Lodetti Zangrandi *et al. Hepatoma Res* 2023;9:32
**DOI:** 10.20517/2394-5079.2023.32

**Hepatoma Research**

**Review**

**Open Access**

# Tumor microenvironment highlighting tumor-associated macrophages and immune cells

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**How to cite this article:** Lodetti Zangrandi G, Tirpanlar D, Pastore M, Soldani C, Lleo A, Raggi C. Tumor microenvironment highlighting tumor-associated macrophages and immune cells. *Hepatoma Res* 2023;9:32. https://dx.doi.org/10.20517/2394-5079.2023.32

**Received:** 3 Apr 2023 **First Decision:** 12 Jun 2023 **Revised:** 4 Jul 2023 **Accepted:** 17 Jul 2023 **Published:** 28 Jul 2023

**Academic Editors:** Giuliano Ramadori, Kenneth Siu Ho Chok **Copy Editor:** Dan Zhang **Production Editor:** Dan Zhang

## Abstract

Cholangiocarcinoma (CCA) grows within a highly desmoplastic microenvironment, exhibiting a continuous interconnection with the immune infiltrate, which is characterized by an abundance of immune cells, including natural killer cells, T lymphocytes, and macrophages. The presence of inflammatory cells within the tumor microenvironment plays a crucial role in determining the aggressiveness and growth of CCA. The immune cell population engages in diverse and dynamic interactions with cancer cells. The balance of different subpopulations within CCA can generate varying responses, either inhibiting or promoting tumoral progression. The purpose of this review is to offer a comprehensive overview of the role of various immune infiltrate subpopulations within the tumor microenvironment, with a particular focus on the actions of tumor-associated macrophages (TAMs) and their critical regulation in the development and progression of CCA. TAMs play vital roles in maintaining homeostasis, facilitating tissue repair, and contributing to immune responses due to their significant functional diversity. Macrophages are present in numerous types of cancer, and their emerging role has also been observed in CCA. Recognizing and attaining a deeper comprehension of the intricate interplay between infiltrating immune cells and CCA cells is essential to identify new opportunities to advance treatment strategies.

**Keywords:** Cholangiocarcinoma (CCA), immune infiltrate, tumor-associated macrophages (TAMs), macrophage, macrophage polarization, tumor microenvironment

## INTRODUCTION

CCA represents the second most prevalent form of primary liver cancer, comprising approximately 10%-15% of all cases of primary liver malignancies. It arises from different sections of the biliary tree and expresses epithelial markers of cholangiocyte differentiation[1,2].

Worldwide, CCA affects more men than women, and there is a substantial increase in the mortality rate with age[3,4].

The incidence and mortality rates of CCA exhibit significant geographical variation worldwide. The incidence ranges from 85 per 100,000 in Eastern countries (such as northeastern Thailand) to 0.4 per 100,000 in Northern countries (like Canada). Similarly, Eastern countries exhibit the highest mortality rates for cholangiocarcinoma, with a rate of 2.5 per 100,000, whereas the lowest mortality rates, ranging from 0.2 to 0.5 per 100,000, are observed in South America[3,5,6].

Geographical variations in incidence and mortality likely reflect a multifactorial etiology based on regional risk factors and genetic predispositions[6].

### Classification and histology of cholangiocarcinoma

The classification of CCA is based on the anatomical site of the biliary ducts and distinguishes among intrahepatic (iCCA), perihilar (pCCA), and distal (dCCA) subtypes[7,8].

*Intrahepatic CCA*

Intrahepatic CCA (iCCA) represents approximately 10% of all Cholangiocarcinoma cases and is associated with a rising global incidence and poor outcomes. It develops within the liver parenchyma, originating from the secondary bile ducts. Following hepatocellular carcinoma, cholangiocarcinoma is the second most common primary intrahepatic liver cancer[9,10].

According to the most recent tumor classification by the World Health Organization (WHO)[11], iCCA can be classified into two distinct types: perihilar large duct type (iCCAphl) and peripheral small duct type (iCCApps).This new classification was introduced following the recognition of distinct tumor cell types and anatomical locations associated with iCCA.

ICCApps arises from intrahepatic bile ductules or Canals of Hering. It has a mass-forming growth pattern within the liver tissue and displays histological features characteristic of a tubular or acinar adenocarcinoma.

