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4-Acetylantroquinonol B from *antrodia cinnamomea* enhances immune function of dendritic cells against liver cancer stem cells

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ABSTRACT

The functions of 4-acetylantroquinonol B (4-AAQB), a ubiquinone derivative isolated from the mycelium of *Antrodia cinnamomea*, in immunotherapy for liver cancer were investigated. We found that 4-AAQB could inhibit liver cancer stem cell related manifestations and activate the antitumor ability of dendritic cells. Specifically, 4-AAQB can inhibit EpCAM, AFP and related pathways of HepG2 cells. It also significantly decreases the expression of β-catenin, inhibits the tumorigenicity and decreases the secretion of immune escape related cytokines. Moreover, 4-AAQB can stimulate the proliferation of immune cells and promote the endocytosis of immature dendritic cells. When co-cultured immature dendritic cells with EpCAM+ HepG2 cells, 4-AAQB enhanced the expression of MHC class I and II on the surface of liver cancer stem cells and dendritic cells, increased the expression of costimulatory molecules CD80 of dendritic cells and cytokines related to immune activation. In conclusion, 4-AAQB from *Antrodia cinnamomea* can enhance immune function of dendritic cells against liver cancer stem cells, and may have the potential to be used for liver cancer prevention and immunotherapy.

1. Introduction

Cancer stem cells (CSCs) are a small number of cells in tumor with self-renewal and differentiation ability, they play an important role in carcinogenesis in various cancers. Hepatocellular carcinoma (HCC) is a malignant tumor with one of the highest mortality rates in solid cancer. Several liver cancer stem cell (LCSC) marker have been identified, including CD133, CD90, CD44, Ov6, EpCAM and AFP, where EpCAM is also defined as the tumor initiating cell markers. EpCAM+ HCC cells can activate the attack and aggressiveness of hepatoma in the mouse model [1]. AFP (α-fetoprotein) is a common marker used in early clinical diagnosis for liver cancer. Low survival rate for HCC patients is often associated with high level of AFP [2]. EpCAM and AFP have been used as clinical prognostic subtypes of HCC. Therefore, we used these two types of LCSCs as the cellular model in this study.

The immune system is the obstacle that cancer cells must face and overcome in order to develop tumor. The immune system under normal conditions can identify the tumor as intruder and activate the immune responses, including both of innate and adaptive immunity. However, cancer cells may secrete immune-escaping cytokines, tame the immune system or hide the antigen-recognition sites to avoid the cells from the attacks of immune system [3,4]. In addition, CSCs may be the main cause of tumor immune escape [5]. CSCs may secrete immunosuppressive cytokine [6], exhibit PD-L1 [7], reduce MHC performance [8], and differentiate into cancer cells that are more adaptable to the immune environment [5]. Thus, study of CSCs vaccine to induce efficient immune response against CSCs has become an important topics in recent years [9]. The dendritic cell (DC) vaccines which target CSCs had shown better therapeutic effects such as decreasing the tumor size, prolonging survival [10,11] and reducing metastasis [12]. However, improvement of the effectiveness of cancer immunotherapy is still the goal that needs continuing endeavor.

It is known that the use of some bioactive compounds along with regular cancer therapy may prolong the life of cancer patients [13,14]. Some compounds isolated from Chinese herbal medicine have been reported for their effects on enhancing the functional activity of DCs, including maturation, cytokine production and MHC class expression. For example, the exopolysaccharide from *Cordyceps sinensis* can increase the expressions of IL-12, TNF- α, CD40, CD80, CD86 and MHC II of DCs in ex vivo animal model [15]. The β –glucan extracted from the widely used medicinal fungus, *Ganoderma lucidum*, can activate DCs and produce high levels of IL-23, TNF- α, IL-6 and IL-10 [16]. Shikonin from Zicao can induce effective immune response through DCs to inhibit tumor growth and metastasis [17]. Hypericin from *Hypericum perforatum* can improve the effectiveness of DC vaccines and increase survival of test animals [18]. Some phytochemicals from plants,

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including terpene compounds, phenols, lipids and alkaloids have also been emphasized for their effects on the modulation of oxidative stress in tumor microenvironment [19], which might also change the tumor-derived immunosuppression from cancer cells and enhance the immune function of DCs.

Antrodia cinnamomea, a precious fungus in traditional herbs, is well known for its liver protection function. In the previous study, we have identified and isolated 4-acetylantroquinonol B (4-AAQB), a ubiquinone derivative, from the mycelium of *Antrodia camphorata*, and found that this compound can inhibit the growth of HepG2 cells with IC_{50} of 0.1 μg/ml [20]. The 4-AAQB arrests proliferation of liver cancer cells by affecting p53, p21 and p27 levels [21], and reduce metastasis in HCC in xenograft model [22]. Although 4-AAQB was found to be able to attenuate stemness-related chemoresistance and negatively regulate stem cell maintenance signal transduction pathways in colorectal carcinoma [23], there is still no information concerning the effect of 4-AAQB on LCSCs and HCC related antitumor immune responses. We suspected that 4-AAQB can not only inhibit LCSCs but also improve the immunosupressive micro-environment to reduce the deterioration of liver cancer. Therefore, this study focused on the inhibitory effect of 4-AAQB on LCSCs, and the activation effect of 4-AAQB on immune cells. We also utilized the model of co-culture of LCSC with immature dendritic cells (iDCs) to reveal the potential application of 4-AAQB in immunotherapy for HCC.

