nature portfolio

Peer Review File

Sphingosine Kinase-2 Inhibition Promotes Immunogenic Differentiation of Myeloid-Derived Suppressor Cells through an Acetyl-CoA Carboxylase-Phosphatidylcholine Axis

Corresponding Author: Dr Shikhar Mehrotra

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)
The present study performed by Chakraborty P. et al aims to clarify the regulatory mechanism of sphingosine-1-phosphate (S1P), an abundant signaling lipid in the tumor microenvironment (TME), on the function and programming of myeloid-derived suppressor cells (MDSCs), addressing the critical issue of MDSCs hindering adoptive T cell therapy. The research employed techniques including gene knockout (SphK1 KO, SphK2 KO), pharmacological inhibition (SphK2 inhibitor ABC294640, ACC inhibitor ND646), bone marrow chimera construction, flow cytometry, LC-MS lipidomics, RNA-seq, ATAC-seq, and proximity ligation assay in murine tumor models (B16-F10, EL4, 4T1, MB49) and human tumor samples (triple-negative breast cancer, prostate cancer, oral cavity squamous cell carcinoma), and found that (1) Inhibition of SphK2 (a key synthase of S1P) significantly reduced the immunosuppressive activity of MDSCs and enhanced their antigen-presenting capacity; (2) SphK2 inhibitor synergized with anti-PD1 therapy in PD1-resistant tumor models to markedly suppress tumor progression; (3) S1P directly binds and inhibits acetyl-CoA carboxylase (ACC1/2); reducing intracellular S1P restores ACC activity, promotes phosphatidylcholine (PC) synthesis, remodels MDSC lipid metabolism, and attenuates their suppressive phenotype; (4) SphK2 expression in human MDSCs positively correlates with an immunosuppressive phenotype, and SphK2 inhibitor reduces the proportion of MDSCs in peripheral blood of patients with advanced prostate cancer while enhancing CD8+ T cell function. The authors finally concluded that the SphK2-S1P-ACC-PC axis is a critical pathway regulating MDSC immunogenicity, providing a novel potential target for cancer immunotherapy.
This study is the first one to reveal the core role of the SphK2-S1P-ACC-PC axis in regulating MDSC immunogenicity, organically combining sphingolipid metabolism, fatty acid synthesis, and tumor immunity. Conclusions are verified from in vitro MDSC-T cell co-culture experiments, in vivo gene knockout, pharmacological inhibition, multi-tumor mouse models, and human tumor samples and clinical trial, with reliable results. Using multiple experimental techniques, the study provided clear clinical application value through a new target pathway and strategy for solving tumor immunotherapy resistance. However, the study only focused on M-MDSCs, with no exploration of the regulatory mechanism of PMN-MDSCs and no explanation of their response differences to SphK2 inhibition, limiting the generalization of conclusions. Human sample size and details are not sufficient. For example, the OCSCC sample size is too small (n=3) with insufficient statistical representativeness; ethical approval information for human samples is not detailed, affecting the credibility of results. Besides, verification of downstream mechanisms is not adequate: Although PC is confirmed as a downstream molecule of the SphK2-ACC axis, "knockout-rescue" experiments are not performed to directly prove that PC is the key to regulating MDSC immunogenicity; the specific functional division of ACC1/2 isoforms (e.g., effects of cytoplasmic/mitochondrial localization) is not deeply studied. Detailed comments are listed below:

Major points:
1. Please provide the ethical approval number (e.g., IRB number) for both human samples and mouse experiments.
2. SphK2 expression levels (flow cytometry or qPCR) in PMN-MDSCs should be provided to explain their non-response to SphK2 inhibition; if possible, conduct experiments on the association between PMN-MDSC-specific metabolic pathways (e.g., neutrophil granule metabolism) and SphK2 to expand the generalization of conclusions.
3. Please carry out CCTα knockout (PC synthase) + exogenous PC rescue experiments to directly prove that PC is the key downstream molecule of the SphK2-ACC axis regulating MDSC immunogenicity. The molecular mechanism of PC affecting MHC-I/CD80/CD86 expression is also missing. Please check the possible pathway or at least provide some related references in Discussion.

4. If possible, please use ACC1/2-single knockout mice, combined with subcellular localization experiments (immunofluorescence), to study the specific roles of ACC1 (cytoplasm) and ACC2 (mitochondria) in regulating PC synthesis, FAO/Glycolysis, and MDSC phenotype, which may explain the mechanism of their synergistic effect.
5. OCSCC patient sample size should be increased (suggest n≥10/group) and in vitro experiments (OCSCC patient-derived MDSCs + SphK2 inhibitor + anti-PD1) should also be better to perform to verify the regulatory role of this axis in PD1 resistance.
6. The binding affinity of S1P to ACC1 (WT vs. Ser1996 mutant) should be detected via surface plasmon resonance or isothermal titration calorimetry to directly prove that Ser1996 is the key site for S1P binding.

