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The role of tumor microenvironment in cholangiocarcinoma

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Abstract

Cholangiocarcinoma (CCA) is an extremely aggressive neoplasia, mostly because of diagnostic delay and lack of effective therapies. CCA is typically surrounded by a peculiar microenvironment that includes abundant desmoplastic stroma and various cell types, which support and enhance CCA development. Among the tumor microenvironment (TME) cells, there are tumor infiltrating lymphocytes (TILs), such as $CD8^+$ and $CD4^+$ cells, Tregs, natural killers (NKs) and B lymphocytes. TILs contribute to an immunosuppressive microenvironment that leads to tumor immune escape. Dendritic cells (DCs) may lead to immunotolerance by maturation or antigen-presentation deficiency. Hepatic stellate cells (HSCs) are one of the major precursors of cancer-associated fibroblast (CAFs), which are distinguished in various subpopulations, each with different functions and interactions with other TME cells. CAFs can promote lymphangiogenesis, early lymph-node metastasis and proinflammatory environment, but they can also provide a physical and chemical barrier to protect CCA. Tumor-associated macrophages (TAMs) could be differentiated between two phenotypes, pro- and anti-inflammatory, and they may sustain invasiveness and immunosuppression. Myeloid-derived suppressor cells (MDSCs) impair cytotoxic T lymphocytes (CTLs) function, stimulating tumor proliferation and angiogenesis. Tumor-associated neutrophils (TANs) function is influenced by the TME, leading to tumor-suppressing or tumor-promoting functions. This paper aims to provide an overview of the CCA microenvironment cells, their role in tumor progression and possible correlated diagnostic, therapeutic and prognostic implications.

Keywords: Tumor microenvironments, cholangiocarcinoma, cancer-associated fibroblast, tumor associated macrophages, myeloid-derived suppressor cells, Tumor-infiltrating lymphocytes, dendritic cells, immunotherapy

INTRODUCTION

Cholangiocarcinoma (CCA) is a rare but aggressive biliary-derived cancer with few therapeutic options. The tumor microenvironment (TME) has a key role in sustaining tumor progression. In fact, CCA has an abundant desmoplastic stroma and it is surrounded by many cell types, such as hepatic stellate cells (HSCs), cancer-associated fibroblasts (CAFs), tumor-infiltrating lymphocytes (TILs), tumor-associated neutrophils (TANs), tumor-associated macrophages (TAMs), myeloid-derived suppressor cells (MDSCs), dendritic cells (DCs) and extracellular matrix (ECM), which have intense crosstalk between themselves and cancer cells [Figure 1]. This interplay provides a good environment for tumor growth, metastasis, chemoresistance, and tumor-specific immune tolerance, which may be a target for new immunotherapy approaches. Here, we will describe the main cell types of CCA TME, and their related pathways, that have been shown to influence tumor development, prognosis or response to current or future treatments.

TUMOR-INFILTRATING LYMPHOCYTES

TILs are involved in immune response against tumor cells, detection of cancer antigens and killing of neoplastic cells[1]. In CCA, TILs consist mainly of $CD8^+$ and $CD4^+$ T lymphocytes but also, to a lesser degree, of NKs and B lymphocytes[2]. Interestingly, TILs from resected CCA differ from their counterparts in tumor-free liver. In fact, in CCA, cytotoxic T cells and natural killer cells (NKs) are reduced while regulatory T cells (Tregs) are increased[3]. In TME, indeed, CAFs, TANs and TAMs produce C-C motif chemokine ligand 2 (CCL2) enrolling Tregs[4-6], while MDSCs and TAMs secrete interleukin-10 (IL10) and transforming growth factor β (TGF-β), which also convert DCs into regulatory DCs. Tregs and regulatory DCs perpetuate this vicious cycle attracting more immunosuppressive immune cells and weakening antitumor defenses[7,8]. However, TGF-β and IL10 production is not dependent only on TME cells but also on CCA cells[4] [Figure 1]. Furthermore, extrahepatic cholangiocarcinoma (eCCA) cells seem to produce prostaglandin E2 and adenosine, reducing T cell activity[9-13] and the expression of CXCL12 by CAFs disrupt T-cells migration into tumors[14].

TILs subpopulations have different localizations. In fact, $CD8^+$ T cells and $CD4^+$ T (Foxp3-) cells are in cancer margins, while Tregs ($Foxp3^+$) infiltrate the core[11]. This setup demonstrates the relegation of the effectors in CCA[15].

