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Original Research

Obatoclax, the pan-Bcl-2 inhibitor sensitizes hepatocellular carcinoma cells to promote the anti-tumor efficacy in combination with immune checkpoint blockade

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ABSTRACT

Bcl-2 family proteins play critical roles in regulating lymphocyte development and maintain homeostasis, and have also been proved to be involved in various cancer types development. However, the role of Bcl-2 in hepatocellular carcinoma (HCC) development has not been clearly studied. Here, we reported the pan-Bcl-2 inhibitor, obatoclax could directly inhibit HCC growth *in vitro*. We further demonstrated in murine HCC model that obatoclax also suppressed HCC development *in vivo*. We also proved that although obatoclax inhibited T cells expansion, it had no influence on T cells activation *in vivo*. Mechanism study revealed that obatoclax sensitized HCC cells to T cell-mediated killing. Combination therapy of obatoclax with anti-PD-1 antibody synergistically suppressed HCC development and prolonged the survival rate of tumor-bearing mice. The combination therapy promoted T cells activation and effector cytokines expression both in spleen and tumor. In summary, our results proved that obatoclax sensitized HCC cells to T cell -mediated killing. Combination of obatoclax with immune checkpoint blockade served as a promising therapeutic strategy for HCC treatment.

Introduction

Hepatocellular carcinoma (HCC) is one of the deadliest diseases, ranking the fifth most common liver malignancy, and the third leading cause of cancer-related death [1]. Multiple risk factors are known to induce the carcinogenesis of HCC, including hepatitis B virus (HBV) infection, alcohol abuse, diabetes, aflatoxin infection, etc. [2,3]. Currently, surgical resection is the most widely-used first-line treatment method for HCC with 5-year survival rate of between 60 and 70% [4,5]. However, most HCC patients are diagnosed at late stage when surgical resection is no longer suitable for treatment. Thus, it's urgent to find new therapeutic method for HCC patients.

B cell leukemia-2 (Bcl-2) family proteins consist of more than 20 different molecules. Based on the structures and functions, the Bcl-2 family proteins can be classified into three main subdivisions: 1) the pro-apoptotic initiators, including Bim (BCL2L11), Puma/BBC3, Bad (Bcl-2/Bcl-x-associated death promoter), Bid (BH-3 interacting-domain death agonist), Bik (Bcl-2-interacting killer), etc.; 2) the pro-apoptotic effectors, including Bax, Bak, and Bok; 3) the anti-apoptotic proteins, including Mcl-1 Bcl-2, Bcl-x, Bcl-w, etc. [6]. The balance between the anti-apoptotic and pro-apoptotic Bcl-2 family proteins is tightly regulated and determined the survival and death of the cells [7].

Bcl-2 family was first discovered in the 1980s, since then, its role in lymphoma has been extensively studied [8–10]. Recently, more and more evidence suggested Bcl-2 also involved in various solid tumor initiation and progression. In triple negative breast cancer, Bcl-2 served as an independent prognostic marker [11]; Bcl-2 upregulation promoted the prostate cancer development by switching the cancer cell from androgen-dependent to an androgen-independent growth stage [12]; in pancreatic ductal adenocarcinoma, Bcl-2 downregulation acted as an independently poor prognostic factor [13]. However, the role of Bcl-2 in HCC development was poorly studied. Zhang et al. found that in andropause-age patients, Bcl-2 expression served as a poor predictor for HCC prognosis [14]. Zhou et al. proved that Bcl-2 played a critical role in glycochenodeoxycholate-induced survival and chemoresistance in HCC [15]. These studies indicated Bcl-2 acted as pro-tumor factors in HCC progression and targeting Bcl-2 might be a potential therapeutic method against HCC.

Obatoclax, a pro-apoptotic BH3 mimic, is known as a pan Bcl-2 inhibitor which antagonizes Bcl-2, Bcl-xL, Bcl-w and Mcl-1 to induce cell apoptosis in cancer cells in preliminary studies [16–19]. Besides, several clinical trials utilizing obatoclax as a single treatment agent have been

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completed, including acute myeloid leukemia, Hodgkin's lymphoma, etc. However, the overall beneficial effects were not satisfying [20]. Interestingly, evidence from clinical trials implicated that obatoclax achieved better anti-tumor efficiency when used in combination with other therapeutic agents, such as bortezomib, fludarabine and rituximab [21,22].

Immune checkpoint therapy targeting the programmed cell death 1 (PD-1) and its ligand PD-L1 has achieved promising therapeutic outcome in various cancer types, including metastatic melanoma, lymphoma, lung cancers, renal cell cancer (RCC), head and neck squamous cell cancer (HNSCC), etc. [23,24]. Although anti–PD-1/PD-L1 therapies are promising, only a small portion of cancer patients respond, especially in HCC patients [25]. Thus, combination therapy of anti-PD-1/PD-L1 antibodies with other therapeutic agents has been extensively studied in the past several years to improve the overall response rate and overall survival rate [26]. Serval clinical trials have been reported to achieve promising outcome: a Phase III study comparing atezolizumab (a PD-L1 inhibitor) and bevacizumab (a VEGF inhibitor) to sorafenib in unresectable HCC, demonstrated that combination PD-L1/VEGF blockade significantly increased overall survival, progression-free survival and response rate [27]; combination therapy of tremelimumab (a CTLA-4 inhibitor) with durvalumab (a PD-1 inhibitor) for patients with advanced HCC has obtained enhanced survival rate compared with single tremelimumab or durvalumab treatment [28].

In this current study, we first examined the effects of obatoclax on HCC tumor cell viability *in vitro*. We found that obatoclax directly inhibited HCC cell expansion. *In vivo* study showed that obatoclax suppressed HCC tumor growth. Combination therapy of obatoclax with anti-PD-1 antibody showed synergistic effects on tumor suppression. Mechanism study revealed that obatoclax sensitized tumor cells to respond to anti-PD-1 therapy. Collectively, our study showed that obatoclax was a promising therapeutic agent against HCC progression.

Material and Methods

Experimental animals

6–8 weeks old specific pathogen-free (SPF) female C57BL/6 mice were purchased from the Experimental Animal Center of Shandong University (Jinan, China). Animal handling and experimental procedures were conducted strictly in accordance with the Provision and General Recommendation of Chinese Experimental Animal Administration Legislation and approved by the Science and Technology Department of Shandong Province.

Cell culture

Murine HCC cell line Hepa1–6 and Hepa1c1c7 were obtained from American Type Culture Collection (ATCC, Manassas, VA). Human HCC cell line HepG2 was obtained from ATCC. The cells were cultured in complete DMEM medium (Hyclone, Piscataway, NJ), containing 10% FBS (GIBCO, Waltham, MA). Cells were incubated at 37 ˚C with 5% CO_2.

Reagents

Obatoclax mesylate (OBAT) was purchased from Selleck Chemicals (Munich, Germany). The OBAT was dissolved in DMSO. Anti-PD-1 antibody (clone RMP1–14) and rat IgG2a isotype control (clone 2A3) were purchased from Bioxcell (Bioxcell, Lebanon, NH).

Cell counting kit 8 (CCK8) assay

Hepa1–6 cells, Hepa1c1c7 or HepG2 cells were seeded into 96 well plate at 5000 cells/well in the presence of different concentrations obatoclax and incubate for 24 h. 10 μl of CCK8 solution was added into each well and incubate for another 4 h. The proliferation rate was measured by the absorbance at 450 nm using a microplate reader (Biorad, Hercules, CA).

