

Supplementary materials

**Tumor cell senescence-induced macrophage CD73 expression is a critical metabolic
immune checkpoint in the aging tumor microenvironment**

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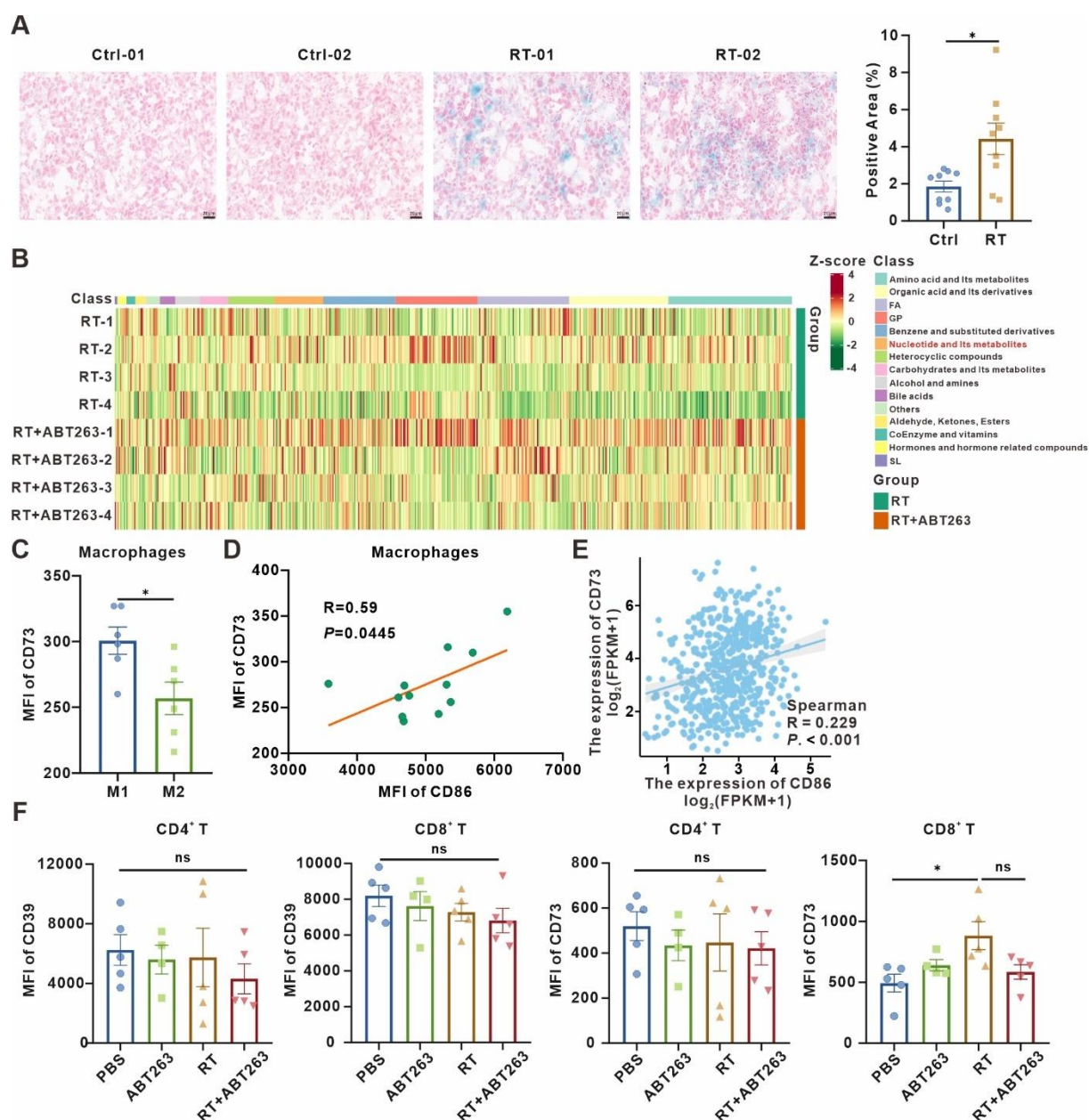


Figure S1. Characterization of the senescent TME in vivo.

