

Harmine derivative ACB1801 induces ferroptosis vulnerability and STAT1 pathway activation to sensitize MSS colorectal cancer to anti-PD-1 immunotherapy

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Abstract

Harmine and its derivatives, a class of β -carboline alkaloids, are promising natural antitumor agents with drug resistance–modulating activities. Emerging evidence suggests that these small molecules can reshape the tumor microenvironment (TME) and modulate antitumor immunity. Immune checkpoint blockade (ICB) therapies provide durable benefit in a subset of colorectal cancers but remain largely ineffective in microsatellite-stable (MSS) subtype, which accounts for approximately 85% of cases. This lack of response is driven by an immune-desert TME characterized by defective antigen presentation and poor cytotoxic T cell infiltration. Whether harmine derivatives can enhance ICB efficacy in MSS colorectal cancer remains unknown, and the underlying molecular mechanisms are poorly defined. We therefore evaluated how ACB1801, a synthetic derivative of the natural harmine, reprograms the TME to restore sensitivity to anti-Programmed cell death protein 1 (PD-1) therapy in MSS CRC. In murine MSS CRC models, ACB1801 markedly enhanced the antitumor efficacy of anti-PD-1, resulting in increased intratumoral CD8⁺ T cell infiltration and reduced regulatory T cell accumulation. Integrated transcriptomic analyses of sorted CD45⁺ immune and CD45⁻ tumor compartments revealed that combination therapy induced coordinated metabolic reprogramming, characterized by suppression of glycolysis and activation of ferroptosis-related pathways. These findings were validated in murine and human MSS CRC cell lines, where ACB1801 reduced glycolytic flux and increased ferroptotic susceptibility. Mechanistically, ACB1801 enhanced C-X-C motif chemokine ligand 10 (CXCL10) secretion in a STAT1-dependent manner and upregulated NOD-like receptor family CARD domain containing 5 (NLRC5)-driven MHC class I antigen presentation, thereby increasing tumor immunogenicity. Together, these effects convert immunologically “cold” MSS tumors into an immune-responsive state. Collectively, our findings position ACB1801 as a clinically relevant metabolic-immunomodulatory agent and provide strong preclinical justification for combining harmine derivatives with anti-PD-1 therapy to overcome resistance in MSS CRC patients.

Introduction

Colorectal cancer (CRC) is the most common malignancy of the gastrointestinal tract and represents the third leading cause of cancer-related mortality worldwide (1). Immunotherapy, particularly ICB targeting PD-1 and its ligand programmed Death-Ligand 1 (PD-L1), has revolutionized the treatment of several cancers, including CRC with deficient mismatch repair (dMMR) and high microsatellite instability (MSI-H) (2). However, the vast majority of CRC cases (approximately 80–85%) exhibit proficient mismatch repair (pMMR) and are either MSS or display low microsatellite instability (MSI-L). These tumors are typically considered immunologically “cold”, characterized by poor immune infiltration and resistance to ICB therapy (3, 4). Therefore, therapeutic strategies designed to convert “cold” MSS CRC tumors into “hot,” immunoresponsive tumors hold great promise for broadening the clinical benefit of ICB in CRC patients.

The TME of MSS CRC is typically characterized by immune exclusion or an immune-desert phenotype, defined by low infiltration of Tumor-infiltrating lymphocytes (TILs), a defect of Cytotoxic T lymphocytes (CTLs), or both (5). This immunologically “cold” state is driven by a combination of low Tumor mutational

burden (TMB), impaired antigen presentation, and reduced surface expression of Major histocompatibility complex class I (MHC-I) molecules on Antigen-presenting cells (APCs) (6). Together, these factors impair immune recognition and limit effective antitumor cytotoxicity. Therefore, therapeutic strategies that restore MHC-I expression and enhance antigen presentation represent a promising strategy to promote CTL infiltration and sensitize MSS CRC tumors to immune checkpoint blockade.

Chemokines are key regulators of the TME, orchestrating the recruitment and spatial distribution of immune cells (7). In CRC, the chemokine network significantly influences both the extent and composition of immune cell infiltration, particularly cytotoxic T lymphocytes and other effector populations. Among these, CXCL10 is an interferon-inducible chemokine secreted by multiple cell types, including monocytes, endothelial cells, fibroblasts, and tumor cells. Through its interaction with the chemokine receptor CXCR3 on activated T cells, CXCL10 plays a pivotal role in anti-tumor immunity by promoting effector T cells trafficking into the TME (8). By facilitating T cell infiltration and accumulation, CXCL10 contributes to the establishment of an inflamed, "hot" TME. Thus, strategies designed to boost CXCL10 expression within tumors may enhance effector T cell recruitment and improve the efficacy of immune checkpoint blockade.

Harmine is a naturally occurring β -carboline alkaloid first isolated from the seeds of *Peganum harmala* L. (Zygophyllaceae) (9). It exhibits diverse biological and pharmacological activities, including anti-inflammatory, antiviral, antifungal, and antitumor effects (10), and has recently been suggested as a potential therapeutic agent for Alzheimer's disease (11). The antitumor properties of harmine have been investigated across multiple cancer types, where it modulates key cellular processes and signaling pathways, including epithelial-mesenchymal transition (EMT) (12), apoptosis (13), cell cycle progression (14–16), angiogenesis (17), and actin cytoskeleton remodeling (18). In addition, harmine can overcome chemotherapeutic drug resistance by inhibiting of Dual-specificity tyrosine-(Y)-phosphorylation regulated kinase 1A (DYRK1A) (19).

ACB1801 is a synthetic derivative of harmine and our previous study showed that ACB1801 enhances anti-PD-1 therapy in a B16-F10 melanoma syngeneic mouse model by upregulating MHC-I antigen presentation (20). However, the potential of ACB1801 to improve anti-PD-1 therapy in MSS CRC and the underlying mechanisms remain largely unexplored.

In this study, we demonstrate that ACB1801 enhances the therapeutic efficacy of anti-PD-1 in a MSS CRC mouse model and reveals how it reshapes tumor cells' metabolism and the tumor microenvironment. By modulating both the immune landscape and tumor cell metabolism, ACB1801 not only fosters immune cell infiltration by activating STAT1 pathway with increased MHC-I expression and CXCL10 release on the tumors but also shifts tumor cells toward a state of metabolic vulnerability, sensitizing them to ferroptotic cell death.

Results

ACB1801 enhances the efficacy of anti-PD-1 therapy in the MSS CRC CT26 mouse model.

We first evaluated the effects of ACB1801 in combination with anti-PD-1 therapy on the MSS CRC CT26 syngeneic mouse model. Mice were treated with isotype control, ACB1801, anti-PD-1, or a combination of ACB1801 and anti-PD-1 (Fig. 1A). Tumor growth curves revealed that monotherapies with either ACB1801 or anti-PD-1 had no significant effects on tumor growth. Remarkably, the co-administration of ACB1801 with anti-PD-1 resulted in a significant reduction in both tumor volumes and tumor weights compared to anti-PD-1 alone (Fig. 1B and 1C), translating into a marked improvement in overall survival (Fig. 1D). Our data indicate that the harmine derivative ACB1801 enhances the efficacy of anti-PD-1 therapy in the MSS CRC mouse model.

We next investigated whether the enhanced therapeutic benefit of anti-PD-1 therapy with ACB1801 was associated with changes in the immune landscape of the tumors. To explore this, comprehensive immune phenotyping was performed on tumors harvested from mice treated with the combination therapy, as well as those from the control and mono-therapy groups. Flow cytometry analysis revealed that tumors treated with the combination therapy showed increased CD8⁺ T cell infiltration, accompanied by a significant reduction in Regulatory T cells (Tregs). Therefore, the CD8/Treg ratio was elevated in tumors treated with combination therapy compared to the control and single-treatment groups (Fig. 1E). The combination therapy did not significantly alter the frequencies of total and activated (CD69⁺) CD4⁺ effector T cells, NK cells, CD11b⁺ myeloid cells, F4/80⁺ macrophages, PMN-MDSCs, monocytes, or CD11c⁺ dendritic cells (**Supplementary Fig. 1A**). Interestingly, however, the proportion of PD-1⁺ CD4⁺ effector T cells was reduced in tumors treated with the combination therapy (**Supplementary Fig. 1B**), suggesting a restoration of CD4⁺ T-cell functionality and alleviation of exhaustion. This finding is consistent with the enhanced antitumor immunity observed in this group of tumors.

Transcriptomic analysis of CD45⁺ immune cells isolated from tumors treated with anti-PD-1 or a combination of anti-PD-1 and ACB1801 showed that pathways related to adaptive immunity, type II interferon signaling, T cell activation, and cytotoxicity were significantly enriched in the combination therapy group versus the group treated with anti-PD-1 alone (Fig. 1F), supporting a shift from an immune-suppressive to an immune-supportive TME. Moreover, enrichment of gamma-delta-T-cell activation (**Supplementary Figs. 2A**) indicates enhanced innate immune responses upon combination therapy. Pathways associated with cytokine production, along with elevated intratumoral IFN γ levels (**Supplementary Figs. 2A and B**) further confirm the activation of adaptive immunity. Interestingly, antigen-processing and presentation pathways were also enriched in the combination group (**Supplementary Figs. 2A**).

ACB1801 induces metabolic reprogramming and downregulates glycolysis in CRC tumor cells.

Cancer cells undergo metabolic reprogramming to support survival, therapy resistance, and immune evasion (21, 22). A dimeric form of β -carboline has been reported to impact sarcoma cell metabolism (23), but the underlying mechanisms remain incompletely understood. To investigate whether and how the β -carboline derivative ACB1801 affects tumor cells in vivo, we performed comparative transcriptomic

analysis of CD45⁺ cells isolated from tumors treated with combination therapy (ACB1801 + anti-PD-1) versus those treated with anti-PD-1 monotherapy.

Remarkably, KEGG enrichment analysis revealed that among the top 30 enriched cellular functions, metabolic pathways were significantly enriched in CD45⁺ cells isolated from combination-treated tumors compared with those treated with anti-PD-1 monotherapy (Fig. 2A). Detailed analysis of KEGG pathway annotations revealed that this metabolic reprogramming was characterized by an enrichment in carbohydrate, lipid, and amino acid metabolism, as well as glycan biosynthesis and metabolism (Fig. 2B). These findings support the hypothesis that ACB1801 drives metabolic remodeling in tumor cells, which may contribute to improved anti-tumor responses.

To further delineate the role of ACB1801 at the cellular level, we performed bulk RNA-sequencing on untreated or ACB1801-treated CT26 cells. Gene set enrichment analysis (GSEA) revealed a significant downregulation of glycolysis along with a concomitant enrichment of fatty acid metabolism and oxidation pathways in ACB1801-treated CT26 cells (Fig. 2C).

We further explored the potential role of ACB1801 in suppressing tumor cell glycolysis. A comparative analysis identified 23 glycolytic genes commonly downregulated by ACB1801 in both mouse CT26 and human HT29 CRC cells, representing core enzymes involved in the 10 steps of the glycolytic pathway (Fig. 2D). Quantitative (q)RT-PCR validation in CT26 and HT29 cells further confirmed the downregulation of key glycolysis-related genes, including HK2, LDHA, SLC16A3, GLUT1, ENO1, and PFKL, following ACB1801 treatment (Figs. 2E).

