

Citalopram exhibits immune-dependent anti-tumor effects by modulating C5aR1⁺ TAMs and CD8⁺ T cells

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eLife Assessment

This **important** study provides **solid** evidence to support the anti-tumor potential of citalopram, originally an anti-depression drug, in hepatocellular carcinoma (HCC). In addition to their previous report on directly targeting tumor cells via glucose transporter 1 (GLUT1), they tried to uncover additional working mechanisms of citalopram in HCC treatment in the current study. The data here suggested that citalopram may regulate the phagocytotic function of TAM via C5aR1 or CD8⁺T cell function to suppress HCC growth in vivo.

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Abstract

Administration of selective serotonin reuptake inhibitors (SSRIs) is associated with a reduced cancer risk and shows significant anti-tumor effects across multiple tumor types, suggesting the potential for repurposing SSRIs in cancer therapy. Nonetheless, the specific molecular

target and mechanism of action of SSRIs remain to be fully elucidated. Here, we reveal that citalopram exerts an immune-dependent anti-tumor effects in hepatocellular carcinoma (HCC). Interestingly, the anti-HCC effects of citalopram are not reliant on its conventional target, the serotonin transporter. Through various drug repurposing approaches, including global reverse gene expression profiling, drug affinity responsive target stability assay, and molecular docking, the complement component 5a receptor 1 (C5aR1) is identified as a new target of citalopram. C5aR1 is predominantly expressed by tumor-associated macrophages (TAMs), and citalopram treatment enhances local macrophage phagocytosis and elicits CD8⁺ T anti-tumor immunity. At a systemic level, citalopram treatment results in decreased serum 5-HT levels, which correlates with increased activation of CD8⁺ T cells. Additionally, citalopram promotes αCD3/αCD28-stimulated CD8⁺ T cell activation, expansion, and cytotoxicity. Depletion of CD8⁺ T cells partially hinders the anti-HCC effects of citalopram. Collectively, our study reveals multiple immunomodulatory roles of citalopram in inducing anti-tumor immunity, and provides a basis for considering the repurposing of SSRIs as promising anticancer agents for HCC treatment.

Introduction

The development of anti-tumor drugs encounters numerous challenges, such as a high failure rate, substantial costs, limited bioavailability, safety concerns, and lengthy design and testing procedures¹. As different diseases may share common pathological mechanisms, there is a growing focus on repurposing existing drugs for new applications, referred to as “new uses for old drugs”^{2,3}. Additionally, exploring new disease-relevant targets for established drugs and broadening their indications offers significant translational value⁴.

Selective serotonin reuptake inhibitors (SSRIs; i.e., citalopram, escitalopram, fluoxetine, fluvoxamine, paroxetine, and sertraline) are frequently prescribed to treat conditions such as depression, anxiety, insomnia, and chronic pain⁵. SSRIs target the serotonin transporter (SERT, encoded by the *SLC6A4* gene), blocking the reuptake of serotonin (5-hydroxytryptamine, 5-HT) into the presynaptic neuron, ultimately increasing 5-HT levels in the brain⁶. Recent population-based epidemiological studies suggest that SSRIs have the potential to reduce the risk of many cancers, including kidney, breast, colorectal, and liver cancers^{7–10}. The anxiety and depression symptoms among cancer patients is increasingly recognized¹¹. Considering the established safety profile and known side effects of SSRIs, repurposing these drugs as potential anti-tumor agents, particularly for cancer patients with comorbid depression, could offer dual benefits. This approach may not only help improve neurological function but also potentially impede tumor progression. Hence, repurposing SSRIs in this context could present a cost-effective option. Nevertheless, the mechanisms of actions responsible for the anti-tumor effects of SSRIs are still largely unknown.

In this study, using hepatocellular carcinoma (HCC) as a model, we revealed that citalopram, an SSRI, exhibits anti-tumor properties not through its traditional target SERT, but instead via partially immune-dependent mechanisms. Through a comprehensive approach involving global reverse gene expression profiling, drug affinity responsive target stability (DARTS)¹², and molecular docking analyses, we identified the complement component 5a receptor 1 (C5aR1), predominantly expressed by tumor-associated macrophages (TAMs), as a target of citalopram. C5aR1 play critical roles in fostering an immunosuppressive microenvironment^{13,14} and boosting the metastatic potential of cancer cells¹⁵. Given its significant impact on cancer progression, C5aR1 has surfaced as a promising therapeutic target for potential cancer interventions. Here, we further elucidated the mechanism underlying the connection between C5aR1⁺ TAMs and citalopram-related phenotypes. Additionally, after citalopram treatment, a decline in serum 5-HT levels was observed, which is linked to the activation of CD8⁺ T cells. More

importantly, citalopram directly stimulates CD8⁺ T cell activation, expansion, and cytotoxicity under *in vitro* conditions. Collectively, the anti-tumor effects of citalopram in HCC involve multiple immune-dependent mechanisms.

Results

The immune-dependent anti-tumor effects of citalopram in HCC

To evaluate the therapeutic effects of citalopram, we engrafted mouse Hepa1-6 and Hep53.4 cells into different strains of recipient mice, including the immune-competent C57BL/6 mice and immune-compromised Rag1^{-/-} mice. Citalopram was given by i.p. administration daily for 30 days after bearing palpable tumors (~50 mm³). Tumor growth was significantly retarded in both two mouse models. Of note, this impairment was more pronounced in immune-competent C57BL/6 mice, indicating an immune-dependent effect of citalopram (Figures 1A–B). This finding was further validated at molecular levels, as evidenced by reduced staining of the proliferation index Ki67 and increased expression of cleaved caspase 3 (CCS3) (Figure S1).

As SERT is the known target of SSRIs, we tested the therapeutic effect of citalopram in the absence of SERT (Figures 1C–D). In mouse HCC cells, SERT knockdown failed to affect cell proliferation or cell apoptosis *in vitro* and *in vivo* (Figures 1E–F and S2A–B). However, citalopram delayed the growth of SERT-silenced HCC tumors, and its suppressive effect was more proficient in immune-competent hosts compared with Rag1^{-/-} ones (Figures 1E–F and S2C–F). Together, these data suggest that citalopram might target other molecules instead of SERT to exhibit an immune-dependent anti-tumor effects in HCC.

C5aR1 is a direct target of citalopram

Previously, using drug-induced gene signature networks and target prediction method, we identified a repertoire of targets related to citalopram treatment¹⁶. The top two hits are glucose transporter 1 (GLUT1) and C5aR1 (Figures 2A–B). Citalopram can reverse the Warburg effect to inhibit HCC by targeting GLUT1 directly¹⁶. Considering the significant role of C5aR1 in immune modulation¹⁷, we investigated whether the immune-dependent anti-tumor effects of citalopram are mediated through C5aR1. Immunohistochemical analysis showed that the positive staining of C5aR1 was predominantly found in scattered immune cells (Figure 2C). Single-cell sequencing data indicated that C5aR1 was expressed at higher levels in monocytes/macrophages within the tumor microenvironment (TME) compared with other cell types (Figure 2D). Co-immunofluorescence analysis corroborated that CD163⁺ macrophages presented significant C5aR1 immunoreactivity (Figure 2E).

The DARTS assay is widely recognized technique used to identify potential protein targets for small molecules. This approach involves conferring proteolytic protection of target proteins by their interaction with small molecules¹⁸. Combined DARTS assay with immunoblotting analysis, we investigated the stability of C5aR1 against pronase in the presence or absence of citalopram treatment. Because C5aR1 is expressed by macrophages, THP-1 cells were employed for the DARTS assay. Citalopram did not affect the basal protein level of C5aR1 in macrophages (Figure 2F). However, citalopram could enhance the stability of C5aR1 against pronase treatment. Of note, this effect was specific to C5aR1, as citalopram did not impact the proteolytic susceptibility of β-actin (Figures 2F). Moreover, the stability of C5aR1 against pronase was enhanced in a dose-dependent manner following treatment with citalopram (Figures 2G). Besides citalopram, other 5 SSRIs (escitalopram, fluoxetine, fluvoxamine, paroxetine, and sertraline) also played protective roles of C5aR1 against pronase (Figure S3A).

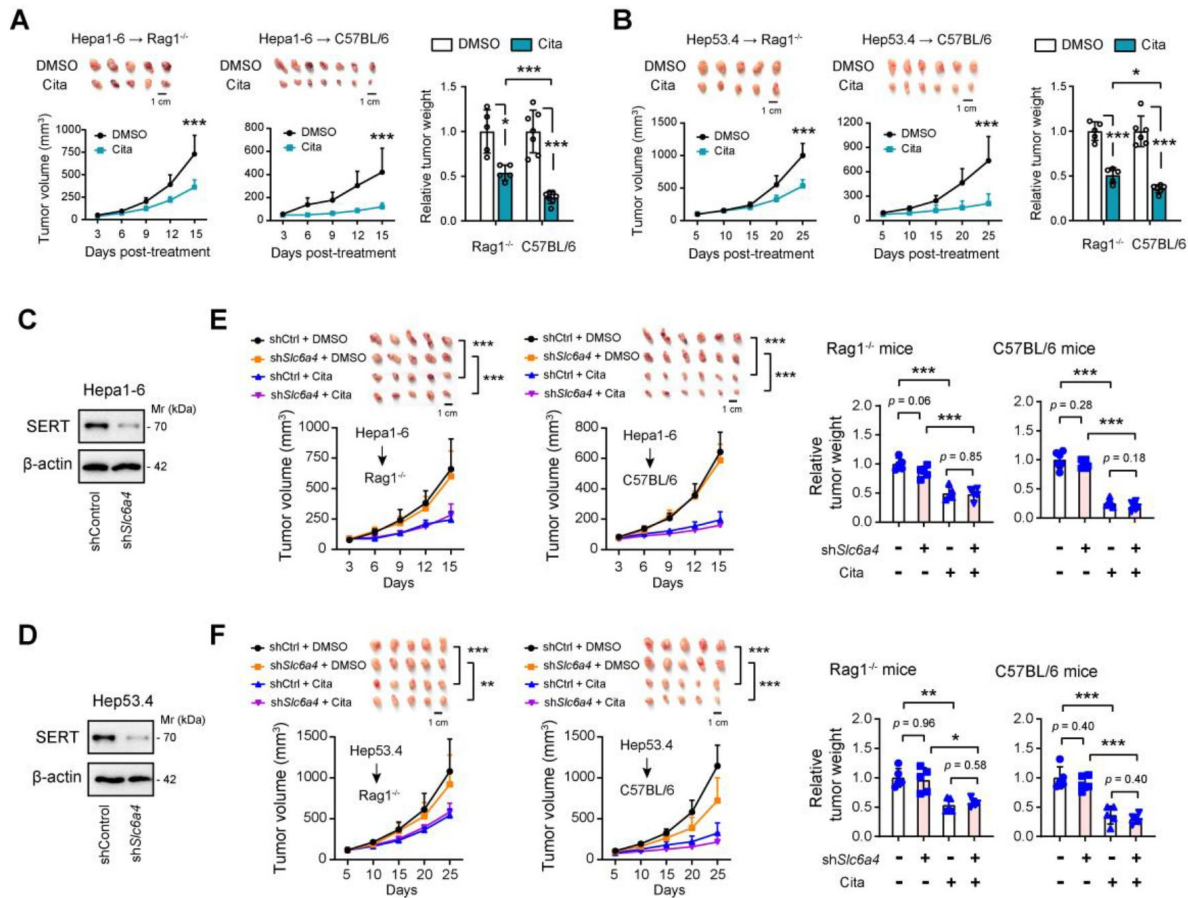


Fig. 1.

The immune-dependent and SERT-independent anti-tumor effects of citalopram in HCC. (A, B) Mouse HCC cells, Hepa1-6 or Hep53.4, were subcutaneously injected into Rag1^{-/-} C57BL/6 or immunocompetent C57BL/6 mice (n = 5-7 per group). When bore visible tumors, 5 mg/kg citalopram was treated daily for 15 or 25 days. Tumors were excised after mice were sacrificed, and the tumor weight was measured. (C, D) Western blotting showed the knockdown efficiency of SERT in Hepa1-6 and Hep53.4 cells. (E, F) shControl and shSlc6a4 Hepa1-6 and Hep53.4 cells were subcutaneously injected into Rag1^{-/-} C57BL/6 or immunocompetent C57BL/6 mice (n = 5-6 per group). When bore visible tumors, 5 mg/kg citalopram was treated daily for 15 or 25 days. Tumors were excised after mice were sacrificed, and the tumor weight was measured. In all panels, **p* < 0.05, ***p* < 0.01, ****p* < 0.001. Values as mean ± SD and compared by one-way ANOVA multiple comparisons with Tukey's method (for bar chart comparison) and two-way ANOVA with Dunnett's multiple comparisons (for survival curve comparison).

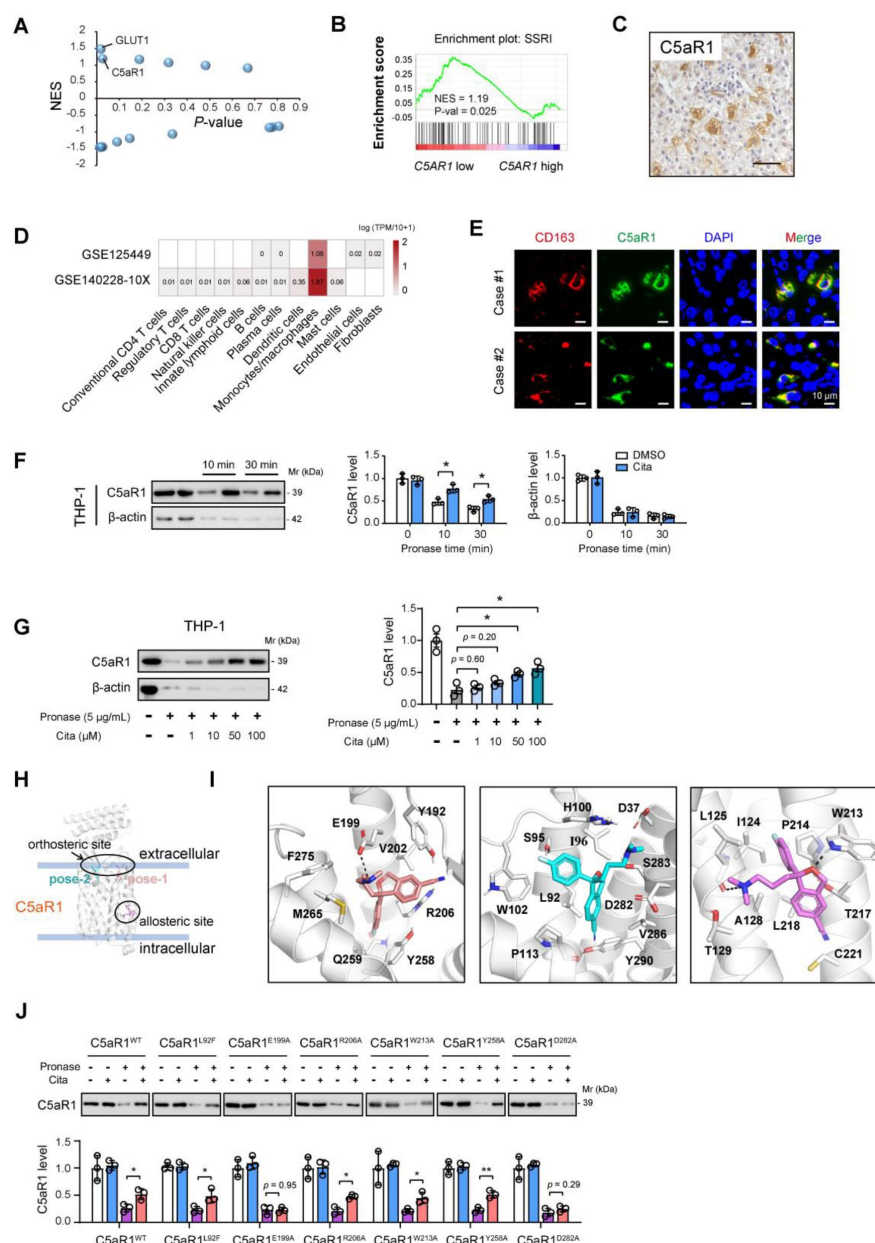


Fig. 2.