2. Materials and methods

2.1. Reagents and chemicals

The 4-AAQB was isolated from the mycelium of *Antrodia cinnamomea* as described before [21]. Dulbecco's Modified Eagle medium (DMEM), Roswell Park Memorial Institute (RPMI), fetal bovine serum (FBS) were obtained from GIBCO/BRL Life Technologies (Grand Island, NY). PBS ingredients were purchased from Merck (Darmstadt, Germany). Trypsin–EDTA, Lipopolysaccharide (LPS), FITC-dextran 7000, 5-Fluorouracil (5-FU) dimethyl sulfoxide (DMSO) and Cell Counting Kit-8 were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). Protocols of cell culture and the related reagents used are described as follows.

2.2. Cell lines and culture

Human liver hepatocellular cells HepG2, human monocytic leukemia cells THP-1 and rat macrophages Raw264.7 were purchased from the Bio-resource Collection & Research Centre (Food Industry Research and Development Institute, Hsinchu, Taiwan). HepG2 cells and Raw264.7 cells were cultured in DMEM medium with 10% FBS supplement. THP-1 cells were maintained in RPMI medium with 10% FBS. Cells were maintained in a humidified incubator at 37 °C in 5% CO_2/95% air. All of the cell culture protocols followed the instruction of ATCC.

2.3. Immunofluorescence assay

The HepG2 cells (4×10^5 cells/ml, 0.5 ml) were seeded on four-well chamber slides (Lab-Tek II, Nunc, Rochester, NY, USA) for 24 h, and then incubated with different concentrations of 4-AAQB for 24 h. Cells were washed with cold PBS and then fixed in 4% formaldehyde for 30 min. Bovine serum albumin (BSA, 5%, with 0.1% Triton X-100) was added to cells and incubated for 60 min. PE-β-catenin (1:200 BD, San Jose, CA, USA) was added to cells and incubated for 1 h. After washing with PBS, FITC- EpCAM (1:200 BD, San Jose, CA, USA) was added to cells and incubated for 1 h. Finally, nuclei were labeled with 300 nM of 4′, 6-diamidino-2- phenylindole (DAPI, Lonza, Walkersville, MD, USA) for 15 min. The chamber was removed and mounting medium was added onto the slide with a coverslip. Confocal microscopy (Leica TCS SP5 II, Solms, Germany) was used for assessment.

2.4. Colony formation assay

The HepG2 cells in 5 ml DMEM (1×10^3 cells) were seeded in 9-cm culture dishes. After incubation for 24 h, cells were treated with various concentrations of 4-AAQB for another 24 h. Then the DMEM was replaced with DMEM containing 10% FBS. After 10 days incubation, cells were observed under the microscope.

2.5. EpCAM and AFP flow cytometry analysis

HepG2 cells (2×10^5 cells/ml, 2 ml) were seeded in 6-well plate and treated with various concentrations of 4-AAQB for 48 h. After treatment, cells were collected in 1.8 ml Eppendorf. Then the FITC-EpCAM (BD, San Jose, CA, USA) was used for surface staining. For intracellular staining, the Pharmingen™ Transcription Factor Buffer Set (BD, San Jose, CA, USA) was applied first, followed by PE- alpha-Fetoprotein (AFP, R&D Systems Inc, Minneapolis, MN) staining. Stained cells were analyzed for two-color immunofluorescence with Beckman Coulter flow cytometer (FC500, Fullerton, CA, USA).

2.6. Isolation of EpCAM + cell subpopulation

Magnetic-activated cell sorting (MACS) method was used to isolate EpCAM+ HCC cells. HepG2 cells ($\sim 1 \times 10^7$) were harvested from culture plate, then incubated with FcR Blocking Reagent and anti-EpCAM microBeads (both from Miltenyi Biotech, Sunnyvale, CA) for 30 min. FITC-EpCAM was then added for staining antibodies to assure that pure EpCAM + can be isolated by flow cytometry. EpCAM + cells were collected by LS magnetic separation columns. After separation, cells were immediately cultured in DMEM with 10% FBS and allowed to stay in incubator for 24 h to revive the cells. This separation process was carried out each time the EpCAM + cells experiment was performed.

2.7. Cell viability assay

EpCAM+ HepG2 cells were seeded in 96-well plate (1×10^5 /ml, 100 μl per well) and incubated overnight to allow cell adherence. After the culture medium was replaced with 4-AAQB containing-medium, the cell culture was incubated again for 48 h. After treatment, Cell Counting Kit − 8 (CCK8; 10 μl) was added to each well, the plate was incubated for 1.5 h at 37 °C, and absorbance was measured at 450 nm by ELISA reader (Denver Jasco Model 7800 UV/VIS Spectrophotometer, Jasco, Tokyo, Japan).

2.8. Analyses of IL-2, 4, 6, 10, TNF-α and IFN-γ

EpCAM+ HepG2 cells (2×10^5 cells/ml, 1 ml) or immature dendritic cells (2×10^5 cells/ml, 1 ml) were seeded and treated with various concentrations of 4-AAQB, then the supernatant was collected to examine the amount of cytokines. The cytokines including IL-2, 4, 6, 10, TNF-α and IFN-γ were quantified using the cytometric bead array kit (human Th1/Th2 inflammatory cytokines, CBA; BD Biosciences) with the Cytoflex flow cytometer (Beckman Coulter, Fullerton, CA, USA).