We next assessed the functional consequences of ACB1801-mediated downregulation of key glycolysis-related genes. Treatment with ACB1801 significantly reduced intracellular glucose and lactate levels as well as extracellular lactate in both CT26 and HT29 cells (Fig. 2F), consistent with the coordinated downregulation of multiple glycolytic enzymes and transporters, including HK2, PFKL, ENO1, LDHA, and SLC16A3. Together, these data indicate that ACB1801 treatment suppresses tumor cells' glycolysis.

ACB1801 induces ferroptotic vulnerability in CRC tumor cells.

Suppression of glycolysis is expected to limit NADPH production, resulting in reduced availability of glutathione(GSH) and impaired antioxidant capacity, thereby increasing tumor cell susceptibility to ferroptosis (24). Consistent with this mechanism, KEGG pathway analysis (Fig. 2B) revealed significant enrichment of lipid metabolism pathways in tumor cells isolated from combination-treated tumors compared with those isolated from anti-PD-1-treated tumors. Furthermore, GSEA of untreated and ACB1801-treated CT26 cells demonstrated enrichment of pathways associated with fatty acid metabolism and oxidation following ACB1801 treatment (Fig. 2C), further supporting a potential shift toward a ferroptosis-prone metabolic state.

In addition, correlation analysis of mouse tumor tissues (CD45⁺ samples) revealed a significant positive correlation between the glycolysis signature and ferroptosis suppressor signatures (**Supplementary**

Fig. 2C). This association was also observed in TCGA colorectal cancer (CRC) patient samples (**Supplementary Fig. 2D**), providing independent clinical support for the relationship between glycolytic activity and ferroptosis resistance. Collectively, these findings suggest that suppression of glycolysis in CRC tumor cells may not only compromise tumor bioenergetic capacity but also increase their vulnerability to ferroptosis. Thus, ACB1801 treatment may promote ferroptotic susceptibility in CRC tumor cells through metabolic reprogramming characterized by reduced glycolysis and enhanced lipid metabolic activity.

To explore the regulation of ferroptosis by ACB1801, we assessed the expression of ferroptosis-related genes in mouse CT26 and human HT29 cells treated with ACB1801, we observed downregulation of several key ferroptosis suppressors, including SLC7A11, HSPA5, ATF4, NRF2, ENO1, and LCN2, along with upregulation of ferroptosis drivers such as ZEB1, STA1, and FADS2 (Fig. 3A). qRT-PCR validation in CT26 and HT29 cells further confirmed the downregulation of SLC7A11, ATF4, NRF2, ENO1, LCN2 and HSPA5 following ACB1801 treatment (Fig. 3B). These coordinated changes support the hypothesis that ACB1801 enhances the sensitivity of MSS CRC cells to ferroptotic cell death.

To assess the functional consequences of the differentially regulated ferroptosis-related genes on ferroptosis sensitivity, we measured the GSH/GSSG ratio, a key indicator of cellular redox status (25). Our results (Fig. 3C) show a decrease in the GSH/GSSG ratio in ACB1801-treated CT26 and HT29 cells, indicating a shift toward increased cellular oxidative stress. We also observed a dose-dependent increase in ROS and lipid peroxidation in these cells following ACB1801 treatment (Fig. 3D). Taken together, these findings strongly suggest that ACB1801 makes tumor cells vulnerable to ferroptotic cell death, a conclusion further supported by the observation that co-treatment with the specific ferroptosis inhibitor ferrostatin-1 completely abrogated ACB1801-dependent induction of cell death (Fig. 3E). ACB1801 increases the expression and release of CXCL10 in CT26 tumor cells via a STAT1-dependent mechanism. Given the elevated IFN γ signature in CT26 tumors treated with the ACB1801 and anti-PD-1 combination (**Supplementary Fig. 2B**), and to determine whether ACB1801 directly influences cytokine and chemokine release from tumor cells, beyond the enrichment observed in CD45⁺ immune cells (**Supplementary Fig. 2A**), we next profiled the cytokines and chemokines secreted by tumor cells in response to ACB1801. These factors likely contribute to reshaping the immune landscape in vivo, as shown in Fig. 1E. We comprehensively profiled 111 cytokines and chemokines simultaneously using supernatants from untreated or ACB1801-treated cells. Under conditions of ACB1801-induced Tap1 upregulation (which served as an internal control), several cytokines and chemokines were differentially regulated, with CXCL10 notably upregulated and LCN2 and CXCL1 downregulated in ACB1801-treated cells compared to controls (Fig. 4A). We focused on CXCL10 due to its well-established role in promoting the infiltration of CXCR3 + CD8⁺ effector T cells into tumors in colorectal cancer models (26). ELISA testing confirmed the increased release of CXCL10 in the supernatants of both mouse CT26 and human HT29 CRC cells treated with ACB1801 (Fig. 4B). Furthermore, ACB1801 treatment also induced a significant increase in CXCL10 mRNA expression in these cells (Fig. 4C), indicating that ACB1801 enhances not only CXCL10 release but also its gene expression. Notably, this increased CXCL10 release was observed not only in vitro, but also in the tumor microenvironment of CT26 tumors treated with

ACB1801 alone, and to an even greater extent in those treated with the combination therapy (Fig. 4D). GSEA of cultured CT26 cells revealed significant enrichment of the IFN γ response signature following ACB1801 treatment (Fig. 4E), suggesting activation of the STAT1 signaling pathway. Consistently, ACB1801 treatment increased STAT1 phosphorylation at Tyr701, pSTAT1(Y701) in both CT26 and HT29 cells, confirming the activation of STAT1 signaling (Fig. 4F). We next assessed whether the upregulated expression of CXCL10 mRNA is a result of ACB1801-induced STAT1 activation, which is well-documented to drive CXCL10 expression (27). Our findings in Fig. 4G and **Supplementary Fig. 3** demonstrate that targeting STAT1 in both mouse CT26 and human HT29 cells abolished the ACB1801-induced increase in CXCL10. These results indicate that ACB1801 promotes CXCL10 overexpression and release in CRC cells through a STAT1-dependent mechanism.

ACB1801 enhances MHC-I expression and antigen presentation in MSS CRC through a STAT1/NLRC5-dependent mechanism.

In addition to the metabolic pathways enriched in tumor cells isolated from combination-treated mice (ACB1801 + anti-PD-1) compared with anti-PD-1 monotherapy, KEGG pathway analysis also revealed significant enrichment of protein processing in the endoplasmic reticulum and ubiquitin-mediated proteolysis pathways (Fig. 2A). These pathways are closely linked to MHC-I antigen processing and presentation, supporting a potential role for ACB1801 in enhancing tumor antigen presentation. By applying Protein-protein interaction (PPI) network construction on differentially expressed genes (DEG), our data revealed that protein processing is the most regulated process upon ACB1801 treatment (Fig. 5A). GSEA further revealed enriched antigen processing and presentation pathways in CT26 cells treated with ACB1801 (Fig. 5B). This finding is further supported by data showing that ACB1801 treatment upregulates the MHC-I genes Tap1, Tap2, and B2M in both CT26 mouse and HT29 human MSS CRC cells (**Supplementary Fig. 4A**) consistent with our previous observations in melanoma cells (20).

As a result, ACB1801-treated mouse and human CRC cells exhibited increased surface expression of H-2K^d and HLA-A, B, C molecules, compared to untreated cells (Fig. 5C). The functional significance of ACB1801-induced upregulation of MHC-I was demonstrated by OVA-derived SIINFEKL peptide loading on MHC-I in MC38 CRC cells (Fig. 5D), which subsequently enhanced OT-I CD8⁺ T cell-mediated killing of OVA-loaded MC38 cells (Fig. 5E).

MHC-I antigen processing and presentation are regulated by various pathways and transcription factors, with NLRC5 protein identified as a key transcriptional regulator of MHC-I-related genes (28). To investigate whether NLRC5 is involved in the regulation of MHC-I by ACB1801, we performed qRT-PCR on mRNA expression of NLRC5 upon ACB1801 treatment. Our data in Fig. 5F demonstrates that ACB1801 significantly increases NLRC5 expression in both CT26 mouse and HT29 human CRC cells. The ACB1801-induced increase in the expression of H-2K α and HLA-A, -B, and -C, as well as the MHC-I-related genes TAP1 and TAP2, requires NLRC5, as this increase was not observed in cells transfected with NLRC5 siRNA (Fig. 5G and **Supplementary Fig. 4B**). These findings further demonstrate that ACB1801 enhances MHC-I through an NLRC5-dependent mechanism in MSS CRC cells.

NLRC5 expression is regulated by various inflammatory signaling pathways, including type I and type II interferons (IFNs) through the activation of STATs (29). We therefore postulated that the ACB1801-dependent increase in NLRC5 may involve an activation of the IFN γ pathway via STAT signaling. This concept is supported by our RNA-seq data derived from untreated and ACB1801-treated CT26 cells, which revealed significant enrichment of the IFN γ response and the increased pSTAT1(Y701) phosphorylation level in treated cells (Fig. 4E **and** F). Consistent with these findings, Fig. 5H shows that treatment of CT26 mouse and HT29 human CRC cells with ACB1801 increases NLRC5 mRNA levels, and targeting STAT1 abolishes the ACB1801-dependent increase in NLRC5. Together, these findings provide compelling evidence that ACB1801 triggers an early event involving STAT1 phosphorylation, positioning pSTAT1 as a key upstream regulator of NLRC5 expression and MHC-I antigen processing and presentation.

Discussion

In this study, we demonstrate that the harmine derivative ACB1801 synergizes with and enhances the efficacy of anti-PD-1 therapy in the MSS CRC CT26 model, extending our previous observations in melanoma (20). More importantly, we provide mechanistic insights into how ACB1801 primes tumor cells and the tumor microenvironment to improve responsiveness to anti-PD-1 therapy. Notably, ACB1801 enhances IFN γ responsiveness and upregulates MHC-I expression, thereby priming tumor cells for immune recognition. However, these effects alone are insufficient to trigger a robust anti-tumor response, as treatment with ACB1801 alone did not significantly inhibit tumor growth, likely because T cell activity remains suppressed by immune checkpoint pathways. When ACB1801 is combined with anti-PD-1, which overcomes this inhibition, the primed tumor microenvironment translates into a robust immune response characterized by an increased frequency of CD8⁺ T cells and a reduced frequency of regulatory T cells, resulting in a significantly higher CD8/Treg ratio within the TME.

Our transcriptomic analysis indicates that the priming effect of ACB1801 on tumor cells is primarily driven by enhanced IFN γ responsiveness, characterized by early STAT1 activation that subsequently engages the NLRC5/CXCL10 axis. Specifically, we observed that ACB1801 treatment upregulates a cluster of interferon-stimulated genes (ISGs), including IRF7, STAT2, DDX58, and OAS1/2, which collectively amplify the IFN γ response and reinforce downstream immune signaling. Several plausible, non-mutually exclusive mechanisms may underline ACB1801's ability to activate STAT1 in tumor cells. ACB1801 induces type I/II IFN-like response under the stress from ferroptotic cell death, with downregulating key genes known to repress IFN γ signaling, thereby promoting STAT1 activation. Our transcriptomic analysis of ACB1801-treated CT26 cells revealed a significant downregulation of TRIB3 (**Supplementary Fig. 5**), a gene well described as pro-survival and repressing the STAT1–CXCL10 axis, reducing CD8⁺ T-cell infiltration, and promoting immune evasion in colorectal cancer (30). Downregulation of TRIB3 will also break the cell survival process and increase ferroptotic susceptibility. However, whether this effect of ACB1801 is linked to its properties as a β -carboline alkaloid derived of harmine (10), as a well known inhibitor of DYRK1A (31), remains to be determined.