Apoptosis assay

1×10^6 Hepa1–6 cells, Hepa1c1c7 cells, HepG2 cells or 1×10^5 T cells were seeded into 24 well plate in the presence of obatoclax. After 24 h, cells were harvested for apoptosis assay by using Apoptosis Detection Kit (Biolegend, San Diego, CA). Briefly, cells were resuspended in AnnexinV binding buffer. Then APC-conjugated Annexin V were added into the cells and incubated for 15 min at room temperature in the dark. Then DAPI (4′,6-Diamidino-2-Phenylindole, Dilactate) was then added. The percentage of early apoptotic ($AnnexinV^+$ $DAPI^-$) and late apoptotic cells ($AnnexinV^+$ $DAPI^+$) were detected by BD FACSCanto II (BD Biosciences, San Jose, CA). The FACS data were further analyzed by Flowjo software (FlowJo LLC, Ashland, Oregon).

Ki67 staining

1×10^6 Hepa1–6 cells were seeded into 24 well plate in the presence of obatoclax. After 24 h, cells were harvested, washed and fixed in ice-cold 70% ethanol. After 2 h incubation in −20 ˚C, cells were stained with APC-conjugated Ki67 antibody (clone 16A8, Biolegend, San Diego, CA) for 20 min at room temperature in dark. The cells were further analyzed by BD FACSCanto II. The FACS data were further analyzed by Flowjo software.

Cell sorting

Antibodies were purchased from BioLegend. T cells were isolated from mouse spleen and stained with anti-CD3 antibody (Clone 17A2), anti-CD69 antibody (Clone H1.2F3), anti-CD44 antibody (Clone IM7) and anti-CD62L (Clone MEL-14) antibody. The $CD3^+$T cells, $CD3^+$ $CD69^+$effector T cells and $CD3^+$ $CD44^-$ $CD62L^+$ naïve T cells were sorted using BD FACSAria^{TM} III cell sorter.

Animal model

To establish murine HCC model, mice were subcutaneously injected with 1.5×10^6 Hepa1–6 cells or 2×10^6 Hepa1c1c7 cells. When the diameter of tumor reached 2–3 mm. Mice were randomly divided into two groups: 1) mice receiving control DMSO as control; 2) mice receiving obatoclax (5 mg/kg) injection three times per week. The tumor size was measured and calculated by the following formula: Volume = (length x width^2)/2. For combination therapy, mice were further injected with 10 mg/kg anti-PD-1 antibody together with obatoclax, the control mice were injected with IgG control.

Flow cytometry analysis

Antibodies were purchased from BioLegend. The general cell staining procedure has been described in previous study [29]. Splenocytes and tumor infiltrating lymphocytes (TILs) were isolated from tumor-bearing mice 2 weeks after treatment. 1×10^5 solenocytes and TILs were stained with fluorescence-conjugated antibodies for 30 min at 4 ˚C in dark. The cells were washed for three times with staining buffer and assessed by BD FACSCanto II cytometry. The antibodies used were anti-mouse CD3 (Clone 17A2), anti-mouse CD4 (Clone GK1.5), anti-mouse CD8 (Clone 53–6.7), anti-mouse TCR$\gamma\delta$ (Clone GL3), anti-mouse CD11B (Clone M1/70), anti-mouse CD11C (Clone N418), anti-mouse NK1.1 (Clone PK136), anti-mouse CD19 anti-mouse (Clone ID3/CD19), TNF-α (Clone MP6-XT22), anti-mouse IFN-γ (Clone XMG1.2). The FACS data were further analyzed by Flowjo software.

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Cytotoxicity Assay

Two weeks after drug treatment, splenocytes were harvested from spleen of the tumor-bearing mice. T cells were then sorted from the splenocytes and used as effector cells. Hepa1–6 cells were pre-treated with obatoclax or control for 8 h. After that, cells were collected and washed three time with PBS to completely remove obatoclax residues. The CytoTox 96 non-radioactive cytotoxicity assay kit (Promega, Madison, WI) was used to measure the cytotoxic activity of T cells against tumor cells.

Western Blot

Hepa1–6 cells were treated with different doses of obatoclax for 8 h. Cells were lysed with NP40 lysis buffer. The protein was resuspended in SDS running buffer, mixed with loading dye (Bio-Rad, Hercules, CA) and heated at 95°C for 5 min. The samples were then loaded into 12% SDS-PAGE gel. Power blotter (Thermo Fisher Scientific, Waltham, MA) was used to transfer the protein bands onto PVDF membrane (Bio-Rad, Hercules, CA). The membrane was blocked for 1 h in 5% BSA. Anti-cleaved-caspase-3 antibody (CST, Danvers, MA), anti-cyclin D1 antibody (CST, Danvers, MA), anti-actin antibody (CST, Danvers, MA) were used as primary antibodies. Goat anti-rabbit IgG-HRP (Invitrogen, Carlsbad, CA) was used as secondary antibody. SuperSignal^{TM} West Pico Chemiluminescent substrate (Thermo Scientific, Waltham, MA) was used and Protein bands were detected with ChemiDoc MP imaging system (Bio-Rad, Hercules, CA).

Immunohistochemistry

Two weeks after tumor implantation, tumor tissues were removed aseptically and immediately fixed in 4% formalin at room temperature for 2 d The fixed tissues were processed through graded concentrations of ethanol and xylene and were then embedded in paraffin wax. Tumor sections were then deparaffinized and treated with 0.08% H_2O_2 for 30 min to block endogenous peroxidase. Slides were incubated with rabbit anti-mouse IL-1R8 antibody (ab228977; Abcam) at 4 C overnight, followed by incubation with HRP-conjugated goat anti-rabbit IGG (AB_2,307,391; Jackson ImmunoResearch, West Grove, PA). Diaminobenzidine was used to develop the staining reaction. Slides were coded and examined by a pathologist who was blinded for the experimental history of the animals.

Two weeks after tumor injection, tumor tissues were removed fixed in 4% formalin followed by graded concentrations of ethanol and xylene. Tumors were then embedded in paraffin wax. Tumor sections were treated with 0.08% H2O2 for 30 min to block endogenous peroxidase. Rabbit anti-mouse cleaved-caspase-3 antibody (CST, Danvers, MA) was used as secondary antibody, followed by incubation with HRP-conjugated goat antirabbit IgG (Invitrogen, Carlsbad, CA). Diaminobenzidine was used to develop the staining reaction.

Statistical analysis

All data were analyzed with student t-test or one-way ANOVA and were expressed as means ± SD; data were analyzed using GraphPad Prism 7 software for Windows (GraphPad, company, San Diego, CA), and differences were considered statistically significant when $p < 0.05$. The significance levels are marked *, $p < 0.05$; **, $p < 0.01$; *** $p < 0.001$.

