

Fibroblast Activation Protein- α -Positive Fibroblasts Promote Gastric Cancer Progression and Resistance to Immune Checkpoint Blockade

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Gastric cancer (GC) is one of the main causes of cancer death. The tumor microenvironment has a profound effect on inducing tumor growth, metastasis, and immunosuppression. Fibroblast activation protein- α (FAP) is a protein that is usually expressed in fibroblasts, such as cancer-associated fibroblasts, which are major components of the tumor microenvironment. However, the role of FAP in GC progression and treatment is still unknown. In this study, we explored these problems based on GC patient samples and experimental models. We found that high FAP expression was an independent prognosticator of poor survival in GC patients. FAP⁺ cancer-associated fibroblasts (CAFs) promoted the survival, proliferation, and migration of GC cell lines in vitro. Moreover, they also induced drug resistance of the GC cell lines and inhibited the antitumor functions of T cells in the GC tumor microenvironment. More importantly, we found that targeting FAP⁺ CAFs substantially enhanced the antitumor effects of immune checkpoint blockades in GC xenograft models. This evidence highly suggested that FAP is a potential prognosticator of GC patients and a target for synergizing with other treatments, especially immune checkpoint blockades in GC.

Key words: Gastric cancer (GC); Fibroblast activation protein (FAP); Prognosis; Tumor progression; Immune checkpoint blockade

INTRODUCTION

Although significant progress has been made in gastric cancer (GC) treatment and reduction of incidence and mortality has been observed in past decades^{1,2}, GC is still a cancer at the top of the list for incidences with an estimate of 723,100 deaths in 2012². The incidence of GC fluctuates among different regions, showing a higher incidence in Eastern Asia, Central and Eastern Europe, and a lower incidence in North America². Risk factors of GC include *Helicobacter pylori* infection, dietary factors (such as high-salt food), tobacco, and obesity, among others³. So far, multiple active treatment strategies are available for GC patients, including surgical approaches, radiotherapy, and chemotherapy⁴. For early stage GC, surgical approaches could achieve a better prognosis^{5,6}; however, for advanced GC patients,

only compromised effects were observed in surgery and chemotherapy due to a large tumor burden, drug resistance, recurrence, and metastasis^{7,8}.

The tumor microenvironment is critical in cancer development and treatment. It is composed of noncancerous cells (fibroblasts, immune cells, and endothelial cells) and an extracellular matrix (ECM). Accumulating evidence has suggested that the tumor microenvironment plays multiple supportive roles for tumor cells through its various components^{9,10}. Fibroblasts are the major cellular members in the tumor microenvironment and the primary source of ECM. Fibroblasts are usually quiescent under normal conditions and are only activated when tissue needs remodeling or healing¹¹. After healing the tissue, the activated fibroblasts undergo apoptosis and are eliminated from the tissue. Unlike in normal conditions,

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fibroblasts in cancers, referred to as cancer-associated fibroblasts (CAFs), remain active in the tumor tissue, and they benefit tumor progression via remodeling ECM, secreting soluble factors, and regulating tumor cell motility, stemness, and metabolism^{10,12,13}.

Fibroblast activation protein- α (FAP) is a membrane protein that is expressed in fibroblasts, especially the reactive CAFs. FAP is a homodimeric integral membrane gelatinase belonging to the serine protease family. It is thought to be involved in the control of fibroblast growth or epithelial–mesenchymal interactions during epithelial carcinogenesis. In pancreatic ductal adenocarcinoma, it has been shown that FAP⁺ CAFs can induce immune suppression in both a xenograft mouse model and spontaneous models^{14,15}. However, the role of FAP⁺ CAFs in GC is still unclear. Here we aimed to explore the role of FAP⁺ CAFs in GC progression as well as its potential influence in antitumor immunity in GC.

MATERIALS AND METHODS

Cell Culture

In this study, we used one human GC cell line (AGS) and one murine GC cell line (424GC). Cell line AGS was purchased from ATCC (Manassas, VA, USA); the 424GC cell line was obtained as a gift from Kammerer's lab¹⁶. These two cell lines were cultured in RPMI-1640 supplemented with 2 mM L-glutamine, 10% heat-inactivated fetal bovine serum, 100 U/ml of penicillin, and 100 μ g/ml of streptomycin. In addition, two kinds of CAFs were isolated from human GC tissue and murine xenograft GC tissue through the outgrowth method as described previously¹⁷. The CAFs were also cultured in RPMI-1640 with the same supplements as cancer cells. All cells were grown in a humidified incubator with 5% CO₂ at 37°C.

Patient Samples

The study included 105 GC patients who were diagnosed between April 2005 and June 2009 in 82nd Hospital of PLA, P.R. China. Informed consents were signed by the patients or their legal representative. The study was approved by the local ethics committee of the 82nd Hospital of PLA. Formalin-fixed, paraffin-embedded (FFPE) GC tissues were collected at surgery. No patients had accepted chemotherapy or radiotherapy before the surgery. Clinicopathological information was collected from the archive of the 82nd Hospital of PLA. TNM classification was based on the UICC TNM classification criteria. The histological grade of these samples was determined based on the World Health Organization (WHO) criteria for GC. Follow-up of each patient started from the date of surgery and ended in January 2014, performed by phone call or personal visit. Overall survival (OS) time was calculated by subtracting the date of surgery from the date

of death. Patients who died due to reasons other than GC were removed from the study.

Cell Viability

Cell counting kit-8 (CCK-8; Sigma-Aldrich, St. Louis, MO, USA) was used to measure cell viability. An equal number of cells (10⁴/well) were seeded into 96-well plates and cultured with 100 μ l of complete medium for 24 h. The cells were then treated with different agents or vehicles. Finally, CCK-8 solution (10 μ l) was added to each well for incubation for 1 h. The absorbance at 450 nm was measured on an MRX II microplate reader (Dy nex Technologies, West Sussex, UK).

GC Xenograft Mouse Model

To explore the role of FAP in vivo, we created a xenograft mouse model using the 424GC cell line and C57BL/6 mice (20–22 g; 6 weeks old; female; Shanghai SLAC Laboratory Animal Co., Ltd.). 424GC cells alone (1 \times 10⁶) or 424GC cells (1 \times 10⁶) plus CAFs (4 \times 10⁶) were injected into the flanks of each mouse subcutaneously. Treatment started 1 week after tumor cell inoculation. The FAP inhibitor (FAPi; linagliptin; 2 mg/mouse)¹⁸ or anti-PD-1 antibody (α -P; 250 μ g/mouse) was injected weekly into the tail vein until the end point of the observation. Tumor volume was measured every 7 days and calculated using the formula: length \times width² \times π /6. All mice were raised in a specific pathogen-free environment with a 12-h light–dark cycle, with free access to clean water and standard food.

Collagen Staining

The amount of collagen was measured in tumor tissues via Sirius red staining. Tissue sections were dewaxed and hydrated. Then sections were stained in picosirius red for 1 h, washed in two changes of acidified water, dehydrated in three changes of 100% ethanol, and mounted in resinous medium. Results were captured in bright-field microscopy.

Cytokine Analysis

Cytokine analysis [for interleukin-2 (IL-2), IL-4, IL-10, IL-13, IL-17, interferon- γ (IFN- γ), tumor necrosis factor- α (TNF- α), PD-L1, and PD-L2] was performed using magnetic Luminex screening assay (R&D Systems; Minneapolis, MN, USA). GC tissues from the xenograft mice were minced into small pieces and then digested by collagenase into cell suspensions. Subsequently, RIPA buffer was used to extract the proteins from these cell suspensions. Protein concentration was tested using a BCA protein assay. The samples were then ready for the bead-based assay, and all the procedures were performed following the manufacturer's instruction. Each sample was measured three times, and the mean was calculated as the final result.