Minor points:
1. Please check and fix all spelling errors throughout the manuscript such as Log_{2}" in the third section of RESULT, "Lys6C" in the second paragraph of DISCUSSION section, "Ly6C^high" in several figure legends and "pITE1a" in Fig. S7 legend at the very last part of the manuscript.
2. Please supply statistical methods (e.g., "mean ± SD, n=5, unpaired t-test, *p<0.05") and experiment repetition times in all figure legends to ensure clear reproducibility. Authors can state these informations together at the end of each legend, like “Data from panel (x, y, z) are from n repeated experiments, in (a) by one-way ANOVA…”.
3. Please submit RNA-seq data to the GEO database and provide a temporary accession number in DATA AVAILABILITY in Method section.
4. Please specify the number of replicates are technical or biological replicates for all experiments in related figure legends.
5. Please add the information of ACC1/2-flox, Lyz-cre, CD45.1 mice in Mice section of Materials and Methods.
6. Authors should supply dose-determination experiments for ABC294640 (50 mg/kg) and ND646 (3 μM) (e.g., the effect of dose gradients on MDSC ACC activity) to explain why this dose was selected and enhance the rationality of experimental design.
7. Does membrane fluidity directly regulate the antigen-presenting capacity of MDSCs like upregulating the CD80/86 or MHC-I expression? Through "membrane fluidity-modifying reagents (e.g., cholesterol carriers) + MDSC phenotype detection" experiments, please verify whether PC influences the expression of these membrane molecules via the membrane fluidity, to improve the mechanistic evidence chain.
8. In TME immune microenvironment, can MDSCs differentiate into M1 macrophages? Please supply expression changes of key molecules (e.g., CD206, CD163, MHC-II) for MDSC-to-TAM transformation in Fig.2D and 2K to clarify whether SphK2 inhibition affects TAM polarization phenotype.
9. Please unify the font, font size, and color labeling (e.g., color correspondence for different groups) of all figures, especially in Fig. 2H and S2D; supply the annotations of each experimental group in the figures especially Fig. S2A-E, S3Fii, S3Fiii, otherwise the readers cannot distinguish which panel belongs to which group at all.
10. Please correct the figure panel labels throughout the manuscript as plenty of labels are missing or incorrect, such as Fig. S3Aii, Fig. S2G “Cells from (c)…”should be (F)?, last paragraph of Result section “…and PERK (Fig. S7G)” should be S7H?, the first paragraph of the fifth section in Result “…Arginase1 expression (Fig. S5C)” missing, the first paragraph of the third section in Result “…healthy individuals (Fig. 3A, S3Ai, Aii)” there is no Aii, the third paragraph of the third section in Result, last sentence “…immune suppression (Fig. S3Giii, S3H)” there is no S3H.
11. Please replace the picture with low resolution, especially Fig. 2H, 3Bi, S2D, can hardly read at present.
12. In Fig. S3A and Ai, labels of cell clusters in Ai “1, 2, 3…” cannot not correlate to the names of cell types in S2A.
13. Please ensure all abbreviations are defined upon first use. Right now, the use is disordered.

Reviewer #2

(Remarks to the Author)
Review: Sphingosine Kinase-2 Inhibition Increases Acetyl-CoA Carboxylase Activity and Phosphatidylcholine Levels to Program Immunogenicity in Myeloid-Derived Suppressor Cells Here, Chakraborty et al. study the molecular impact of S1P on MDSC function in preclinical models of breast, bladder, and melanoma cancers. The authors focus on the receptor independent intracellular S1P signaling, and suggest that S1P directly interacts with ACC1 to inhibit its function, altering fatty acid and glycolytic pathways. Lowering intracellular S1P restores ACC activity and promotes PC synthesis, which improves membrane fluidity, reduces lipid droplet (LD) formation and ER stress, reducing the immunosuppressive phenotype in MDSCs. These findings are impactful as they suggest testing SphK2/ACC/phospholipid axis as a metabolic target in cancer immunotherapy. Conceptually, targeting metabolic pathways to cause nutrient/energy shortage in MDSCs would be a novel therapeutic strategy against different types of cancers. The manuscript is well-structured, methodology is robust, and the proteomic + lipidomic focus is especially appreciated. However, questions surrounding the specific cell type impacted, as well as the plasticity of reprogramming, detract from the final story.

Major comments:
1) How does SphK2 KO impact survival of myeloid cells? Or lead to change in their proliferation? The role played by SphK2 on autophagosome machinery and lysosomal clearance will probably have immediate impact on their pro-survival pathways. Is this different for ‘MDSC’ isolated from tumor vs spleen, given the difference in tissue cues + inflammation?
2) Conceptually, is there a functional difference between the role of SphK2 in neutrophils, monocytes, vs mo-MDSC? Currently, the use of GR1+ to mark MDSCs (non-specific nomenclature) can lead to confusion in the field, as it would imply this signaling pathways is unique to these cell states found in inflammatory contexts. Hence, comparison of SphK2 level and ACC activity across steady-state myeloid populations and cells denoted tumoral macrophages or MDSCs in cancer context will be important. Maybe this is a generalizable pathway for macrophage states too?
3) Relatedly, it will be important to check how acute SphK2 KO influences MDSC-to-macrophage differentiation bottleneck in cancer. For this, the authors could do short window of differentiation using MDSC-like cells isolated from tumor-bearing SphK2 KO mice and transferred into congenic host.
4) Also, is there a correlation between intratumoral myeloid cell SphK2 levels and circulating monocyte SphK2 levels? How early (in circulation vs tissue) is this metabolic change occurring?
5) In Fig 3B, the TMA analysis of SphK2 is insufficient without clear visualization of localization in HLA-DRlow MDSC-like myeloid cells? For robustness and completeness, can the authors show SphK2 in HLA-DRlow MDSC-like cells, vs HLA-DRhi macrophages and/or neutrophils? If the assertion is true, then ideally there is correlation for MDSCs but not for other myeloid cells.
6) Can the authors describe what are other regions impacted, beyond H2 loci (Fig. S1H)? Potentially using tools such as GREAT to annotate genomic regions can help. Currently this analysis is under-developed and does not explain why cells become immunosuppressive beyond increase in MHC.
7) Is phosphatidylcholine (PC) species upregulation observed in lipidomics directly a result of SphK2 inhibition or indirect due to chronic effect of KO? Here, comparing acute S1P imbalance using small molecule inhibitor ABC294640 vs SphK2 KO could dissect the clear reason for PC enrichment.
8) What are the consensus lipid species upregulated upon KO of SphK2? The current heatmaps in Fig. S4C or Fig. S5E are not informative, it would be helpful to synthesize the data into dominant/consensus classes (diacyl-PCs, alkylacyl-PCs, alkenylacyl-PCs, lyso-PCs etc).
9) scRNA analysis (in fig 1C or fig 3A) is not upto standard for the level of this publication, it will be important to show scaled expression (with scale shown). Ideally, instead of UMAP/TSNE based visualization, the authors should show scaled expression on a violin plot for populations of interest.
10) The reviewer is surprised by the absence of in vivo functional tumor study (burden/survival/immunophenotyping) using LysMCre;ACC-DKO mice. That will add weight to current findings.
11) While not critical, it would be pertinent and valuable to the field to compare how conditioned media (CM) from different tumor lines (with different driver mutations) can lead to differential ACC activity– as a way to assess cues initiating this metabolic change.