(A) LLC murine subcutaneous tumors senescence detected by SA-β-Gal staining assay and the statistics of staining positive area. Scale bar: 20 μm. Mean ± SEM (3 microscopic fields at 400× magnification per mice to evaluate). (B) Heatmap showing the differential metabolites between the senescence (RT) and senescence-clearing (RT+ABT263) TIF detected by LC-MS/MS. (C) Flow cytometric analysis of CD73 expression on M1- and M2-type macrophages in LLC subcutaneous tumor under senescent TME (n = 6). (D) Linear regression analysis between the MFI of CD86 and CD73 in macrophages in the TME (n = 12). (E) Association between the expression of CD86 and CD73 in TCGA lung adenocarcinoma dataset. (F) Flow cytometric analysis of CD39 and CD73 expression on CD4⁺ T cells and CD8⁺ T cells in LLC

23 subcutaneous tumor under various treatment conditions (n = 4 to 5 per group), analyzed by one-way
24 ANOVA. * $p < 0.05$; ns, not statistically significant.
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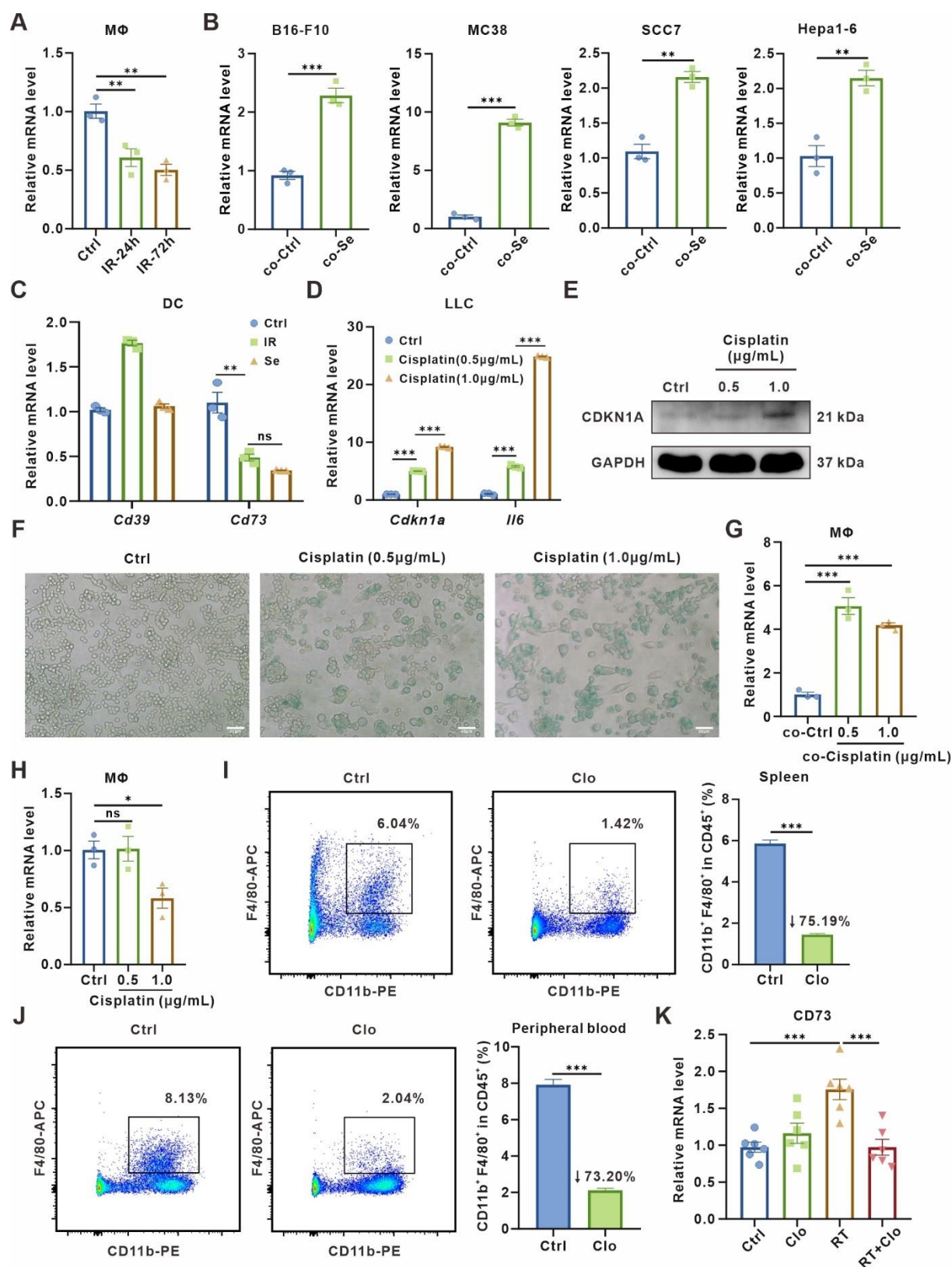


Figure S2. Senescent tumor cells cause upregulation of CD73 expression in macrophages.

