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Pharmacological Activation of STAT1-GSDME Pyroptotic Circuitry Reinforces Epigenetic Immunotherapy for Hepatocellular Carcinoma

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Abstract

Background: Genomic screening uncovered interferon-gamma (IFNγ) pathway defects in tumors refractory to immune-checkpoint blockade (ICB). However, its non-mutational regulation and reversibility for therapeutic development remain less understood.

Objective: We aimed to identify ICB resistance-associated druggable histone deacetylases (HDACs) and develop a readily-translatable combination approach for patients with hepatocellular carcinoma (HCC).

Design: We correlated the prognostic outcomes of HCC patients from a pembrolizumab trial (NCT03419481) with tumoral cell expressions of all HDAC isoforms by single-cell RNA sequencing. We investigated the therapeutic efficacy and mechanism-of-action of selective HDAC inhibition in 4 ICB-resistant orthotopic and spontaneous models using immune profiling, single-cell multiomics and chromatin immunoprecipitation-sequencing, and verified by genetic modulations and co-culture systems.

Results: HCC patients showing higher *HDAC1/2/3* expressions exhibited deficient IFNγ signaling and poorer survival upon ICB therapy. Transient treatment of a selective class-I HDAC inhibitor CXD101 re-sensitized HDAC1/2/3^{high} tumors to ICB therapies, resulting in CD8^{+}T cell-dependent antitumor and memory T cell responses. Mechanistically, CXD101 synergized with ICB to stimulate STAT1-driven antitumor immunity through enhanced chromatin accessibility and H3K27 hyperacetylation of IFNγ-responsive genes. Intratumoral recruitment of IFNγ^{+}GZMB^{+}cytotoxic lymphocytes further promoted cleavage of CXD101-induced Gasdermin E (GSDME) to trigger pyroptosis in a STAT1-dependent manner. Notably, deletion of GSDME micmicked STAT1 knockout in abolishing the antitumor efficacy and survival benefit of CXD101-ICB combination therapy by thwarting both pyroptotic and IFNγ responses.

Conclusion: Our immuno-epigenetic strategy harnesses IFNγ-mediated network to augment the cancer-immunity cycle, revealing a self-reinforcing STAT1-GSDME pyroptotic circuitry as the mechanistic basis for an ongoing phase-II trial to tackle ICB resistance (NCT05873244).

Key words: Immune-checkpoint blockade, epigenetics, HDAC, interferon-gamma, pyroptosis, hepatocellular carcinoma

Significance of this study

What is already known on this topic?

- ICB-resistant HCC has been primarily linked to the immunosuppressive tumor microenvironment (TME) characterized by immune exclusion of cytotoxic lymphocytes.
- The deficiency of IFNγ signaling confers immune evasion through affecting multiple steps of the cancer-immunity cycle, especially the immune effector cell trafficking/infiltration, antigen presentation and tumor cell recognition.
- The output of IFNγ signaling in HCC is lower than other solid tumors, but genetic mutations in the IFNγ pathway and its downstream effectors are rarely reported in patients with HCC.
- Although the HDAC family represents promising druggable targets to reprogram TME, the lack of in-depth characterization of epigenomic reprogramming hinders the development of selective HDAC-targeted immunotherapy.

What are the new findings?

- HCC patients with *HDAC1/2/3*^{high} tumors exhibited lower levels of IFNγ and T-cell exclusion gene signatures and poorer survival upon ICB therapy.
- A selective class-I HDAC inhibitor CXD101 re-sensitized HDAC1/2/3^{high} tumors to ICB by concomitant restoration of multiple rate-limiting steps of the cancer-immunity cycle.
- CXD101 synergized with ICB to stimulate STAT1-driven antitumor immunity through enhanced chromatin accessibility and H3K27 hyperacetylation of IFNγ-responsive genes.
- CXD10I-ICB combination therapy induced tumor cell pyroptosis by cooperative functions of CXD101-induced GSDME expression and IFNγ/STAT1-mediated cleavage by cytotoxic lymphocytes.

How might it impact on clinical practice in the foreseeable future?

- The successful conversion of the immune-excluded into an inflamed immunotype through epigenetic activation of STAT1-GSDME pyroptotic circuitry provides a mechanistic basis of CXD101 plus anti-PD-1 treatment in patients with ICB-resistant HCC.

- The application of single-cell multiomics analysis in the new phase-II clinical trial (NCT05873244) will advance precision medicine with immuno-epigenetic therapy through identification of predictive biomarkers for responsiveness and durability.

INTRODUCTION

Hepatocellular carcinoma (HCC), currently the third leading cause of cancer death worldwide, is estimated to directly affect ~1 million people annually by 2025 [1]. Although immune checkpoint blockade (ICB) therapies such as antibodies against Programmed cell death-1 (PD-1) or its ligand (PD-L1) have revolutionized the treatment paradigm for HCC, the immunosuppressive tumor microenvironment (TME) characterized by immune exclusion of cytotoxic lymphocytes (CTLs) in tumor stroma restricts the clinical benefits of ICB therapies to a minority of HCC patients [2]. The clinical success of atezolizumab (anti-PD-L1) plus bevacizumab (anti-vascular endothelial growth factor A) [3], which has become the new standard of care of HCC [4], highlights the importance of co-targeting by combination immunotherapy in the context of TME. However, the fact that less than one-third of patients respond remains a major challenge, which demands more effective strategies against the rate limiting steps of the cancer-immunity cycle for the generation of antitumor immune responses [5].

Among cancer-intrinsic and -extrinsic mechanisms underlying immunotherapy resistance [6], the deficiency of interferon-gamma (IFNγ) signaling confers immune evasion through affecting multiple steps of antitumor immunity, especially the immune effector cell trafficking/infiltration, antigen presentation and tumor cell recognition [7, 8, 9]. In HCC, the output of IFNγ signaling is lower than other solid tumors [10]. Importantly, patients with higher baseline IFNγ responsive genes (IRGs) expression have been found to exhibit good response to ICB therapy [11]. Since genetic mutations in the IFNγ pathway and its downstream effectors are rarely reported in patients with HCC, understanding the non-mutational regulation of IFNγ responses may help reinforce the cancer-immunity cycle and augment efficacy of immunotherapy.

Alterations in epigenomic landscapes have now been recognized to drive the development and progression of cancers [12]. Histone deacetylases (HDACs) responsible for histone deacetylation have become important targets for cancer therapeutic development [13]. Several HDAC inhibitors (HDACi) such as vorinostat, panobinostat and romidepsin had been approved by Food and Drug Administration (FDA) for the treatment of hematological malignancies [14]. However, the non-selective HDACi used in most studies have also shown immunosuppressive side effects in patients [15]. We and others have

recently demonstrated the potential of selective HDACis in enhancing ICB efficacy through TME remodeling using preclinical models of solid tumors including HCC [16, 17, 18, 19]. Further in-depth characterization of the cellular and molecular mechanisms by which specific epigenomic reprogramming elicits antitumor immunity is instrumental in rational development and clinical translation of selective HDAC-targeted immunotherapy.

Elucidating the tumor ecosystem at single-cell resolution has markedly improved our understanding of intratumor heterogeneity, cellular and molecular wiring of TME, and therapeutic resistance [20]. Here we charted the prognostic outcomes of HCC patients in relationship with all the human HDAC isoforms using single-cell RNA sequencing (scRNA-seq) data from an ICB therapy cohort and identified epigenetic drivers associated with ICB resistance. Elucidating the treatment-induced epigenomic remodeling in our preclinical models through single-cell assays for transposase-accessible chromatin-sequencing (scATAC-seq) and chromatin immunoprecipitation-sequencing (ChIP-seq) further revealed the mechanism-of-action of a selective HDAC inhibitor plus ICB in revitalizing IFNγ responses and a highly immunogenic cell death, thus providing a readily-translatable strategy to expand the spectrum of patients who can benefit from ICB therapy.

MATERIALS AND METHODS

A detailed description of all methods used in this study can be found in the online supplemental information.

RESULTS

Single-cell transcriptomics of HCC patient biopsies identifies *HDAC1/2/3* as predictive markers for poor responders of ICB therapy

To develop a mechanism-based combinatory ICB strategy with selective HDACi, we integrated single-cell analysis of an HCC patient cohort with functional and mechanistic delineation using our established ICB-resistant mouse models and co-culture systems (**figure 1A**). Based on scRNA-seq of tumor biopsies from our previous phase-II study of pembrolizumab in patients with HBV-related HCC (NCT03419481) [21], we correlated the baseline expression levels of all 18 human HDAC isoforms in tumor cells with the survival outcomes of patients by Cox proportional hazard models with optimal cut-offs (**figure 1B**). The results showed that patients with higher expression of *HDAC10*, NAD-dependent deacetylase *Sirtuin* (*SIRT*)*1* and *SIRT5* were correlated with better patient survival upon anti-PD-1 treatment (**figure 1B and supplementary figure 1**), which are consistent with their immuno-regulatory roles in cancer [22, 23, 24].