2.9. Generation of immature dendritic cells

THP-1 cell suspension was centrifuged and the precipitate was resuspended in RPMI medium supplemented with 10% FBS at a concentration of 2×10^5 cells/ml. To induce differentiation, rhIL-4 (100 ng/ml = 1500 IU/ml) and rhGM-CSF (100 ng/ml = 1500 IU/ml) (BioVision, Mountain View, CA) were added [24]. Culture medium was replaced every 2 days with fresh cytokine-supplemented medium. Cells

were cultured for 5 days to acquire the immature dendritic cells.

2.10. Endocytosis of immature dendritic cells

After 5 days differentiation, immature dendritic cells were centrifuged and re-suspended in RPMI medium at a concentration of 2×10^5 cells/ml. The cell suspension was charged into 6-well plate, and then treated with various concentrations of 4-AAQB in an incubator for 24 h at 37 °C. Then 1 mg/ml FITC- 70 kDa dextran was added and incubated for 15 min [25]. As a blank control, cells were incubated in the same medium without FITC- 70 kDa dextran at 37 °C. The cells were washed three times with cold PBS and analyzed with FC500 flow cytometer (Beckman Coulter, Fullerton, CA, USA).

2.11. Effect of 4-AAQB on proliferation of THP-1 and Raw264.7 cells

The cell proliferation assay was carried out using CCK-8 kit. THP-1 or Raw264.7 cells (2×10^4 cells /per well) were grown in 96-well plate in a final volume of 100 µL culture medium overnight. Cells were then exposed to 4-AAQB at different concentrations for 24 h. After incubation, 10 µl of CCK-8 solution was added to each well and the cells were further incubated for 1.5 h at 37 °C. Absorbance at 450 nm was measured using a ELISA reader.

2.12. Co-culture of iDCs with 4-AAQB-treated EpCAM + HepG2 cells

After 24 h treatment with different concentrations of 4-AAQB in 12-well plate, 4-AAQB-treated EpCAM + HepG2 cells were co-cultured with iDCs at a ratio of 1:1. After incubation for 24 h, the co-culture was collected in 1.8 ml Eppendorf. The supernatant was collected by centrifugation to examine immune activated cytokines. And the phenotype of the cells was then analyzed by FACS.

2.13. CD80 and MHC class I, II surface marker staining

The co-cultured cells were surface stained with antibodies of MHC class I, II and CD80 (all purchased from BD Bioscience) and then analyzed by FC500 flow cytometer (Beckman Coulter, Fullerton, CA, USA). To determine the phenotype of iDCs and EpCAM + HepG2 cells, each type of cells was analyzed individually via gating the FSC/SSC of each group of cells.

2.14. Statistical analysis

All of the experiments were carried out in triplicates, and results were expressed as mean ± standard error. Comparative analysis was done using either the Duncan's multiple comparison tests or one-way analysis of variance (ANOVA) using GraphPad (San Diego, CA, USA; * for $P < 0.05$, ** for $P < 0.01$ and *** for $P < 0.001$).

3. Results

3.1. Effect of 4-AAQB on hepatocellular carcinoma stem cell and tumorigenicity

The effects of 4-AAQB on two hepatocellular carcinoma stem cell markers including AFP and EpCAM were evaluated. Result showed that 4-AAQB inhibited the expression of EpCAM and AFP in a dose-dependent manner (Fig. 1A). When the concentration of 4-AAQB increased, the EpCAM + AFP + decreased and the EpCAM − AFP − increased. Fig. 1B shows the Mean Fluorescence Intensity (MFI) of Fig. 1A. The MFI of EpCAM and AFP of 4-AAQB-treated HepG2 cells was significantly lower than the untreated group ($p < 0.01$).

β-catenin is a necessary factor for activating EpCAM and it involves in a variety of carcinogenic mechanisms. The confocal microscopy showed the β-catenin (red), EpCAM (green), and DAPI (blue) in HepG2 cells after 1 µg/ml 4-AAQB treatment for 24 h (Fig. 1C). A high level expression of EpCAM in the cell membrane area and β-catenin in the cytoplasm was observed in HepG2 cells. However, after treating the cells with 1 µg/ml 4-AAQB for 24 h, they exhibited less EpCAM as well as β-catenin. In the meantime, the apparent nucleus condensation was observed, as shown in white circle area of Fig. 1C.

From the confocal microscopy images, it can be assumed that 4-AAQB has the ability to inhibit the formation of swelling tumors. Therefore, HepG2 cells (1×10^3) were seeded in 9-cm petri dish, so that each cell could attach and grow. After 10 days, the colony formation of the HepG2 was obviously noticeable. However, the cells treated with 0.1 or 1 µg/ml 4-AAQB ceased to grow after two or three days, even under rich nutritional condition. After 10 days, the colony formation of the 4-AAQB treated cells was obviously less than the untreated cells. In addition, cell shrinkage was observed as compared with the untreated group.

3.2. 4-AAQB inhibits EpCAM + liver cancer stem cells and improves LCSCs tumor immune environment

EpCAM + and EpCAM- HepG2 cells were separated and cultured for 4 days. At the same cell seeding concentration, the EpCAM + HepG2 cells showed a better vitality, more diverse and aggressive than the EpCAM- HepG2 cells (Fig. 2A). As expected, 4-AAQB exhibited significant cytotoxicity to the EpCAM + HepG2 cells. When treated with 1 µg/ml 4-AAQB for 48 h, the viability of the cells decreased by approximately 50%.