Various molecular mechanisms underlying TILs regulation have been described. Intrahepatic cholangiocarcinoma (iCCA) cells induce T and NK lymphocyte death via Fas/Fas ligand (Fas L) high expression levels[16]. 67-kDa laminin receptor induces FasL expression in human CCA cells with subsequent activation of the FasL promoter via the extracellular signal-regulated kinase (ERK) pathway, which is a possible target of specific mitogen-activated protein kinase (MAPK)-ERK cascade inhibitor[17]. Killer cell immunoglobulin-like receptors (KIRs) regulate NK cells function and KIR genes were found altered in CCA, possibly affecting NK cell tumor surveillance[18,19]. Moreover, Wingless and Int-1(Wnt)/-catenin and TGF-signaling pathways are correlated to a reduced number of tissue-resident memory-like $CD8^+$ TILs, which are involved in immune response against tumor cells[20]. The expression of B7-H1 and its receptor programmed death 1 (PD-1) in iCCA leads to immune escape due to $CD8^+$ TILs apoptosis[21] [Figure 2]. The atypical protein kinase C-iota (aPKC-i)/Ser59-phosphorylated specificity protein 1 (P-Sp1)/Snail signaling stimulates the differentiation in T regulatory-like cluster of CD25- cells which have an immunosuppressive function in CCA[22].

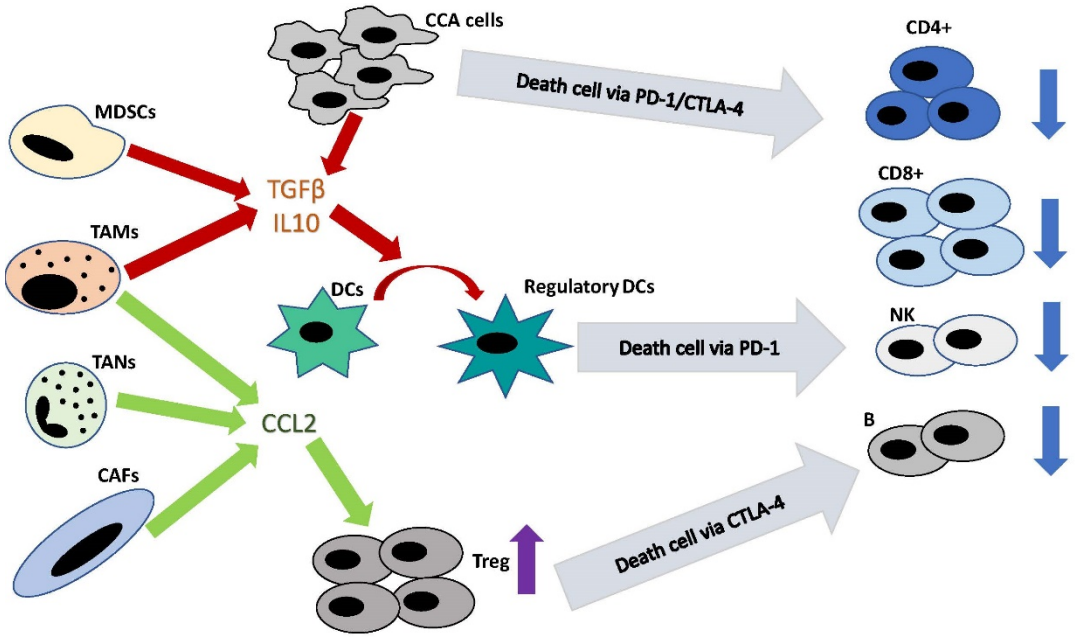

Figure 1. Tumor microenvironment influence on effector lymphocytes. CCA: cholangiocarcinoma; PD-1: programmed cell death; CTLA4: cytotoxic T-lymphocyte-associated protein 4; NKs: natural killers; IL-10: interleukin-10; TGFβ: transforming growth factor β; MDSCs: myeloid-derived suppressor cells; B: B lymphocytes; CD 8^+: CD 8^+ lymphocytes; CD 4^+: CD 4^+ lymphocytes; Treg: regulatory T cells; DCs: dendritic cells; CAFs: cancer-associated fibroblasts; TAMs: tumor-associated macrophages; TANs: tumor-associated neutrophils; CCL2: C-C motif chemokine ligand 2.