Another potential mechanism is that ACB1801 promotes the autocrine secretion of chemokines, such as CXCL10, thereby establishing a positive feedback loop that sustains STAT1 activation. This mechanism is supported by our transcriptomic data, which show that ACB1801 downregulates DUSP5, a nuclear phosphatase that specifically dephosphorylates MAPK (ERK1/2) (32). Downregulation of DUSP5 leads to increased ERK1/2 phosphorylation, which in turn promotes CXCL10 expression through transcription factors such as AP-1 and NF- κ B (33). This upregulation of CXCL10 reinforces the autocrine loop, further sustaining STAT1 activation. Collectively, ACB1801 appears to engage multiple overlapping mechanisms that converge on enhanced IFN responsiveness, STAT1 activation, NLRC5 upregulation, and increased MHC-I expression.

One important consequence of STAT1 activation by ACB1801 is the modulation of the chemokine network released by tumor cells, which shapes the tumor immune microenvironment. The simultaneous upregulation of CXCL10 and downregulation of LCN2 upon ACB1801 treatment may represent a synergistic mechanism driving the remodeling of the immune landscape and regulating tumor cell ferroptosis. We believe that CXCL10 upregulation (Fig. 4B) is likely established a chemotactic gradient promoting CD8 + T-cell infiltration (8, 34), while reduced LCN2 expression, as shown in Fig. 3B and **Supplementary Fig. 6A**, enhanced tumor cell susceptibility to ferroptosis, as previously reported in colon cancer cells (35). Although the molecular mechanisms underlying CXCL10 upregulation via the STAT1/NLRC5 axis are well established (36), the mechanisms responsible for LCN2 downregulation in this context remain to be elucidated.

The reduction in Treg frequency in tumors treated with ACB1801 and anti-PD-1 combination therapy may be mechanistically linked to ACB1801-mediated downregulation of LCN2 as demonstrated in **Supplementary Fig. 6A**. Recent work has shown that tumor-derived LCN2 promotes intratumoral Treg accumulation by binding SLC22A17 on macrophages, driving their polarization toward an immunosuppressive phenotype that supports Treg recruitment and stability (37). Although ACB1801 downregulates LCN2, a significant reduction in Treg frequency was observed only with combination therapy, not with ACB1801 alone. This suggests that LCN2 suppression primarily limits de novo Treg recruitment, whereas established Tregs, characterized by high PD-1 expression, require PD-1 blockade for their destabilization and functional impairment. Thus, restricting Treg influx via LCN2 suppression while disrupting Treg maintenance through anti-PD-1 likely explains the synergistic increase in the CD8⁺/Treg ratio observed in the combination group.

Another important and unexpected function of ACB1801 is its ability to reprogram tumor cell metabolism. This metabolic shift is characterized by decreased intracellular glucose and reduced extracellular lactate levels, driven by the coordinated downregulation of key glycolytic enzymes and transporters (38). Such reprogramming has significant therapeutic relevance in colorectal cancer, where MSS CRC tumors are highly dependent on aerobic glycolysis. The resulting accumulation of lactate acidifies the TME and profoundly impairs CD8⁺ T-cell proliferation, cytokine secretion, and cytotoxic activity (39, 40), while concurrently promoting Treg differentiation and stability (41). By lowering intracellular glucose, reducing lactate production, and decreasing the expression of key ferroptosis

suppressor genes, we believe that ACB1801 alleviates these immunosuppressive pressures, restores CD8⁺ T-cell fitness, and increases tumor cell vulnerability to ferroptosis; these factors collectively shift the TME toward a more immune-permissive state.

Several lines of evidence from our study support this mechanism. First, transcriptomic profiling of CD45⁺ cells isolated from tumors treated with ACB1801 + anti-PD-1 revealed enrichment of adaptive immune activation, cytotoxic pathways, and IFN γ signaling, consistent with a more metabolically favorable niche for effector T cells. Second, analyses of TCGA CRC datasets showed that ferroptosis suppressor genes and glycolysis signature genes were negatively correlated with CD8⁺ T-cell markers and positively correlated with Treg markers (**Supplementary Fig. 6B**), supporting the metabolic and immunological remodeling effect of ACB1801. CD8⁺ T cells is known to mediate tumor ferroptosis via IFN γ process (42), ferroptosis can further elicit features of immunogenic cell death, as lipid peroxidation-driven membrane damage promotes the release of danger-associated molecular patterns (DAMPs) and inflammatory mediators that can enhance dendritic cell activation and antitumor immune responses (43). Thus, enhancing tumor cells' ferroptosis susceptibility by ACB1801 would magnify this effect during anti-tumor therapy. Together, these findings support a dual mechanism for ACB1801: enhancing tumor immunogenicity through increased IFN responsiveness and MHC-I expression, while simultaneously reprogramming tumor metabolism to counteract glycolysis- and lactate-driven immunosuppression (Fig. 6). By integrating these effects, ACB1801 promotes a more immune-supportive TME and increases tumor susceptibility to cytotoxic T-cell attack.

Materials and Methods

Cell lines, cell culture, siRNA transfection, and reagents

CT26, MC38, and HT29 cell lines (ATCC) were cultured in RPMI-1640, DMEM high-glucose, and McCoy's 5a medium, respectively, supplemented with 10% FCS, 2 mM L-glutamine, penicillin, and streptomycin. All cells were maintained at 37°C with 5% CO₂ and routinely tested for mycoplasma contamination. siRNA transfections were performed using Lipofectamine RNAiMAX (Invitrogen, CAT# 13778150) following manufacturer instructions (siRNA details in **Supplementary Table 1**). ACB1801 was provided by AC Bioscience (Switzerland), and ferrostatin-1 was purchased from Selleck Chemicals (CAT# S7243).

In vivo experiments

All animal experiments were conducted in accordance with European Union guidelines and approved by the Luxembourg Institute of Health ethical authorities and the Luxembourg Ministry of Agriculture, Viticulture, and Rural Development (Approval No. LECR-2018-12). Six-week-old BALB/c mice (Janvier Laboratories) were housed under controlled temperature and 12-h light/dark cycles with free access to food and water. For tumor models, CT26 cells (0.5×10^6 in 100 μ L PBS) were injected subcutaneously into the right flank. Treatments began when tumors became palpable. Mice received vehicle (0.5%

methylcellulose) or ACB1801 (50 mg/kg, oral gavage daily), and isotype control (BioXCell, CAT# BE0089, Clone 2A3) or anti-PD-1 antibody (BioXCell, CAT# BE0146, Clone RMP1-14) at 1 mg/kg intraperitoneally every other day. Tumor size was measured twice weekly, and volumes were calculated using the formula: $\text{length} \times \text{width}^2 \times 0.52$. Tumors were collected at endpoint for analysis.

Flow cytometry

For in vitro staining, cells were washed with PBS, detached using 0.05% Trypsin-EDTA (Thermo Fisher Scientific, CAT# 25300062), quenched with FBS-containing medium, centrifuged ($400 \times g$, 5 min), and resuspended in MACS buffer (Miltenyi Biotec, CAT# 130-091-221). For tumor immune profiling, tumors were dissociated using a tumor dissociation kit (Miltenyi Biotec, CAT# 130-096-730) following manufacturer instructions. Single-cell suspensions were blocked with anti-mouse CD16/32 (BioLegend, CAT# 101319) and stained with antibody panels (30 min, 4°C). Samples were analyzed using a NovoCyte Quanteon Flow Cytometer and FlowJo software. The following antibodies from BioLegend were used: PE anti-mouse H-2Kb (CAT# 116507), PE anti-mouse H-2Kb-SIINFEKL (CAT# 141603), APC anti-mouse H-2Kd (CAT# 116619), PE anti-human HLA-A,B,C (CAT# 311405), BV421 anti-mouse FoxP3 (CAT# 126419), BV570 CD44 (CAT# 103037), BV605 CD69 (CAT# 104529), BV650 CD4 (CAT# 100469), BV785 CD3 (CAT# 100231), APC CD8a (CAT# 100711), PE PD-1 (CAT# 135205), PE/Cy5 CD25 (CAT# 102010), PerCP/Cy5.5 TCR β (CAT# 109227), PE/Cy7 CD19 (CAT# 152417), APC NK1.1 (CAT# 156505), BV421 Ly-6C (CAT# 128031), BV510 I-A/I-E (CAT# 107635), BV605 F4/80 (CAT# 123133), BV785 CD11b (CAT# 101243), PE CD206 (CAT# 141705), PE/Cy5 CD11c (CAT# 117316), PE/Cy7 Ly-6G (CAT# 127617). Zombie NIR (CAT# 423105) was used for viability assessment.

In vitro Ovalbumin (OVA)257–264(SIINFEKL) antigen presentation assay

MC38 cells were seeded at a density of 0.3×10^6 cells per well in 6-well plates. After 24 h, cells were pulsed with 1 nmol OVA257–264 (SIINFEKL) peptide (InvivoGen, CAT# vac-sin) for 8 h, followed by treatment with DMSO or ACB1801 for an additional 24 h. Cells were then harvested, and surface expression of OVA-bound MHC-I was analyzed by flow cytometry as described above.

CD8 + OT-I T cell isolation and cytotoxicity assay

CD8⁺ OT-I T cells were isolated from spleens and lymph nodes of OT-I mice (Charles River) using the MojoSort Mouse CD8 T Cell Isolation Kit (BioLegend, CAT# 4800008). Cells were cultured in complete RPMI-1640 supplemented with 10% FBS, 20 mM HEPES, 2 mM L-glutamine, 1 mM sodium pyruvate, 1% penicillin–streptomycin, and 50 μM 2-mercaptoethanol. T cells were activated with Dynabeads CD3/CD28 (Thermo Fisher Scientific, CAT# 11452D) at a 1:1 bead-to-cell ratio for 48 h, followed by recombinant mouse IL-2 (20 ng/ml; PeproTech, CAT# 212-12-20UG). For cytotoxicity assays, MC38 cells (5×10^3 /well) were seeded in 96-well plates and pulsed with 1 nmol OVA257–264 (SIINFEKL) peptide (InvivoGen, CAT# vac-sin) for 8 h. Activated OT-I CD8⁺ T cells were added at effector-to-target ratios of

0:1, 1:2.5, 1:1, and 2.5:1 and co-cultured for 48 h. Tumor cell viability was quantified by flow cytometry using Precision Count Beads (BioLegend, CAT# 424902).

RNA extraction and quantitative RT-PCR

Total RNA was extracted using the NucleoSpin RNA Kit (MACHEREY-NAGEL, CAT# 740984.250) according to the manufacturer's instructions. First-strand cDNA was synthesized using the Maxima First Strand cDNA Synthesis Kit. Quantitative real-time PCR was performed using SYBR qPCR Blue Master Mix (Eurogentec, CAT# UF-LSMT-B0701) on a StepOnePlus Real-Time PCR System (Applied Biosystems). Relative gene expression was determined using the comparative Ct ($\Delta\Delta C_t$) method. Primer sequences are provided in **Supplementary Table 1**.