Previous reports pointed out that cancer stem cells have the potential to worsen tumor immune environment [26]. Thus, the secretion of immunosuppressive cytokine IL-6 and IL-10 was also assessed in this study, and the potential cancer immunosuppressive agent 5-FU was used for comparison [27,28]. As shown in Fig. 2C and D, the expression of these two inflammatory cytokines in 4-AAQB-treated HepG2 cells were significantly less than the untreated group. 4-AAQB reduced the secretion of IL-6 in EpCAM + HepG2 cells by 42–66% as compared with untreated group, and the IL-10 secretion of the 4-AAQB-treated EpCAM + HepG2 cells was only 55–99% of the untreated group. A dose dependent inhibitory effect of 4-AAQB on IL-6 and IL-10 was also observed. In contrast, there was no significant difference ($p > 0.05$) between the 5-FU treatment group and the untreated group in IL-6 and IL-10 secretion.

3.3. 4-AAQB can promote immune cell proliferation and activation

Besides inhibiting the liver cancer stem cells, 4-AAQB can also enhance immunity. As shown in Fig. 3A, 4-AAQB can stimulate the growth of immune cells such as THP-1 and Raw264.7. It is worth noting that THP-1 is an immortalized cell line which helps reproducibility of findings and safe to use [29]. The average increments at the dose of 1 µg/mL and one-day exposure of 4-AAQB for THP-1 and Raw264.7 cells were 1.56-fold and 1.16-fold higher than the untreated control, respectively. Attempts were also made to use GM-CSF/IL-4 to differentiate THP-1 into iDCs. After 5 days of culture, the THP-1 cells began to form the surface spines and folds, and the number of the iDCs cells was approximately 1.5 ~ 2.0 fold of the THP-1 cells. We have further confirmed that the differentiation was successful because the expressions of CD40, CD80 and CD86 were also increased (data not shown) [24]. Then we cultivated the iDCs with GM-CSF/IL-4 and TNF-α to obtain mature dendritic cells (mDCs). After 10 days, the mDCs developed slender synapses attached to the bottom of the petri dish (Fig. 3B). Thus we have confirmed that the THP-1 cells were indeed differentiated into iDCs.

Dendritic cells are the most potent antigen presenting cells and they are highly associated with innate and adaptive immunity. The iDCs displayed high endocytotic activity, thus the uptake of FITC-labeled 70 kDa dextran by iDCs after 4-AAQB treatment was examined. As

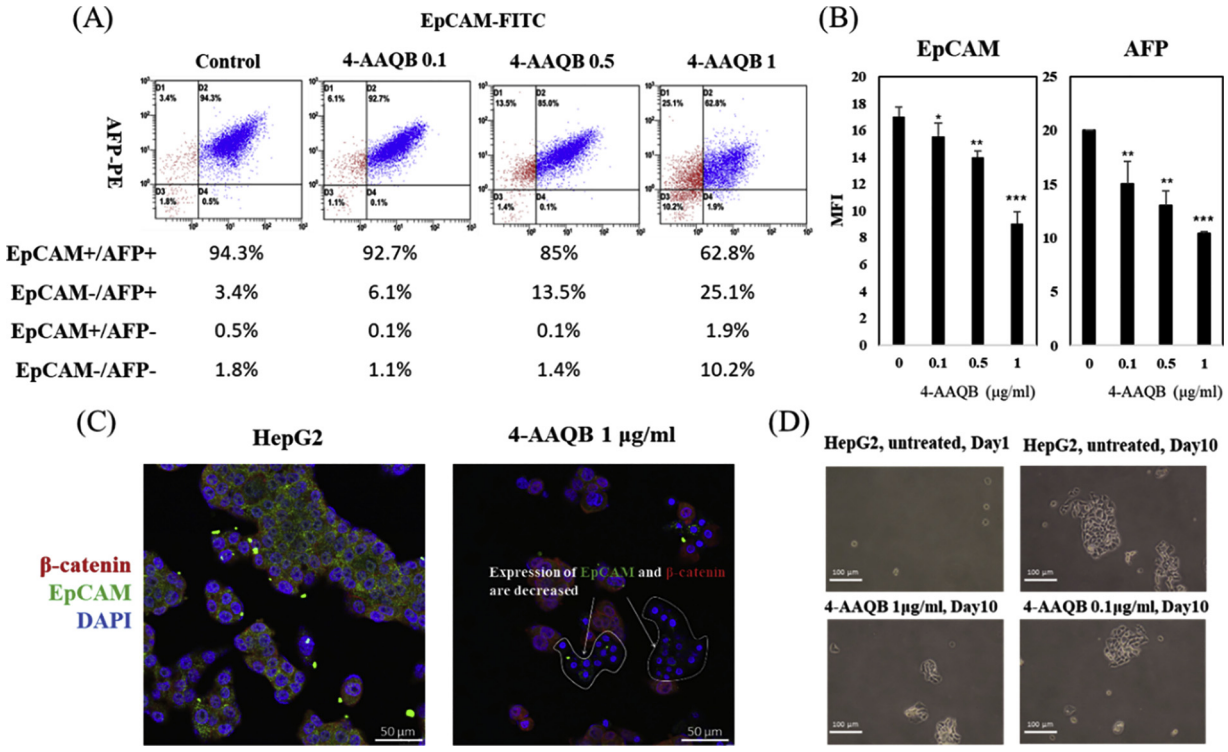

Fig. 1. Effects of 4-AAQB on hepatocellular carcinoma stem cell inhibition. (A) Effects of 4-AAQB on expression of EpCAM and AFP in HepG2 cells. (B) The MFI of EpCAM and AFP of 4-AAQB-treated HepG2 cells. (C) The confocal microscopic view of β-catenin (red), EpCAM (green), DAPI (blue) of HepG2 cells. (D) Colony formation of 4-AAQB-treated HepG2 cells. Data are expressed as mean ± SD (n = 3).*, P < 0.05, **, P < 0.01, ***, P < 0.001, Treated vs. Untreated.