In contrast, higher expression of three class-I HDACs, namely *HDAC1*, *HDAC2* or *HDAC3* were significantly associated with poorer survival of HCC patients (**figure 1, B and C**). In line with the observed differences in survival outcome, we also noted that *HDAC1/2/3* was highly expressed in the tumor cells of ICB non-responders compared with responders (**figure 1D**). Notably, we found that *HDAC1/2/3*^{high} tumor cells uniformly exhibited lower levels of IFNγ gene signature (**figure 1E**). Moreover, Tumor Immune Dysfunction and Exclusion (TIDE) analysis [25] of bulk RNA-seq dataset from The Cancer Genome Atlas (TCGA) also demonstrated highly significant associations of *HDAC1/2/3*^{high} patients with predicted ICB non-responsiveness (**figure 1F**), further evidenced by the higher TIDE, T cell exclusion and myeloid-derived suppressor cell (MDSC) gene signatures (**figure 1G**). Overall, these data support the isoform-specific roles of HDAC1/2/3 in antagonizing the immune responses of HCC patients to ICB therapy.

A selective class-I HDAC inhibitor re-sensitizes HDAC1/2/3^{high} tumors to ICB by triggering robust antitumor immunity

To recapitulate the insensitivity of ICB in HCC patients, we have established Hepa1-6 and RIL-175 derived anti-PD-L1-resistant (PD-L1R) models by serial orthotopic implantation of HCC cells through anti-PD-L1-treated syngeneic, immunocompetent mice [21]. We also generated RIL-175 derived anti-PD-1-resistant (PD-1R) model using the same *in vivo* selection approach (**supplementary figure 2A**), in which anti-PD-1 treatment no longer exerted significant effect on the PD-1R tumors (**supplementary figure 2B**). Compared to the parental ICB-sensitive HCC cells, the PD-(L)1R-induced TME was comprised of fewer CTLs, namely $CD4^{+}$T, $CD8^{+}$T, natural killer (NK) and NKT cells with less cytotoxic marker expressions but more MDSCs (**supplementary figure 2, C and D**) [21]. Notably, HDAC1/2/3 was highly expressed in the tumor cells of ICB-resistant mouse models (**figure 2A and supplementary figure 3, A and B**), which also showed reduced levels of IRG expressions (**supplementary figure 2E**) as observed in $HDAC1/2/3^{high}$ HCC patients exhibiting poor response to ICB therapy.

Given the pivotal role of IFNγ signaling in cancer immunotherapy [7, 8, 9], we hypothesized that HDAC1/2/3 may confer ICB resistance through dysregulated hepatoma-intrinsic IFNγ signaling. We first investigated the functional effects of a selective HDAC1/2/3 inhibitor, CXD101/zabadinostat [26, 27], in combination with PD-(L)1 blockade in 3 ICB-resistant orthotopic models (**figure 2B**). While CXD101 monotherapy showed limited effect, the tumor growth was substantially abrogated when CXD101 treatment was combined with anti-PD-(L)1 antibody (**figure 2, C and D, and supplementary figure 4**), without observable side effect of weight loss or internal organ abnormality (**supplementary figure 5, A-G**). Moreover, the combination therapy significantly decreased the serum alanine transaminase (ALT) and aspartate transaminase (AST) levels to the extent comparable to that of age-matched normal mice (**supplementary figure 5H**). As evidence of CTL-mediated antitumor immunity, co-blockade of HDAC1/2/3 and PD-(L)1 significantly increased the intratumoral levels of a key chemokine CXCL10 [28] (**supplementary figure 6**) and CD45 positive leukocytes (**figure 2E, supplementary figure 7A and 8A**), mainly comprised of $CD8^{+}$T, $CD4^{+}$T, NK and NKT cells (**figure 2F, supplementary figure 7A and 8A**) which exhibited significantly negative correlations with tumor burden (**figure 2G**). Of note, the combination of CXD101 and anti-PD-(L)1 also significantly augmented the antitumor functions of $CD8^{+}$T cells as demonstrated by the increased proportions of

IFNγ$^+$ and granzyme B$^+$ (GZMB$^+$) cells (**figure 2H** and **supplementary figure 7B and 8B**). Notably, depletion of CD8$^+$T cells, but not NK cells, significantly impeded the combination therapy-induced intratumoral CD45$^+$immune cells infiltration and lytic tumor cell death, leading to abolishment of the therapeutic efficacy (**Supplementary figure 9**). These findings suggest that the antitumor CD8$^+$T cell functions mediate the combination therapy.

Remarkably, the combination treatment significantly prolonged the mouse survival and resulted in tumor eradication in >50% of mice in the Hepa1-6-PD-L1R model (**figure 2, I and J**). These findings suggest that transient treatment of CXD101 synergizes with ICB to induce long-lasting protection that may prevent future recurrence. Indeed, in contrast to the treatment-naïve mice of the same age, all mice cured by prior co-blockade survived after a secondary tumor challenge implanted into their livers (**figure 2K**), which were accompanied by significant increases in effector memory CD8$^+$T cells (CD8$^+$T$_{EM}$) and CD4$^+$T$_{EM}$ cells in the peripheral blood (**figure 2L**). Overall, these data suggest that HDAC1/2/3 inhibition induces strong antitumor immunity for effective and durable ICB therapies via promoting CTL recruitment, activation, and memory formation.

Single-cell multiomics reveals reactivation of IFNγ/STAT1 signaling by CXD101-ICB combination therapy

To decode the molecular and epigenetic reprogramming leading to the therapy-induced antitumor immunity, we performed single-cell multiomics [29] using tumor tissues from the Hepa1-6-PD-L1R model at an earlier treatment time-point (day 11) (**figure 3A and supplementary figure 10**). We obtained transcriptomic and open chromatin profiles for ~5,700-7,000 single-cells in each of the 4 control/single/combination treatment groups, and identified clusters of tumor cells, lymphocytes, myeloid cells, endothelial cells, and fibroblasts based on the canonical markers (**figure 3, B-D and supplementary figure 11, A and B**). While the immune cell proportions were markedly increased by the combination treatment, the tumor cell proportion was greatly reduced (**figure 3E**). However, reclustering of the tumor cells based on RNA expression (**figure 3F and supplementary figure 12A**) and chromatin accessibility profiles (**figure 3G and supplementary figure 12B**) revealed distinctive increases in the C6-RNA and C6-ATAC sub-clusters, respectively (**figure 3, H-K**), which were

enriched in interferon-related immue effector functions (**figure 3, L and M**). Moreover, >70% (433) of genes associated with the C6-ATAC sub-cluster was overlapped with the C6-RNA genes (**figure 3N**) and similarly enriched in response to type II interferon and MHC class I peptide loading complex (**figure 3O**). Transcriptional Regulatory Relationships Unraveled by Sentence-based Text mining (TRRUST) [30] further predicted that the overlapping genes were predominantly regulated by transcription factors crucial for IFNγ-dependent immunity (**figure 3P**), namely Signal Transducer and Activator of Transcription 1 (STAT1) and Interferon Regulatory Factor 1 (IRF1) that were concomitantly upregulated in the C6-RNA sub-cluster (**figure 3Q**).

Gene Set Enrichment Analysis (GSEA) confirmed the significant enrichment of IFNγ response as the top pathway induced by the CXD101-ICB combination therapy when compared with the control group (**figure 3R**). In concord, the expressions of IRGs related to antigen processing and presentation (*B2m*, *Cd74*, *Tap1* and *Tapbp*), lymphocyte recruitment (*Cxcl9* and *Cxcl10*) and signaling transduction (*Stat1* and *Irf1*) were synergistically up-regulated (**figure 3S and supplementary figure 13, A-C**) and accompanied by increased chromatin accessibilities (**figure 3T**). ATAC-based motif analysis further highlighted remarkable enrichments of the STAT/IRF families, which were not observed in the comparisons with single treatment by CXD101 or anti-PD-L1 (**figure 3U and supplementary figure 14**). Collectively, these data demonstrate that CXD101 synergizes with ICB to reactivate IFNγ/STAT1 signaling to induce IRG expressions in a subset of tumor cells, which may contribute to the antitumor responses.

