Tumor Derived Extracellular Vesicles Drive T Cell Exhaustion in Tumor Microenvironment through Sphingosine Mediated Signaling and Impacting Immunotherapy Outcomes in Ovarian Cancer

Prachi Gupta¹, Ishaque Pulikkal Kadamberi¹, Sonam Mittal¹, Shirng-Wern Tsaih¹, Jasmine George¹, Sudhir Kumar¹, Dileep K. Vijayan²,⁵, Anjali Geethadevi¹, Deepak Parashar¹, Paytsar Topchyan³, Lindsey McAlarnen¹, Brian F Volkman⁴, Weiguo Cui³, Kam Y. J. Zhang⁵, Dolores Di Vizio⁶, Pradeep Chaluvally-Raghavan¹,⁷,⁸, Sunila Pradeep¹,⁷,⁸

Supplementary Figures
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Supplementary Methods
**Sup Figure 1.** A) Representative TEM image of purified EVs from HeyA8 cells. Scale bar, 50nm. B) Characterization of purified EVs using nanoparticle tracking (NTA). C) Western blotting for EV positive markers (TSG101, CD63, ALIX, HRS) and negative marker GM130. D) Western blotting for SPHK1 and the EV markers CD63 and Alix in different sucrose gradient fractions. E) GFP-tagged SPHK1 was ectopically expressed in HeyA8 cells, and its presence in EVs was observed by western blotting. Relative SPHK1 activity was measured by an ELISA based method in whole cell lysate and EVs from HeyA8 (F) and OVCAR5 (G) cells. All results are means of three technical replicates ± SEM. ***p<0.001 compared to negative control by one-way ANOVA. H) S1P concentration was measured by mass spectrometry in culture media incubated with EVs. Media without EVs severed as negative control. Results are mean of two technical replicates ± SEM. **p<0.01 compared to negative control by student’s t-test. I) Schema showing the interaction of TSG101 with SPHK1 during EVs biogenesis that leads to SPHK1 secretion into EVs.
Sup Figure 2. A) A human immune checkpoint qPCR array was performed using cDNA prepared from OVCAR5 cells treated with the S1P signaling inhibitor PF543 (10 µM). Gene expression is represented as Log2 of Ct values; p<0.05 compared to untreated control. Only genes that were ≥1.5-fold downregulated (blue) or upregulated (red) were included in the heat map. Ovarian cancer cell lines B) HeyA8 and C) OVCAR4 were treated with S1P (100 nM) or PF543 (10 µM) for 24 h, and a qPCR array was performed to identify the top four genes upregulated by S1P. Mean ± SEM fold-change values of the indicated genes were shown with respect to control. **p<0.01 ***p<0.001 ****p<0.0001 (one-way ANOVA). The ovarian cancer cell line ID8 Trp53+/−:Brca2−/− was stimulated with the indicated doses of S1P for 24 h, and expression of PD-L1 was detected by D) flow cytometry, Mean fluorescent intensity (MFI) were calculated by Flowjo for triplicate experiments. Data is presented as mean ± SEM. ****p<0.0001 (one-way ANOVA) and E) western blotting. F) ID8 Trp53−/−:Brca2−/− cells were treated with PF543 for 24 h at the indicated doses, and changes in PD-L1 expression were detected by western blotting. G) Schematic diagram of a PD-L1-promotor luciferase reporter assay performed with S1P (10 µM), PF543 (10 µM), FTY720 (10 µM), and JTE013 (10 µM) alone or in combination for 24 h after transfection with vector. H) Luciferase activity was measured in culture medium after OVACR5 cells were treated with the indicated drugs. **p<0.01 ***p<0.001 ****p<0.0001 (one-way ANOVA). I) Bar graph gives the results of a transcription factor array assay performed with OVCAR5 cells stimulated with S1P (100 nM) for 6 h. The x-axis shows the transcription factors, and the y-axis shows the luminescence reading at the end of the assay. J) GFP-tagged SPHK1 was ectopically expressed in HeyA8 cells, and its presence in whole-cell lysates was observed by western blotting. K) Ovarian cancer xenograft mouse models using 1.2x10^6 luciferase-tagged HeyA8 cells were orthotopically injected into the right ovary bursa of female athymic nude mice (N=9 mice/group). Controls were empty vector and SPHK1-expressing vector. The mice from both groups were imaged using biophotonic.
IVIS, and representative photographs were presented. (L) Bar graph indicates the average radiance of luminescence intensity from mice (N=9) in each group. Result is shown as mean with 95% CI. ****p<0.0001 (Student’s t-test). (M) Representative image of a nude mouse from the control and SPHK1-overexpression groups that were surgically opened. Areas circled in blue indicate tumor growth and metastatic locations. (N) Primary and disseminated tumors were collected from M, and total tumor weight was recorded and mean± SEM was plotted. ***p<0.001(Student’s t-test). (O) Bar graph shows the mean of number of tumor nodules in each group. ***p<0.001(Student’s t-test). (P) Western blot analysis of lysates collected from tumor tissues selected from each group (N=3), using the indicated antibodies. β-actin was used as a loading control. (Q) Immunohistochemical (IHC) analysis of tumor tissues selected from each group (N=3) was performed for the indicated antibodies. Positivity is shown as brown staining of DAB. All results are means of three technical replicates ± SEM. **p<0.01 ***p<0.001 ****p<0.0001 compared to control by Student’s t-test otherwise indicated.
Sup Figure 3. A) Bar graph indicates average mouse weight with the indicated EVs. **p<0.01 compared to control by one-way ANOVA. Implantation of RAB27a−/− ID8 cells into the ovary of C57BL/6 mice and subsequent treatment with the indicated EVs (N=5 mice/group). Flow cytometric quantification of the percentages of B) CD8+ and C) CD4+ cells among CD45+ and CD3+ cells in ascites after the mice were injected with indicated EVs. Quantification of Ki67+ cells among (D) CD8+ and E) CD4+ T cells in ascites (N=5/group). Quantification of Granzyme B+ cells among F) CD8+ cells and G) CD4+ T cells in ascites (N=5/group). Quantification of TNF alpha+ cells among H) CD8+ cells and I) CD4+ T cells in ascites (N=5/group). Quantification of PD-1+ cells among J) CD8+ cells and K) CD4+ T cells in ascites (N=5/group). Quantification of TIM3+ cells among L) CD8+ cells and M) CD4+ T cells in ascites (N=5/group). Percentages of FOXP3+ cells, N) CD8+ cells, and O) CD4+ T cells among CD45+ and CD3+ cells in ascites of mice (N=5/group). All results are shown as means ± SEM *p<0.05 **p<0.01 ***p<0.001 compared to control EVs by one-way ANOVA.
Sup Figure 4. A) Implantation of SPHK1<sup>+/+</sup> ID8 cells into the ovary of C57BL/6 mice treated with the indicated EVs (N=5 mice/group). B) By the end of treatment, the mice treated with ectopically expressed EVs containing SPHK1 had a high volume of ascites, whereas the mice treated with control EVs had a smaller volume of ascites. C) Peritoneal cavity of mice showing tumor locations (blue circles) in all treatment groups. Bar graphs indicate mean D) tumor weight, E) ascites volume, and F) body weight with SEM for the mice treated with the EVs. *p<0.05 **p<0.01 compared to control by Student’s t-test. G) Implementation of parental ID8 cells, RAB27a<sup>+/−</sup> ID8 cells, SPHK1<sup>−/−</sup> ID8 cells and SPHK1 ectopic expressing (EE) ID8 cells (N=5 mice/group) into the ovary of C57BL/6 mice. General appearance of mice at the end of treatment. Mice implanted with SPHK1 EE cells showed higher volume of ascites compared to RAB27a<sup>+/−</sup> ID8 cells and SPHK1<sup>−/−</sup> ID8 cells group. H) Peritoneal cavity of mice showing tumor locations (blue circles) in all groups. Bar graphs indicate mean I) ascites volume, J) tumor weight, and K) body weight with SEM for the mice injected with indicated ID8 cells. **p<0.01 ***p<0.001 compared to parental ID8 cells by one-way ANOVA.
Sup Figure 5. A) Human blood CD8+ T cells were stimulated with S1P, and Ki67 was measured by flow cytometry. Percentages of CD8+ Ki67+ cells were determined by FlowJo. Adjacent bar diagrams represent means ± SEM of cumulative data from three experiments. ****p<0.0001 (one-way ANOVA). (B-G) A human cytokine bead array was performed to determine levels of the indicated cytokines in indicated EVs treated human CD8+ T cells. Results are presented as means ± SEM of technical triplicates. *p<0.05 **p<0.01 ***p<0.001 compared to stimulated and untreated T cells by one-way ANOVA. (H-M) A human cytokine bead array was performed to determine levels of the indicated cytokines in S1P-treated human CD8+ T cells. Results are presented as means ± SEM of technical triplicates. **p<0.01 ***p<0.001 compared to stimulated and untreated T cells by one-way ANOVA. N) Schema is showing isolation of dendritic cells and CD8 T cells from mice and activation of T cells in the presence of S1P in preparation for an IncuCyte and flow cytometry experiment. O) An IncuCyte experiment was performed with activated T cells with or without S1P and ID8 tumor cells. Red color shows staining by annexin-v dye, indicating cell death. P) The total red object area is the IncuCyte count indicating cell death at different time points. Median and error was plotted with 95% CI. 2way ANOVA is applied to know the significance. Q-S) Mouse CD8+ T cells were isolated from spleen and stimulated with S1P. Staining of Ki67, granzyme B, and PD-1 was measured by flow cytometry. Percentages of CD8+ Ki67+ cells, CD8+ granzyme B+ cells, and CD8+ PD-1+ cells were determined by FlowJo. Results are means of three technical replicates ± SEM. ****p<0.0001 compared to control by one-way ANOVA.
Sup Figure 6. A-B) Flow cytometric quantification of the percentages of TCF1+ CD8+ and CD4+ cells in ascites from control vs PF543 treated group. (N=3) C) IHC staining of S1P in tumor tissues from control and PF543 treatment group. D-H) Flow cytometric quantification of the percentages of CD8+ and CD4+ cells among CD45+ and CD3+ cells in the (D) spleen, (E) lymph nodes, (F) bone marrow, (G) liver, and (H) lung in control vs. PF543- treated mice bearing ID8 tumors (N=3/group). I-N) Flow cytometric quantification of the percentages of CD8+ and CD4+ cells among CD45+ and CD3+ cells in the (I) bone marrow, (J) liver (K) lung, (L) lymph nodes, (M) spleen, and (N) blood in control and PF543-treated normal mice (N=3/group). O) Representative contour plots showing the general gating strategy used to identify CD8+ and CD4+ T cells in the mouse samples. Adjacent bar diagrams represent cumulative data from three experiments. Results are presented as means ± SEM. **p<0.01 ***p<0.001 ****p<0.0001 in comparison to control or normal (Student’s t test).
A. TCGA OV RNA seq
   SPHK1: high vs low group