TILs are also important for potential immunotherapy for CCA. Tumor-induced immunological checkpoints control [e.g., PD-1 and cytotoxic T-lymphocyte-associated protein 4 (CTLA-4)] establish an immunosuppressive microenvironment, which leads to tumor immune escape[23,24] [Figure 1]. A combination of anti-CD40 and anti-PD-1 in mice iCCA results in myeloid cells, $CD4^+$ and $CD8^+$ T cells and NK cells activation, with a cancer burden reduction[25]. Pan *et al.* showed that the development of specific antibodies, which inhibit iCCA growth in rats, may be elicited by CTLA4 - anti-programmed cell death ligand (PD-L1) DNA immunization[26]. Moreover, the combination of gemcitabine with cytotoxic T-lymphocytes (CTLs) increases the cytotoxic activity of effector T cells against chemo-resistant CCA cells *in vitro*, suggesting a potential benefit by this combination therapy[27]. Cetuximab is found to stimulate the activity of cultured cytokine-activated (with a high dose of IL-2 and anti-CD3 monoclonal antibodies) killing cells against CCA[28]. Conversely, Kirsten rat sarcoma (KRAS) alteration is associated, in PD-1/PD-L1 blockade-treated patients, with resistance to immunotherapy. This seems to be related to a low TILs density in the TME of KRAS-altered neoplasia, suggesting a correlation between KRAS and low immunogenicity in iCCA[29]. Furthermore, high-level microsatellite instability (MSIH) in CCA predicts response to immune checkpoint blockade, in particular to anti-PD-1 or PD-L1 therapy[30]. MSIH is also associated with a longer overall survival (OS) and the presence of a more numerous population of $CD8^+$ T cells, $FOXP3^+$ regulatory T cells, and $CD20^+$ B cells[31]. Moreover, MSIH correlates with a higher level of BRCA-mutated iCCA[32]. Immune checkpoint inhibitors may represent a promising strategy for CCA treatment. Durvalumab (anti-PD-L1), which is now under investigation for advanced biliary tract cancer, showed interesting results with a 36-month OS rate of 30.7% and a manageable safety profile as second-line in monotherapy and in combination with other molecules[33].

Other than for position, $CD8^+$ and $CD4^+$ T cells and Tregs differ for prognostic value. The former and $CD20^+$ B cells are related to a better prognosis; the latter, when abundant, are associated with worse overall