H3K27 hyperacetylation by CXD101 primes IRG activation in response to ICB-induced IFNγ

IFNγ augments the transcriptional activation of IRGs by opening and priming chromatin with positive histone marks at the gene regulatory elements [8]. Surprisingly, the intratumoral IFNγ levels induced by anti-PD-(L)1 were not significantly different from anti-PD-(L)1 plus CXD101 in ICB-resistant models (**supplementary figure 15, A and B**), indicating that IFNγ immune stimulus alone is not sufficient to elicit full antitumor responses, which may be due to alterations of the epigenomic landscape in the ICB-resistant tumor cells. To understand how CXD101 re-sensitizes tumoral responsiveness to ICB-induced IFNγ stimulation, we investigated the chromatin remodeling of tumor

tissues from the Hepa1-6-PD-L1R model by ChIP-seq of histone H3 lysine 27 acetylation (H3K27ac), H3K4 monomethylation (H3K4me1) and H3K4me3 that distinguish active from inactive/poised enhancer and promoter elements [31]. Intriguingly, treatment with CXD101 alone or in combination with anti-PD-L1 did not obviously alter the genomic distribution of H3K27ac abundance (**figure 4A**), but substantially increased the H3K27ac levels around the transcription start site (TSS) of the IRGs (**figure 4B**) whose chromatin was highly accessible after the combination therapy (**figure 3T**). For example, CXD101 elevated the H3K27ac level of the H3K4me3-marked promoter of *Cd74*, a crucial effector for antigen presentation [32], and its H3K27ac level and accessibility were further increased by combination therapy (**figure 4C**). These results suggest that CXD101-induced H3K27 hyperacetylation primes IRGs for increased chromatin accessibility and robust transcription in response to ICB-induced IFNγ.

The expression, phosphorylation and the subsequent auto-regulation of the primary IFNγ signaling mediator STAT1 play a critical role in IRG expressions [33]. As ChIP-seq revealed a CXD101-induced H3K27 hyperacetylation pattern in the open chromatin of the *Stat1* promoter and enhancer (**figure 4D**), we further investigated the regulatory effect of CXD101 on STAT1 in PD-(L)1R cells with or without IFNγ stimulation that modelled ICB *in vivo*. We found that CXD101 dose-dependently amplified STAT1 expression and phosphorylation in the presence of IFNγ (**figure 4E, supplementary figure 16, A and B**), leading to synergistic upregulation of IRGs (**figure 4F, supplementary figure 16, C and D**) that mimicked the combination therapy *in vivo* (**supplementary figure 13, A-C**). However, CXD101 treatment alone could not activate STAT1 signaling and IRG transcription to the same extent (**figure 4, E and F and supplementary figure 16**), suggesting that chromatin priming by CXD101 is necessary but not sufficient for full IRG activation in PD-(L)1R cells. Notably, CRISPR-mediated knockout (KO) of STAT1 (**figure 4G**) abrogated H3K27ac induction by IFNγ and diminished H3K27ac hyperacetylation in the combined treatment with CXD101 in the *Stat1* promoter and enhancer (**figure 4H**), leading to the abolishment of synergistic activation of *Stat1* and the other IRG expressions (**figure 4I**). Altogether, these results provide a synergy mechanism whereby CXD101 cooperatively creates a primed chromatin environment with IFNγ/STAT1 signaling to augment IRG transcription.

CXD101 and IFNγ/STAT1 signaling coordinate CD8⁺T cell-induced pyroptosis

Given the synergistic activation of IRGs, we next determined whether and how CXD101 and IFNγ/STAT1 signaling coordinate tumor cell killing by CTLs. We briefly cultured the pretreated Hepa1-6-PD-L1R tumor cells with activated $CD8^+$T cells (6-hr) isolated from mice bearing the same parental tumor (**figure 5A**). We noted that single or combined CXD101 and IFNγ treatment *per se* did not affect tumor cell viability, whereas concomitant treatment with the $CD8^+$T cells significantly induced tumor cell killing (**figure 5B**). Notably, the dying tumor cells exhibited swelling and membrane blebbing resembling pyroptotic cell death [34] (**figure 5C**). Indeed, CXD101 and IFNγ synergistically increased the extracellular level of lactate dehydrogenase (LDH), an enzyme released through membrane permeabilization by dead or dying cells during pyroptosis, in the presence of $CD8^+$T cells (**figure 5D**). Moreover, the tumor cell viability was restored in STAT1-KO cells (**figure 5E**), while the membrane blebbing (**figure 5F**) and LDH release were also abolished (**figure 5G**), illustrating that IFNγ/STAT1 signaling is instrumental in $CD8^+$T cell-induced pyroptosis.

Tumor cell recognition by $CD8^+$T cells mediated through IFNγ/STAT1 signaling is fundamental to successful ICB therapy [7, 8, 9]. Given the synergistic upregulation of IRGs crucial for MHC class-I complex and antigen presentation (**figure 3S and supplementary figure 13**), we next determined the functional effects of CXD101 and IFNγ treatment on tumor cell recognition using an ovalbumin (OVA)-specific, $CD8^+$T (OT-I) cell model (**supplementary figure 17, A and B**). We found that single or combined CXD101 and IFNγ treatment *per se* did not affect tumor cell viability, whereas concomitant treatment with the OT-I cells significantly induced OVA-specific tumor cell killing (**supplementary figure 17C**). Moreover, the tumor cell viability was restored in STAT1-KO cells (**supplementary figure 17D**), illustrating that IFNγ/STAT1 signaling is instrumental in tumor cell recognition by $CD8^+$T cells for effective combination treatment.

As a lytic pro-inflammatory type of cell death, pyroptosis is induced by the activation and cleavage of pore-forming effector proteins called gasdermins (GSDMs) [35]. The family comprises *GSDMA*, *GSDMB*, *GSDMC*, *GSDMD* and *GSDME* in humans, while mice lack *Gsdmb* [35]. Integrated omics analysis of the PD-L1R model revealed chromatin accessibility and H3K27ac occupancy in the

regulatory elements of *Gsdmd* and *Gsdme* (**supplementary figure 18**). While GSDMD was constitutively expressed in PD-L1R tumor cells *in vitro* (**figure 5H**), GSDME was inducible by CXD101 (**figure 5I and supplementary figure 19**) with elevated H3K27ac levels at its promoter and enhancer (**figure 5J**). Intriguingly, GSDME was further cleaved to form its cytotoxic N-terminal domain when the CXD101 and IFNγ-pretreated cells were exposed to $CD8^{+}$T cells (**figure 5H**). Moreover, deletion of STAT1 abrogated GSDME cleavage without influencing its induction (**figure 5K**). Collectively, these results suggest that tumor cell pyroptosis occurs by cooperative functions of CXD101-induced GSDME expression and IFNγ/STAT1-mediated cleavage by $CD8^{+}$T cells upon tumor cell recognition.

GSDME upregulation by CXD101 renders CTL-induced pyroptosis

We further investigated the role of CXD101-induced GSDME expression on CTL-mediated tumor cell pyroptosis using the NK92 cell model [36], which acquires the capability to recognize and kill mouse tumor cells [37] (**figure 2F**). We found that pretreatment of Hepa1-6-PD-L1R cells with CXD101 markedly enhanced NK92 cell-induced morphological changes (**figure 6A**) and LDH release (**figure 6B**), which phenocopied the pyroptotic effects of GSDME overexpression (**figure 6, C-E**). Consistent with the $CD8^{+}$T cell model, CXD101 induced cleavage of GSDME, but not GSDMD, and release of a pyroptosis marker high mobility group box-1 (HMGB1) [38] in the medium when the pretreated Hepa1-6-PD-L1R cells were co-cultured with NK92 cells (**figure 6F**). Notably, CRISPR-mediated KO of GSDME abolished CXD101-induced release of HMGB1 (**figure 6G**) and LDH (**figure 6H**) as well as pyroptotic morphological changes even in the presence of NK92 cells (**figure 6I**). These data suggest that GSDME upregulation by CXD101 renders CTL-induced pyroptosis.

We next examined whether tumor cell pyroptosis occurred upon CXD101 and ICB treatment *in vivo*, by which tumor regression was accompanied with significant recruitment of CTLs (**figure 2H**). In PD-(L)1R models, we found that the combination treatment induced upregulation and cleavage of GSDME (**figure 6, J and K and supplementary figure 20**) and increased proportions of 7-aminoactinomycin $(7\text{-AAD})^{+}$Annexin $V^{+}CD45^{-}$ cells (**figure 6, L and M**) representing pyroptotic tumor cells [39], which were accompanied with elevated HMGB1 levels in the TME (**figure 6, N and**

O). Taken together with the *in vitro* data, these findings suggest that intratumoral recruitment and activation of CTLs promote cleavage of CXD101-induced GSDME to trigger pyroptosis in the ICB combination therapy.