376 unique subjects

High SPHK1 group (>median value 863.8)  N=94
Low SPHK1 group  N=282

Differential Expression analysis

Total of 11,036 genes were statistically differentially expressed between SPHK1 high group and SPHK1 low group with FDR 0.05 cutoff

Upregulated =5,478 genes  Down regulated =4,325 genes

B. log2 (PD-L1)

C. log2 (PDCD1)

D. log2 (FOXP3)

E. log2 (E2F1)

F. Enrichment plot:
   Hallmark_EMT
   NES=3.11  P=0.001

   ECM1  TGFBI  ECM2  SPOKC1
   COL5A3  LOX  NID2  TNFAIP3
   ADAM12  SNAI2  SFRP4  SPARC
   COL5A2  COL16A1  FSTL1  LUM
   ITGA5  SERPINE1  F3H1  CTHRC1
   FAP  COL4A1  CCN2  CCN1
   LRR15  TIMP3  COMP  FBLN2
   COL11A1  NT5E  CXCL12  LRP1
   THBS2  DCN  ITGB5  EMP3
   FN1  VCAM1  TNC  ITGB1
   COL5A1  SCG2  PMEPAL1  THY1
   GREM1  BMP1  TAGLN  LOX1L2
   POSTN  GEM  SDC1  FBN1
   MMP14  DAB2  NNMT  COL1A2
   PRX1  CDH11  COL8A2  THBS1
   INHBI  FSTL3  SERPINH1  BGN
   COL1A1  EDIL3  MATN3  WIF1
   COL3A1  PLAU  GADD45B  COL4A2
   NTM  GLIFR1  COL7A1  MXRA5
   PDGFRA  PMP22  HTRA1  IGFBP4
   MMP2  ACTA2  GJA1  GAS1
   COL6A2  PCOLCE  TGM2  MMP1
   VCAN  RBM4  MMP3  TPM1
   COL6A3  LOXL1  DPYSL3

   SPHK1  STAB1  TNFRSF1B
   ITGA5  ICAM1  PVR
   MMP14  ABCA1  IL18R1
   INHBA  CMKL1  OSM
   PTGIR  PDE4B  NFKB1
   IL7R  CD14  BDKRB1
   TNFAIP6  LCP2  KLF6
   SERPINE1  IL10  CCL2
   RGS16  FPR1  PTAFL
   PDPN  TL2  KON3
   APLNR  CCRL2  ITGB3
   PLAU  CYBB  CD48
   AXI  SELE  SELE
   TNFRSF9  IL2RB  IL18RAP
   PIK3R5  IL4R  EREG
   SCARF1  CDKN1A  P2RX7
   NLRP3  NOD2  HER1F
   PTPRE  OSM  MARCO
   IL10RA  RGS1  SLAMF1
   NLR3  EMP3  NOD2
   HBEGF  MSR1  PTGER2
   GPR183  GPR132  EB13
   LIF  PTGER4  LCK
   OLR1  CLEC5A  HIF1A
   C3AR1  IL1B  IL6
   SLC33A2  TLR1  TPGB
   HAS2  MEFV  CXCL9
   GNA15  CCR7  CCL2
   TNSF9  LPAR1  LTA
   MMP11  CMKL1
   PLAU  CSF2RA
   PRRX1  MMP9
   INHBA  CD37
   IL7R  ITGB2
   TMEM158  LAPT5
   PRDM1  TL8
   RS16  ENG
   GFPT2  TRAF1
   ITGB1  ADAM8
   GUCY1A1  LAT2
   PLAU  CLEC4A
   MAFB  NIN
   ETS1  IRF8
   ET1  TNFAIP3
   ADGRA2  ADAMDEC1
   PLAV  IL1B
   PDCD1L2  TNFRSF1B
   PECAM1  NRP1
   DOCK2  CIDEA
   GPNMB  CFH
   EBF4L3  GLRX
   IL10RA  IL12R
   IL1B
   HBEVF  EMP1
   TMEM376B
   ADGR4  SPP1
   I2F1  ALDH1A3
**Sup Figure. 7.** A) Flow chart shows the strategy used to analyze TCGA ovarian cancer RNA seq data for SPHK1 expression. B-E) Box plot analysis, using TCGA dataset, of expression of the indicated genes in the patient groups with low vs. high SPHK1 expression. **p<0.001 (Student's t-test)** F) Gene set enrichment analysis of the genes with high SPHK1 expression, using cancer hallmarks database using the Molecular Signatures Database Hallmark Gene Set Collection (GSEA 4.1.0.).
Sup Figure. 8. IHC staining of tissue sections from Human A) Normal ovary and B) Tumor ovary from different subjects. Brown staining is DAB showing positive expression of S1P in tumor microenvironment. 20x images were taken by case viewer. Scale bar=50μM.
| Position | Gene Symbol | Fold Change (comparing to control) | Group 1 (S1P) | Group 2 (PF543) |
|----------|-------------|-------------------------------------|---------------|----------------|
| A01      | TAP1        | 0.67                               | 0.1054        | 0.49           | 0.2223        |
| A02      | TAP2        | 0.41                               | 0.1288        | 1.06           | 0.8918        |
| A03      | JAK         | 1.15                               | 0.3399        | 0.64           | 0.4559        |
| A04      | CASP10      | 0.63                               | 0.2326        | 0.4            | 0.1018        |
| A05      | CASP3       | 0.55                               | 0.0974        | 0.92           | 0.5772        |
| A06      | CASP6       | 0.63                               | 0.1255        | 0.27           | 0.0328        |
| A07      | CASP7       | 0.59                               | 0.0804        | 0.74           | 0.1888        |
| A08      | JAK1        | 0.92                               | 0.6318        | 0.48           | 0.1359        |
| A09      | DAXX        | 0.81                               | 0.6100        | 0.23           | 0.0513        |
| A10      | FADD        | 0.9                                | 0.3937        | 1.63           | 0.0079        |
| A11      | FAS         | 0.7                                | 0.5770        | 0.55           | 0.2365        |
| A12      | HMOX1       | 0.64                               | 0.3293        | 0.36           | 0.1638        |
| B01      | STAT3       | 0.83                               | 0.4385        | 0.28           | 0.0718        |
| B02      | TNFRSF10A   | 0.6                                | 0.2173        | 0.79           | 0.4233        |
| B03      | TNFRSF10B   | 0.69                               | 0.3311        | 0.38           | 0.1857        |
| B04      | TNFRSF18    | 0.76                               | 0.1775        | 0.13           | 0.0025        |
| B05      | TNFRSF9     | 0.89                               | 0.6999        | 0.37           | 0.0042        |
| B06      | TRIM39      | 0.88                               | 0.2745        | 0.6            | 0.0749        |
| B07      | GUCY1A3     | 1.62                               | 0.3049        | 0.99           | 0.7716        |
| B08      | ADORA2A     | 0.67                               | 0.0314        | 0.08           | 0.0021        |
| B09      | ICOSLG      | 0.58                               | 0.0286        | 0.24           | 0.0309        |
| B10      | IDO1        | 0.98                               | 0.7360        | 0.28           | 0.0863        |
| B11      | OR2H2       | 0.55                               | 0.0010        | 0.25           | 0.0046        |
| B12      | IL6         | 1.48                               | 0.1503        | 0.21           | 0.0003        |
| C01      | PDCD1       | 0.65                               | 0.1736        | 0.43           | 0.0422        |
|   |   |   |   |   |
|---|---|---|---|---|
| C02 | PTPN6 | 1.04 | 0.1343 | 0.50 | 0.0002 |
| C03 | BTLA | 0.82 | 0.7283 | 0.62 | 0.0026 |
| C04 | CD24 | 0.95 | 0.7286 | 0.17 | 0.0001 |
| C05 | CD27 | 0.37 | 0.0358 | 0.09 | 0.0020 |
| C06 | CD274 | 2.14 | 0.0533 | 0.30 | 0.0547 |
| C07 | CD276 | 0.17 | 0.0575 | 0.37 | 0.0410 |
| C08 | CD70 | 0.83 | 0.1734 | 0.53 | 0.0006 |
| C09 | HAVCR2 | 0.96 | 0.8751 | 0.66 | 0.1284 |
| C10 | HLA- DMA | 0.94 | 0.2514 | 0.58 | 0.0016 |
| C11 | HLA- DMB | 3.21 | 0.3087 | 0.42 | 0.5881 |
| C12 | ICOS | 1.36 | 0.1796 | 1.02 | 0.8328 |
| D01 | IL10 | 0.74 | 0.2854 | 0.64 | 0.0361 |
| D02 | IL10RA | 1.49 | 0.0412 | 0.56 | 0.0011 |
| D03 | IL10RB | 0.85 | 0.0878 | 0.73 | 0.0093 |
| D04 | IL18 | 1.07 | 0.9068 | 0.80 | 0.3451 |
| D05 | IL1A | 2.92 | 0.1853 | 1.36 | 0.8832 |
| D06 | JAK3 | 0.56 | 0.0717 | 0.24 | 0.0120 |
| D07 | MICA | 0.80 | 0.7379 | 0.07 | 0.0032 |
| D08 | MICB | 0.95 | 0.8508 | 0.20 | 0.0120 |
| D09 | PDCD1LG2 | 1.14 | 0.5236 | 0.31 | 0.0023 |
| D10 | TNFRSF4 | 0.47 | 0.0273 | 0.39 | 0.0086 |
| D11 | TNFSF14 | 1.28 | 0.6939 | 0.18 | 0.0528 |
| D12 | TNFSF9 | 0.43 | 0.0208 | 0.43 | 0.2257 |
| E01 | CCL2 | 1.37 | 0.0007 | 0.37 | 0.0000 |
| E02 | HRH4 | 1.30 | 0.0032 | 0.57 | 0.1566 |
| E03 | NT5E | 0.97 | 0.6688 | 0.61 | 0.0006 |
| E04 | PDCD4 | 1.21 | 0.0908 | 0.99 | 0.8662 |
| E05 | CD244 | 1.15 | 0.5033 | 0.97 | 0.9809 |
| E06 | CD28 | 0.42 | 0.0515 | 0.35 | 0.0293 |
| E07 | CD4 | 0.34 | 0.0191 | 0.22 | 0.0026 |
|   |   |   |   |   |   |
|---|---|---|---|---|---|
| E08 | CD80 | 0.45 | 0.0445 | 0.46 | 0.0244 |
| E09 | CD86 | 0.59 | 0.2063 | 0.33 | 0.0728 |
| E10 | PIK3CA | 1.24 | 0.0184 | 0.75 | 0.0434 |
| E11 | PSMB8 | 0.97 | 0.6618 | 0.88 | 0.0850 |
| E12 | PSMB9 | 0.7 | 0.2857 | 0.88 | 0.5589 |
| F01 | PTPN11 | 1.01 | 0.9729 | 0.74 | 0.0513 |
| F02 | LGALS9 | 0.81 | 0.2338 | 0.13 | 0.0041 |
| F03 | VTCN1 | 0.61 | 0.2937 | 0.53 | 0.0408 |
| F04 | IL12B | 0.94 | 0.6828 | 0.44 | 0.2421 |
| F05 | TUBB | 0.68 | 0.0141 | 0.78 | 0.0706 |
| F06 | CTLA4 | 0.52 | 0.1494 | 0.44 | 0.0882 |
| F07 | FOXP3 | 0.53 | 0.0710 | 0.35 | 0.0059 |
| F08 | STAT1 | 1.24 | 0.0275 | 1.13 | 0.1467 |
| F09 | FLOT1 | 1.06 | 0.2079 | 0.79 | 0.0147 |
| F10 | TNFRSF14 | 0.89 | 0.1791 | 0.43 | 0.0068 |
| F11 | LAG3 | 1.2 | 0.7175 | 2.92 | 0.4969 |
| F12 | CD160 | 0.55 | 0.0457 | 0.17 | 0.0018 |
| G01 | CD247 | 1 | 0.8961 | 0.25 | 0.0692 |
| G02 | STAT5A | 0.85 | 0.3886 | 0.36 | 0.0424 |
| G03 | CD40LG | 0.66 | 0.6016 | 0.13 | 0.2048 |
| G04 | CD96 | 1.4 | 0.4901 | 0.72 | 0.4029 |
| G05 | IL6ST | 1.24 | 0.7144 | 0.81 | 0.3857 |
| G06 | IFNG | 0.75 | 0.4155 | 0.63 | 0.2111 |
| G07 | KIR3DL1 | 0.41 | 0.1786 | 0.18 | 0.1322 |
| G08 | KLRG1 | 0.78 | 0.2697 | 0.24 | 0.0010 |
| G09 | TGFBRII | 0.84 | 0.0696 | 0.74 | 0.0240 |
| G10 | TNFSF4 | 1.12 | 0.4610 | 0.85 | 0.0004 |
| G11 | FOXO1 | 1.12 | 0.4345 | 0.97 | 0.8297 |
| G12 | IRF1 | 0.95 | 0.3523 | 0.88 | 0.9515 |
| H01 | ACTB | 0.65 | 0.1031 | 0.72 | 0.1794 |
| H02  | B2M  | 1.2  | 0.0353 | 0.86  | 0.0982 |
|------|------|------|--------|-------|--------|
| H03  | GAPDH| 0.87 | 0.1051 | 1.63  | 0.0074 |
| H04  | HPRT1| 1.15 | 0.0477 | 1     | 0.9573 |
| H05  | RPLP0| 1.28 | 0.0070 | 0.98  | 0.8534 |
| H06  | GDC  | 0.98 | 0.9351 | 0.82  | 0.3768 |
Sup Table 2 E2F1 binding sites predicted by MatInspector on PD-L1 promotor

