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CPT1C-positive cancer-associated fibroblast facilitates immunosuppression through promoting IL-6-induced M2-like phenotype of macrophage

Rongyuan Wei[a,b*], Junquan Song[a,b*], Hongda Pan[a,b*], Xiaowen Liu[a,b], and Jianpeng Gao[a,b]

[a]Department of Gastric Surgery, Fudan University Shanghai Cancer Center, Shanghai, China; [b]Department of Oncology, Shanghai Medical College, Fudan University, Shanghai, China

ABSTRACT

Cancer-associated fibroblasts (CAFs) exhibit remarkable phenotypic heterogeneity, with specific subsets implicated in immunosuppression in various malignancies. However, whether and how they attenuate anti-tumor immunity in gastric cancer (GC) remains elusive. CPT1C, a unique isoform of carnitine palmitoyltransferase pivotal in regulating fatty acid oxidation, is briefly indicated as a protumoral metabolic mediator in the tumor microenvironment (TME) of GC. In the present study, we initially identified specific subsets of fibroblasts exclusively overexpressing CPT1C, hereby termed them as CPT1C$^+$CAFs. Subsequent findings indicated that CPT1C$^+$CAFs fostered a stroma-enriched and immunosuppressive TME as they correlated with extracellular matrix-related molecular features and enrichment of both immunosuppressive subsets, especially M2-like macrophages, and multiple immune-related pathways. Next, we identified that CPT1C$^+$CAFs promoted the M2-like phenotype of macrophage *in vitro*. Bioinformatic analyses unveiled the robust IL-6 signaling between CPT1C$^+$CAFs and M2-like phenotype of macrophage and identified CPT1C$^+$CAFs as the primary source of IL-6. Meanwhile, suppressing CPT1C expression in CAFs significantly decreased IL-6 secretion *in vitro*. Lastly, we demonstrated the association of CPT1C$^+$CAFs with therapeutic resistance. Notably, GC patients with high CPT1C$^+$CAFs infiltration responded poorly to immunotherapy in clinical cohort. Collectively, our data not only present the novel identification of CPT1C$^+$CAFs as immunosuppressive subsets in TME of GC, but also reveal the underlying mechanism that CPT1C$^+$CAFs impair tumor immunity by secreting IL-6 to induce the immunosuppressive M2-like phenotype of macrophage in GC.

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Introduction

Gastric cancer (GC) is a leading global malignancy in terms of both incidence and mortality rates.[1] Despite recent progress in comprehensive treatments, especially targeted therapy and immune checkpoint inhibitors (ICIs)-based immunotherapy, challenges persist as treatment benefits are still limited and the rates of recurrence and metastasis remain high.[2] The immunosuppressive factors in the tumor microenvironment (TME) contribute significantly to these challenges, enabling cancer cells to evade immune surveillance and make progression.[3] Therefore, there is an urgent need to explore the modulatory mechanism of tumor immunity in GC.

Carnitine Palmitoyltransferase (CPT), a rate-limiting enzyme of fatty acid oxidation (FAO), is responsible for the transportation of fatty acid into mitochondria for further beta-oxidation.[4] CPT1C, an atypical isoform with limited catalytic activity, stands out due to its role as a nutrient sensor regulating cancer cell metabolic reprogramming in response to the dynamic TME.[5] In GC, studies have highlighted the association of elevated CPT1C expression with unfavorable prognosis, enhanced cancer cell proliferation, and distant metastasis.[6,7] However, whether CPT1C correlates with any key components in TME and its potential implication in immunomodulation are largely unknown in GC.

Cancer-associated fibroblasts (CAFs), constituting a major component of TME, represent a phenotypically heterogeneous group actively involved in various aspects of tumor progression such as cell proliferation, metastasis, remodeling of extracellular matrix, and treatment resistance through several mechanisms.[8] Of note, some CAF subsets contribute to an immunosuppressive microenvironment by releasing a plethora of immunomodulatory molecules to regulate the activity of key immune cell components that would otherwise attack or protect the tumor.[9] In particular, CAFs essentially regulate macrophages by not only recruiting them and their precursors to tumor sites,[10] but also promote their differentiation toward the immunosuppressive and protumoral M2-like phenotype,[11] which is featured with anti-inflammatory response, angiogenesis, and suppressed immune effector cells.[12] Notably, CAF-secreted molecules like interleukin-6 (IL-6) are implicated in inducing the M2-like phenotype of macrophages in several cancer types.[13] In GC, although CAFs have been extensively characterized in various aspects of tumor progression, their specific roles in regulating tumor immunity are rarely addressed.[14] Some studies demonstrated that CAFs were enriched in deep layer of stomach, while others found the

CONTACT Xiaowen Liu liuxw1129@hotmail.com; Jianpeng Gao jianpeng.gao@shca.org.cn Department of Gastric Surgery, Fudan University Shanghai Cancer Center, No. 270 Dong'an Road, Xuhui District, Shanghai 200032, China
*These authors contributed equally to this work.
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correlation between the suppression of CAF markers and the accumulation of M2-like macrophage.[15,16] However, the interplay between key immunomodulatory molecules secreted by specific CAF subsets and M2-like phenotype of macrophages in the regulation of anti-GC immunity remains largely unknown.

In the present study, we initially identified CPT1C$^+$CAFs as a novel subset of fibroblasts uniquely overexpressing oncogenic CPT1C in GC. CPT1C$^+$CAFs contributed to shaping the stroma-enriched and immunosuppressive TME and more intriguingly, promoted the protumoral and immunosuppressive M2-like phenotype of macrophages via secreting IL-6. Clinical cohort analyses revealed their adverse prognostic implications, therapeutic resistance, and poor response to immunotherapy. Our findings present a new discovery of functionally distinctive CPT1C$^+$CAF subset, characterizing its immunosuppressive traits and the potential molecular mechanisms involved. This underscores the promise of targeting CPT1C$^+$CAF as a treatment strategy to counter CAF-induced immunosuppression in upcoming GC therapies.

Material and methods

Study populations

GC specimens were collected from patients who received surgery in the Gastric surgery department at Fudan University Shanghai Cancer Center. The resected specimens were immediately collected and subjected to cytological and pathological diagnosis. Informed consent was obtained from all patients and the study protocol was approved by the Clinical Research Ethics Committee of Fudan University Shanghai Cancer Center.

Gene expression profiles and corresponding clinical data of the Asian Cancer Research Group (ACRG) cohort (GSE66229, $n = 300$), GSE26942 cohort ($n = 217$), Samsung Medical Center (SMC) cohort (GSE26253, $n = 432$) were included for analysis. The ACRG cohort of GC patients receiving adjuvant chemotherapy ($n = 106$) was selected from the GSE26942 cohort for survival analysis.[17] All patients in the SMC cohort underwent curative gastrectomy and received 5-fluorouracil/leucovorin and radiation. The TCGA cohort was composed of 375 GC patients, with 5 patients excluded due to unavailable survival information. Gene expression profiles and corresponding clinical data were downloaded from GEO (https://www.ncbi.nlm.nih.gov/geo/) and UCSC XENA website (https://xenabrowser.net/datapages/). Additionally, expression profiles and clinical responses of the pembrolizumab, an FDA-approved immune checkpoint inhibitor, treatment cohorts with metastatic GC were obtained from the Tumor Immune Dysfunction and Exclusion (TIDE) database (http://tide.dfci.harvard.edu).[18] Following the RECIST 1.1 guidelines,[19] patients classified in the complete response (CR) and partial response (PR) groups were considered responders, whereas those in the stable disease (SD) and progressive disease (PD) groups were considered non-responders.