Deletion of GSDME abolishes CXD101-ICB combination therapeutic effects by thwarting pyroptotic and IFNγ responses

To investigate the significance of GSDME-triggered pyroptosis and STAT1-mediated IFNγ responses on the combination therapy, we determined the therapeutic efficacy in the Hepa1-6-PD-L1R model derived from wild-type (WT), GSDME-KO and STAT1-KO tumor cells (**figure 7A**). Notably, deletion of GSDME abolished the antitumor effect of CXD101 and anti-PD-L1 combination treatment to the same extent as the STAT1 KO (**figure 7B**). Intriguingly, GSDME KO not only abrogated tumor cell pyroptosis as reflected by 7-AAD$^+$Annexin V$^+$CD45$^-$cell proportions (**figure 7, C and D**), but also attenuated the intratumoral IFNγ induction (**figure 7E**) and IRG upregulations for antigen processing and presentation (*Cd74*, *H2-k1*, *Tap2* and *Tapbp*) by the combination therapy (**figure 7F**). In addition, the intratumoral recruitment and activation of CTLs were dramatically reduced (**figure 7, G and H**) that ultimately culminated in worsened survival of mice (**figure 7I**). These findings emphasize the crucial role of GSDME-triggered pyroptosis in tumor cell killing and IFNγ responses for the therapeutic benefits of CXD101 plus anti-PD-L1 treatment.

On the other hand, although deletion of STAT1 did not significantly affect *Gsdme* induction by the combination treatment (**figure 7C**), the tumor cell pyroptosis was still abolished (**figure 7D**) which may be attributable to the effect on IFNγ/STAT1-mediated cleavage of GSDME by CD8$^+$T cells as observed *in vitro* (**figure 5**). In concord, STAT1 KO dampened the IFNγ induction in TME and IRG upregulations for antitumor immunity (**figure 7, E-H**), leading to a significant reduction in survival benefit of the combination therapy (**figure 7I**). Taken together, our findings uncover a reciprocal regulation between GSDME-triggered pyroptosis and STAT1-mediated IFNγ responses that plays a pivotal function in the TME remodeling for the effectiveness of the CXD101-ICB combination therapy.

CXD101 averts ICB resistance in spontaneous HDAC1/2/3^{high} HCC model

Given the importance of tumour heterogeneity for resistance to cancer therapies [40], finally we tested the efficacy of the combination immunotherapy in a spontaneous HCC model induced by hydrodynamic tail-vein injection (HDTVi) of MYC and CTNNB1 encoding plasmids together with a sleeping beauty transposase construct [41]. We employed this model because it recapitulates the high-frequency mutation and activation of β-catenin pathway observed in human HCCs which are characterized by T cell exclusion and immunotherapy resistance [42, 43]. Importantly, data from transcriptomic analysis of 9 HDTVi models [41] showed that the MYC/CTNNB1 tumor model highly expressed *Hdac1/2/3* (**figure 8A**), which could be validated by Western blot analysis (**figure 8B**).

Consistent with the previous findings, we first confirmed that the HDAC1/2/3^{high} HCC tumors of the MYC/CTNNB1 model were resistant to anti-PD-L1 therapy (**figure 8, C and D**). Moreover, single CXD101 treatment also did not influence the tumor burden of this aggressive model, which was indicated by the liver weight, liver/body weight ratio and tumor area assessed by H&E staining (**figure 8, D and E**). Remarkably, concurrent CXD101 treatment could re-sensitize HDAC1/2/3^{high} HCC tumors to PD-L1 blockade, resulting in significant reduction in tumor burden (**figure 8, D and E**) and prolongation of mouse survival when compared to the untreated and single treatment groups (**figure 8F**) without signs of toxicity (**supplementary figure 21**). These findings demonstrate that selective class-I HDAC inhibition can overcome ICB resistance in a preclinical model representing the highly prevalent MYC^{high} and CTNNB1^{mut} human HCCs.

DISCUSSION

Recent progresses from therapeutic checkpoint inhibition in the clinic have provided new insights into the rate limiting steps of the cancer-immunity cycle [44]. One of the key mechanisms that renders the immune system unable to control tumor growth is the deficiency in IFNγ signaling. Despite intensive research in the perturbations of this pathway in cancer patients unresponsive to ICB [11, 45, 46], its non-mutational regulation and potential reversibility for therapeutic development remain less understood. Here, our integrated scRNA-seq and clinical interrogation of a PD-1 blockade cohort reveals that HCC patients with *HDAC1/2/3*^{high} tumors exhibited deficient IFNγ signaling and poor survival upon ICB therapy. Using our anti-PD-(L)1-resistant orthotopic HCC models and multiomics analysis, we demonstrate the rationality, effectiveness, and mechanism-of-action of selective HDAC1/2/3 inhibition by CXD101, which epigenetically overcomes ICB resistance by activating a self-reinforcing circuitry of IFNγ/STAT1 signaling and GSDME-mediated pyroptosis (**figure 8G**). More importantly, the CXD101-ICB combination therapy elicited strong antitumor efficacy and survival benefit in a preclinical model of the highly prevalent MYC^{high} and CTNNB1^{mut} human HCCs, thus providing a proof-of-concept for our ongoing phase-II trial to tackle resistance to ICB treatment in HCC (NCT05873244).

The iterative nature of the antitumor immune responses where the recognition and killing of tumor cells by T cells initiates subsequent rounds of antigen presentation, T cell stimulation, trafficking and infiltration into tumors reflects not only the complexity of human immunity, but also the array of tumor immune evasion capabilities [5, 6, 44]. The strong therapeutic activities of CXD101-ICB combination therapy therefore stems from the concomitant restoration of multiple rate limiting steps of the cancer-immunity cycle. First, CXD101 systemically hyperacetylases the regulatory regions of IRGs including *STAT1*, the crucial meditator of IFNγ pathway. This epigenomic priming works in tandem with the IFNγ signal released by CTLs [47], which are prevented from the development of the exhausted phenotype by checkpoint blockade [48], to synergistically activate IRGs encoding chemokines and MHC class-I complex critical for lymphocyte recruitment and tumor cell visibility, respectively. Second, CXD101 treatment also opens and primes chromatin with H3K27ac at the *GSDME* promoter and enhancer for transcriptional reactivation, leading to CTL-mediated GSDME cleavage and

induction of pyroptosis, an inflammatory form of cell death that further promotes infiltration and activation of antitumor innate and adaptive immune cells [49]. As GSDME silencing by promoter hypermethylation also occurs in some human cancers [50], pyroptosis may be epigenetically exploited in a tumor-specific manner to reinstate the cancer-immunity cycle and amplify the antitumor responses to ICB therapy.

Intriguingly, we found that IFNγ/STAT1 signaling is prerequisite for GSDME-mediated pyroptosis *in vitro* and *in vivo*. Unlike GSDMB expression which can be directly upregulated by IFNγ [51], the cleavage of CXD101-induced GSDME is dependent on the IFNγ/STAT1 axis, possibily due to the necessity of tumor cell recognition before GZMB release by cytotoxic $CD8^+$T cells [52]. The immunogenic cell death in turn fuels more lymphocyte-derived IFNγ and GZMB in the TME to reinforce the STAT1-GSDME pyroptotic circuitry. Notably, deletion of either reciprocally regulated player in the circuit abolished the therapeutic efficacy of our combination targeting strategy, further highlighting the significance of the cooperative and synergistic remodeling of cancer-intrinsic epigenomic landscape and immune-excluded TME in the generation of an optimal antitumor immunity.

There are limitations to our study. The relatively small cohort of patients in our phase-II study (NCT03419481) may preclude the potential predictive power of HDACs for ICB responsiveness, although TIDE analysis of TCGA dataset was performed to verify the findings. While we have applied single-cell multiomics to characterize the role of chromatin accessibility in the therapy-induced antitumor immunity, we cannot decode the histone modification profiles in tumor heterogeneity using the current bulk approach. Recent advancements of high-throughput single-cell ChIP-seq [53] may enable identification of a sub-cluster of IFNγ-responsive tumor cells with specific chromatin states and transcription factors responsible for epigenetic reprogramming through motif analysis of the enriched regions. As the majority of HCC develops in liver fibrosis, in which the peritumoral stromal and myeloid cell components form a barricade to restrict T cell immunity [54, 55], orthotopic mouse models using cell lines may not fully reflect the immune-excluded immunotype [44]. The validated therapeutic benefits of CXD101 plus ICB treatment in the HDTVi spontaneous HCC model warrants further investigation in the context of fibrosis-associated HCC.