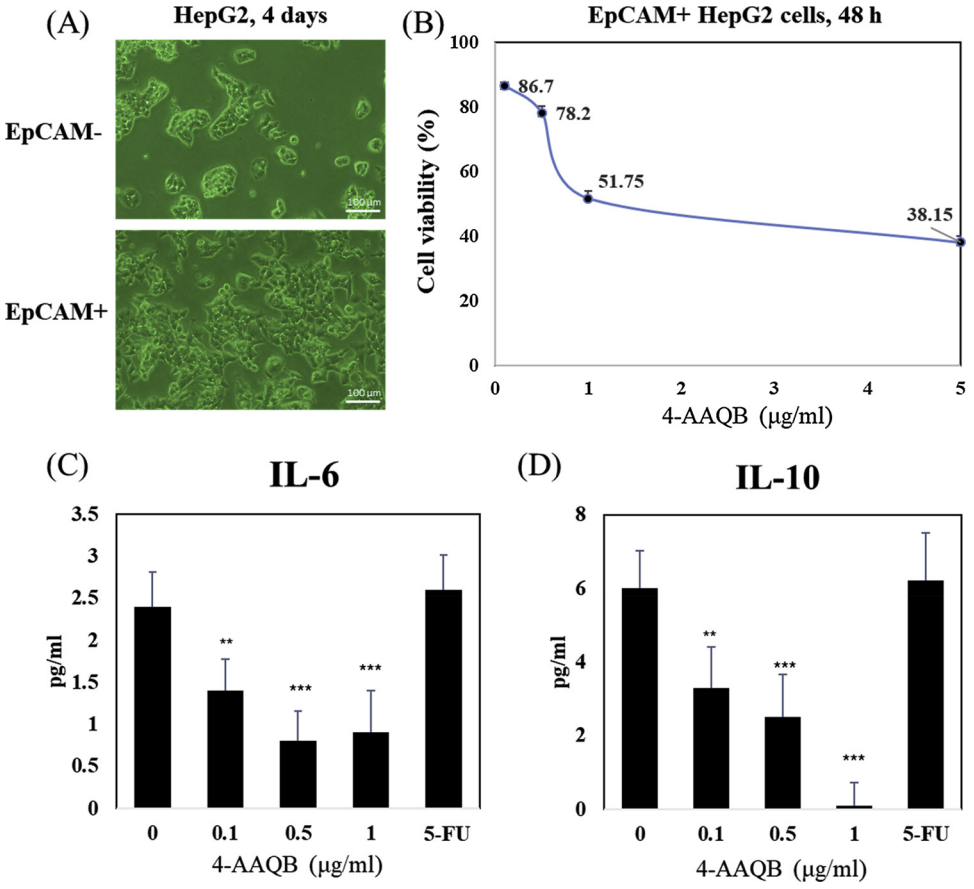

Fig. 2. Effect of 4-AAQB on the growth and the immune escape ability of EpCAM+ HepG2 cells. (A) Effects of 4-AAQB on morphology of EpCAM+/- HepG2 cells after 48 h (light microscopy, 400X) (B) Cell viability of EpCAM+ HepG2 cells following treatment with 4-AAQB for 48 h. (C) IL-6 productions from EpCAM+ HepG2 cells with and without 4-AAQB treatment. (D) IL-10 productions from EpCAM+ HepG2 cells with and without 4-AAQB treatment. Data are expressed as mean ± SD (n = 3).*, P < 0.05, **, P < 0.01, ***, P < 0.001, Treated vs. Untreated.