(A) Relative mRNA expression of *Cd73* in BMDMs 24 h and 72 h after 10 Gy-radiation (IR). The indicated results represent the mean \pm SEM of 3 independent experiments. (B) Relative mRNA expression

of *Cd73* in BMDMs after co-culture with various senescent tumor cells (B16-F10, MC38, SCC7, and Hepa1-6). The indicated results represent the mean \pm SEM of 3 independent experiments. (C) Relative mRNA expression of *Cd39* and *Cd73* in BMDCs after co-culture with Ctrl LLCs (Ctrl), LLCs one day after 10 Gy-radiation (IR), or LLCs three days after 10 Gy-radiation (Se) for 24 h. The indicated results represent the mean \pm SEM of 3 independent experiments. (D) Relative mRNA expression of senescence-associated genes in LLCs three days after pretreated with 0.5 μ g/mL or 1.0 μ g/mL cisplatin for 24 h. The indicated results represent the mean \pm SEM of 3 independent experiments. (E) Western blot identifying the expression changes of CDKN1A in LLCs described in (D). (F) LLCs described in (D) senescence detected by SA- β -Gal staining assay. Scale bar: 50 μ m. (G) Relative mRNA expression of *Cd73* in BMDMs after co-culture with LLCs as described in (D). The indicated results represent the mean \pm SEM of 3 independent experiments. (H) Relative mRNA expression of *Cd73* in BMDMs after treated with 0.5 μ g/mL or 1.0 μ g/mL cisplatin. The indicated results represent the mean \pm SEM of 3 independent experiments. (I) Macrophage clearance efficiency in mouse spleen detected by flow cytometry (n = 3 per group). The indicated results represent the mean \pm SEM. (J) Macrophage clearance efficiency in mouse peripheral blood detected by flow cytometry (n = 3 per group). The indicated results represent the mean \pm SEM. (K) Relative mRNA expression of *Cd73* in LLC murine subcutaneous tumors in the corresponding treatment groups (n = 6 per group). The indicated results represent the mean \pm SEM. * p < 0.05; ** p < 0.01; *** p < 0.001; ns, not statistically significant.