As we have recently shown that CXD101 treatment could increase MHC class-I and -II gene expressions in dendritic cells leading to antigen presentation [56], it is worthwhile to determine the direct effects of CXD101 on immune cells integral in the cancer-immunity cycle. While the T_{EM} cells were elevated in the tumor re-challenge model, our pilot *ex vivo* study did not show obvious changes in memory T cell proportions by direct CXD101 and ICB treatment (**supplementary figure 22**). CXD101 treatment also did not exhibit direct effect on $CD8^+$T cell cytotoxicity in the OVA-specific OT-I cell model (**supplementary figure 23**). Further studies using T cell-selective knockout approach are warranted to clarify the impact of class-I HDACs on T cell responses.

The identification of clinically druggable targets that restrict antitumor immunity is required to develop potential combination therapies. Using single-cell transcriptomic data on patients with HCC treated with anti-PD-1 therapy and newly established preclinical ICB-resistant models, we identified class-I HDACs as key epigenetic drivers restricting the amplification of the antitumor immunity cycle, thereby contributing to the immune-refractory feature of TME and ICB resistance. The successful conversion of the immune-excluded into an inflamed immunotype through epigenetic activation of STAT1-GSDME pyroptotic circuitry provides a mechanistic basis of CXD101 plus anti-PD-1 treatment in patients with ICB-resistant HCC. We envision that application of single-cell multiomics analysis in the new phase-II clinical trial (NCT05873244) will advance precision medicine with immuno-epigenetic therapy through identification of predictive biomarkers for responsiveness and durability.

Contributors

Study concept and design: Y.T., S.L.C. and A.S.-L.C.; Data acquisition and analysis: Y.T., H.W., C. Z., Y.L., Z.X., S.C, J.W., P.P.-C.W., W.Y., Z.L., J.L., S.C., L.Z., Y.F., W.W.-Y.S.-T., B.Y., Y.L., J.L., L.L., J.S.L.V., W.R., T.T.K., H.H.W.L., J.Z., H.S., S.L.C., and A.S.-L.C.; Clinical resources: S.L.C. and K.-F.T.; Bioinformatics analysis: H.W.; Writing of manuscript: Y.T. and A.S.-L.C.; Material support: S.M., D.K. and N.L.T.; Critical review of manuscript: J.Z. and A.S.-L.C.; Supervision: M.T., Q.X., J.J.-Y.S., S.L.C. and A.S.-L.C.; Funding acquisition: S.M., D.K., N.L.T., J.J.-Y.S., S.L.C. and A.S.-L.C. A.S.-L.C. is the guarantor.

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Competing interests

The authors declare no conflicts of interest that pertain to this work except the following declarations. SLC serves an advisory member for AstraZeneca, MSD, Eisai, BMS, Ipsen, and Hengrui, received research funds from MSD, Eisai, Ipsen, SIRTEX, and Zailab, and honoraria from AstraZeneca, Eisai, Roche, Ipsen, and MSD.

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Patient and Public Involvement

Patients or the public were not involved in the design, or conduct, or reporting, or dissemination plans of our research.

Ethics Statement

This study involves human participants and animal subjects, which was approved by the Joint CUHK-NTEC Clinical Research Ethics Committee (CREC 2017.465-T) and The Chinese University of Hong Kong Animal Experimentation Ethics Committee (20-038-GRF), respectively.

References

1 Llovet JM, Kelley RK, Villanueva A, Singal AG, Pikarsky E, Roayaie S, *et al.* Hepatocellular carcinoma. Nat Rev Dis Primers 2021;**7**:6.

2 Donne R, Lujambio A. The liver cancer immune microenvironment: Therapeutic implications for hepatocellular carcinoma. Hepatology 2023;**77**:1773-96.

3 Finn RS, Qin S, Ikeda M, Galle PR, Ducreux M, Kim TY, *et al.* Atezolizumab plus Bevacizumab in Unresectable Hepatocellular Carcinoma. The New England journal of medicine 2020;**382**:1894-905.

4 Sangro B, Sarobe P, Hervas-Stubbs S, Melero I. Advances in immunotherapy for hepatocellular carcinoma. Nat Rev Gastroenterol Hepatol 2021;**18**:525-43.

5 Chen DS, Mellman I. Elements of cancer immunity and the cancer-immune set point. Nature 2017;**541**:321-30.

6 Sharma P, Hu-Lieskovan S, Wargo JA, Ribas A. Primary, Adaptive, and Acquired Resistance to Cancer Immunotherapy. Cell 2017;**168**:707-23.

7 Parker BS, Rautela J, Hertzog PJ. Antitumour actions of interferons: implications for cancer therapy. Nat Rev Cancer 2016;**16**:131-44.

8 Ivashkiv LB. IFNgamma: signalling, epigenetics and roles in immunity, metabolism, disease and cancer immunotherapy. Nat Rev Immunol 2018;**18**:545-58.

9 Kalbasi A, Ribas A. Tumour-intrinsic resistance to immune checkpoint blockade. Nat Rev Immunol 2020;**20**:25-39.

10 Liu H, Golji J, Brodeur LK, Chung FS, Chen JT, deBeaumont RS, *et al.* Tumor-derived IFN triggers chronic pathway agonism and sensitivity to ADAR loss. Nat Med 2019;**25**:95-102.

11 Haber PK, Castet F, Torres-Martin M, Andreu-Oller C, Puigvehi M, Miho M, *et al.* Molecular Markers of Response to Anti-PD1 Therapy in Advanced Hepatocellular Carcinoma. Gastroenterology 2023;**164**:72-88 e18.

12 Terekhanova NV, Karpova A, Liang WW, Strzalkowski A, Chen S, Li Y, *et al.* Epigenetic regulation during cancer transitions across 11 tumour types. Nature 2023;**623**:432-41.

13 Mohammad HP, Barbash O, Creasy CL. Targeting epigenetic modifications in cancer therapy: erasing the roadmap to cancer. Nat Med 2019;**25**:403-18.

14 West AC, Johnstone RW. New and emerging HDAC inhibitors for cancer treatment. J Clin Invest 2014;**124**:30-9.

15 Kelly-Sell MJ, Kim YH, Straus S, Benoit B, Harrison C, Sutherland K, *et al.* The histone deacetylase inhibitor, romidepsin, suppresses cellular immune functions of cutaneous T-cell lymphoma patients. Am J Hematol 2012;**87**:354-60.

16 Truong AS, Zhou M, Krishnan B, Utsumi T, Manocha U, Stewart KG, *et al.* Entinostat induces antitumor immune responses through immune editing of tumor neoantigens. The Journal of clinical investigation 2021;**131**:e138560.

17 Oh SJ, Lee HJ, Song KH, Kim S, Cho E, Lee J, *et al.* Targeting the NANOG/HDAC1 axis reverses resistance to PD-1 blockade by reinvigorating the antitumor immunity cycle. J Clin Invest 2022;**132**:e147908.

18 Que Y, Zhang XL, Liu ZX, Zhao JJ, Pan QZ, Wen XZ, *et al.* Frequent amplification of HDAC genes and efficacy of HDAC inhibitor chidamide and PD-1 blockade combination in soft tissue sarcoma. Journal for immunotherapy of cancer 2021;**9**:e001696.

19 Yang W, Feng Y, Zhou J, Cheung OK, Cao J, Wang J, *et al.* A selective HDAC8 inhibitor potentiates antitumor immunity and efficacy of immune checkpoint blockade in hepatocellular carcinoma. Sci Transl Med 2021;**13**:eaaz6804.

20 Lim B, Lin Y, Navin N. Advancing Cancer Research and Medicine with Single-Cell Genomics. Cancer cell 2020;**37**:456-70.

21 Xiong Z, Chan SL, Zhou J, Vong JSL, Kwong TT, Zeng X, *et al.* Targeting PPAR-gamma counteracts tumour adaptation to immune-checkpoint blockade in hepatocellular carcinoma. Gut 2023; **72**:1758-1773.

22 Sun R, Zhang Z, Bao R, Guo X, Gu Y, Yang W, *et al.* Loss of SIRT5 promotes bile acid-induced immunosuppressive microenvironment and hepatocarcinogenesis. J Hepatol 2022;**77**:453-66.

23 Li Y, Zhang X, Zhu S, Dejene EA, Peng W, Sepulveda A, *et al.* HDAC10 Regulates Cancer Stem-Like Cell Properties in KRAS-Driven Lung Adenocarcinoma. Cancer Res 2020;**80**:3265-78.

24 Li G, Choi JE, Kryczek I, Sun Y, Liao P, Li S, *et al.* Intersection of immune and oncometabolic pathways drives cancer hyperprogression during immunotherapy. Cancer Cell 2023;**41**:304-22 e7.

25 Jiang P, Gu S, Pan D, Fu J, Sahu A, Hu X, *et al.* Signatures of T cell dysfunction and exclusion predict cancer immunotherapy response. Nat Med 2018;**24**:1550-8.