| Matrix ID | Name | Score | Relative score | Start | End | Strand | Predicted sequence |
|-----------|------|-------|----------------|-------|-----|--------|-------------------|
| MA0024.2  | E2F1 | 11.7459 | 0.944735071336 | 450   | 460 | +      | CTCGCGGGAAC       |
| MA0024.2  | E2F1 | 7.51648 | 0.881105040123 | 406   | 416 | -      | CGCGCGGAAAG       |
| MA0024.1  | E2F1 | 8.83194 | 0.87025055575  | 125   | 132 | -      | TTTGGCAC          |
| MA0024.1  | E2F1 | 8.83194 | 0.87025055575  | 319   | 326 | -      | CTTGGGC            |
| MA0024.1  | E2F1 | 8.32861 | 0.852930971432 | 408   | 415 | +      | TTCCGCG            |
| MA0024.2  | E2F1 | 5.62925 | 0.85271214037  | 61    | 71  | +      | ATGGTGGAAC         |
| MA0024.3  | E2F1 | 8.68073 | 0.834170048106 | 121   | 132 | +      | GATGGTGACAAA       |
| MA0024.2  | E2F1 | 3.31535 | 0.817900176445 | 122   | 132 | +      | ATGGTGQCAAAA       |
| MA0024.2  | E2F1 | 3.29836 | 0.817644509905 | 449   | 459 | -      | TTCCGC            |
| MA0024.3  | E2F1 | 7.17397 | 0.809082621095 | 121   | 132 | -      | TTTGGGACCATC       |
| MA0024.2  | E2F1 | 2.67957 | 0.808335054732 | 371   | 381 | -      | GGAGCGGC           |
| Female Age | Organ/Anatomic Site | Pathology diagnosis                  | TNM     | Grade | Stage | Type      | SPHK 1 staining intensity | PDL1 staining intensity |
|------------|---------------------|--------------------------------------|---------|-------|-------|-----------|---------------------------|------------------------|
| 65         | Ovary               | Low grade serous carcinoma           | T1N0M0  | -     | I     | Malignant | 3                         | 3                      |
| 38         | Ovary               | Low grade serous carcinoma           | T3cN1M0 | -     | IIIC  | Malignant | 3                         | 3                      |
| 51         | Ovary               | High grade serous carcinoma          | T3cN1M0 | -     | IIIC  | Malignant | 3                         | 3                      |
| 22         | Ovary               | High grade serous carcinoma          | T2bN0M0 | -     | IIB   | Malignant | 3                         | 3                      |
| 48         | Ovary               | Low grade serous carcinoma           | T1N0M0  | -     | I     | Malignant | 3                         | 3                      |
| 26         | Ovary               | High grade serous carcinoma          | T3cN1M0 | -     | IIIC  | Malignant | 3                         | 3                      |
| 25         | Ovary               | Low grade serous carcinoma           | T1N0M0  | -     | I     | Malignant | 3                         | 3                      |
| 50         | Ovary               | High grade serous carcinoma          | T2N0M0  | *     | II    | Malignant | 2                         | 3                      |
| 26         | Ovary               | High grade serous carcinoma          | T1cN0M0 | -     | IC    | Malignant | 2                         | 3                      |
| 47         | Ovary               | High grade serous carcinoma          | T1N0M0  | -     | I     | Malignant | 3                         | 3                      |
| 58         | Ovary               | High grade serous carcinoma          | T1N0M0  | -     | I     | Malignant | 3                         | 3                      |
|   | Org | Diagnosis                          | Stage | Grade | TNM | Stage   | Malignant |
|---|-----|-----------------------------------|-------|-------|-----|---------|-----------|
| 57 | Ovary | High grade serous carcinoma      | T1cN0M0 | -     | IC  | Malignant | 3         |
| 51 | Ovary | High grade serous carcinoma      | T1aN0M0 | *     | IA  | Malignant | 3         |
| 52 | Ovary | High grade serous carcinoma      | T2N0M0  | -     | II  | Malignant | 3         |
| 54 | Ovary | High grade serous carcinoma      | T3cN1M0 | -     | IIIC | Malignant | 3         |
| 33 | Ovary | High grade serous carcinoma      | T1N0M0  | -     | I   | Malignant | 2         |
| 56 | Ovary | High grade serous carcinoma      | T2N0M0  | -     | II  | Malignant | 2         |
| 41 | Ovary | High grade serous carcinoma      | T1N0M0  | -     | I   | Malignant | 2         |
| 46 | Ovary | High grade serous carcinoma      | T3aN0M0 | -     | III | Malignant | 3         |
| 46 | Ovary | High grade serous carcinoma      | T2cN1M0 | *     | IIIC | Malignant | 3         |
| 57 | Ovary | High grade serous carcinoma      | T3cN1M0 | -     | IIIC | Malignant | 3         |
| 75 | Ovary | High grade serous carcinoma      | T2N0M0  | -     | II  | Malignant | 2         |
| 54 | Ovary | High grade serous carcinoma      | T3cN1M0 | -     | IIIC | Malignant | 3         |
| 49 | Ovary | High grade serous carcinoma      | T2N0M0  | -     | II  | Malignant | 2         |
| 50 | Ovary | High grade serous carcinoma      | T1N0M0  | -     | I   | Malignant | 3         |
| 52 | Ovary | High grade serous carcinoma      | T2N0M0  | *     | II  | Malignant | 1         |
|   | Tissue  | Histologic Type                         | Stage   | Grade | Stage  | Malignancy | 2 | 2 |
|---|---------|----------------------------------------|---------|-------|--------|------------|---|---|
| 47| Ovary   | High grade serous carcinoma            | T3cN1M0 | -     | IIIC   | Malignant  | 2 | 2 |
| 34| Ovary   | Mucinous adenocarcinoma                 | T1bN0M0 | 2     | IB     | Malignant  | 2 | 1 |
| 63| Ovary   | Mucinous adenocarcinoma                 | T1aN0M0 | 1     | IA     | Malignant  | 1 | 1 |
| 69| Ovary   | Mucinous adenocarcinoma                 | T1bN0M0 | 2     | IB     | Malignant  | 1 | 1 |
| 46| Ovary   | Endometrioid adenocarcinoma             | T2N0M0  | 1-2   | II     | Malignant  | 3 | 2 |
| 47| Ovary   | Endometrioid adenocarcinoma             | T2aN0M0 | 1-2   | IIA    | Malignant  | 1 | 2 |
| 54| Ovary   | Endometrioid adenocarcinoma             | T1bN0M0 | 1-2   | IB     | Malignant  | 2 | 2 |
| 65| Ovary   | Adenocarcinoma (sparse)                | T1cN0M0 | 2     | IC     | Malignant  | 2 | 2 |
| 55| Ovary   | Endometrioid adenocarcinoma             | T1N0M0  | 2     | I      | Malignant  | 3 | 2 |
| 54| Ovary   | Endometrioid adenocarcinoma             | T1bN0M0 | 1     | IB     | Malignant  | 2 | 2 |
| 43| Ovary   | Endometrioid adenocarcinoma             | T1cN0M0 | 2     | IC     | Malignant  | 2 | 2 |
| 55| Ovary   | Endometrioid adenocarcinoma with necrosis | T1N0M0  | 2     | I      | Malignant  | 1 | 2 |
| 53| Ovary   | Endometrioid adenocarcinoma             | T2aN0M0 | 3     | IIA    | Malignant  | 3 | 2 |
| 50| Ovary   | Endometrioid adenocarcinoma             | T3bN1M0 | 2     | IIIC   | Malignant  | 2 | 2 |
|   | Location | Tumor Type                                           | Tumor Stage | Grade | Grade Category | Histology |   |
|---|----------|-----------------------------------------------------|-------------|-------|----------------|-----------|---|
| 51 | Ovary    | Invasive urothelial carcinoma                       | T1bN0M0     | -     | IB             | Malignant | 2 |
| 39 | Ovary    | Invasive urothelial carcinoma                       | T1aN0M0     | -     | IA             | Malignant | 3 |
| 38 | Ovary    | Invasive urothelial carcinoma                       | T1N0M0      | -     | I              | Malignant | 3 |
| 66 | Ovary    | Invasive urothelial carcinoma with squamous metaplasia | T1aN0M0     | -     | IA             | Malignant | 1 |
| 53 | Ovary    | Invasive urothelial carcinoma                       | T1N0M0      | -     | I              | Malignant | 1 |
| 47 | Mesentery| Metastatic papillary adenocarcinoma from ovary      |             | -     | 2              |           | 2 |
| 57 | Greater omentum | Metastatic papillary adenocarcinoma from ovary |             | -     | 2              |           | 3 |
| 65 | Greater omentum | Metastatic papillary adenocarcinoma from ovary |             | -     | 2              |           | 3 |
| 59 | Mesentery| Metastatic papillary adenocarcinoma from ovary      |             | -     | 2              |           | 3 |
| 28 | Greater omentum | Metastatic papillary adenocarcinoma with calcification from ovary | | -     | 2              |           | 2 |
| 64 | Greater omentum | Metastatic papillary adenocarcinoma from ovary |             | -     | 2              |           | 3 |
|      |          |                                                                 |    |    |                     |
|------|----------|-----------------------------------------------------------------|----|----|---------------------|
| 50   | Greater  | Metastatic papillary adenocarcinoma from ovary                  | -  | 2  | Metastasis          |
|      | omentum  |                                                                 |    |    |                     |
| 58   | Greater  | Metastatic adenocarcinoma from ovary                            | -  | 3  | Metastasis          |
|      | omentum  |                                                                 |    |    |                     |
| 47   | Peritoneum| Metastatic adenocarcinoma from ovary                           | -  | 3  | Metastasis          |
|      | um       |                                                                 |    |    |                     |
| 49   | Peritoneum| Metastatic adenocarcinoma from ovary                           | -  | 3  | Metastasis          |