Minor comments:
1) Schematic of signaling pathways proposed in these myeloid cells will be extremely helpful for readers.
2) Can the authors provide histograms in colorblind-friendly format, as well as add color legends (for example, Fig. 4C).
3) Please show %variance on PC1 vs PC2 axis (for example, in Fig. 4F or Fig. 5F)
4) What does AVG refer to, in Fig. S6A? Please improve representation of this data, and provide statistics (False Discovery Rate or similar).

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
The authors have addressed most of my concerns. But the following informations are still missing at present and please supply them:

1. The ethical approval number for the experiments using human samples should be provided in Method section (all procedures should be approved by the institutional ethics committee with an approval number).
2. Authors should state experiment repetition times in all figure legends to ensure clear reproducibility (for example“Data from panel (x, y, z...) are representative from 2/3/4... repeated experiments).

Reviewer #2

(Remarks to the Author)
The authors have addressed most of my scientific concerns, and I understand the time/resource limitations for in vivo data in LysMCre;ACC-DKO mice. This manuscript is now much improved.

There are a few minor typos throughout manuscript (for example, word 'fluidity' is misspelt in schematic Fig S8), please proof-read.

Response to Reviewer's comments (NCOMMS-25-76017-T)

We thank the Reviewers for their helpful critiques and suggestions. We especially thank the Reviewers for finding t*he manuscript well-written and well-structured and being of high interest to the readers*. The additional concerns raised by the specific reviewers have been addressed, and the details for these changes are mentioned below. All concerns are summarized in *italics,* while responses are in regular font.

REVIEWER #1

Major points:

1. *Please provide the ethical approval number (e.g., IRB number) for both human samples and mouse experiments.*

Thanks for pointing out on this missing information. We have now included the details of the IACUC approval (IACUC-2018-00628-2) under which all experiments were performed.

2. *SphK2 expression levels (flow cytometry or qPCR) in PMN-MDSCs should be provided to explain their non-response to SphK2 inhibition; if possible, conduct experiments on the association between PMN-MDSC-specific metabolic pathways (e.g., neutrophil granule metabolism) and SphK2 to expand the generalization of conclusions.*

We thank the reviewer for this important point. As suggested, we directly assessed SphK2 expression in PMN-MDSCs isolated from both spleen and tumor-bearing mice. Using flow cytometry, we found that SphK2 expression levels were not significantly different between splenic and tumor-derived PMN-MDSCs, in contrast to monocytic MDSCs, where SphK2 expression was markedly elevated in the tumor context. These data have been added to the revised manuscript (***Fig. S1H***). Consistent with these findings, PMN-MDSCs did not exhibit significant phenotypic or functional changes following SphK2 inhibition, suggesting that tumor-induced upregulation of SphK2 is selective for the monocytic MDSC lineage.

3. *Please carry out CCTα knockout (PC synthase) + exogenous PC rescue experiments to directly prove that PC is the key downstream molecule of the SphK2-ACC axis regulating MDSC immunogenicity. The molecular mechanism of PC affecting MHC-I/CD80/CD86 expression is also missing. Please check the possible pathway or at least provide some related references in the Discussion.*

We thank the reviewer for this important and constructive suggestion. We have now directly addressed this point through complementary loss-of-function and rescue experiments targeting choline cytidylyltransferase-α (CCTα), the rate-limiting enzyme in phosphatidylcholine (PC) biosynthesis. Specifically, siRNA-mediated knockdown of CCTα in SphK2-deficient MDSCs significantly reduced the expression of MHC-I, CD80, and CD86 and partially restored their suppressive phenotype (new ***Fig. 7A–D***), indicating that *de novo* PC synthesis is required for the immunogenic reprogramming induced by SphK2 inhibition. Importantly, exogenous supplementation with PC restored antigen-presenting molecule expression and T-cell stimulatory capacity (***Fig. 7E–H***), supporting the conclusion that PC functions as a critical downstream effector of the SphK2–ACC axis in regulating MDSC immunogenicity. Together, these loss-and-rescue experiments provide direct functional evidence that PC availability is required for the acquisition of the immunogenic phenotype following SphK2 inhibition.

Regarding the mechanism, we now provide additional discussion addressing how increased PC levels may influence antigen-presentation machinery. Our data indicate that elevated PC content enhances membrane fluidity, reduces lipid-droplet accumulation, and alleviates endoplasmic reticulum stress, processes known to impact MHC-I trafficking, co-stimulatory molecule surface expression, and immune synapse formation. Increased membrane fluidity may also facilitate the lateral mobility and clustering of antigen-presenting and costimulatory receptors, promoting the formation of functional signaling domains required for effective immune activation. We have incorporated relevant references linking membrane phospholipid composition and biophysical properties to antigen presentation, receptor organization, and immune synapse formation.