C5aR1 is a direct target of citalopram.

(A) Gene Set Enrichment Analysis (GSEA) identified GLUT1 and C5aR1 as two top hits related to SSRI-induced gene changes. (B) GSEA of HCC RNAseq data (TCGA cohort) with the SSRI-related gene signature. Sample grouping was made based on the median expression of C5aR1. (C) Representative immunohistochemical images showed the expression pattern and cellular distribution of C5aR1 in human HCC tissues. Scale bar, 50 μ m. (D) Single cell RNA sequencing analysis showed the expression pattern of C5aR1 with the immune microenvironment of HCC. (E) Co-immunofluorescence of C5aR1 (green) with CD163 (red) in HCC samples. Scale bar, 10 μ m. (F) The DARTS assay and immunoblot analysis showed C5aR1 protein stability against 5 μ g/mL pronase in the presence and absence of 100 μ M citalopram treatment. (G) The DARTS assay and immunoblot analysis showed C5aR1 protein stability against 5 μ g/mL pronase in the presence of different concentration of citalopram treatment. (H) The overall conformation of citalopram binding to C5aR1. (I) Representative models of citalopram in pose-1 (left), pose-2 (middle) and allosteric site (right). Several polar interactions were indicated by black dashed lines. (J) HEK293T cells were transfected with either WT or mutant C5aR1 expression plasmids for 48 h, followed by DARTS assay with immunoblotting analysis of C5aR1 protein levels. In all panels, * p < 0.05, ** p < 0.01. Values as mean $\pm$ SD and compared by the Student's t test (F) or one-way ANOVA multiple comparisons with Tukey's method among groups (G, J).

To address citalopram/C5aR1 interaction, *in silico* docking analysis was conducted. Previous studies revealed two different inhibitor-binding sites in C5aR1, namely, the orthosteric site on the extracellular side and the allosteric site outside the transmembrane bundle ¹⁹. Therefore, we docked the structure of citalopram into both orthosteric and allosteric sites of human C5aR1 (PDB id: 6C1Q), respectively. *In silico* docking of citalopram into the orthosteric site predicted two binding conformations without overlap (**Figures 2H** and S3B). In pose-1, the fluorophenyl and the cyanophthalane group of citalopram stacked with the side chains of Y258 and R206, and the amino group of citalopram interacted with the negatively charged residue E199 (**Figure 2I**, left). In pose-2, two negatively charged residues, D282 and D37, surrounded the amino group (**Figure 2I**, middle). Considering the accessibility, D282 orienting towards the binding cavity of C5aR1 might play a more pivotal role in the citalopram binding event proposed by pose-2. Additionally, the cyanophthalane group of citalopram interacted with hydrophobic residues L92 and P113 of C5aR1. The fluorine was inserted into a narrow space lying over the side chain of W102 (**Figure 2I**, right). For the allosteric site, residue W213 played a critical role in inhibitor binding via hydrogen-bond interactions established by the amino group in the indole ring ²⁰. According to the best pose from cluster 2, the citalopram formed a hydrogen-bond via the oxygen atom in the cyanophthalane group with W213 (**Figure 2I**, right). Inspired by these molecular docking results, we generated six C5aR1 mutants to figure out the real binding site of citalopram in HEK293T cells (**Figure S3C**). Indeed, citalopram had no protective effects on C5aR1 stability with the transfection of HA- C5aR1 E199 and D282 mutant against pronase (**Figure 2J**). Consistently, mutations on E199 and D282 reduce C5a binding affinity to C5aR1 ²¹. Furthermore, docking analysis and DARTS assay revealed that other four SSRIs could bind to the D282 site of C5aR1 (**Figures S3D-E**). These findings demonstrate that SSRIs adopt the orthosteric site to bind to C5aR1 and electrostatic interactions between C5aR1 D282 and the amino groups of SSRIs play an instrumental role in the binding mechanisms.

Citalopram targets C5aR1 in tumor-associated macrophages (TAMs)

C5aR1, the receptor for anaphylatoxin C5a, is profoundly implicated in tumor progression via inducing an immunosuppressive microenvironment in which TAMs are involved ¹⁴. As C5aR1 is localized in TAMs within the TME of HCC, we explored whether citalopram exerted anti-tumor functions by targeting C5aR1 on TAMs. Given that citalopram also targets GLUT1 in HCC ¹⁶, we generated a Hepa1-6 clone with complete knockdown of GLUT1 (GLUT1^{KD}) to dissect C5aR1-dependent roles (**Figure 3A**). Interestingly, citalopram displayed an inhibitory effect on GLUT1^{KD} tumors in immune-component C57BL/6 mice but not Rag1^{-/-} ones (**Figure 3B**), underscoring the contribution of C5aR1. To delineate the involvement of macrophages, we first depleted mouse macrophages with clodro-Liposomes and found that citalopram was ineffective in controlling GLUT1^{KD} tumors (**Figure S4**), implying the influences of citalopram on macrophages. As the second line of evidence, we engrafted GLUT1^{KD} Hepa1-6 cells into the syngeneic C5aR1^{+/-} and C5aR1^{-/-} mice. Compared with C5aR1^{+/-} recipients, tumor growth was attenuated in C5aR1^{-/-} recipients (**Figure 3C**), whereas C5a deposition remained unchanged (**Figure 3D**). Third, we co-injected C5aR1^{+/-} versus C5aR1^{-/-} bone marrow-derived macrophages (BMDMs) with GLUT1^{KD} Hepa1-6 cells into syngeneic recipient C5aR1^{-/-} mice to analyze the therapeutic effect of citalopram. Implantation of C5aR1^{+/-} BMDMs into C5aR1^{-/-} recipients restored tumor growth compared with their C5aR1^{-/-} counterparts and citalopram cannot restrict tumor growth in rC5aR1^{-/-}; dC5aR1^{-/-} mice; in contrast, tumor growth kinetics were considerably diminished in rC5aR1^{-/-}; dC5aR1^{+/-} mice upon citalopram treatment (**Figure 3E**), suggesting that C5aR1⁺ TAM rewiring enables the tumoricidal effects of citalopram. Notably, C5a deposition was not affected by citalopram treatment (**Figure 3F**).

To answer how C5aR1⁺ macrophages mediate citalopram-induced anti-tumor effects, we probed macrophage functions. Phagocytosis assay showed that isolated TAMs from C5aR1^{-/-} recipients had higher phagocytic capacity of HCC cells than that of C5aR1^{+/-} recipients (**Figure 3G**), and

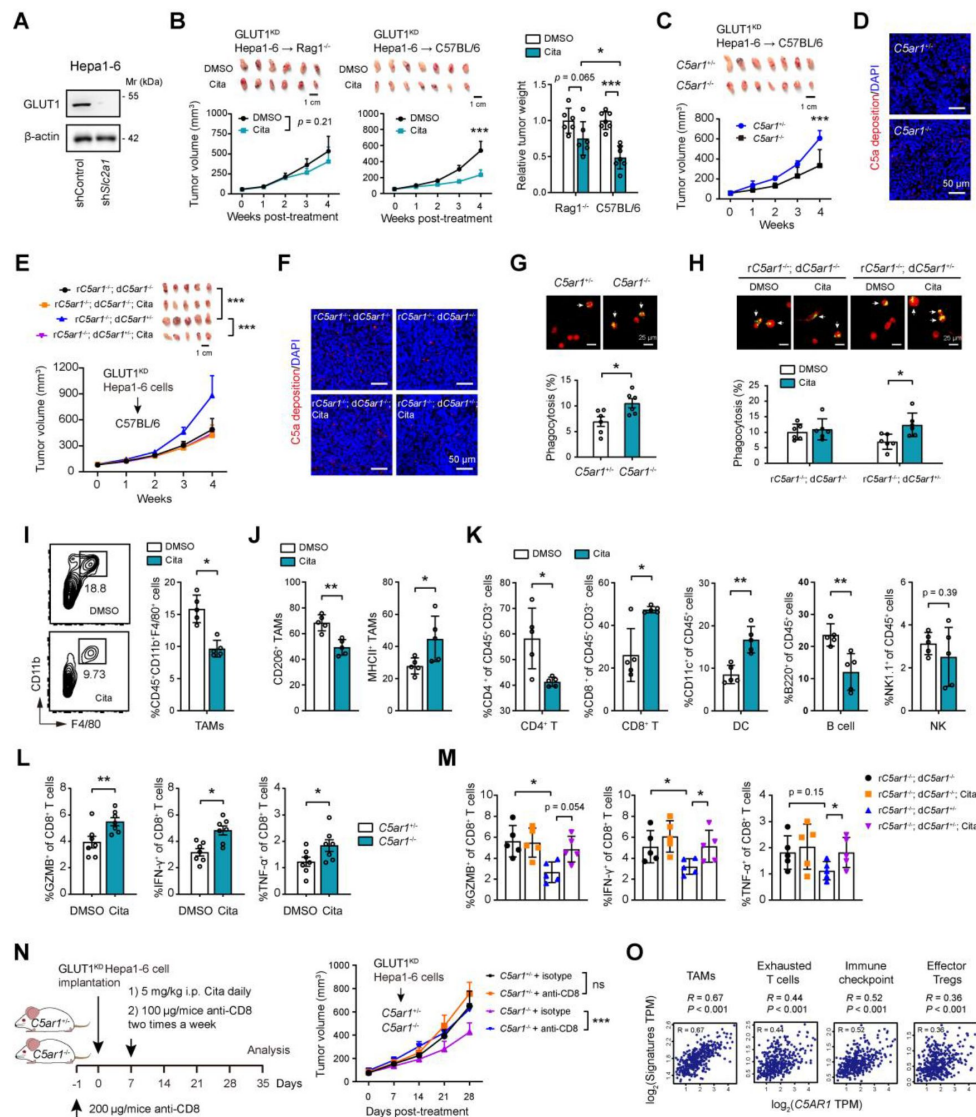


Fig. 3.

Citalopram targets C5aR1⁺ TAMs

(A) Western blotting showed the knockdown efficiency of GLUT1 in mouse Hepa1-6 cells. (B) GLUT1^{KD} Hepa1-6 cells were subcutaneously injected into the Rag1^{-/-} or immunocompetent C57BL/6 mice, and mice were treated with 5 mg/kg citalopram when bore visible tumors; three weeks later, tumor burden was examined (n = 6-7 per group). (C) The growth kinetics of GLUT1^{KD} Hepa1-6 tumors in C5aR1^{+/-} and C5aR1^{-/-} C57BL/6 host (n = 7). (D) Immunofluorescence analysis of C5a deposition in GLUT1^{KD} Hepa1-6 tumors from C5aR1^{+/-} and C5aR1^{-/-} C57BL/6 host. Scale bar, 50 μm. (E, F, H) GLUT1^{KD} Hepa1-6 cells admixed with C5aR1^{+/-} and C5aR1^{-/-} BMDMs from donor (d) mice were subcutaneously implanted into syngeneic recipient (r) mice. The therapeutic effect of citalopram (E), C5a deposition (F), and macrophage phagocytosis (H) in this model were analyzed. Scale bar, 50 μm. (G) The phagocytic capacity of macrophages isolated from GLUT1^{KD} Hepa1-6 tumors in C5aR1^{+/-} and C5aR1^{-/-} C57BL/6 host. (I-K) Flow cytometry showed the infiltration of CD45⁺CD11b⁺F4/80⁺ macrophages (I), CD206⁺ TAMs and CD11b⁺ TAMs (J), tumor-infiltrating lymphocytes (K) in tumor tissues from orthotopic xenograft model, which generated in immunocompetent C57BL/6 mice with Hepa1-6 cells (n = 5 per group). (L, M) Measurement of CD8⁺ T cell function in tumor tissues from the groups mentioned in C and E. (N) The growth kinetics of GLUT1^{KD} Hepa1-6 tumors in C5aR1^{+/-} and C5aR1^{-/-} C57BL/6 host upon CD8⁺ T cell deletion (n = 7). (O) Correlation analysis of C5aR1 expression and immune checkpoint molecules, gene signatures of TAMs, exhausted T cells, and effector Tregs in the TCGA cohort (n = 371). In all panels, *p < 0.05, **p < 0.01, ***p < 0.001; ns, non-significant. Values as mean ± SD and compared by two-way ANOVA with Dunnett's multiple comparisons (B, C, E, N), Student's t test (G, H, I-L), one-way ANOVA multiple comparisons with Tukey's method (B, M), and the Spearman's rank correlation methods (O).

citalopram increased the phagocytosis of TAMs from *rC5ar1^{-/-}*; *dC5ar1^{+/-}* mice (**Figure 3H** [↗](#)). Moreover, C5a-mediated macrophage phagocytic impairment was reversed by C5aR1 knockdown or treatment with citalopram or different SSRIs (Figures S5A-C). C5a was able to suppress, while citalopram was effective in bolstering phagocytosis in macrophages expressing C5aR1^{WT} but not C5aR1^{D282A} (Figures S5D-E). Despite several residues of mouse C5aR1 protein being different from that of human C5aR1, citalopram shared a similar binding model with them (Figure S6). In orthotopic GLUT1^{KD} Hepa1-6 models, flow cytometry revealed a significant reduction of TAMs in the citalopram group compared with the control group (**Figure 3I** [↗](#)). Moreover, TAM polarization was shifted to a proinflammatory state (**Figure 3J** [↗](#)). Further phenotyping of tumor immune subsets in immunocompetent C57BL/6 mice showed that citalopram increased the infiltration of CD8⁺ T and dendritic cells while decreasing the infiltration of CD4⁺ T and B220⁺ B cells (**Figure 3K** [↗](#)). No significant changes were observed in splenic immune subsets upon citalopram treatment (Figure S7).

Next, we sought to understand whether C5aR1⁺ TAMs impact on CD8⁺ T cells. Intratumoral CD8⁺ T cells from tumor-bearing *C5ar1^{-/-}* mice had increased frequency and expression of GZMB, IFN- γ , and TNF- α compared with that from *C5ar1^{+/-}* mice (**Figure 3L** [↗](#)).