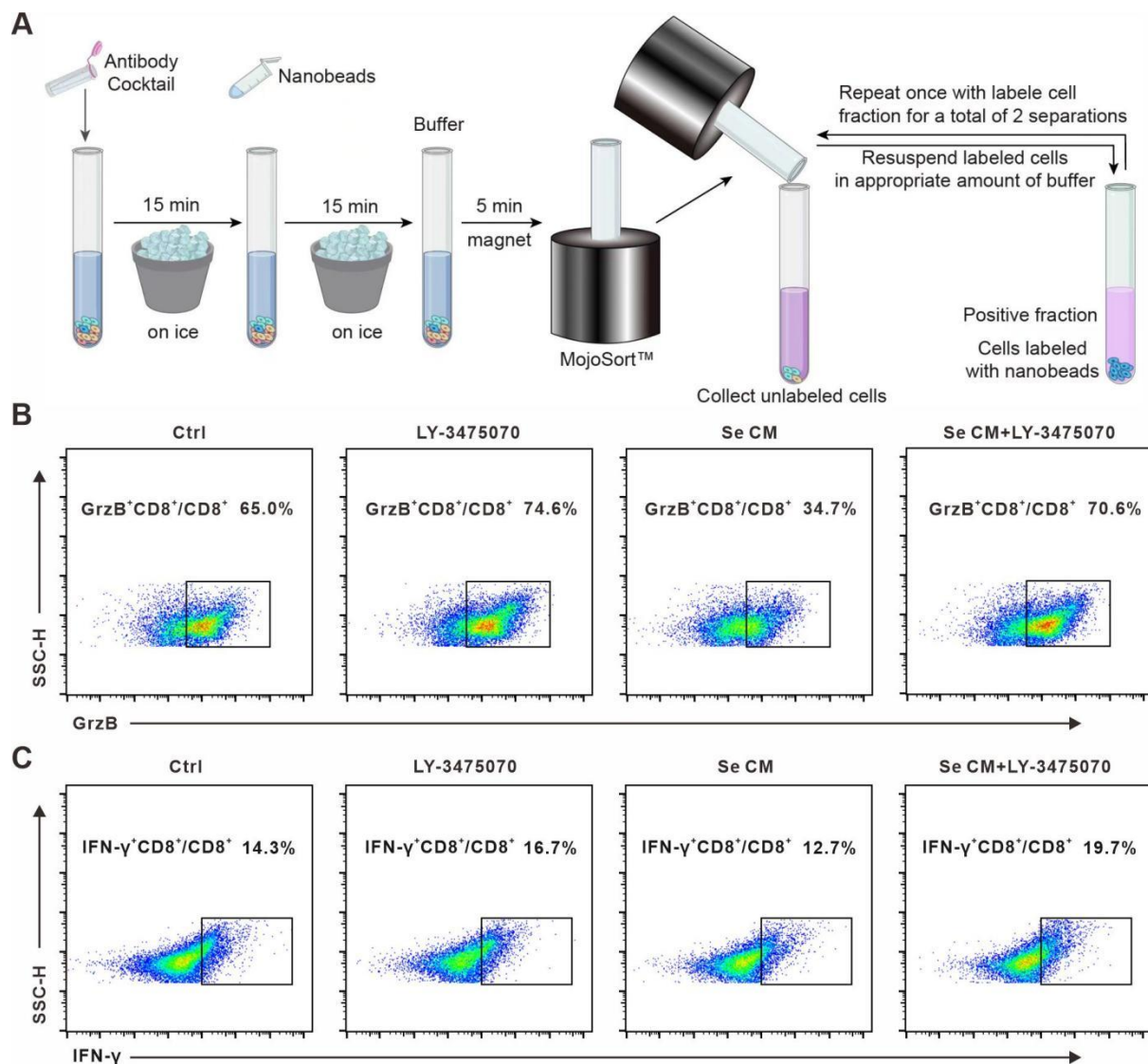


Figure S3. Macrophages co-cultured with senescent tumor cells inhibits the function of T cells.

(A) Schematic diagram of the CD8⁺ T cells sorting process. (B-C) Representative flow cytometry dot plots of the percentage of GrzB⁺CD8⁺ T cells in CD8⁺ T cells (B) and the percentage of IFN-γ⁺CD8⁺ T cells in CD8⁺ T cells (C) in the corresponding group.

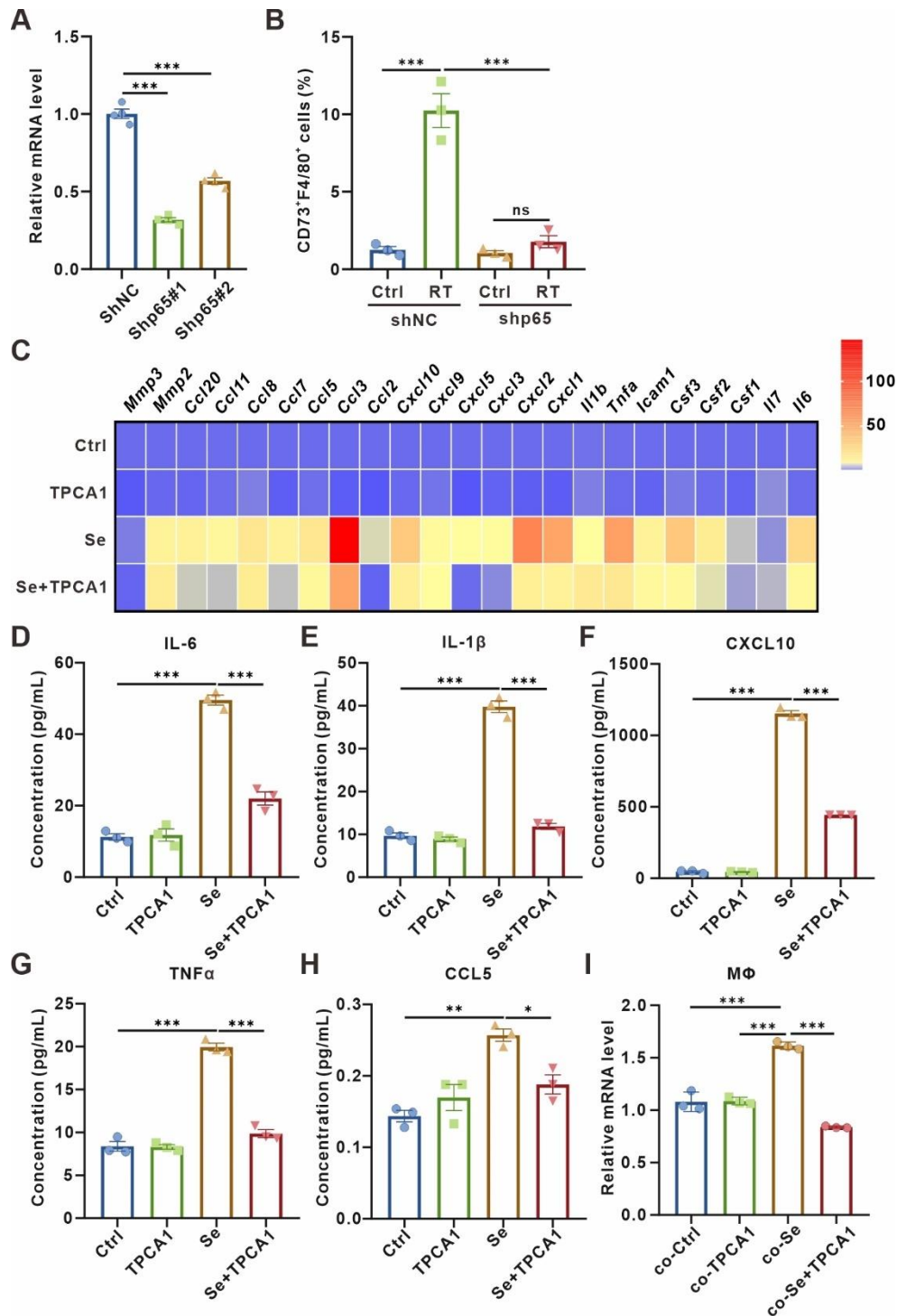


Figure S4. TPCA1 inhibits the upregulation of BMDMs CD73 by senescent LLCs.