|      | um       |                                                                 |    |    |                     |
| 34   | Ovary    | Borderline serous papillary cystadenoma                         | -  | -  | Borderline          |
|      |          |                                                                 |    |    |                     |
| 34   | Ovary    | Borderline serous papillary cystadenoma                         | -  | -  | Borderline          |
|      |          |                                                                 |    |    |                     |
| 28   | Ovary    | Borderline serous papillary cystadenoma                         | -  | -  | Borderline          |
|      |          |                                                                 |    |    |                     |
| 22   | Ovary    | Borderline mucinous papillary cystadenoma                       | -  | -  | Borderline          |
|      |          |                                                                 |    |    |                     |
| 60   | Ovary    | Borderline serous papillary cystadenoma                         | -  | -  | Borderline          |
|      |          |                                                                 |    |    |                     |
| 50   | Ovary    | Borderline serous papillary cystadenoma                         | -  | -  | Borderline          |
|   |   |                   |   |   |                     |   |   |        |
|---|---|-------------------|---|---|---------------------|---|---|--------|
|37 | Ovary | Borderline mucinous papillary cystadenoma | - | - | Borderline         | 1 | 1 |        |
|62 | Ovary | Serous cystadenoma | - | - | Benign              | 2 | 2 |        |
|70 | Ovary | Serous cystadenoma | - | - | Benign              | 2 | 2 |        |
|49 | Ovary | Serous cystadenoma | - | - | Benign              | 2 | 2 |        |
|16 | Ovary | Serous cystadenoma | - | * | Benign              | 2 | 2 |        |
|34 | Ovary | Serous cystadenoma | - | - | Benign              | 2 | 2 |        |
|22 | Ovary | Serous cystadenoma | - | - | Benign              | 2 | 2 |        |
|19 | Ovary | Mucinous cystadenoma | - | - | Benign              | 2 | 2 |        |
|17 | Ovary | Mucinous cystadenoma | - | - | Benign              | 2 | 2 |        |
|41 | Uterus | Mucinous cystadenoma | - | - | Benign              | 2 | 2 |        |
|26 | Ovary | Mucinous cystadenoma | - | - | Benign              | 2 | 2 |        |
|22 | Ovary | Mucinous cystadenoma | - | * | Benign              | 2 | 2 |        |
|38 | Ovary | Mucinous cystadenoma | - | * | Benign              | 2 | 2 |        |
|47 | Ovary | Mucinous cystadenoma | - | - | Benign              | 2 | 2 |        |
| Case | Ovary       | Diagnosis               | - | - | - | Status       | Count |
|------|-------------|-------------------------|---|---|---|--------------|-------|
| 70   | Ovary       | Mucinous cystadenoma    | - | - | - | Benign       | 2     |
| 51   | Ovary       | Mucinous cystadenoma    | - | - | - | Benign       | 2     |
| 29   | Ovary       | Mucinous cystadenoma    | - | - | - | Benign       | 2     |
| 35   | Ovary       | Mucinous cystadenoma    | - | - | - | Benign       | 2     |
| 18   | Ovary       | Mucinous cystadenoma    | - | - | - | Benign       | 2     |
| 30   | Ovary       | Adjacent normal ovary tissue | - | - | - | NAT         | 1     |
| 39   | Ovary       | Adjacent normal ovary tissue | - | - | - | NAT         | 1     |
| 29   | Ovary       | Adjacent normal ovary tissue | - | - | - | NAT         | 1     |
| 41   | Ovary       | Adjacent normal ovary tissue | - | - | - | NAT         | 1     |
| 62   | Ovary       | Adjacent normal ovary tissue | - | - | - | NAT         | 1     |
| 63   | Ovary       | Adjacent normal ovary tissue | - | - | - | NAT         | 1     |
| 45   | Ovary       | Adjacent normal ovary tissue | - | - | - | NAT         | 1     |
| 48   | Ovary       | Adjacent normal ovary tissue | - | - | - | NAT         | 1     |
| 53   | Ovary       | Adjacent normal ovary tissue | - | - | - | NAT         | 1     |
| 53   | Ovary       | Adjacent normal ovary tissue | - | - | - | NAT         | 1     |
| No. | Tissue | Description | Comments | Grade | Confirmation |
|-----|--------|-------------|----------|-------|--------------|
| 57  | Ovary  | Adjacent normal ovary tissue | - | - | - | NAT | 1 1 |
| 38  | Ovary  | Adjacent normal ovary tissue | - | - | - | NAT | 1 1 |
| 53  | Ovary  | Adjacent normal ovary tissue | - | - | - | NAT | 1 1 |
| 59  | Ovary  | Adjacent normal ovary tissue | - | - | - | NAT | 1 1 |
| 48  | Ovary  | Adjacent normal ovary tissue | - | - | - | NAT | 1 1 |
| 50  | Ovary  | Adjacent normal ovary tissue | - | - | - | NAT | 1 1 |
| 52  | Ovary  | Adjacent normal ovary tissue | - | - | - | NAT | 1 1 |
| 27  | Ovary  | Ovary tissue | - | * | - | Normal | 1 0 |
| 34  | Ovary  | Ovary tissue | - | * | - | Normal | 1 0 |
| 19  | Ovary  | Ovary tissue | - | - | - | Normal | 1 0 |
| 42  | Adrenal gland | Pheochromocytoma (tissue marker) | - | - | - | Malignant |
| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| Antibodies          |        |            |
| Anti-mouse CD4-BV711| Biolegend | Clone: GK1.5; Cat# 100447; RRID: AB_2564586 |
| Anti-mouse CD8-BV605| Biolegend | Clone: 53-6.7; Cat# 100744; RRID:AB_2562609 |
| Anti-mouse CD45-BV510| Biolegend | Clone: 30-F11; Cat# 103138; RRID:AB_2563061 |
| Anti-mouse CD3-BUV395| BD Horizon | Clone: 145-2C11 (RUO) Cat# 563565; RRID:AB_2738278 |
| Anti-mouse CD3-PE/Cy7| Biolegend | Cat# 100220 |
| Anti-mouse Ki67 Alexa Fluor 647 | BD Pharmingen | Clone: N/A; Cat# 561126; RRID:AB_10611874 |
| Anti-mouse Granzyme B efour 405 | Thermo Fisher Scientific | Clone: NGZB; Cat# 48-8898-82; RRID:AB_11149362 |
| Anti-mouse PD1-PE | BD Pharmingen | Clone: N/A; Cat# 561788; RRID:AB_10895570 |
| Anti-mouse TIM3-BV421 | BD Biosciences | Clone: 5D12; Cat# 747626; RRID:AB_2744192 |
| Anti-mouse Foxp3-FITC | Thermo Fisher Scientific | Clone: FJK-16s; Cat# 11-5773-82; RRID:AB_465243 |
| Anti-mouse CD45-PE | Thermo Fisher Scientific | Clone: 30-F11; Cat# 12-0451-83; RRID:AB_465669 |
| Anti-mouse CD8-APC eflour 780 | Thermo Fisher Scientific | Clone: 53-6.7; Cat# 47-0081-82; RRID:AB_1272185 |
| Anti-mouse CD3-BV510 | BD Horizon | Clone: 145-2C11; Cat# 563024; RRID:AB_2737959 |
| PPARg-Alexa Fluor 405 | Santa Cruz Biotechnology | Clone: N/A; Cat# SC-7273; RRID:AB_628115 |
| Anti-mouse EpCam-FITC | Thermo Fisher Scientific | Clone: G8.8; Cat# 11-5791-82; RRID:AB_11151709 |
| Anti-mouse PDL1-BV605 | Biolegend | Clone: 10F.9G2; Cat# 124321; RRID:AB_2563635 |
| Anti-human CD8-eFlour450 | Thermo Fisher Scientific | Clone: RPA-T8; Cat# 48-0088-42; RRID: AB 1272062 |
| Anti-human Ki67-PE-Cy7 | BD Pharmingen | Clone: B56; Cat# 561283; RRID: AB 10716060 |
| Anti-human Granzyme B-PE | Thermo Fisher Scientific | Clone: GB11; Cat# GRB04; RRID: AB 2536538 |
| Antibody          | Manufacturer          | Clone/ID Number          |
|------------------|-----------------------|--------------------------|
| Anti-human PD-1-PE | Thermo Fisher Scientific | Clone: MIH4; Cat# 12-9969-42; AB 10736473 |
| Anti-human Foxp3-PE | Thermo Fisher Scientific | Clone: PCH101; Cat# 12-4776-42; RRID: AB 1518782 |
| SPHK1            | Proteintech           | Clone: N/A; Cat# 10670-I-AP; RRID:AB 2195809 |
| PDL1 (IHC)       | Proteintech           | Cat# 17952-I-AP          |
| TSG101           | Proteintech           | Clone: N/A; Cat# 14497-I-AP; RRID:AB_2208090 |
| CD63             | Proteintech           | Clone: N/A; Cat# 25682-I-AP; RRID:AB_2208090 |
| PDL1             | Cell Signaling Technology | Clone: E1L3N®; Cat# 13684S; RRID:AB_2687655 |
| GAPDH            | Cell Signaling Technology | Clone: 14C10; Cat# 2118S; RRID:AB_561053 |
| Alix             | Santa Cruz Biotechnology | Clone: 1A12; Cat# sc-53540; RRID:AB_673819 |
| Ki67 (IHC)       | Cell Signaling Technology | Clone: D3B5; Cat# 12202S; RRID:AB_2620142 |
| Beta-Actin       | Cell Signaling Technology | Clone: 13E5; Cat#4970; RRID:AB_2223172 |
| TSG101           | Thermo Fisher Scientific | Clone: 4A10; Cat# MA1-23296; RRID:AB_2208088 |
| GFP              | Santa Cruz Biotechnology | Clone: NA; Cat# Sc-9996; RRID:AB_627695 |
| Anti-Rabbit HRP  | Cell Signaling Technology | Cat#: 7074S; RRID:AB_2099233 |
| Anti-Mouse HRP   | Cell Signaling Technology | Cat#: 7076P2; RRID:AB_330924 |
| Anti-Rabbit STAR 580 | Abberior            | Cat#ST580-1002          |
| Anti-Mouse STAR 635 | Abberior            | Cat#ST635-1001          |
| inVivoMAB anti-mouse PD-1 | BioxCell           | Clone RMPI 14;Cat# BE0146; RRID: AB_10949053 |
| CD3 (IHC)        | Abcam                 | Clone:NA; Cat# ab5690: RRID:AB_305055 |
| CD4 (IHC)        | Cell Signaling Technology | Clone D7D2Z;Cat# 25229; RRID:AB_2798898 |
|                |                          |                          |                          |
|----------------|--------------------------|--------------------------|--------------------------|
| **CD8 (IHC)**  | Cell Signaling Technology | Clone D4W2Z; Cat# 98941: RRID:AB_2756376 |                          |
| Anti S1P antibody | Echelon Biosciences      | Cat# Z-P300               |                          |
| Power Vision poly HRP anti rabbit | Leica Biosystems | Cat# PV6119: RRID:AB_1307590 |                          |
### Sup Table 5 List of Primers and siRNAs

