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Original Research

Cancer-associated fibroblasts induce immunotherapy resistance in hepatocellular carcinoma animal model

LiBao Yu[1#], Qiyu Liu[2#], Jiege Huo[3], Furong Wei[4], Wei Guo[5*]

[1] Department of Hepatobiliary Surgery, the 309th Hospital of Chinese People`s Liberation, Army, Beijing, China
[2] Ganmei Affiliated Hospital of Kunming Medical University the First People's Hospital of Kunming, Kunming, Yunnan, China
[3] Department of Oncology, Affiliated Hospital of Integrated Traditional Chinese and Western Medicine, Nanjing University of Chinese Medicine, Nanjing, Jiangsu, China
[4] Department of Gastroenterology, Central Hospital of Haining, Haining, Zhejiang, China
[5] Department of General Surgery, the Ninth People's Hospital of Chongqing, No. 69, Jialing village, Beibei District, Chongqing, China

*Correspondence to: drcorespond@gmail.com

# They contributed equally to this work.

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Abstract: Hepatocellular carcinoma is known to be a common predominant cancer in adults, especially in eastern countries. Immune response and cancer-associated fibroblasts (CAFs) have significant influences on tumor development. However, the interaction between CAFs and immunotherapy is unclear in hepatocellular carcinoma. We measured the number of activated fibroblasts in hepatocellular carcinoma samples and samples taken from normal liver tissues. A total of 20 patients' fresh hepatocellular carcinoma and normal tissues which were surrounding the tumor were obtained from the surgery and used for evaluating alpha-SMA expression. We investigated the effects of CAFs in anti-tumor immunity in hepatocellular carcinoma animal model. The effects of CAFs in inducing anti-PD-1 treatment resistance were also measured in a preclinical animal model. Activated fibroblasts were highly accumulated in hepatocellular carcinoma tissues but not in surrounding normal tissues. CAFs showed a significant tumor-promoting effect in an immunocompetent model. The infiltration and function of some immune cells like myeloid-derived suppressive cells and T-cells were increased by CAFs. CAFs also reduced the number and activation of tumor-infiltrating cytotoxic T-cell in tumor tissue. In the treatment model, tumors with a higher amount of CAFs had been insensitive to therapy with anti-PD-1. CAFs are potent inducers of immunosuppression in hepatocellular carcinoma. Depleting CAFs rescued the antitumor immunity in the hepatocellular model and could be a novel treatment to combine with the existing immunotherapy.

Key words: Hepatocellular carcinoma; Cancer-associated fibroblasts; Immune response; Anti-PD-1 treatment; Preclinical model.

Introduction

Hepatocellular carcinoma is known to be one of the most common malignant tumors in Asian counties (1). The introduction of combinational chemotherapy and surgery for localized hepatocellular carcinoma may increase the survival rate of patients (2). However, the cure rate for patients with the metastatic or relapsed disease remains dismal with short long-term survival (2). Hence, understanding the mechanisms of hepatocellular development is urgent.

Tumors are communities of malignant cells as well as surrounding stromal cells, as well as fibroblasts and infiltrating immune cells (3). The significance of immune cells in determining the cancer patient's survival and treatment has been widely studied (4, 5). Recently, the US FDA has approved the immunotherapies, such as immune checkpoint blockades, to treat several types of tumors, including melanoma, lung cancer, and renal cancer (6-8). However, immune checkpoint blockades' efficiency is determined by the overall immune cell function, which can be regulated by non-immune cells, such as cancer-associated fibroblasts (CAFs) (9).

CAFs in cancerous tissues are as like as myofibroblasts in morphology (10). Functionally, CAFs are perpetually activated in cancer tissue and don't undergo apoptosis-like non-cancerous fibroblasts (10). Designing efficient medications for cancers needs more knowledge about CAFs. Herein, we investigated the immunoregulatory roles of CAFs in a hepatocellular carcinoma model.

Materials and Methods

Cell culture and transfection

Murine hepatocellular carcinoma cell line H22 was received from the Chinese Academy of Sciences (Shanghai, China) Cell Bank. Cell lines were cultured in DMEM medium (Thermo Fisher Scientific, IL, USA) comprising 5% fetal bovine serum (FBS), 100 mg/ml streptomycin, and 100 U / ml penicillin in a 5% CO2 incubator at 37 ° C humidified. At a confluence of 70 percent of the growing cell, layer subculture took place. Main cancer-associated fibroblasts (CAFs) have been derived from hepatocellular fresh H22 murine tissue. Standard hepatic stellate cells (HSCs) were isolated

from the liver of BALB / c mice. A procedure that was previously published was used to separate and classify CAFs14. The CAFs were medium cultivated in DMEM and subcultured to ten lines.