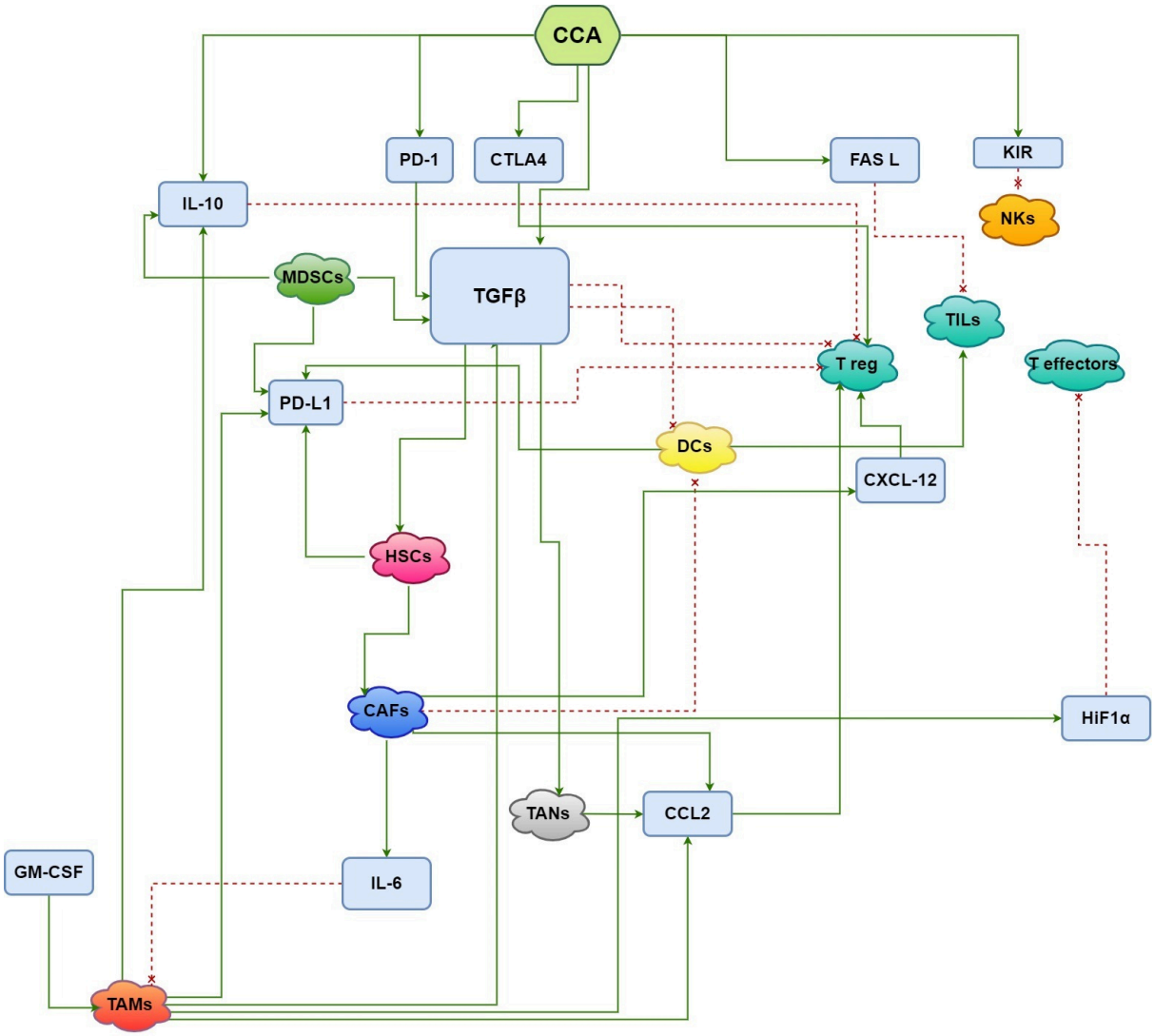

Figure 2. Interactions of cells involved in cholangiocarcinoma development and tumor microenvironment. The green arrows indicate induction, while the red dotted lines suggest inhibition. CCA: cholangiocarcinoma; PD-1: programmed cell death; CTLA4: cytotoxic T-lymphocyte-associated protein 4; FasL: Fas ligand; KIR: killer cell immunoglobulin-like receptor; NKs: natural killers; IL-10: interleukin-10; TGFβ: transforming growth factor β; MDSCs: myeloid-derived suppressor cells; PD-L1: programmed cell death ligand; T reg: regulatory T cells; TILs: tumor infiltrating lymphocytes; DCs: dendritic cells; CXCL-12: C-X-C motif chemokine ligand 12; HSCs: hepatic stellate cells; CAFs: cancer-associated fibroblasts; TAMs: tumor-associated macrophages; TANs: tumor-associated neutrophils; CCL2: C-C motif chemokine ligand 2; IL-6: interleukin-6; GM-CSF: granulocyte-macrophage colony-stimulating factor; HiF1α: hypoxia-inducible factor 1 subunit alpha.

survival (OS)[2,3,20,34-40]. A recent systematic review showed that high levels of $CD8^+$ and $CD4^+$ T cells are associated with better prognosis in CCA, regardless of their position. Recently, Alvisi *et al.* demonstrated that in iCCA $CD4^+$ Tregs are hyperactivated in comparison with $CD8^+$ T cells, suggesting that $CD8^+$ effector functions are reduced and $CD4^+$ Tregs immunosuppressive functions are amplified[41]. This evidence comes from the hyperexpression of mesenchyme homeobox 1 (MEOX1) by Tregs and the consequent evolution of circulating Tregs in tumor-infiltrating Tregs. Therefore, these finding correlate with a poor prognosis due to immunosuppression[41]. However, the prognostic value of $Foxp3^+$ T cells is still not clear and requires further research[2,34-39,42-46]. Instead, B cells seem to be associated with an improved prognosis[2,15]. Finally, high C-X-C motif ligand 9 (CXCL9) expression, which in animal models stimulates NK cell recruitment, enhancing

antitumor immunity, is correlated with better survival after resection[6].

DENDRITIC CELLS

Tumor-infiltrating DCs are abundant in TME and are characterized by an elevated expression of CD40 on their surface[25,47]. They may contribute to immunotolerance by maturation or antigen-presentation deficiency, which leads to an inhibition of $CD8^+$ and $CD4^+$ T-cell priming[7,8]. Moreover, DCs produce PD-L1[8] and attract Tregs which express (CTLA-4) that sustains the regulatory phenotype of DCs[7,8]. In CCA, CAFs can attract DCs and reduce the expression of human leukocyte antigen (HLA) molecules, weakening the activation of TILs[48]. Interestingly, Martìn-Sierra *et al.* found that in CCA patients, the immunomodulation may not be relegated only to the peritumoral area[49]. In fact, in these patients, there are low levels of circulating classical dendritic cells and monocytes positive for the Fc fragment of IgE high affinity I receptor (FceRI), which are suspected to eventually differentiate into classical dendritic cells and tumor necrosis factor α(TNF-α) -producing proinflammatory DCs[49,50].

IL-10 and TGFβ, produced by CCA cells, have an immunosuppressive effect on dendritic cells: they depress antigen presentation and the activation of effector T lymphocytes, leading to tumor evasion of immune surveillance[51-53] [Figure 2]. Thepmalee *et al.* demonstrated that the blockage of IL-10 and TGF-β receptors on DCs by specific neutralizing antibodies enhances cytolytic activity of effector T-cells against CCA and increases the level of interferon-γ (IFN-γ)[54].

The use of postoperative DCs vaccine plus activated T-cell transfer could prevent recurrence and improve survival in patients affected by iCCA, as demonstrated by a clinical trial. Five years progression-free survival (PFS) and OS were superior in patients treated with DCs vaccine plus activated T-cell transfer than in patients who did not receive that treatment[55].

Intracellular protein kinase CAMP-dependent type I regulatory subunit alpha (PRKAR1A) is overexpressed in CCA. PRKAR1A-presenting self-differentiated monocyte-derived dendritic cells (SD-DC) activates effector T cells killing ability versus the tumor, which is nearly doubled than those stimulated with control DC *in vitro*[56].