Immunostaining for Paraffin Sections

Immunohistochemistry (IHC) was performed to detect the expression of FAP protein in GC patient tumor tissue, and immunofluorescence (IF) staining was used to detect the expression of CD3 and FAP in GC xenograft mouse tumor tissue. The primary antibodies of FAP (1:100 dilution) and CD3 (1:100 dilution) were purchased from Boster Biological Technology (Pleasanton, CA, USA) and Abcam (Cambridge, MA, USA). Standard procedures of both IHC and IF were followed. Tissue samples were incubated with primary antibodies under 4°C overnight. The grade of FAP expression was determined following the principle: 1 for <30% positive, 2 for 30%–60% positive, and 3 for >60% positive. Each slide was evaluated by two people without knowing the clinical data.

Enzyme-Linked Immunosorbent Assay

Enzyme-linked immunosorbent assay (ELISA) was performed to measure the granzyme B and IFN- γ levels in T cells. T cells were harvested after being cultured with conditioned medium (CM) of CAFs, followed by lysing with RIPA buffer containing the protease inhibitor. All procedures were conducted according to the manufacturer's instructions (Abcam).

Fluorescence-Activated Cell-Sorting Analysis

Fluorescence-activated cell-sorting (FACS) analysis was conducted following the standard procedures. In short, for membrane markers, cells were washed and stained for 15 min on ice. For intracellular proteins, cells were fixed and permeabilized after membrane staining, and then stained intracellular markers were placed on ice for 15 min. Primary antibodies of FOXP3, CD markers (CD19, CD3, CD8, CD8, CD4, and CD25), and FAP were purchased from BioLegend (San Diego, CA, USA). BrdU incorporation was detected with the BD Pharmingen™ BrdU Flow Kits (BD Biosciences, San Jose, CA, USA), according to the manufacturer's instructions. All samples were analyzed on BD FACSCANTO II flow cytometers (BD Biosciences). Data were visualized with FlowJo software.

Conditioned Medium Production

The CM of CAFs was produced using FBS-free basal media. One hundred percent confluent FAP⁺ CAFs or FAP⁻ CAFs were cultured in FBS-free basal media for 48 h. The resulting CM was centrifuged for 10 min at 1,000 rpm after collection and stored at -80°C for no more than 2 months before use.

Invasion Assay

Tumor cell invasion was measured by counting the number of tumor cells that invaded through the Matrigel-precoated Transwell chambers with 8- μ m pores (BD Biosciences). GC tumor cells (AGS/424GC, 1.5×10^5 each)

were placed on top of the inserts. CAFs (FAP⁺ CAFs or FAP⁻ CAFs, 1.5×10^5 each) in FBS-free media were added on the bottom chamber. FBS-free media were added to the negative control group on the bottom. For positive control, 10% FBS media were added to the bottom. After incubation for 24 h, the invaded cells were fixed with 70% ethanol, stained with crystal violet, and counted in five random fields under a light microscope. Each experiment was repeated twice.

T-Cell Activation Assay

Human peripheral blood monocytes (PBMCs) and mouse spleen tissue were used as the source of T cells. Dynabeads™ Untouched™ Human T Cells Kit and Dynabeads® Untouched™ Mouse T Cells Kit were used to isolate T cells from human PBMCs and mouse spleen. Then T-cell purity was checked by FACS analysis. Finally, mouse and human Dynabeads® T-Activator CD3/CD28 for T-Cell Expansion and Activation was used to induce T-cell activation. T-cell proliferation and granzyme B were used as the T-cell activation markers.

FAP Gene Knockdown

We knocked down the FAP gene (Genbank accession No. U09278) in CAFs isolated from human and mouse GC tumor tissue using BLOCK-iT™ Lentiviral RNAi Expression System (Invitrogen™, Carlsbad, CA, USA). All the procedures followed the manufacturer's instruction. The sequences of the double-stranded oligonucleotide (ds oligo) used to construct pENTR™/U6 vector (Invitrogen™) were 5'-CACCGGTCGCCTGTTGGGA GTAAATCGAAATTTACTCCCAACAGGCGACC-3' ("top strand" for human FAP); 5'-AAAAGGTCGCCT GTTGGGAGTAAATTTTCGATTTACTCCCAACAGGC GACC-3' ("bottom strand" for human FAP); 5'-CAC CGCATAAAGCTTGGCTGTTTCTCGAAAGAAA CAGCCAAGCTTTATGC-3' ("top strand for mouse FAP"); 5'-AAAAGCATAAAGCTTGGCTGTTTCTTTC GAGAAACAGCC AAGCTTTATGC-3' ("bottom strand" for mouse FAP). These oligonucleotides were annealed to make double-stranded oligonucleotides with a 5' overhang (CACC) and a 3' overhang (AAAA). Expression vectors were kept in One Shot® Stbl3™ Chemically Competent *E. coli*. Lentivirus was produced in 293FT cells.

Statistical Analysis

All statistical analysis was performed by SPSS 17.0 software (SPSS Inc., Chicago, IL, USA) and GraphPad Prism software (GraphPad Software, La Jolla, CA, USA). Chi-squared test and one-way ANOVA method were used to analyze the statistical difference between two groups or multiple groups appropriately. For the comparison between two treatment groups after one-way ANOVA,

Bonferroni's pairwise comparison was used. Survival analysis of the GC patients and the xenograft mouse model was performed using the Kaplan–Meier method. The log-rank test was used to evaluate the difference of survival between different cohorts of GC patients. To evaluate the prognosis value of different parameters, a Cox regression model was used. For all the statistical tests, a value of $p < 0.05$ was considered to be significant.

RESULTS

FAP Is a Prognosticator for Poor Survival of GC Patients

In order to evaluate the FAP expression level in GC tissue and its prognostic value, we collected FFPE tissue samples from 105 GC patients diagnosed between April 2005 and June 2009 in 82nd Hospital of PLA (mean age=64). There were 74 out of 105 (70.47%) GC patients with advanced TNM stage (III+IV), and 65 out of 105 (61.90%) patients had a poorly differentiated structure; 55.23% of these patients survived to the end of our follow-up. Using the IHC method, we measured the FAP expression in each sample and analyzed its relationship with the clinicopathological features of GC patients. FAP was expressed in the tumor stroma area of GC tumor tissue (Fig. 1A and B). According to the ROC analysis, the samples with the original FAP score of 2 and 3 were assigned to the high-FAP-expression group, while samples with the original FAP score of 1 were assigned to the low-FAP-expression group (Fig. 1C). A total of 44

(41.9%) GC patients had high FAP expression and 61 (58.1%) patients had low FAP expression.