Bioinformatic analyses of single cell RNA sequencing (scRNA-seq) database

The processed expression matrices were obtained from GEO under accession ID: GSE183904.[20] The scRNA-seq data in this study was analyzed using the R package "Seurat". Normalization of the data was achieved using a scaling factor of 10,000, and subsequent identification of the top 2000 variable genes was performed through the "FindVariableFeatures" function for principal component analysis. The "FindNeighbors" algorithm utilized the first 30 principal components, and the clusters were determined using the "FindClusters" function with resolution set to 0.3. Visualization of the identified clusters was accomplished using the Uniform Manifold Approximation and Projection (UMAP) algorithm.[21,22] Differentially expressed genes (DEGs) within the clusters were identified by "FindAllMarkers" function. To systematically investigate cell–cell communication, the "CellChat" and "NicheNet" R packages were employed. Furthermore, Monocle2 and Cytotrace were leveraged to infer the pseudotime progression. Assessment of the infiltration levels of CPT1C$^+$CAFs in bulk RNA sequencing (bulk-RNA seq) data was conducted by the mean expression of the top 50 markers of CPT1C$^+$CAF derived from the scRNA-seq data. The top 50 markers were sorted based on the fold change calculated by the "FindMarkers" function, which compared the differential expression genes between CPT1C$^+$CAFs and CPT1C$^-$CAFs in scRNA-seq data.

Isolation of CAFs from surgically resected GC tissues

CAFs were isolated from human GC tissues of patients from the same department. They were rinsed three times with phosphate buffer saline (PBS) supplemented with 100 U/mL penicillin and 100 μg/mL streptomycin before being cut into small pieces. Then tissues were digested with 0.1% Type IV collagenase (Sigma-Aldrich, #C5138) at 37°C and 5% CO_2 for 1.5 h. Fetal bovine serum was added to stop digestion followed by centrifugation at 800 × g for 5 min, and the cells were then transferred to a new culture dish. Purified after two generations, CAFs were collected for purity verification and subsequent co-culture system with THP-1 cell lines or human peripheral blood mononuclear cell (PBMC)-derived macrophages.

Macrophages differentiation from monocytes of PBMC and THP-1 cell line

Macrophages derived from THP-1 cell line (American Type Culture Collection, ATCC) were obtained by exposure to 100 ng/mL PMA (MedChemExpress, #HY-18739) for 24 h. PBMCs were isolated by density gradient centrifugation with a leukocyte separation solution (Solarbio, #P8680), followed by purification of CD14$^+$ monocytes via MACS CD14 MicroBeads (Miltenyi Biotec, #130-050-201) and stimulation with macrophage colony-stimulating factor (M-CSF, 100 ng/ml) for 6 days. The processed macrophages co-cultured with CAFs or cultured in supernatants of CAFs for 72 h were collected for subsequent experiments.

Flow cytometry

Following being stained with APC anti-mouse/human CD11b Antibody and PerCP/Cyanine5.5 anti-human CD163 Antibody, single-cell suspensions were resuspended in permeabilization wash buffer (Biolegend, #421002) after fixation and then stained with for APC/Cyanine7 anti-human CD206 (MMR) for 30 min. The purity of CAFs was assessed by flow cytometry with positive selection for FITC anti-human Podoplanin Antibody. All flow cytometry antibodies were purchased from Biolegend. CytoFLEX S Flow Cytometer (Beckman Coulter) was utilized for data acquisition, and results were analyzed with FlowJo 10.

Enzyme-linked immunosorbent assay (ELISA)

The supernatants of transfected CAFs were collected at 72 h for ELISA assay. Human Elisa kit was purchased from Shanghai Weiao Biotech Ltd., Shanghai, China, and the concentration of IL-6 were measured following the manufacturer's instruction.

Luminex liquid suspension chip detection

After culturing macrophages in conditioned medium from CAFs for 36 h, they were washed once with PBS and then incubated with fresh serum-free medium for another 36 h. The collected medium was used for cytokine profile. Luminex liquid suspension chip detection was conducted by utilizing the Bio-Plex Pro Human Chemokine Panel 40-plex kit as per the manufacturer's instructions. The conditioned medium from each group was incubated in 96-well plates embedded with microbeads for 1 h, followed by incubation with detection antibodies for 30 min. Subsequently, streptavidin-PE was added into each well for 10 min, while the values were read using the Bio-Plex MAGPIX System (Bio-Rad).

Ethical statement

The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). The study was approved by the local ethics committee of the Fudan University Shanghai Cancer Center (Number: 050432–4-1911D) and informed consent was taken from all patients.

Statistical analysis

All statistical analyses were performed using GraphPad Prism 9 and R software 4.3.1. Statistical significance was determined by two-tailed Student's t-test, one-way analysis of variance or Spearman correlation coefficient as appropriate. Survival analysis was calculated using the Kaplan–Meier method and analyzed with log-rank test. The data were presented as the mean ± SEM and $p < 0.05$ was considered statistical significance.

Results

Identification of CPT1C-positive cancer associated fibroblasts

CPT1C has been shown to exacerbate the severity of GC previously.[5–7] To characterize the role of CPT1C in the tumor microenvironment (TME), we analyzed the scRNA-seq data encompassing 118,027 cells from 40 samples of 29 GC patients and divided them into 9 distinct categories based on their transcriptional features (Figure 1(a)). Next, we assessed the expression of CPT1C across these cell types and observed that some subpopulations of fibroblasts exhibited a remarkably high expression of CPT1C, followed by endothelial and smooth muscle cells (Figure 1(b,c)). We also noted the correlation between CPT1C expression and CAF markers (Figure 1(d)). Next, we categorized CAFs into 11 subclusters (Figure 1(e)) and observed the overwhelmingly high expression of CPT1C in subcluster 4 than other subclusters (Figure 1(f)). Co-localization of CPT1C-expressing cells and a fraction of CAFs was observed by multiplex immunofluorescence on GC specimens (Figure 1(g)). For the first time, we identified the overexpression of protumoral CPT1C by a unique subset of CAFs, termed as CPT1C$^+$CAFs, prompting us to focus on its implications in GC in the following investigations.