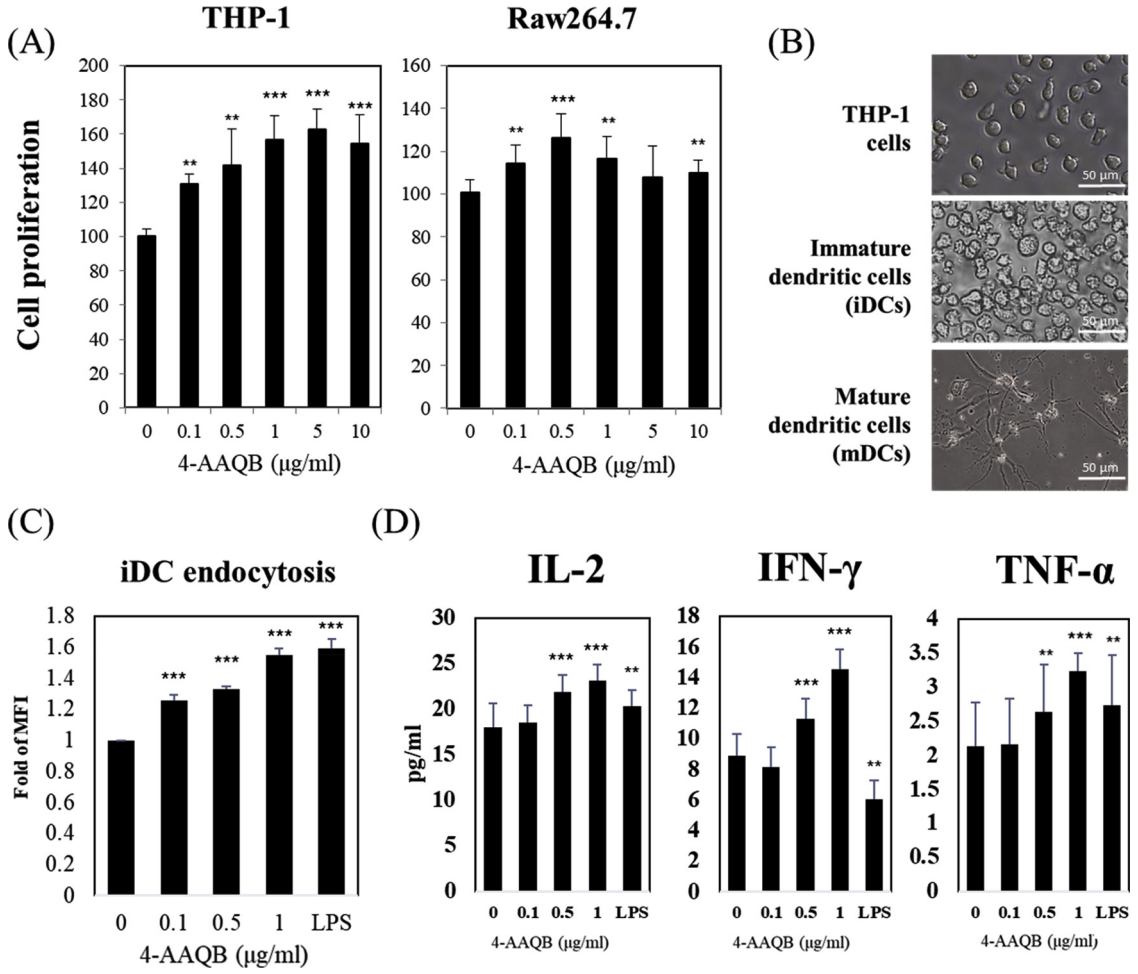

Fig. 3. Stimulatory effects of 4-AAQB on immune function of some immune cells. (A) Proliferation of THP-1 and Raw264.7 cells. (B) Differentiation of human mononuclear cells THP-1 into immature and mature dendritic cells. (C) Endocytosis of FITC-dextran by iDCs with and without 4-AAQB treatment. (D) IL-2, TNF-α, IFN-γ productions from iDCs with and without 4-AAQB treatment. Data are expressed as mean ± SD (n = 3).*, $P < 0.05$, **, $P < 0.01$, ***, $P < 0.001$, Treated vs. Untreated.

shown in Fig. 3C, 4-AAQB can enhance endocytosis of iDCs in a dose dependent manner. When treated with 1 μg/ml 4-AAQB for 24 h, the MFI was 1.55-fold of the untreated group. In addition, we also found that 4-AAQB can stimulate immune-activating cytokine secretion in iDCs. As shown in Fig. 3D, 4-AAQB has led to a significant increase of IFN-γ, TNF-α, and IL-2 secretion. These results revealed that 4-AAQB has a role in promoting the immune system toward Th1 antitumor immune response.

3.4. 4-AAQB activates immune response between dendritic cells and EpCAM + liver cancer stem cells

To further investigate the enhancement effect of 4-AAQB on immune activity, we co-cultured iDCs with EpCAM+ HepG2 cells and treated them with 4-AAQB. It is known that MHC plays an important role for cancer cells in antigen presentation. MHC class I can activate dendritic cell identification, present tumor antigen, and induce specific immune bridge. And MHC class II can directly induce CD4 + T cells' tumor poisoning reaction without dendritic cell activation [30]. Fig. 4A shows the expression of MHC class I and II of EpCAM+ HepG2 cells after co-culture with iDCs. A dose of 1 μg/ml of 4-AAQB increased the MFI of MHC class I from 13.3 cps (untreated control) to 19.1 cps, and increased the MHC class II from 15.8% to 36.9%. In addition, 4-AAQB increased the MHC expression in a dose dependent manner.

Besides EpCAM+ HepG2 cells, we also assessed the expression of MHC class I, II and co-stimulatory molecule (CD80) in iDCs. In line with expectations, the expression of MHC class I, II and CD80 increased with increasing treatment dosage of 4-AAQB in the co-cultured iDCs. As shown in Fig. 4B, the MFI of MHC class II was from 20.2 cps (untreated group) to 27.2 cps (1 μg/ml 4-AAQB) and MHC class I from 6.6% to 14%. The expression of co-stimulatory molecule CD80 also increased from 5.1% (untreated group) to 16% (1 μg/ml 4-AAQB).

IFN-γ and TNF-α can stimulate cellular maturation and exert anti-tumor effect of dendritic cells. Fig. 4C and D show the effect of 4-AAQB on IFN-γ and TNF-α extracellular secretion in the co-cultured medium. We found that 4-AAQB has led to a significant increase of IFN-γ and TNF-α secretion in a dose dependent manner. When cells were treated with 1 μg/ml 4-AAQB, 2.5- and 3-fold increment of IFN-γ and TNF-α were observed as compared with control.

4. Discussion

HCC has been one of the leading cause of cancer death worldwide. One of the main reason that HCC is so serious is its ability of chemotherapy resistance associated with the presence of CSCs. Nowadays, LCSC is regarded as the therapeutic target for the treatment of liver cancer [31]. In this study, we investigated the effect of 4-AAQB on LCSCs and its possible uses in liver cancer immunotherapy. We found that 4-AAQB can inhibit LCSCs and improve the antitumor immune responses via dendritic cells.