(A) Relative mRNA expression of *P65* in LLCs harboring control (shNC) or p53 shRNAs (shp65). The indicated results represent the mean \pm SEM of 4 independent experiments. (B) The proportion of CD73⁺F4/80⁺ cells in LLCs shNC or shp65 subcutaneous tumor sections. (C) Heatmap of cytokine array results from senescent LLCs and/or treated with TPCA1 (1 μ M) for 24 h. Data presented as mean of 3 biological replicates. (D-H) The content of IL-6 (D), IL-1 β (E), CXCL10 (F), TNF α (G) and CCL5 (H) in

64 the supernatant of LLCs in the corresponding groups detected by ELISA. (I) Relative mRNA expression of
65 *Cd73* in BMDMs after co-culture with senescent LLCs which were pretreated with TPCA1 (1 μ M) for 24
66 h. The indicated results represent the mean \pm SEM of 3 independent experiments. * $p < 0.05$; ** $p < 0.01$;
67 *** $p < 0.001$; ns, not statistically significant.

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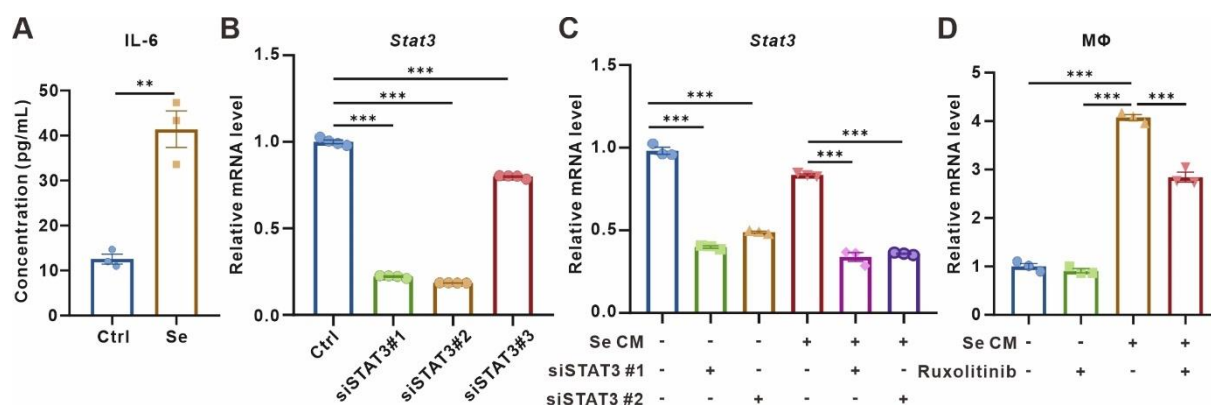


Figure S5. Upregulation of macrophage CD73 is mediated by JAK/STAT3 pathway.

(A) IL-6 content in the supernatant of senescent LLCs detected by ELISA. (B) RT-qPCR analysis to verify the expression levels of *Stat3* mRNAs in BMDMs harboring control (Ctrl) or STAT3 siRNAs (siSTAT3). The indicated results represent the mean \pm SEM of 4 independent experiments. (C) RT-qPCR analysis to verify the mRNAs expression of *Stat3* in BMDMs in the corresponding groups. The indicated results represent the mean \pm SEM of 3 independent experiments. (D) Relative mRNA expression of *Cd73* in BMDMs after co-culture with Se CM and treated with Ruxolitinib (5 μ M) for 24 h. The indicated results represent the mean \pm SEM of 3 independent experiments. ** $p < 0.01$; *** $p < 0.001$.