Next, we explored the association between CPT1C$^+$CAF infiltration and clinicopathological parameters of GC patients. CPT1C$^+$CAF infiltration was significantly higher in tumors than normal mucosae (Figure 1(h)). We further unveiled the differential CPT1C$^+$CAFs infiltration in four molecular subtypes of TCGA GC cohort. The immune "cold" genome-stable (GS) subtype exhibited the highest infiltration of CPT1C$^+$CAFs, whereas the EBV-positive subtype, known for heightened immunotherapy responsiveness, showcased the lowest infiltration (Figure 1(i)).[23,24] Moreover, GC patients with low CPT1C$^+$CAF infiltration exhibited significantly prolonged survival time than those with high infiltration (Figure 1(j–k)), indicating its unfavorable prognostic value in GC.

CPT1C$^+$CAFs were associated with stroma-rich and immunosuppressive TME in GC

Next, we investigated the potential influence of CPT1C$^+$CAFs on the microenvironment of GC. Firstly, we conducted pseudotime analysis, a widely-applied approach to dynamically study cell differentiation and other biological process,[25] to reveal the differentiation trajectory of CPT1C$^+$CAFs in GC (Figure 2(a,b)). Notably, CPT1C$^+$CAFs were predominantly enriched at the terminal stage of differentiation (Figure 2(c)), signifying them as a subgroup of well-differentiated CAFs. Differential gene expression analysis highlighted the overexpression of extracellular matrix (ECM)-related genes (COL1A1, COL1A2, and MGP) in CPT1C$^+$CAFs when compared to CPT1C$^-$CAFs (Figure 2(d)). Gene ontology (GO) and pathway enrichment analysis also underscored that highly expressed genes in CPT1C$^+$CAFs were primarily associated with ECM (Figure 2(e)). Additionally, we found that CPT1C$^+$CAFs were significant correlated with both ECM receptor binding pathway (Figure 2(f)) and the tumor stromal score (Figure 2(g)), collectively demonstrating its association with the enriched stroma in GC microenvironment.

Although CAF have been implied to shape an immunosuppressive microenvironment in various malignancies[26], whether and how it exerts any immunomodulatory influence on TME of GC remains to elucidate. Consequently, we explored the

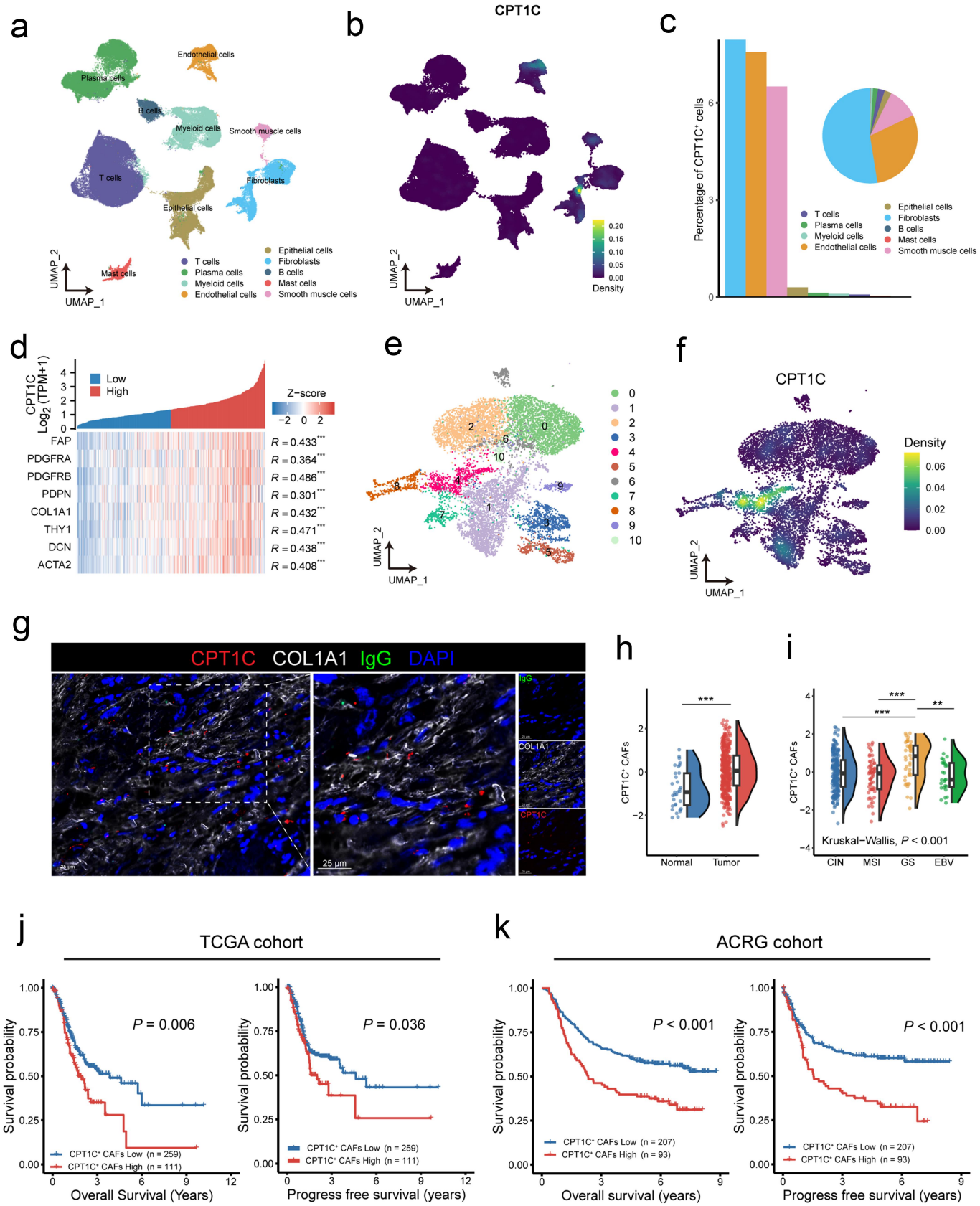

Figure 1. Identification of CPT1C$^+$CAFs and their prognostic significance in GC. (a) UMAP plot represented 118,027 single cells categorized into cell types with distinct color codes. (b) UMAP plot illustrated the distribution and expression level of CPT1C. (c) Proportion of CPT1C-expressing cells detected in each cell type. (d) The correlation of CPT1C with CAFs markers in bulk RNA-seq data. (e-f) UMAP plot illustrated the distribution and expression level of CPT1C in 11 subclusters of CAFs. (g) Multiplex immunofluorescence staining with anti-CPT1C, anti-COL1A1 antibodies and IgG identified the co-localization of CPT1C-expressing cells and CAFs in GC tissue. (h) Differential infiltration of CPT1C$^+$CAFs between normal and tumor tissues from TCGA cohort (***$p < 0.001$, Wilcoxon test). (i) Differential infiltration of CPT1C$^+$CAFs between four molecular subtypes of GC in TCGA cohort (**$p < 0.01$; ***$p < 0.001$, Wilcoxon test). (j, k) Survival analysis of OS and PFS of low and high CPT1C$^+$CAF group stratified in TCGA and ACRG cohort, respectively. OS, overall survival. PFS, progression-free survival.