26 Eyre TA, Collins GP, Gupta A, Coupe N, Sheikh S, Whittaker J, *et al.* A phase 1 study to assess the safety, tolerability, and pharmacokinetics of CXD101 in patients with advanced cancer. Cancer 2019;**125**:99-108.

27 Booth SW, Eyre TA, Whittaker J, Campo L, Wang LM, Soilleux E, *et al.* A Phase 2a cohort expansion study to assess the safety, tolerability, and preliminary efficacy of CXD101 in patients with advanced solid-organ cancer expressing HR23B or lymphoma. BMC Cancer 2021;**21**:851.

28 Karin N, Razon H. Chemokines beyond chemo-attraction: CXCL10 and its significant role in cancer and autoimmunity. Cytokine 2018;**109**:24-8.

29 Ma S, Zhang B, LaFave LM, Earl AS, Chiang Z, Hu Y, *et al.* Chromatin Potential Identified by Shared Single-Cell Profiling of RNA and Chromatin. Cell 2020;**183**:1103-16 e20.

30 Han H, Cho JW, Lee S, Yun A, Kim H, Bae D, *et al.* TRRUST v2: an expanded reference database of human and mouse transcriptional regulatory interactions. Nucleic Acids Res 2018;**46**:D380-D6.

31 Creyghton MP, Cheng AW, Welstead GG, Kooistra T, Carey BW, Steine EJ, *et al.* Histone H3K27ac separates active from poised enhancers and predicts developmental state. Proc Natl Acad Sci U S A 2010;**107**:21931-6.

32 Basha G, Omilusik K, Chavez-Steenbock A, Reinicke AT, Lack N, Choi KB, *et al.* A CD74-dependent MHC class I endolysosomal cross-presentation pathway. Nat Immunol 2012;**13**:237-45.

33 Platanias LC. Mechanisms of type-I- and type-II-interferon-mediated signalling. Nature Reviews Immunology 2005;**5**:375-86.

34 Chen X, He WT, Hu L, Li J, Fang Y, Wang X, *et al.* Pyroptosis is driven by non-selective gasdermin-D pore and its morphology is different from MLKL channel-mediated necroptosis. Cell Res 2016;**26**:1007-20.

35 Broz P, Pelegrín P, Shao F. The gasdermins, a protein family executing cell death and inflammation. Nature Reviews Immunology 2020;**20**:143-57.
36 Lu C, Guo C, Chen H, Zhang H, Zhi L, Lv T, *et al.* A novel chimeric PD1-NKG2D-41BB receptor enhances antitumor activity of NK92 cells against human lung cancer H1299 cells by triggering pyroptosis. Mol Immunol 2020;**122**:200-6.
37 Yang HG, Kang MC, Kim TY, Hwang I, Jin HT, Sung YC, *et al.* Discovery of a novel natural killer cell line with distinct immunostimulatory and proliferative potential as an alternative platform for cancer immunotherapy. J Immunother Cancer 2019;**7**:138.
38 Volchuk A, Ye A, Chi L, Steinberg BE, Goldenberg NM. Indirect regulation of HMGB1 release by gasdermin D. Nat Commun 2020;**11**:4561.
39 Yu P, Zhang X, Liu N, Tang L, Peng C, Chen X. Pyroptosis: mechanisms and diseases. Signal Transduct Target Ther 2021;**6**:128.
40 Dagogo-Jack I, Shaw AT. Tumour heterogeneity and resistance to cancer therapies. Nat Rev Clin Oncol 2018;**15**:81-94.
41 Molina-Sanchez P, Ruiz de Galarreta M, Yao MA, Lindblad KE, Bresnahan E, Bitterman E, *et al.* Cooperation Between Distinct Cancer Driver Genes Underlies Intertumor Heterogeneity in Hepatocellular Carcinoma. Gastroenterology 2020;**159**:2203-20 e14.
42 Sia D, Jiao Y, Martinez-Quetglas I, Kuchuk O, Villacorta-Martin C, Castro de Moura M, *et al.* Identification of an Immune-specific Class of Hepatocellular Carcinoma, Based on Molecular Features. Gastroenterology 2017;**153**:812-26.
43 Ruiz de Galarreta M, Bresnahan E, Molina-Sanchez P, Lindblad KE, Maier B, Sia D, *et al.* beta-Catenin Activation Promotes Immune Escape and Resistance to Anti-PD-1 Therapy in Hepatocellular Carcinoma. Cancer Discov 2019;**9**:1124-41.
44 Mellman I, Chen DS, Powles T, Turley SJ. The cancer-immunity cycle: Indication, genotype, and immunotype. Immunity 2023;**56**:2188-205.
45 Benci JL, Johnson LR, Choa R, Xu Y, Qiu J, Zhou Z, *et al.* Opposing Functions of Interferon Coordinate Adaptive and Innate Immune Responses to Cancer Immune Checkpoint Blockade. Cell 2019;**178**:933-48.e14.
46 Litchfield K, Reading JL, Puttick C, Thakkar K, Abbosh C, Bentham R, *et al.* Meta-analysis of tumor- and T cell-intrinsic mechanisms of sensitization to checkpoint inhibition. Cell 2021;**184**:596-614.e14.
47 Qiao Y, Giannopoulou EG, Chan CH, Park SH, Gong S, Chen J, *et al.* Synergistic activation of inflammatory cytokine genes by interferon-gamma-induced chromatin remodeling and toll-like receptor signaling. Immunity 2013;**39**:454-69.
48 Sharma P, Goswami S, Raychaudhuri D, Siddiqui BA, Singh P, Nagarajan A, *et al.* Immune checkpoint therapy-current perspectives and future directions. Cell 2023;**186**:1652-69.
49 Rosenbaum SR, Wilski NA, Aplin AE. Fueling the Fire: Inflammatory Forms of Cell Death and Implications for Cancer Immunotherapy. Cancer Discov 2021;**11**:266-81.
50 Zhang Z, Zhang Y, Lieberman J. Lighting a Fire: Can We Harness Pyroptosis to Ignite Antitumor Immunity? Cancer Immunol Res 2021;**9**:2-7.
51 Zhou Z, He H, Wang K, Shi X, Wang Y, Su Y, *et al.* Granzyme A from cytotoxic lymphocytes cleaves GSDMB to trigger pyroptosis in target cells. Science 2020;**368**.

52 Zhang Z, Zhang Y, Xia S, Kong Q, Li S, Liu X*, et al.* Gasdermin E suppresses tumour growth by activating anti-tumour immunity. Nature 2020;**579**:415-20.
53 Grosselin K, Durand A, Marsolier J, Poitou A, Marangoni E, Nemati F*, et al.* High-throughput single-cell ChIP-seq identifies heterogeneity of chromatin states in breast cancer. Nature genetics 2019;**51**:1060-6.
54 Liu M, Zhou J, Liu X, Feng Y, Yang W, Wu F*, et al.* Targeting monocyte-intrinsic enhancer reprogramming improves immunotherapy efficacy in hepatocellular carcinoma. Gut 2020;**69**:365-79.
55 Liu X, Zhou J, Wu H, Chen S, Zhang L, Tang W*, et al.* Fibrotic immune microenvironment remodeling mediates superior anti-tumor efficacy of a nano-PD-L1 trap in hepatocellular carcinoma. Molecular therapy : the journal of the American Society of Gene Therapy 2023;**31**:119-33.
56 Liu G, Barczak W, Lee LN, Shrestha A, Provine NM, Albayrak G*, et al.* The HDAC inhibitor zabadinostat is a systemic regulator of adaptive immunity. Commun Biol 2023;**6**:102.

Figure legends

Figure 1. **Tumor cell-intrinsic HDACs correlate with ICB therapy efficacy and survival outcome in patients with HCC**. **A**, Overview of this study. **B**, Dot plot depicting the relationship between the baseline expression levels of tumor cell-intrinsic *HDAC* isoforms and the survival outcomes of HCC patients treated with pembrolizumab. Each plot represents a *P* value obtained from the Kaplan-Meier survival analysis of the patients according to their baseline tumoral cell *HDAC* expression levels. **C**, Kaplan-Meier survival analyses of patients with HCC undergone pembrolizumab treatment according to their baseline tumoral cell *HDAC1*, *HDAC2* and *HDAC3* expression levels. **D**, Expression levels of *HDAC1*, *HDAC2* and *HDAC3* in tumor cells of the patients according to pembrolizumab treatment outcomes. R, responders; NR, non-responders. **E**. GSEA of the genes between the $HDAC1/2/3^{high}$ and $HDAC1/2/3^{low}$ tumor cells. Tumor cells were stratified by top (high) and bottom (low) 25% based on the expression levels of *HDAC1*, *HDAC2* or *HDAC3*. **F** and **G**, TCGA HCC samples with high (n=92) and low (n=92) mRNA levels of *HDAC1*, *HDAC2* or *HDAC3* stratified by top and bottom 25 % in 369 patients were selected for subsequent analysis. (F) Prediction of potential clinical ICB response in patients between the $HDAC1/2/3^{high}$ and $HDAC1/2/3^{low}$ tumor cells using the TIDE signature. R, responders; NR, non-responders. (G) Analysis of TIDE, T cell exclusion and MDSC scores by TIDE algorithm in patients between the $HDAC1/2/3^{high}$ and $HDAC1/2/3^{low}$ tumor cells. Statistical significance was assessed by two-sided log-rank (Mantel-Cox) test for (B and C), Wilcoxon rank sum test for (D), Kolmogorov–Smirnov test for (E), two-sided χ^2 test for (F) or unpaired two-tailed Student's t-test for (G). ***$P < 0.001$; ****$P < 0.0001$.