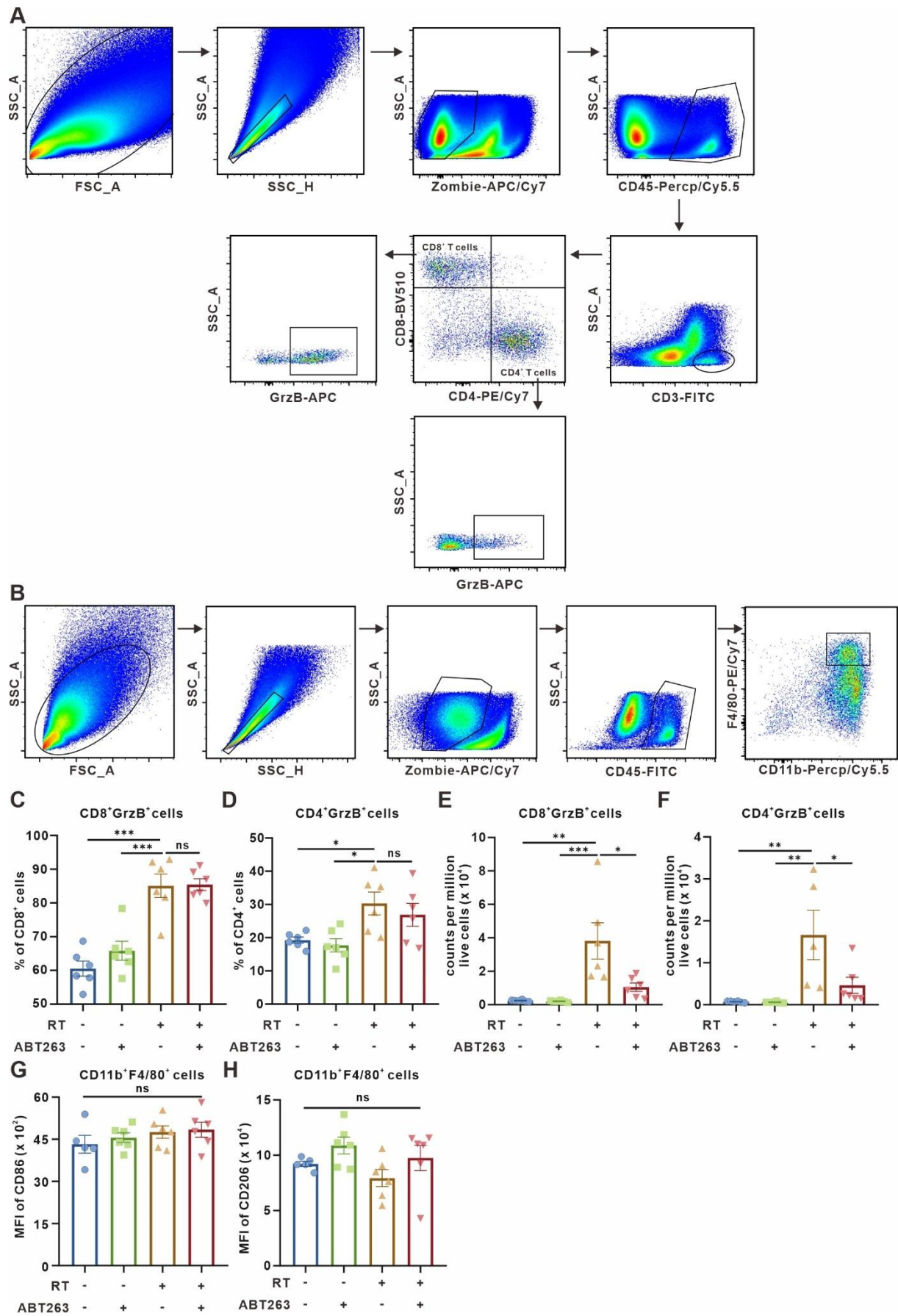


Figure S6. Effects of senescence removal treatment on the immune microenvironment.

(A) Gating strategy for detection of the CD4⁺ T cells and CD8⁺ T cells by flow cytometry. (B) Gating strategy for detection of the TAMs by flow cytometry. (C-D) Flow cytometry analysis of changes in the CD8⁺GrzB⁺ T (C) cells and CD4⁺GrzB⁺ T cells (D) in the TME of LLC subcutaneous transplant model that underwent different treatments (n = 6 per group). (E-F) Flow cytometry analysis of changes in the absolute counts of CD8⁺GrzB⁺ T (E) cells and CD4⁺GrzB⁺ T cells (F) in the TME of LLC subcutaneous transplant model that underwent different treatments (n = 5 to 6 per group). (G-H) Flow cytometric analysis of CD86 (G) and CD206 (H) expression in TAMs of LLC subcutaneous transplant model that underwent different treatments (n = 5 to 6 per group). The indicated results represent the mean ± SEM, analyzed by one-way ANOVA. **p* < 0.05; ***p* < 0.01; ****p* < 0.001; ns, not statistically significant.