Additionally, iCCApps is associated with poor or absent mucin production[12,13]. On the contrary, iCCAphl originates from large intrahepatic bile ducts or their peribiliary glands and is characterized by extended mucin production. Histologically, iCCAphl demonstrates a growth pattern with large tubular or papillary architecture[12,14]

ICCApps typically exhibits larger tumor size, less frequent lymph node metastasis, lower expression of Ki67, CA19-9, and CEA, and a better prognosis compared to iCCAphl[13,15]. ICCA can be further macroscopically divided into different subtypes, including mass-forming (60%-80%), periductal-infiltrating (15%-35%), intraductal (8%-29%), and undefined and mixed subtypes[14]. This classification is based on distinct growth patterns observed in CCA. These classes differ in terms of prognosis and their association with specific CCA types.

The most common growth pattern observed in iCCA is the mass-forming type. On the other hand, intraductal iCCA is a papillary tumor with a slow growth rate, which is associated with a more favorable prognosis. Conversely, periductal infiltrating CCA exhibits a growth pattern along the bile duct without forming a distinct mass. Instead, it manifests as diffuse bile duct thickening or small lesions. This rare condition is often found concurrently with mass-forming CCA[16,17].

*Perihilar CCA and Distal CCA*

Perihilar CCA (pCCA) arises in the common bile duct, specifically below the secondary biliary branches and above the cystic duct. It represents the most prevalent subtype of cholangiocarcinoma, accounting for an incidence of 50%-60%.

Distal CCA (dCCA) is localized to the region between the origin of the cystic duct and Vater's ampulla. It represents approximately 20%-30% of all cholangiocarcinoma cases[18].

Both pCCA and dCCA often present with similar characteristics, appearing as flat or poorly defined nodular sclerosing tumors or, less commonly, as intraductal papillary masses. Histologically, the majority of both pCCA and dCCA cases are conventional mucin-producing adenocarcinomas or papillary tumors[19].

PCCA and dCCA are often regarded as single entity due to their similarities and are collectively referred to as extrahepatic cholangiocarcinoma (eCCA).

PCCA, dCCA, and large bile duct iCCA share a common origin from columnar mucous cholangiocytes, as well as some typical mutations, including frequent mutations of KRAS and/or P53[12,14,20,21].

Moreover, they share a typical presentation characterized by periductal infiltration or an intraductal growth pattern[18,19].

On the other hand, iCCA pps is characterized by a mass-forming pattern and the presence of fibroblast growth receptor 2 (FGFR2) fusions and isocitrate dehydrogenase (IDH 1-2)[22,23].

## Risk factors

Currently, cholangiocarcinoma is predominantly observed to have a sporadic origin, however, Multiple risk factors have been associated with the development of CCA[18].

Some risk factors for CCA, such as autoimmune disease (type I diabetes), inflammatory bowel disease (IBD), and peptic ulcers can be considered common to all the subtypes, while others specifically influence certain CCA subtypes or are region-specific[6,24].

Likewise, alcohol consumption, non-alcoholic fatty liver diseases (NAFLD), lupus, obesity, alcohol-related disorders, HBV and HCV infections, and hemochromatosis were more strongly associated with iCCA. On the other hand, cholangitis, chronic pancreatitis, choledochal cysts, cholelithiasis, choledocholithiasis, and tobacco smoking tended to be more strongly related to eCCA[24-26].

Caroli disease (CD) demonstrates the most significant associations with CCA compared to any other medical condition, resulting in a 38-fold higher risk of iCCA and a 97-fold higher risk of eCCA[24].