EpCAM and AFP are defined as prognostic subtypes of HCC. Yamashita proposed an easy classification system defined by EpCAM

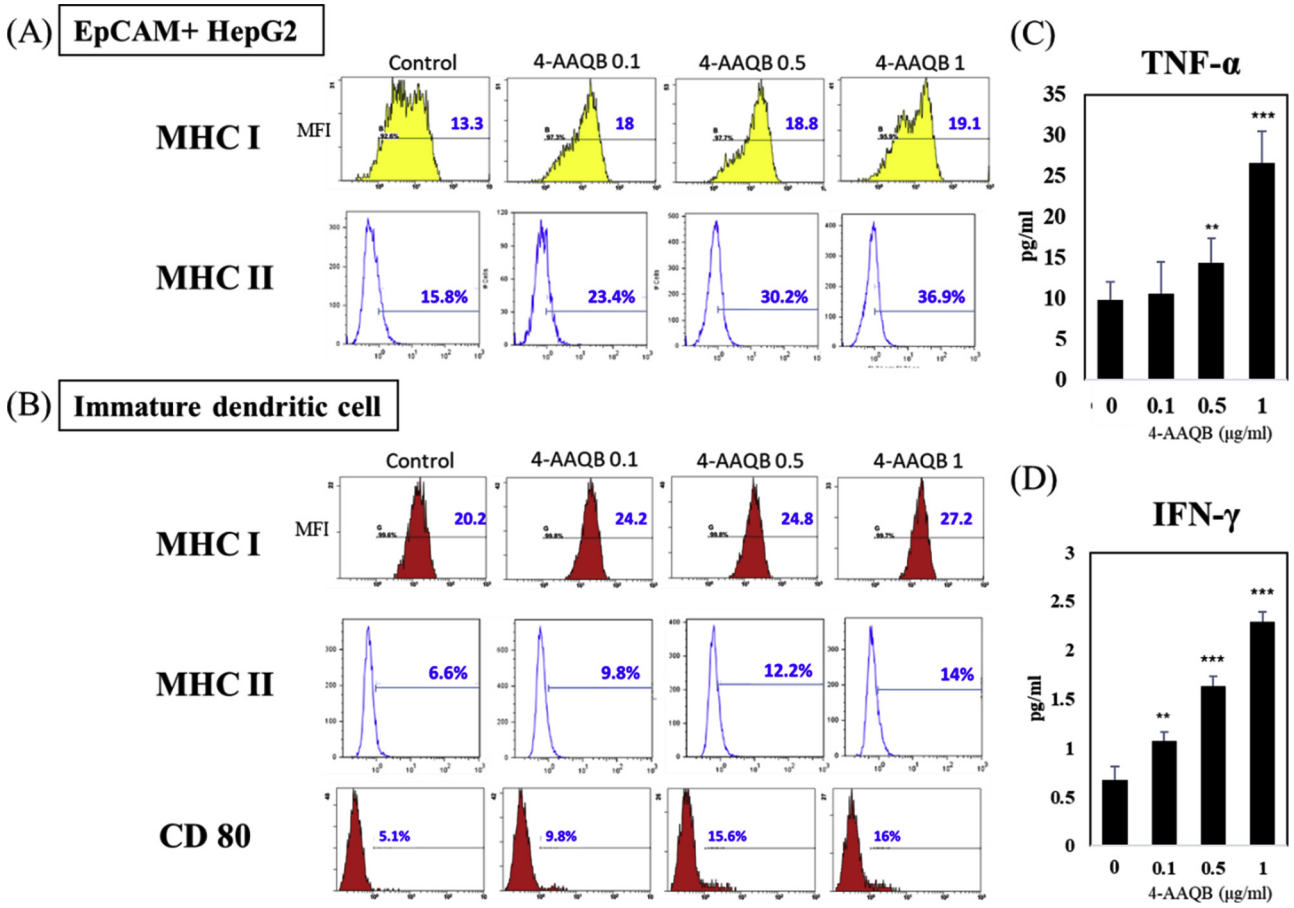

Fig. 4. Activation effect of 4-AAQB on EpCAM+ HepG2 cells co-cultured with iDCs. (A) Expression of MHC class I and II of EpCAM+ HepG2 cells after co-culturing with iDCs. (B) Expression of MHC class I, II and CD 80 of iDCs after co-culturing with EpCAM+ HepG2 cells. (C) Expression of TNF-α in the co-culture of iDCs and EpCAM+ HepG2 cells. (D) Expression of IFN-γ in the co-culture of iDCs and EpCAM+ HepG2 cells. Data are expressed as mean ± SD (n = 3).*, $P < 0.05$, **, $P < 0.01$, ***, $P < 0.001$, Treated vs. Untreated.