Likewise, the frequency and cytotoxicity of CD8⁺ T cell from *rC5ar1^{-/-}*; *dC5ar1^{+/-}* mice were reduced compared with *rC5ar1^{-/-}*; *dC5ar1^{-/-}* mice, and this effect was partially reversed by citalopram treatment (**Figure 3M** [↗](#)). Furthermore, depletion of CD8⁺ T cells abrogated the effect of macrophage C5aR1 deficiency on tumor growth (**Figure 3N** [↗](#)), suggesting that CD8⁺ T cells are needed for the pro-tumor phenotype of C5aR1⁺ TAMs. Clinically, data from TCGA cohort reinforced the link between C5aR1 expression and an immunosuppressive TME as demonstrated by the associations with TAMs, exhausted T cells, immune checkpoint molecules, and effector Tregs (**Figure 3O** [↗](#)).

Citalopram-induced activation of CD8⁺ T cells involves a serotonergic-dependent mechanism

Considering the fact that citalopram targets GLUT1 and CD8⁺ T cell function is reliant on glycolytic metabolism, we investigated the *in vivo* effects of citalopram on T cells. As a result, citalopram significantly invigorated CD8⁺ T cell function and glycolytic metabolism in intrahepatic tumors from WT C57BL/6 mice (**Figures 4A-B** [↗](#)). To simulate the role of metabolic-associated fatty liver disease (MAFLD) in the development of cirrhosis and HCC^{22, 23}, we fed C57BL/6 mice a choline-deficient, amino acid-defined high-fat diet (CDAHFD) to induce metabolic dysfunction-associated steatohepatitis (MASH)/fibrosis prior to orthotopic Hepa1-6 cell implantation. Likewise, CD8⁺ T cell function and glycolytic metabolism were enhanced in MASH mice (**Figures 4C-D** [↗](#)). GLUT1-deficient cytotoxic T cells remain phenotypically and functionally unaffected²⁴, indicating that other glucose uptake mechanisms exist in cytotoxic T cells. GLUT1 and GLUT3 are essential for the activation and effector function of T cells^{24, 25}. We revealed that intratumoral CD8⁺ T cells massively upregulated GLUT3 but not GLUT1 (Figure S8A), and this finding was supported by single cell sequencing analysis (Figure S8B), suggesting the GLUT3-dependent glucose uptake in CD8⁺ T cells.

Given the wide range of peripheral 5-HT actions, we hypothesized that citalopram modulates the immune system through the serotonergic mechanisms. Indeed, citalopram administered for 2 weeks in tumor-bearing mice led to reduced serum 5-HT levels (**Figure 4E** [↗](#)), which were accompanied with a reduction of low-grade systemic inflammatory responses in the MASH mice (**Figure 4F** [↗](#)). Compared to the control C57BL/6 mice, tumor-bearing *Tph1^{-/-}* mice, which lack peripheral 5-HT, exhibited slowed tumor growth and increased effector function of intratumoral CD8⁺ T cells (**Figures 4G-I** [↗](#)). Interestingly, citalopram reduced serum 5-HT to a level as observed in *Tph1^{-/-}* mice, and displayed a superior inhibitory effect on tumor growth and CD8⁺ T cell function compared with that induced by *Tph1* deficiency (**Figures 4G** [↗](#) - I). This finding suggests a

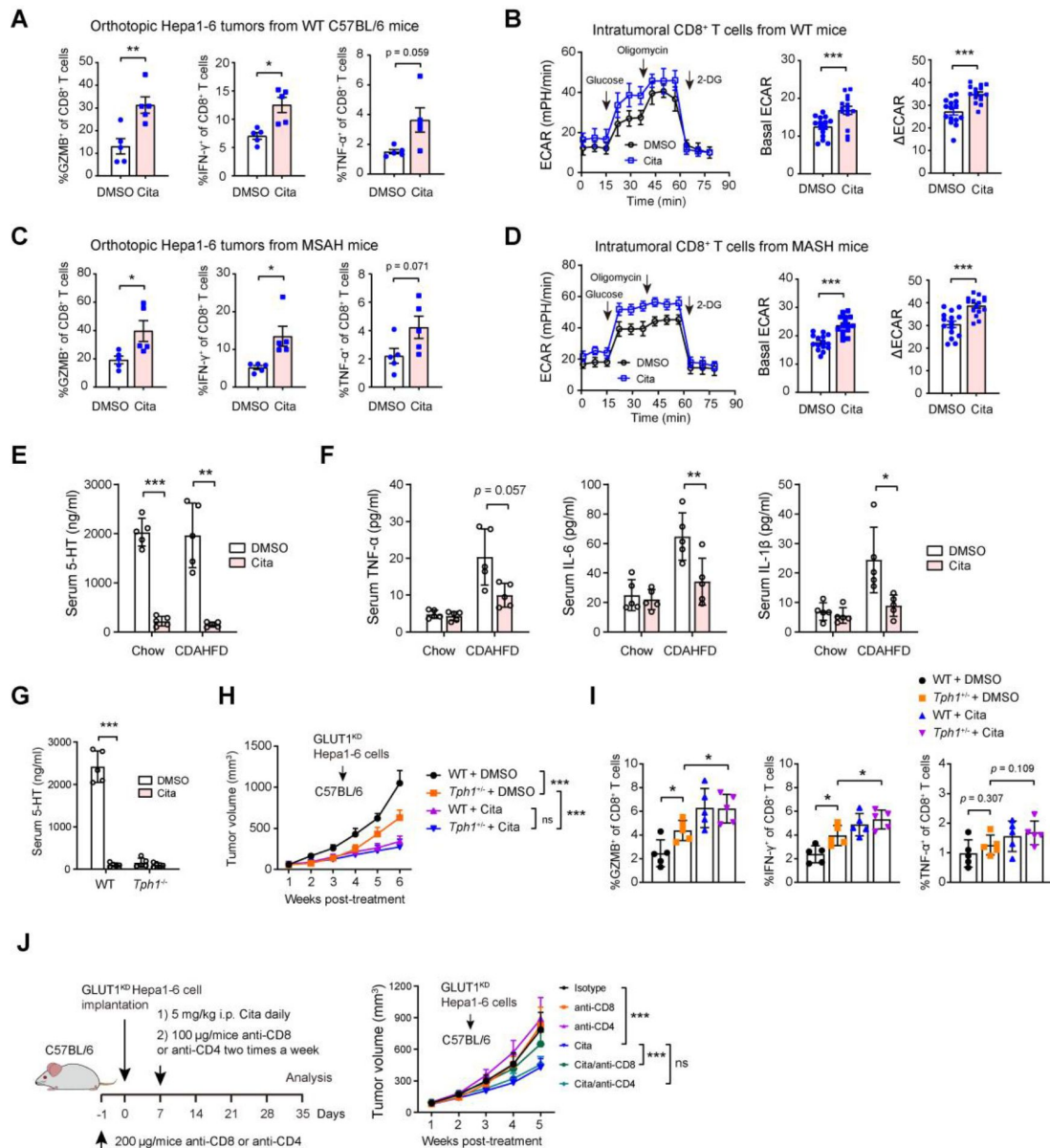


Fig. 4.

Citalopram activates CD8⁺ T cells.

(A, B) Measurement of CD8⁺ T cell function and glycolysis in orthotopic tumor tissues from WT C57BL/6 mice ($n = 5$ per group). (C, D) Measurement of CD8⁺ T cell function and glycolysis in orthotopic tumor tissues from MASH mice ($n = 5$ per group); Basal ECAR indicates glycolysis after the addition of glucose, and Δ ECAR represents the difference between oligomycin-induced ECAR and 2-DG-induced ECAR. (E) Serum 5-HT levels in GLUT1^{KD} Hepa1-6 tumor-bearing mice fed with chow diet or CDAHFD, with the presence or absence of citalopram treatment ($n = 5$ per group). (F) Serum TNF- α , IL-6, and IL-1 β levels in GLUT1^{KD} Hepa1-6 tumor-bearing mice fed with chow diet or CDAHFD, with the presence or absence of citalopram treatment ($n = 5$ per group). (G) Serum 5-HT levels in WT C57BL/6 and *Tph1*^{-/-} mice, with the presence or absence of citalopram treatment ($n = 5$ per group). (H) Tumor growth of WT and *Tph1*^{-/-} mice after subcutaneous injection of Hepa1-6 cells and treatment with citalopram. (I) Measurement of CD8⁺ T cell function in tumor tissues from the groups mentioned in H. (J) The therapeutic effect of citalopram on GLUT1^{KD} Hepa1-6 tumor was tested in the presence or absence of CD4⁺ T or CD8⁺ T cell deletion. In all panels, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$; ns, non-significant. Values as mean $\pm$ SD and compared by the Student's *t* test (A-F), one-way ANOVA multiple comparisons with Tukey's method (I), and two-way ANOVA with Dunnett's multiple comparisons (H, J).

dual immunostimulatory roles of citalopram on CD8⁺ T cells, particularly the serotonergic-dependent mechanism. Next, we investigated the direct effects of citalopram on T cell activation, expansion, and cytotoxicity. Citalopram enhanced αCD3/αCD28-stimulated CD8⁺ T cell proliferation as indicated by CFSE staining (Figure S9A). Moreover, citalopram promoted the TCR-instigated activation of CD8⁺ T cells and the generation of effector CD8⁺ T cells as evidenced by the cell surface expression of CD44 and CD62L (Figure S9B). Likewise, the cytotoxic activity of activated CD8⁺ T was enhanced by citalopram, as reflected by GZMB, IFNγ, and TNF-α staining (Figures S9C-E).

Finally, to examine the immune-dependent anti-tumor effects of citalopram, we depleted either CD8⁺ or CD4⁺ T cells from GLUT1^{KD} Hepa1-6 tumor-bearing mice and found that depletion of CD8⁺ T cells, rather than CD4⁺ T cells, increased tumor growth in citalopram-treated mice but had marginal effects in control mice (Figure 4J [↗](#)), showing that CD8⁺ T cells are needed in the effector mechanisms of citalopram.

Discussion

Repurposing approved non-oncology drugs for cancer therapy offers cost-effectiveness, an accelerated development timeline, and a known safety profile as key advantages. Accumulating studies have deciphered the inverse link between SSRIs use and cancer risk. However, there is a scarcity of studies investigating the potential repurposing SSRIs for cancer treatment and their mechanisms of action. Non-oncology drugs may have mechanisms of action that are different from traditional cancer treatments. Previously, we and others have uncovered several new targets of SSRIs, including GLUT1 in HCC, SMPD1 in glioblastoma [26](#)[↗](#), and VDAC1 in autophagy [27](#)[↗](#). Here, we further broaden the knowledge of how SSRIs impact the immune system to exert anti-tumor effects. By targeting C5aR1 on TAMs, citalopram enhances the phagocytic activity of TAMs and boosts local anti-tumor immune responses. Furthermore, treatment with citalopram results in a reduction in circulating 5-HT levels and this phenomenon is akin to the deficiency of *Tph1* and enhances the activation of CD8⁺ T cells. Therefore, these insights shed light on the immunomodulatory role of SSRIs in the context of cancer treatment (Figure 5 [↗](#)).

C5aR1 is expressed on TAMs within the TME, which helps to explain why citalopram has better anti-tumor effects in immune-competent mice. In addition to TAMs, C5aR1 is also expressed by neutrophils, mast cells, and myeloid-derived suppressor cells in cancers [14](#)[↗](#), [28](#)[↗](#), [29](#)[↗](#). Although the functional impacts of citalopram on these C5aR1-expressing immune cells have not been formally determined, macrophage depletion assay and C5aR1^{-/-} BMDM co-injection experiment suggested that citalopram exerted anti-tumor functions *in vivo* through C5aR1-expressing TAMs. C5aR1⁺ macrophages are profoundly implicated in squamous carcinogenesis and targeting C5aR1 reprograms macrophages to recruit cytotoxic CD8⁺ T cells [14](#)[↗](#). In alignment with this, citalopram treatment increased CD8⁺ T cell filtration and their cytotoxic activities in HCC, and this effect was largely dependent on C5aR1⁺ macrophages. One possible explanation is that citalopram targets C5aR1 to enhance macrophage phagocytosis and subsequent antigen presentation and/or cytokine production, which promotes T cell recruitment and activity as well as modulate other aspects of tumor immunity. Moreover, citalopram induces TAM phenotypic polarization towards to a proinflammatory state, which supports anti-tumor immune response within the TME. Therefore, we provide a mechanistic insight regarding how citalopram target C5aR1 activity in TAMs, the central findings are important to our understanding of citalopram-induced anti-tumor activities and may lay a basis for upcoming mechanistic studies aimed to decipher specific tumor-promoting roles of this defined TAM population.

While we highlight the anti-HCC effects of SSRIs through targeting C5aR1, it is possible that SSRIs impact tumor progression by modulating the systemic environment via serotonergic mechanisms, especially concerning the activation of CD8⁺ T cells. Apart from its role in regulating hemostasis, 5-

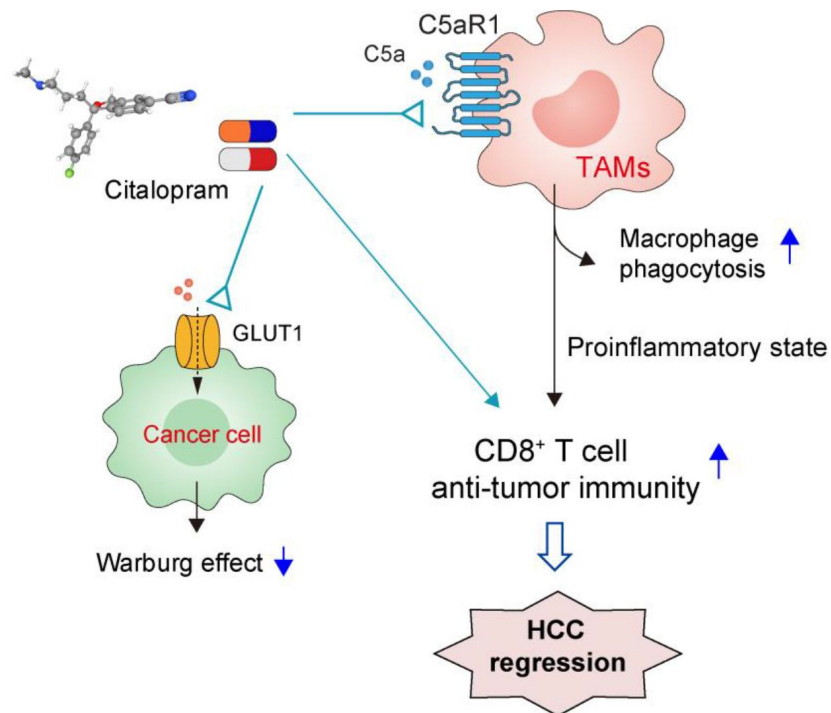


Fig. 5.

Mechanism model.

Model depicting the molecular mechanism by which citalopram inhibit the Warburg effect and elicit an anti-tumor response in HCC. Citalopram not only inhibits glycolytic metabolism of cancer cells by targeting GLUT1 (Ref 16) but also enhances macrophage-driven anti-tumor immunity and induces a systemic immunostimulatory effect on CD8⁺ T cell functions through serotonergic mechanisms.