4. *If possible, please use ACC1/2-single knockout mice, combined with subcellular localization experiments (immunofluorescence), to study the specific roles of ACC1 (cytoplasm) and ACC2 (mitochondria) in regulating PC synthesis, FAO/Glycolysis, and MDSC phenotype, which may explain the mechanism of their synergistic effect.*

We thank the reviewer for this insightful suggestion and agree that isoform-specific dissection of ACC1 and ACC2 is mechanistically important, particularly given their distinct subcellular localization and metabolic functions. While generation and validation of myeloid-specific ACC1- or ACC2-single knockout mice, together with complementary subcellular immunofluorescence analyses, would be highly informative, these experiments were not feasible within the revision timeframe. Instead, we performed isoform-selective loss-of-function studies in MDSCs using siRNA-mediated knockdown of ACC1 or ACC2 individually, as well as combined ACC1/2 knockdown (***Fig. S5***).

Our data demonstrates distinct functional contributions of each isoform. ACC1 knockdown preferentially reduced phosphatidylcholine (PC) enrichment associated with the *SphK2*-KO phenotype (***Fig. 5E-F***), supporting a primary role for ACC1-derived cytosolic malonyl-CoA in driving *de novo* lipid synthesis required for PC generation. In contrast, ACC2 knockdown more prominently impaired mitochondrial fatty-acid oxidation (FAO)-linked bioenergetic parameters, as assessed by Seahorse oxygen consumption rate (OCR) measurements (***Fig. 5G–H***), consistent with its established role in regulating CPT1 activity and mitochondrial substrate utilization. Importantly, dual ACC1/2 knockdown produced the most pronounced reversal of the immunogenic phenotype, including reduced MHC-I, CD80, CD86, and IL-12 expression and restoration of suppressive features (***Fig. 5A–E***), indicating complementary contributions of both isoforms. We have clarified these isoform-specific functional conclusions in the revised Results and Discussion and now cite prior literature supporting the predominant cytosolic localization of ACC1 and the mitochondria-associated regulatory role of ACC2. Collectively, these isoform-selective knockdown data provide mechanistic support for distinct yet complementary roles of ACC1 in PC synthesis and ACC2 in FAO-linked metabolic control in shaping MDSC programming downstream of SphK2 signaling.

5. *OCSCC patient sample size should be increased (suggest n≥10/group) and in vitro experiments (OCSCC patient derived MDSCs + SphK2 inhibitor + anti-PD1) should also be better to perform to verify the regulatory role of this axis in PD1 resistance.*

We thank the reviewer for this important suggestion. As this is a retrospective study, all available materials consist of archived fixed tissue samples, which limits both sample expansion and the ability to perform additional functional *in vitro* assays. Nevertheless, we have increased the cohort to include eight PD-1–sensitive and six PD-1–resistant OCSCC cases (***Fig. 3F***). We acknowledge that larger cohorts and functional validation using patient derived MDSCs would further strengthen the findings, and we are actively pursuing sample collection to enable such studies in the future.

6. The binding affinity of S1P to ACC1 (WT vs. Ser1996 mutant) should be detected via surface plasmon resonance or isothermal titration calorimetry to directly prove that Ser1996 is the key site for S1P binding.

We thank the reviewer for this important suggestion. To directly quantify S1P–ACC1 interaction, we performed complementary biochemical and cellular binding assays.

First, we performed saturation binding assays using partially purified FLAG-tagged WT or mutant ACC1 expressed in HEK 293T cells and isolated with anti-FLAG agarose beads. The immobilized proteins were incubated in the presence of increasing concentrations of biotinylated S1P (starting at 0.3 nM) with or without excess of unlabeled S1P (10 uM) to define nonspecific binding. Bound biotinylated S1P was measured by using a biotin-sensitive ELISA. Nonlinear regression analysis revealed an equilibrium dissociation constant (Kd) of approximately 61 nM for WT ACC1 and 60 nM for the mutant protein (***Figs. 6C, S6A***). Interestingly, while Kd values remain in a similar range, the binding stoichiometry derived from Bmax values demonstrates an approximate 2:1 ratio of S1P to WT ACC1 compared to 1:1 for the mutant protein (***Fig. 6C***). Collectively, these data show that S1P binds directly to ACC1 with modest affinity in vitro and suggest that this interaction may regulate ACC1 function by modulating its enzymatic activity. Moreover, these results indicate that the Ser1996 residue is critical for optimal S1P-ACC1 interaction.

To further validate this interaction in cells, we reconstituted FLAG-tagged WT or mutant ACC1 in ACC1-deficient (LysM-CreAcc1$^{flox/flox}$) MDSCs and treated cells with C17-Sphingosine. Following FLAG

immunoprecipitation, associated lipids were quantified by targeted lipidomics. WT ACC1 exhibited significantly greater C17-S1P association compared to the mutant protein, confirming reduced binding capacity in a cellular context (Fig. 6D).

Together, these data demonstrate direct nanomolar-affinity binding of S1P to ACC1 and identify Ser1996 as a critical residue required for full binding stoichiometry and optimal ligand engagement, supporting a residue-specific regulatory mechanism governing ACC1 activity in MDSCs.

Minor comments:

6. *Authors should supply dose-determination experiments for ABC294640 (50 mg/kg) and ND646 (3 μM) (e.g., the effect of dose gradients on MDSC ACC activity) to explain why this dose was selected and enhance the rationality of experimental design.*

For ABC294640, we conducted *in vivo* dose-finding experiments in tumor-bearing mice comparing 25 mg/kg, 50 mg/kg, and 100 mg/kg administered every other day. We assessed pharmacodynamic modulation of the SphK2–S1P–ACC axis in tumor-infiltrating MDSCs, using intracellular S1P levels, ACC activity, and immunophenotypic markers of monocytic MDSCs (MHC-I, CD80, CD86) as primary readouts. We observed that 50 mg/kg produced a substantially more robust modulation of these parameters than 25 mg/kg, while remaining well tolerated. Increasing the dose to 100 mg/kg did not further enhance pathway modulation, indicating a plateau effect. These data support 50 mg/kg as a biologically effective, non-toxic dose, and they have been added to the revised manuscript (***Fig. S1I***).