#### Primer

| Human PDL1 | F 5’- CCA AGG CGC AGA TCA AAG AGA-3’  
| R 5’- AGG ACC CAG ACT AGC AGA -3’  |
| Mouse PDL1 | F 5’-GACCAGCTTTTTGAAGGGAATG 3’  
| R 5’ CTGGTGTATTGGCGGTATGG 3’ |
| E2F1 chip primer | F 5’-ATT GGG CGT TTC TCT TGG T-3’  
| R 5’-GAG TAA GCG CCT GGG ATA TTT-3’  |
| Human GUCY1A3 | F 5’-TCAGCCCCCTACTTGTTGA CTCC-3’  
| R 5’-CAGAATAGCGATGTGGGAATCAC-3’  |
| Human HLA-DMB | F 5’-ACCTGTCCTGTTGGATGATG CCT-3’  
| R 5’-CGCAAGGGCCATCTTATTCT-3’ |
| Human IL1A | F 5’-TGGTAGTAGCAACCAACGGGA-3’  
| R 5’-ACTTTGATTGAGGGCGTCATTC-3’ |

#### siRNA

| TSG101 | 5’-GGU UAC CCG UUU AGA UCA A[dT][dT]-3’  
| 5’-UUG AUC UAA ACG GGU AAC C[dT][dT]-3’  |
| E2F1 | SiRNA Id- SASI_Hs01_00162220 (SIGMA-AlDRICH)  |
| ETS1 | SiRNA Id- SASI_Hs01_00173246 (SIGMA-AlDRICH)  |
| MYB | SiRNA Id- SASI_Hs01_00127047 (SIGMA-AlDRICH)  |
| STAT1 | SiRNA Id- SASI_Hs02_00343387 (SIGMA-AlDRICH)  |
| IRF1 | SiRNA Id- SASI_Hs01_00143090 (SIGMA-AlDRICH)  |
| FOXH1 | SiRNA Id- SASI_Hs01_00108856 (SIGMA-AlDRICH)  |
| POU4F1 | SiRNA Id- SASI_Hs02_00341153 (SIGMA-AlDRICH)  |
| TFAP2A | SiRNA Id- SASI_Hs01_00080715 (SIGMA-AlDRICH)  |
| HIF1A | SiRNA Id- SASI_Hs02_00332063 (SIGMA-AlDRICH)  |
| CEBPA | SiRNA Id- SASI_Hs01_00058601 (SIGMA-AlDRICH)  |
| SPHK1 | ON-TARGET plus SMARTpool SiRNA-L-004172-00-0005 (DHARMACON)  |
| S1PR1 | ON-TARGET plus SMARTpool SiRNA-L-003655-00-0005 (DHARMACON)  |
| S1PR2 | ON-TARGET plus SMARTpool SiRNA-L-003952-00-0005 (DHARMACON)  |
MATERIALS AND METHODS

In vivo Extracellular vesicle treatment

RAB27a−/− ID8 cells and SPHK1−/− ID8 cells were orthotopically injected into the right ovary of C57BL/6 mice. EVs isolated by ultracentrifugation from culture media of ID8 cells, ID8 SPHK1−/− cells, and ID8 cells with SPHK1 overexpressed were injected into mice (20 μg/mouse) via the tail vein twice a week until the control mice became moribund.