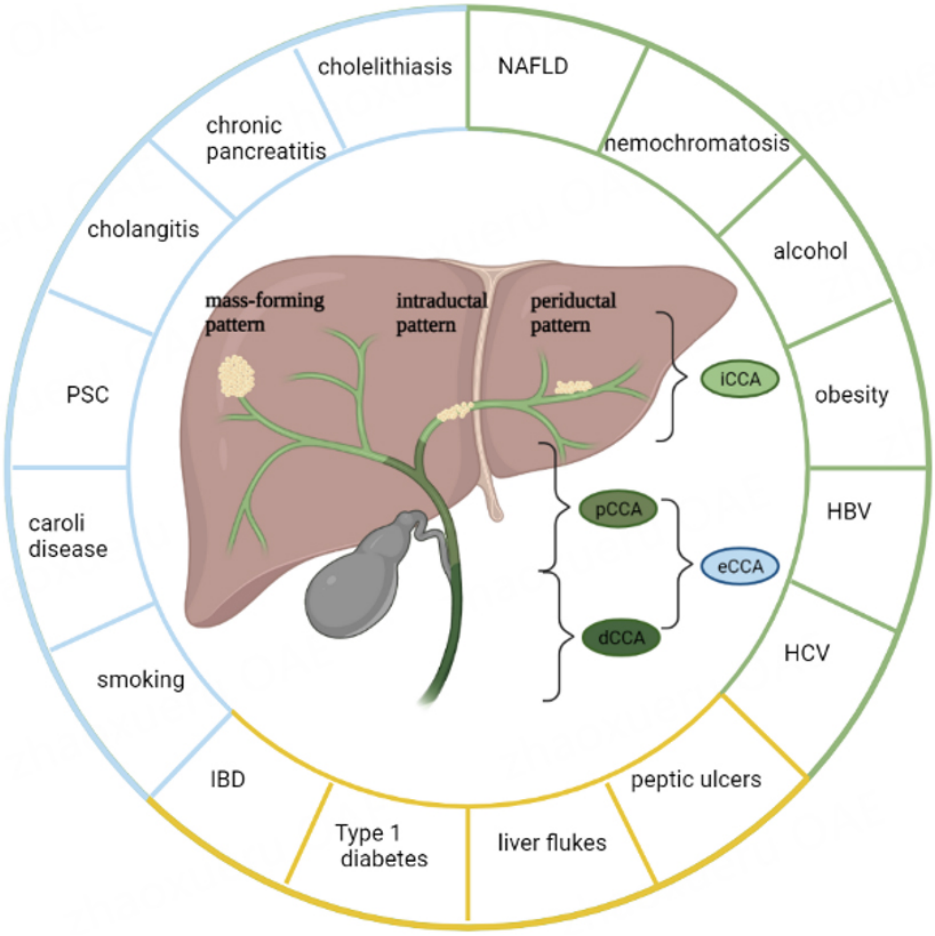


**Figure 1.** The image represents the anatomical classification of CCA into iCCA and eCCA, further divided into pCCA and dCCA subtypes[7]. Additionally, the image illustrates the different gross patterns observed in intrahepatic CCA, including mass-forming, intraductal, and periductal patterns[14]. The surrounding circle highlights the main risk factors associated with cholangiocarcinoma, which are common to all CCA types but may have a more typical cause for each subtype. These risk factors are categorized into three groups: light blue indicates the primary risk factors for extrahepatic CCA, light green signifies the main risk factors for intrahepatic CCA, and yellow represents the shared risk factors between the two types of CCA[6,29]. IBD: inflammatory bowel disease; PSC: primary sclerosing cholangitis; NAFLD: non-alcoholic fatty liver diseases; eCCA: extrahepatic cholangiocarcinoma; iCCA: intrahepatic cholangiocarcinoma; pCCA: perihilar cholangiocarcinoma; dCCA: distal cholangiocarcinoma.

Caroli disease is an extremely rare liver disease, with an incidence of 1 in 1,000,000. It is characterized by non-obstructive cystic dilatations of the embryonic bile ducts, leading to an anomalous expansion[27].

Furthermore, Primary sclerosing cholangitis (PSC) is a rare condition strongly related to CCA, particularly eCCA in northern Europe and the United States. Approximately 10% of all the patients with PSC develop CCA, resulting in an overall average risk of cholangiocarcinoma that is approximately 400 times higher than the non-affected population. Cholangiocarcinoma affects approximately 10% of all patients with PSC[28].

The distinct relationship between CCA subtypes and specific risk factors has provided insight into regional incidence trends.

Liver flukes, such as *Opisthorchis viverrini* and *Clonorchis sinensis*, are prevalent in eastern countries, influencing the rates of CCA in those regions. However, in Western countries, liver fluke infection is an

unlikely factor. Infections caused by liver flukes induce biliary epithelial hyperplasia, potentially facilitating changes in cholangiocarcinogenesis and exerting a promoting effect[29].

Underlining the importance of regional risk factors, different studies have demonstrated that the rates of iCCA in different countries correspond to the trends in alcohol consumption. For example, in countries such as Italy and France, where a decrease in alcohol-associated liver disease has been observed, there has also been a reduction in the incidence of iCCA. Conversely, countries that have experienced an increase in HCV infections, obesity, or NAFLD, have a higher incidence of iCCA[24,30,31] [Figure 1].

### Genetics alterations in cholangiocarcinoma

Cholangiocarcinoma (CCA) exhibits considerable genetic heterogeneity attributed to chromosomal aberrations, as well as genetic and epigenetic mutations.

The mutations are likely to arise as a result of chronic inflammation, which directly damages the DNA molecules and impairs DNA repair mechanisms. These DNA lesions give rise to molecular abnormalities, leading to the accumulation of additional mutations and the subsequent development of the tumor[32].

Despite the diverse phenotypic presentations and activated molecular pathways in CCA, several common chromosomal alterations have been identified. These include losses at 1p, 4q, 8p, 9p, 17p, and 18p, as well as gains at 1q, 5p, 7p, 8q, 17q, 19p, and 20q[33].