Results

Obatoclax inhibited Hepa1–6 cell proliferation and promoted apoptosis *in vitro* To dissect the effects of obatoclax on tumor cell viability. We firstly treated mouse HCC cell line Hepa1–6 with different concentrations of obatoclax. CCK-8 results showed that obatoclax inhibited Hepa1–6 cell proliferation in a dose dependent manner (Fig. 1A). This effect was further confirmed by KI67 staining, cells treated with higher concentration of obatoclax showed lesser KI67 staining compared with the untreated cells (Fig. 1B). Previously report suggested that obatoclax downregulated cyclin D1 expression to suppress human colorectal carcinoma cells expansion. To investigate whether obatoclax had similar effects on HCC cells, the expression level of cyclin D1 in Hepa1–6 cells were detected after obatoclax treatment. The results showed that obatoclax suppressed cyclin D1 expression in Hepa1–6 cells in a dose-dependent manner, suggesting obatoclax suppressed Hepa1–6 cells by arresting the cell cycle (Fig. S1A). Apoptosis assay demonstrated obatoclax induced Hepa1–6 cell apoptosis. The early apoptosis rate increased from 4.59 ± 1.02% to 8.41± 0.42%, the late apoptosis rate increased from 1.84 ± 0.19% to 8.19 ± 1.07% upon 200 nM obatoclax treatment (Fig. 1C). To further confirm that obatoclax induced caspase-3 dependent apoptosis in Hepa1–6 cells. Western blot was performed to detect the cleavage of caspase-3 in Hepa1–6 cells. The results showed that obatoclax induced higher level of caspase-3 cleavage, proving that Hepa1–6 cells underwent caspase-3 dependent apoptosis (Fig. S1B). To overcome the bias of one cell line, another murine HCC cell line Hepa1c1c7 was used to validate the results obtained from Hepa1–6 cells. As expected, obatoclax treatment also suppressed Hepa1c1c7 expansion (Fig. S1C) and induced higher level of cell apoptosis (Fig. S1D). Furthermore, HepG2 cell line was also used to study the effects of obatoclax on human HCC cells. The results also proved that obatoclax treatment significantly suppressed HepG2 cells expansion (Fig. 1D). The overall apoptosis rate was also increased after obatoclax treatment (Fig. 1E & Fig. S1E).

Taken together, these *in vitro* results proved obatoclax impaired both human and murine HCC cell viability.

Obatoclax differentially affected t cell subsets in vitro

Next, we investigated the potential influence of obatoclax on murine T cell subsets. $CD3^+$ T cells were isolated from WT mouse and treated with 200 nM obatoclax. The results showed that the total number of T cells were slightly decreased after obatoclax treatment (Fig. 2A). Previous studies reported other Bcl-2 inhibitors differently affected T cell subsets [30,31]. To examine whether obatoclax had similar effects on different T cell subsets. Naïve T cells and effector T cells were isolated and treated with obatoclax, respectively. We found that obatoclax inhibited both naïve T cells and effector T cells expansion (Fig. 2B). Importantly, 200 nM obatoclax induced a 24% reduction of viable naïve T cells, while only induced a 13% decrease of viable effector T cells. This result suggested naïve T cells are more sensitive to obatoclax treatment. Apoptosis assay also showed that obatoclax induced lesser cell death in effector T cells compared with native T cells (Fig. 2C&D). Upon 200 nM obatoclax treatment, the total apoptosis rate increased from 6.91 ± 1.55% to 29.9 ± 2.69% in naïve T cells (Fig. 2C), while the total apoptosis rate increased from 8.95 ± 0.60% to 12.01 ± 0.56% in effector T cells (Fig. 2D). Taken together, these data indicated effector T cells are more resistant to obatoclax cytotoxicity than naïve T cells.

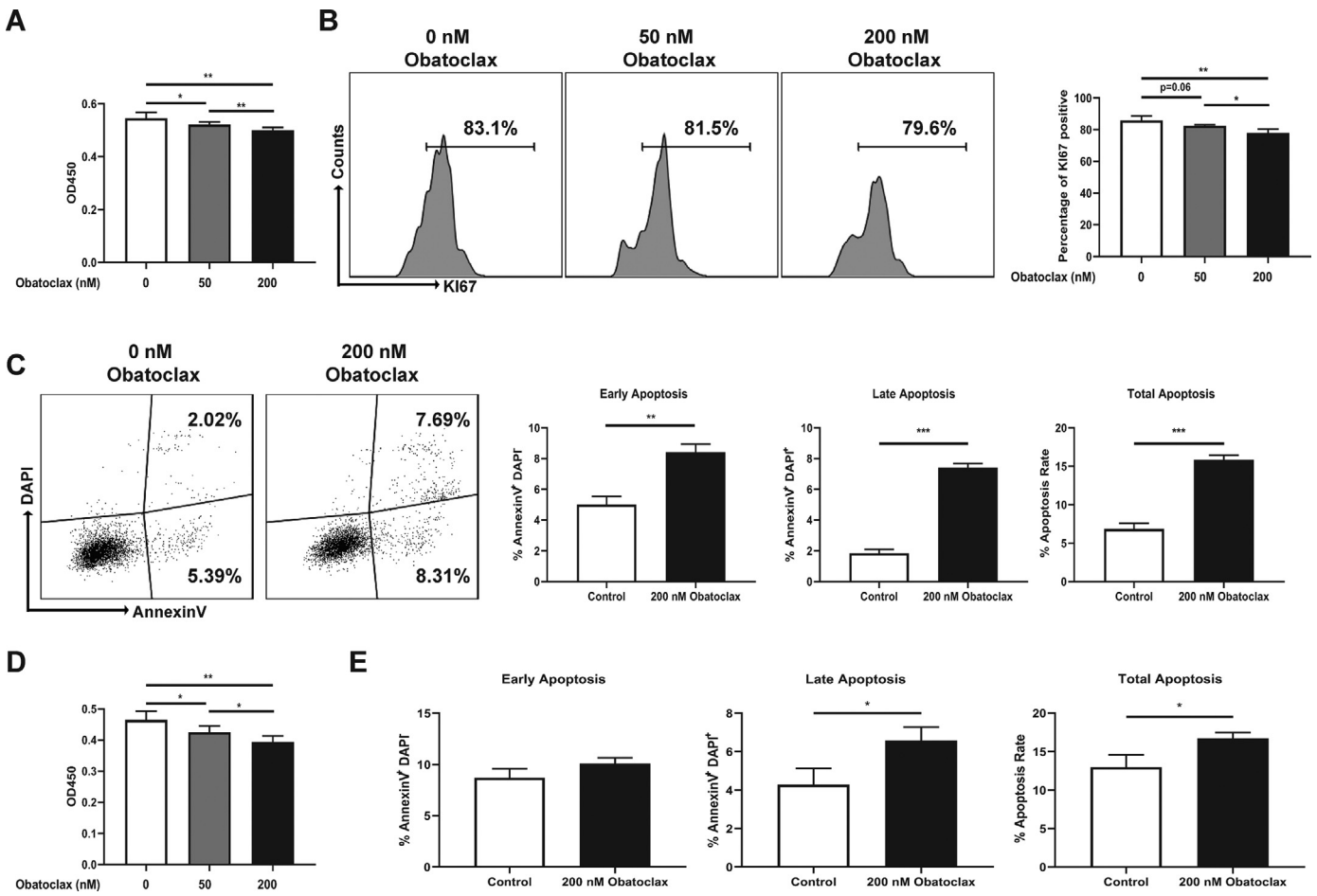

Fig. 1. Obatoclax directly impaired HCC cell viability *in vitro*. (A) 5×10^4 Hepa1–6 cells were seeded into 96-well plate and treated with 50 nM and 200 nM obatoclax, respectively. The proliferation of Hepa1–6 cells was determined by CCK8. (B) Representative graphs of Ki67 staining assay. 1×10^6 Hepa1–6 cells were treated with 50 nM and 200 nM obatoclax, respectively. The expression of the proliferate marker Ki67 was assessed by flow cytometry. (C) Representative flow graphs of apoptosis assay. 1×10^6 Hepa1–6 cells were treated with 200 nM obatoclax for 8 h. The apoptosis rates were assessed by AnnexinV & DAPI staining. The early apoptotic cells were defined as AnnexinV$^+$ DAPI$^-$, the late apoptotic cells were defined as AnnexinV$^+$ DAPI$^+$. The total apoptosis cells were defined as AnnexinV$^+$. (D) 5×10^4 HepG2 cells were seeded into 96-well plate and treated with 50 nM and 200 nM obatoclax, respectively. The proliferation of HepG2 cells was determined by CCK8. (E) 1×10^6 HepG2 cells were treated with 200 nM obatoclax for 8 h. The apoptosis rates were assessed by AnnexinV & DAPI staining. Data are presented as means + SD. $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