The chi-square test indicated that there was a significant difference in FAP expression between patients with advanced T stage (T3+T4) and low T stage (T1+T2), and between patients with a high number of lymph node invasion (N3–N4) and a low number of lymph node invasion (N0–N2), with a value of $p = 0.030$ and 0.011 , respectively. The patients with higher T stage or higher numbers of regional lymph node metastasis tended to have high FAP expression. Most importantly, the FAP expression was highly associated with the survival status of GC patients ($p = 0.002$). There was a higher proportion of GC patients who died of GC during our follow-up period who expressed a high level of FAP (55.2%) than those who survived (25.5%). In summary, these data suggested that FAP expression might be harmful to GC patients. Thus, we further analyzed the prognostic value of FAP in GC by survival analysis and multivariate Cox proportional hazard model. The Kaplan–Meier plot showed that GC patients with high FAP tended to have shorter OS time than those with low FAP expression (Fig. 1C). The log-rank test indicated a significant difference in survival curves between high-FAP patients and low-FAP patients. Furthermore, multivariate Cox proportional hazard model analysis suggested that high FAP expression was an independent prognosticator of poor survival in GC patients with a hazard ratio (HR) of 1.943 and 95% confidential interval (CI) of 1.083–3.484. In addition, the T stage (HR: 2.071, 95% CI: 1.039–4.129) and the lymph node

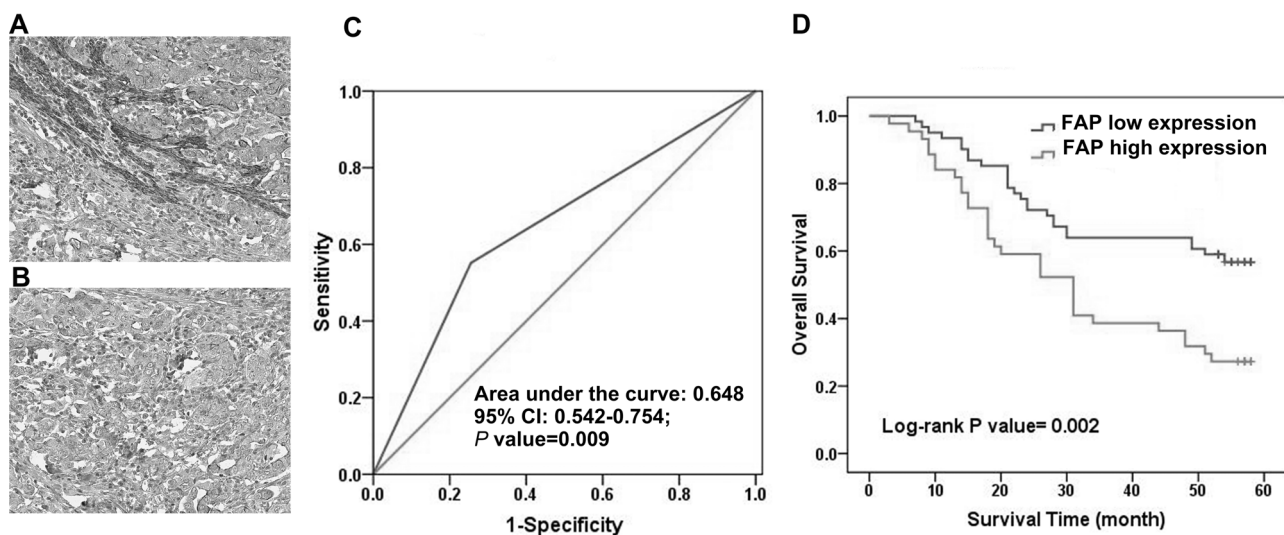


Figure 1. High FAP expression in GC tumor tissue predicted poor survival. (A, B) Representative pictures of tissue samples of GC patients that were stained by the IHC method. The dark color indicates FAP⁺ cells. (C) The ROC curve shows the sensitivity and specificity of the cutoff point of FAP expression. The cutoff point was determined by ROC analysis of the original score of FAP expression. (D) Survival analysis of GC patients with high FAP expression and low FAP expression. The patients with high FAP expression tended to have shorter survival time than those with low FAP expression ($p = 0.002$, log-rank test).

metastasis (HR: 2.212, 95% CI: 1.183–4.137) were also indicated as independent prognosticators in GC patients.

FAP⁺ CAFs Promote GC Progression In Vitro

In order to further explore the role of FAP⁺ CAFs in GC development, we created FAP knockdown CAFs (FAP⁻ CAFs) by shRNA gene silencing technology (Fig. 2A) and evaluated its influence on GC cell lines. The proliferation of GC cell lines AGS and 424GC was measured by BrdU incorporation. Under the presence of FAP⁺ CAF CM, the GC cells showed higher proliferation activity than those under the FAP⁻ CAF CM or normal medium (Fig. 2B). Also, the migration of GC cells was increased by the FAP⁺ CAF CM, while GC cells cultured with FAP⁻ CAF CM only had a compromised migration ability (Fig. 2C). We then tested the effects of FAP⁺ CAFs on GC cells' response to chemotherapy. GC cell lines (AGS and 424GC) cultured with FAP⁺ CAF CM had a worse response to increasing concentrations of 5-fluorouracil (5-FU) or paclitaxel treatment compared with those cultured with FAP⁻ CAF CM or normal medium (Fig. 2D–G). Collectively, these data suggested that FAP⁺ CAFs could promote GC progression by increasing their proliferation, migration, and drug resistance.

FAP⁺ CAFs Enhance GC Tumor Growth In Vivo

Upon observing the influence of FAP⁺ CAFs on GC cells in vitro, we decided to further confirm this influence in an animal model. We established a GC xenograft mouse model using 424GC inoculation in combination with CAFs. Tumor volume and survival status were measured every 7 days. The tumor volume increased much slower in the 424GC plus FAP⁻ CAF coinoculated group compared with the 424GC plus FAP⁺ CAF coinoculated group, which suggested that the FAP⁺ CAFs accelerated GC tumor growth (Fig. 3A). Consistently, the survival curve indicated that mice inoculated with 424GC plus FAP⁺ CAFs had a shorter survival time than those inoculated with 424GC plus FAP⁻ CAFs (Fig. 3B). The GC tumor tissue from the xenograft mouse model was then collected to measure the BrdU incorporation rate by FACS analysis. The results showed that tumor tissue from 424GC plus FAP⁺ CAF coinoculated xenograft mice had an increased rate of BrdU incorporation compared with those from the 424GC plus FAP⁻ CAF coinoculated group or the 424GC-only group (Fig. 3C). Therefore, we believe that FAP⁺ CAFs play an essential role in promoting GC growth.

FAP⁺ CAFs Inhibit T-Cell Activation and Infiltration in GC

The immune system is essential in restricting tumor growth. In order to explore the influence of FAP⁺ CAFs

on tumor immune response, we evaluated the proliferation and activity of T cells cultured by FAP⁺ CAF or FAP⁻ CAF CM in vitro. First, we purified T cells from human peripheral blood and mouse spleen (the result of purity checking is shown in Fig. 4A). Apparently, T cells cultured with FAP⁺ CAF CM had a significantly decreased proliferation ability compared to those cultured with FAP⁻ CAF CM (Fig. 4B). In line with this, the granzyme B and IFN- γ levels were also significantly decreased in the FAP⁺ CAF CM-cultured group (Fig. 4C and D). Moreover, we detected the number of T cells infiltrating into the tumor tissue in the xenograft mouse model. Interestingly, the number of infiltrating T cells in the GC tissue was also decreased in the 424GC plus FAP⁺ CAF coinoculated mice (Fig. 4E). In summary, this evidence suggests that FAP might inhibit the antitumor immunity of GC by suppressing T-cell activation and infiltration.

Inhibition of FAP Enhances the Effects of Immune Checkpoint Blockade Therapy in GC

Based on the knowledge that FAP⁺ CAFs could influence tumor immunity in GC, we further explored the possibility of FAPi therapy and immune checkpoint blockade therapy in our xenograft mouse model. We found that anti-PD-1 treatment alone did not cause obvious inhibition of tumor growth in 424GC plus FAP⁺ CAF xenograft mouse model. However, blocking both FAP and PD-1 simultaneously induced apparent inhibition of tumor growth. Particularly, in the 424GC plus FAP⁻ CAF coinoculating group, the greatest inhibition of tumor growth was achieved by the anti-PD-1 treatment (Fig. 5A). Survival analysis confirmed this phenomenon (Fig. 5B). FAP is crucial for FAP⁺ CAF proliferation and ECM secretion. Therefore, we checked whether FAPi could directly target FAP⁺ CAFs. The in vitro data showed that FAPi could inhibit the cell viability of FAP⁺ CAFs but not that of tumor cells and FAP⁻ CAFs (Fig. 5C). The staining of xenograft tumors revealed that FAPi could inhibit FAP⁺ CAFs and desmoplasia in vivo (Fig. 5D).