HT exerts immunoregulatory functions on circulating immune cells ³⁰. In this study, it was observed that both citalopram treatment and *Tph1* deficiency enhanced CD8⁺ T cell cytotoxic activity. Consistently, peripheral 5-HT has been shown to dampen the effector functions of CD8⁺ T cells within tumors ³¹. Similarly, treatment with SSRIs such as fluoxetine increased the influx of CD8⁺ T cells and reduced the growth of syngeneic pancreatic and colorectal tumors ³¹. In addition to the traditional receptor-dependent signaling transduction, 5-HT can modulate CD8⁺ T activity through post-translational modification known as serotonylation ³². It remains to be explored whether serotonylation contributes to the immunomodulatory effects of citalopram, as citalopram directly stimulates CD8⁺ T cell proliferation, activation, and cytotoxicity *in vitro*. The observed reduction in serum 5-HT levels and activation of CD8⁺ T cells following SSRI treatment further highlight the complex interplay between neurotransmitters, the immune system, and cancer progression. Immunotherapy, especially immune checkpoint inhibitors (ICIs), has revolutionized the management of HCC ³³. The immune microenvironments play crucial roles in the response or resistance of HCC to immunotherapies ³⁴. However, limited molecular biomarkers are available to guide clinical decision-making for ICI therapy of HCC. Given SSRIs can increase CD8⁺ T cell infiltration as well as cytotoxic activities by targeting C5aR1⁺ TAMs, one can suppose that C5aR1⁺ TAMs within the HCC microenvironment might be used as a molecular marker for SSRIs use in combination with ICI therapy.

In conclusion, our study suggests the potential repurposing of SSRIs for the treatment of HCC and highlights C5aR1 as a direct target of citalopram. We elucidate the immune-dependent mechanisms of action of citalopram, involving the regulation of both TAMs and CD8⁺ T cells. Combining SSRIs with ICIs or other first-line therapies could enhance the treatment efficacy of current standard therapies.

Materials and Methods Animal models

In the subcutaneous xenograft model, Rag1^{-/-} C57BL/6N mice (male, 6-8-week-old, Cyagen Biosciences, C001197), wild type immune-competent C57BL/6 mice (male or female, 6-8-week-old) or *Tph1*^{-/-} mice (male, 6-8-week-old, Cyagen Biosciences, S-KO-05541), were maintained on a 12-hour day/night cycle with ad libitum access to food and water. For the pharmacological inhibition study, 5 × 10⁶ mouse HCC cells (wild type, shControl, sh*Slc6a4* or sh*Slc2a1* Hepa1-6 and Hep53.4) in 100 µl HBS were injected subcutaneously in the lower back. When bore visible tumors, mice were given intraperitoneal injections of vehicle or citalopram (5 mg/kg body weight, Selleck, Shanghai, China) daily (n = 5-7 per group).

In the orthotopic xenograft model, 5 × 10⁶ Hepa1-6 cells transfected with luciferase-expressing lentiviruses were orthotopically implanted into the liver of WT or MASH C57BL/6 mice. The day following tumor cell implantation, mice were treated with citalopram (5 mg/kg body weight) or DMSO. In the diet-induced MASH model, mice were fed a choline-deficient, amino acid-defined high-fat diet (45 kcal% fat) supplemented with 0.1% methionine (CDAHFD diet, A06071309, from Research Diets) for a duration of 6 weeks.

For the C5aR1 knockdown study, C57BL/6J-*C5ar1*^{em1Cya} mice were purchased from Cyagen Biosciences (S-KO-01274, Suzhou, China). 5 × 10⁶ sh*Slc2a1* Hepa1-6 cells were injected into the lower back of 6-8-week-old male *C5ar1*^{+/-} or *C5ar1*^{-/-} C57BL/6 mice. Tumor volume was measured every week using a digital caliper. Four weeks later, mice were sacrificed and xenograft tumors were analyzed.

To determine the association between citalopram and C5aR1, 2 × 10⁶ GLUT1^{KO} Hepa1-6 cells were mixed 1:1 with BMDMs (*C5ar1*^{+/-} or *C5ar1*^{-/-}) in growth factor-reduced Matrigel (#354230, Corning, Shanghai, China) and injected into the lower back of 6-8-week-old male *C5ar1*^{+/-} C57BL/6 mice. To acquire BMDMs, bone marrow was harvested from the femurs and tibias of *C5ar1*^{+/-} or *C5ar1*^{-/-} mice. Then, isolated cells were cultured in RPMI 1640 supplemented with 10% FBS, stimulated with recombinant CSF-1 (20 ng/ml, 416-ML, R&D systems), and maintained at 37°C in a humidified

atmosphere with 5% CO₂. Differentiation of BMDMs was confirmed by flow cytometric analysis of F4/80 (28463-1-AP, Proteintech) and CD45 (20103-1-AP, Proteintech) expression. One week after cancer cell implantation, mice were subjected to treatment with citalopram (5 mg/kg body weight) or DMSO. At indicated time point, mice were sacrificed and xenograft tumors were analyzed.

In all animal studies, tumor volume was assessed using caliper measurements and calculated using the formula: tumor = 1/2(length × width²), where length represents the largest diameter and width indicates the smallest diameter. At the experimental endpoint, mice were humanely euthanized, tumors were excised, and tumor weight was recorded. All animals received proper care in accordance with the guidelines outlined in the “Guide for the Care and Use of Laboratory Animals” established by the National Academy of Sciences and published by the National Institutes of Health (NIH publication 86-23 revised 1985). The protocols for animal studies were approved by the ethics committee of the Ren Ji Hospital, School of Medicine, Shanghai Jiao Tong University (approval number 202201427 and RA- 2021-096).

Cell culture and reagents

The following cell lines were included: Hepa1-6, Hep53.4, THP-1, and HEK293T. All cells were cultured in a medium recommended by ATCC, supplemented with 10% fetal bovine serum (FBS; Gibco, USA) and 1% (v/v) streptomycin-penicillin (Sigma-Aldrich, Shanghai, China), and maintained at 37°C in a humidified atmosphere with 5% CO₂. Prior to conducting cell experiments, all the mentioned cell lines underwent testing for short tandem repeat profiling and mycoplasma contamination to ensure their authenticity and purity. Citalopram (S4749), escitalopram (S4064), fluoxetine (S1333), fluvoxamine (S1336), paroxetine (S3005), and sertraline (S4053) were all obtained from Selleck (Shanghai, China).

Plate colony formation assay

For cell proliferation analysis, a total of 1,000 indicated HCC cells were seeded into 6-well plates with three replicates. After treatment with citalopram for 10-14 days, the plates were washed with phosphate-buffered saline (PBS) twice, fixed with methanol for 10 min, and stained with 0.5% (w/v) crystal violet solution for 10 min. Colonies containing more than 30 cells were counted under the microscope.

Detection of Caspase-3/7 activity

Cell apoptosis in HCC cells upon citalopram treatment was assessed using the Apo-ONE® Homogeneous Caspase-3/7 Assay (Promega, Madison, WI, USA) as described previously³⁵. To normalize the data, the cell number in the same plate was detected using CellTiter-Blue (Promega, G8081). The relative Caspase-3/7 activity was determined by calculating the ratio of the signals obtained from the Apo-ONE assay to those from the CellTiter-Blue assay.

Gene knockdown and knockout

The specific shRNA oligonucleotides targeting *Slc6a4* and *Slc2a1* were custom-synthesized by Genepharma (Shanghai, China). Lentivirus packaging was carried out in HEK293T cells using Lipofectamine 2000 (Invitrogen, Carlsbad, CA), and virus titers were determined following standard procedures. HCC cells (Hep53.4 and Hepa1-6) were infected with the virus-containing supernatant in the presence of 6 µg/ml polybrene (Sigma-Aldrich, H9268, St. Louis, MO) when they reached 70% confluence. Cells expressing the shRNAs were selected using puromycin (2 µg/ml, Gibco, A1113802).

For CRISPR/Cas9-mediated gene knockout, THP-1 cells were initially transfected with the lentiCas9-Blast vector to facilitate the expression of Cas9. To generate *C5aR1*^{-/-} THP-1 cells, a lentiviral system utilizing guide RNA (sgRNA) targeting C5AR1 was utilized. The lentivirus was produced in HEK293T cells by transfecting lentiGuide-Puro along with packaging plasmids pSVg and psPAX2.

The transfection was carried out using Fugene6 (E2693, Promega) in OptiMEM. After two days, the supernatants were filtered through a 0.45 µm filter, and Cas9-expressing cells were infected with the lentivirus in the presence of 6 µg/ml polybrene. Individual colonies were then seeded into 96-well plates, allowed to reach confluence, and subsequently subjected to knockout efficiency analysis. The sequences for shRNA and sgRNA are provided below: sh*Slc6a4*, CACCGCCTCCTACTATAACACCATCCGAAGATGGTGTATAGTAGGAGGC; sh*Slc2a1*, CACCGGGAGAAGAAGGTCACCATCTCGAAAGATGGTGACCTTCTTCTCCC; C5AR1 sgRNA, CTTCACTCAACACGTTCCGG.

Quantitative real-time PCR (qRT-PCR)

RNA was extracted from CD8⁺ T cells using the RNAiso Plus reagent (Takara, Japan) and subjected to reverse transcription using the PrimeScript RT-PCR kit (Takara, Japan). Then, qRT-PCR was performed using the 7500 Real-time PCR system (Applied Biosystems, USA). The housekeeping gene *Gapdh* was used to normalize gene expression. Primer sequences used in this study were shown below: *Slc2a1* forward, 5'-CAGTTCGGCTATAACACTGGTG-3'; *Slc2a1* reverse, 5'-GCCCGGACAGAGAAGATG-3'; *Slc2a3* forward, 5'-ATGGGGACAACGAAGGTGAC-3'; *Slc2a3* reverse, 5'-GTCTCAGGTGCATTGATGACTC-3'; *Gapdh* forward, 5'-AGGTCGGTGTGAACGGATTG-3'; *Gapdh* reverse, 5'-TGTAGACCATGTAGTTGAGGTCA-3'.

Immunoblotting

Cellular lysates were extracted using RIPA lysis buffer (P0013B, Beyotime, Shanghai, China) supplemented with protease and phosphatase inhibitor cocktails (ab201119, Abcam, Shanghai, China). The protein concentration was quantified with a BCA Protein Assay Kit (Pierce Biotechnology, USA). The proteins were then separated by sodium dodecyl sulfate- polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto polyvinylidene difluoride (PVDF, Millipore) membranes. Subsequently, the membranes were blocked with 5% (m/v) skim milk for 1 h at room temperature before being incubated with specific antibodies overnight at 4°C. On the following day, the membranes were washed three times with PBS and then incubated with HRP-conjugated secondary antibodies for 45 min. Subsequently, an ECL chemiluminescence assay (SB-WB012, Share-bio, Shanghai, China) was performed using a Bio-Spectrum Gel Imaging System (Bio-Rad). The antibodies utilized in this study included: SERT (1:500, ProteinTech, 19559-1-AP), GLUT1 (1:2,000, Cell Signaling Technology, #73015), C5aR1 (1:1,500, ProteinTech, 21316-1-AP), and β-actin (1:2,000, Abcam, ab8226). The information for secondary antibodies is as follows: goat anti-rabbit IgG (H+L) cross-adsorbed secondary antibody (1:5,000, Thermo Fisher Scientific, G-21234) and goat anti-mouse IgG (H+L) cross-adsorbed secondary antibody (1:5,000, Thermo Fisher Scientific, G-21040).

Immunohistochemistry and immunofluorescence analysis

Paraffin-embedded sections of mouse HCC tissues were subjected to immunohistochemical analysis of Ki67, cleaved caspase 3, and C5aR1 expression. Immunohistochemical analysis was performed as reported previously³⁵. The primary antibodies used in this study were shown as follows: Ki67 (1:400, Cell Signaling Technology, #9449), cleaved caspase 3 (1:400, Cell Signaling Technology, #9661), and C5aR1 (1:1,200, ProteinTech, 21316-1-AP). For immunofluorescence analysis, paraffin sections (5 µm) of HCC samples were deparaffinized and rehydrated with graded ethanol, followed by microwave heating-based antigen retrieval. Then tissue sections were blocked with 10% donkey serum (Sigma-Aldrich, D9663, USA) for 1 h at room temperature and incubated with primary antibodies at 4°C overnight. The following antibodies were used: C5aR1 (1:50, Proteintech, 10375-1-AP) and CD163 (1:200, Abcam, ab182422). The next day, tissue sections were incubated with donkey anti-rabbit Alexa Fluor 594 (1:400, Jackson ImmunoResearch, #711-585-152) and donkey anti-mouse Alexa Fluor 488 (1:400, Jackson ImmunoResearch, #715-545-150).

for 30 min at room temperature. Finally, 4',6-diamidino-2-phenylindole (DAPI) staining was performed to visualize the nuclei. Confocal microscope equipped with a digital camera (Nikon, Japan) was used for high-resolution imaging.

Bioinformatics analysis and single cell sequencing data

Correlation analysis was performed with GEPIA2 (<http://gepia2.cancer-pku.cn/#index>)³⁶. The following gene signatures were included for analysis, TAMs (*CD33*, *MARCO*, *CXCL5*, *SULT1C2*, *MSR1*, *CTSK*, *PTGDS*, *COLEC12*, *GPC4*, *PCOLCE2*, *CHIT1*, *CLEC5A*, *CCL7*, *FN1*, *CD163*, *GM2A*, *BCAT1*, *RAI14*, *COL8A2*, *CHI3L1*, *ATG7*, *CD84*, *MS4A4A*, *EMP1*, *CYBB*, *CD68*, and *CD11b*), exhausted T cells (*HAVCR2*, *TIGIT*, *LAG3*, *PDCD1*, *CXCL13* and *LAYN*), immune checkpoint (*IDO1*, *IDO2*, *PDCD1*, *CD274*, *PDCD1LG2*, *CTLA4*, *LAG3*, *HAVCR2*, *C10orf54*, *BTLA*, *ICOS*, and *TNFRSF9*), and effector Treg cells (*FOXP3*, *CTLA4*, *CCR8*, and *TNFRSF9*). The single-cell transcriptome expression of C5aR1 and GLUT family members in the tumor microenvironment was identified with GSE125449 and GSE140228-10X.

DARTS experiments

THP-1 or HEK293T cells were lysed using NP40 lysis buffer (Thermo Fisher Scientific, 89842Y). Following centrifugation at 12,000 rpm for 10 min, the supernatant containing the proteins was collected, and the protein concentration was determined using a BCA Protein Assay Kit (Pierce Biotechnology, USA). Samples of equivalent quality were then treated with specific SSRIs and a vehicle at 37°C for 4 h. Subsequently, pronase (10 µg/mL, Roche, 10165921001) or distilled water was added and incubated at room temperature for specified time points (10 and 30 min). The reaction was terminated by adding protease inhibitors, and the resulting products were harvested and subjected to analysis using Western blotting.