Obatoclax suppressed HCC development in vivo

Next, we investigate the effects of obatoclax on HCC development *in vivo*. Murine HCC model was established by injecting Hepa1–6 cells subcutaneously into the WT male mice. When the tumor size reached 2–3 mm in diameter, mice were randomly divided into two groups: one group were given 5 mg/kg obatoclax injection three times per week; the other group were given DMSO as control. As shown in Fig. 3A, mice receiving obatoclax injection had smaller tumor size compared with the control group. The survival curve also showed that obatoclax treatment prolonged the survival time post tumor injection. The average survival time increased from 37.7d to 45d post tumor injection (Fig. 3B). Collectively, these data proved that obatoclax served as a tumor suppressor against HCC development.

Obatoclax sensitized tumor cell to T cell-mediated cytotoxicity

Our previous results proved obatoclax inhibited HCC growth both *in vitro* and *in vivo*. To examine whether the anti-tumor activity is solely dependent on the direct inhibition of obatoclax on tumor cell viability. We established the murine HCC model in immunodeficiency NOD/SCID mice. To our surprise, the anti-tumor activity of obatoclax on HCC growth was diminished (Fig. 4A). This result suggested immune system was required for obatoclax-mediated HCC suppression.

T cells played a critical role in the anti-tumor immune responses. To reveal the potential effects of obatoclax on immune system mediated anti-tumor activity, splenocytes and tumor infiltrating lymphocytes (TILs) were isolated from tumor-bearing mice, the distributions of the major immune cell subsets were assessed by FACS. We found that obatoclax didn't alter the macrophages, DCs, T cells, B cells and NK cells distribution in both spleen and TILs (Fig. 4B&C). Intracellular staining also showed that obatoclax had no influence on effector cytokines expressions by T cells (Fig. 4D&E). Interestingly, we found that T cells isolated from HCC tumor-bearing mice showed higher killing capacity against obatoclax pre-treated Hepa1–6 cells (Fig. 4F). These results indicated although obatoclax had no effects on immune cells function *in vivo*, it sensitized tumor cells to T cell-mediated cytotoxicity.

Obatoclax augmented the anti-tumor activity of anti–PD-1 antibody in vivo

The previous results suggested obatoclax inhibited HCC progression by sensitizing the tumor cells to T cell-mediated cytotoxicity. PD-1 is a key molecule expressed on activated T cell surface that negatively regulates the anti-tumor T cell activity. Engagement of PD-1 by its ligands PD-L1 or PD-L2 induces the signal transduction that inhibits T-cell

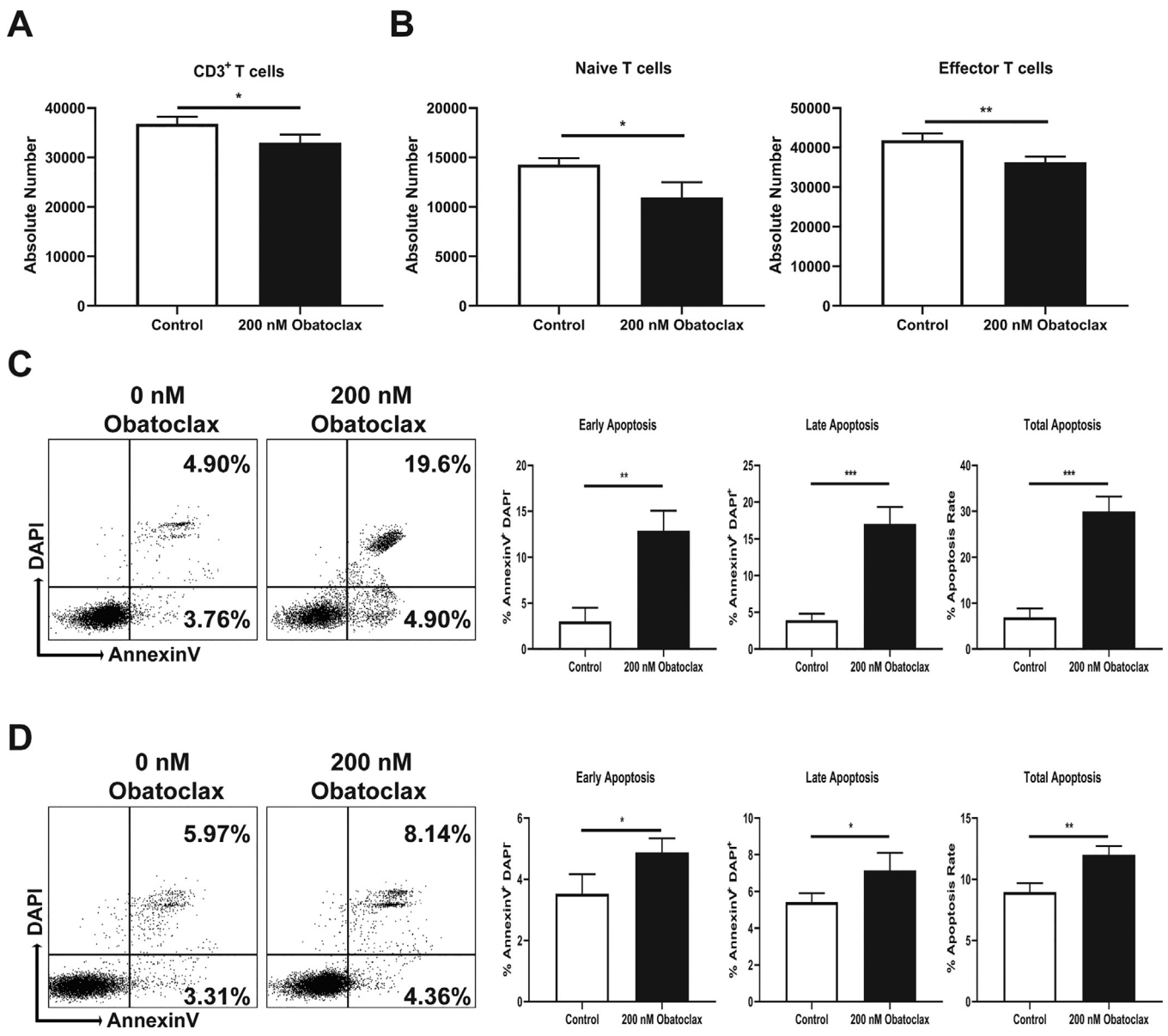

Fig. 2. Obatoclax differentially affected T cells subsets viability *in vitro*. (A) T cells were isolated from WT mouse and were treated with 200 nM obatoclax in the presence of anti-CD28 and 100 U/ml rIL-2. The total number of T cells were counted after 24 h. (B) Naïve T cells and effector T cells were isolated from WT mice and were treated with 200 nM obatoclax in the presence of anti-CD28 and 100 U/ml rIL-2. The total number of naïve T cells and effector T cells were counted after 24 h, respectively. (C) Representative flow graphs of apoptosis assay. Naïve T cells were treated with 200 nM obatoclax for 24 h. The apoptosis rates were assessed by AnnexinV & DAPI staining. (D) Representative flow graphs of apoptosis assay. Effector T cells were treated with 200 nM obatoclax for 24 h. The apoptosis rates were assessed by AnnexinV & DAPI staining. Data are presented as means ± SD. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