We further analyzed the immune component in the xenograft tumors. We found that FAP inhibition and anti-PD-1 treatment increased the CD8⁺ T cell versus Treg ratio in both the tumor tissue and the tumor-draining lymph node tissue of the 424GC plus CAF coinoculated xenograft mouse model (Fig. 6A and B). However, this effect was not observed in the anti-PD-1 single-treated group.

Inhibition of FAP and Anti-PD-1 Treatment Change Cytokine Expression in GC Tissue

Our data suggested that the combination of FAP inhibition and PD-1 antibody enhanced antitumor immunity, so we analyzed the expression of key cytokines that regulate

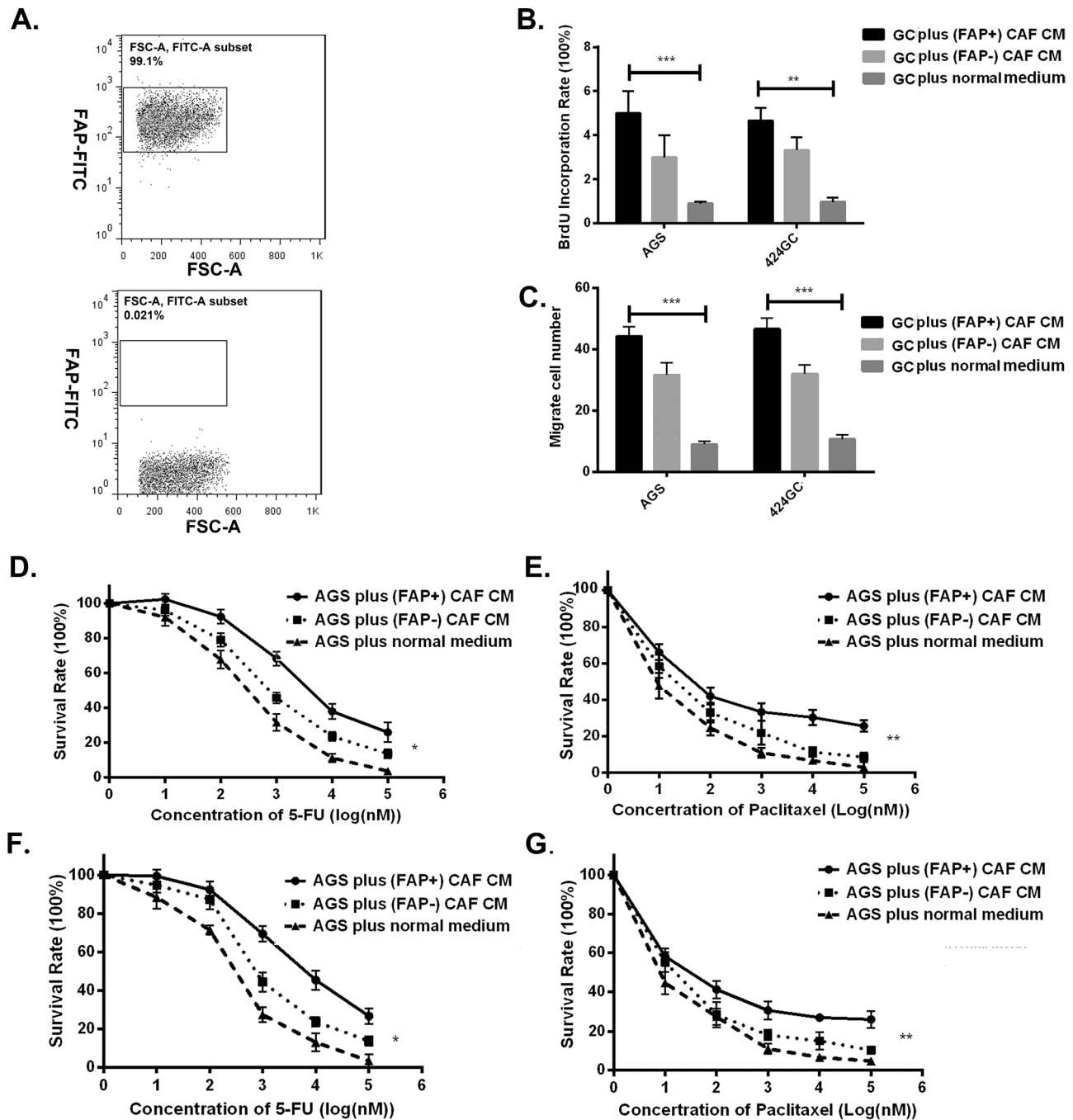


Figure 2. FAP⁺ CAFs promoted GC cell line migration, survival, and drug resistance. (A) The purity of FAP⁺ CAFs and FAP⁻ CAFs was checked by FACS. The upper part shows the FAP⁺ CAFs, and the lower part shows the FAP⁻ CAFs. (B) BrdU incorporation of GC cell lines AGS and 424GC cultured with different CM was tested. AGS and 424GC cultured with CM of FAP⁺ CAFs had higher BrdU incorporation than those cultured with CM from FAP⁻ CAFs. (C) The migration ability of AGS and 424GC cells cultured with the CM from FAP⁺ CAFs was significantly higher than that of those cultured with FAP⁻ CAF CM. (D, E) The survival curve of AGS cells under the treatment of increasing concentrations of 5-FU and paclitaxel. Under both of these drug treatments, the AGS cells cultured with FAP⁺ CAFs had higher survival rate than those cultured with the CM from FAP⁻ CAFs. (F, G) The survival curve of 424GC cells under the treatment of increasing concentrations of 5-FU and paclitaxel. Under both of these drug treatments, the 424GC cells cultured with FAP⁺ CAFs had higher survival rate than those cultured with the CM from FAP⁻ CAFs. One-way ANOVA was performed to evaluate the difference between multiple groups.