Exogenous S1P treatment

S1P (Avanti Polar Lipids) was dissolved in fat-free bovine serum albumin (BSA) (4 mg/ml) in 1X phosphate buffer saline (PBS) (pH 7.4) at 125 mM concentration according to the protocol utilized by Panneer Selvam et al., 2015 [1]. The solution was added to culture medium at the required concentration.

Purification of EVs

Cells were cultured in serum-free media, and supernatants were collected at 48–72 h. EVs were purified from the supernatants by standard differential centrifugation [2]. In brief, the supernatants were centrifuged at 2,000g for 20 min to remove cell debris and dead cells; then they were centrifuged at 16,500g for 45 min. In turn, those supernatants were centrifuged at 100,000g for 2 h at 4 °C (Beckman Coulter, Optima XPN-100). The pelleted EVs were suspended in PBS and collected by ultracentrifugation at 100,000g for 2 h. Also, EVs were isolated from culture medium with Total Exosome Isolation Reagent (Invitrogen, Cat# 4478359).

To purify circulating EVs from blood using the exosome isolation kit, we first centrifuged cell-free plasma at 16,500g for 45 min (Eppendorf, 5418R) to pellet large membranous vesicles. EVs were then purified from the supernatant, using the Exosome Isolation Kit (Invitrogen, Cat# 4484450).
**Measurement of S1P in plasma and SPHK1 activity**

We used the Sphingosine 1-Phosphate ELISA Kit (Echelon, Salt Lake City, UT, USA) to quantify S1P in plasma samples from ovarian cancer patients and healthy controls, following the manufacturer's instructions. Sphingosine kinase activity was measured with the Sphingosine Kinase Activity Assay (Echelon, Salt Lake City, UT, USA) according to the manufacturer's instructions. In brief, protein extracts from EVs or WCL (5μg) were incubated in reaction buffer, 100 μm sphingosine, and 10μm ATP for 2h at RT, and then luminescence attached ATP detector was added to stop the kinase reaction. Kinase activity was measured using a luminometer (TECAN SPARK, Switzerland). All samples were prepared in triplicate, and the assay was repeated at least three times. Negative controls were prepared by heating samples at 90°C for 10 min to inactivate all enzymatic activity.

**Measurement of S1P in culture media by Mass Spectrometry**

S1P concentrations were quantified by the method of Berdyshev et. al. [3], with 20 pg/μl of C_{17}S1P as the internal standard. After extraction and derivatization, bisacetylated S1P eluted from an Agilent Eclipse XDB-LB column (4.6 mm×150 mm, 5-μm particle) and detected using by an Agilent 6460 Triple Quadrupole mass spectrometer running in the negative ion mode and equipped with a JetStream source as previously reported [4]. Bisacetylated sphingolipids were eluted using the following gradients: 2-min hold of solvent A, ramp to 100% solvent B over 3 min, hold at 100% solvent B for 3 min, and then regenerate the column with solvent A for 4 min with the following settings: sheath gas temp =325°C, sheath gas flow =11 L/min, CE=13 V, fragmentor =210, acceleration voltage =7 V, capillary =3500 V, nozzle =1000 V. Solvent A = water/methanol/formic acid (20/80/0.5 v/v/v) mmol/L in ammonium formate. Solvent B = methanol/acetonitrile/formic acid (60/40/0.5 v/v/v) 5 mmol/L in ammonium formate. Multiple reaction monitoring transitions monitored were C_{17}S1P m/z 448/388 and S1P m/z 462/402 [5].
**Western blotting**

To prepare whole cell lysates, we washed cells twice with ice-cold PBS and lysed them on ice in 1x RIPA lysis buffer containing a freshly added protease inhibitor cocktail (Thermo Fisher Scientific Inc., Rockford, IL, USA) and 1 mM PMSF. To prepare tissue lysates, we homogenized tumor tissues in 1x RIPA lysis buffer over ice. After the homogenates were incubated for 30 min, lysates were collected by centrifugation at 4°C for 10 min at 10,000 rpm. The amount of total protein was determined using a BCA protein assay kit (Thermo Fisher Scientific Inc., Waltham, MA). An equal amount of total protein (30 μg) was resolved on precast 4%–12% SDS-PAGE gels (Biorad, Hercules, CA, USA), and the protein was transferred onto PVDF membranes and incubated with the desired primary antibodies. The preparation was then washed and incubated with HRP-conjugated secondary antibodies, and protein bands were detected with a chemiluminescence kit (Thermo Fisher Scientific Inc., Waltham, MA). List of the antibody used in this study is given is S Table 4.

**siRNA transfection**

Predesigned siRNAs for human SPHK1 (Cat# L-004172-00-0005), S1PR1 (Cat# L-003952-00-0005), and S1PR2 (Cat# L-003655-00-0005) were purchased from Dharmacon, Lafayette, CO. Negative siRNAs universal controls (siCont) were obtained from Sigma-Aldrich, which also supplied us with predesigned siRNAs. Transfections were performed using the Lipofectamine RNAiMAX transfection reagent (Thermo Fisher Scientific Inc., Waltham, MA). At 48 h post-transfection, cells were harvested for further analysis. siRNA sequences for all the genes are listed in S Table 5.

**Tissue microarrays (TMAs) and immunohistochemistry (IHC)**

SPHK1 and PD-L1 protein levels in human ovarian cancer tissues were analyzed using TMAs (Cat# OV1005b, US Biomax Inc., Rockville, MD). For this purpose, the slides were dewaxed in
xylene, and rehydrated through graded ethanol to distilled water. Antigen retrieval for the slide specimens was performed using IHC-Tek epitope retrieval solution and steamer set (IHC World, LLC.). The slides were then immersed in 3% H₂O₂ for 10 min to quench endogenous peroxidase, and then blocked with 10% goat serum for 1 h. The Vectastain ABC-AP Kit (Vector Labs, Burlingame, CA) and Vector Red Alkaline Phosphatase Substrate Kit I (Vector Labs, Burlingame, CA) were used for tissue staining as per the manufacturer's protocol. Both the SPHK1 primary antibody (Proteintech, Cat# 10670-I-AP) and the PD-L1 primary antibody (Proteintech, Cat# 17952-I-AP) were used at a 1:50 dilution. Following Vector red staining, the slides were counterstained with Harris modified hematoxylin (Thermo Fisher Scientific Inc., Rockford, IL), dehydrated with graded ethanol and xylene, and finally mounted with mounting medium. The TMA slides were then digitally scanned, using a Pannoramic 250 FLASH III scanner (3D HISTECH Ltd. Version 2.0). Case Viewer software (3D HISTECH Ltd. Version 2.0) was used to view and analyze the images. For other IHC, DAB sating was used according to the manufacturer's protocol (DAB Substrate Kit, Vectors Labs, Cat# SK-4100, Burlingame, CA).

To stain CD3, CD4, and CD8 in mouse tissue, we preheated slides at 60°C for 10 min and transferred them to a staining rack with xylene (3X 5 min each). Then the slides were hydrated through an alcohol gradient to distilled water. They were then placed in citrate buffer (Vector Labs) and then in antigen unmasking solution (Cat# H-3300) for 5 min. Next, the slides were placed in EDTA buffer (Sigma-Aldrich, Cat# E1161), and steamed for 45 min. When they had cooled, they were rinsed with PBST, and sections were incubated with blocking solution (Dako-dual endogenous enzyme block, Cat# S2003) for 5 min. After a rinse with PBST, the sections were incubated with primary antibodies CD3 (Abcam, Cat# ab5690, 1:500 dilution), CD4 (Cell Signaling, Cat# 25229, 1:100 dilution), and CD8 (Cell Signaling, Cat# 98941, 1:100 dilution) overnight. After a rinse with PBST, the slides were incubated with HRP-conjugated secondary antibody (Leica Biosystems, Power Vision poly HRP-anti rabbit, Cat# PV6119) for 1h, and then
DAB was applied. After a rinse with PBST, the slides were stained with hematoxylin, dehydrated with graded ethanol and xylene, and finally mounted with mounting medium (Sigma-Aldrich, Cat# 06522).

We performed S1P staining on frozen tissue sections according to Visentin B et. al. protocol [6]. Briefly, frozen tissue sections were place at RT for 20mins and fixed in 10% neutral buffered formalin for exactly 2 min. Slides were washed with water for 2 min. Endogenous peroxidase blocking was performed for 10 min in 0.3% (v/v) H$_2$O$_2$ in PBS. Slides were washed with PBS and blocked in 2x casein solution for 10min at RT. Then sections were incubated with S1P specific antibody (LT1002, Echelon Biosciences) overnight at 4°C. ImmPRESS anti-mouse IgG polymer kit (Vector Laboratories, Cat# MP-7802) was used to develop the DAB staining.