For ND646, we performed an *in vitro* dose titration (0.03, 0.3, and 3 μM) in SphK2-deficient monocytic MDSCs. We evaluated ACC activity alongside cell viability. This analysis revealed a graded inhibition of ACC activity, with near-maximal functional effects at 3 μM and minimal impact on cell viability, supporting our selection of 3 μM as the working concentration for mechanistic experiments (***Fig. S5A***). Together, these dose-response studies provide a clear pharmacodynamic rationale for the concentrations of ABC294640 and ND646 used in this study.

7. *Does membrane fluidity directly regulate the antigen-presenting capacity of MDSCs like upregulating the CD80/86 or MHC-I expression? Through "membrane fluidity-modifying reagents (e.g., cholesterol carriers) + MDSC phenotype detection" experiments, please verify whether PC influences the expression of these membrane molecules via the membrane fluidity, to improve the mechanistic evidence chain.*

We thank the reviewer for this important suggestion. To directly test whether membrane biophysical properties contribute to regulation of antigen-presentation/co-stimulatory molecules, we modulated membrane composition using phosphatidylcholine (PC) supplementation together with methyl-β-cyclodextrin (MβCD), a cholesterol-depleting reagent commonly used to alter membrane order. Under our experimental conditions, MβCD alone had minimal effects on MHC-I, CD80, or CD86 expression. In contrast, PC supplementation significantly enhanced these markers, and the combination of PC + MβCD produced the most robust induction (new ***Fig. S7E***). Mechanistically, PC (particularly unsaturated PC species) can increase membrane fluidity by disrupting tight lipid packing, whereas MβCD reduces membrane cholesterol content; together, these manipulations lower the cholesterol-to-phospholipid ratio and favor a more disordered bilayer. The observed functional synergy supports a model in which PC-driven membrane remodeling—augmented by reduced cholesterol constraint—promotes increased surface expression of antigen-presentation and co-stimulatory molecules, thereby strengthening the mechanistic chain linking the SphK2–ACC–PC axis to immunogenic MDSC programming.

8. *In TME immune microenvironment, can MDSCs differentiate into M1 macrophages? Please supply expression changes of key molecules (e.g., CD206, CD163, MHC-II) for MDSC-to-TAM transformation in Fig.2D and 2K to clarify whether SphK2 inhibition affects TAM polarization phenotype.*

To address this point, we have now included additional analyses of TAM-associated markers in the tumor experiments shown in ***Fig. 2D*** and ***2K***. Specifically, we evaluated the expression of CD163, CD206, and MHC class II. Our results show that SphK2 inhibition alters the expression of CD163, and MHC class II whereas CD206 expression was not significantly changed in F4/80+ macrophages. The additional data have now been incorporated into the revised figures (***Fig.S2B, C, E,F***) and corresponding text.

Minor points:

1. Please check and fix all spelling errors throughout the manuscript such as Log2" in the third section of RESULT, "Lys6C" in the second paragraph of DISCUSSION section, "Ly6C^high" in several figure legends and "pITE1a" in Fig. S7 legend at the very last part of the manuscript.

We thank the reviewer for pointing this out. All spelling and typographical errors throughout the manuscript, including those noted (e.g., “Log2”, “Lys6C”, “Ly6C^high”, and “pITE1a”), have been carefully checked and corrected in the revised manuscript.

*2. Please supply statistical methods (e.g., "mean ± SD, n=5, unpaired t-test, *p<0.05") and experiment repetition times in all figure legends to ensure clear reproducibility. Authors can state these informations together at the end of each legend, like “Data from panel (x, y, z) are from n repeated experiments, in (a) by one-way ANOVA…”.*

We appreciate this helpful suggestion. Statistical methods, including the type of analysis, number of replicates, and data presentation format, have now been clearly specified in all relevant figure legends to improve transparency and reproducibility

3. Please submit RNA-seq data to the GEO database and provide a temporary accession number in DATA AVAILABILITY in Method section.

As suggested by the reviewer, the RNA-seq dataset has now been deposited in the GEO database. The accession number has been included in the Data Availability section of the revised manuscript.

4. Please specify the number of replicates are technical or biological replicates for all experiments in related figure legends.

The figure legends have been revised to clearly specify whether replicates correspond to biological or technical replicates.

5. Please add the information of ACC1/2-flox, Lyz-cre, CD45.1 mice in Mice section of Materials and Methods.

The requested information regarding ACC1/2-flox, Lyz-Cre, and CD45.1 mice has now been added to the Mice subsection of the Materials and Methods.

9. Please unify the font, font size, and color labeling (e.g., color correspondence for different groups) of all figures, especially in Fig. 2H and S2D; supply the annotations of each experimental group in the figures especially Fig. S2A-E, S3Fii, S3Fiii, otherwise the readers cannot distinguish which panel belongs to which group at all.

The formatting of all figures has been standardized, including font type, font size, and color schemes for experimental groups. In addition, group annotations have been added to the relevant figures to ensure that each panel can be clearly interpreted.

10. Please correct the figure panel labels throughout the manuscript as plenty of labels are missing or incorrect, such as Fig. S3Aii, Fig. S2G “Cells from (c)…”should be (F)?, last paragraph of Result section “…and PERK (Fig. S7G)” should be S7H?, the first paragraph of the fifth section in Result “…Arginase1 expression (Fig. S5C)” missing, the first paragraph of the third section in Result “…healthy individuals (Fig. 3A, S3Ai, Aii)” there is no Aii, the third paragraph of the third section in Result, last sentence “…immune suppression (Fig. S3Giii, S3H)” there is no S3H.

All figure panel labels and references throughout the manuscript have been thoroughly reviewed and corrected where necessary to ensure consistency and accuracy.

11. Please replace the picture with low resolution, especially Fig. 2H, 3Bi, S2D, can hardly read at present.

As suggested, figures with low resolution have been replaced with higher-resolution versions to improve readability.