Some of these common chromosomal alterations involved important genes, such as ERBB2 and MAP2K2/MEK, underlying the significance of constitutive activation of the growth factors pathway in cancer development[34,35].

The major oncogenic networks that are deregulated in CCA, involve key molecules including transforming growth factor (TGF-β), mitogen-activated protein kinase-1/2 (MAPK 1/2), and in genes enriched in key networks controlled by VEGF/ERRB, CTNNB1/MYC, and tumor necrosis factor (TNF)[36].

Genetic studies have identified a direct relationship between gene expression and patient prognosis. Mutations in KRAS, TP53, and ARID2 were found to be significantly associated with poorer patient outcomes[37].

Furthermore, distinct distributions of mutations can be observed among the different subtypes of CCA. This highlights the importance of categorizing CCA based on subtype to understand the specific mutation profiles and their implications.

Genetic alterations such as FGFR2 (fibroblast growth factor receptor 2) fusion genes, hotspot IDH mutations in IDH1 and IDH2, BAP1, and RNF43 genes are much more present in iCCA[38].

Additionally, the JAK-STAT signaling pathway, which is important for the activation of immunosuppressive effects, is specifically expressed in iCCA due to a mutation of STAT3[39].

On the other hand, mutations in ARID1B and ELF3 are more frequently observed in eCCA compared to iCCA. Additionally, specific gene fusions such as ATP1B-PRKACA and ATP1B-PRKACB have been detected exclusively in eCCA cases.

Furthermore, when considering dCCA and pCCA subtypes, a significant differentiation is observed primarily in the expression of TP53, which is more frequently mutated in distal cases[37].

It is important to emphasize how these mutations can result in alterations not only within the tumor cells but also in the surrounding tumor microenvironment. Mutations in genes such as FGFR2, and the

JAK-STAT pathway can disrupt the immune infiltrate and influence the tumor microenvironment (TME). The differential expression of mutations in various subtypes of cholangiocarcinoma has the potential to play a pivotal role in elucidating the distinct characteristics of the tumor microenvironment associated with each subtype.

## IMMUNE INFILTRATE

The progression of the tumor is maintained by the interplay between inflammatory cells and tumor cells, where inflammatory cells play a crucial role at different stages of tumor growth, including initiation, promotion, malignant transformation, invasion, and metastasis[40]. Within the tissue, there exists a complex and ever-changing network of cytokines, chemokines, growth factors, inflammatory mediators, and enzymes involved in modifying the extracellular matrix. This network facilitates intercellular communication amidst notable alterations in the physical and chemical properties of the tissue. This leads to the reprogramming of the surrounding cells, enabling them to assume a vital role in the survival and advancement of the tumor[41].

In TME, various types of cellular components are present: cancer-associated fibroblasts (CAFs), vascular endothelial cells, lymphatic endothelial cells, and immune cells. The immune cell composition includes various types such as tumor-infiltrating lymphocytes (TILs), tumor-associated macrophages (TAM), tumor-associated neutrophils (TAN), dendritic cells (DC), natural killer cells (NK), and myeloid-derived suppressor cells (MDSC), has been observed[2].

CCA grows in a pro-inflammatory microenvironment in which CAFs, ECM, and the immune cells, play a pivotal role in keeping these conditions that promote tumor progression and diffusion[42].

CAFs play a central role in shaping the TME in CCA by promoting increased production of extracellular matrix proteins and collagen. This leads to the development of a desmoplastic TME and a hypovascularized stroma, characterized by the formation of adhesions and fibrous connective tissue within the tumor[43,44].

The desmoplastic and hypo vascularized microenvironment poses a barrier to the infiltration of immune cells, leading to a higher concentration of immune cells in the peritumor area surrounding the tumor rather than within the tumor itself.

However, as evidenced in multiple studies outlined below, immune cells capable of infiltrating the tumor site play a pivotal role in the tumor's development and progression. Modulating the immune response in CCA is a primary target for future therapeutic interventions, aiming to activate the immune response and reduce genetic instability and tumor development.

### Tumor-infiltrating lymphocytes

Of the various inflammatory cells that infiltrate the tumor, tumor-infiltrating lymphocytes (TILs) are recognized as the primary contributors to the host immune response against tumor cells[45].

TILS is composed of two main populations: T cells and B cells[46].

T cells can be classified based on their T cell receptor (TCR) subunits, which are responsible for recognizing membrane-bound peptides presented on the Major Histocompatibility Complex (MHC). The two major populations of T cells are distinguished by the expression of cell surface molecules CD4 and CD8, respectively.