Animal model

A syngeneic animal model was developed using 6-week old female BALB / c mice (21-23 g, Shanghai SLAC Laboratory Animal Center at Chinese Academy of Sciences, China) and H22 cells to examine the in vivo immunoregulatory function of CAFs. Each mouse was injected on the hind legs flank with 2 x 105H22 cells with or without 8 x 105CAFs or 8 x 105 HSCs. Each group included ten mice. The productivity of the tumors was tracked every five days. The size of the tumor was determined on the basis of the generally known formula: tumor volume=length * width2 *π/6. For the orthotopic model, the same number of cells were injected. The detailed procedure was reported previously (11). The animal work was approved by the local Animal Care and Use Committee. Every mouse was kept in a specific pathogen-free area with free exposure to autoclaved water, regular food, and a day and night period of 12 hours. The treatment plan for each experiment was included in the corresponding Figure legend.

Flow cytometry

A study of the flow cytometry was used in an experimental model to analyze the immune infiltration. $CD8^+$ T cells ($CD19^-$, $CD3^+$, $CD4^-$, and $CD8^+$), regulatory T cells (Treg, $CD19^-$, $CD3^+$, $CD4^+$, $CD8^-$, $CD25^+$ and $FOXP3^+$), helper T-cells (Th1: $CD19^-$, $CD3^+$, $CD4^+$, $CD8^-$, and IFN-γ^+, Th2: $CD19^-$, $CD3^+$, $CD4^+$, $CD8^-$, and $IL4^+$), and myeloid-derived suppressive cells (MDSCs, $CD45^+$, $CD11b^+$, and $Gr1^+$) were classified and analyzed. We isolated single cells from animal tumor tissues and washed them with PBS once. To extract red blood cells the red blood cell lysis buffer was applied. Cells were then washed with PBS once and resuspended in blocking buffer for 10min. Cell membrane staining was then performed, and cells were incubated 15min at room temperature. After cell membrane staining, cells were fixed by fixation/permeabilization buffer for 30min at room temperature. The cytoplasm proteins were then stained at room temperature for 30min. We used the FACSCanto II equipment (Becton Dickinson and Company, San Jose, CA) for data acquiring. Flow Jo software was used to visualize the data.

Patient sample

A total number of 20 hepatocellular carcinoma tissues and 20 tumor-adjacent normal liver tissues were included in this study. These patients were diagnosed from January 2016 to December 2016. All the tissues were obtained during the surgery before chemotherapy or radiotherapy. This study was approved by the local ethics committee. All patients assigned written informed consent.

Western blotting

The protein content of fresh human tissue and cell lysates was calculated utilizing the BCA (Thermo Scientific) protein measurement test. Anti-smooth muscle actin antibody (1:1000 dilution, Abcam) was used for Western blotting to test the rates of various proteins in the lysates. Beta-actin (Abcam, 1:2000 dilution) has been used as a charge buffer. The general western blotting technique was implemented. Pierce ECL Western Blotting Substrate (Thermo Scientific) was also used to generate the signals.

Statistical analysis

Graph Pad software (CA, USA) was used for statistical analyses and data visualization. The data were shown as mean ± SEM. One-way ANOVA was used to analyze the difference of means between more than two different groups. T-test was performed for two-group comparison. Differences with a two-tailed P-value<0.05 were considered as statistically significant.

Results

Activated fibroblasts are accumulated in hepatocellular carcinoma

A total of 20 patients' fresh hepatocellular carcinoma and surrounding normal tissues were obtained from the surgery and used for evaluating alpha-SMA expression. The hepatocellular carcinoma tissues showed higher alpha-SMA expression than tumor-adjacent normal tissues (Figure 1A and B). Alpha-SMA is expressed in activated fibroblasts, but not in quiescent fibroblasts. Thus, our data suggested that CAFs, a subtype of activated fibroblasts were accumulated in hepatocellular carcinoma.