12. In Fig. S3A and Ai, labels of cell clusters in Ai "1, 2, 3…" cannot correlate to the names of cell types in S2A.
We thank the reviewer for highlighting this issue. The figures have been corrected and updated in the revised manuscript.

13. Please ensure all abbreviations are defined upon first use. Right now, the use is disordered.

All abbreviations have now been carefully reviewed and defined upon their first appearance to ensure consistency throughout the manuscript.

REVIEWER #2

Major comments:

1. *How does SphK2 KO impact survival of myeloid cells? Or lead to change in their proliferation? The role played by SphK2 on autophagosome machinery and lysosomal clearance will probably have immediate impact on their pro-survival pathways. Is this different for 'MDSC' isolated from tumor vs spleen, given the difference in tissue cues + inflammation?*

We appreciate this important question. To determine whether SphK2 deletion affects myeloid cell survival or proliferation, we quantified total cell numbers, assessed viability by Live/Dead staining, and evaluated cell cycle/autophagic activity using Cyto-ID staining in both tumor- and spleen-derived MDSCs. Across independent experiments, we did not observe significant differences in overall cell survival, accumulation, or proliferative capacity between WT and SphK2-deficient MDSCs. Although Cyto-ID signal was modestly elevated in *SphK2*-KO cells, this did not correspond to altered viability. Instead, given that SphK2 loss promotes maturation of MDSCs toward a more inflammatory, antigen-presenting TAM-like phenotype, the observed change in Cyto-ID staining may reflect increased cellular remodeling associated with enhanced antigen processing rather than altered pro-survival signaling. Importantly, our data do not support a survival advantage or proliferative defect as the driver of the observed immunogenic reprogramming. A detailed mechanistic dissection of autophagic flux and lysosomal dynamics in this context, while of interest, is beyond the scope of the present study.

2. *Conceptually, is there a functional difference between the role of SphK2 in neutrophils, monocytes, vs mo-MDSC? Currently, the use of GR1+ to mark MDSCs (non-specific nomenclature) can lead to confusion in the field, as it would imply this signaling pathways is unique to these cell states found in inflammatory contexts. Hence, comparison of SphK2 level and ACC activity across steady-state myeloid populations and cells denoted tumoral macrophages or MDSCs in the cancer context will be important. Maybe this is a generalizable pathway for macrophage states too?*

We thank the reviewer for this important conceptual point. In the revised manuscript, we have now included additional data examining SphK2 expressions in both monocytic and neutrophil-like PMN MDSCs rather than relying solely on GR1+ gating (see revised Fig. S1H and corresponding text). Specifically, we compared polymorphonuclear MDSCs (PMN-MDSCs) and monocytic MDSCs (M-MDSCs) isolated from spleen and tumor. Our analysis shows that SphK2 expression does not significantly differ between splenic and tumor-derived PMN-MDSCs. In contrast, SphK2 expression is significantly increased in tumor-derived M-MDSCs compared to their splenic counterparts. These findings indicate that SphK2 upregulation is preferentially associated with the monocytic MDSC subset within the tumor microenvironment. Consistent with this, the metabolic and antigen-presenting reprogramming described in our study was most pronounced in M-MDSCs, supporting the conclusion that the SphK2–ACC–PC axis is particularly relevant to tumor-conditioned monocytic MDSCs.

3. *Relatedly, it will be important to check how acute SphK2 KO influences MDSC-to-macrophage differentiation bottleneck in cancer. For this, the authors could do short window of differentiation using MDSC-like cells isolated from tumor-bearing SphK2 KO mice and transferred into congenic host.*

To determine whether SphK2 KO influences MDSC-to-macrophage differentiation *in vivo*, we performed congenic adoptive transfer experiments (***Figs. S2I-K***). In vitro–generated WT or SphK2 KO CD45.2$^+$ MDSCs were transferred into B16 tumor-bearing CD45.1$^+$ hosts, and donor-derived CD45.2$^+$ cells were analyzed within the tumor microenvironment. We observed that WT CD45.2$^+$ MDSCs exhibited limited maturation toward Ly6C^{low} F4/80$^+$ macrophage-like cells. In contrast, SphK2 KO donor MDSCs showed enhanced differentiation toward this more mature TAM-like phenotype. Furthermore, Ly6C$^+$ WT donor cells expressed lower levels of CD80, CD86, and MHC-I compared to SphK2 KO donor cells.
Consistent with these *in vivo* findings, *in vitro* differentiation of WT or SphK2 KO bone marrow–derived cells into macrophages under tumor explant supernatant conditions revealed that SphK2 KO macrophages (CD11b+ F4/80+) exhibited increased expression of CD40, CD80, CD86, MHC-I (H-2Kb), iNOS, and IL-12, along with decreased Arginase-1 and IL-10 (***Figs. S2L-M***). Together, these data indicate that SphK2 KO promotes M-MDSC differentiation into an immunogenic TAM-like state, characterized by enhanced antigen-presenting capacity and the potential to induce stronger anti-tumor T-cell responses.

4. *Also, is there a correlation between intratumoral myeloid cell SphK2 levels and circulating monocyte SphK2 levels? How early (in circulation vs tissue) is this metabolic change occurring?*

We thank the reviewer for this insightful question. To determine whether SphK2 upregulation occurs systemically or is primarily induced within the TME, we analyzed SphK2 expression in splenic M-MDSCs and compared it with intratumoral M-MDSCs isolated from the same tumor-bearing mice. As shown in the revised ***Fig. S1E***, intratumoral M-MDSCs consistently exhibited significantly higher SphK2 expression than their splenic counterparts across all tumor size groups. While splenic M-MDSCs showed a modest increase in SphK2 expression with increasing tumor burden, the magnitude of this change was substantially lower than that observed in tumor-infiltrating cells. To further address when this metabolic change occurs, we assessed SphK2 expression in intratumoral M-MDSCs across tumors of increasing size. We observed a progressive increase in SphK2 expression with tumor growth, suggesting that SphK2 upregulation is initiated locally after myeloid cell infiltration into the tumor and becomes more pronounced as the tumor microenvironment evolves. Together, these findings indicate that although tumor progression may modestly influence systemic myeloid cells, the strongest induction of SphK2 occurs within the tumor microenvironment, supporting a model in which tumor-derived cues drive SphK2-mediated metabolic reprogramming of infiltrating MDSCs.