With regards to the prognostic role of DCs, peritumoral plasmacytoid DCs (pDCs) correlate with wider local and distal extension, higher chance of recurrence and shorter OS. Furthermore, larger numbers of pDCs are associated with increased $Foxp3^+$ regulatory T-cell infiltration[57].

$CD83^+$ mature dendritic cells are located primarily on invasive front of CCA, while $CD1a^+$ immature DCs are gathered within the tumor tissue. $CD83^+$ DCs density was associated with a greater number of $CD4^+$ or $CD8^+$ T cells infiltrating the tumor and was correlated with a good prognosis and lower incidence of metastases[50,58].

CANCER-ASSOCIATED FIBROBLASTS

CAFs are a group of various cells, in which the predominant type is the activated myofibroblasts. CAFs express several phenotypic markers such as α-smooth muscle actin (α-SMA), platelet-derived growth factor receptor β (PDGFRβ), fibroblast specific protein-1 (FSP-1 or S100A4), mucin-like transmembrane glycoprotein podoplanin, and the cell surface metalloprotease cluster of differentiation 10 (CD10). CAFs origin is still partially unclear and controversial; it is likely that CAFs derive from HSCs[59], periductal or portal fibroblasts (PFs)[60], pericytes, mesenchymal stem cells, circulating bone marrow-derived mesenchymal cells and adipocytes[61,62].

Various clusters of CAFs have been described. The first is *vascular CAFs*, which were found in the tumor core and in the microvascular region. *Vascular CAFs* are characterized by the presence of microvascular genes and the production of IL-6 and CCL8, implying a possible interaction with cancer[63]. *Matrix CAFs* express high levels of ECM molecules [e.g., collagen molecules and periostin (POSTN)] and lower levels of α -SMA. *Inflammatory CAFs* produce low levels of α-SMA and high levels of fibulin 1 (FBLN1), insulin-like growth factor 1 (IGFI), insulin-like growth factor binding protein 6 (IGFBP6), secretory leukocyte peptidase inhibitor (SLPI), serum amyloid A1 (SAA1), C3 and C7, intimating a role in cancer immunity. *Myofibroblastic CAFs* and *mesothelial CAFs* coexpress portal fibroblast/mesothelial markers[64]. These different phenotypes are probably involved in the CCA progression through the release of biochemical signals such as TGF-β1, connective tissue growth factor (CTGF), stromal cell-derived factor-1 (SDF-1), ECM components such as POSTN, collagen type I, osteopontin, IL-6 and IL-33, and matrix metalloproteases (1, 2, 3,9)[65,66] [Table 1 and Figure 2].

In iCCA, low tissue expression of osteopontin was associated with lymph node metastasis and worse prognosis, while serum osteopontin levels were elevated in patients with CCA compared to healthy controls and patients with primary sclerosing cholangitis. Moreover, high concentrations of serum osteopontin before and after surgery are associated with poor postoperative survival.

Similarly, high POSTN, produced by α-SMA$^+$ CAFs, can be evaluated to discriminate CCA from normal/cirrhotic liver or hepatocellular carcinoma and is correlated with a shorter 5-year survival in post-resected iCCA.

PDGF D domain (PDGF-DD) produced by CCA cells binds to PDGFRβ and stimulates fibroblasts motility[67] and vascular endothelial growth factor (VEGF)-C and VEGF-A secretion, thus contributing to early metastasis to lymph nodes[68]. Nevertheless, imatinib mesylate (suppressor of the migration on myofibroblast) has shown disappointing preliminary results[69].

CAF-released heparin-binding (HB) EGF, which binds the EGF receptor on CCA cells, activates signal transducers and activators of transcription 3 (STAT3); this promotes the formation of a proinflammatory microenvironment through activation of the IL6/STAT3 axis[70] with subsequent tumor cell migration, motility, and invasion[71].

Another interesting peculiarity about activated CAFs is the enhanced susceptibility to apoptosis. In fact, BH3-only proteins initiate apoptosis by the ignition of Bax and Bak (multidomain proapoptotic Bcl-2 proteins) in activated CAFs. CCA cells widely express antiapoptotic multidomain Bcl-2 proteins, such as Mcl-1, which inhibits this pathway[72]. Navitoclax, a BH3-only protein mimetic, leads to selective apoptosis in α-SMA$^+$ CAFs but not in CCA cells and quiescent fibroblasts. The downregulation of Mcl-1 and the upregulation of Bax protein sensitize activated CAFs to navitoclax-mediated apoptosis, inducing a reduction in neoplastic burden and metastatization, due to a reduced lymphatic vascularization. Thus, navitoclax leads to an improvement in survival in animal models[68,73].