Cytokine/chemokine profiling and ELISA

CT26 cells (1×10^6 per 60 mm dish) were treated with DMSO or ACB1801 for 24 h. Supernatants were collected, centrifuged to remove debris, and protein concentrations were measured using the Pierce BCA Protein Assay Kit (Thermo Fisher Scientific, CAT# 23227) for normalization. In parallel, cells were harvested in RNA lysis buffer for qRT-PCR analysis of corresponding gene expression. Broad cytokine profiling was performed using the Mouse XL Cytokine Array Kit (Bio-Techne, CAT# ARY028) according to the manufacturer's instructions. Membranes were exposed to X-ray films, scanned, and quantified using ImageJ software. For CXCL10 quantification, supernatants were analyzed by ELISA using the Human CXCL10/IP-10 DuoSet Kit (Bio-Techne, CAT# DY266) for HT29 cells and the Mouse CXCL10/IP-10/CRG-2 DuoSet Kit (Bio-Techne, CAT# DY466) for CT26 cells, following the manufacturer's protocols.

Tumor plasma collection

Harvested tumors were minced and enzymatically dissociated using a tumor dissociation kit (Miltenyi Biotec, CAT# 130-096-730) according to the manufacturer's instructions. Following centrifugation (1,500 rpm, 10 min, 4°C), supernatants were collected and clarified by a second centrifugation. For concentration, 10 mL of supernatant was loaded into 3K MWCO protein concentrators (Thermo Fisher Scientific, CAT# 88526) and centrifuged at $8,000 \times g$ for 2–5 h to a final volume of ~ 1 mL. Concentrated tumor plasma was aliquoted and stored at – 80°C for downstream analyses.

Immunoblotting analysis

Cells were washed with PBS and lysed in RIPA buffer (Sigma, CAT# R0278). After centrifugation, protein concentrations were determined, and equal amounts were separated by SDS–PAGE and transferred to PVDF membranes. Membranes were blocked in 5% milk/TBST and incubated overnight at 4°C with primary antibodies, followed by HRP-conjugated secondary antibodies (2 h, room temperature). Signals were detected using chemiluminescent substrate (Millipore, CAT# WBKLS0500) and X-ray films (FUJIFILM).

Primary antibodies included anti-STAT1 (Cell Signaling Technology, CAT# 9172), anti-phospho-STAT1 (Tyr701; Cell Signaling Technology, CAT# 9167), and anti- β -actin (Invitrogen, CAT# MA5-15739-HRP).

Intracellular glucose measurement

Cells were seeded in 96-well plates (2×10^4 cells/well) and treated with DMSO or ACB1801 for 24 h. Intracellular glucose levels were measured using the Glucose-Glo Assay kit (Promega, CAT# J6021) following the manufacturer's instructions. Briefly, cells were washed with PBS, lysed with inactivation and neutralization solutions, and incubated with glucose detection reagent for 60 min at room temperature. Luminescence was recorded and glucose concentrations were calculated using a standard curve.

Lactate measurement

Both extracellular and intracellular lactate levels were measured using the Lactate-Glo Assay Kit (Promega, CAT# J5021) following the manufacturer's instructions. Cells were seeded in 96-well plates (2×10^4 cells/well) and treated with DMSO or ACB1801 for 24 h. For extracellular lactate, 5 μ L of culture medium was diluted in 95 μ L PBS and stored at -20°C until analysis. Fifty microliters of sample were mixed with 50 μ L detection reagent, incubated for 60 min at room temperature, and luminescence was recorded. For intracellular lactate, cells were washed with PBS, lysed using inactivation and neutralization solutions, incubated with detection reagent for 60 min, and lactate levels were quantified using a standard curve.

Glutathione (GSH)/ Glutathione disulfide (GSSG) ratio measurement

Reduced (GSH) and oxidized (GSSG) glutathione levels were measured using the GSH/GSSG-Glo Assay Kit (Promega, CAT# V6611) following manufacturer instructions. Cells were seeded in 96-well plates (5×10^3 cells/well) and treated with DMSO or ACB1801 for 24 h. Parallel plates were lysed using total or oxidized glutathione lysis reagents (50 μ L/well) and shaken for 5 min at room temperature. Luciferin generation reagent (50 μ L) was added, incubated for 30 min, followed by luciferin detection reagent (100 μ L) for 15 min before luminescence recording. GSH/GSSG ratios were calculated from standard luminescence signals as recommended by the manufacturer.

Cellular Reactive Oxygen Species (ROS) measurement

Cells were seeded in 60 mm dishes (1×10^6 cells/dish) and treated with DMSO or ACB1801 for 24 h. For total ROS detection, cells were stained with CellROX Green (Thermo Fisher Scientific, CAT# C10492) for 30 min at 37°C , harvested, filtered, and analyzed by flow cytometry (488 nm excitation) using a NovoCyte Quanteon system and FlowJo software. For lipid peroxidation analysis, cells were stained with C11-BODIPY 581/591 (Thermo Fisher Scientific, CAT# D3861) for 30 min at 37°C , harvested, filtered, and analyzed using 488 nm and 561 nm lasers with FlowJo software.

Cell viability assay

Cells were seeded in 96-well plates (5×10^3 cells/well) and treated with DMSO, ACB1801, or ACB1801 plus ferrostatin-1 for 24 h. Cell viability was measured using the CellTiter-Glo 2.0 Assay (Promega, CAT# G9241) according to manufacturer instructions. Equal volumes of reagent were added to each well, plates were shaken to induce lysis, incubated for 10 min at room temperature, and luminescence was recorded.

RNA-sequencing analysis

Total RNA was extracted in biological triplicates using the MACHEREY-NAGEL RNA Extraction Kit (CAT# 740984.250). RNA quality and quantity were assessed using a fragment analyzer (Labgene) and Quanti-iT™ RiboGreen RNA Assay Kit (Thermo Fisher Scientific). Libraries were prepared from 200 ng RNA using the TruSeq Stranded Total RNA Kit (Illumina) and sequenced (81 bp, single-end) on an Illumina HiSeq 2500. Reads were aligned to the human genome (hg19/GRCh37.75) using RNA-STAR, retaining uniquely mapped reads. Gene expression was quantified using QuasR, and differential expression analysis was performed with edgeR ($p < 0.05$ and $|\log_2FC| \geq 0.5$). Selected genes were subjected to pathway enrichment analysis.

Functional enrichment analysis

Functional enrichment of differentially expressed genes was performed using Bioconductor packages in R (org.Mm.eg.db, GO.db, GOstats, KEGG.db, ReactomePA). Biological process and pathway enrichment were assessed using Fisher's exact test, with $P \leq 0.05$ considered significant.

Gene set enrichment analysis (GSEA)

GSEA was performed using a Java application from the Broad Institute (v3.0, <http://www.broadinstitute.org/gsea>). Gene sets were derived from gene ontology (GO) annotations, and pathway information was obtained from the Kyoto Encyclopedia of Genes and Genomes (KEGG, <http://www.genome.jp/kegg/>).

Re-analysis of transcriptomic profiling data

RNA-sequencing gene expression data were retrieved from the TCGA Colon adenocarcinoma (COAD) dataset (Cancer Genome Atlas Research Network, 2017) via cBioPortal (<http://www.cbioportal.org>).

Statistical analysis

All statistical tests were two-sided. Data are presented as means, with error bars representing $\pm$ standard error of the mean (SEM). Analyses were performed using GraphPad Prism 9.0.

Use of large language models (LLMs)

LLMs, including ChatGPT and Perplexity, were used to assist with text summarization and editing. All content was critically reviewed, validated, and revised by the authors.

Availability of data and materials

The sequencing files are deposited on GEO database (<https://www.ncbi.nlm.nih.gov/geo/browse/>) with the accession numbers: GSE (To be included upon acceptance).

Declarations

Conflict of Interest

AI and CA are employees of AC Bioscience. The remaining authors declare that the research was conducted without any commercial or financial relationships that could be understood as a potential conflict of interest.

Primers for human MHC-I related genes

hRPL19-F GATGCCGGAAAAACACCTTG

hRPL19-R TGGCTGTACCCTTCCGCTT

hTAP1-F GCAGTCAACTCCTGGACCACTA

hTAP1-R CAAGGTTCCCACTGCTTACAGC

hTAP2-F TCTTGCGAAGCCTGGTGAAAGTG

hTAP2-R ACTTCCTGATGGCGGGTGTTGT

hB2M-F CTGGGTTTCATCCATCCGACA

hB2M-R TTCACACGGCAGGCATACTCATC

hCXCL10-F GGTGAGAAGAGATGTCTGAATCC

hCXCL10-R GTCCATCCTTGGAAGCACTGCA

hSTAT1-F ATGGCAGTCTGGCGGCTGAATT

hSTAT1-R CCAAACCAGGCTGGCACAATTG

hNLRC5-F AGTGGCTCTTCCGCTTGGACAT

hNLRC5-R CGGAACCCTAAGAACTTGGCTG

Primers for mouse MHC-I related genes

mRpl19-F CTCGTTGCCGGAAAAACA

mRpl19-R TCATCCAGGTCACCTTCTCA
mTap1-F GACTCCTTGCTCTCCACTCAGT
mTap1-R AACGCTGTCACCGTTCCAGGAT
mTap2-F CCTGGGATACGAAAAGGAGACG
mTap2-R TATCTAGTCATACGGAGGGTGA
mB2m-F CGGCCATACTGTCATGCTTAACTC
mB2m-R AGCCGAACATACTGAACTGCTACG
mStat1-F GCCTCTCATTGTCACCGAAGAAC
mStat1-R TGGCTGACGTTGGAGATCACCA
mNlrc5-F CTTCCCGCCTCTCCTTCCACAAT
mNlrc5-R CTCCACCTGCCCACATCCTACCA
mCycl10-F GGCTCGCAGGGATGATTTCAA
mCycl10-R CCAAGTGCTGCCGTCATTTTC

Primers for human Glycolysis-related genes

hGLUT1-F TTGCAGGCTTCTCCAACCTGGAC
hGLUT1-R CAGAACCAGGAGCACAGTGAAG
hHK2-F GAGTTTGACCTGGATGTGGTTGC
hHK2-R CCTCCATGTAGCAGGCATTGCT
hLDHA-F GGATCTCCAACATGGCAGCCTT
hLDHA-R AGACGGCTTTCTCCCTCTTGCT
hPDK1-F CATGTCACGCTGGGTAATGAGG
hPDK1-R CTCAACACGAGGTCTTGGTGCA
hGAPDH-F GTCTCCTCTGACTTCAACAGCG
hGAPDH-R ACCACCCTGTTGCTGTAGCCAA