$CD8^+$ T cells are capable of recognizing peptides presented by MHC class I molecules, whereas $CD4^+$ T cells rely on MHC class II molecules for peptide recognition[47].

Both $CD4^+$ and $CD8^+$ T cells are present in the tumor microenvironment of cholangiocarcinoma, but their distribution differs. Studies revealed that $CD4^+$ cells are predominantly located in the interface area surrounding the tumor, while $CD8^+$ cells infiltrate the tumor tissue[48].

$CD4^+$ is an antigenic peptide expressed in approximately 60% of mature T cells. $CD4^+$ cells differentiate into T helper lymphocytes, which can activate and stimulate B cells, $CD8^+$ cells, and TAMs. T-helper lymphocytes involvement in the immune response against cancer has been associated with better overall survival in patients[49].

Moreover, the immunosuppressive effects are mediated by a subgroup of T cells expressing $CD4^+CD25^+$ $Foxp3^+$, known as regulatory T cells (Tregs). Tregs represent approximately 5%-10% of the total number of $CD4^+$.

Tregs suppress T cells' activity by inhibiting the costimulatory molecule CD28. This is achieved through the overexpression of $Foxp3^+$, a transcription factor associated with the up-regulation of CTLA-4 on the cell surface[50].

Single-cell RNA-sequencing analysis of T cells comparing the tumoral and peritumoral areas of iCCA has shown an altered network of transcription factors. This altered network leads to increased infiltration of hyperactivated $CD4^+$ Tregs and a reduction in the effector functions of$CD8^+$ T cells. This dysregulated immune response is likely the cause of the negative impact of Tregs in CCA[51].

Tregs have been implicated in cancer progression and a TME that is rich in Treg cells and has low levels of $CD8^+$ T cells is associated with poor overall survival. This suggests that the presence of Tregs in the TME contributes to tumor progression and is indicative of a worse prognosis in CCA[52].

On the other hand, cytotoxic $CD8^+$ T lymphocytes (CTL) can selectively detect and induce tumor cell death through the release of cytotoxic granules.

Among the antigens expressed by tumor cells, tumor-specific neoantigens play a significant role. Upon encountering antigens in an acute inflammatory environment, naïve antigen-specific $CD8^+$ T cells undergo clonal expansion and differentiate into cytolytic effector T cells. Despite the presence of cytolytic effector T cells in a neoantigen-enriched tumor microenvironment, cancer can develop evasive strategies that enable its progression[53,54].

Based on immunohistochemical staining, $CD8^+$ T cells are found to be the most abundant tumor-infiltrating lymphocytes (TILs) within the tumor regions.

In contrast to regulatory T cells (Tregs), $CD8^+$ cytotoxic T lymphocytes exhibit anti-cancer activities, and a higher density of CTLs is associated with longer overall survival or disease-free survival[49].

Lymphocyte B cells play a significant role in inhibiting tumor development through various mechanisms. They are involved in the production of tumor-reactive antibodies, priming $CD4^+$ and $CD8^+$ T cells, and releasing cytokines that influence the activities of other immune cells such as NK cells, DCs, and TAMs[55,56]. Although B cells are less represented among TILs, a high infiltration of B cells in CCA has been associated with better overall survival[57].

### Natural killer cells

NK cells, a type of innate lymphoid cells, possess the capacity to identify and eradicate tumor cells without requiring prior antigenic exposure. In humans, NK cells are characterized as $CD3\text{-}CD56^+$ lymphocytes.

NK cell recognition is mediated by multiple receptor families, such as CD94-NKG2 and KIRs, which specifically bind to MHC class I molecules. These receptors exhibit either inhibitory or activating actions. Inhibitory receptors function to inhibit the killing of healthy cells, whereas activating receptors interact with ligands expressed on rapidly proliferating cells, infected cells, transformed cells, and tumor cells.

NK cell binding between activating receptors and their ligands leads to the formation of an immunological synapse, triggering the release of granules containing perforin and granzymes. Additionally, NK cell activation results in the expression of death-inducing ligands like FAS ligand and TNF-related apoptosis-inducing ligand (TRAIL), which initiate the apoptosis cascade[58].

The liver, due to its exposure to a large number of antigens, is rich in NK cells, accounting for nearly 50% of all liver lymphocytes[59,60].

Currently, only a limited number of studies have been conducted to fully characterize the role of NK cells in CCA. However, the current study has demonstrated the cytolytic activity of NK cells against CCA cells through *in vitro* and in vivo experiments[61,62].

Despite the demonstrated cytotoxic activity of NK cells, cancer cells have been reported to employ evasive strategies. Carnevale *et al.* have shown that iCCA cells can induce apoptosis in NK cells and T cells ($CD4^+$ and $CD8^+$) through the Fas/FasL pathway[63].