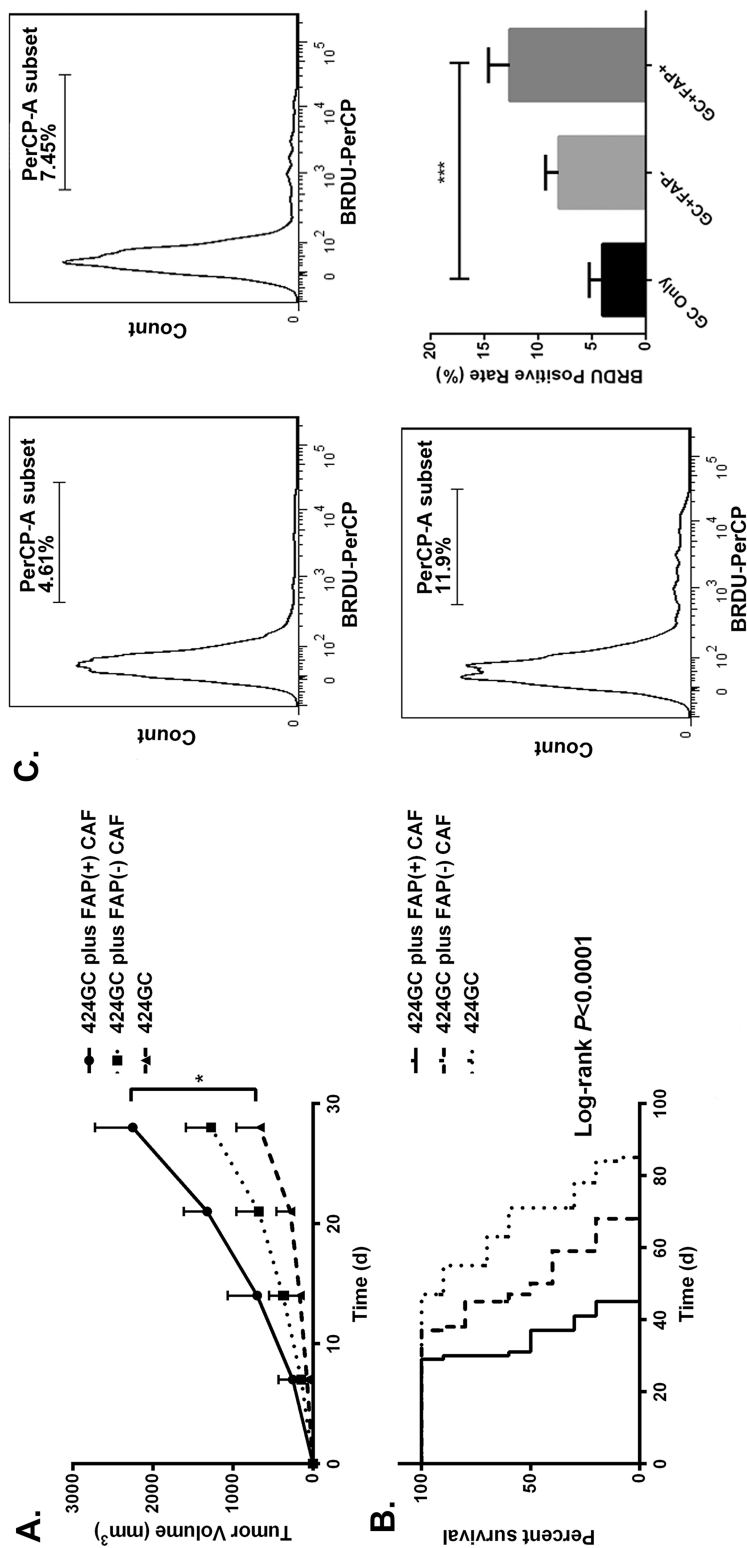


Figure 3. FAP⁺ CAFs promoted GC tumor progression in 424GC xenograft mouse model. (A) GC xenograft mouse model was created using the 424GC cell line. Tumor volume was measured every 7 days. The tumors of mice inoculated with 424GC and FAP⁺ CAFs grew more quickly than those of mice inoculated with 424GC and FAP⁻ CAFs (one-way ANOVA was performed to evaluate the difference between multiple groups). (B) Survival analysis indicated that mice bearing 424GC and FAP⁺ CAFs had a shorter overall survival time than the mice with 424GC and FAP⁻ CAFs ($p < 0.0001$). (C) BrdU incorporation was tested in the tumor tissues from the xenograft mouse model. FACS analysis was used. Tumor tissues containing 424GCs and FAP⁺ CAFs had higher BrdU incorporation than those containing 424GC and FAP⁻ CAFs.

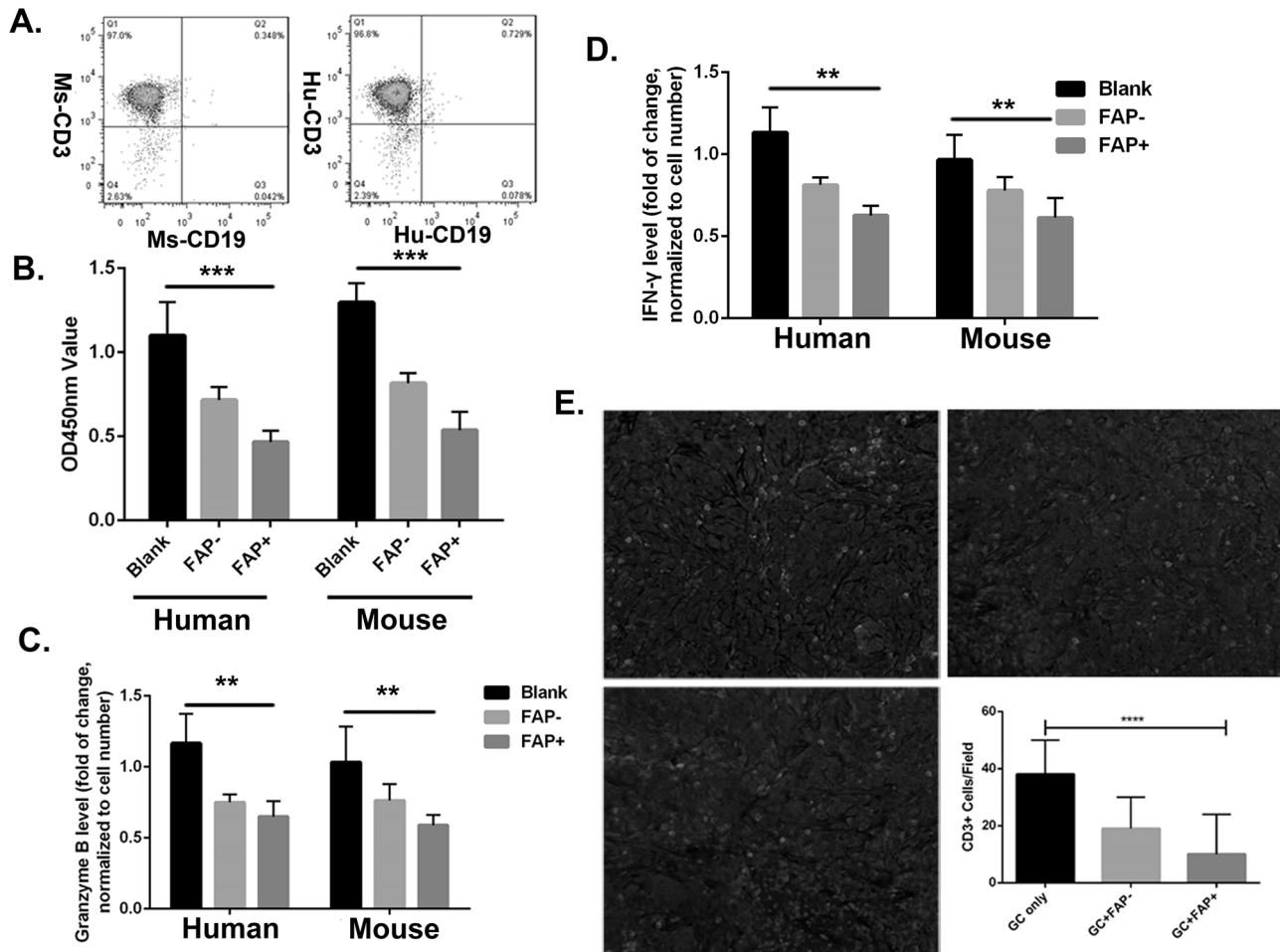


Figure 4. FAP inhibited T-cell activation in vivo. (A) T cells were isolated from human PBMCs and mouse spleen tissues. FACS was performed to check the purity of these isolated T cells using CD3 and CD19 markers. (B) Proliferation was evaluated by checking the cell density via the CCK-8 assay. T cells cultured with FAP⁺ CAF CM had higher proliferative ability than the ones cultured with FAP⁻ CAF CM. (C, D) The amount of granzyme B produced and IFN- γ by T cells was evaluated by ELISA. The T cells cultured by FAP⁺ CAF CM produced more granzyme B and IFN- γ than those cultured by FAP⁻ CAF CM. (E) Representative pictures of CD3 marker in tumor tissue from GC xenograft mouse models (upper left: mice bearing 424GC plus FAP⁺ CAFs; upper right: mice bearing 424GC plus FAP⁻ CAFs; lower left: mice bearing 424GC only; lower right: the quantitative data of CD3 staining). One-way ANOVA was performed to evaluate the difference between multiple groups.