expansion, effector cytokines production, as well as cytolytic functions [32]. Thus, we studied whether the combinations of obatoclax with anti–PD-1 antibody could enhance the anti-tumor function in murine HCC models. As expected, anti–PD-1 antibody treatment significantly suppressed tumor growth compared with isotype control. Notably, this anti-tumor activity was further enhanced by co-treatment with obatoclax (Fig. 5A). Mice body weight was also monitored to dissect the potential side effects of obatoclax. No significantly body weight reduction was observed, suggesting obatoclax treatment is safe for *in vivo* application (Fig. 5B). Survival analysis also revealed that the combination therapy of obatoclax with anti–PD-1 antibody significantly prolonged the HCC tumor-bearing mice survival time (Fig. 5C).

Immune phenotyping showed that the distribution of T cell subpopulations was not significantly altered in the tumor microenvironment (Fig. S2A). Notably, anti-PD-1 treatment significantly increased T cell activation. The percentage of activated T cells ($CD69^{+}$) were increased in both spleen (Fig. 5D) and TILs (Fig. 5E) in anti-PD-1 group and the combination group. Furthermore, the effector cytokines expressions were also increased in T cells in the anti-PD-1 group and the combination group. The percentage of TNF-α^{+} IFN-γ^{-} *T* cells were increased from 41.6 ± 4.68% in control group and 42.4 ± 3.31% in obatoclax group to 60.3 ± 3.21% in anti-PD-1 group and 61.5 ± 6.38% in combination group in spleen (Fig .5F). Similar increase of effector cytokines expressing T cells were also found in the TILs after been treated with anti–PD-1 alone or in combination with obatoclax (Fig. 5G). We further performed immunohistochemistry to confirm that the combination therapy induced tumor cell apoptosis in the tumor microenvironment. We observed an increased level of cleaved caspase-3 in the tumor of combination treatment group, suggesting that tumor cells underwent apoptosis after treatment (Fig. S2B). Taken together, these results demon-

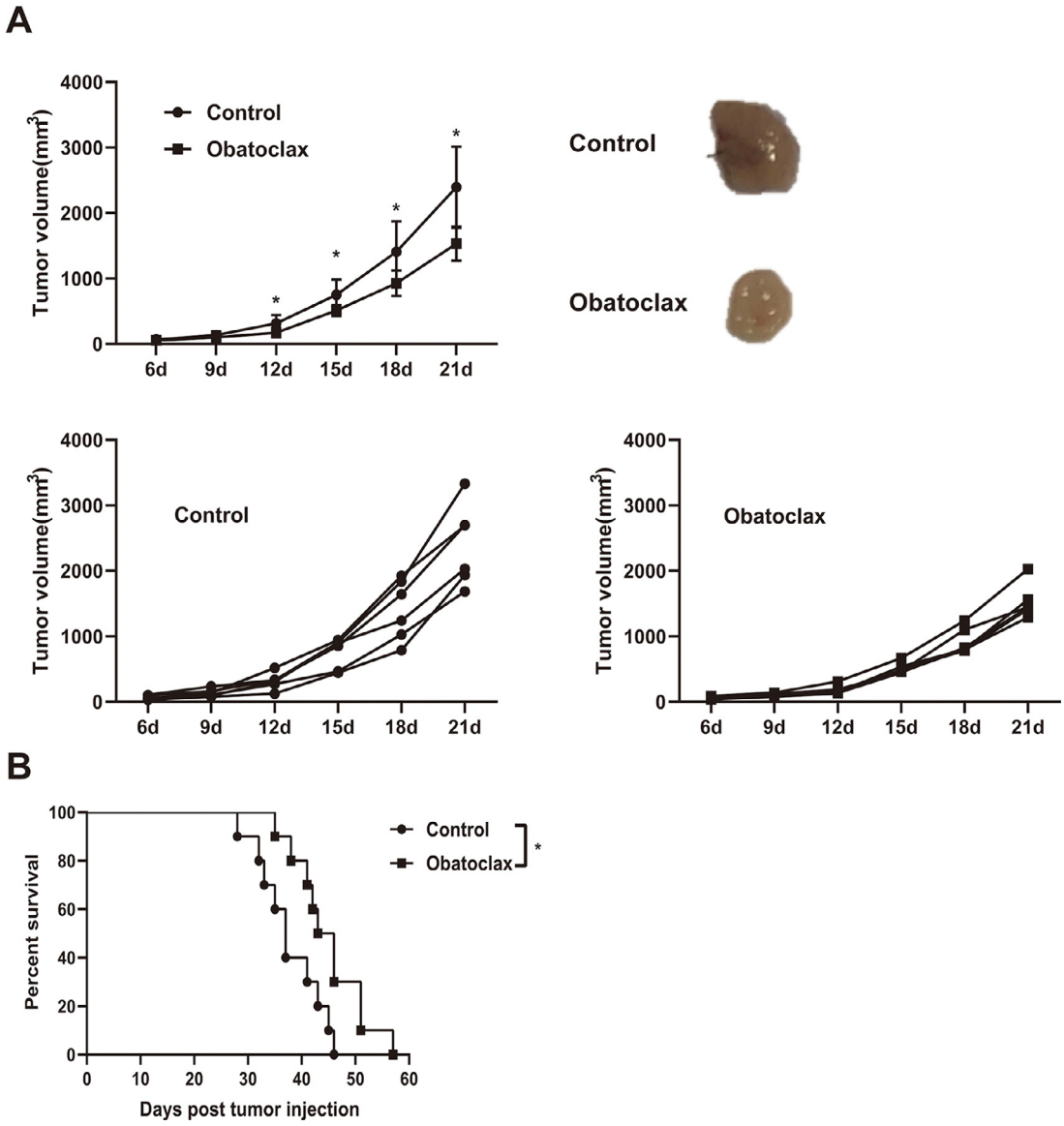

Fig. 3. Obatoclax inhibited HCC development *in vivo*. (A) Mice (n = 6) were injected subcutaneously with 1.5×10^6 Hepa1–6 cells. When the diameter of tumor reached 2–3 mm, mice were intraperitoneal injected with DMSO control or obatoclax (5 mg/kg) three times per week. The tumor size was measured and calculated by the following formula: Volume = (length x width^2)/2. The representative images of tumor and tumor growth curve were shown. (B) The survival rate of HCC tumor-bearing mice (n = 8) was monitored and compared by log-rank test. The data shown are representative of three experiments. Data are presented as means ± SD. * $p < 0.05$.

strated anti–PD-1 treatment induced T cells activation to suppress the tumor growth.