hSLC16A1-F TTGTTGGTGGCTGCTTGTTCAGG

hSLC16A1-R TCATGGTCAGAGCTGGATTCAAG

hSLC16A3-F CCACAAGTTCTCCAGTGCCATTG

hSLC16A3-R CGCCAGGATGAACACGTACATG

hGLUT2-F ATGTCAGTGGGACTTGTGCTGC

hGLUT2-R AACTCAGCCACCATGAACCAGG

hPCK2-F TAGTGCCTGTGGCAAGACCAAC

hPCK2-R GAAGCCGTTCTCAGGGTTGATG

hPFKL-F AAGAAGTAGGCTGGCACGACGT

hPFKL-R GCGGATGTTCTCCACAATGGAC

Primers for human ferroptosis-related genes

hGCLC-F GGAAGTGGATGTGGACACCAGA

hGCLC-R GCTTGTAAGTCAGGATGGTTTGCG

hGCLM-F TCTTGCCTCCTGCTGTGTGATG

hGCLM-R TTGGAACTTGCTTCAGAAAGCAG

hSLC3A2-F CCAGAAGGATGATGTCGCTCAG

hSLC3A2-R GAGTAAGGTCCAGAATGACACGG

hAIFM2-F GACTCCTTCCACCACAATGTGG

hAIFM2-R CAGCACCATCTGGTTCTTCAGG

hHSPA5-F CTGTCCAGGCTGGTGTGCTCT

hHSPA5-R CTTGGTAGGCACCACTGTGTTC

hLCN2-F CCACCTCAGACCTGATCCCA

hLCN2-R CCCCTGGAATTGGTTGTCCTG

hSLC7A11-F TCCTGCTTTGGCTCCATGAACG

hSLC7A11-R AGAGGAGTGTGCTTGCGGACAT

hATF4-F TTCTCCAGCGACAAGGCTAAGG

hATF4-R CTCCAACATCCAATCTGTCCCG

hNRF2-F CACATCCAGTCAGAAACCAAGTGG

hNRF2-R GGAATGTCTGCGCCAAAAGCTG

hGPX4-F ACAAGAACGGCTGCGTGGTGAA

hGPX4-R GCCACACACTTGTGGAGCTAGA

hEN01-F TGTTCCTTCCAGGTGTCTC

hEN01-R CGTTCAGTTTCTTGCTAACCA

Primers for mouse Glycolysis-related genes

mHk2-F CCCTGTGAAGATGTTGCCCACT

mHk2-R CCTTCGCTTGCCATTACGCACG

mLdha-F ACGCAGACAAGGAGCAGTGGA

mLdha-R ATGCTCTCAGCCAAGTCTGCCA

mPdk1-F CCACTGAGGAAGATCGACAGAC

mPdk1-R AGAGGCGTGATATGGGCAATCC

mGapdh-F CATCACTGCCACCCAGAAGACTG

mGapdh-R ATGCCAGTGAGCTTCCCGTTCAG

mGlut1-F GCTTCTCCAACCTGGACCTCAAAC

mGlut1-R ACGAGGAGCACCGTGAAGATGA

mPck2-F AACGTGGCTGAGACCAGTGATG

mPck2-R AAGCGCGAGTTTGGATGTGCAC

mPfk1-F CCATCAGCAACAATGTGCCTGG

mPfk1-R TGAGGCTGACTGCTTGATGCGA

m*Slc16a1*-F GACCATTGTGGAATGCTGCCCT

m*Slc16a1*-R CGATGATGAGGATCACGCCACA

m*Slc16a3*-F TCCATCCTGCTGGCTATGCTCT

m*Slc16a3*-R CAGAAGGACGCAGCCACCATTC

m*Glut2*-F GTTGGAAGAGGAAGTCAGGGCA

m*Glut2*-R ATCACGGAGACCTTCTGCTCAG

Primers for mouse ferroptosis-related genes

m*Eno1*-F TACCGCCACATTGCTGACTTGG

m*Eno1*-R GCTTGTTGCCAGCATGAGAACC

m*Slc7a11*-F CTTTGTTGCCCTCTCCTGCTTC

m*Slc7a11*-R CAGAGGAGTGTGCTTGTGGACA

m*Nrf2*-F TCTTGGAGTAAGTCGAGAAGTGT

m*Nrf2*-R GTTGAAACTGAGCGAAAAAGGC

m*Gclc*-F ACACCTGGATGATGCCAACGAG

m*Gclc*-R CCTCCATTGGTCGGA ACTCTAC

m*Gclm*-F TCCTGCTGTGTGATGCCACCAG

m*Gclm*-R GCTTCCTGGAACTTGCCTCAG

m*Slc3A2*-F GAGCGTACTGAATCCCTAGTCAC

m*Slc3a2*-R GCTGGTAGAGTCGGAGAAGATG

m*Aifm2*-F GCGACCTTCAAGGACAACTTCC

m*Aifm2*-R GCCAGGATAAGATGTGAGAAGGG

m*Hspa5*-F TGTCTTCTCAGCATCAAGCAAGG

m*Hspa5*-R CCAACACTTCCTGGACAGGCTT

m*Atf4*-F AACCTCATGGGTCTCCAGCGA

mAtf4-R CTCCAACATCCAATCTGTCCCG

mLcn2-F ATGTCACCTCCATCCTGGTCAG

mLcn2-R GCCACTTGCACATTGTAGCTCTG

mGpx4-F CCTCTGCTGCAAGAGCCTCCC

mGpx4-R CTTATCCAGGCAGACCATGTGC

siRNAs (Horizon Discovery)

ON-TARGET plus Non-targeting Control Pool

D-001810-10-05, Horizon Discovery

ON-TARGET plus Human *NLRC5* siRNA (LQ-018267-02-0005, Horizon Discovery)

si*NLRC5*#11: J-018267-11-0005;

si*NLRC5*#12: J-018267-12-0005

ON-TARGET plus Mouse *Nlrc5* siRNA (LQ-067620-01-0005, Horizon Discovery)

si*Nlrc5*#17: J-067620-17-0005

si*Nlrc5*#18: J-067620-18-0005

ON-TARGETplus Human *STAT1* siRNA (LQ-003543-00-0005, Horizon Discovery)

si*STAT1*#7: J-003543-07-0005

si*STAT1*#8: J-003543-08-0005

ON-TARGETplus Mouse *Stat1* siRNA (LQ-058881-00-0005, Horizon Discovery)

si*Stat1*#8: J-058881-09-0005

si*Stat1*#9: J-058881-12-0005

Author Contributions

BJ, CA, AI, MM, ED, GB and RG initiated the project and designed the research plan. RG, KVM, EB and MGT performed the in vitro experiments. CP, AO, RG, MP, TLR, DM and KVM carried out the in vivo experiments under the supervision of BJ. RG, CL and XQ conducted the bioinformatics analysis. Data

analysis was performed by BJ, RG, AI and CA. The manuscript was written by BJ and RG and revised with input from all authors.

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Tables

Table 1 is available in the Supplementary Files section.

Figures

Figure 1

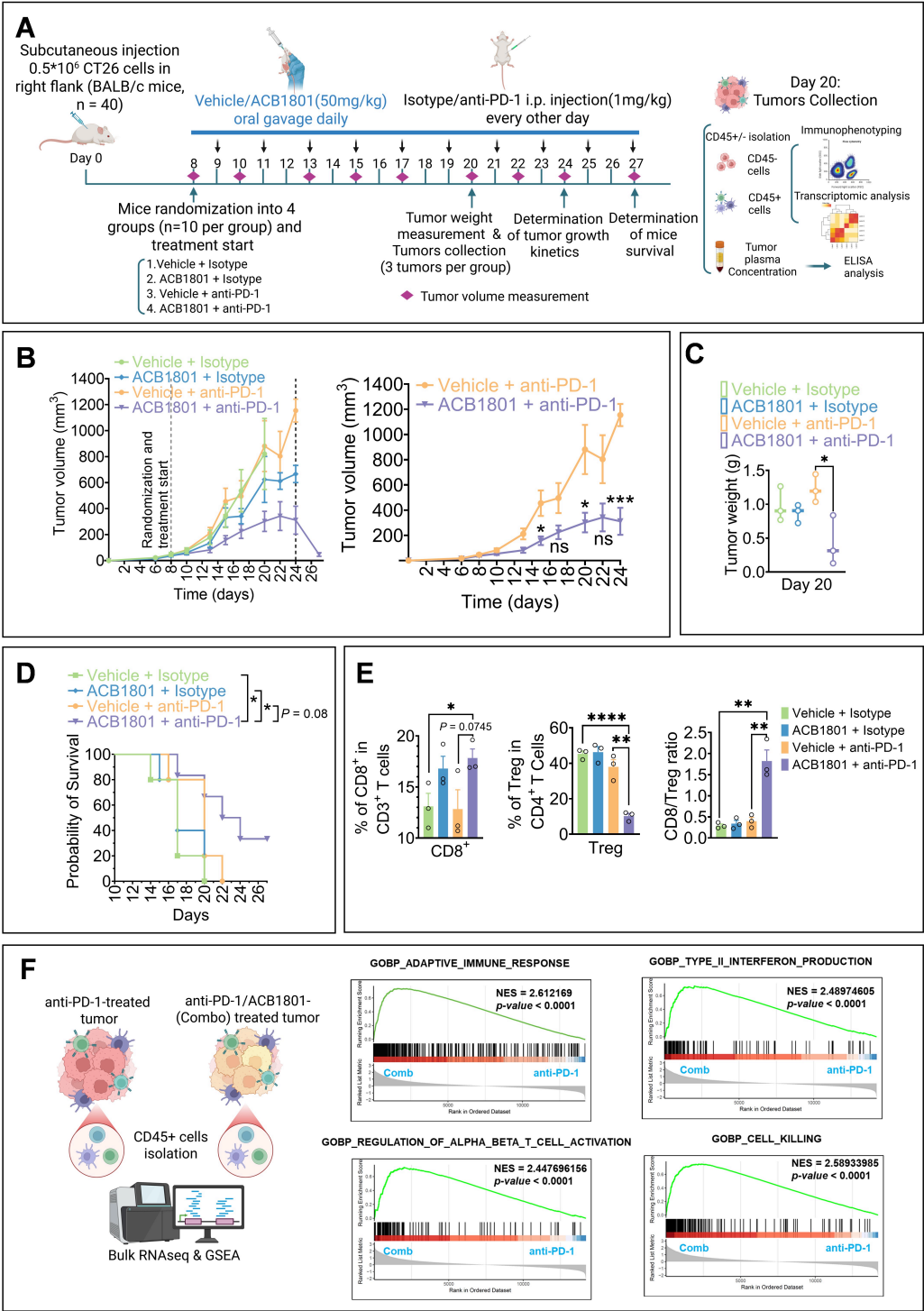


Figure 1

ACB1801 enhances anti-PD-1 efficacy in the MSS CRC CT26 syngeneic mouse model

(A) In vivo experimental design. CT26 cells (0.5×10^6 cells per mouse) were injected subcutaneously into the right flank of 40 BALB/c mice. On day 8, mice were randomized into 4 treatment groups (n = 10 per group): (1) vehicle + isotype control, (2) ACB1801 + isotype control, (3) vehicle + anti-PD-1, and (4)

ACB1801 + anti-PD-1, and treatments were initiated. Vehicle or ACB1801 (50 mg/kg) was administered daily by oral gavage, while isotype control or anti-PD-1 antibody (1 mg/kg) was administered intraperitoneally (i.p.) every other day. On day 20, group 1 was discontinued because 60% of mice reached humane endpoints or exhibited tumor necrosis or health deterioration. On the same day, three tumors per group were harvested, weighed, and dissociated for isolation of CD45- and CD45+ cells for transcriptomic analysis and immune phenotyping; corresponding tumor plasma samples were collected for cytokine and chemokine profiling. Tumor growth kinetics were assessed up to day 24, and overall survival was monitored until day 27, when the remaining mice were sacrificed.

(B) Tumor growth kinetics of CT26 tumors in BALB/c mice treated with ACB1801 and/or anti-PD-1. Tumor volumes were monitored longitudinally and are presented as mean $\pm$ SEM. Upper panel represent the tumor growth kinetic of the four group plotted until day 27. Lower panels represent the comparison of: vehicle + isotype control versus ACB1801 + isotype control (left panel); vehicle + isotype control (group 1) versus vehicle + anti-PD-1 (middle panel); vehicle + anti-PD-1 versus the combination of ACB1801 and anti-PD-1 (right panel) plotted until day 24. Statistical significance difference in the tumor volume was determined using on day 24 was determined using unpaired two-tailed t test with Welch's correction (ns = not significant; * $p < 0.05$; ** $p < 0.005$).