### Tumor-associated neutrophils

Neutrophils are the most abundant leukocyte in human blood, constituting approximately 50%-70% of the total leukocyte population. Neutrophils are the initial responders to cellular damage, and their infiltration contributes to persistent inflammation.

Under various cytokine stimulations, neutrophils can differentiate into two distinct phenotypes: antitumoral (N1) or protumor (N2). N1 neutrophils exert their antitumoral activity through direct cytotoxicity, antibody-dependent recognition, and immune cell activation via cytokine release. Conversely, N2 neutrophils activation leads to a persistent release of enzymes, resulting in inflammatory angiogenesis and the production of reactive oxygen species. These conditions collectively facilitate cancer growth[64-66].

Another crucial aspect of neutrophil action is their interaction with and production of chemokines. Zhou *et al*. have demonstrated that CXCL5, a member of the CXC-type chemokine family, is overexpressed in cholangiocarcinoma cells. This chemokine plays a role in promoting the growth and metastasis of iCCA by recruiting infiltrating neutrophils within the tumor microenvironment[67].

Numerous studies have extensively highlighted the significance of neutrophil infiltration, with the Neutrophil-to-Lymphocyte Ratio (NLR) emerging as a valuable prognostic marker for long-term outcomes. Independent associations have been observed between NLR and worse Overall Survival and Disease-Free Survival following curative resection of both pCCA and iCCA. A high NLR is thought to reflect an aberrant inflammatory response with a pro-tumor activity, contributing to an unfavorable prognosis[68,69].

### Dendritic cells

Within healthy liver tissue, Dendritic cells DCs are typically found in limited quantities, yet they retain their specialized role as antigen-presenting cells (APCs) capable of initiating adaptive immune responses. Like other APCs, when activated, DCs capture antigens and migrate to the lymph nodes. There, they present the antigens on their cell membrane, leading to the activation of specific B and T cells. Additionally, DCs play a crucial role in facilitating the selection of T cell receptors (TCRs) and B cell receptors (BCRs)[70].

DCs can be categorized into two primary subgroups: conventional DCs (cDCs) and plasmacytoid DCs (pDCs). Among cDCs, there are two distinct subclusters: cDC1s, which play a role in sustaining T cell restimulation within the TME, and cDC2s, which are responsible for presenting antigens to naive $CD4^+$ T cells and inducing their differentiation[70,71].

On the other hand, the activity of pDCs remains unclear, but they are believed to have primarily tolerogenic functions. This is partially supported by evidence of poor prognosis in various types of cancer[72].

Currently, limited research has been conducted to thoroughly characterize DCs in CCA. However, recent studies have revealed intriguing findings regarding their functionality. Observations have indicated that blocking IL-10 and TGF-β receptors on DCs can result in elevated IFN-γ levels and heightened cytolytic activity of effector T cells in CCA. This suggests a potential role for DCs in promoting an immune response against CCA.

Furthermore, DCs have been found to stimulate the proliferation of NK cells through a combination of cytokine signaling and direct cell-to-cell contact. This highlights the significance of DCs in facilitating NK cell activation and their potential impact on the immune response in CCA[73,74].

### Myeloid-derived suppressor cells

Myeloid-derived suppressor cells (MDSCs) are a heterogenous population of cells that undergo expansion in inflammatory conditions, including the TME. MDSCs can be classified into two main groups based on their origins: granulocytic or polymorphonuclear MDSCs (PMN-MDSCs) and monocytic MDSCs (M-MDSCs). The primary characteristic of MDSCs is their capacity to suppress immune responses, thereby directly impacting crucial immune cells[75].

The two populations, PMN-MDSCs, and M-MDSCs employ different pathways of action. PMN-MDSCs release reactive oxygen species (ROS), peroxynitrite, arginase 1, and prostaglandin E2 (PGE2) into the environment to mediate immune suppression. On the other hand, M-MDSCs secrete nitric oxide (NO), and immunosuppressive cytokines such as IL-10 and TGFβ, and upregulate the expression of programmed

death-ligand 1 (PDL1)[76].

An increase in circulating MDSCs has been reported in patients with cholangiocarcinoma[77].

Furthermore, the potential clinical importance of MDSCs is underlined by Ma *et al.* in their study, which demonstrated, using an in vivo model of cholangiocarcinoma, that concurrent depletion of PMN-MDSCs and macrophages sensitized the tumor to anti-PD-L1 therapy[75] [Figure 2].