T-cell activity in the GC xenograft tumor. We found that several important cytokines, such as IL-2, IL-4, IL-10, IFN- γ , and TNF- α , were increased in the group receiving combination treatment (Fig. 6C). On the contrary, some other cytokines, such as PD-L1 and PD-L2, were decreased. This evidence suggested that the cytokines that increase the T-cell activity were increased, but the cytokines with immunosuppressive functions were decreased.

DISCUSSION

Although surgical treatment significantly extended the OS of early stage GC patients, invasion and metastasis are still challenges in the survival of advanced stage patients^{6,19}. Previous data have shown that more than

half of GC patients were at stage IV at their diagnosis, which means these GC patients may need comprehensive treatments²⁰. However, current strategies such as adjuvant chemotherapy or radiotherapy could not achieve an obvious survival benefit due to low sensitivity or drug resistance^{8,20}. Thus, new treatment strategies are quite necessary for increasing the survival of GC patients. The tumor microenvironment has been widely accepted to be an active participant throughout the entire process of tumor development^{10,21,22}. Many factors closely related to tumor progression and prognosis have been found within the tumor microenvironment, such as immune cell infiltration, CAFs, and tumor stroma autophagy level^{13,21,23}. Among these factors, CAFs promote tumor development

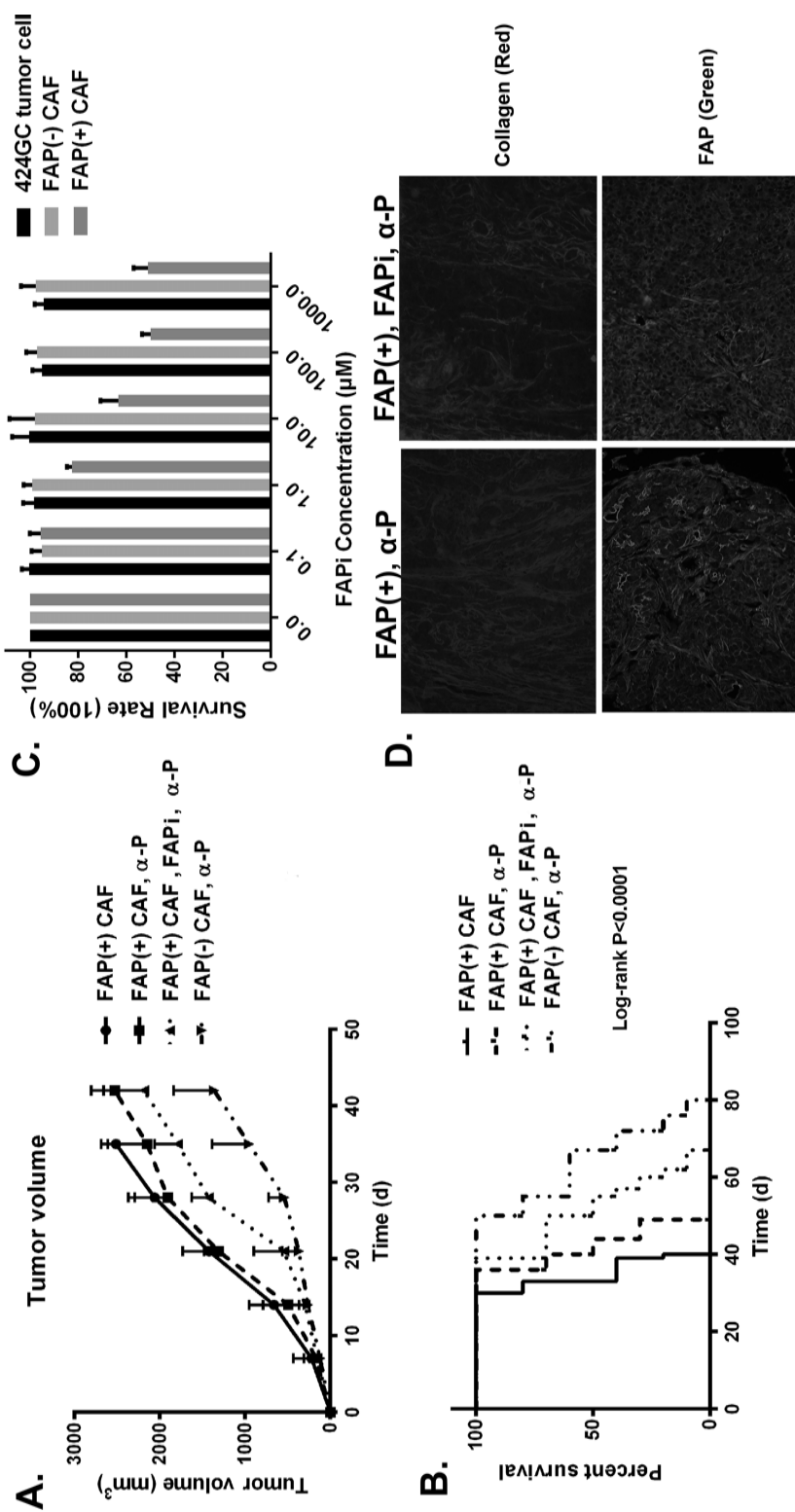


Figure 5. FAP inhibition enhanced the therapeutic effects of immune checkpoint blockade in GC xenograft mice. (A) Tumor volume of the 424GC xenograft mice under the FAPi or anti-PD-1 treatment was plotted. The combination of anti-PD-1 and FAPi treatment obviously inhibited tumor growth, while anti-PD-1 treatment under the presence of FAP⁺ CAFs did not significantly inhibit tumor growth. Anti-PD-1 treatment achieved excellent inhibition of tumor growth in mice with FAP⁻ CAFs. (B) 424GC xenograft mice with FAP⁻ CAFs treated by anti-PD-1 and the ones with FAP⁺ CAFs treated by both anti-PD-1 Abs and FAPi had the best survival compared to mice with FAP⁺ CAFs treated by anti-PD-1 only. (C) In vitro cell viability assay showed that FAPi targets only FAP⁺ CAFs, but not tumor cells and FAP⁻ CAFs. (D) FAP and collagen were stained in xenograft tumors. FAPi decreased FAP expression and the amount of collagen in treated tumor tissues.