In Silico docking study

To predict the binding mode of citalopram/C5aR1, molecular docking was performed using the AutoDock 4.2.6 software package³⁷. The receptor structure for C5aR1 (PDB id: 6C1Q)²⁰ was sourced from the RCSB protein data bank (<https://www.rcsb.org/>). Due to the lack of available structural information, the structure of mouse C5aR1 was predicted using a local version of the ColabFold (AlphaFold2) software^{38,39}. The original substrates and other heteroatoms in these crystal structures were removed for further docking procedure. The absolute structure of citalopram (CID: 2771), fluoxetine (CID: 3386), fluvoxamine (CID: 5324346), paroxetine (CID: 43815), and sertraline (CID: 68617) were sourced from the PubChem database (<https://pubchem.ncbi.nlm.nih.gov/>). In each docking scenario, molecular docking was conducted following a standard protocol⁴⁰, with a modification in the number of genetic algorithm runs to 500 to comprehensively explore the binding modes. The binding energy and cluster information were directly extracted from the AutoDock output.

Biochemical analysis

The serum levels of inflammatory cytokines (TNF-α, IL-1β, and IL-6) were analyzed using commercial ELISA kits following the manufacturer's instructions (R&D Systems). The cytokine concentrations were calculated using a standard curve, and the data were presented in picograms per milliliter. Serum 5-HT levels were assessed using the 5-HT ELISA Kit from Abcam (ab133053), following the manufacturer's guidelines.

Isolation of TAMs from xenograft tumor tissues

Fresh sterile tumor tissues from subcutaneous xenograft model were washed with PBS two times, minced with scalpels, and then digested using a Tumor Dissociation Kit (Miltenyi Biotec, cat #130-095-929) at room temperature for 30 min. Digested tumor tissues were filtered through Falcon 70Micrometer Cell Strainer (Corning, #352350) and centrifuged at 1,500 rpm for 5 min. Red blood

cells were removed using Red Blood Cell Lysis Solution (Miltenyi Biotec, cat #130-094-183). Then, TAMs were selected by CD11b microbeads (Miltenyi Biotec, cat #130-049-601) according to the manufacturer's instructions.

Isolation of immune cells and flow cytometry

Mice were euthanized, and fresh tumor tissues and spleen were harvested. Spleens were mechanically disrupted through 70 μ m Nylon mesh (Corning, #352350) to release single cells. The cells were washed with PBS, followed by the lysis of red blood cells with Red Blood Cell Lysis Solution (Miltenyi Biotec, cat #130-094-183). Tumor tissues were cut into small pieces with surgical scissors and digested with 2 mg/mL collagenase IV (Sigma) for 20 min and filtered with 70 μ m Nylon mesh. The isolated cells were collected with PBS and subjected to centrifuged at 1,500 rpm for 5 min. To enrich immunocytes from tumor tissues, harvested cells were centrifuged through different concentrations of discontinuous gradients of 40/80 Percoll (GE Healthcare, cat #17-0891-01) at 350 $\times$ g for 30 min. The middle pellet was washed with PBS, subjected to red blood cell lysis for 3 min.

For staining molecules expressed on cell surface, single cell suspensions from spleens and tumor tissues were labeled with fluorophore-conjugated antibodies: PerCP-Cyanine 5.5 7AAD (Biolegend, Cat #420404, 1:1,000), FITC anti-mouse CD4 (Biolegend, Cat #100406, 1:1,000), PE anti-mouse Ly6G (Biolegend, Cat #127608, 1:200), APC anti-mouse CD11c antibody (Biolegend, Cat #117310, 1:200), PE-cy7 anti-mouse CD8 α (Biolegend, Cat #100722, 1:200), Brilliant Violet 421 anti-mouse CD3 (Biolegend, Cat #100227, 1:200), Alex Fluor 647 anti-mouse F4/80 (Invitrogen, Cat #2604384, 1:200), Brilliant Violet 510 anti-mouse CD45 (Biolegend, Cat #103138, 1:200), PE-cy7 anti-mouse NK1.1 (Biolegend, Cat #156513, 1:200), Brilliant Violet 421 anti-mouse B220 (Biolegend, Cat #103240, 1:200), Brilliant Violet 605 anti-mouse CD11b (Biolegend, Cat #101257, 1:200), APC-CY7 Stain 780 (BD Bioscience, Cat #565388, 1:1,000).

For intracellular staining, single cell suspensions from tumor tissues were stimulated with Cell Stimulation Cocktail (Invitrogen) for 4 h, and labeled with PE anti-mouse CD45 antibody (Biolegend, Cat #103106, 1:200), PerCP-Cyanine 5.5 CD3 (Biolegend, Cat #100325, 1:200), PE-cy7 anti-mouse CD8 α (Biolegend, Cat# 100722, 1:200), APC-CY7 Stain 780 (BD Bioscience, Cat #565388, 1:1,000). After fixation and permeabilization using the Fix/Perm kit (ThermoFisher, GAS004), cells were labeled with APC anti-human/mouse Granzyme B Recombinant antibody (Biolegend, Catalog no.372203, 1:200), Brilliant Violet 421 anti-mouse IFN- γ (Biolegend, Cat #505830, 1:200), Brilliant Violet 605 anti-mouse TNF- α (Biolegend, Cat #506329, 1:200). Flow cytometry was performed in BD FACSCanto II.

T cell activation, expansion and cytotoxicity

Single-cell suspensions were freshly dissected from the spleen and lymph nodes, and red blood cells were removed using Red Blood Cell Lysis Solution (Miltenyi Biotec, cat #130-094-183). CD8⁺ T cells were isolated from the resulting cell suspensions by CD8⁺ magnetic bead selection (Miltenyi Biotec, cat #130-117-044). For T cell proliferation assay, isolated CD8⁺ T cells were resuspended in PBS supplemented with carboxy fluorescein succinimidyl ester (CFSE, Invitrogen, 1:10,000) for 8 min at room temperature and washed with RPMI 1640, supplemented with 10% heat-inactivated FBS. Then, CD8⁺ T cells were activated by plate-bound α CD3 ϵ (5 μ g/mL, Biolegend, Catalog no.100359) and α -CD28 (2 μ g/mL, Biolegend, Catalog no.102121) in complete RPMI 1640 medium at 37°C and 5% CO₂ for 72 h in the presence or absence of 5 μ M citalopram treatment. Finally, cell proliferation was assessed by flow cytometry. For assessment of cellular activation and cytotoxicity, CD8⁺ T cells were stimulated by Leukocyte Activation Cocktail (GolgiPlug™ BD, Catalog no.550583) for 4 h, resuspended in PBS/2% FBS containing PE anti-mouse CD62L antibody (Biolegend, Catalog no.104407, 1:200) and Brilliant Violet 510 anti-mouse/human CD44 Antibody (Biolegend, Catalog no.103044, 1:200), followed by incubation at room temperature for 30 min and washed in PBS/2% FBS. After fixation and permeabilization using the Cytofix/Cytoperm solution

(BD Biosciences) for 20 min at 4°C, Brilliant Violet 421 anti-mouse IFN- γ antibody (Biolegend, Catalog no.505830, 1:200), APC anti-human/mouse Granzyme B Recombinant antibody (Biolegend, Catalog no.372203, 1:200) and Alexa Fluor 488 anti-mouse TNF- α antibody (Biolegend, Catalog no. 506313, 1:200) were employed for intracellular staining. Then, cell activation and cytotoxicity were assessed by flow cytometry.

Macrophage phagocytosis assay

Phagocytosis assays of tumor cells were performed as previously described ⁴¹. Briefly, 5×10^4 phorbol myristate acetate (PMA, 100 ng/ml) primed THP-1 cells and harvested TAMs were seeded overnight in a 24-well tissue culture plate. The next day, HCC-LM3 cells and Hepa1-6 cells were labelled with pHrodo™ Green (Thermo, Cat #P35373, 1:1,000). After incubating macrophages in serum-free medium for 2 h, the macrophages were labeled with CellTracker™ Deep Red Dye (Thermo, Cat #C34565, 6 ng/mL). Then, 5×10^4 predyed tumor cells were added to the macrophages, in the presence or absence of C5a stimulation and different SSRIs (5 μ M). After incubation at 37 °C for 4 h, macrophages were extensively washed and imaged with Confocal Laser Scanning Microscope (Leica).

Macrophage depletion assay

The method for eliminating macrophages was reported previously ⁴². Briefly, C57BL/6 mice were intraperitoneally injected with clodronate liposomes (1.4 mg/20 g body weight) to remove macrophages. Clodronate liposomes (Yeasen, 40337ES10, Shanghai, China) were given every three days for 12 days before tumor inoculation. After inoculation of shSlc2a1 (GLUT1^{KD}) Hepa1-6 cells, clodronate liposomes were also administered every three days until the end of the experiment. When the mice were sacrificed, liver and tumor tissues were extracted, and F4/80 staining (1:250, Cell Signaling Technology, #70076) was performed to determine the efficiency of macrophage deletion.

CD4⁺ T cell and CD8⁺ T cell depletion experiments

For CD4⁺ T and CD8⁺ T cell depletion, mice were treated intraperitoneally with 200 μ g of specific monoclonal antibodies (Ultra-LEAF™ Purified anti-mouse CD8a Antibody, Biolegend, cat #100763; and Ultra-LEAF™ Purified anti-mouse CD4 Antibody, Biolegend, cat #100457) the day before the implantation of cancer cells and then with 100 μ g twice a week for the entire duration of the experiment.

Statistical analysis

The sample sizes for in vitro and in vivo studies determined based on similar studies in the field and through pilot studies. Details regarding sample sizes and biological replicates can be found in the figure legends. Continuous variables are presented as means $\pm$ SD. For comparisons between two independent groups, a two-tailed Student's t-test was employed. Statistical analyses involving more than two groups were conducted using either a one-way analysis of variance (ANOVA) with Tukey's method or two-way ANOVA with Dunnett multiple comparisons, as specified. GraphPad Prism 9 (GraphPad Software Inc., San Diego, CA) was used for statistical analyses. Kaplan-Meier survival curves were generated and analyzed using the log-rank test. A p-value less than 0.05 was considered statistically significant. In all figures, statistical significance is represented as follows: *p < 0.05; **p < 0.01; ***p < 0.001.

Supplementary Materials

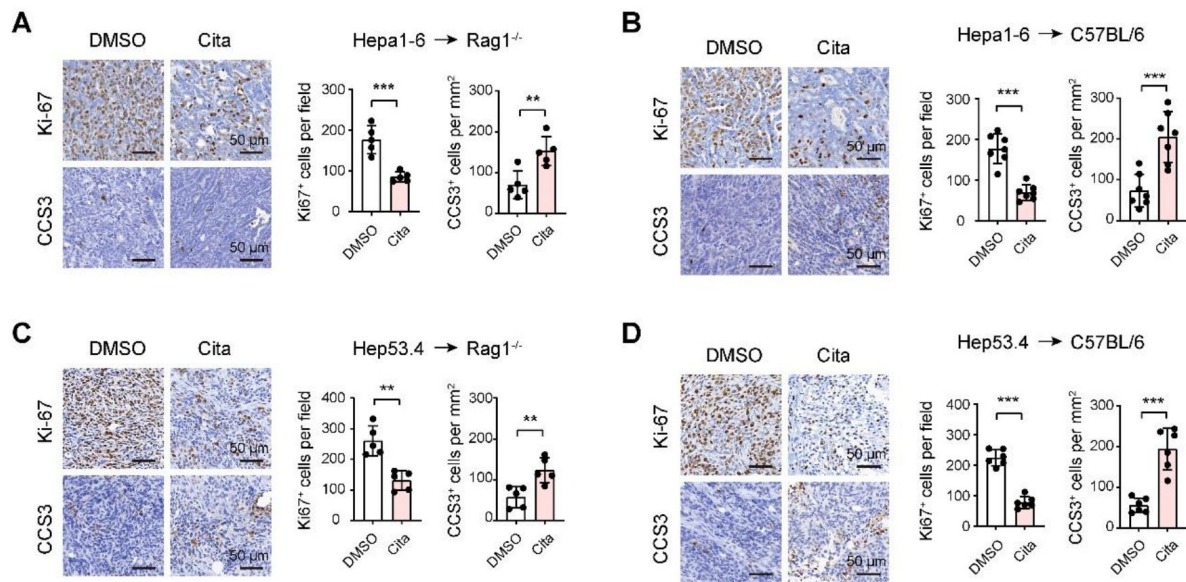


Figure S1.

Citalopram inhibits HCC cell proliferation and promotes cell apoptosis in immune-competent and immune-deficient mouse models.

(A, B) Immunohistochemical analysis of cleaved caspase-3 (CCS3) and Ki67 in Hepa1-6-bearing subcutaneous xenograft tumors from Rag1^{-/-} or immunocompetent C57BL/6 mice, treated with DMSO or 5 mg/kg citalopram. (C, D) Immunohistochemical analysis of CCS3 and Ki67 in Hep53.4-bearing subcutaneous xenograft tumors from Rag1^{-/-} or immunocompetent C57BL/6 mice, treated with DMSO or 5 mg/kg citalopram. In all panels, ***p* < 0.01, ****p* < 0.001. Scale bar, 50 μm. Values as mean ± SD and compared by the Student's *t* test.

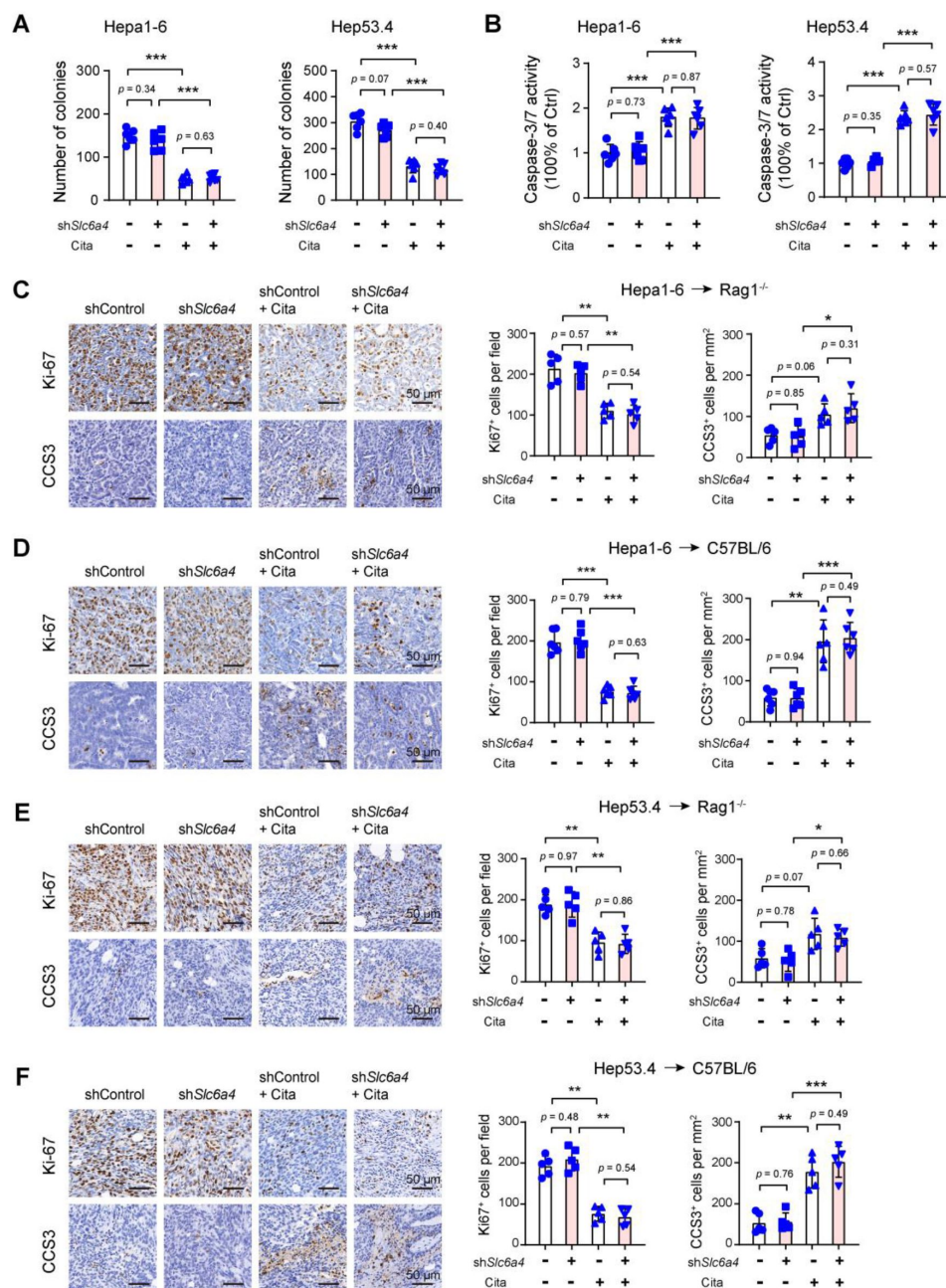


Figure S2.