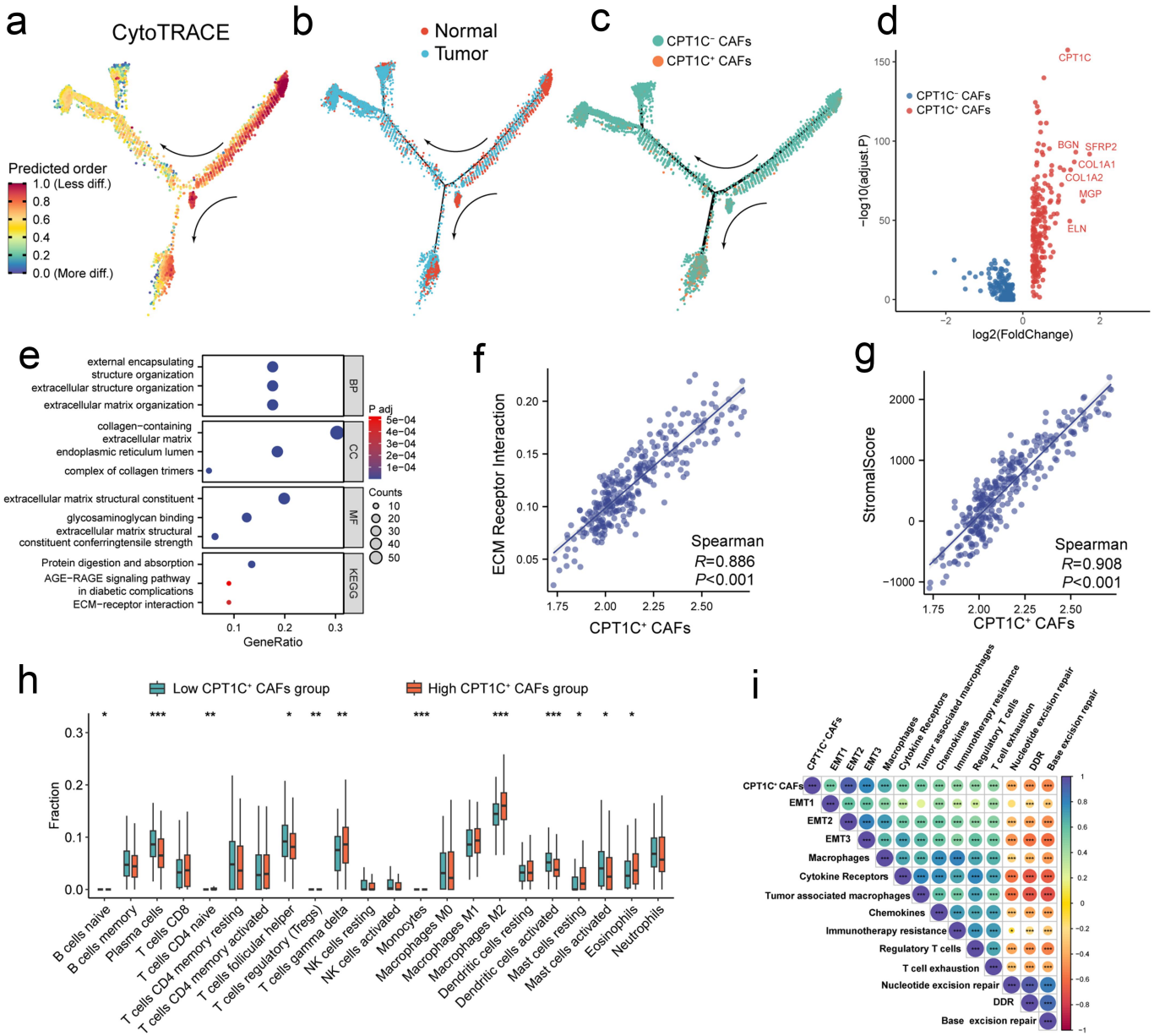

Figure 2. CPT1C⁺CAFs were associated with enriched stroma and immunosuppressive TME of GC. (a) trajectory of fibroblasts along pseudotime in a two-dimensional space. (b, c) trajectory of fibroblasts of normal mucosa versus GC tissues and CPT1C⁺CAFs versus CPT1C⁻CAFs along pseudotime in a two-dimensional space, respectively. (d) Differentially expressed genes between CPT1C⁺CAFs and CPT1C⁻CAFs. (e) GO and KEGG pathways enrichment analysis of highly expressed genes in CPT1C⁺CAFs. (f, g) correlation of CPT1C⁺CAFs with ECM receptor interaction pathway and tumor stromal score, respectively. (h) Differential infiltration of immune cell subsets between high and low CPT1C⁺CAFs group. (i) Correlation between CPT1C⁺CAFs and cancer- and immune-associated pathways. GO, Gene Ontology. ECM, extracellular matrix.

potential correlation between CPT1C⁺CAFs and key components in TME. Notably, our analysis revealed that the high CPT1C⁺CAFs group exhibited significant enrichment of macrophage with M2-like phenotype, known as a key negative regulator of anti-tumor immunity, along with other immunosuppressive subsets such as γδ and regulatory T cells. In contrast, the low CPT1C⁺CAFs group displayed an accumulation of activated dendritic cells and other effector immune cell subsets (Figure 2(h)). We also demonstrated that CPT1C⁺CAFs correlated with pathways related to macrophages, cytokine receptors, tumor-associated macrophages (TAMs), and immunotherapy resistance (Figure 2(i)). These findings strongly suggested the potential role of CPT1C⁺CAFs in fostering the immunosuppressive TME in GC.

CPT1C⁺CAFs promoted the M2-like phenotype of macrophages in GC

Accumulating evidence suggests that the CAFs facilitate immunosuppression by regulating key immune factors in malignancies.[27] Consequently, we further explored whether CPT1C⁺CAFs shaped the immunosuppressive TME by exerting modulatory effects on macrophages in GC. Firstly, we categorized myeloid cells into five clusters (Figure 3(a)) and observed a significant negative and positive correlation of CPT1C⁺CAFs infiltration with M1 and M2-like macrophages, respectively (Figure 3(b,c)). GC patients exhibiting high CPT1C⁺CAFs levels displayed significantly increased abundance of M2-like macrophages in the TME (Figure 3(d)), paralleled by the enrichment of M2-like macrophage signature in

the high CPT1C$^+$CAFs group in the ACRG cohort (Figure 3(e)). To further validate the observed association, we established an *in vitro* co-culture system of CAFs freshly isolated from surgical specimens with THP-1 cell line- or human peripheral blood monocytes (PBMCs)-derived macrophages (Figure 3(f)) and demonstrated that siRNA-based down-regulation of CPT1C expression in CAFs (Figure 3(g)) resulted in a significant reduction of M2-like macrophage represented by its distinctive CD163 and CD206 marker (Figure 3(h,i)). Taken together, these findings highlighted the promotive role of CPT1C$^+$CAFs in inducing the M2-like phenotype of macrophages in GC.