(C) Tumor weights on day 20 in each treatment group. Data represent the mean $\pm$ SEM of three tumors per group. Statistical significance was assessed using an unpaired two-tailed t-test with Welch's correction (* $p < 0.05$). Only statistically significant differences are shown.

(D) Kaplan–Meier survival curves of CT26 tumor-bearing mice. Loss of survival was defined as death, extensive tumor necrosis, significant body weight loss, deterioration of health status, or tumor volume exceeding 1500 mm³. Survival percentages were calculated using GraphPad Prism, and statistical significance was determined using the log-rank (Mantel–Cox) test (* $p < 0.05$).

(E) Flow cytometry analysis of tumor-infiltrating immune cells. Left: CD8+ T cells; middle: Tregs; right: CD8/Treg ratio. Tumors were harvested on day 20, and immune populations were gated on live CD45+ cells. Each dot represents an individual tumor (n = 3 per group). Data are presented as the mean of 3 experiments $\pm$ SEM. Statistical significance was assessed using ordinary one-way ANOVA (* $p < 0.05$; ** $p < 0.001$; *** $p < 0.00001$).

(F) Gene set enrichment analysis (GSEA) of RNA-seq data from isolated intratumoral CD45+ cells comparing the combination (combo) treatment group (ACB1801 + anti-PD-1) with the single-agent anti-PD-1 group. The enrichment plots show significant upregulation of gene sets associated with adaptive immune response, type II interferon production, regulation of alpha/beta T cell activation, cell killing in the combination group. For each pathway, the green line represents the running enrichment score, vertical black bars indicate the positions of genes from the gene set in the ranked list, and the normalized enrichment score (NES) and p-value are reported within each panel.

Figure 2

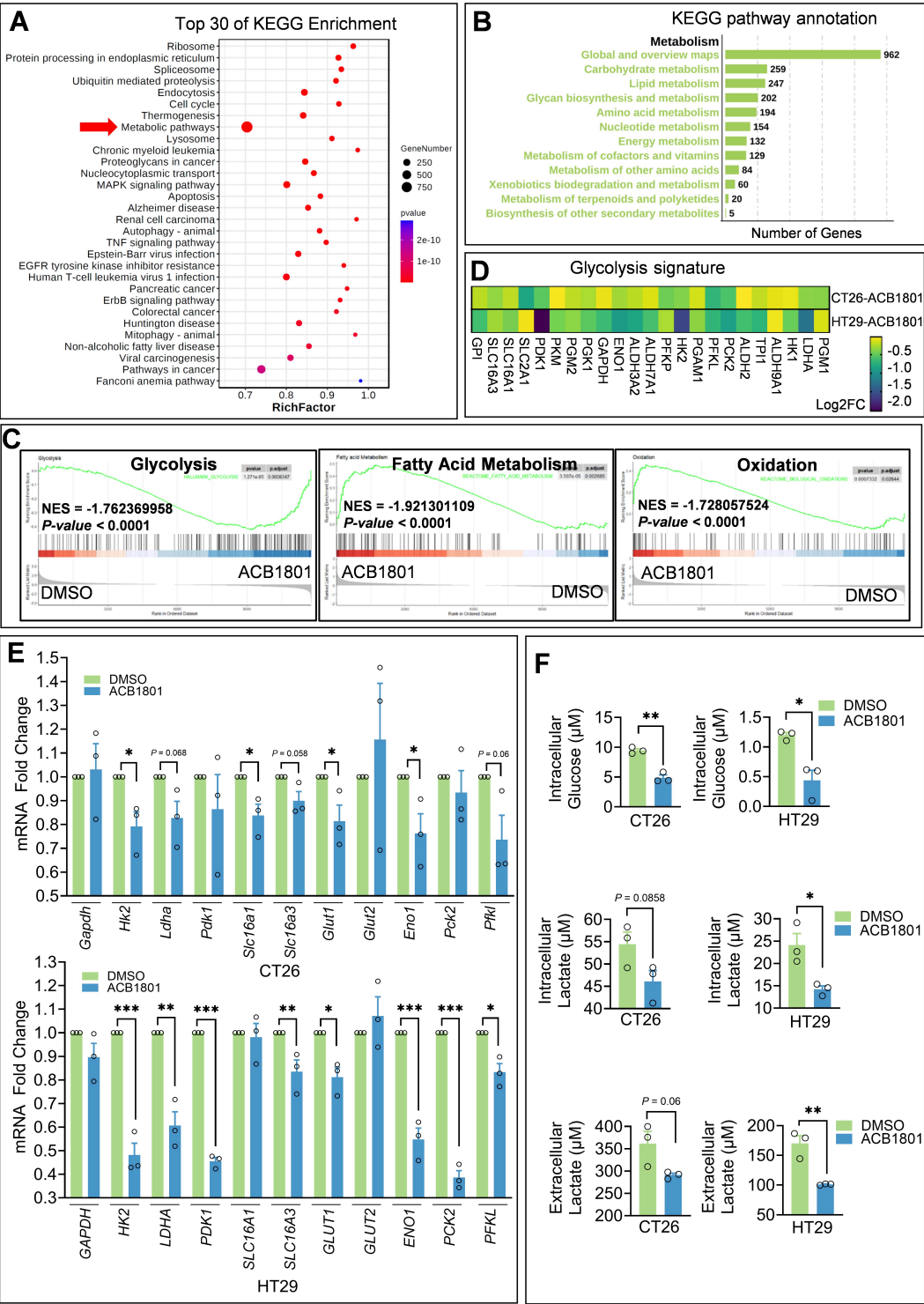


Figure 2

ACB1801 inhibits glycolytic and metabolic pathways in tumor and cancer cells

(A) KEGG pathway enrichment analysis of CD45- cells isolated from tumors treated with anti-PD-1 alone or with combination anti-PD-1/ACB1801. The dot plot shows the top 30 enriched KEGG pathways ranked by RichFactor, with dot size indicating the number of genes per pathway and color representing the

adjusted p-value for significance (blue to red scale). Pathways associated with ACB1801-related functional changes in tumor CD45⁺ cells are highlighted, including those related to metabolic processes.

(B) KEGG pathway annotation of metabolic processes in CD45⁺ cells from tumors treated with anti-PD-1 alone or in combination with ACB1801. The bar graph illustrates the number of genes involved in key metabolic pathways, grouped by category. The greatest gene representation is found in global and overview maps, with additional subdivisions including carbohydrate, lipid, glycan, amino acid, nucleotide, and energy metabolism, as well as biosynthetic and biodegradation pathways. This profile highlights metabolic processes most affected by ACB1801 treatment in the combination setting.

(C) Gene set enrichment analysis (GSEA) comparing metabolic pathway activity between ACB1801- and DMSO-treated CT26 cells. The enrichment plots show normalized enrichment scores (NES) and p-values for glycolysis, fatty acid metabolism, and oxidation pathways. In all cases, metabolic pathway gene sets are significantly downregulated in ACB1801-treated cells relative to the DMSO control ($p < 0.0001$), indicating inhibition of glycolytic and oxidative metabolic programs by ACB1801.

(D) Common glycolysis signatures in ACB1801-treated CT26 and HT29 cells. The heatmap displays log₂ fold change (Log₂FC) for key glycolytic genes in CT26 and HT29 cells following ACB1801 treatment. Each column represents a different gene implicated in glycolysis, while rows correspond to the 2 cell lines. Color scale indicates the degree of gene expression change, highlighting consistent downregulation of glycolytic pathways across both cell types in response to ACB1801.

(E) ACB1801 suppresses glycolytic gene expression in CT26 and HT29 cells. The bar graphs show mRNA fold changes of key glycolytic genes in CT26 (top) and HT29 (bottom) cells treated with DMSO or ACB1801 (10 μ M for CT26; 20 μ M for HT29, 24 hours), as measured by quantitative RT-PCR. ACB1801 consistently downregulates multiple glycolytic genes, including HK2, LDHA, SLC16A1, PDK1, GLUT1, ENO1, and PFKL, in both cell lines. Data are presented as the fold change (FC) relative to the control (DMSO), with mean $\pm$ SEM from 3 independent experiments. Statistical significance was determined by an unpaired t-test (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

Figure 3

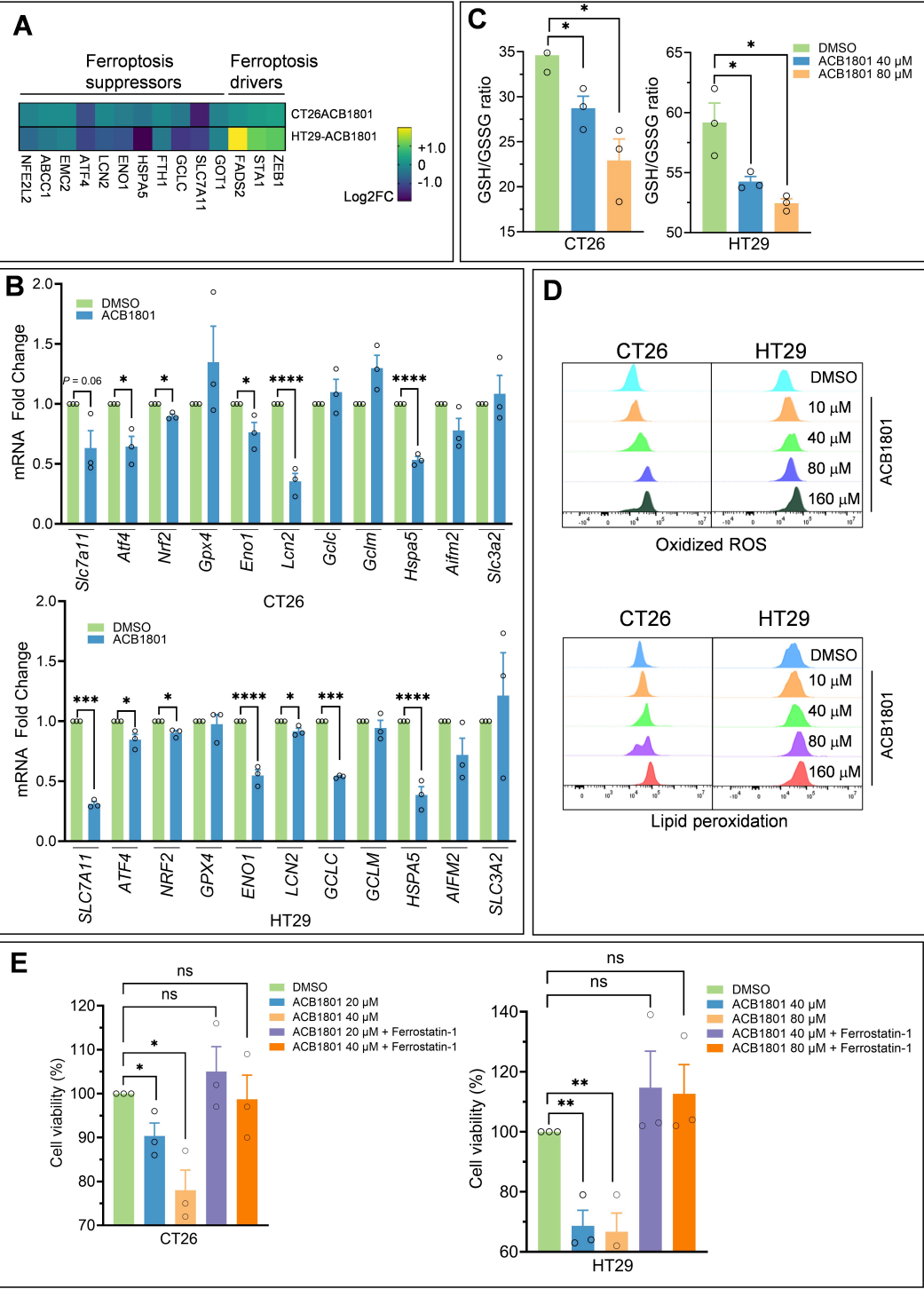


Figure 3

ACB1801 facilitates MSS CRC cells' sensitivity to ferroptosis to modulate the tumor microenvironment

(A) Expression analysis of ferroptosis suppressor and driver genes in CT26 and HT29 cells following ACB1801 treatment (10 μ M for CT26; 20 μ M for HT29, 24hours). The heatmap illustrates log2 fold change (Log2FC) in the expression of key genes associated with ferroptosis suppression and induction

for CT26 and HT29 cells. The color scale reflects relative up- or downregulation of each gene. Distinct expression patterns highlight the impact of ACB1801 on ferroptosis regulatory networks in both cell lines.