Moreover, CCA cells overexpress CXC chemokine receptor-4 (CXCR4)- SDF-1, the cognate receptor of SDF-1, which is widely expressed by CAFs in the peritumoral stroma. The binding between CXCR4 and SDF-1 stimulates the antiapoptotic protein Bcl-2 and activates ERK1/2 and PI3K/Akt pathways, permitting CCA cells survival and invasiveness and enhancing HSCs differentiation, supporting further CAFs enrichment[67]. Furthermore, Okamoto *et al.* demonstrated that the wide expression of SDF-1 is correlated with cancer fibrogenesis and epithelial-to-mesenchymal transition (EMT), predicting poor prognosis[74].

Table 1. Different CAFs phenotypic subpopulation, their location in the tumor and roles

CAFs phenotypic subpopulation	Location	Expression	Role
Vascular CAFs	tumor core and microvascular region	production of IL-6 and CCL8	interaction with malignant cells
Matrix CAFs	invasive front of intrahepatic CCA	high levels of ECM molecules, low level of α-SMA	Invasiveness/ECM and collagen fibril organization
Inflammatory CAFs	no specific spatial distribution	low levels of α-SMA, high levels of FBLN1, IGFI, IGFBP6, SLPI, SAA1, C3 and C7,	immune modulation
Myofibroblastic CAFs	no specific spatial distribution	Express portal fibroblast/mesothelial markers	Promotion of tumor growth
Mesothelial CAFs	no specific spatial distribution	Express portal fibroblast/mesothelial markers	Fibrogenic effect

CAFs: Cancer associated fibroblast; IL-6: interleukin-6; CCL8: Chemokine (C-C motif) ligand 8; CCA: cholangiocarcinoma; ECM: extracellular matrix; α-SMA: α-smooth muscle actin; FBLN1: fibulin 1; IGFI: insulin-like growth factor 1; IGFBP6: insulin-like growth factor binding protein 6; SLPI: secretory leukocyte peptidase inhibitor; SAA1: serum amyloid A1.

Interestingly, CAFs could also inhibit CCA progression. It has been demonstrated that high IL-33 content in CAFs and cancer cells is associated with a better prognosis. Therefore, IL-33 may be considered as a valuable prognostic marker and a potential future treatment target[75].

Finally, high expression of α-SMA is correlated to larger tumor size, lymph node metastasis, higher histological grade and a worse 5-year survival rate (6% *vs.* 29%)[67]. Nintedanib (a tyrosine kinase inhibitor of PDGFR, VEGFR, and FGFR) seems to be a promising treatment in refractory iCCA by inhibiting activation, proliferation and αSMA expression in CAFs and reducing cancer-promoting cytokines, such as IL-6 and IL-8[76-78].

HEPATIC STELLATE CELLS

HSCs have an established role in liver tumor carcinogenesis[79] and activated HSC/myofibroblasts are relevant in TME development. The activation of HSCs into tumor-promoting myofibroblasts is induced by TGFβ through the binding with its receptors TGFβR1/TGFβR2 and the subsequent nuclear translocation of small mothers against decapentaplegic homolog (SMAD)[80]. Furthermore, TGFβ induces HSCs expression of α-SMA, fibronectin and CTGF, markers of HSC activation and paracrine factors, that enhance liver progression and metastatization[81,82]. Recently, Sun *et al.* showed how PD-L1, produced by HSCs, stabilizes TGFβR2 and TGFβR1 supporting TGF-β-stimulated activation of HSCs into myofibroblasts[83]. Interestingly, an extracellular domain of PD-L1 halts the lysosomal degradation of TGFβR2 protein and the RNA exosome complex degradation of TGFβR1 mRNA [Figure 2]. Moreover, PD-L1 is a possible target for suppressing HSC activation in iCCA microenvironment due to its role in CAFs differentiation[83]. Furthermore, focal adhesion kinase (FAK) drives TGFβR2 to the HSCs membrane which protects the receptor from degradation, perpetuating HSC activation. Thus, targeting FAK could have a role in the suppression of HSC activation[84].

TUMOR-ASSOCIATED MACROPHAGES

Environmental stimuli, such as IFN-γ, TNF-α, granulocyte-macrophage colony-stimulating factor (GM-CSF) or bacterial endotoxin, may enhance the differentiation of macrophages towards the M1 inflammatory subtype. In contrast, the anti-inflammatory M2 macrophage phenotype is triggered by IL-4, IL-10, IL-13 IL-34, osteoactivin, GM-CSF and immune complexes (IC). M1 macrophages are characterized by high expression of CXCL9 and proinflammatory cytokine (IL-1β, IL-6, IL-12, and IL-23), and low expression of IL-10 and strong tumoricidal activity[85-87] [Figure 2]. M2 macrophages are found to be more represented in

CCA and to facilitate tissue remodeling, tumor progression and immunomodulation[88]. Moreover, M2 macrophages promote EMT by the secretion of cytokines and chemokines, which stimulate IL-10/STAT3 and AKT3/PRAS40 pathways[89,90].