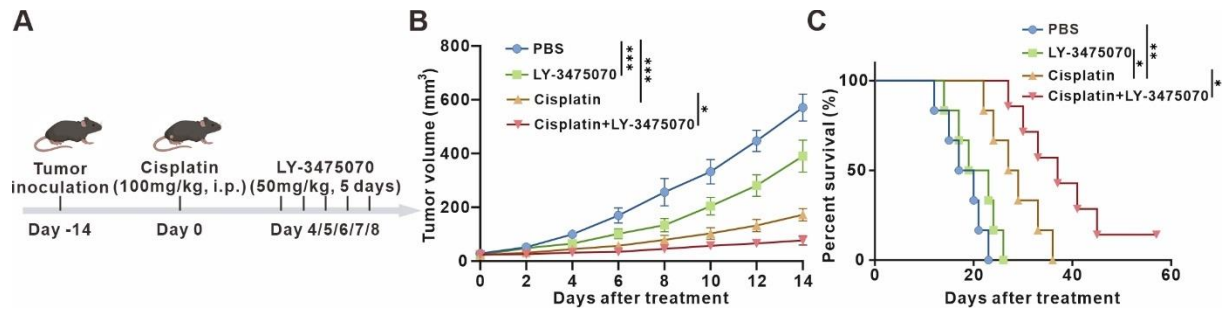


Figure S7. Cisplatin chemotherapy-induced senescence combined with CD73 inhibitor suppresses tumor growth.

(A) Schematic of experiment to assess efficacy of LY-3475070 in the cisplatin chemotherapy-induced senescence mouse model. (B) Tumor growth curves of LLC subcutaneous transplant model in corresponding treatment groups (n = 6 to 7 per group), analyzed by 2-way ANOVA. (C) Kaplan-Meier survival plot of LLC lung cancer-bearing mice in the corresponding treatment groups described in (B) (n = 6 to 7 per group). * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

Figure S8. Bioinformatics analysis to verify the correlation between senescence and macrophage CD73 expression.

(A) Association between CDKN1A expression and immune score in TCGA lung adenocarcinoma dataset. (B) Association between CDKN1A expression and cytotoxic cells enrichment in TCGA lung adenocarcinoma dataset. (C) The expression correlation between CDKN1A and CD73 in TCGA lung adenocarcinoma dataset. (D) Left panel, tissue preference of each cluster. Right panel, dot plot of mean expression of canonical marker genes for ten major lineages from tissues of each origin, as indicated. (E) Heatmap of selected myeloid cell marker genes in each cell cluster. (F) Linear regression analysis between the SASP gene set (R.HSA.2559582) expression of tumor cells and the CD73 expression of macrophages in the single-cell dataset.

Table S1. Sequences of designed siRNA targeting STAT3.

siRNA	SenseSeq (5'-3')	Seq (5'-3')
#1	CGACUUUGAUUUAACUACAA	UUGUAGUUGAAAUCAAGUCG
#2	CCUGAGUUGAAUUAUCAGCUU	AAGCUGAUAAUUAACUCAGG
#3	CACCAUUCAUUGAUGCAGUUU	AAACUGCAUCAAAUGAUGGUG

Table S2. Sequences of designed shRNA targeting p65.

shRNA	Sequence (5'-3')	Company
#1	GGAGTACCCTGAAGCTATAAC	Vigene Biology
#2	CTGTCCTCTCACATCCGATTT	Vigene Biology

118 **Table S3. Antibodies used in this article.**

Antibodies	Source	Detail information
GAPDH	ABclonal	GAPDH Rabbit pAb (AC001)
CDKN1A	Proteintech	P21 Polyclonal antibody #10355-1-AP
STAT3	Proteintech	STAT3 Polyclonal antibody #10253-2-AP
p-STAT3	CST	Phospho-Stat3 (Tyr705) (D3A7) XP® Rabbit mAb #9145
Second antibody	ABclonal	HRP Goat Anti-Rabbit IgG (H+L) (AS014)
Second antibody	ABclonal	HRP Goat Anti-Mouse IgG (H+L) (AS003)

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Table S4. Sequences of primers for RT-qPCR analysis.