**Quantitative real time-PCR (qRT-PCR)**

Total RNA was isolated from the cells using the RNeasy Mini Kit (Qiagen, Valencia, CA, USA), and first-strand cDNA was transcribed using iScript reverse transcription supermix (Biorad, Hercules, CA, USA). qRT-PCR was performed using CFX Connect Real-Time PCR systems (Biorad, Hercules, CA, USA) and SYBR Premix Ex Taq II (Biorad, Hercules, CA, USA). List of the qPCR primers used in the study is given as S Table 5.

A customized PCR array for human immune checkpoints was purchased from Qiagen (Valencia, CA, USA), and used according to the manufacturer's instructions. Data from the PCR array were analyzed using SABiosciences RT$^2$ Profiler PCR Data Analysis software (http://pcrdataanalysis.sabiosciences.com/pcr/arrayanalysis.php), and were considered significant at > 1.5 fold change and $p<0.05$. Five housekeeping genes, $B2M$, $HPRT1$, $RPLP0$, $GAPDH$, and $ACTB$, were used to normalize the data, and fold change was calculated relative to the control untreated OVACR5 cells.

**Lentiviral SPHK1 knockdown**
To create the lentiviral delivery system, HEK293T cells were transfected with packaging vectors pLP1, pLP2 and VSVG plasmids, including control empty vector pLKO.1 (Cat#SHC001V) and two different SPHK1-targeting short hairpin RNAs (shRNA) (TRC number 1: TRCN0000333028, Clone ID:NM_182965.2-1922s21c1; 2: TRCN0000333675, Clone ID: NM_182965.2-1104s21c1) purchased from Sigma-Aldrich (Saint Louis, MO). Competent lentiviruses were collected 48 h after transfection. HeyA8 cells were passaged to 40% confluence, and viral medium was added to them the next day along with 8 μg/ml of polybrene. The efficacy of individual SPHK1 shRNA constructs was checked by Western blot analysis. Puromycin (8 μg/ml; for 2 weeks) was used to select the most effective shRNA construct for generating a cell line in which SPHK1 was stably knocked down. The clones were picked and subjected to expansion culture for further selection. Western blot analysis was performed to identify the stable clone with the most efficiently downregulated SPHK1 protein. That clone was used in further experiments.

**CRISPR/CAS9 knockdown of genes**

SPHK1 and RAb27a genes knockout ID8 cells were engineered by Synthego (CA, USA) using CRISPR/CAS9 technology. The following guide sequence were used:

**SPHK1**: GCCGUGGGAGCAGUCCUGA

**RAB27a**: CCUGCAGUUAUGGGACACGG

**Transfections for overexpression**

To establish the stable overexpression of SPHK1 and TSG101 in HeyA8 cells, we used Lipofectamine 2000 (Invitrogen, Carlsbad, CA) to transfect HeyA8 cells with a control vector or a pReceiver-M29 vector expressing SPHK1 (Cat# EX-H5064-M29), SPHK1 mutant 1 (PAAA) (Cat# CS-H5064-M29-01), SPHK1 mutant 2 (PTAA) (Cat# CS-H5064-M29-02), SPHK1 mutant 3 (ATAP) (Cat# CS-H5064-M29-03), and TSG101(Cat# EX-A6458-M29) were purchased from GeneCopoeia, Rockville, MD. 48 h after transfection, the cells were transferred to culture medium
containing G418 (800 μg/ml) for 2 weeks to ensure that the control sequences or SPHK1 would become stably incorporated. Western blotting was then performed to check GFP-SPHK1 and GFP-TSG101 expression. We also synthesized a viral vector to express mouse SPHK1 (pLCP-GFP+SPHK1-Neo), using a gene from the plasmid EX-Mm05392-M68 (GenCopoeia, Rockville, MD). The viral plasmid was transfected into ID8 cells with polybrene (8 μg/μl) for 48 h, and selection was performed by culturing the cells in medium containing G418 (1000 μg/ml) for 1 week.

**Proximity ligation assay**

The DuoLink® In Situ Red Starter Kit Mouse/Rabbit (Sigma-Aldrich) was used to detect proximity between SPHK1 and TSG101 proteins according to the manufacturer’s protocol. Briefly, HeyA8 cells were seeded in 8-well chamber slides (ibidi USA, Madison, WI, USA) and cultured overnight. The slides were then washed with cold 1×PBS, fixed in 4% paraformaldehyde for 30 min, and blocked with Duolink Blocking Solution in a pre-heated humidified chamber for 1 h at 4°C. The primary antibodies for detecting SPHK1 (Proteintech, Cat# 10670-I-AP) and TSG101 (Invitrogen Cat#, MA1-23296) were added, and the slides were incubated overnight at 4°C. The slides were then washed with 1×Wash Buffer A, and subsequently incubated with the PLA probes (1:5 dilution in antibody diluents) for 1h, then with Ligation-Ligase solution for 30 min, and finally with the Amplification-Polymerase solution for 100 min in a pre-heated humidified chamber at 37°C. Before imaging, the slides were washed with 1×Wash Buffer B and mounted with a coverslip, using Duolink In Situ Mounting Medium with DAPI. Fluorescence images were acquired with a confocal laser scanning microscope (LSM 510; Zeiss, Oberkochen, Germany).

**STED imaging and image processing**

Cells grown on cover slips (#1.5) were washed twice with PBS, and fixed with 2% paraformaldehyde (PFA) (15 min). The cells were rinsed with PBS three times, and permeabilized with 0.1% Triton X-100 (10 min). After washing, blocking was performed with 1X PBS/2% BSA
for 1 h. The primary antibodies of SPHK1 (Cat# 10670-I-AP, Proteintech, USA) and TSG101 (Cat# MA1-23296, Invitrogen, USA) were used for immunostaining in PBS for overnight at 4°C. After the slides were rinsed in PBS slides three times, cells were incubated with secondary antibodies goat anti-rabbit STAR 580 (Cat#ST580-1002, Abberior, Germany) and goat anti-mouse STAR 635 (Cat#ST635-1001, Abberior, Germany) for 1 h at RT. Cover slips were mounted using ProLong Gold Antifade Reagent (Life Technologies, Carlsbad, CA). Images were acquired with a 100X objective with 4.55 zoom, using a STED microscope (Leica). Image analysis was performed by Image J software, using the JACoP plugin.

Co-immunoprecipitation GFP Trap

HeyA8 cells stably expressing either GFP fusion proteins or SPHK1 variants were washed with PBS and then mechanically detached in PBS. The cells were pelleted by centrifugation at 500 × g for 3 min at 4 °C and rinsed twice with PBS. Cell lysis was achieved by adding lysis buffer (10 mM Hepes, 150 mM NaCl, 0.5 mM EDTA, 1% Triton X-100, 0.5% SDS, and proteinase inhibitor mixture, pH 7.5). We avoided Tris buffers because we wanted to use DTSSP cross-linker. Cleared lysates (17,000 × g, 15 min, 4°C) were incubated with GFP-nanobody agarose (GFP-Trap, Chromotek) for 1 h at 4°C. After the fusion proteins were bound to the beads, we applied 3 mM DTSSP cross-linker (Thermo Fisher Scientific) for 2 h at 4°C for nonspecific stabilization of protein–protein interactions. Finally, the beads were rinsed four times with Co-IP washing buffer (10 mM Hepes, 150 mM NaCl, 0.5 mM EDTA), and boiled in SDS-PAGE loading buffer for Western blot analysis.

Sucrose gradient for EVs purity

Culture medium was isolated and centrifuged at 4°C (2000 rpm, 20 min) to remove cell debris. The supernatant was transferred into a new tube and centrifuged at 16,500rpm for 45 min at 4°C to remove any large vesicles such as microvesicles. Supernatant was further transferred into
Beckman tubes (38.5 ml ultra-clear tubes) and ultracentrifuged at 4°C (100,000 g for 2 h) using a SW32 Ti rotor to pellet crude EVs, which were then resuspended in 100µl PBS.

We prepared stock solutions of sucrose ranging from 1%-60% in PBS and added them to a Beckman tube in the order of highest to lowest sucrose concentration. Crude EVs pellet was placed on top of the sucrose gradient and ultracentrifuged at 4°C (100,000g for 16 h) in a SW32 Ti rotor. We then collected 2 ml of each of the fractions, and ultracentrifuged them at 4°C (100,000g for 2 h). The isolated pellets were individually suspended in RIPA buffer, and western blotting was used to check the CD63, SPHK1, and ALIX expression.

**Nanoparticle tracking analysis (NTA)**

The EVs’ size distribution and concentrations were determined by Nanoparticle Tracking Analysis (NTA), using the NanoSight LM10 instrument (Malvern Panalytical, Malvern, UK) with a 488 nm laser and NTA3.1 software. Three 30 s measurements were recorded for each sample, with automated analysis settings for blur, track length, and minimum expected particle size. The camera level was set at 12 and the detection threshold at 10.

**Transcription factor array**

OVACR5 cells were treated with 100 nM S1P for 6 h, and nuclear protein was extracted from treated and untreated cells. We measured the activity of 96 transcription factors in 15 µg of each protein extract, using the TF Activation Profiling Plate Array II (Signosis) according to manufacturer’s instructions, Relevant TFs were selected by the fold change (>2 fold) between S1P-treated and untreated OVCAR5 cells.