Lymphocyte T $CD8^+$, T $CD4^+$, and CCA have a mutual inhibiting action, high-density $CD4^+$ and $CD8^+$ determine a longer OS and CCA cells can induce apoptosis in NK cells and T-cells ($CD4^+$, $CD8^+$), via the Fas/FasL pathway[49,63]. Lymphocyte Treg overexpression of CTLA 4 promotes CCA proliferation through the inhibition of lymphocyte T; An environment rich in Treg has poor overall survival[50,52]. High Lymphocyte B infiltration in CCA was related to better overall survival[57]. NLR and neutrophil infiltration combined with intratumor IL-17 cells are related to worse overall survival, demonstrating the importance of TANs infiltration as a promoter of CCA cell proliferation[68,69,78]. On the other hand, CCA cells overexpress CXCL5, which promotes iCCA growth and metastasis by recruiting infiltrative intertumoral neutrophils[67]. Has been demonstrated an *in vitro* and in vivo cytolytic activity of NK cells against CCA[61,62]. Dendritic cells interact with lymphocyte T and Natural Killer cells to promote their activities against CCA cells[73,74]. MDSCs act on the activity of the fibroblasts and of the macrophages causing an inhibition of CCA cells[75].

## MACROPHAGES

Macrophages, known for their remarkable plasticity, represent the most versatile cells within the hematopoietic system. They are present in all tissues[79] and demonstrate significant functional diversity, playing vital roles in processes such as development, tissue homeostasis, repair, and immune responses[80]. They regulate immune responses by pathogen phagocytosis and antigen presentation and additionally, they contribute to tissue formation and reorganization, wound healing, coagulation, and inflammation[81,82].

Macrophages originate from hematopoietic stem cells (HSCs) located in the bone marrow and undergo a series of sequential differentiation stages. These stages include the common myeloid progenitor (CMP), the granulocyte-macrophage progenitor (GMP)[83], the common macrophage and DC precursor (MDP)[84], and finally, the committed monocyte progenitor (cMoP)[85]. The homeostatic regulation of macrophage development is influenced by macrophage colony-stimulating factor (M-CSF), also referred to as colony-stimulating factor-1 (CSF-1)[86]. During the inflammatory state, granulocyte-macrophage colony-stimulating factor (GM-CSF) involves in the development of macrophages[87]. Macrophages are referred to by various names depending on which tissue they are found in, such as osteoclasts in bone, alveolar macrophages in the lung, microglial cells in the central nervous system, histiocytes in connective tissue, and Kupffer cells in the liver. These tissue-resident macrophage populations have such diverse transcriptional characteristics that they could potentially be classified as distinct classes of macrophages[88].

As previously stated, macrophages are plastic[79] and " exist in a continuum of functional states "[89]. The mononuclear phagocyte system is widely recognized for its functional versatility, and the M1 and M2 polarization paradigm represents the two extremes of the entire spectrum of macrophage functional activity[90,91]. As a result, macrophages present in distinct portions of TME display properties of both M1 and M2, depending on the tumor type[92]. The diverse signals within the TME can influence the diversity and function of TAMs, leading to their dual role in tumor progression, which can either promote or suppress tumor growth[93]. TAMs can be classified into classically activated (M1) and alternatively activated (M2) phenotypes[94,95] analogous to T helper type-1 (Th1) and T helper type-2 (Th2) of T cells[91].