(B) ACB1801 downregulates the expression of ferroptosis-related genes in HT29 cells. The bar graphs show mRNA fold changes of key genes involved in ferroptosis regulation following treatment with DMSO or ACB1801 (10 μ M for CT26; 20 μ M for HT29, 24hours), as measured by quantitative PCR. Significant decreases are observed for several ferroptosis suppressors and antioxidants, including SLC7A11, ATF4, NRF2, GPX4, ENO1, LCN2, GCLC, and GCLM. Data represent the mean $\pm$ SEM of the fold change relative to the DMSO-treated controls. Statistical significance was determined by an unpaired t-test (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$).

(C) ACB1801 lowers the GSH/GSSG ratio in CT26 and HT29 cells. The bar graphs show the ratio of reduced glutathione (GSH) to oxidized glutathione (GSSG) in cells treated with DMSO or ACB1801 (40 μ M and 80 μ M). ACB1801 significantly decreases the GSH/GSSG ratio in a dose-dependent manner, indicating increased oxidative stress and reduced cellular antioxidant capacity. Data represent the mean $\pm$ SEM from 3 independent experiments. Statistical significance was determined by an unpaired t-test (* $p < 0.05$).

(D) ACB1801 increases oxidized ROS and lipid peroxidation in CT26 and HT29 cells. The flow cytometry histograms show dose-dependent changes in oxidized reactive oxygen species (ROS, top) and lipid peroxidation (bottom) in CT26 and HT29 cells treated with escalating concentrations of ACB1801 compared to DMSO controls. Both cell lines exhibit increased oxidative stress and membrane lipid damage upon 24hours with ACB1801.

(E) ACB1801 reduces cell viability via ferroptosis in CT26 and HT29 cells. The bar graphs show cell viability following treatment with DMSO or ACB1801 (20, 40, or 80 μ M) in the absence or presence of the ferroptosis inhibitor ferrostatin-1. ACB1801 significantly decreases cell viability in both cell lines, an effect that is reversed by ferrostatin-1 co-treatment, indicating ferroptosis involvement. Data represent the mean $\pm$ SEM from 3 independent experiments. Statistical significance was determined by an ordinary one-way ANOVA (* $p < 0.05$; ** $p < 0.01$; ns = not significant).

Figure 4

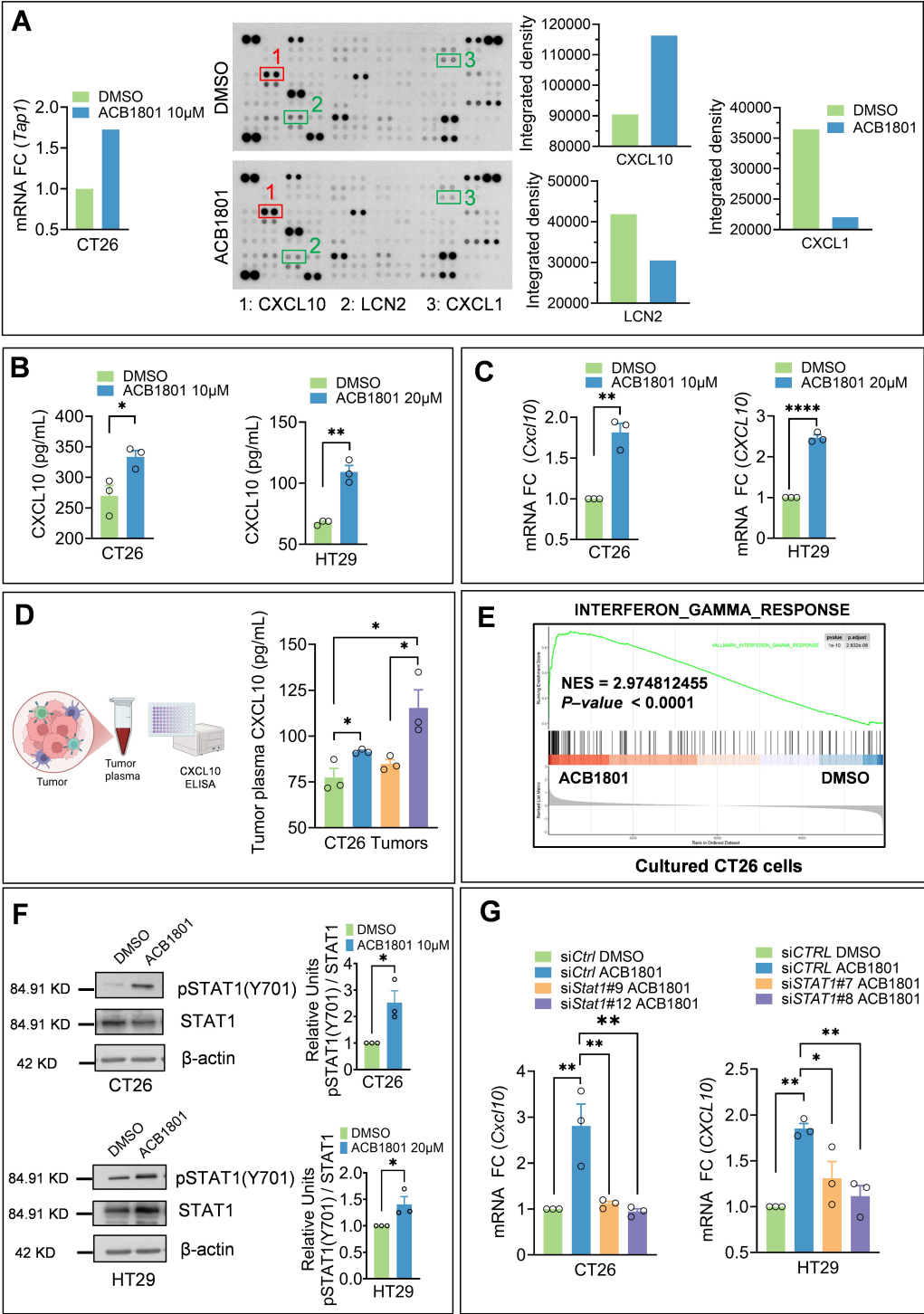


Figure 4

ACB1801 regulates CXCL10 expression in vitro and in vivo through STAT1

(A) Cytokine profiling of CT26 cell culture supernatants after treatment with DMSO or ACB1801 (10 μ M) for 24 hours. The left panel shows an increase in Tap1 expression under these conditions. Each duplicate spot represents the relative abundance of an individual cytokine or chemokine. Highlighted

boxes mark CXCL10 (1, red), LCN2 (2, green), and CXCL1 (3, green). The adjacent bar graphs show the integrated density quantification for CXCL10, LCN2, and CXCL1. ACB1801 treatment enhances CXCL10 secretion while reducing LCN2 and CXCL1 levels relative to the DMSO control.

(B and C) Quantification of CXCL10 expression at the protein (B) and mRNA (C) levels following treatment with DMSO or ACB1801. (B) ELISA measurement of CXCL10 protein secretion in CT26 and HT29 cells treated with ACB1801 (10 μ M for CT26; 20 μ M for HT29) for 24 hours shows a significant increase in CXCL10 levels compared with DMSO. (C) Quantitative RT-PCR analysis of Cxcl10 (CT26) and CXCL10 (HT29) mRNA expression reveals elevated transcript levels in ACB1801-treated samples. Data represent the mean $\pm$ SEM from 3 independent experiments. Statistical significance was determined by an unpaired t-test (**p < 0.01, ***p < 0.001).

(D) Quantification of CXCL10 levels in tumor plasma from CT26 tumors following the indicated treatments. CXCL10 concentrations were measured by ELISA. Data represent the mean $\pm$ SEM from 3 tumors per treatment group. Statistical significance was determined by an ordinary one-way ANOVA (*p < 0.05).

(E) Gene set enrichment analysis (GSEA) of CT26 cells treated with ACB1801 versus DMSO control. ACB1801 treatment significantly enriches the interferon-gamma response gene signature, as reflected by a high normalized enrichment score (NES) and strong statistical significance (P < 0.0001).

(F) Western blot analysis of STAT1 phosphorylation at Tyr701 (Y701) in CT26 and HT29 cells following 24 h treatment with DMSO or ACB1801 (10 μ M for CT26; 20 μ M for HT29). Whole-cell lysates prepared in RIPA buffer were immunoblotted for phospho-STAT1 (Y701), total STAT1, and β -actin as a loading control. Right panels show the ratio of phospho-STAT1 (Y701) to total STAT1, expressed as the mean $\pm$ SEM of three independent experiments. Statistical significance was assessed using an unpaired two-tailed t-test with Welch's correction (p < 0.05).

(G) Quantitative RT-PCR analysis of Cxcl10/CXCL10 mRNA expression in CT26 and HT29 cells after 24-hour treatment with DMSO or ACB1801 (10 μ M for CT26; 20 μ M for HT29), following transfection with control siRNA (siCTRL) or STAT1-targeting siRNAs (CT26: siStat1#9 and #12; HT29: siSTAT1#7 and #8). ACB1801-induced upregulation of Cxcl10/CXCL10 mRNA is attenuated upon STAT1 knockdown. Data are presented as the fold change (FC) relative to the control, with mean $\pm$ SEM from three independent experiments. Statistical significance was determined by an ordinary one-way ANOVA (*p < 0.05; **p < 0.01).