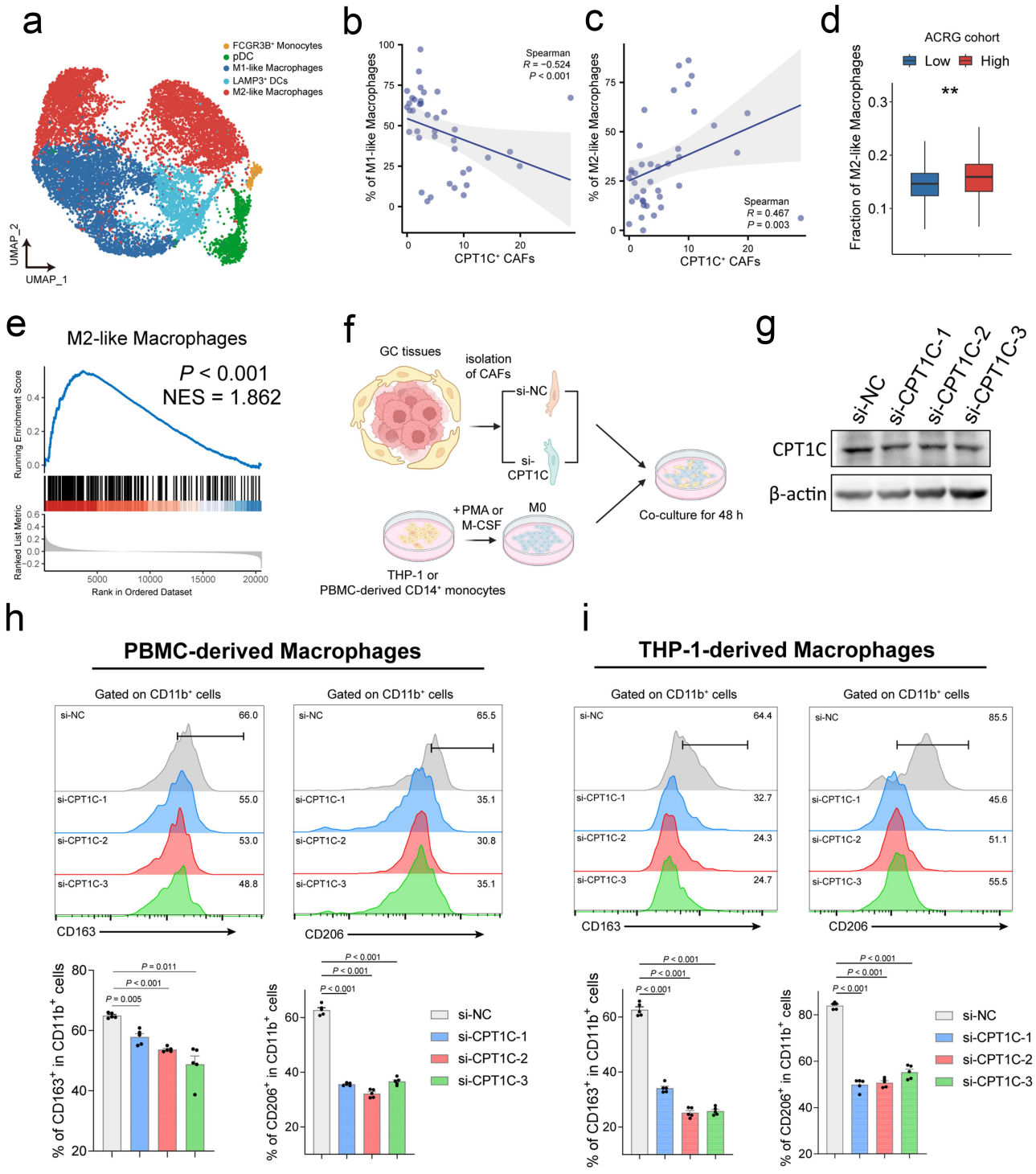

Figure 3. The effect of CPT1C+CAFs on promoting M2-like phenotype of macrophage in GC. (a) UMAP plot illustrated five clusters of myeloid cells with distinctive colors. (b, c) Correlation of CPT1C$^+$CAFs with M1-like and M2-like macrophage, respectively. (d) Differential infiltration of M2-like macrophages in high and low CPT1C$^+$CAFs group. (e) Enrichment of M2-like macrophage signature in high CPT1C$^+$CAFs group shown by GSEA. (f) Schematic diagram of co-culturing CAFs and THP-1 cell line- or PBMC-derived macrophages of GC. (g) Differential expression of CPT1C in control group (si-NC) and CPT1C-knockdown group (si-CPT1C). (h, i) Differential expression of CD163 and CD206 in PBMC-derived macrophages (h) and THP-1-derived macrophages (i) in control group (si-NC) and CPT1C-knockdown group (si-CPT1C) determined by flow cytometry. PBMC, peripheral blood monocytes. GSEA, Gene set enrichment analysis.

CPT1C$^+$CAFs promoted the M2-like phenotype of macrophage in GC by secreting IL-6

A variety of cytokines secreted by CAFs are known to promote the M2-like phenotype of macrophage in several cancer types.[28] We therefore wondered if any cytokines derived from CPT1C$^+$CAFs critically involved in such process. Firstly, we noted that down-regulation of CPT1C expression in CAFs significantly reduced the proportion of M2-like macrophages in the conditioned medium (CM) with enriched cytokines (Figure S1A and B). By profiling the cytokines in the supernatant of macrophages pre-treated with conditioned medium, we observed that the control group exhibited higher levels of M2-like macrophages-secreted cytokines, such as IL-10, CCL1, CCL17, CCL20, and CCL22. On the contrary, the CPT1C-knockdown group demonstrated increased levels of IFN-γ, TNF-α, IL-1β, CXCL10, CXCL11 which were considered to be secreted by M1-like macrophages (Figure 4(a)).[29,30] Compared with control group, CM from CPT1C-knockdown group showed significantly increased expression levels of CD86, HLA-DRA, and TNF-α (M1-related markers) but reduced expression levels of VEGFA, CD163, CD206, TGF-β, and ARG1 (M2-related markers) (Figure 4(b) and S1C). Intriguingly, cell–cell communication analysis of above-mentioned scRNA-seq data revealed a robust IL-6 signaling communication between myeloid cells and CPT1C$^+$CAFs, contrasting with much weaker IL-6 signaling between myeloid cells and CPT1C$^-$CAFs (Figure 4(c,d)). In fact, IL-6 is known for promoting M2-like phenotype of macrophages and impeding anti-tumor immunity.[31–33] Similarly, our results also indicated that neutralizing interleukin-6 in CM from CAFs could attenuate M2-like phenotype of macrophage (Figure S2). Notably, we observed the strongest IL-6 signaling intensity between CPT1C$^+$CAFs and M2-like macrophages among various myeloid cell clusters (Figure 4(e)) and identified that CPT1C$^+$CAFs interacted with various ligands of M2-like macrophages through IL-6 (Figure 4(f)), highlighting the rich regulatory capacity of IL-6 secreted by CPT1C$^+$CAFs.