CAFs induced immunosuppressive cells accumulation in tumor tissue

Flow cytometry quantified the major innate immune cell types in tumor tissues. The gating strategy of myeloid-derived suppressive cells (MDSCs) has been shown in Figure 2A. The number of tumor-infiltrating MDSCs was highest in tumors containing exogenous CAFs (Figure 2B). However, the frequency of tumor-infiltrating macrophages and dendritic cells (DCs) were not changed by the exogenous CAFs (Figure 2E and F). The immunosuppressive cytokines, IL-10 and PD-L1, were also accumulated in tumor-infiltrating MDSCs from the tumors with a high amount of exogenous CAFs (Figure 2C and D). When dasatinib, the functional inhibitor of

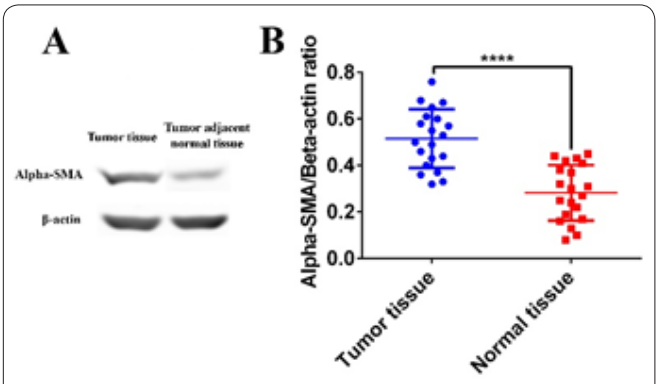

Figure 1. CAFs in hepatocellular carcinoma. (A)alpha-SMA expression in the representative case of hepatocellular carcinoma and surrounding normal liver tissues. (B) Quantification of alpha-SMA expression in 20 hepatocellular carcinomas and surrounding normal liver tissues. The data were normalized to β-actin expression. (**** P-value < 0.0001).

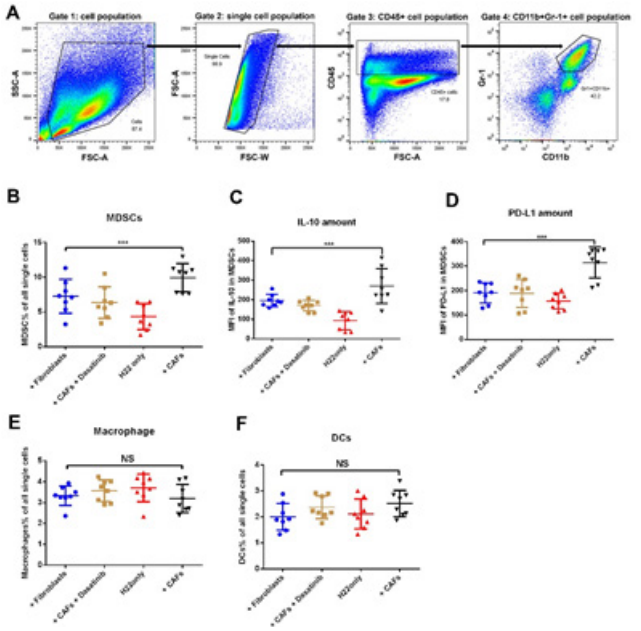

Figure 2. Effects of CAFs on MDSCs infiltration in hepatocellular carcinoma model. (A)The flow cytometry gating plots of MDSCs. (B)Quantification of MDSCs number in tumor tissues with and without exogenous CAFs. (C & D) Expression of IL-10 and PD-L1 in MDSCs. (E & F) Quantification of macrophages (E) and DCs (F) number in tumor tissues with and without exogenous CAFs. (n=8 in each group). For the groups with dasatinib treatment, CAFs were pre-incubated with dasatinib (0.5uM) for 48h before injected with tumor cells. (NS: None-significance, * P-value < 0.05, ** P-value < 0.001, and *** P-value < 0.001).

CAFswas administrated, the immunosuppressive effects of CAFs were neutralized.

CAFs reduced T-cell infiltration but enhanced Treg accumulation in tumor tissue

T-cell infiltration and phenotype are key factors of antitumor immunity. We found that tumors with exogenous CAFs had less $CD8^+$ T-cell (Figure 3B). We then investigated the subtype of $CD4^+$ T-cell: T-helper cells (Th1 and Th2) and regulatory T-cell (Treg) in the tumor tissue. The frequency of tumor-infiltrating Th1 and Th2 cells was very close in all groups (Figure 3C and E). However, the Tregwas accumulated in tumors with exogenous CAFs (Figure 3D). However, when the function of CAFs was inhibited by dasatinib, the number of tumor-infiltrating T-cells was also rescued. These data suggested that CAFs significantly influenced the quantity and phenotype of T-cell.

CAFs suppressed tumor-infiltrating T-cell function

We further measured the function of tumor-infiltrating T-cell in each group. The expression of IFN-γ and granzyme B by $CD8^+$ T-cell was significantly inhibited by exogenous CAFs (Figure 4A and B). However, dasatinib neutralized the inhibitory effects of CAFs on IFN-γ and granzyme B expression (Figure 4A and B).