To further confirm the anti-tumor effects of obatoclax in combination with anti–PD-1 antibody, another murine HCC model was established by injecting Hepa1c1c7 cells subcutaneously into the mice. Tumor growth curve exhibited that obatoclax or anti-PD-1 treatment alone showed the trend of tumor inhibition (Fig. 6A). Importantly, cotreatment of anti-PD-1 with obatoclax dramatically enhanced the anti-tumor effects compared with the solo treatment (Fig. 6A). FACS analysis also showed that T cells were activated in the anti-PD-1 group and the combination group (Fig. 6B). Collectively, our data demonstrated obatoclax augmented the anti-tumor activity of anti-PD-1 antibody *in vivo* by activating T cells.

Discussion

In the present study, for the first time, we evaluated the anti-tumor function of obatoclax in combination with anti-PD-1 monotherapy in HCC. We found that obatoclax could directly suppress HCC cell growth *in vitro*. Further experiments utilizing two different murine HCC model demonstrated obatoclax suppressed tumor development *in vivo*. Moreover, obatoclax, in combination with anti-PD-1 antibody achieved enhanced anti-tumor efficiency compared with solo treatment through sensitizing tumor cells and promoting. T cells activation.

Immune checkpoint blockade promotes anti-tumor T cells immune responses and has achieved long-term remission in patients with different tumor types, especially for leukemia and melanoma [33,34]. Over the past decades, immune checkpoint blockade has been a great booster for advanced malignant tumors treatment. PD-1 is expressed on T cells upon activation and negatively regulate T cells immune responses. Using antibodies targeting PD-1 to block its interaction with PD-L1/PD-L2 stimulates the immune system to keep the tumor in surveillance. So far, the anti-PD-1 antibodies, nivolumab and pembrolizumab have been approved by the US Food and Drug Administration (FDA) for treating HCC. In a singlearm clinical trial which HCC patients were previously treated

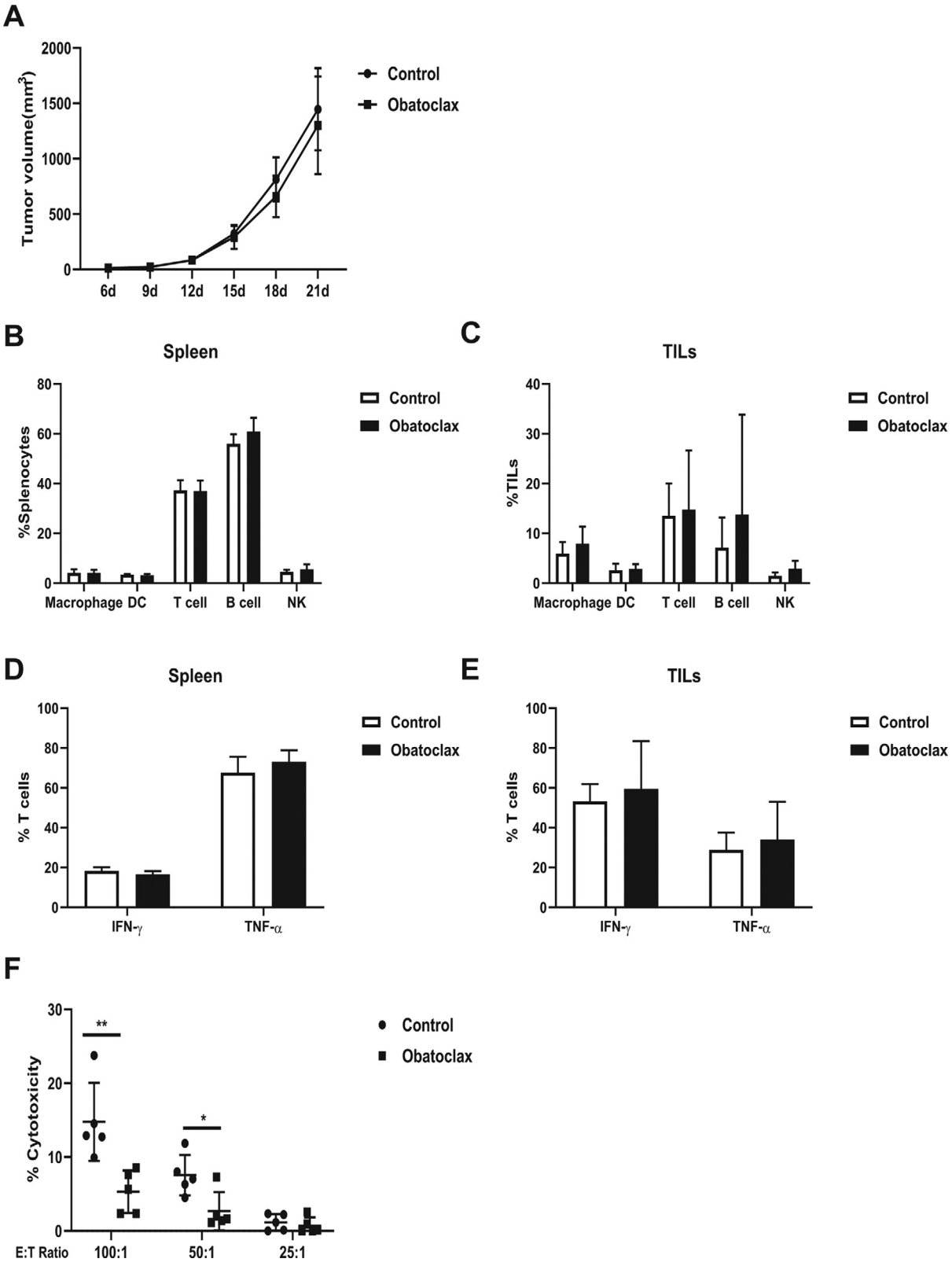

Fig. 4. Obatoclax sensitized HCC cells for T cells-mediated killing. (A) NOD/SCID mice ($n = 6$) were injected subcutaneously with 1.5×10^6 Hepa1–6 cells. When the diameter of tumor reached 2–3 mm, mice were intraperitoneal injected with DMSO control or obatoclax (5 mg/kg) three times per week. The tumor size was measured and calculated by the following formula: Volume = (length x width^2)/2. (B) Splenocytes and (C) TILs were isolated from tumor bearing mice 2 weeks after tumor injection. The percentages of CD11B$^+$ macrophages, CD11C$^+$ DCs, CD3$^+$ T cells, CD19$^+$ B cells and NK1.1$^+$ NK cells were detected by flow cytometry. The percentages of TNF-α, IFN- γ expressing T cells in (D) spleen and (E) TILs were assessed by flow cytometry. (F) T cells isolated from HCC tumor-bearing mice were co-cultured with obatoclax pre-treated Hepa1–6 cells at the indicated E/T ratio. The cytotoxicity was assessed. The data shown are representative of three experiments. Data are presented as means ± SD. $^*p < 0.05$, $^{**}p < 0.01$.