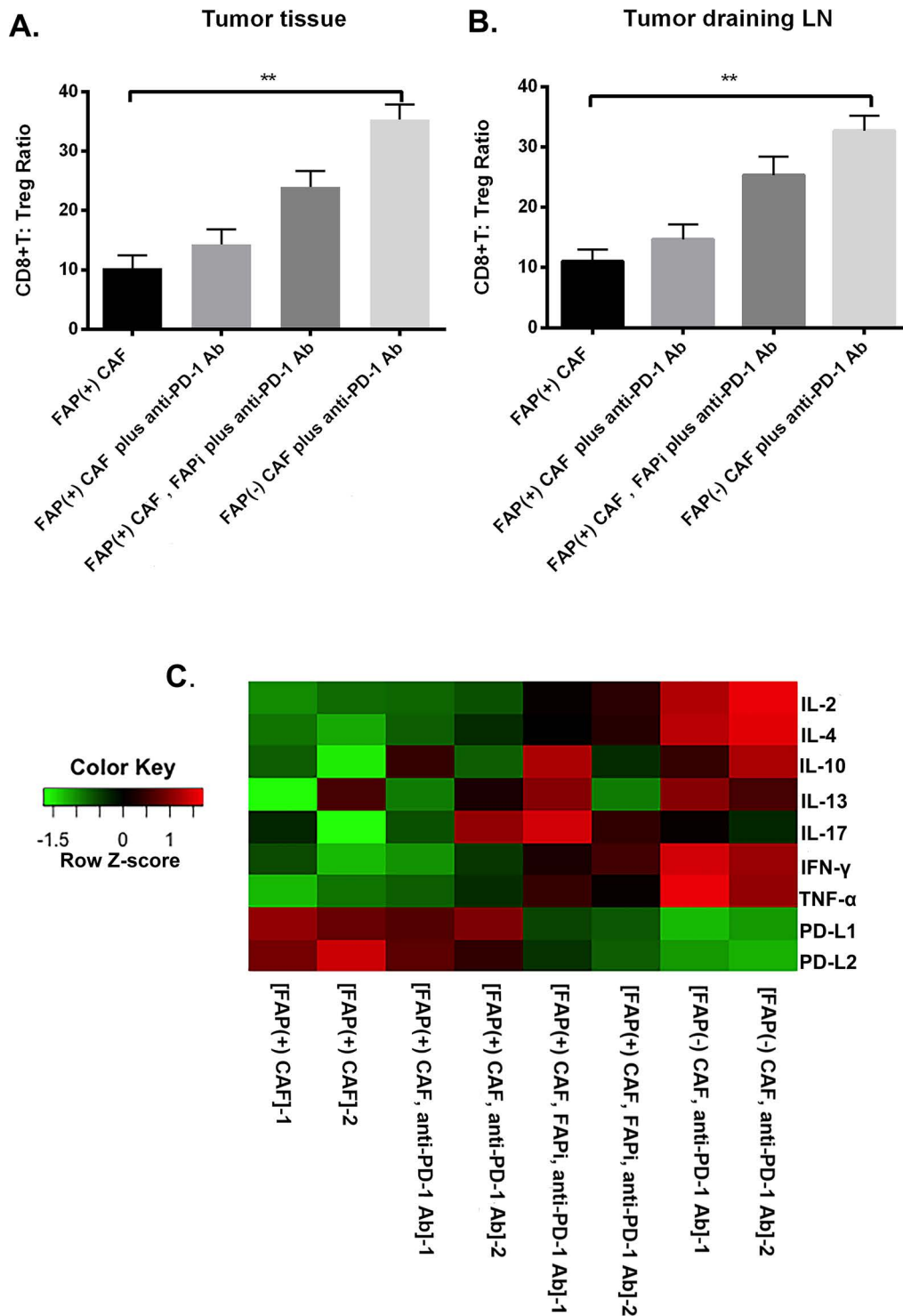


Figure 6. Inhibition of FAP and anti-PD-1 treatment changed the T-cell distribution and cytokine expression in tumor tissue of GC in vivo. (A, B) The combination of blocking FAP and PD-1 significantly increased the ratio of CD8⁺ T cells versus Tregs in both the tumor tissue and the tumor-draining LNs of 424GC xenograft mice (one-way ANOVA was performed to evaluate the difference between multiple groups). (C) Some key cytokines that can promote T-cell activation were increased by the FAP blocking and anti-PD-1 treatment in 424GC xenograft mice. Red color means high level; green color means low level.

through many mechanisms, including promoting tumor cell survival, proliferation, angiogenesis, drug resistance, inhibiting tumor apoptosis, and tumor immunity^{10,13,24,25}. Thus, there should be a focus on CAFs for exploring new antitumor strategies.

FAP is a membrane-bound serine protease that is selectively expressed on activated fibroblasts during tissue healing and remodeling, as well as in some malignant tumor cells^{26,27}. Cancers are usually thought to be “never-healed” tissue; thus, the role of FAP expressed by CAFs becomes intriguing. Previous studies suggested that a larger proportion of CAFs in malignant cancer tissues expressed FAP, including breast, colorectal, skin, and pancreatic cancers²⁸. In colon cancer, increased FAP expression was associated with aggressive disease²⁹. Moreover, FAP expression was also related to poor prognosis in many other cancers, such as pancreatic cancer and hepatocellular cancer^{30,31}. In our study, the survival analysis of 105 GC patients indicated that patients with low FAP expression tended to have a longer survival time compared with patients with high FAP expression. Multivariate analysis also suggested that high FAP expression was an adverse prognosticator of GC patients. Taking these data together, we believe that FAP has a tumor-promoting role during the development of GC and is worth targeting.

It is widely accepted that dynamic reciprocal interactions exist between tumor cells and their microenvironment. Many studies have suggested that CAFs actively participated in these interactions, mostly through intercellular cytokines. However, factors that could regulate the behaviors of CAFs are still under discussion. Here we observed a higher migration ability, drug resistance, and proliferation in GC cells cultured by FAP⁺ CAF CM. In addition, the 424GC xenograft mouse model with FAP⁻ CAFs or FAP⁺ CAFs also showed different results: mice with FAP⁺ CAFs had higher tumor growth speed and shorter OS time. This evidence suggested that FAP could influence the behavior of CAFs and thus promote the development of GC.

Antitumor immunity is critical in restricting cancer development. A previous study has shown that FAP⁺ CAFs have an important role in suppressing antitumor immunity in pancreatic cancer¹⁵. In our GC model, we also found profound immunosuppressive roles in FAP⁺ CAFs by inhibiting T-cell activation and infiltration. More interestingly, when combined with FAPi, immune checkpoint blockades became more efficient in eliminating GC tumors. FAP activity is involved in fibroblast growth and ECM secretion control. Therefore, we proposed that by inhibiting FAP activity, the viability and ECM secretion function of FAP⁺ CAFs would be compromised³². Further analysis found that FAPi could inhibit FAP⁺ CAFs in vitro and in vivo. More importantly, desmoplasia in the

tumor microenvironment was also reduced by FAPi. The cytokine analysis revealed that an antitumor immunity boosting cytokine network was established by FAPi. These data are important as it suggests that a combination of FAPi could break the insensitivity of GC toward immune checkpoint blockades by reducing the FAP⁺ CAFs and desmoplasia-induced immunosuppressive tumor microenvironment, therefore enhancing antitumor immunity of immune checkpoint blockades.

In conclusion, for the first time, our study indicated that high expression of FAP in CAFs is an independent prognosticator and has a profound effect on promoting GC progression, immunosuppression, and drug resistance. More importantly, our data indicated that by inhibiting FAP in CAFs, GC would be more sensitive to immune checkpoint blockades, which showed a very promising effect on certain cancers. Based on the results of our study, further studies that evaluate the therapeutic effects of FAP inhibitors would be highly valued.

ACKNOWLEDGMENTS: This work was supported by the internal fund from the Huai'an City, Jiangsu Province 223001, P.R. China. The authors declare no conflicts of interest.