Inhibition of functional CAFs sensitized anti-PD-1 treatment in hepatocellular carcinoma

Since we showed that CAFs were able to induce immunosuppression in the tumor microenvironment and inhibition of CAFs function can neutralize this effect,

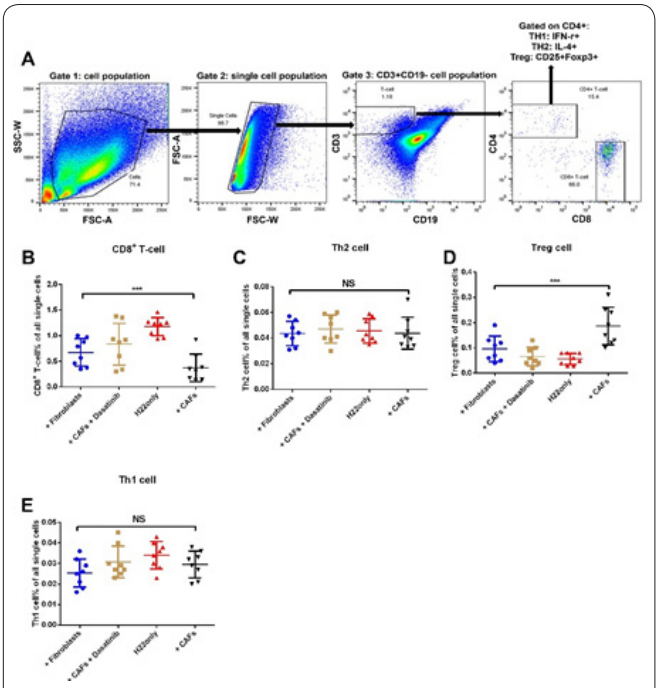

Figure 3. Effects of CAFs on T-cell infiltration. (A)The flow cytometry gating plots of total T-cell and subtypes. (B)The frequency of tumor-infiltrating $CD8^+$ T-cell was increased by down inhibiting the function of CAFs. (C & E) The frequency of tumor-infiltrating Th cell was not influenced by exogenous CAFs. (D)The frequency of tumor-infiltrating Teg cells was increased by exogenous CAFs. (n=8 in each group). For the groups with dasatinib treatment, CAFs were pre-incubated with dasatinib (0.5uM) for 48h before injected with tumor cells. (NS: None-significance, ** P-value < 0.001, *** P-value < 0.001, and **** P-value < 0.0001).

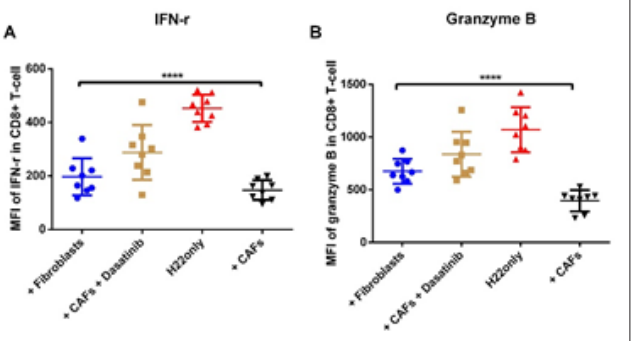

Figure 4. IFN-γ and granzyme B expression on T-cell. Exogenous CAFs significantly reduced IFN-γ and granzyme B expression on tumor-infiltrating T-cell. The inhibiting function of CAFs rescued IFN-γ and granzyme B expression. For the groups with dasatinib treatment, CAFs were pre-incubated with dasatinib (0.5uM) for 48h before injected with tumor cells. (*** P-value < 0.001, and **** P-value < 0.0001).

we further investigated the therapeutic role of dasatinib in hepatocellular carcinoma. As shown in Figure 5A, the administration of dasatinib delayed tumor growth in tumors with a high number of CAFs. In an orthotopic model, we found that anti-PD-1 treatment alone didn't dramatically increase mice survival time. However, when dasatinib was combined, the survival time was dramatically increased (Figure 5B).