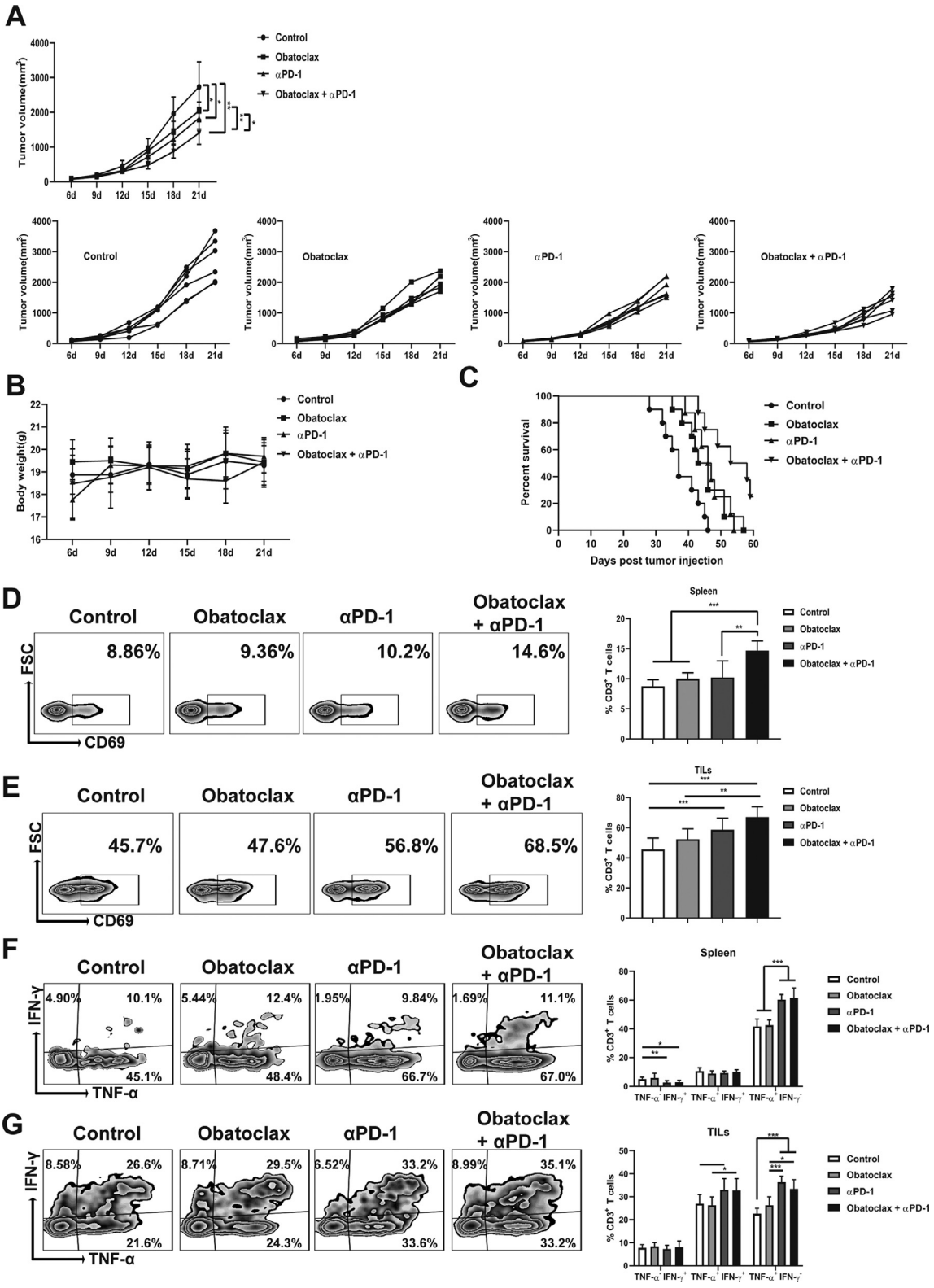

Fig. 5. Obatoclax enhanced the anti-tumor efficacy of anti-PD-1 monotherapy in murine Hepa1–6 HCC model
(A) Mice ($n = 6$) were injected subcutaneously with 1.5×10^6 Hepa1–6 cells. When the diameter of tumor reached 2–3 mm, mice were intraperitoneal injected with DMSO control, obatoclax (5 mg/kg), anti–PD-1 antibody (10 mg/kg) alone or in combination with obatoclax three times per week. The tumor size was measured and calculated by the following formula: Volume = (length x width^2)/2. (B) The body weight was monitored. (C) The survival rate of HCC tumor-bearing mice ($n = 8$) was monitored and compared by log-rank test. (D) Splenocytes and (E) TILs were isolated from tumor bearing mice 2 weeks after treatment. The percentages of CD69$^+$ activated T cells were detected by flow cytometry. The percentages of TNF-α, IFN- γ expressing T cells in (F) spleen and (G) TILs were assessed by flow cytometry. The data shown are representative of three experiments. Data are presented as means ± SD. $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