TAMs are induced by costimulation by toll-like receptor (TLR) ligands and A2 adenosine receptor (A2R) agonists or by IL-6. TAMs highly express IL-10, TGF-β, VEGF and angiopoietins, increasing tumor aggressivity[91], while they express low levels of IL-12, TNF-α, and IL-1β. These molecules trigger cholangiocytes proliferation, fibrogenesis, angiogenesis and biliary carcinogenesis[92-95]. In fact, TAMs are negatively correlated with prognosis in CCA[96].

TAMs have multiple functions due to their proinflammatory activity, such as invasiveness, adhesion, and immunosuppression. For instance, TAMs activate Wnt/β-catenin pathway due to Wnt3a and Wnt7b, which contributes to CCA proliferation[97,98]. Moreover, TAMs may suppress T cells effector antitumor activity via hypoxia-inducible factor-1 (HIF-1α) expression, which is expressed in about 66% of CCA and modulates the production of VEGF-A through hypoxia and other autophagy modulators such as PI3KC3, which is highly expressed in CCA and correlates with worse prognosis. Hypoxia-associated autophagy, in fact, is associated with CCA metastasis and a worse prognosis[99,100]. Interestingly, in a recent study, Ruffolo *et al.* demonstrated that anti-GM-CSF antibodies stimulate the repolarisation of immunosuppressive TAMs and MDSCs, promoting anti-tumoral T cell immunity and depressing inflammatory networks[87]. Indeed, a lower expression of GM-CSF in CCA is associated with improved overall survival after tumor resection[78,87].

Liver macrophages express TNF-like weak inducer of apoptosis (TWEAK). In case of damage, Fn14 modulates TWEAK, which supports the proliferation, migration, and polarization of both macrophages and CAFs. Furthermore, TWEAK builds up a proinflammatory environment in CCA, and it is hypothesized to induce NF-kB-driven mitogen, which stimulates neoplastic proliferation[101].

TNFα is widely produced by Kupffer cells surrounding CCA. TNFα stimulates cholangiocyte proliferation, differentiation and carcinogenesis through the activation of c-Jun N-terminal kinase (JNK) signaling. In fact, mitochondrial dysfunction and oxidative stress, due to reactive oxygen species (ROS) secretion from Kupffer cells, are demonstrated to trigger cholangiocellular growth. Thus, ROS/Tnf/JNK axis may be a possible target of therapy in iCCA[102]. Another interesting pathway under study is PCAT6/miR-326/RohA, which has a role in M2 polarization of TAMs since prostate cancer-associated transcript 6 (PCAT6) is an oncogene highly expressed by macrophages in CCA patients[103].

Finally, exosome Circ_0020256 has been recently described in TAM-secreted exosomes and is involved in neoplastic progression *in vivo*[104]. Tumor-derived exosomal miR-183-5p stimulates, through the miR-183-5p/PTEN/AKT/PD-L1 pathway, the expression of macrophage PDL-1, of which major source are TAMs. Thereby exosomal miR-183-5p may be a target against immunotolerance in CCA[105].

MYELOID-DERIVED SUPPRESSOR CELLS

MDSCs derive from bone marrow and are divided into two groups: polymorphonuclear MDSCs (PMN-MDSCs) and monocytes (M-MDSCs). The first group includes differentiated neutrophils, basophils, eosinophils and mast cells. The second group is composed of macrophages and DCs[106]. Specifically, PMN-MDSCs recruitment is permitted by the bond of chemokine CXCL1 to its receptor CXCR2, whereas M-MDSCs are inducted by CCL2-CCR2[107]. These cells are mainly triggered by inflammatory cytokines, and one of their major functions is the suppression of CTLs by PD-L1, permitting tumor proliferation [Figure 2]. In fact, it has been shown that the blockage of TAMs and PD-L1 alone was not sufficient to

slower CCA progression, because PMN-MDSCs bypass the PD/L1 and TAMs blockage through the impaired T-cells mediated immune responses, therefore dual blockage of TAMs and MDSCs may be a goal for CCA treatment[108].