Figure 5

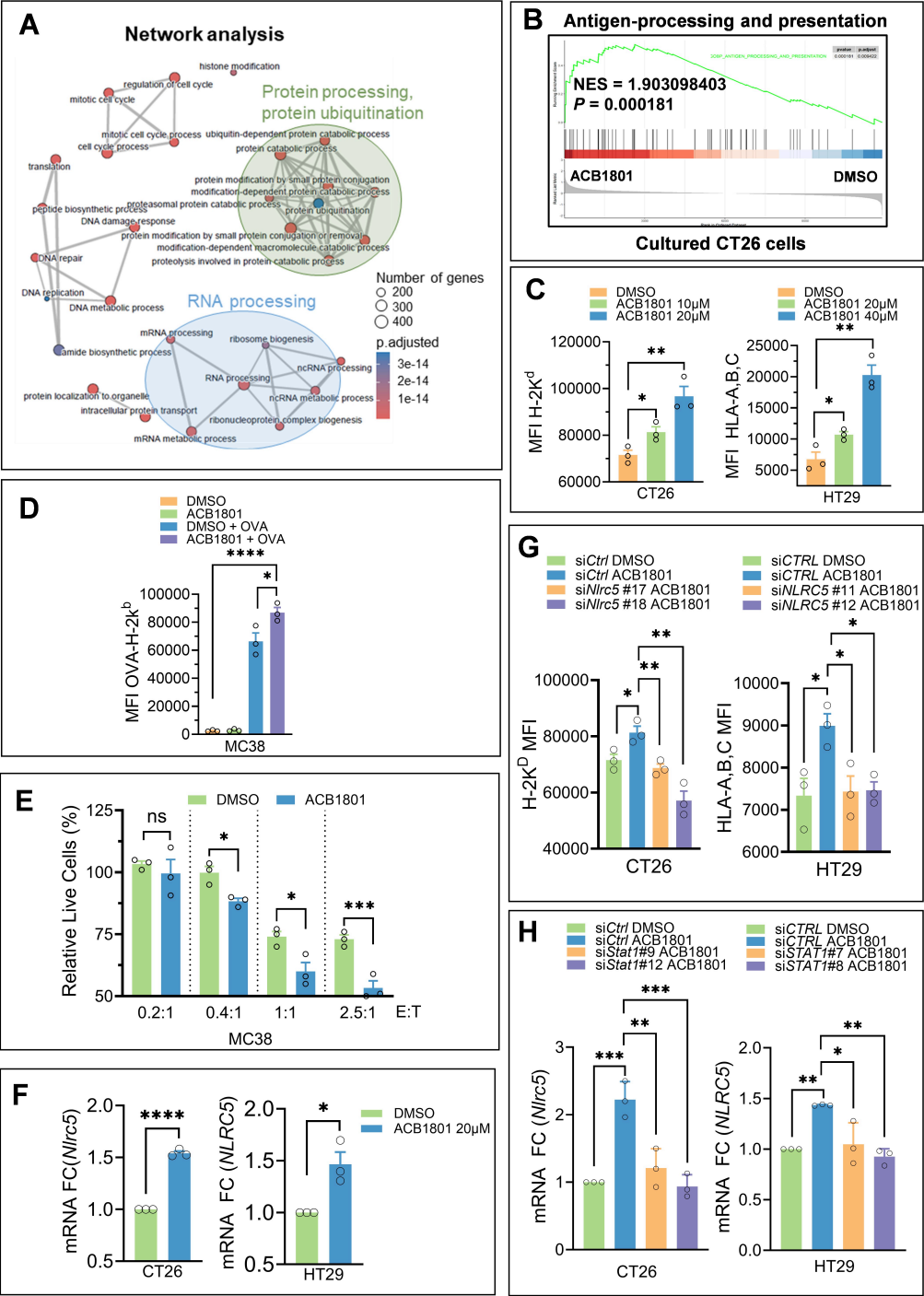


Figure 5

ACB1801 upregulates antigen processing and presentation pathways through STAT1-dependent induction of NLRC5

(A) Gene ontology (GO) enrichment network analysis of differentially expressed genes in CT26 tumors treated with ACB1801. Network nodes correspond to enriched GO biological processes, with node size

proportional to the number of associated genes and edges indicating functional relationships between GO terms. Adjusted p-values are color-coded to reflect enrichment strength. Major functional clusters center on protein modification, ubiquitin-dependent protein catabolism, and protein processing (highlighted in blue), consistent with coordinated regulation of pathways involved in antigen processing and presentation.

(B) Gene set enrichment analysis (GSEA) comparing CT26 tumors treated with ACB1801 versus DMSO reveals significant enrichment of the antigen processing and presentation pathway. The enrichment plot shows a high normalized enrichment score (NES = 1.90) and strong statistical significance ($p = 0.000181$), indicating robust upregulation of antigen-processing genes following ACB1801 treatment.

(C) ACB1801 increases cell-surface MHC-I expression in CT26 and HT29 cells. The representative flow cytometry histograms show H-2Kd (CT26) and HLA-A, B, and C (HT29) expression after treatment with DMSO or ACB1801 (20 μ M, 40 μ M) for 24 hours. The bar graphs represent the mean fluorescence intensity (MFI) from 3 independent experiments $\pm$ SEM. Statistical significance was determined by an unpaired t-test: * $p < 0.05$, ** $p < 0.01$.

(D) ACB1801 enhances the presentation of the OVA_{257–264} (SIINFEKL) antigen in MC38 cells. Cells were pulsed with SIINFEKL peptide for 8 hours, followed by treatment with either DMSO or ACB1801 for 24 hours. Surface OVA–H-2Kb complexes were quantified by flow cytometry using PE–OVA–H-2Kb staining. The representative flow cytometry histograms (top) and bar graphs (bottom) show the mean fluorescence intensity (MFI) from 3 independent experiments $\pm$ SEM. Statistical significance was determined by an unpaired t-test (* $p < 0.05$; **** $p < 0.0001$), demonstrating that ACB1801 significantly increases SIINFEKL presentation.

(E) ACB1801 enhances OT-I CD8⁺ T cell-mediated killing of MC38-OVA tumor cells. MC38-OVA cells were pulsed with SIINFEKL peptide for 8 hours and co-cultured with activated OT-I CD8⁺ T cells at the indicated effector: target (E:T) ratios in the presence of DMSO or ACB1801. After 48 hours, tumor cell numbers were quantified using Precision Count Beads. The bar graphs show MC38-OVA cell viability (% $\pm$ SEM) from 3 independent experiments. Statistical significance was determined by unpaired t-test (ns = not significant; * $p < 0.05$; *** $p < 0.001$), demonstrating that ACB1801 significantly enhances OT-I-mediated cytotoxicity, particularly at higher E:T ratios.

(F) Quantitative RT-PCR analysis of NLRC5 mRNA levels in CT26 and HT29 cells after treatment with ACB1801 (20 μ M) or DMSO for 24 hours. Data are presented as mean fold change (FC) relative to DMSO-treated samples from 3 independent experiments ($\pm$ SEM). Statistical significance was determined by an unpaired t-test (* $p < 0.05$; **** $p < 0.0001$).

(G) Flow cytometry analysis of cell surface MHC-I expression in CT26 and HT29 cells following 24-hour treatment with ACB1801 (20 μ M) or DMSO. Cells were transfected with either control siRNA (siCtrl) or NLRC5-targeted siRNAs (siNLRC5#17 and #18 for CT26; siNLRC5#11 and #12 for HT29). Representative flow cytometry histograms show H-2KD (CT26, APC conjugated) and HLA-A, B, C (HT29, PE conjugated)

staining. The bar graphs show mean fluorescence intensity (MFI), presented as mean $\pm$ SEM from 3 independent experiments. Statistical significance was calculated using an ordinary one-way ANOVA : **p < 0.01, *p < 0.05.

(H) Quantitative RT–PCR analysis of NLRC5 expression in CT26 and HT29 cells following STAT1 knockdown and ACB1801 treatment. Cells were transfected with either control siRNA (siCtrl) or STAT1-targeting siRNAs (CT26: siSTAT1#9 and #12; HT29: siSTAT1#7 and #8), then treated for 24 hours with DMSO or ACB1801. NLRC5 mRNA levels are presented as the fold change (FC) relative to the control. Data represent the mean $\pm$ SEM from 3 independent experiments. Statistical significance was determined by an ordinary one-way ANOVA (*p < 0.05, **p < 0.01, ***p < 0.001).

Figure 6

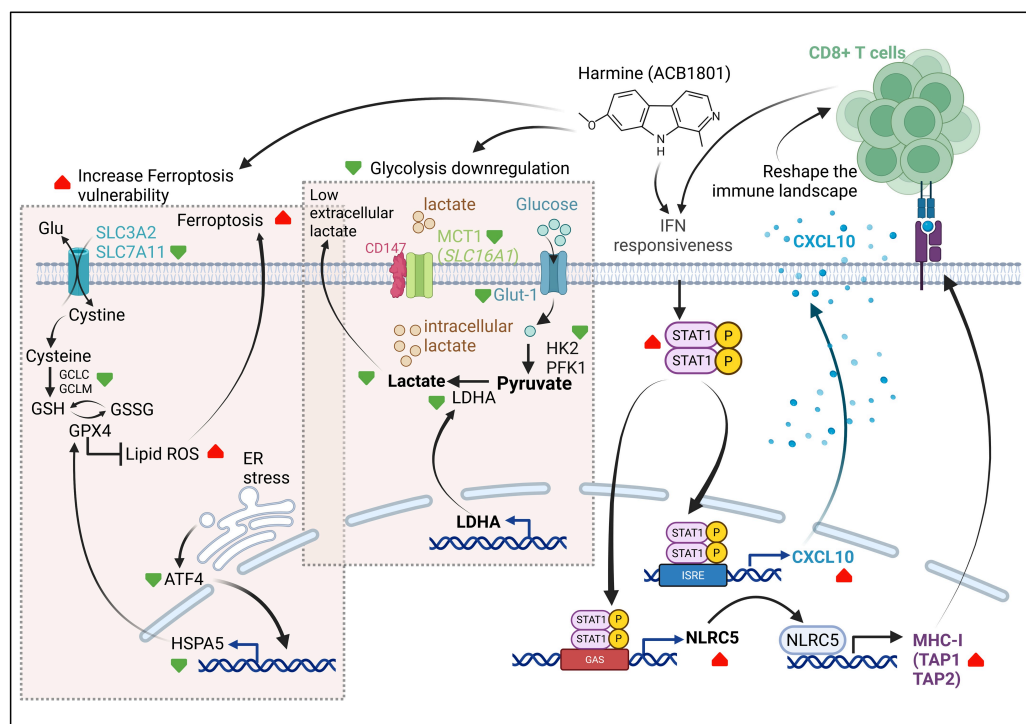


Figure 6

Proposed mechanism of action of ACB1801

ACB1801 enhances tumor immunogenicity by activating the IFN γ /STAT1 signaling pathway, leading to the upregulation of NLRC5 and CXCL10. Increased NLRC5 expression promotes the transcription of MHC class I–related genes (including TAP1 and TAP2), resulting in enhanced MHC-I surface expression

and improved antigen loading. In parallel, STAT1 activation induces CXCL10 expression and secretion, thereby reshaping the tumor immune microenvironment through the recruitment of CD8⁺ T cells. ACB1801 also reprograms tumor cell metabolism to counteract glycolysis- and lactate-driven immunosuppression. This effect is associated with the downregulation of key glycolytic and lactate transport genes, including SLC16A1 (MCT1), GLUT1, HK2, PFK1, and LDHA, leading to reduced intracellular and extracellular lactate levels. In addition, ACB1801 increases tumor cell vulnerability to ferroptosis by downregulating several genes directly or indirectly involved in redox homeostasis and ferroptotic resistance, including SLC7A11, GCLC/GCLM, ATF4, and HSPA5. Suppression of the SLC7A11–GCLC–GPX4 axis impairs glutathione-dependent detoxification of lipid peroxides, resulting in enhanced lipid ROS accumulation. Furthermore, inhibition of the ATF4/HSPA5 stress-response pathway further alleviates GPX4-mediated protection, collectively amplifying ferroptotic susceptibility. *Created in BioRender. Janji, B. (2026) <https://BioRender.com/898neq5>*

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- [Table1CDD.docx](#)
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