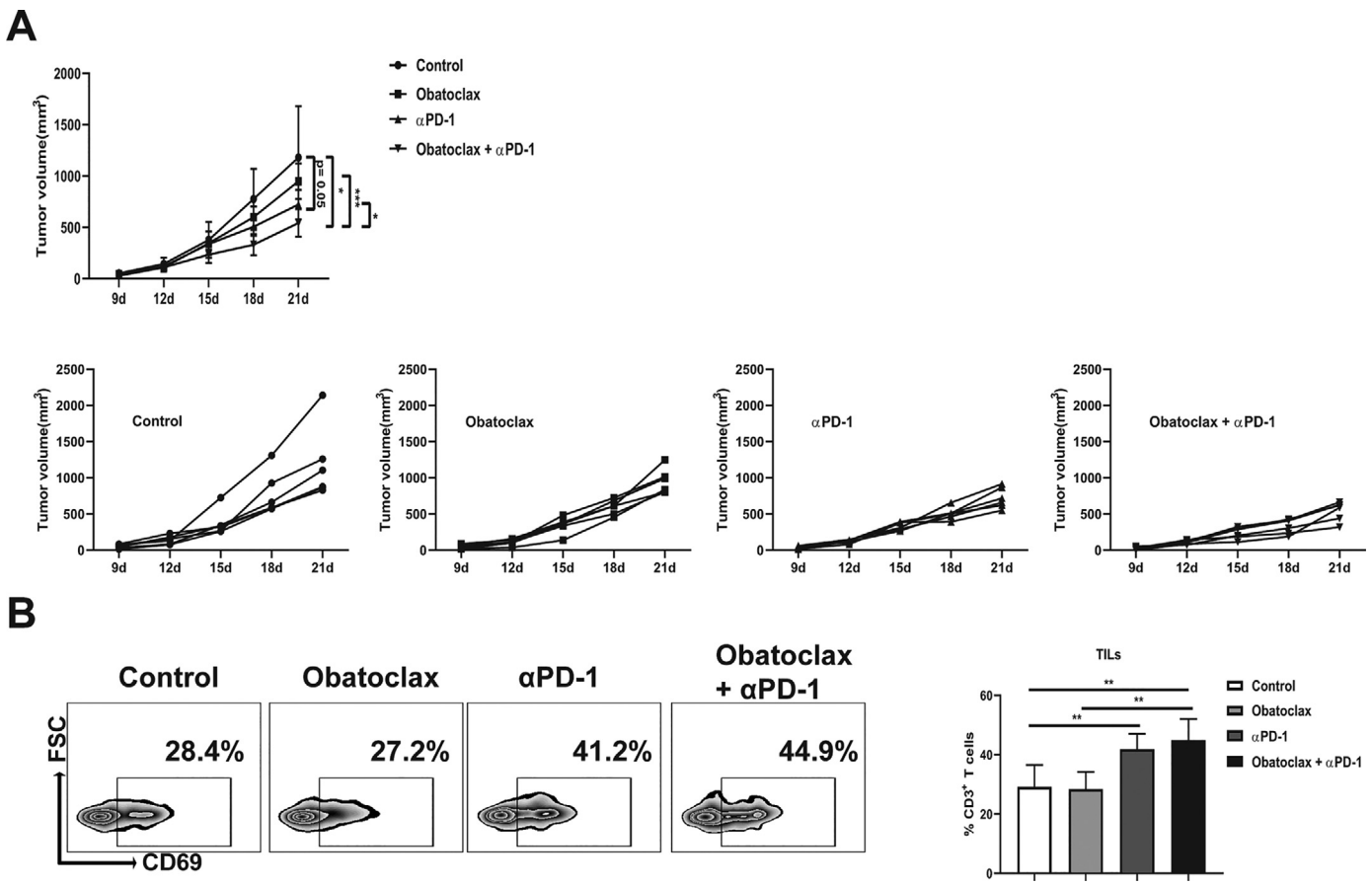

Fig. 6. Obatoclax enhanced the anti-tumor efficacy of anti-PD-1 monotherapy in murine Hepa1c1c7 HCC model (A) Mice (n = 6) were injected subcutaneously with 2 × 10^6 Hepa1c1c7 cells. When the diameter of tumor reached 2–3 mm, mice were intraperitoneal injected with DMSO control, obatoclax (5 mg/kg), anti–PD-1 antibody (10 mg/kg) alone or in combination with obatoclax three times per week. The tumor size was measured and calculated by the following formula: Volume = (length x width^2)/2. (B) TILs were isolated from tumor bearing mice 2 weeks after treatment. The percentages of CD69$^+$ activated T cells were detected by flow cytometry. Data are presented as means ± SD. $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

with sorafenib, the overall response rate (ORR) and overall survival (OS) were nearly 15% and 12 months after been treated with anti-PD-1 antibody, respectively [35,36].

Despite the beneficial effects of anti-PD-1 antibody treatment for HCC, randomized clinical trials of anti-PD-1 monotherapy in either first-line (nivolumab *vs*. sorafenib) or second-line (pembrolizumab *vs*. placebo) settings did not achieve significant improvement in OS [28,37]. Therefore, it is urgent to develop the combination therapeutic strategies to improve beneficial efficacy of anti-PD-1 monotherapy for HCC.

The correlation of Bcl-2 family members and various cancer types have been well established. Utilizing Bcl-2 inhibitors alone or in combination with other therapeutic agents for cancer treatment has shown promising outcome in acute leukemia, lymphomas, as well as solid tumors [38]. For example, the selective small molecule inhibitor of Bcl-2, Venetoclax has been approved for treating chronic lymphocytic leukemia (CLL) or small lymphocytic lymphoma (SLL) in combination with immune checkpoint blockade [39]. The pan Bcl-2 inhibitor, obatoclax infusion combined with carboplatin–etoposide showed improved efficacy in treating patients with extensive-stage small cell lung cancer [40].

Besides tumor cells, Bcl-2 family proteins also played essential roles for T cells development and survival. However, accumulating evidence has suggested that Bcl-2 knockout had no influence on T cells activation [41]. These results were consistent with our findings that obatoclax reduced T cells proliferation. Notably, we demonstrated that activated T cells were more tolerated to obatoclax treatment compared with the naive T cells. This obatoclax-induced cytotoxicity to T cells raised the concern that obatoclax would reduce the anti-tumor activity of T cells. Interestingly, our data showed that although obatoclax induced T cells apoptosis *in vitro*, it didn't affect the T cells activation *in vivo*. By comparing the T cells distribution and activation between control and obatoclax solo treatment group, no significant difference of T cells distribution and activation was observed in the tumor microenvironment, suggesting T cells are tolerant to obatoclax *in vivo*. One possible mechanism underlying this might be the different expression level of Bcl-XL in different immune cells. Frederick et al. proved that activated T cells expressed higher level of Bcl-XL compared to naive T cells, making the activated cells more resistant to Blc-2 inhibitor treatment [31]. Further experiments are needed to be done to test this hypothesis.

Tumor cell intrinsic genetic mutations evolved rapidly to evade and resist to immune surveillance and elimination through multiple mechanisms. For example, 1), tumor cells downregulated tumor antigen and MHC molecules expressions to escape from immune recognition; 2), tumor cells upregulated the immunosuppressive receptor or cytokines (*e.g.*, IL-10, TGF-β) to suppress immune responses; 3), solid tumor generated an immunosuppressive tumor microenvironment to escape from immune elimination [42,43]. Thus, sensitizing the tumor cells to immune response is a promising therapeutic strategy for cancer treatment. Here, we found that upon obatoclax treatment, tumor cells are more sensitive to T cells-mediating killing, suggest obatoclax could sensitize tumor cells for cytotoxic T cells. The possible mechanism might be that obatoclax could increase the expressions of Fas, TRAIL and TNF death receptors on tumor cells [44]. Further experiments should be done to test our hypothesis.

Though we provided evidences that obatoclax could enhance the anti-tumor activity of anti-PD-1 monotherapy in murine HCC model, there are still several limitations. First, two murine subcutaneous HCC models were used in this current study, however, the subcutaneous model couldn't completely reflect the complexity and heterogenicity of the liver microenvironment. Further studies should be done by using murine orthotopically HCC model to validate our current findings. Second, as a pan-Bcl-2 inhibitor, obatoclax are known to inhibit multiple Bcl-2 family members [20], thus raising the question which specific Bcl-2 member is dominantly suppressed by obatoclax. Third, besides obatoclax, various Bcl-2 inhibitors have been tested in clinical trials against different types of cancer [45], whether other Bcl-2 inhibitors could also inhibit HCC development in combination with anti-PD-1 monotherapy needs further investigation. Last but not least, whether obatoclax could enhance the anti-tumor activity of anti-PD-1 monotherapy in other cancer types needs to be studied.

Despite the recent advances in immunotherapy against HCC, HCC still remains one of the deadliest diseases globally. Targeting angiogenesis by blocking VEGF has been widely used in clinical settings in combination with chemotherapy and/or immune checkpoint blockade. However, none of these strategies was specific to HCC. In our perspective, explore the specific targets for HCC is of great importance for HCC immunotherapy. With the rapid advances in next-generation sequencing (NGS) technology, identifying one or more specific targets using NGS for personalized treatment is extremely promising to enhance the anti-tumor effects of chemo therapy or immune checkpoints blockade. However, numerous obstacles still need to be solved. For example, the high cost of NGS and personalized therapy; the precision of NGS results; the availability of drugs against the identified target's by NGS, etc.

Taken together, we provided evidence for the first time that obatoclax could enhance the anti-tumor activity of anti-PD-1 monotherapy in murine HCC model. Although obatoclax could directly inhibit HCC cell growth *in vitro*, this direct inhibitory effect was insufficient to induce a significant tumor remission *in vivo* in NOD/SCID mice. Mechanism study revealed that obatoclax sensitized tumor cell to T cells-mediated cytotoxicity. Combination therapy of obatoclax and anti-PD-1 antibody synergically reduced HCC growth *in vivo* by promoting T cells activation. Thus, obatoclax could serve as a potential therapeutic agent for HCC treatment.

Declaration of Competing Interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

CRediT authorship contribution statement

Jingye Li: Methodology, Writing - review & editing, Supervision, Funding acquisition. **Jinrong Xu:** Investigation, Formal analysis, Writing - original draft. **Zhibing Li:** Investigation, Writing - original draft.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.tranon.2021.101116.

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