Figure 2. A selective class-I HDAC inhibitor overcomes ICB resistance in HCC models. **A**, Western blot analysis of HDAC1, HDAC2 and HDAC3 levels in orthotopic Hepa1-6-PD-L1R (left) and RIL-175-PD-1R (right) tumor and non-tumor liver tissues isolated from mice. GAPDH was used as loading control. **B**, Treatment schedule of CXD101 and anti-PD-(L)1 antibody in C57BL/6 mice bearing PD-(L)1R tumors. **C** and **D**, Tumor weights and representative images of livers and tumors from indicated groups in Hepa1-6-PD-L1R (C) and RIL-175-PD-1R (D) models (n = 8 to 10). **E**, Representative CD45 immunohistochemistry images and statistical analysis of positively stained cells in Hepa1-6-PD-L1R tumors from indicated groups (n = 4). Scale bars, 100 μm. Black boxes indicate regions shown in enlarged inset. **F**, Proportions (%) of tumor infiltrating $CD45^{+}$cells, $CD45^{+}CD3^{+}CD8^{+}$T cells, $CD45^{+}CD3^{+}CD4^{+}$T cells, $CD45^{+}NK1.1^{+}$NK cells, $CD45^{+}CD3^{+}NK1.1^{+}$NKT cells, $CD45^{+}CD11b^{+}Gr1^{+}$MDSCs, $CD45^{+}CD11b^{+}Gr1^{-}CD11c^{+}$dendritic cells, $CD45^{+}CD11b^{+}Gr1^{-}F4/80^{+}Ly6C^{-}$macrophages of total cells in Hepa1-6-PD-L1R tumors from indicated groups were determined by flow cytometry (n = 7 to 9). **G**, Correlation analysis between tumor weights and the proportions of indicated cells in Hepa1-6-PD-L1R tumors. **H**, Representative flow cytometry dot plots and proportions of $IFN\gamma^{+}$ or $GZMB^{+}$ cells in tumor infiltrating $CD8^{+}$T cells from indicated groups in Hepa1-6-PD-L1R model (n = 7 to 9). **I** and **J**, Kaplan-Meier survival analysis of mice from indicated groups in RIL-175-PD-1R (I) and Hepa1-6-PD-L1R (J) ICB-resistant models (n = 12 to 15). **K**, Combination therapy cured mice (from J) and naïve mice with same age were challenged with Hepa1-6-PD-L1R cells at 140-day after initial tumor cell inoculation (n = 7). Kaplan-Meier survival analysis of mice from indicated groups are shown. **L**, Representative flow cytometry dot plots and proportions of $CD8^{+}CD44^{+}CCR7^{+}CD62L^{+}$central memory $CD8^{+}$T cells ($CD8^{+}T_{CM}$) and

$CD8^+CD44^+CCR7^-CD62L^-$effector memory $CD8^+$T cells ($CD8^+T_{EM}$) in circulating $CD8^+$T cells, and the corresponding $CD4^+T_{CM}$ and $CD4^+T_{EM}$ in circulating $CD4^+$T cells from combination therapy cured mice and naive mice (n = 7). Data represent as mean ± SD. Statistical significance was assessed by one-way ANOVA with Tukey's multiple comparisons correction for (C-F, H), single-tailed Pearson's correlation for (G), two-sided log-rank (Mantel-Cox) test for (I-K) or unpaired two-tailed Student's t-test for (L). ns, no significance; $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

Figure 3. Single-cell multiomics reveals reactivation of IFNγ/STAT1 signaling by CXD101-ICB combination therapy. **A**, Treatment schedule of CXD101 and anti-PD-L1 antibody in mice bearing Hepa1-6-PD-L1R tumors for single-cell multiomics analysis. Fresh tumor tissues from three representative mice per group were employed to avoid the interindividual variability. **B**, t-SNE plot showing identified cell clusters within tumor from all merged groups. Cell annotations were derived from RNA analysis according to representative lineage markers. **C**, Dot plot showing the RNA expression levels of representative marker genes in annotated cell clusters. **D**, Visualization of the pseudo bulk chromatin accessibility tracks of representative marker gene loci in annotated cell clusters. **E**, Distribution fraction of distinct cells in the indicated groups. **F** and **G**, t-SNE plot showing the identified sub-clusters of tumor cells from RNA (F) and ATAC (G). **H** and **I**, t-SNE plots (H) and proportions (I) of the identified tumor sub-clusters in indicated groups from RNA analysis. **J** and **K**, t-SNE plots (J) and proportions (K) of identified tumor sub-clusters in indicated groups from ATAC analysis. **L** and **M**, Pathway enrichment of differentially expressed genes for RNA-based cluster 6 (C6 (RNA)) and ATAC-based cluster 6 (C6 (ATAC)), respectively. **N**, Overlapping genes between C6 (RNA) and C6 (ATAC). **O**, Pathway enrichment of overlapping genes in (N). **P**, TRRUST analysis showing the key transcription factors for regulating the overlapping genes. **Q**, Violin plots showing the RNA expression levels of *Stat1* and *Irf1* across the six tumor cell sub-clusters based on scRNA-seq analysis. **R**, GSEA of the genes in combination group versus control group. **S** and **T**, Heatmap for the RNA expression (S) and chromatin accessibility (T) levels of the top 30 IRGs (upregulated in combination therapy-treated group) in tumor cells from indicated groups. **U**, Transcription factor-binding motif enrichment analysis showing the key transcription factors in tumor cells of the indicated groups versus control group.

Figure 4. CXD101-induced histone hyperacetylation primes IRG activation in response to IFNγ. **A** and **B**, Heatmaps depicting the H3K27ac intensity in genome-wide scale (A) and in IRGs (B) in tumor tissues from the indicated groups as shown in figure 3A. **C** and **D**, Overlapping tracks of ATAC-seq and nanoscale ChIP-seq (H3K27ac, H3K4me1 and H3K4me3) at the *Cd74* (C) and *Stat1* (D) loci. ATAC-seq data were extracted from the *in vivo* scATAC-seq of tumor cells. **E**, Western blot analysis of STAT1 and p-STAT1 levels in Hepa1-6-PD-L1R cells treated with the indicated concentrations of CXD101 or vehicle control in the presence or absence of IFNγ (10 ng/ml) for 48 h. GAPDH was used as loading control. **F**, RT-qPCR analyses of mRNA levels of the indicated genes in Hepa1-6-PD-L1R cells treated with CXD101 (2 μM) or vehicle control in the presence or absence of IFNγ (10 ng/ml) for 48 h. *Gapdh* was used as normalization control. **G**, Western blot analysis of STAT1 levels in Hepa1-6 derived PD-L1R-WT and STAT1-KO cells treated with IFNγ (10 ng/ml) for 48 h. GAPDH was used as loading control. **H**, ChIP-qPCR analyses of H3K27ac occupancy in the enhancer and

promoter regions of *Stat1* in Hepa1-6 derived PD-L1R-WT or STAT1-KO cells treated with CXD101 (2 μM) or vehicle control in the presence or absence of IFNγ (10 ng/ml) for 48 h. *Stat1* enhancer (site 1) and promoter (site 2) loci for ChIP-qPCR analysis as shown in figure 4D. **I**, RT-qPCR analyses of mRNA levels of the indicated genes in Hepa1-6 derived PD-L1R-WT or STAT1-KO cells treated with CXD101 (2 μM) or vehicle control in the presence or absence of IFNγ (10 ng/ml) for 48 h. *Gapdh* was used as normalization control. Data represent as mean ± SEM. Statistical significance was assessed by one-way ANOVA with Tukey's multiple comparisons correction. **P* < 0.05; ***P* < 0.01; ****P* < 0.001.