Citalopram suppresses HCC cell proliferation and promote cell apoptosis in a SERT-independent manner.

(A) Plate colony formation assay revealed the effect of SERT knockdown alone or combined with citalopram treatment on the long-term cell proliferation of Hepa1-6 and Hep53.4 cells (n = 6 per group). **(B)** Caspase-3/7 activity in Hepa1-6 and Hep53.4 cells upon SERT knockdown or combined treatment with 5 μ M citalopram (n = 6 per group). **(C, D)** Immunohistochemical analysis of CCS3 and Ki67 in shControl and sh*Slc6a4* Hepa1-6-bearing subcutaneous xenograft tumors from Rag1^{-/-} or immunocompetent C57BL/6 mice, treated with DMSO or 5 mg/kg citalopram. Scale bar, 50 μ m. **(E, F)** Immunohistochemical analysis of CCS3 and Ki67 in shControl and sh*Slc6a4* Hep53.4-bearing subcutaneous xenograft tumors from Rag1^{-/-} or immunocompetent C57BL/6 mice, treated with DMSO or 5 mg/kg citalopram. Scale bar, 50 μ m. In all panels, * p < 0.05, ** p < 0.01, *** p < 0.001. Scale bar, 50 μ m. Values as mean $\pm$ SD and compared by one-way ANOVA multiple comparisons with Tukey's method among groups.

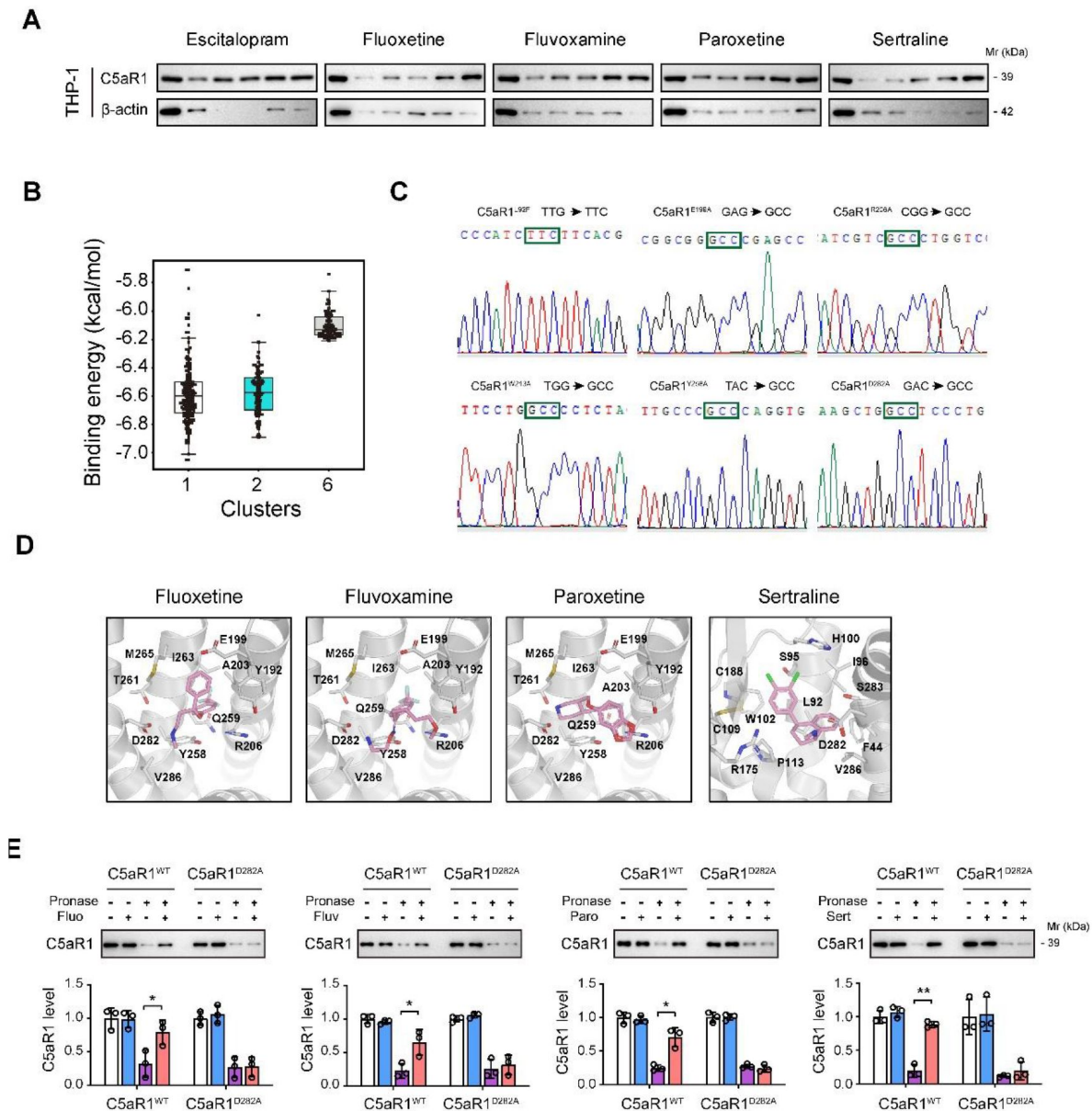


Figure S3.

Identification of C5aR1 as a direct target for citalopram. (A) The DARTS assay and immunoblot analysis showed C5aR1 protein stability against 5 $\mu\text{g/mL}$ pronase in the presence of different concentrations of SSRIs treatment (0, 1, 10, 50, and 100 μM). (B) For C5aR1, the predicted binding energy distribution of the clusters with poses more than 50. (C) Sequencing analysis showed the successful generation of six C5aR1 mutants. (D) The best-scored complex models of C5aR1 with other four different SSRIs. (E) HEK293T cells were transfected with either WT or mutant C5aR1 (D282A) expression plasmids for 48 h, followed by DARTS assay with immunoblotting analysis of C5aR1 protein levels. In all panels, $*p < 0.05$. Values as mean $\pm$ SD and compared by one-way ANOVA multiple comparisons with Tukey's method among groups (E).

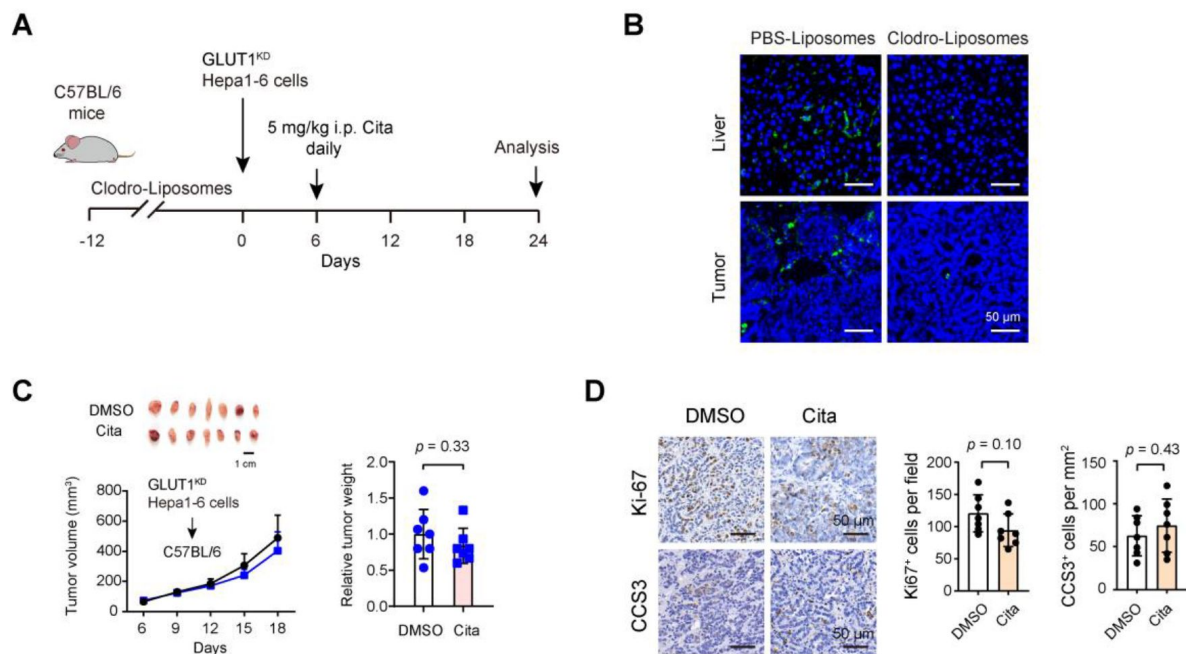


Figure S4.

Macrophage depletion mitigates the anti-tumor effects of citalopram.

(A) Schematic depicting macrophage blockade with clodronate liposomes. 12 days before tumor inoculation, C57BL/6 mice were pretreated with clodronate liposomes or phosphate-buffered saline (PBS) liposomes. Subsequently, GLUT1^{KD} Hepa1-6 cells were subcutaneously injected into the C57BL/6 mice. The effect of citalopram (5 mg/kg) on the tumor burden was evaluated after 18 days of drug treatment. (B) Immunofluorescence analysis of F4/80⁺ macrophages in the liver and tumor tissues of indicated groups. (C) In C57BL/6 mice, the effect of citalopram on the GLUT1^{KD} Hepa1-6 xenograft tumors was measured in the presence of macrophage deletion (n = 7 per group). (D) Immunohistochemical analysis of CCS3 and Ki67 in GLUT1^{KD} Hepa1-6-bearing subcutaneous xenograft tumors, treated with DMSO or 5 mg/kg citalopram (n = 7 per group). Scale bar, 50 μ m. Values as mean $\pm$ SD and compared by the Student's t test (C, D) and two-way ANOVA with Dunnett's multiple comparisons (C).

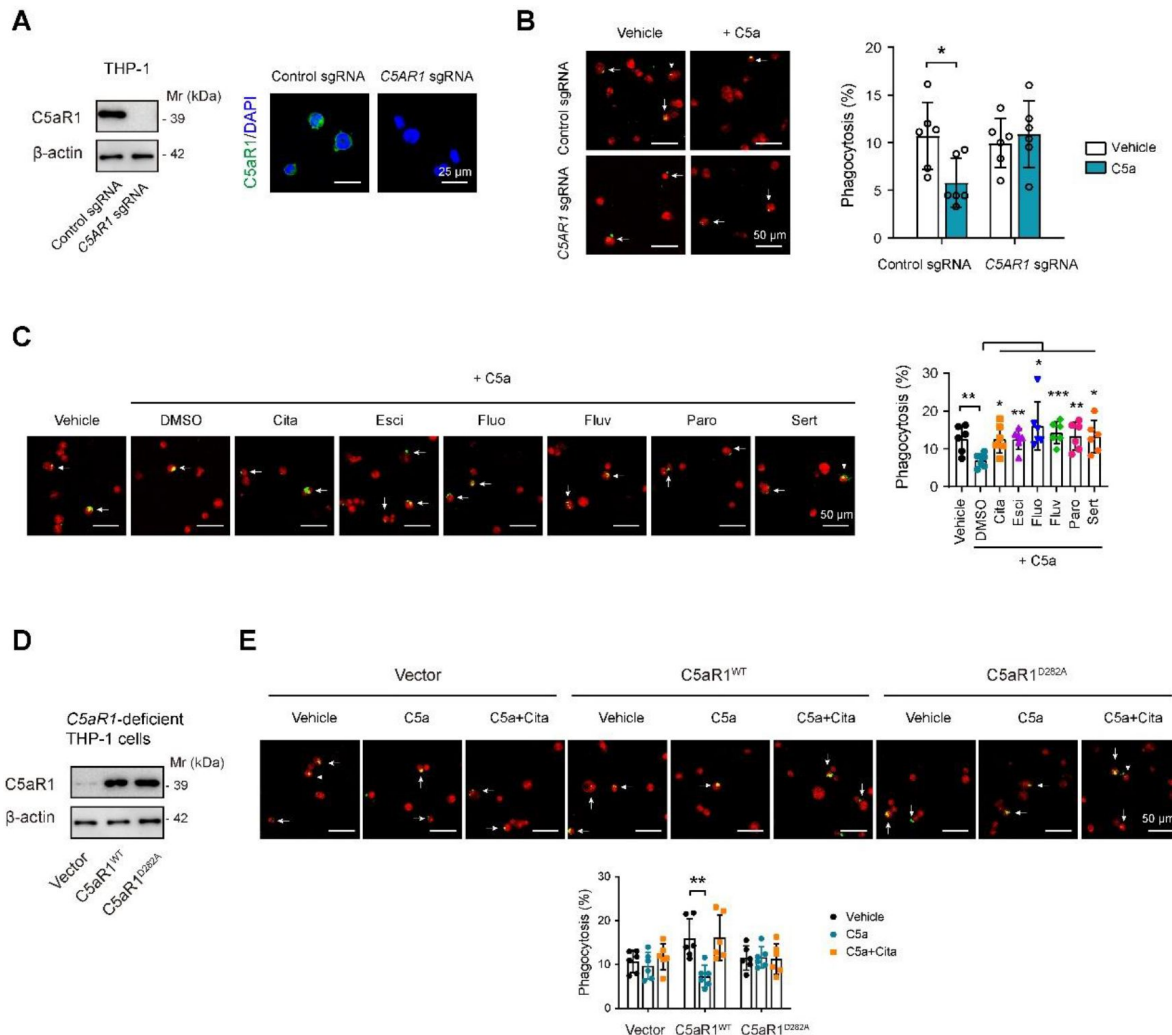


Figure S5.

Citalopram reverses C5a-mediated macrophage phagocytic impairment via targeting C5aR1.