REFERENCES

1. Siegel RL, Miller KD, Jemal A. Cancer statistics, 2015. *CA Cancer J Clin.* 2015;65:5–29.
2. Torre LA, Bray F, Siegel RL, Ferlay J, Lortet-Tieulent J, Jemal A. Global cancer statistics, 2012. *CA Cancer J Clin.* 2015;65:87–108.
3. Brenner H, Rothenbacher D, Arndt V. Epidemiology of stomach cancer. In: Verma M, editor. *Cancer epidemiology: Modifiable factors*. Totowa (NJ): Humana Press; 2009. p. 467–77.
4. Japanese Gastric Cancer Association. Japanese gastric cancer treatment guidelines 2010 (ver. 3). *Gastric Cancer* 2011;14:113–23.
5. Spataro V, Genoni M, Maurer C, Muller W. Stomach cancer: 10 Years experiences with surgical treatment and possibilities for improving the prognosis. *Helv Chir Acta* 1993;59:589–95.
6. Songun I, Putter H, Kranenbarg EM, Sasako M, van de Velde CJ. Surgical treatment of gastric cancer: 15-Year follow-up results of the randomised nationwide Dutch D1D2 trial. *Lancet Oncol.* 2010;11:439–49.
7. Glimelius B, Ekstrom K, Hoffman K, Graf W, Sjoden PO, Haglund U, Svensson C, Enander LK, Linne T, Sellstrom H and others. Randomized comparison between chemotherapy plus best supportive care with best supportive care in advanced gastric cancer. *Ann Oncol.* 1997;8:163–8.
8. Hallissey MT, Dunn JA, Ward LC, Allum WH. The second British Stomach Cancer Group trial of adjuvant radiotherapy or chemotherapy in resectable gastric cancer: Five-year follow-up. *Lancet* 1994;343:1309–12.
9. Li H, Fan X, Houghton J. Tumor microenvironment: The role of the tumor stroma in cancer. *J Cell Biochem.* 2007;101:805–15.
10. Zhao X, He Y, Chen H. Autophagic tumor stroma: Mechanisms and roles in tumor growth and progression. *Int J Cancer* 2013;132:1–8.

11. Gabbiani G, Ryan GB, Majne G. Presence of modified fibroblasts in granulation tissue and their possible role in wound contraction. *Experientia* 1971;27:549–50.
12. Cirri P, Chiarugi P. Cancer-associated-fibroblasts and tumour cells: A diabolic liaison driving cancer progression. *Cancer Metastasis Rev.* 2012;31:195–208.
13. Zhao X, He Y, Gao J, Fan L, Li Z, Yang G, Chen H. Caveolin-1 expression level in cancer associated fibroblasts predicts outcome in gastric cancer. *PLoS One* 2013; 8:e59102.
14. Fearon DT. The carcinoma-associated fibroblast expressing fibroblast activation protein and escape from immune surveillance. *Cancer Immunol Res.* 2014;2:187–193.
15. Feig C, Jones JO, Kraman M, Wells RJ, Deonarine A, Chan DS, Connell CM, Roberts EW, Zhao Q, Caballero OL. Targeting CXCL12 from FAP-expressing carcinoma-associated fibroblasts synergizes with anti-PD-L1 immunotherapy in pancreatic cancer. *Proc Natl Acad Sci USA* 2013;110:20212–7.
16. Nockel J, van den Engel NK, Winter H, Hatz RA, Zimmermann W, Kammerer R. Characterization of gastric adenocarcinoma cell lines established from CEA424/SV40 T antigen-transgenic mice with or without a human CEA transgene. *BMC Cancer* 2006;6:57.
17. Normand J, Karasek MA. A method for the isolation and serial propagation of keratinocytes, endothelial cells, and fibroblasts from a single punch biopsy of human skin. *In Vitro Cell Dev Biol Anim.* 1995;31:447–55.
18. Jansen K, Heirbaut L, Cheng JD, Joossens J, Ryabtsova O, Cos P, Maes L, Lambeir AM, De Meester I, Augustyns K, Van der Veken P. Selective inhibitors of fibroblast activation protein (FAP) with a (4-quinolinoyl)-glycyl-2-cyanopyrrolidine scaffold. *ACS Med Chem Lett.* 2013;4: 491–6.
19. Lee SR, Kim HO, Son BH, Shin JH, Yoo CH. Prognostic significance of the metastatic lymph node ratio in patients with gastric cancer. *World J Surg.* 2012;36:1096–101.
20. Alberts SR, Cervantes A, van de Velde CJ. Gastric cancer: Epidemiology, pathology and treatment. *Ann Oncol.* 2003;14(Suppl 2):ii31–6.
21. Liotta LA, Kohn EC. The microenvironment of the tumour-host interface. *Nature* 2001;411:375–9.
22. Whiteside TL. The tumor microenvironment and its role in promoting tumor growth. *Oncogene* 2008;27:5904–12.
23. Curiel TJ, Coukos G, Zou L, Alvarez X, Cheng P, Mottram P, Evdemon-Hogan M, Conejo-Garcia JR, Zhang L, Burow M, Zhu Y, Wei S, Kryczek I, Daniel B, Gordon A, Myers L, Lackner A, Disis ML, Knutson KL, Chen L, Zou W. Specific recruitment of regulatory T cells in ovarian carcinoma fosters immune privilege and predicts reduced survival. *Nat Med.* 2004;10:942–9.
24. Wang W, Kryczek I, Dostal L, Lin H, Tan L, Zhao L, Lu F, Wei S, Maj T, Peng D, He G, Vatan L, Szeliga W, Kuick R, Kotarski J, Tarkowski R, Dou Y, Rattan R, Munkarah A, Liu JR, Zou W. Effector T cells abrogate stroma-mediated chemoresistance in ovarian cancer. *Cell* 2016;165:1092–105.
25. Loeffler M, Kruger JA, Niethammer AG, Reisfeld RA. Targeting tumor-associated fibroblasts improves cancer chemotherapy by increasing intratumoral drug uptake. *J Clin Invest.* 2006;116:1955–62.
26. Acharya PS, Zukas A, Chandan V, Katzenstein AL, Pure E. Fibroblast activation protein: A serine protease expressed at the remodeling interface in idiopathic pulmonary fibrosis. *Hum Pathol.* 2006;37:352–60.
27. Goodman JD, Rozypal TL, Kelly T. Seprase, a membrane-bound protease, alleviates the serum growth requirement of human breast cancer cells. *Clin Exp Metastasis* 2003;20:459–70.
28. Garin-Chesa P, Old LJ, Rettig WJ. Cell surface glycoprotein of reactive stromal fibroblasts as a potential antibody target in human epithelial cancers. *Proc Natl Acad Sci USA* 1990;87:7235–9.
29. Liu R, Li H, Liu L, Yu J, Ren X. Fibroblast activation protein: A potential therapeutic target in cancer. *Cancer Biol Ther.* 2012;13:123–9.
30. Cohen SJ, Alpaugh RK, Palazzo I, Meropol NJ, Rogatko A, Xu Z, Hoffman JP, Weiner LM, Cheng JD. Fibroblast activation protein and its relationship to clinical outcome in pancreatic adenocarcinoma. *Pancreas* 2008;37:154–8.
31. Ju MJ, Qiu SJ, Fan J, Xiao YS, Gao Q, Zhou J, Li YW, Tang ZY. Peritumoral activated hepatic stellate cells predict poor clinical outcome in hepatocellular carcinoma after curative resection. *Am J Clin Pathol.* 2009;131:498–510.
32. Brennan WN, Isaacs JT, Denmeade SR. Rationale behind targeting fibroblast activation protein-expressing carcinoma-associated fibroblasts as a novel chemotherapeutic strategy. *Mol Cancer Ther.* 2012;11:257–66.