Given that IL-6 was reportedly involved in inducing the differentiation of macrophage into M2-like phenotype in GC,[34] we further explored the source of IL-6 within TME and identified fibroblasts, particularly CPT1C$^+$CAFs rather than CPT1C$^-$CAFs, as major contributors in GC (Figure 4(g,h)). Meanwhile, we identified that the amount of IL-6 was positively correlated with both CPT1C$^+$CAFs infiltration and CPT1C expression level (Figure 4(i)), and GSEA unveiled that IL-6 signaling pathway was significantly enriched in patients in high CPT1C$^+$CAFs group (Figure 4(j)). In line with these findings, we observed that knockdown of CPT1C expression in CAFs correspondingly reduced the secretion of IL-6 *in vitro* (Figure 4(k)). Viewed as a whole, our mechanism studies unveiled that CPT1C$^+$CAFs facilitated the induction of M2-like phenotype of macrophage in GC by promoting the secretion of IL-6 and its signaling.

CPT1C$^+$CAFs were associated with resistance to adjuvant chemotherapy and immunotherapy in GC

Given the crucial role of adjuvant therapy in patients with advanced GC,[2] we examined the correlation between CPT1C$^+$CAFs infiltration and treatment efficacy. In both ARCG and GSE26942 adjuvant chemotherapy cohorts, patients in high CPT1C$^+$CAFs group displayed significantly worse long-term outcome than those with low CPT1C$^+$CAFs (Figure 5(a,b)). Similar trends were also observed in the SMC cohort of GC patients receiving chemoradiotherapy (Figure 5(c)). These results suggested that heightened CPT1C$^+$CAFs infiltration indicated resistance to adjuvant chemotherapy and chemoradiotherapy in GC.

While immune checkpoint inhibitor (ICI)-based immunotherapy has emerged as a promising treatment for late-stage GC patients, its effectiveness can be hindered by immunosuppressive cell subsets within TME.[35] As the above-mentioned data indicated the immunosuppressive role of CPT1C$^+$CAFs by promoting M2-like phenotype of macrophages via heightened secretion of IL-6, we hereby investigated the potential association between CPT1C$^+$CAFs infiltration and patient response to immunotherapy (Figure 5(d)). In fact, it was noted in KIM immunotherapy cohort that GC patients in the high CPT1C$^+$CAFs group exhibited significantly lower response rate to immunotherapy compared to those with low CPT1C$^+$CAFs (Figure 5(e)), and CPT1C$^+$CAF infiltration level of immunotherapy responders was remarkably lower than non-responders (Figure 5(f)). In summary, these cohort-based analyses demonstrated the immunosuppressive role of CPT1C$^+$CAF in the clinical practice in the treatment of GC, especially in the realm of immunotherapy.

Discussion

Our study presents a novel perspective on how phenotypically heterogenous CAFs negatively modulate tumor immunity in GC. For the first time, we identified CPT1C$^+$CAFs as a unique subset characterized by CPT1C overexpression and the immunosuppressive traits. Elevated expression of CPT1C$^+$CAFs indicated both unfavorable prognosis and therapeutic resistance of GC patients. By analyzing scRNA-seq datasets, we demonstrated that CPT1C$^+$CAFs contributed to shaping the stroma-enriched and immunosuppressive TME. Mechanism studies revealed that CPT1C$^+$CAFs-derived immunosuppressive cytokine IL-6 promoted M2-like phenotype of macrophage in GC. Clinical cohort study demonstrated GC patients with high infiltration of CPT1C$^+$CAFs responded poorly to multiple treatments, especially immunotherapy.

Carnitine palmitoyltransferase 1 (CPT1) catalyzes the rate-limiting step of FAO which fuels tumor growth under metabolic stress, and inhibition of CPT1 expression significantly reduced tumor progression.[36,37] It also functionally intertwines with key molecules in certain pathways to promote the growth and metastasis of many malignancies.[38] Notably, the intriguing isoform CPT1C, although characterized by low enzymatic activity, influences energy homeostasis and acts as a metabolic mediator to adapt to the constantly changing TME.[39] Interestingly, recent studies have addressed the potential link between CPT1-mediated metabolism and CAFs. For instance, Peng