(A) Western blotting and immunofluorescence analysis showed SERT protein levels in Cas9-sgControl, -sgC5aR1 THP-1 subclones. (B) Effects of C5aR1 deficiency on the macrophage phagocytosis of HCC-LM3 in the presence or absence of C5a stimulation. (C) Effects of different SSRIs on the macrophage phagocytosis of HCC-LM3 in the presence of C5a stimulation. (D) Reconstituted expression of WT and D282A mutant C5aR1 in C5aR1^{KO} THP-1 cells. (E) The effects of citalopram on macrophage phagocytosis in the absence of C5aR1 with reconstituted expression of C5aR1^{WT} or C5aR1^{D282A}. In all panels, $p < 0.05$, $**p < 0.01$, $***p < 0.001$. Values as mean $\pm$ SD and compared by one-way ANOVA multiple comparisons with Tukey's method among groups. Data are representative of three independent experiments (B, C, E).

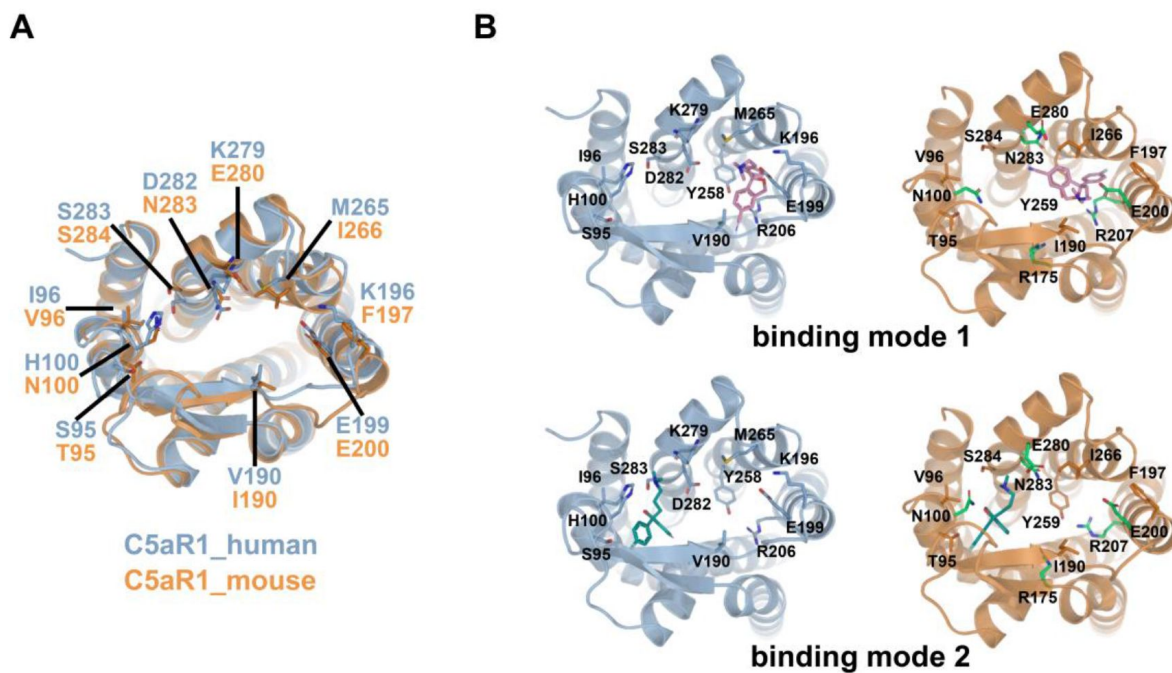


Figure S6.

Predicted binding modes of citalopram to human and mouse C5aR1. (A) Conformations of orthosteric binding sites in human (light blue) and mouse (orange) C5aR1. The conformation of human C5aR1 was obtained from the crystal structure (PDB id: 6c1q). The structure of mouse C5aR1 was predicted using the ColabFold (AlphaFold2) software. (B) The predicted binding modes of citalopram to human (light blue) and mouse (orange) C5aR1. The conformations of citalopram were shown in pink (binding mode 1) or deep green (binding mode 2) sticks. For mouse C5aR1, green sticks indicate residues set to flexible in the molecular docking process.

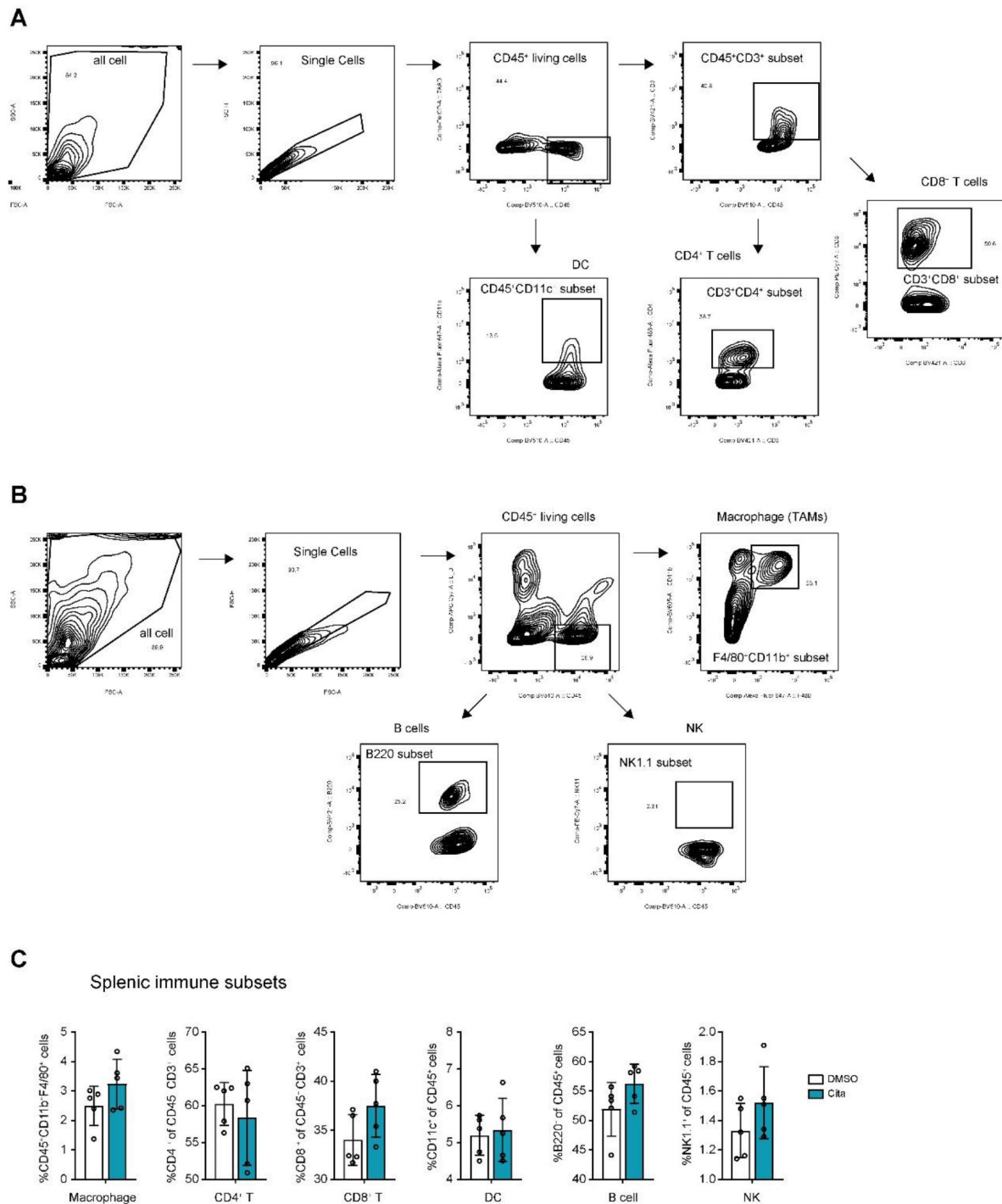


Figure S7.

Flow cytometry analysis of immune cells in tumor tissues and spleen.

(A, B) Gating strategies used for flow cytometry analysis of tumor and splenic lymphocytes. Panel A: Identification of CD4⁺ T cells, CD8⁺ T cells, and dendritic cells (DC). Panel B: Identification of B220⁺ B cells, tumor-associated macrophages (TAMs), and nature killer (NK) cells. (C) Flow cytometry showed the infiltration of CD45⁺CD11b⁺F4/80⁺ macrophages, CD4⁺ T cells, CD8⁺ T cells, B220⁺ B cells, CD11c⁺ DC cells, and NK1.1⁺ NK cells in spleen tissues from orthotopic xenograft model, which generated in immunocompetent C57BL/6 mice with Hepa1-6 cells (n = 5 per group). Values as mean ± SD and compared by the Student's t test.

Figure S8.

The expression pattern of *Glut1* and *Glut3* in CD8⁺ T cells.

(A) Real-time qPCR analysis of *Glut1* and *Glut3* expression in intratumoral CD8⁺ T cells (n = 3 per group). (B) Single cell analysis of the expression of glucose transporter members in two HCC cohorts. Values as mean $\pm$ SD and compared by the Student's t test (A), ***p* < 0.01.

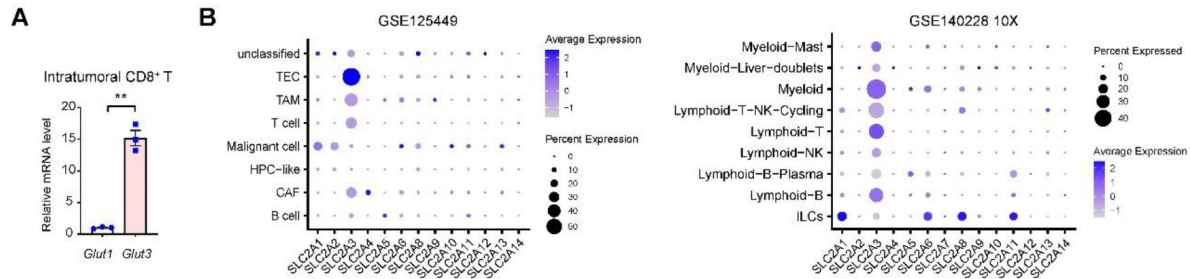
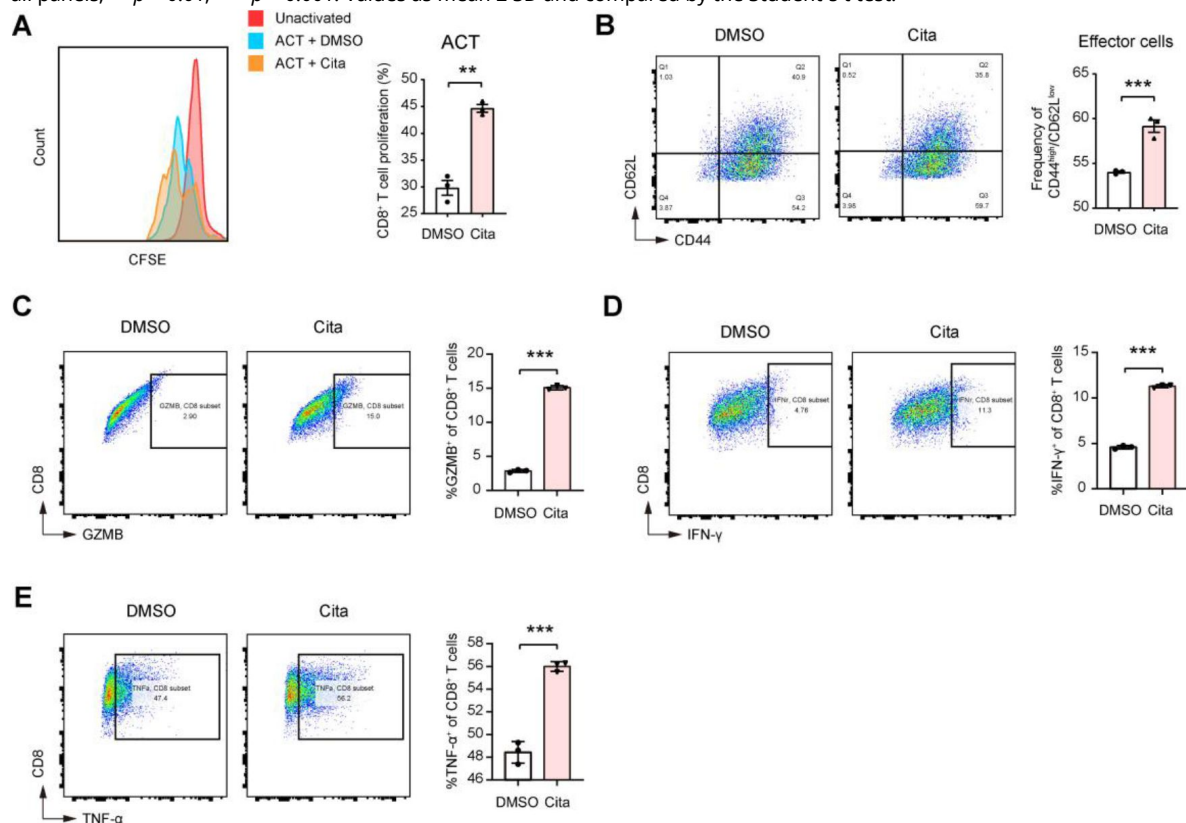


Figure S9.

The *in vitro* effects of citalopram treatment on CD8⁺ T cell function.

(A) Splenic CD8⁺ T cells isolated from C57BL/6 mice were stimulated with plate-bound α -CD3 plus α -CD28 for 72 h, and T-cell proliferation (CFSE staining) upon citalopram treatment was determined by flow cytometry (n = 3 per group); ACT: activated T cells. (B) Splenic CD8⁺ T cells from C57BL/6 mice were stimulated with plate-bound α -CD3 and α -CD28 for 72 h, and CD44 and CD62L levels in activated CD8⁺ T cells upon citalopram treatment were determined by flow cytometry (n = 3 per group). (C-E) Intracellular GZMB, IFN- γ , and TNF- α were detected in activated CD8⁺ T cells upon citalopram treatment (n = 3 per group). In all panels, ***p* < 0.01, ****p* < 0.001. Values as mean $\pm$ SD and compared by the Student's t test.



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Reviewer #1 (Public review):

Summary:

In their previous publication (Dong et al. Cell Reports 2024), the authors showed that citalopram treatment resulted in reduced tumor size by binding to the E380 site of GLUT1 and inhibiting the glycolytic metabolism of HCC cells, instead of the classical citalopram receptor. Given that C5aR1 was also identified as the potential receptor of citalopram in the previous report, the authors focused on exploring the potential of the immune-dependent anti-tumor effect of citalopram via C5aR1. C5aR1 was found to be expressed on tumor-associated macrophages (TAMs) and citalopram administration showed potential to improve the stability of C5aR1 in vitro. Through macrophage depletion and adoptive transfer approaches in HCC mouse models, the data demonstrated the potential importance of C5aR1-expressing macrophage in the anti-tumor effect of citalopram in vivo. Mechanistically, their in vitro data suggested that citalopram may regulate the phagocytosis potential and polarization of macrophages through C5aR1. Next, they tried to investigate the direct link between citalopram and CD8⁺T cells by including an additional MASH-associated HCC mouse model. Their data suggest that citalopram may upregulate the glycolytic metabolism of CD8⁺T cells, probability via GLUT3 but not GLUT1-mediated glucose uptake. Lastly, as the systemic 5-HT

level is down-regulated by citalopram, the authors analyzed the association between a low 5-HT and a superior CD8⁺T cell function against a tumor. Although the data is informative, the rationale for working on additional mechanisms and logical links among different parts is not clear. In addition, some of the conclusion is also not fully supported by the current data.