**Electron microscopy and immuno-gold staining of isolated exosomes**
The exosome suspension was fixed for 30 min on ice by adding an equal volume of fixative (4% paraformaldehyde + 0.2% glutaraldehyde in 200 mM sodium phosphate buffer) to give a final concentration of 2% paraformaldehyde + 0.1% glutaraldehyde in 100mM buffer containing 3.5% sucrose and 0.5 mM CaCl$_2$. The EVs were then pelleted at 16,000 g for 10 min. The supernatant was discarded, and the pellet, still adhering to the Eppendorf centrifugation tube, was processed following the protocol of Bulreigh et. al [7]. Essentially, the pellets were washed 3x20 min in 0.1M phosphate buffer containing 3.5% sucrose and 0.5 mM CaCl$_2$. Then unreacted aldehydes were quenched in 100 mM glycine in phosphate buffer for 1h on ice before the pellets were returned to PO$_4$ buffer. The pellets were then rinsed 4 times (for 15 min each time) in 100 mM tris maleate buffer + 3.5% sucrose pH 6.5 at 4°C and then in 2% uranyl acetate (w/v) in tris buffer pH6 for 2h at 4°C. After that, the pellets were given a final rinse 2x5 min in tris maleate buffer pH 6.5. They were then processed by the progressive lowering of temperature method of Berryman et al [8] into Lowicryl K4M resin, and polymerized by UV irradiation. Ultrathin sections (70 nm) were cut onto Formvar/carbon-coated grids. The grids were immunolabeled by floating them on 100mM phosphate buffer containing 5% BSA (PB-BSA) and then incubating them for 90 min with rabbit SPHK1 polyclonal antibody (Proteintech, Cat# 10670-I-AP) diluted 1:50. Non-immune rabbit polyclonal serum was used as the negative control. This step was followed by 3x5 min washes in PBS-BSA. The sections were then incubated with goat anti-rabbit IgG conjugated to 10nm colloidal gold for 90 min at room temp, rinsed in distilled water, stained with 2% aqueous uranyl acetate, and examined in a Hitachi H600 TEM at 75 kV.

**T cell Isolation, treatment with EVs, and CFSE labeling, cytokine bead array**

Human CD8+ T cells were purified from blood, using the EasySep Direct Human CD8+T Cell Isolation Kit (STEMCELL Technologies). The cells were stimulated with anti-CD3 (2µg/ml) and anti-CD28 (2 µg/ml) antibodies, and incubated with or without EVs for 72 h. The ratio of T cells and EVs was kept at 1:1500. The treated cells were then collected, stained, and analyzed by flow cytometry. Expression of Ki67, Granzyme B, PD-1, and FOXP3 were analyzed by flow cytometry.
To assay the proliferation of CD8+ T cells, we used CFSE dye. T cells were stained with 5µM CFSE and incubated at 37°C for 20 min. The reaction was stopped by adding 5 volumes of cold medium with 10% FBS. The cells were then treated with EVs as above. Unstimulated CFSE-labeled cells served as a non-dividing control.

Stimulated T cells were either treated with EVs or S1P and cytokine bead array was performed according to manufacture protocol (BD, Cat# 560484, Human Th1/Th2/Th17 cytokine kit).

**Luciferase reporter assay**

Plasmid vector containing the promotor of PD-L1 was purchased from GeneCopoeia (Cat# HPRM40139-PG02 and transfected into cells by Lipofectamine-2000 reagent. The cells were treated with S1P or drugs at a given concentration for the next 24 h. Culture medium was then collected and centrifuged at 2000g for 10 min at 4°C, and 10 µl samples of supernatant were transferred into white-walled 96-well plates in triplicate. Luciferase intensity in each well was immediately measured, using a luminometer, as described in the Secrete-Pair Gaussia Luciferase Assay Kit (GeneCopoeia, Cat# LF061).

**Preparation of ascites sample and flow cytometry**

Ascites collected from euthanized mice were centrifuged at 2000 rpm. ACL lysis buffer was added according to the volume of the pellet (10 ml of buffer for 2 ml of pellet) and incubated for 5 min to lyse red blood cells. 1X PBS was added, and the preparation was centrifuged again to compact the cell pellet. The mixture was filtered through a 70 µm filter to collect tumor cells and TILs. These cells were then washed and counted, and 2x10^6 cells were collected to be stained for flow cytometry. The single-cell suspension was first incubated with Fc block for 10 min, then live-dead staining was performed, and then the cells were washed with stain buffer (PBS+ 0.5% BSA). Cell-surface antigens were stained by co-incubation with antibodies for 30 min on ice followed by washing with stain buffer. Staining of intracellular FOXP3, Ki67, and Granzyme B was performed.
with a BD Pharamingen kit (Cat# 562574) according to the manufacturer’s protocol. Flow cytometry was performed on a Fortessa X20, and data were analyzed by FlowJo software. List of antibodies used for flow cytometry is given in S Table 4.

**Activation of T cells with dendritic cells (DCs)**

Femurs and tibias were isolated from wildtype C57BL/6 mice and flushed with 10% FBS containing RPMI medium. RBCs were lysed with ACK lysis buffer for 5 min; then medium was added, and cells were spun down. The cells were resuspended in 10% FBS containing RPMI at about 1–1.5x 10^6 per ml. Then FLT3L (100 ng/ml) was mixed into the suspension. Cells were plated into a 24-well plate at 1ml per well and cultured for the next 7 days. Meanwhile, ID8 cells were grown in a large flask and collected in PBS. 10x10^6 cells were suspended per 1.5 ml tube in PBS, and 5 cycles of freezing (dry ice) and thawing (56°degree water bath) were performed. When 100% of the cells were dead (as confirmed with trypan blue staining), the tubes were spun at 1700g for 5 min and supernatants were collected and stored at −80°C for later use.

After 7 days of cell culture, the DC cells in each well were counted, and their percentage was checked using CD11c+MHCII+ antibodies to stain cells for flow cytometry. Around 60%–70% DCs were found per well. 180 μl of ID8 lysate (made as above) was added to each well, and the cells were maintained overnight in an incubator. The next day, LPS (100 ng/ml per well) was added to stimulate the DCs to mature. After about 6 h, DCs were collected and washed twice with PBS.

**Activation of T cells with DC and IncuCyte experiment and flow cytometry**

Spleen was isolated from one normal C57BL/6 mice and CD8 T cells were isolated using MojoSort™ Mouse CD8 T Cell Isolation Kit (Biolegend). T cells were mixed with DCs in the ratio of 5:1 (T cells:1 DCs) and divided in two group. 1) Untreated, 2) S1P (5 μM) and culture for next 3 days before performing IncuCyte experiment. Expression of Ki67, GranzymB and PD-1 was determined by flowcytometry on CD8+ T cells.
For IncuCyte experiment, ID8 cells (1000/well) were seeded in 96 well plate and T cells from both groups were added in ratio of 5:1 along with Annexin V IncuCyte reagent (red). This cocultures were then incubated in IncuCyte for three days and data was collected.

**CHiP assay**

We used the MatInspector (Genomatix) Analysis to determine if the PD-L1 promoter contains a binding site for the transcription factor E2F1. HeyA8 cells were treated with 100 nM S1P for 24 h. We performed the CHiP assay (using the protocol followed by Parashar D. et al [9] by adding E2F1 antibody (Cat No# 3742, CST) to both samples. Crude DNA (Input), control IGG, and no antibody samples were the controls. Primers amplifying the E2F1 binding region on the PD-L1 promoter were designed by PrimerQuest software (IDT). Real-time PCR were used to analyze the amplification of region when E2F1 binds to the PD-L1 promoter. Real-time PCR was used to amplify the binding region. Amplicons were also analyzed on agarose gel. The PCR results from the CHiP assay were analyzed by the fold enrichment method.

**QUANTIFICATION AND STATISTICAL ANALYSIS**

**RNA-sequencing data from the TCGA and bioinformatics**

The R/Bioconductor package TCGAbiolinks was used to retrieve RNA-Seq gene expression data (workflow HTSeq-Counts) of 376 patients in The Cancer Genome Atlas Ovarian Cancer (TCGA–OV) database [https://portal.gdc.cancer.gov/]. Patients were ordered by the expression value of SPHK1, and the 3rd quartile value (863.8) was used as the cutoff to split patients into high and low groups. Thus, those with a raw count above 863.8 (i.e., the highest 25%) were in the high group, whereas those with a raw count below the cutoff were in the low group. Differential expression between the low and high SPHK1 groups was analyzed with the edgeR-limma suite of statistical methods. Genes with FDR <0.05 were considered to be differentially expressed (DE). Pre-ranked gene set enrichment analysis (GSEA) with DE genes pre-ranked by limma’s t-statistics was performed to discover potential biological pathways associated with SPHK1 expression [10]. All analyses were performed using R software v4.0.4, and figures were produced
using the ggplot2 package (Wickham, 2016). A heatmap was drawn using the heatmap.3 function (https://github.com/obigriffith/biostar-tutorials/blob/master/Heatmaps/heatmap.3.R). The relative expression levels of selected genes between the high and low SPHK1 groups were analyzed with Student's t-test. $P < 0.05$ was considered statistically significant unless otherwise indicated.

We analyzed data from three biological replicates unless indicated otherwise in the figure legends. Statistical significance between two groups was defined as $^*p<0.05$ $^{**}p<0.01$ $^{***}p<0.001$ $^{****}p<0.0001$, as determined by unpaired Student's t-tests. We compared multiple groups by one-way analysis of variance (ANOVA) with statistical significance defined as $^*p<0.05$ $^{**}p<0.01$ $^{***}p<0.001$ $^{****}p<0.0001$ compared to control group as mentioned in the respective figure legends. Data are presented as mean ± standard error (SEM) as also indicated in the figure legends. To determine correlation coefficients, we calculated Mendoir’s correlation coefficients ($r$). GraphPad Prism 9 (GraphPad, San Diego, CA) was used to perform all statistical analyses and determine $p$-values.
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