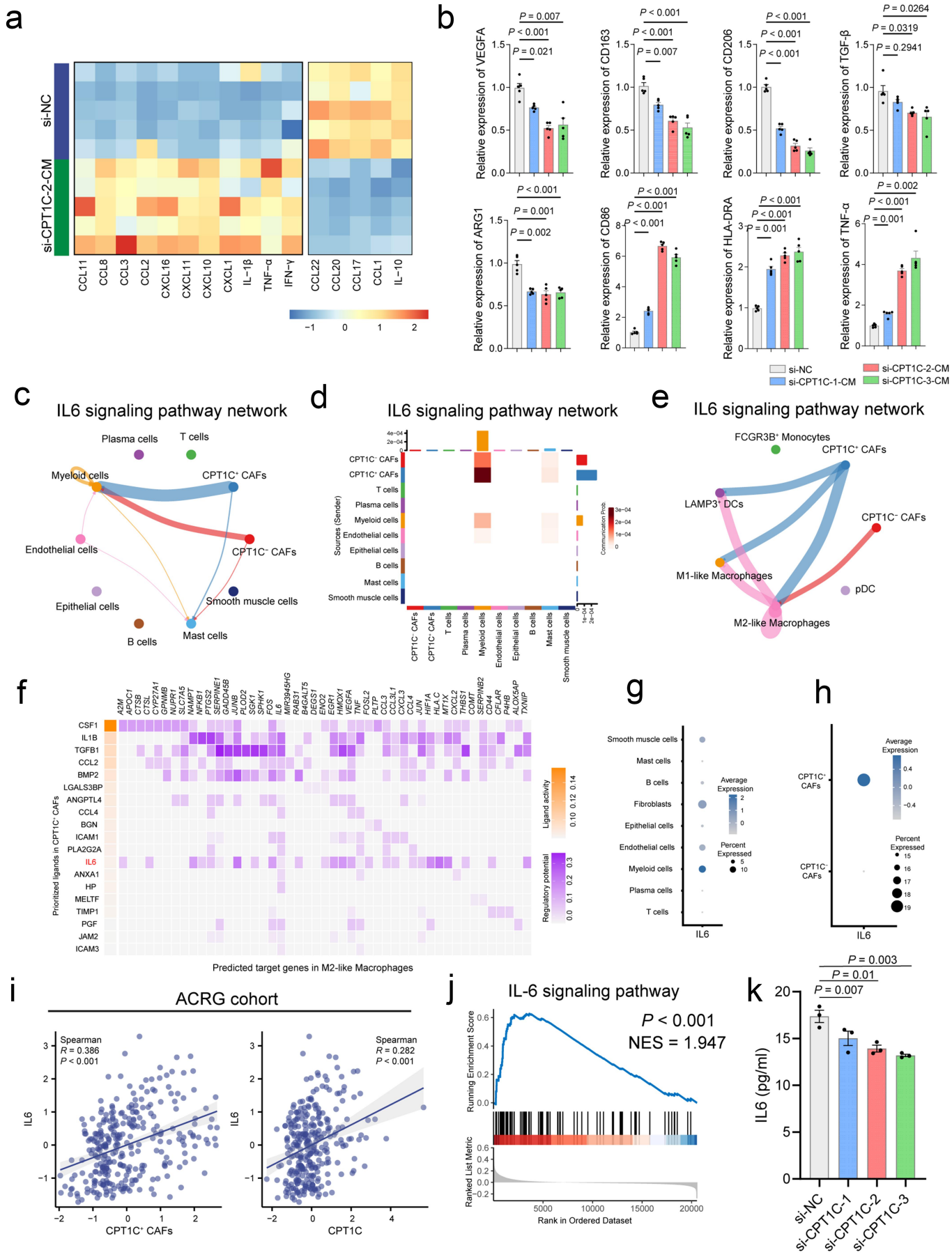

Figure 4. CPT1C^{+}CAFs promoted M2-like phenotype of macrophage in GC through IL-6. (a) cytokine profiling of PBMC-derived macrophages pre-treated with CM. (b) The relative expression of VEGFA, CD163, CD206, TGF-β, and ARG1 (M2-related markers), as well as CD86, HLA-DRA, and TNF-α (M1-related markers), in PBMC-derived

et al. revealed that CAF-derived lipids facilitated peritoneal metastasis of colorectal cancer through increased lipid uptake in cancer cells, dependent on CPT1A overexpression by CAFs.[40] Inhibition of CPT1A activity disrupted the fatty acid metabolism in the CAF and resulted in tumor proliferation and metastasis.[41] In GC, although studies indicated its protumoral role,[6,7] any specific subsets in TME that correlate with CPT1C and their potential association with immunomodulation in GC are yet to characterize.

Our bioinformatics analyses of scRNA-seq datasets revealed that CPT1C-expressing cells were predominantly clustered within subsets of fibroblasts, which were further validated by multiplex immunofluorescence. Consequently, we speculated that CPT1C$^+$CAFs constituted a unique subset influencing GC severity in certain ways. In fact, CAFs are composed of phenotypically diverse cellular populations within TME,[42] and recent advancements in scRNA-seq have underscored this heterogeneity by delineating distinct cell types and subpopulations in TME.[43] By dissecting distinctive subpopulations of CAFs with various origins, activation states and differential gene expression profiles, these subpopulations were linked to various aspects of tumor progression through the scRNA-seq analyses.[44,45] Of note, certain subsets of CAFs have been associated with modulating tumor immunity by secreting immunosuppressive factors, recruiting immunosuppressive cells, altering cytokine and chemokine milieu within TME in various cancer types.[46,47] In GC, however, limited studies have characterized the immunomodulatory roles of CAFs in this regard. A recent bioinformatic study reported the positive correlation of CAF infiltration with M2-like macrophage accumulation whereas its canonical markers, CD163 and CD206, varied in accordance with quantified CAFs in clinical cohorts, indicating the immunosuppressive aspect of CAFs in GC.[48] Considering the significant immunosuppressive role of macrophage with M2-like phenotype in cancer,[49,50] we explored and validated the potential correlation between CPT1C$^+$CAF and M2-like phenotype of macrophage in GC with both bioinformatic analyses of public datasets and *in vitro* assays. Additionally, we found that CPT1C$^+$CAF were more enriched in the GS and CIN subtypes associated with more immunosuppressive phenotype and less responsive to immunotherapy when compared to EBV and MSI subtypes.[24,51] In light of these findings, we not only identified CPT1C$^+$CAF as a functionally unique subset of CAFs, but also demonstrated its immunosuppressive role through promoting M2-like phenotype of macrophage in GC.

Further mechanism interrogation uncovered IL-6 secreted by CPT1C$^+$CAF played a key role in the elevation of M2-like phenotype of macrophage. In fact, CAFs-derived IL-6 is reportedly involved in immunomodulation through interacting with key tumor-infiltrating components in TME. Kato et al. identified that IL-6 secreted by CAFs inhibited CD8$^+$ T cells and FoxP3$^+$ T cells and neutralizing antibody targeting IL-6 significantly improved immunosuppression.[52] They further interrupted CAF-induced immunosuppression *in vivo* using anti-IL-6 receptor antibody, suggesting the possibility of overcoming CAF-induced immunosuppression by targeting IL-6 receptor.[53] Importantly, CAF-derived IL-6 has been repeatedly shown to facilitate the polarization of macrophage into the M2-like phenotype. For instance, Cho et al. found that CAFs-secreted IL-6 and GM-CSF cooperatively induce the differentiation of monocyte into M2-like macrophages in colon cancer.[33] Co-transplantation of CAFs and cancer cells increased the level of IL-6 and GM-CSF and promoted TAM infiltration and tumor metastases, such effects could be significantly reversed by joint antibody blockade of IL-6 and GM-CSF.[33] Kim et al. proved that CAFs secreted IL-6 and IL-8 to promote the differentiation of myeloid cells into M2-like macrophage in colorectal cancer.[54] In pancreatic cancer, stellate cells, a subset of pancreatic CAFs, produce IL-6 and other factors to facilitate the recruitment of monocyte, differentiation of macrophage and its M2 polarization.[55] In GC, CAF-derived IL-6 promoted cancer metastasis and reduced chemotherapy-induced apoptosis by mediating key cancer pathway.[56,57] IL-6 was also indicated as a key player in the crosstalk between CAFs and cancer cells.[57] However, neither the immunomodulatory roles of CAF-derived IL-6 nor the specific key immune subsets they interact with have been addressed before. Notably, our novel findings highlighted the immunosuppressive activity of CPT1C$^+$CAFs through promoting IL-6-induced M2-like phenotype of macrophage in GC. So far as we know, this is the first study to report that IL-6 is a key cytokine underlying the CAF-mediated promotion of M2-like phenotype of macrophage in GC.