5. *In Fig 3B, the TMA analysis of SphK2 is insufficient without clear visualization of localization in HLA-DRlow MDSC-like myeloid cells? For robustness and completeness, can the authors show SphK2 in HLA-DRlow MDSC-like cells, vs HLA-DRhi macrophages and/or neutrophils? If the assertion is true, then ideally there is correlation for MDSCs but not for other myeloid cells.*

We thank the reviewer for this thoughtful suggestion. In our human prostate and TNBC tumor specimens, HLA-DR^{low}CD14$^+$ monocytic MDSC-like cells represented the predominant suppressive myeloid population within the tumor microenvironment. In contrast, CD15$^+$ PMN-MDSC–like cells and HLA-DR^{hi} mature macrophage populations were present at very low frequency in these tumor sections, limiting our ability to perform a robust quantitative comparison across all subsets. Given the relative abundance of monocytic MDSC-like cells in these tumor types, our analysis focused on this dominant suppressive population, where SphK2 expression was clearly enriched. Notably, limited infiltration of PMN-MDSC–like cells in certain human tumor types has also been reported in prior high-impact studies (e.g., PMID: 32294407), where consistent analysis of PMN-MDSCs was similarly constrained by low abundance in human ovarian tumors. In our study, SphK2 expression was clearly enriched in monocytic MDSC-like cells, the dominant suppressive subset in these tumors. Importantly, in our murine tumor models, we directly compared SphK2 expression between PMN-MDSCs and M-MDSCs and found that SphK2 expression was significantly increased in tumor-derived M-MDSCs but not in PMN-MDSCs. These findings across species

indicate that SphK2 upregulation is preferentially associated with the monocytic MDSC subset rather than representing a universal feature of all myeloid populations.

6. *Can the authors describe what are other regions impacted, beyond H2 loci (Fig. S1H)? Potentially using tools such as GREAT to annotate genomic regions can help. Currently this analysis is under-developed and does not explain why cells become immunosuppressive beyond increase in MHC.*

We thank the reviewer for this important suggestion. To comprehensively characterize genomic regions impacted beyond the H2 locus, we performed GREAT (Genomic Regions Enrichment of Annotations Tool) analysis on the differentially accessible chromatin regions identified in SphK2 KO MDSCs. A total of 11,356 open chromatin regions were analyzed, of which only 33 (0.3%) were not associated with any gene, indicating that most differential regions are linked to gene regulatory domains. Given that the dataset covered a substantial fraction of the genome, we focused primarily on region-based (binomial) enrichment metrics, which are more appropriate for large chromatin datasets.

Importantly, GREAT annotation revealed that chromatin remodeling in *SphK2*-KO MDSCs extends well beyond the H2 locus and is significantly enriched in biological processes related to regulation of myeloid leukocyte–mediated immunity, myeloid leukocyte activation, leukocyte degranulation, Toll-like receptor signaling, response to lipopolysaccharide, leukocyte migration, and MAPK pathway regulation. In addition, enriched cellular component categories included phagocytic vesicle membranes, autophagosomes, and mitochondrial membranes. These findings indicate that SphK2 deletion induces broad transcriptional reprogramming of immune regulatory and metabolic pathways, rather than selectively affecting MHC loci. Together, these results demonstrate that SphK2-mediated chromatin remodeling influences multiple immune and metabolic programs that collectively contribute to the immunosuppressive phenotype of tumor-associated MDSCs.

7. *Is phosphatidylcholine (PC) species upregulation observed in lipidomics directly a result of SphK2 inhibition or indirect due to chronic effect of KO? Here, comparing acute S1P imbalance using small molecule inhibitor ABC294640 vs SphK2 KO could dissect the clear reason for PC enrichment.*

To determine whether the phosphatidylcholine (PC) enrichment observed in SphK2-deficient MDSCs reflects a direct consequence of acute SphK2 inhibition rather than a chronic adaptation to genetic deletion, we isolated tumor-derived monocytic MDSCs from EL-4 tumor-bearing mice treated with the SphK2 inhibitor ABC294640 or vehicle control and measured PC abundance using a commercial kit. We found that total PC abundance was significantly increased in tumor-derived MDSCs from ABC294640-treated mice compared with untreated controls. We have now incorporated this data into the revised manuscript (***Fig. S5J***) and clarified this point in the Results and Discussion.

8. *What are the consensus lipid species upregulated upon KO of SphK2? The current heatmaps in Fig. S4C or Fig. S5E are not informative, it would be helpful to synthesize the data into dominant/consensus classes (diacyl-PCs, alkylacyl-PCs, alkenylacyl-PCs, lyso-PCs etc).*

We thank the reviewer for this suggestion. As suggested, we reorganized the lipid data at the lipid-class level and generated consensus heatmaps summarizing dominant lipid subclasses (revised ***Fig. 4E*** and ***Fig. S5I***). We agree that this has certainly helped to improve the interpretability of this data set.

9. *scRNA analysis (in fig 1C or fig 3A) is not upto standard for the level of this publication, it will be important to show scaled expression (with scale shown). Ideally, instead of UMAP/TSNE based visualization, the authors should show scaled expression on a violin plot for populations of interest*.