Discussion

There is abundant evidence indicating that natural

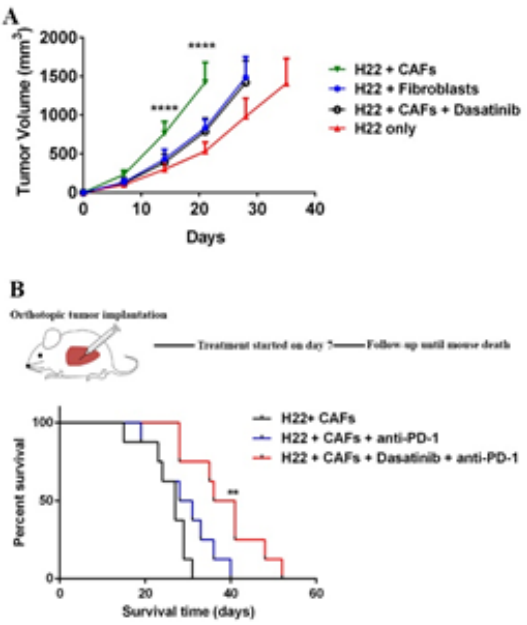

Figure 5. *CAFs functional inhibitor in combination with anti-PD-1.* (A) Exogenous CAFs significantly accelerated tumor development *in vivo*. Mice were treated with dasatinib (1.25mg/kg) twice a week until the observation endpoint. (B) In an orthotopic hepatocellular carcinoma model, inhibition of CAFs' function synergized with anti-PD-1 treatment. Mice were treated with anti-PD-1 (0.2mg/kg) and dasatinib (1.25mg/kg) twice a week until death. (** P-value < 0.01, and **** P-value < 0.0001).

and/or therapy-induced antitumor immune responses dictate a better prognosis for patients across diverse histological types of neoplasia (12). The function of the cytotoxic T-cell, the major immune cell type that kills tumor cells, is affected by both immune and non-immune factors (9). CAFs are characterized by unchecked pro-fibrotic and pro-inflammatory signaling, which can suppress T-cell function (13). In the present study, we aimed to understand the roles of CAFs activation on hepatocellular carcinoma anti-tumor immune response.

In our study, we first measured the amount of CAFs in hepatocellular carcinoma tissues and compared with the adjacent normal tissues. In line with the previous report (14), the amount of activated fibroblast (alpha-SMA positive) was much higher in tumor tissue than in the normal liver. These observations provided the rationale to study the effects of CAFs on immunoregulation and targeting CAFs in hepatocellular carcinoma.

Innate immune cells are major cell types of the tumor microenvironment. We investigated the frequency of tumor-infiltrating macrophages, DCs, and MDSCs in tumors with and without functional exogenous CAFs. We found that MDSC is the only cell type that was affected by functional CAFs in the tumor microenvironment. MDSCs are potent suppressors of anti-tumor immunity and significant impediments to cancer immunotherapy (15). We noticed that exogenous CAFs significantly enhanced MDSCs tumor infiltration. The immunosuppressive phenotype of MDSCs was also enlarged with higher IL-10 and PD-L1 expression. Dasatinib is a functional inhibitor of activated fibroblasts (16). When the activation of CAFs was inhibited by dasatinib, the immunosuppressive phenotype of MDSCs was diminished.

T-cell infiltration and classification in tumor tissue are major indicators of antitumor immunity (17). Via flow cytometry, we systematically studied the infiltration and classification of T-cell in the hepatocellular carcinoma model. The number of $CD8^+$ T-cell in tumor tissue was decreased by exogenous CAFs. However, the number of Treg in tumor tissue was enlarged by exogenous CAFs. This data suggested that CAFs induced immunosuppression via excluding cytotoxic T-cell and accumulating Treg in tumor tissue. This is in line with the previous study in gastric cancer that activated CAFs can shift the ratio of cytotoxic T-cell to Treg (18). We also checked the functional markers of T-cell, IFN-γ and granzyme B, which are downregulated by functional CAFs as well. These data strongly supported that functional CAFs are immunosuppressive in hepatocellular carcinoma and reversing CAFs activation may release the suppression.

The immune checkpoint blockades have achieved impressive effects in melanoma patients (19, 20). However, when tested in solid tumors, the immune checkpoint blockades alone showed limited efficacy. This is partly due to the immunosuppressive microenvironment in solid tumors, such as hepatocellular carcinoma (21). Combinatory immunotherapy has been widely tested in clinical trials (22). Here, we showed that inhibiting the activation of CAFs could reduce exogenous CAFs mediated hepatocellular carcinoma growth. More importantly, the efficacy of anti-PD-1 treatment was enhanced by dasatinib treatment. These data highlighted the clinical value of targeting CAFs in hepatocellular carcinoma.

In conclusion, our study indicated that activated CAFs promoted hepatocellular development via inducing strong immunosuppression. Inhibition of activated CAFs released the immunosuppression in the tumor microenvironment and thus might be a promising target for combining with immunotherapies.

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Conflict of interests

None.

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