Figure 5. CXD101 and IFNγ/STAT1 signaling coordinate $CD8^{+}$T cell-induced pyroptosis. A, Schematic illustration of $CD8^{+}$T cell/tumor cell co-culture cytotoxicity system. **B-D**, Hepa1-6-PD-L1R cells were pre-treated with CXD101 (2 μM) or vehicle control in the presence or absence of IFNγ (10 ng/ml) for 42 h. $CD8^{+}$T cells at an effector/target ratio (E/T) of 10:1 were co-cultured with drug washed out cells for 6 h. Cell viabilities (B), morphological changes (C) and LDH release (D) of tumor cells were measured by the corresponding assays. (C) Red arrowheads indicate pyroptotic cells, while blue arrowheads indicate representative $CD8^{+}$T cells binding on pyroptotic tumor cells. Scale bar = 20 μm. **E**-**G**, Hepa1-6 derived PD-L1R-WT or STAT1-KO cells were pre-treated with CXD101 (2 μM) or vehicle control in the presence or absence of IFNγ (10 ng/ml) for 42 h, $CD8^{+}$T cells at an E/T of 10:1 were co-cultured with drug washed out cells for 6 h. Cell viabilities (E), morphological changes (F) and LDH release (G) of tumor cells were measured by the corresponding assays. (F) Red arrowheads indicate pyroptotic cells, scale bar = 20 μm. **H**, Hepa1-6-PD-L1R cells were pre-treated with CXD101 (2 μM) or vehicle control in the presence or absence of IFNγ (10 ng/ml) for 42 h. $CD8^{+}$T cells at an E/T of 10:1 were co-cultured with drug washed out cells for 6 h, followed by Western blot analysis of the indicated proteins. **I**, Hepa1-6-PD-L1R cells were treated with DMSO or CXD101 at the indicated concentrations for 48 h, the *Gsdme* mRNA and GSDME protein levels were measured by RT-qPCR and Western blot assays, respectively. **J**, ChIP-qPCR analysis of H3K27ac occupancy in the enhancer and promoter regions of *Gsdme* in Hepa1-6-PD-L1R cells treated with CXD101 (2 μM) or vehicle control for 48 h. *Gsdme* enhancer and promoter loci for ChIP-qPCR analysis are shown in **supplementary figure 18**. **K**, Hepa1-6 derived PD-L1R-WT or STAT1-KO cells were pre-treated with CXD101 (2 μM) or vehicle control in the presence or absence of IFNγ (10 ng/ml) for 42 h. $CD8^{+}$T cells at an E/T of 10:1 were co-cultured with drug washed out cells for 6 h, followed by Western blot analysis of the indicated proteins. Data represent as mean ± SEM. Statistical significance was assessed by one-way ANOVA with Tukey's multiple comparisons correction. ****P* < 0.001.

Figure 6. Epigenetic upregulation of GSDME renders CTL-induced tumor cell pyroptosis. **A**, Hepa1-6-PD-L1R cells were pretreated with DMSO or CXD101 at 2 μM for 42 h. NK92 cells at an E/T of 2:1 were co-cultured with drug washed out cells for 5 h. The representative images are shown. Red arrowheads indicate pyroptotic cells. Scale bar = 20 μm. **B**, Hepa1-6-PD-L1R cells were pretreated with DMSO or CXD101 at 2 μM for 42 h. NK92 cells at indicated E/T were co-cultured with drug washed out cells for 5 h. The LDH release levels of tumor cells are shown. **C**-**E**, Hepa1-6 derived PD-L1R-empty vector (EV) and GSDME-overexpressed (OE) cells were co-cultured with NK92 cells at an E/T of 2:1 for 5 h. The indicated protein levels (C), morphological changes (D) and LDH release (E) of tumor cells were measured by the corresponding assays. (D) Red arrowheads

indicate pyroptotic cells. Scale bar = 20 μm. **F**, Hepa1-6-PD-L1R cells were pretreated with DMSO or CXD101 at 2 μM for 42 h. NK92 cells at an E/T of 2:1 were co-cultured with drug washed out cells for the indicated times, followed by Western blot analysis of the indicated proteins. **G**, Hepa1-6 derived PD-L1R-WT or GSDME-KO cells were pretreated with DMSO or CXD101 at 2 μM for 42 h. NK92 cells at an E/T of 2:1 were co-cultured with drug washed out cells for 5 h. The indicated protein levels (G), LDH release (H) and morphological changes (I) of tumor cells were measured by the corresponding assays. #, nonspecific bands. (I) Red arrowheads indicate pyroptotic cells. Scale bar = 20 μm. **J** and **K**, The total and cleaved GSDME levels in Hepa1-6-PD-L1R (J) and RIL-175-PD-1R (K) tumors from indicated groups were measured by Western blot assays. GAPDH was used as loading control. **L** and **M**, The proportions of $CD45^{-}7AAD^{+}Annexin\text{-}V^{+}$cells in Hepa1-6-PD-L1R (L) and RIL-175-PD-1R (M) tumors from the indicated groups were detected by flow cytometry assay (n = 5 - 8). **N** and **O**, HMGB1 levels in Hepa1-6-PD-L1R (N) and RIL-175-PD-1R (O) tumors from the indicated groups are shown (n = 6 to 9). Data represent as mean ± SEM (B, E and H) or SD (L-O). Statistical significance was assessed by one-way ANOVA with Tukey's multiple comparisons correction. ***P* < 0.01; ****P* < 0.001.

Figure 7. Deletion of tumoral STAT1 or GSDME abolishes the antitumor effects of CXD101 and anti-PD-L1 combination therapy. **A**, Treatment schedule of CXD101 and anti-PD-L1 antibody in C57BL/6 mice bearing the indicated Hepa1-6-PD-L1R tumors. **B**, Tumor weights and representative images of livers and tumors from indicated groups are shown (n = 9 to 12). **C**, RT-qPCR analyses of mRNA levels of *Stat1* and *Gsdme* in tumors from the indicated groups (n = 7 to 8). **D**, The proportions of $CD45^{-}7AAD^{+}Annexin\text{-}V^{+}$cells in tumors from the indicated groups were detected by flow cytometry assay (n = 8 to 9). **E**, IFNγ levels in tumors from the indicated groups were measured by ELISA assay (n = 6 to 7). **F**, RT-qPCR analyses of mRNA levels of the indicated genes in tumors from indicated groups (n = 7 to 8). **G**, Proportions (%) of tumor infiltrating $CD45^{+}$cells, $CD45^{+}CD3^{+}CD8^{+}$T cells, $CD45^{+}CD3^{+}CD4^{+}$T cells, $CD45^{+}NK1.1^{+}$NK cells and $CD45^{+}CD3^{+}NK1.1^{+}$NKT cells of total cells in the indicated tumors were determined by flow cytometry (n = 8 to 10). **H**, Representative flow cytometry dot plots and proportions of $IFN\gamma^{+}$ or $GZMB^{+}$ cells in tumor infiltrating $CD8^{+}$T cells from the indicated groups are shown (n = 8 to 10). **I**, Kaplan-Meier survival analysis of mice from the indicated groups (n = 10 to 12). Data represent as mean ± SD. Statistical significance was assessed by one-way ANOVA with Holm-Sidak's multiple comparisons correction. **P* < 0.05; ***P* < 0.01; ****P* < 0.001.

Figure 8. CXD101 synergies with anti-PD-L1 antibody to suppress tumorigenicity in spontaneous HDAC1/2/3^{high} HCC model. **A**, mRNA levels of *Hdac1*, *Hdac2* and *Hdac3* in normal livers or the indicated HCC tumors were extracted from the published RNA-seq data (GSE148379). **B**, Western blot analysis of HDAC1, HDAC2 and HDAC3 protein levels in MYC^{high} and CTNNB1^{mut} tumor and non-tumor liver tissues isolated from mice. GAPDH was used as loading control. **C**, Treatment schedule of CXD101 and anti-PD-L1 antibody in C57BL/6 mice bearing MYC^{high} and CTNNB1^{mut} tumors by HDTVi of the indicated plasmids. **D**, Representative photos (top) and H&E staining images (bottom) of livers and tumors from the indicated groups are shown. Scale bars, 1000 μm. Tumor areas are circled by black dotted lines. **E**, Tumor burden in indicated groups was evaluated

by liver weight, liver weight versus body weight ratio and tumor area per slide calculated from H&E images. **F**, Kaplan-Meier survival analysis of mice from the indicated groups in MYC^{high} and $CTNNB1^{mut}$ HCC model (n = 13 to 14). **G**, A summary schematic of this study. Data represent as mean ± SD. Statistical significance was assessed by one-way ANOVA with Tukey's multiple comparisons correction for (E) and two-sided log-rank (Mantel-Cox) test for (F). $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.