We thank the reviewer for this insightful comment. As suggested by the reviewer, in the revised ***Fig. 1C*** and ***Fig. 1D***, we show the scaled mean and percentage expression of Sphk1 and Sphk2 in different immune cell types in GEM models using dot plots and violin plots with scale bars, which are standard, highly used methods to represent scRNA-seq data (PMID: 32066986, PMID: 38782901). In the revised ***Fig. 3A*** and ***Fig. 3B***, we show the scaled mean and percentage expression of Sphk2 and the MDSC markers in different immune cell types in TNBC tumors and normal PBMCs using dot plots. The overall distribution of the cell types within each dataset were shown by UMAP plots as supplementary data (revised ***Fig. S1C, S3A, S3B***).

10. *The reviewer is surprised by the absence of in vivo functional tumor study (burden/survival/immunophenotyping) using LysMCre;ACC-DKO mice. That will add weight to current findings.*

We thank the reviewer for this important suggestion and agree that *in vivo* studies using LyMCreACC double-knockout mice would further strengthen the conclusions. At the time these experiments were performed, the LyMCreACC DKO line was not available, as the colony required re-derivation and is currently being regenerated by our collaborator. However, as per reviewer suggestion and to functionally evaluate the role of myeloid ACC *in vivo*, we performed tumor growth studies using $LysM^{Cre}Acc1^{fl/fl}$ mice in the setting of SphK2 inhibition (ABC294640), with or without dual ACC1/2 inhibition (ND646) (***Figs. 5I, S5H***). Tumor-bearing mice were assigned to the following groups: $Acc1^{fl/fl}$, $Acc1^{fl/fl}$ + ABC294640, $LysM^{Cre}Acc1^{fl/fl}$ + ABC294640, and ABC294640, $LysM^{Cre}Acc1^{fl/fl}$ + ABC294640 + ND646. Myeloid-specific deletion of ACC1 significantly attenuated the tumor control achieved by SphK2 inhibition (**Fig. 5I**: *p* value = 0.0103). Moreover, dual ACC1/2 inhibition with ND646 resulted in an even greater loss of tumor control compared with the ACC1-intact group (*p* value = 0.0001). These findings provide *in vivo* functional validation of the dependence of SphK2 inhibition on myeloid ACC activity.

11. *While not critical, it would be pertinent and valuable to the field to compare how conditioned media (CM) from different tumor lines (with different driver mutations) can lead to differential ACC activity– as a way to assess cues initiating this metabolic change.*

We thank the reviewer for this thoughtful suggestion. To address this point, we evaluated the effect of conditioned media (CM) derived from multiple tumor cell lines with distinct oncogenic drivers on ACC activity in myeloid cells (see attached data). We observed that CM from different tumor lines was capable of modulating ACC activity; however, the magnitude of the effect varied modestly between lines and did not clearly segregate according to specific driver mutations. Given that these experiments were exploratory and not designed to mechanistically dissect mutation-specific signaling pathways, we are cautious not to overinterpret these findings. While the data suggests that tumor-derived soluble factors can influence ACC activity, defining the precise oncogenic cues responsible would require a focused investigation beyond the scope of the current study. As the central aim of this manuscript is to define the functional requirement of the SphK2–ACC axis in regulating anti-tumor immunity, we have not incorporated these exploratory CM comparisons into the main text. We believe such analyses represent an important and interesting direction for future investigations.

Minor comments:

1. *Schematic of signaling pathways proposed in these myeloid cells will be extremely helpful for readers.*
   As suggested, we have included a schematic diagram (*Fig. S8*).
2. *Can the authors provide histograms in colorblind-friendly format, as well as add color legends (for example, Fig. 4C).*
   As suggested, we have changed the color scheme for all figures to make them color-blind friendly.

3. *Please show %variance on PC1 vs PC2 axis (for example, in Fig. 4F or Fig. 5F)*
   As suggested, this has been included in the revised figures.

4. *What does AVG refer to, in Fig. S6A? Please improve the representation of this data and provide statistics (False Discovery Rate or similar).*
   We thank the reviewer for pointing this out. In the original figure, “AVG” referred to the average peptide abundance across biological replicates obtained from the acetylome mass spectrometry analysis. To improve clarity and presentation, we have revised the figure (now shown as Fig. S5K) and replaced the previous table-based representation with a graphical plot showing the total number of unique peptides detected for the indicated proteins in WT MDSCs. The revised figure now clearly presents peptide coverage to justify the selection of candidate proteins for further validation.

We thank the reviewers for their constructive suggestions, which have helped strengthen the manuscript. We hope that our revisions and responses satisfactorily address the concerns raised and that the manuscript is now suitable for publication in *Nature Communications*.

Response to Reviewer's comments (NCOMMS-25-76017A)

We thank the reviewers for their careful evaluation of our revised manuscript and for their positive comments. We have addressed the remaining concerns as outlined below. All concerns are summarized in *italics,* while responses are in regular font.

REVIEWER #1

1. *The ethical approval number for the experiments using human samples should be provided in Method section.*
   We have now included the IRB approval information and protocol numbers for all studies involving human samples in the Methods section.

2. *Authors should state experiment repetition times in all figure legends to ensure clear reproducibility.*
   We appreciate the reviewer's suggestion. We have revised the figure legends throughout the manuscript to indicate the number of biologically independent experiments and/or mice used for each analysis, where applicable.

REVIEWER #1

1. *There are a few minor typos throughout manuscript (for example, word 'fluidity' is misspelt in schematic Fig S8), please proof-read.*
   We thank the reviewer for pointing this out. We have carefully proofread the manuscript and corrected typographical and spelling errors throughout the text and figures, including the error in *Supplementary Fig. 8.*

We thank the reviewers for their constructive suggestions, which have helped strengthen the manuscript. We hope that our revisions and responses satisfactorily address the concerns raised and that the manuscript is now suitable for publication in *Nature Communications*.