity of CD300ld-mediated mechanisms linking our previous and current work. To address the reviewer's concern, we clarify that these pathways are distinguished by both PS-dependent and PS-independent: the former relies on CD300ld's direct physical interaction with PS on PS-high $CD8^+$ T cells to mediate contact-dependent suppression, while the latter requires CD300ld binding to yet-uncharacterized ligands to trigger intracellular STAT3-S100A8/A9 signaling for PMN-MDSC intrinsic suppressive function.

Crucially, our data show these pathways operate independently: as PS does not induce STAT3-S100A8/A9 activation intact (**For reviewer Figure 1a**), whereas inhibiting STAT3/S100A9 does not disrupt CD300ld-PS-mediated interactions (**For reviewer Figure 1b**). Regarding the reviewer's query about whether CD300ld could signal independently of PS, our tumor lysate experiments provide direct evidence for a PS-independent mechanism: tumor lysate directly activates CD300ld, as shown by elevated STAT3 phosphorylation and S100A8 expression, yet this activation remains unperturbed by AnnexinV addition (**For reviewer Figure 1c**). This strongly implicates a yet-unidentified, PS-independent ligand in the tumor microenvironment that binds CD300ld to drive downstream signaling. While we are actively pursuing its characterization using co-immunoprecipitation, mass spectrometry, and functional validation, conclusive identification remains an ongoing effort.

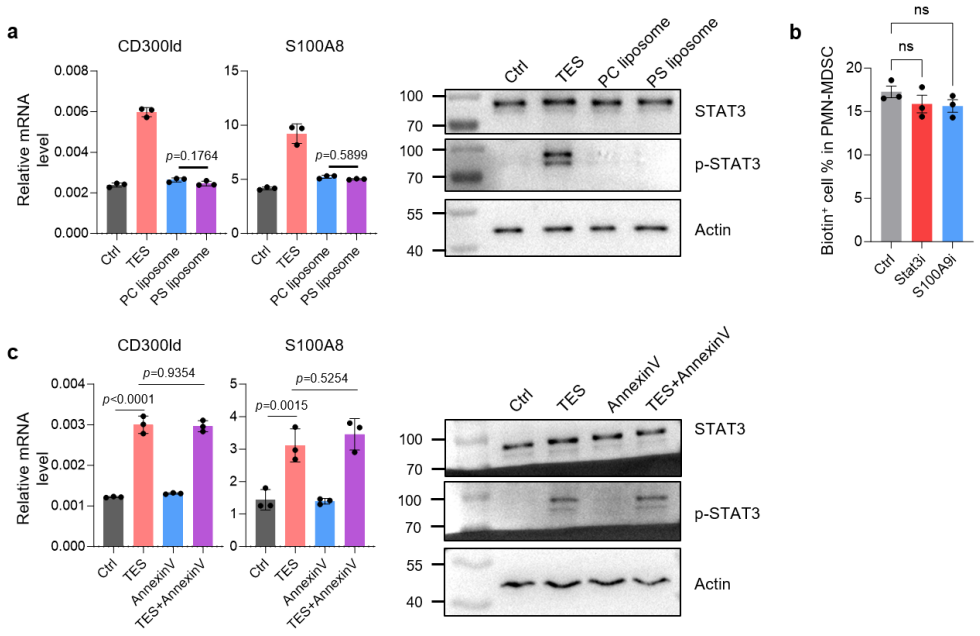
We acknowledge that engagement of distinct ligands by a single receptor is not a widespread phenomenon, though precedents exist. In fact, a part of PS binding receptors are known to interact with ligands other than PS: examples include Tim4 (binds PS[1] and Tim-1[2]), Tim3 (binds Galectin-9[3], Hmgb1[4] and PS[5]), Trem2 (binds Apoe[6], HDL, LDL[7] and PS[8]), and CD300lb (binds LPS[9] and PS[10]). These receptors collectively coordinate interactions with multiple ligands to initiate context-specific signaling. Taking Tim4 as an example, which Tim4 exemplifies this multifunctionality: a 2005 *Nature Immunology* study reported that Tim1 binds Tim4, and soluble Tim1 protein can engage Tim4 to induce T cell hyperactivation[2]; additionally, Tim4 acts as a PS-binding protein, with a 2021 *Cancer Cell* study demonstrating that macrophage-expressed Tim4 recognizes PS on activated T cells to suppress T cell activity-a interaction specifically mediated by Tim4-PS binding[11]. Given these precedents, we therefore consider it highly plausible that CD300ld may also bind novel ligands beyond PS.

We can now propose a mechanistic hypothesis for how CD300ld orchestrates immunosuppression: Following tumor initiation, an uncharacterized tumor-inducing factor (presumed to be a secreted or membrane-bound protein) binds to and activates CD300ld, inducing CD300ld upregulation and activating the STAT3-S100A8/A9 signaling axis, thus conferring enhanced immunosuppressive capacity on neutrophils. In this context, a secondary function of CD300ld comes into play: it recognizes PS signals on activated $CD8^+$ T cells, reduces spatial proximity to T cells, and consequently suppresses T cell activity. Within our proposed hypothesis,

CD300ld-PS binding constitutes a critical prerequisite for immune suppression. While the unidentified ligand amplifies neutrophil-mediated immunosuppression via CD300ld, this inhibitory function is not operational enough to manifest without CD300ld-PS interaction. Our previous work focused on upstream elements of this immunosuppressive cascade, whereas current findings highlight downstream mechanisms, findings that are complementary rather than conflicting. We contend that this model is logically coherent and strongly supported by our existing data. Furthermore, we believe this nuanced framework addresses the reviewer's concern about mechanistic complexity and artificiality, anchoring our conclusions in both experimental evidence and biological plausibility.

Given the limited understanding of this uncharacterized ligand, we have exercised scientific prudence by avoiding extensive discussion in the Discussion section. We anticipate that once this ligand is identified, refining the mechanistic framework of CD300ld-mediated immunosuppression will enable us to develop a more integrated and holistic understanding, thereby gaining deeper insights into this pathway.

We thank the reviewer for his/her contributions to improving the rigor and clarity of our study.



For Reviewer Fig.2: a, RT-qPCR detect CD300ld, S100A8 mRNA expression and western blot detection of phosphorylated STAT3 (p-STAT3) in neutrophils isolated from mouse bone marrow and treated with B16-F10 tumor explant supernatant (TES), PC liposomes and PS liposomes. **b**, Flow cytometric analysis of Biotin$^+$ labeling in PMN-MDSCs pre-treated with Stat3i (WP1066, 5 μM) or S100A9i (Tasquinimod, 50 μM) after co-incubation with SrtA$^+$ CD8$^+$ T cells. **c**, RT-qPCR detect CD300ld, S100A8 mRNA expression and western blot detection of phosphorylated STAT3 (p-STAT3) in neutrophils isolated from mouse bone marrow and treated with B16-F10 tumor TES, AnnexinV (10ug/ml) and combine.

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