and AFP via the analyses of 238 independent HCC cases [1]. Pathway analysis indicates that the molecular biological activities differ significantly between EpCAM + AFP + and EpCAM − AFP+ HCC subtypes. The EpCAM + AFP + cells are generally recognized as stem/progenitor cells of HCC and EpCAM − AFP − as mature cells of HCC. In addition, the activation of Wnt-β-catenin signaling is mainly associated with EpCAM + AFP+ HCC. In Fig. 1A and B, we found that 4-AAQB can inhibit EpCAM and AFP expression. It can be inferred that 4-AAQB may have inhibitory effect on HCC stem cell related genes' expression. Wnt/β-catenin can activate a variety of carcinogenic mechanisms, including cell proliferation, regulation of cell apoptosis, regulation of differentiation and change of liver metabolic mechanism, to make liver cancer cells have more growth advantage [32], and EpCAM can activate β-catenin [1] and induce a variety of stem cell-related genes such as oct4, sox2, klf4 and c-myc [33]. In this study, we confirmed that the decreases of the expression of EpCAM was associated with the decrease of β-catenin by confocal microscopy. After 4-AAQB treatment, β-catenin and EpCAM decreased with the condensation of nucleus and decrease in the size of cell colony. And the development of liver cancer cell essentially stopped due to 4-AAQB treatment (Fig. 1C, D). These results can echo our past findings, i.e. 4-AAQB can inhibit proliferation of HepG2 cells via cell cycle arrest [21]. Besides, another study also showed that 4-AAQB can inhibit Akt, PI3K and reduce metastasis in HCC in xenograft model [22]. We suspect that 4-AAQB may have changed the dynamics of cell cytoskeleton, but this speculation needs further works to confirm.

Cancer stem cells may be the culprit in tumor immune evasion because they have the ability to inhibit anti-tumor immunity [5]. CSCs can express anti-apoptotic proteins such as BCL-2 and BCL-XL to resist apoptosis-inducing immune killing, or secrete Immunosuppressive cytokines to inhibit immune response [6]. In this study, we isolated EpCAM + cancer stem cells from HepG2 cells and showed that 4-AAQB has a potent inhibitory ability against these cells, its IC_{50} is close to 1 ppm. In addition, 4-AAQB significantly changes the morphology and growth characteristics of the EpCAM+ HepG2 cells, as observed under microscopy (data not shown). The immunosuppressive cytokine IL-6 and IL-10 are the key cytokines for the remodeling of tumor microenvironment in cancer stem cells. Tumor cells can survive and block the maturation of DC by autocrine IL-6. Furthermore, IL-6 may suppress Th1 immunity of tumor poisoning. 4-AAQB has significant inhibitory effect on IL-6 and IL-10 of EpCAM+ HepG2 cells at very low dose, 0.1 μg/ml. In contrast, the common cancer chemotherapy drug 5-FU at dose of 10 μg/ml can neither suppress the expression of IL-6 nor the IL-10. This finding further suggests the value of 4-AAQB.

Immunity is very important for cancer patients. Many functional molecules, like polysaccharides from *Ganoderma lucidum*, have been proven to be able to induce human monocytic leukemia cells into dendritic cells with immuno-stimulatory function. In this study, we found that 4-AAQB could enhance endocytosis of iDCs, same as *Ganoderma lucidum* polysaccharides [34,35]. Then we tested 4-AAQB on different kinds of immune cells, and confirmed that 4-AAQB can stimulate the proliferation of immune cells, including monocyte (THP-1) and macrophage (Raw264.7). Additionally, we have also observed an increase in immune-related cytokines secretion by iDCs, including IL-2, TNF-α, IFN-γ, due to 4-AAQB treatment (Fig. 3). IL-2 can phosphorylate STAT5 to drive IFN-γ production and activate dendritic cells

[36]. IFN-γ and TNF-α, which bring about Th1 polarization, are now clinically used in monocyte-derived dendritic cells in cancer therapy [37]. The results of this study suggested that 4-AAQB can not only suppress the liver cancer stem cells but also enhance the compromised immunity caused by liver cancer stem cells.

Immunotherapy for cancer has developed rapidly in recent years. It is known that DCs in cancer patients may be dysfunctionally regulated [38]. On the other hand, immunotherapy using DC-based vaccination is an approved approach to enhance patients' own immune system to eliminate cancer cells [39]. But some clinical responses were still not as expected [40], probably because of immunosuppressive microenvironment caused by tumor, and the stimulatory DCs are reprogrammed to become regulatory DCs. However, recent research has developed dendritic cell vaccines for targeting CSCs. The CSCs vaccines have shown significant antitumor ability, such as decreasing metastasis [26] and increasing secretion of tumor immune response cytokines [41]. Results of this study showed that, in the co-culture of iDCs and EpCAM+ HepG2 cells, 4-AAQB can enhance the expression of MHC class I, II of both iDCs and EpCAM+ HepG2 cells (Fig. 4). The increased expression of MHC class I, II indicated that 4-AAQB can enhance the antigen presenting function of these cells. In addition, when the MHC class II on the surface of the dendritic cell interact with the receptor on T cells, the CD80 on dendritic cells would also interact with the CD28 receptor on the T cells, and activate T cells effectively [42]. Without further stimulation by CD80, the T cell becomes impotent. This study indicated that 4-AAQB can increase the expression of CD80 in iDCs. This was also confirmed by extracellular secretion of IFN-γ and TNF-α. Both of these cytokines can increase the expression of MHC class II, CD40, CD80, and secretion of IL-12, and prolong the survival time of iDCs [43,44]. The aforementioned mechanisms may explain how 4-AAQB promotes the poisoning of liver cancer cells.

In conclusion, this study explored the effects of 4-AAQB on three aspects of HCC. This bioactive compound can inhibit EpCAM + AFP + cancer stem cells, increase immune activity as well as upregulate antigen presentation of DCs. Thus, 4-AAQB can enhance immune function of DCs by increasing the antigen presenting ability of these cells. Thus 4-AAQB may have potential to be used in HCC immunotherapy.

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