Strengths:

The idea of repurposing clinical-in-used drugs showed great potential for immediate clinical translation. The data here suggested that the anti-depression drug, citalopram displayed an immune regulatory role on TAM via a new target C5aR1 in HCC.

Weaknesses:

- (1) The authors concluded that citalopram had a 'potential immune-dependent effect' based on the tumor weight difference between Rag^{-/-} and C57 mice in Figure 1. However, tumor weight differences may also be attributed to a non-immune regulatory pathway. In addition, how do the authors calculate relative tumor weight? What is the rationale for using relative one but not absolute tumor weight to reflect the anti-tumor effect?
- (2) The authors used shSlc6a4 tumor cell lines to demonstrate that citalopram's effects are independent of the conventional SERT receptor (Figure 1C-F). However, this does not entirely exclude the possibility that SERT may still play a role in this context, as it can be expressed in other cells within the tumor microenvironment. What is the expression profiling of Slc6a4 in the HCC tumor microenvironment? In addition, in Figure 1F, the tumor growth of shSlc6a4 in C57 mice displayed a decreased trend, suggesting a possible role of Slc6a4.
- (3) Why did the authors choose to study phagocytosis in Figures 3G-H? As an important player, TAM regulates tumor growth via various mechanisms.
- (4) The information on unchanged deposition of C5a has been mentioned in this manuscript (Figures 3D and 3F), the authors should explain further in the manuscript, for example, C5a could bind to receptors other than C5aR1 and/or C5a bind to C5aR1 by different docking anchors compared with citalopram.
- (5) Figure 3I-M - the flow cytometry data suggested that citalopram treatment altered the proportions of total TAM, M1 and M2 subsets, CD4⁺ and CD8⁺T cells, DCs, and B cells. Why does the author conclude that the enhanced phagocytosis of TAM was one of the major mechanisms of citalopram? As the overall TAM number was regulated, the contribution of phagocytosis to tumor growth may be limited.
- (6) Figure 4 - what is the rationale for using the MASH-associated HCC mouse model to study metabolic regulation in CD8⁺T cells? The tumor microenvironment and tumor growth would be quite different. In addition, how does this part link up with the mechanisms related to C5aR1 and TAM? The authors also brought GLUT1 back in the last part and focused on CD8⁺T cell metabolism, which was totally separated from previous data.
- (7) Figure 5, the authors illustrated their mechanism that citalopram regulates CD8⁺T cell anti-tumor immunity through proinflammatory TAM with no experimental evidence. Using only CD206 and MHCII to represent TAM subsets obviously is not sufficient.

<https://doi.org/10.7554/eLife.103016.1.sa2>

Reviewer #2 (Public review):

Summary:

Dong et al. present a thorough investigation into the potential of repurposing citalopram, an SSRI, for hepatocellular carcinoma (HCC) therapy. The study highlights the dual mechanisms by which citalopram exerts anti-tumor effects: reprogramming tumor-associated macrophages (TAMs) toward an anti-tumor phenotype via C5aR1 modulation and suppressing cancer cell metabolism through GLUT1 inhibition while enhancing CD8⁺ T cell activation. The findings emphasize the potential of drug repurposing strategies and position C5aR1 as a promising immunotherapeutic target. However, certain aspects of experimental design and clinical relevance could be further developed to strengthen the study's impact.

Strength:

It provides detailed evidence of citalopram's non-canonical action on C5aR1, demonstrating its ability to modulate macrophage behavior and enhance CD8⁺ T cell cytotoxicity. The use of DARTS assays, in silico docking, and gene signature network analyses offers robust validation of drug-target interactions. Additionally, the dual focus on immune cell reprogramming and metabolic suppression presents a thorough strategy for HCC therapy. By emphasizing the potential for existing drugs like citalopram to be repurposed, the study also underscores the feasibility of translational applications.

Major weaknesses/suggestions:

The dataset and signature database used for GSEA analyses are not clearly specified, limiting reproducibility. The manuscript does not fully explore the potential promiscuity of citalopram's interactions across GLUT1, C5aR1, and SERT1, which could provide a deeper understanding of binding selectivity. The absence of GLUT1 knockdown or knockout experiments in macrophages prevents a complete assessment of GLUT1's role in macrophage versus tumor cell metabolism. Furthermore, there is minimal discussion of clinical data on SSRI use in HCC patients. Incorporating survival outcomes based on SSRI treatment could strengthen the study's translational relevance.

By addressing these limitations, the manuscript could make an even stronger contribution to the fields of cancer immunotherapy and drug repurposing.

<https://doi.org/10.7554/eLife.103016.1.sa1>

Author response:

Public Reviews:

Reviewer #1 (Public review):

Summary:

In their previous publication (Dong et al. Cell Reports 2024), the authors showed that citalopram treatment resulted in reduced tumor size by binding to the E380 site of GLUT1 and inhibiting the glycolytic metabolism of HCC cells, instead of the classical citalopram receptor. Given that C5aR1 was also identified as the potential receptor of citalopram in the previous report, the authors focused on exploring the potential of the immune-dependent anti-tumor effect of citalopram via C5aR1. C5aR1 was found to be expressed on tumor-associated macrophages (TAMs) and citalopram administration showed potential to improve the stability of C5aR1 in vitro. Through macrophage depletion and adoptive transfer approaches in HCC mouse models, the data demonstrated the potential importance of C5aR1-expressing macrophage in the anti-tumor effect of citalopram in vivo. Mechanistically, their in vitro data suggested that citalopram may regulate the phagocytosis potential and polarization of macrophages through C5aR1.

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Thanks very much for your insightful evaluation and the constructive suggestions. We have thoroughly studied the comments and a provisional point-to-point response is shown as follows.

Strengths:

The idea of repurposing clinical-in-used drugs showed great potential for immediate clinical translation. The data here suggested that the anti-depression drug, citalopram displayed an immune regulatory role on TAM via a new target C5aR1 in HCC.

Thank you for your constructive comments. We believe that further investigation into the mechanisms by which citalopram modulates TAM function could provide valuable insights into its potential role in HCC therapy.

Weaknesses:

(1) The authors concluded that citalopram had a 'potential immune-dependent effect' based on the tumor weight difference between Rag-/- and C57 mice in Figure 1. However, tumor weight differences may also be attributed to a non-immune regulatory pathway. In addition, how do the authors calculate relative tumor weight? What is the rationale for using relative one but not absolute tumor weight to reflect the anti-tumor effect?

We appreciate your insights into the potential contributions of non-immune regulatory pathways to the observed tumor weight differences between Rag-/- and C57 mice, and we will further address this issue in our discussion. The relative tumor weight was calculated by assigning an arbitrary value of 1 to the Rag1^{-/-} mice in the DMSO treatment group, with all other tumor weights expressed relative to this baseline. As suggested, we will include absolute tumor weight data in our revised manuscript.

(2) The authors used shSlc6a4 tumor cell lines to demonstrate that citalopram's effects are independent of the conventional SERT receptor (Figure 1C-F). However, this does not entirely exclude the possibility that SERT may still play a role in this context, as it can be expressed in other cells within the tumor microenvironment. What is the expression profiling of Slc6a4 in the HCC tumor microenvironment? In addition, in Figure 1F, the tumor growth of shSlc6a4 in C57 mice displayed a decreased trend, suggesting a possible role of Slc6a4.

To identify the expression patterns of Slc6a4 in different cellular contexts within the HCC tumor microenvironment, we will conduct a thorough screening of HCC datasets that include single-cell sequencing analysis. The possible role of Slc6a4 on tumor growth will be verified with in vitro loss-of-function experiments.

(3) Why did the authors choose to study phagocytosis in Figures 3G-H? As an important player, TAM regulates tumor growth via various mechanisms.

Thank you for your question. We focused on this aspect because citalopram targets C5aR1-expressing TAM. C5aR1 is a receptor for complement component C5a, and complement components play a significant role in mediating the phagocytosis process in macrophages. In the revised manuscript, we will emphasize this rationale clearly.

(4) The information on unchanged deposition of C5a has been mentioned in this manuscript (Figures 3D and 3F), the authors should explain further in the manuscript, for example, C5a could bind to receptors other than C5aR1 and/or C5a bind to C5aR1 by different docking anchors compared with citalopram.

Thank you for your insightful comment. First, we will investigate the docking anchors involved in the binding of C5a to C5aR1 and compare these interactions with those of C5aR1 and citalopram. Additionally, we will discuss the potential binding of C5a to other receptors, providing a broader perspective on the signaling mechanisms.

(5) Figure 3I-M - the flow cytometry data suggested that citalopram treatment altered the proportions of total TAM, M1 and M2 subsets, CD4+ and CD8+T cells, DCs, and B cells. Why does the author conclude that the enhanced phagocytosis of TAM was one of the major mechanisms of citalopram? As the overall TAM number was regulated, the contribution of phagocytosis to tumor growth may be limited.

As suggested, we will restate the conclusion to enhance clarity and better articulate the relationship between citalopram treatment, TAM populations, and their phagocytic activity. Thank you for your valuable input.

(6) Figure 4 - what is the rationale for using the MASH-associated HCC mouse model to study metabolic regulation in CD8+T cells? The tumor microenvironment and tumor growth would be quite different. In addition, how does this part link up with the mechanisms related to C5aR1 and TAM? The authors also brought GLUT1 back in the last part and focused on CD8+T cell metabolism, which was totally separated from previous data.

We chose the MASH-associated HCC mouse model because it closely mimics the etiology of metabolic-associated fatty liver disease (MAFLD), which is a significant contributor to the development of cirrhosis and HCC. The inclusion of CD8⁺ T cells in our study is based on the understanding that citalopram targets GLUT1, which plays a crucial role in glucose uptake. CD8⁺ T cell function is heavily reliant on glycolytic metabolism, making it essential to investigate how citalopram's effects on GLUT1 influence the metabolic pathways and functionality of these immune cells. The data presented in this section primarily aim to demonstrate how citalopram influences peripheral 5-HT levels, which subsequently affects CD8⁺ T cell functionality. By linking these findings, we will clarify how citalopram impacts both TAM and CD8⁺ T cells. In the revised manuscript, we will enhance the background information and provide relevant data support to avoid any gaps.

(7) Figure 5, the authors illustrated their mechanism that citalopram regulates CD8+T cell anti-tumor immunity through proinflammatory TAM with no experimental evidence. Using only CD206 and MHCII to represent TAM subsets obviously is not sufficient.

As suggested, more relevant experimental data will be included in the revised manuscript to better characterize the TAM populations and their roles in mediating the effects of citalopram on CD8⁺ T cells.

Reviewer #2 (Public review):*Summary:*

Dong et al. present a thorough investigation into the potential of repurposing citalopram, an SSRI, for hepatocellular carcinoma (HCC) therapy. The study highlights the dual mechanisms by which citalopram exerts anti-tumor effects: reprogramming tumor-associated macrophages (TAMs) toward an anti-tumor phenotype via C5aR1 modulation and suppressing cancer cell metabolism through GLUT1 inhibition while enhancing CD8+ T cell activation. The findings emphasize the potential of drug repurposing strategies and position C5aR1 as a promising immunotherapeutic target. However, certain aspects of experimental design and clinical relevance could be further developed to strengthen the study's impact.

Thank you for your thoughtful review and constructive feedback, and we look forward to improving our manuscript accordingly.

Strength:

It provides detailed evidence of citalopram's non-canonical action on C5aR1, demonstrating its ability to modulate macrophage behavior and enhance CD8+ T cell cytotoxicity. The use of DARTS assays, in silico docking, and gene signature network analyses offers robust validation of drug-target interactions. Additionally, the dual focus on immune cell reprogramming and metabolic suppression presents a thorough strategy for HCC therapy. By emphasizing the potential for existing drugs like citalopram to be repurposed, the study also underscores the feasibility of translational applications.

Your insights reinforce the significance of our findings, and we will ensure that these points are clearly articulated in the revised manuscript to enhance its impact.

Major weaknesses/suggestions:

The dataset and signature database used for GSEA analyses are not clearly specified, limiting reproducibility. The manuscript does not fully explore the potential promiscuity of citalopram's interactions across GLUT1, C5aR1, and SERT1, which could provide a deeper understanding of binding selectivity. The absence of GLUT1 knockdown or knockout experiments in macrophages prevents a complete assessment of GLUT1's role in macrophage versus tumor cell metabolism. Furthermore, there is minimal discussion of clinical data on SSRI use in HCC patients. Incorporating survival outcomes based on SSRI treatment could strengthen the study's translational relevance.

By addressing these limitations, the manuscript could make an even stronger contribution to the fields of cancer immunotherapy and drug repurposing.

We appreciate your valuable suggestions. As suggested, we will take the following actions:

- (1) GSEA analysis: we will clearly specify the datasets and signature databases used for the GSEA in the revised manuscript.
- (2) Exploration of binding selectivity: we recognize the importance of exploring the potential promiscuity of citalopram's interactions across GLUT1, C5aR1, and SERT1. As suggested, we will include a more detailed analysis of these interactions, which will help elucidate binding selectivity and its implications for therapeutic outcomes.
- (3) GLUT1 knockdown in macrophages: to address the gap in our assessment of GLUT1's role in macrophages, we will incorporate GLUT1 knockdown or knockout experiments in

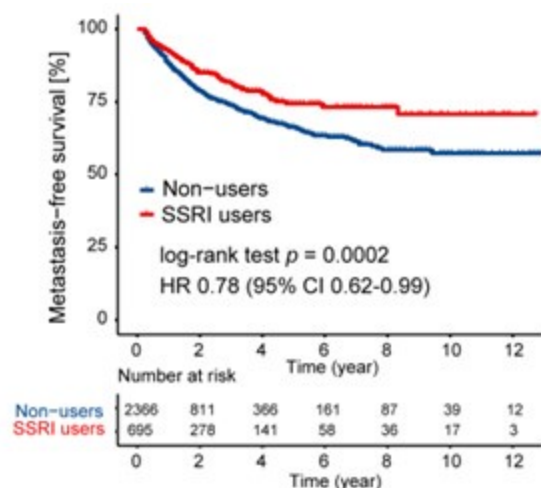
macrophages upon citalopram treatment. Moreover, a DARTS assay for GLUT1 in THP-1 cells will be conducted.

(4) Clinical data on SSRI use in HCC patients: Related data have been reported previously in PMID: 39388353 (Cell Rep. 2024 Oct 22;43(10):114818.). As detailed below:

“SSRIs use is associated with reduced disease progression in HCC patients

We determined whether SSRIs for alleviating HCC are supported by real-world data. A total of 3061 patients with liver cancer were extracted from the Swedish Cancer Register. Among them, 695 patients had been administrated with post-diagnostic SSRIs. The Kaplan-Meier survival analysis suggested that patients who utilized SSRIs exhibited a significantly improved metastasis-free survival compared to those who did not use SSRIs, with a P value of log-rank test at 0.0002. Cox regression analysis showed that SSRI use was associated with a lower risk of metastasis (HR = 0.78; 95% CI, 0.62-0.99).”

Author response image 1.



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