Gene name	Forward Primer	Reverse Primer
<i>Cdkn1a</i>	CCTGGTGATGTCCGACCTG	CCATGAGCGCATCGCAATC
<i>Il6</i>	TAGTCCTTCCTACCCCAATTTCC	TTGGTCCTTAGCCACTCCTTC
<i>Gapdh</i>	TGACCTCAACTACATGGTCTACA	CTTCCCATTCTCGGCCTTG
<i>Cd39</i>	AAGGTGAAGAGATTTTGCTCCAA	TTTGTCTGGGTCAGTCCCAC
<i>Cd73</i>	GGACATTTGACCTCGTCCAAT	GGGCACTCGACACTTGGTG
<i>Rela</i>	AGGCTTCTGGGCCTTATGTG	TGCTTCTCTCGCCAGGAATAC
<i>Il7</i>	GCTGCTTTTCTAAATCGTGCTGCTC	G TTCACCAGTGTTTGTGTGCCTTG
<i>Il8</i>	TCGGGAGACCTCTAGACACTTTGC	GCCTGTCAAGCTGACTTCACTGG
<i>Csf1</i>	CCAATGCTAACGCCACCGAGAG	GCCTTGTTCTGCTCCTCATAGTCC
<i>Csf2</i>	GCATTGTGGTCTACAGCCTCTCAG	GGCATGTCATCCAGGAGGTTTCAAG
<i>Csf3</i>	TGCCAACTTTGCCACCACCATC	CGCTGGAAGGCAGAAAGTGAAGG
<i>Icam1</i>	TCCGCTGTGCTTTGAGAACTGTG	AGGGTGAGGTCCTTGCCTACTTG
<i>Tnfa</i>	CGCTCTTCTGTCTACTGAACTTCGG	GTGGTTTGTGAGTGTGAGGGTCTG
<i>Il1b</i>	GCAACTGTTCTGAACTCAACT	ATCTTTTGGGGTCCGTCAACT
<i>Cxcl1</i>	GGCTGGGATTACCTCAAGAACATC	TGAGTGTGGCTATGACTTCGGTTTG
<i>Cxcl2</i>	AACATCCAGAGCTTGAGTGTGACG	GGGCTTCAGGGTCAAGGCAAAC
<i>Cxcl3</i>	GCCACTCTCAAGGATGGTCAAGAAG	GGACTTGCCGCTCTTCAAGTATCTTC
<i>Cxcl5</i>	TGCGTTGTGTTTGCTTAACCGTAAC	TGACTTCCACCGTAGGGCACTG
<i>Cxcl9</i>	TCCTTTTGGGCATCATCTTCC	TTTGTAGTGGATCGTGCCTCG
<i>Cxcl10</i>	CCAAGTGCTGCCGTCATTTTC	GGCTCGCAGGGATGATTTCAA
<i>Ccl2</i>	CACTCACCTGCTGCTACTCATTAC	CTTCTTTGGGACACCTGCTGCTG
<i>Ccl3</i>	CTCCCAGCCAGGTGTCATTTTCC	CAGGCATTCAAGTTCAGGTCAGTG
<i>Ccl5</i>	GACACCACTCCCTGCTGCTTTG	CTCTGGGTGGCACACACTTGG
<i>Ccl7</i>	GCTTCTGTGCCTGCTGCTCATAG	GCTCTTGAGATTCTCTTGGGGATC
<i>Ccl8</i>	GCTCCAGTCACCTGCTGCTTTC	ACACAGAGAGACATAACCTGCTTGG
<i>Ccl11</i>	CTATTCCTGCTGCTCACGGTCAC	GCTCTTCAGTAGTGTGTTGGGGATC
<i>Ccl20</i>	AGGCAGAAGCAAGCAACTACGAC	ATCGGCCATCTGTCTTGTGAAACC
<i>Mmp2</i>	ACCATGCGGAAGCCAAGATGTG	AGGGTCCAGGTCAGGTGTGTAAC
<i>Mmp3</i>	GACGATGATGAACGATGGACAGAGG	TGTGGAGGACTTGTAGACTGGGTAC
<i>Ccl19</i>	TGCTAATGATGCGGAAGACTGCTG	TCCTTCTGGTGCTGTTGCCTTTG
<i>Ccl1</i>	GCCGTGTGGATACAGGATGTTGAC	TAGCAGGGGTTTACCTTCTTCAGG
<i>Ccl17</i>	GGTCACTTCAGATGCTGCTCCTG	CCTGGACAGTCAGAAACACGATGG
<i>Ccl21a</i>	GAGCCTCCTTAGCCTGGTCCTG	TGTTCAAGTCTCTTGCAGCCCTTG
<i>Ccl22</i>	CTCCTGGTGGCTCTCGTCCTTC	CGGCACAGATATCTCGGTTCTTGAC

Gene name	Forward Primer	Reverse Primer
<i>Ccl25</i>	TTTGAAGACTGCTGCCTGGGTTAC	CACTCCTCACGCTTGTACTGTTGG
<i>Ccl27a</i>	CTGGCATCCGTGGAACAAGACTAAG	GTCCCTTGGAGCCTTTTCCCTTG
<i>Cx3cl1</i>	TGGAAGACCTTGCTTTGGAAGTAC	AAGTAGTGGACACCTGAGGAGATGG
<i>Ccl27</i>	CTACCGAAAGCCACTCTCAGACAAG	TCAGCCCATTTTCCTTAGCATCCC
<i>Ccl28</i>	GTGTGTGTGGCTTTTCAAACCTCAG	AAGTACGATTGTGCGGGCTGATG
<i>Stat3</i>	CAATACCATTGACCTGCCGAT	GAGCGACTCAAACCTGCCCT

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