TUMOR-ASSOCIATED NEUTROPHILS

Neutrophils are part of the innate immune system and play an active function in inflammation. Previous studies have shown that neutrophils seem also involved in the development of cancer as tumor-associated neutrophils (TANs)[109]. Their function in cholangiocarcinogenesis has not been deeply understood and investigated. They seem able to have both tumor-suppressing and tumor-promoting functions. TANs may have two different phenotypes, depending on the signals coming from the TME. In mouse models of different cancers, Fridlender *et al.* showed that type 1 ("N1 phenotype") is induced by IFNs and has an antitumor role, while type 2 ("N2 phenotype") is stimulated by TGFβwith tumor-promoting features[110]. In iCCA, both cancer and stromal cells produce CXCL5, which strongly attracts TANs to tumor and promote metastatization as a result of the PI3K-AKT and ERK1/2 pathways activation[5]. TANs expressing CCL2 and CCL17 foster immunosuppression by the recruitment of TAMs and Tregs[4] [Figure 2]. TANs and TAMs interactions favor iCCA development through OSM/IL-11/STAT3 signaling pathway activation. TANs and TAMs interaction has been abolished by STAT3 knockdown or STAT3 inhibitors, as demonstrated *in vitro* and *in vivo*[111].

Conversely, N1 may have an antitumor function. In fact, Gao *et al.* studied the effect of the administration, via percutaneous transhepatic biliary drainage, of tumor-cell-derived microparticles loaded with methotrexate into the bile-duct lumen above biliary obstruction from eCCA[112]. Tumor-cell-derived microparticles may serve as a carrier of chemotherapeutic drugs and simultaneously act as an immune modulator. Mobilization and activation of neutrophils and relief of biliary obstruction were observed in 25% of cases. Neutrophils showed an N1 phenotype, and they were able to attack and kill eCCA cells[78,112].

The prognostic role of TANs has been evaluated in various studies. Kitano *et al.* analyzed eCCA microenvironment and found that TANs were directly correlated with $FOXP3^+$ (T-regs) and inversely with $CD8^+$ T cells[45]. Moreover, a high number of TANs and T-regs are significantly related to poor OS. Mao *et al.* studied neutrophils in CCA and adjacent tissues, using CD15 as their marker[113]. They found that patients with deep neutrophils infiltration (high CD15 expression) had a reduced disease-free survival time and OS[99,113]. Interestingly, a systematic review showed a correlation between neutrophil to lymphocyte ratio (NLR) and OS: a high NLR is associated with significantly poorer OS in CCA[114,115]. Finally, epithelial expression of CXCL15 in CCA was found to correlate with TANs recruitment and α-SMA expression and it is related to a worse prognosis due to shorter survival after resection[116-118].

CONCLUSION

Growing evidence shows how CCA microenvironment plays a key role in multiple aspects of tumor progression. Although a reduction of lymphocyte effector cells leads to immune escape, the development of an immunotolerant environment is the final step of non-tumoral and tumoral cell crosstalk. Specifically, TILs are switched to an immunoregulatory phenotype, DCs have their antigen-presenting activity reduced, CAFs provide both a physical and a chemical barrier to protect CCA, TAMs differentiate themselves in a pro- and anti-inflammatory phenotype and MDSCs impair the cytotoxic activity [Figure 1]. Moreover, CAFs and TAMs support lymphangiogenesis and angiogenesis through VEGF, IL10 and TGFβ production.

The late diagnosis of CCA and the frequent struggle to obtain a diagnostic biopsy have stimulated the research of novel diagnostic biomarkers. Liquid biopsy seems a promising tool to achieve this aim. In fact, the serum or bile evaluation of circulating tumor DNA and miRNA could play a future role as minimally invasive screening, diagnostic, prognostic and therapeutic monitoring biomarkers[119].

Other than the evaluation of circulating genetic material, also proteins, cytokines and serum metabolites could have a relevant role in the diagnostic and prognostic assessment of CCA. As listed above, POSTN and osteopontin may discriminate CCA from healthy controls and offer a stratification of post-surgical survival[119-122].

Furthermore, non-tumoral cells and various molecular signaling are associated with different prognostic values. For instance, $CD8^+$ and $CD4^+$ T cells are correlated with a better prognosis in CCA as well as high IL-33 levels in CAFs, while Tregs are correlated with worse OS. High levels of pDCs, TAMs and NLR and the expression of α-SMA and SDF-1 by CAFs are correlated with worse prognosis.

Targeting TME could be a strategy for the development of more effective therapies against CCA. In particular, immunotherapy seems to offer a promising option in clinical practice. Novel biomarkers could assist in a diagnostic and prognostic evaluation of CCA. However, further studies are needed to achieve a better comprehension of the relationship between CCA and TME and to deliver new findings in clinical practice.

DECLARATIONS

Authors' contributions

Conceptualization and resources: Argenziano ME, Montori M
Methodology: Argenziano ME, Montori M, Maroni L
Software and project administration: Argenziano ME
Data curation, writing-original draft preparation: Argenziano ME, Montori M, Scorzoni C
Writing-review and editing: Argenziano ME, Montori M, Scorzoni C, Marzioni M, Maroni L
Visualization: Argenziano ME, Montori M, Scorzoni C, Benedetti A
Supervision: Benedetti A, Marzioni M, Maroni L
All authors have read and agreed to the published version of the manuscript.

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Conflicts of interest

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Ethical approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

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