In summary, we herein present compelling evidence from both bioinformatic analyses and experimental assays to demonstrate the significance of a novel functionally distinctive CAF subset, CPT1C$^+$CAFs, in fostering immunosuppressive TME in GC. Mechanistically, CPT1C$^+$CAFs secrets IL-6 to promote the immunosuppressive M2-like phenotype of macrophage to negatively regulate anti-tumor immunity. Although this is the first study to report the immunosuppressive role of CPT1C$^+$CAFs via IL-6-mediated elevation of M2-like macrophage in GC, we are aware that the complicated process of macrophage differentiation and polarization involves a variety of cytokines and chemokines from TME beyond IL-6 alone, not to mention the molecules and their corresponding signaling pathways that IL-6 interact with in GC. Future studies should focus on not only identifying CPT1C$^+$CAFs-derived key cytokines and factors other than IL-6, but also investigating

macrophages cultured with different CM. (c) Cell-cell communication network analysis illustrated IL-6 signaling intensity between CPT1C$^\pm$CAFs and 9 distinct cell types categorized by their transcription features. (d) Differential IL-6 signaling intensity between CPT1C$^\pm$CAFs and 9 distinct cell types shown in heatmap. (e) Cell-cell communication network analysis illustrated the IL-6 signaling intensity between CPT1C$^\pm$CAFs and five clusters of myeloid cells. (f) The prioritized ligands in CPT1C$^+$CAFs and the predicted targeted genes in M2-like macrophages shown in heatmap. (g) Differential expression level of IL-6 between 9 distinct cell types. (h) Differential expression level of IL-6 between CPT1C$^+$CAFs and CPT1C$^-$CAFs. (i) Correlation of IL-6 with CPT1C$^+$CAFs infiltration and CPT1C expression in ACRG cohort ($n = 300$). (j) IL-6 signaling signature enriched in high CPT1C$^+$CAFs group shown by GSEA. (k) Differential level of IL-6 in control group (si-NC) and CPT1C-knockdown group (si-CPT1C) in CM determined by ELISA kit. CM, conditioned medium. ELISA, enzyme-linked immunosorbent assay.

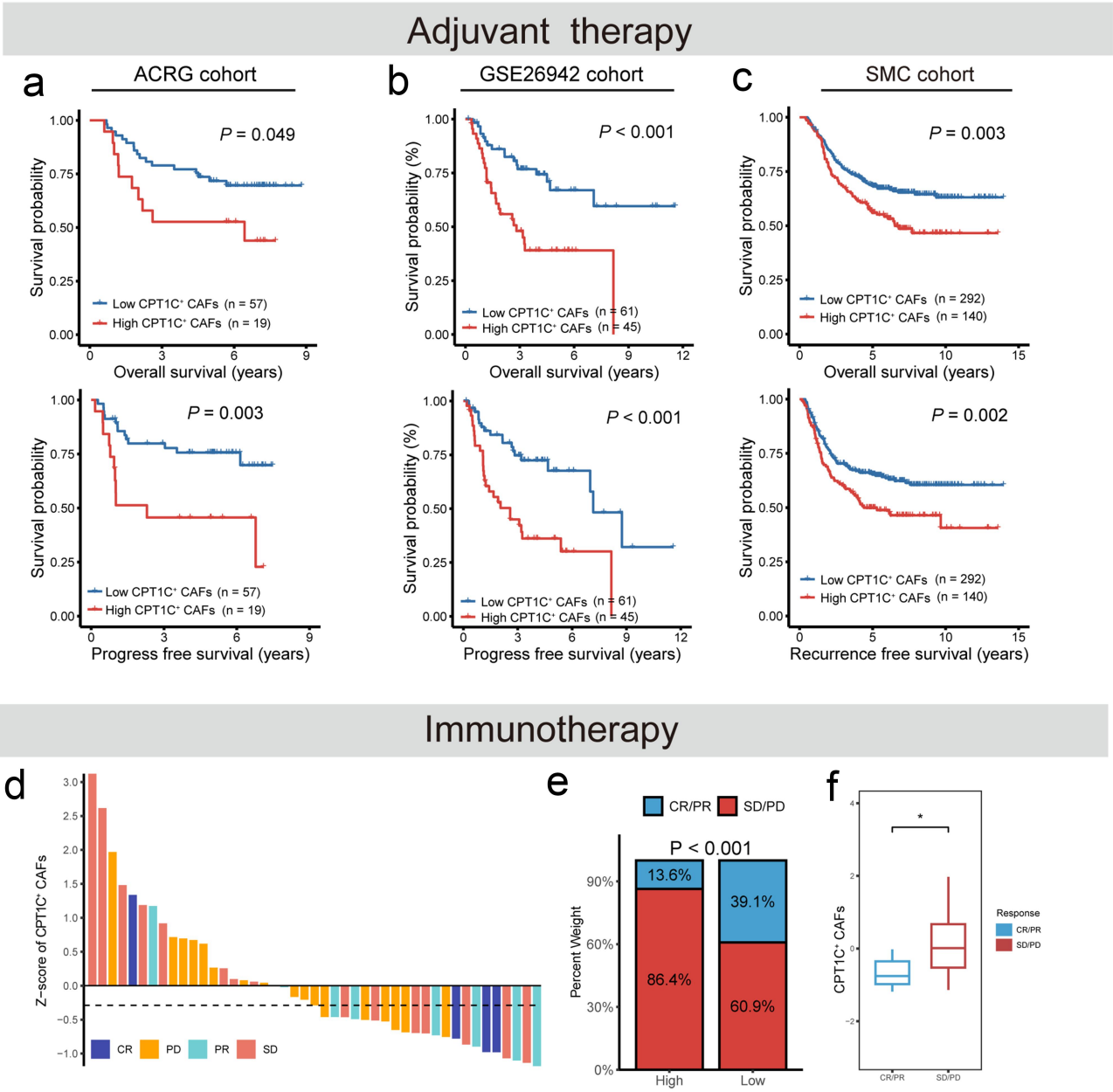

Figure 5. CPT1C$^+$CAFs were associated with resistance to adjuvant therapy and immunotherapy in GC. (a, b) Kaplan – Meier plots illustrated the survival of CPT1C$^+$CAFs high and low patients receiving adjuvant chemotherapy in ACRG cohort ($n = 76$) and GSE26942 cohort ($n = 106$), respectively. (c) Kaplan – Meier plots illustrated the survival of CPT1C$^+$CAFs high and low patients receiving adjuvant chemoradiotherapy in SMC cohort ($n = 432$). (d) The landscape of CPT1C$^+$CAFs infiltration level in GC patients with differential immunotherapy responses ($n = 45$) in KIM cohort. (e) Differential proportion of CR/PR and SD/PD in high and low CPT1C$^+$CAFs group. (f) Differential infiltration of CPT1C$^+$CAFs in CR/PR group and SD/PD group (* $p < 0.05$). CR, complete response. PR, partial response. SD, stable disease. PD, progressive disease.

the underlying mechanisms that determine the differentiation and polarization of macrophage in GC. Such effort could pave the way for improving the efficacy of immunotherapy by overcoming CAF-induced immunosuppression.

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ORCID

Jianpeng Gao http://orcid.org/0000-0002-4627-6278

Author contributions

X. Liu and J. Gao conceptualized and designed the study, H. Pan, X. Liu, and J. Gao provided the surgical specimen from gastric cancer patients, R. Wei, J. Song, H. Pan, and J. Gao performed experiments and analyzed

and interpreted data, X. Liu and J. Gao supervised the study, R. Wei and J. Gao wrote the manuscript with input from all authors.

Data availability statement

The authors confirm that all data used and analyzed in the current study are available from the corresponding author on reasonable request.

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