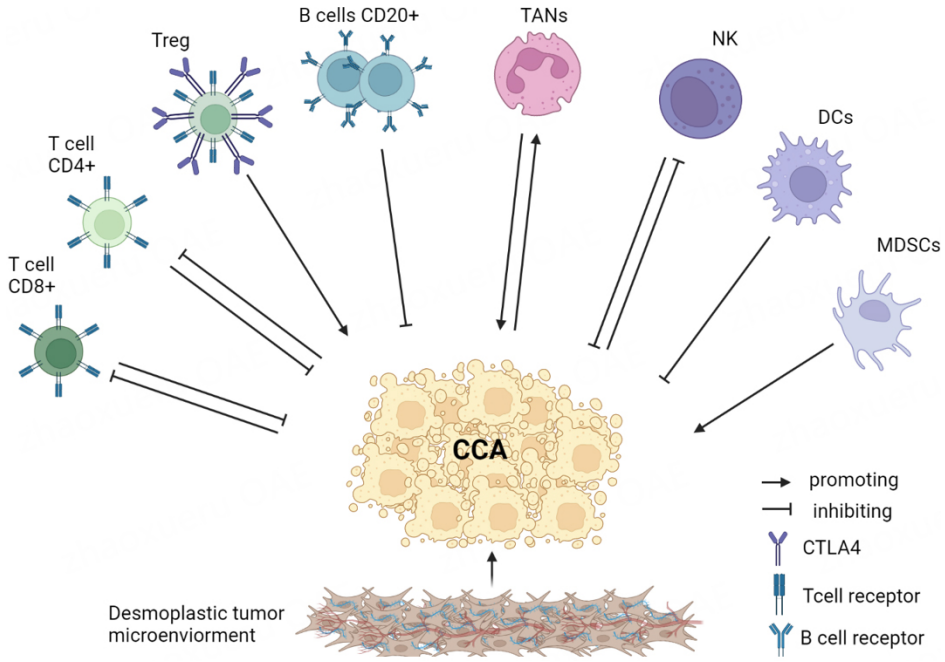


**Figure 2.** Representation of the immune infiltrate interaction with CCA and its microenvironment. TANs: tumor-associated neutrophils; NK: natural killer; DCs: dendritic cells; MDSCs: myeloid-derived suppressor cells; CCA: cholangiocarcinoma.

M1 macrophages, also called classically activated M1 macrophages, are activated under the condition of Th1 cytokines, such as interferon-gamma (INF-γ)[96], tumor necrosis factor (TNF-α), GM-CSF[97,98] and toll-like receptor (TLR) ligands[99] alone or together with lipopolysaccharide (LPS)[100] [Figure 3]. Their activation is characterized by high antigen presenting[101] and high phagocytic capacity[102], expressing high levels of pro-inflammatory cytokines such as interleukin (IL)-1β, IL-6, IL-12, IL-23, C-X-C motif chemokine (CXCL)9, and CXCL10, and major histocompatibility complex (MHC) molecules[103]. The expression of surface proteins CD68, CD80, CD86[104], and intracellular protein suppressor cytokine signaling 3 (SOCS3)[105] are upregulated. M1 macrophages are involved in the Th1 response to infection[106].

M2s are activated by M-CSF[107], prostaglandin F (PGF), prostaglandin E2 (PGE2)[79], and vitamin D3[108], and they produce transforming growth factor beta (TGF-β), vascular endothelial growth factor A (VEGF-A), and matrix metalloproteinase-2 (MMP-2)[109]. M2 macrophages exhibit elevated IL-10, scavenger receptor A (CD163, CD204)[99], arginase-1[40], mannose receptor-1 (CD206), and c-type lectin (CD301) expression[110]. M2 macrophages downregulate the expression of MHC class II and IL-12 expression[40]. M2 macrophage induction can be enhanced by IL-25 and IL-33 indirectly through Th2 cells[111,112].

TAMs appear in the majority of human and experimental murine cancer models[113]. The release of macrophage components into the tumor stroma, such as epidermal growth factor (EGF), fibroblast growth factor (FGF) family proteins, TGF-β, VEGF, distinct chemokines and cytokines, enhances tumor progression and promotes cell migration and metastasis[91]. The phenotype of TAMs is defined by their gene expression profile rather than deterministic differentiation pathways and lineage choices, as seen in Th1 and Th2 cells[40]. TAMs undergo M1-like or M2-like activation in response to the effects of tumor-derived growth factors, especially M-CSF[91], after being derived from peripheral blood monocytes and recruited to TME[103]. TAMs are associated with elevated levels of anti-inflammatory cytokine expression, which can alter the immunosuppressive capacity of TME and promote tumor development via TAM-derived

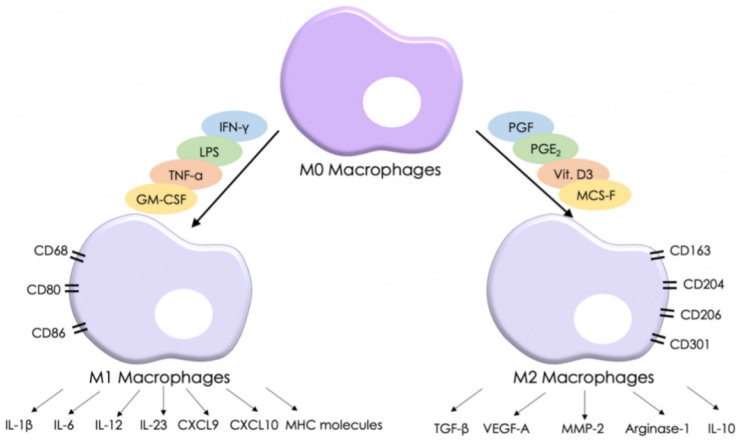


**Figure 3.** Classically activated M1 macrophages are activated by interferon-gamma (INF-γ)[96], tumor necrosis factor (TNF-α), GM-CSF[97,98] and lipopolysaccharide (LPS)[100], whereas alternatively activated macrophages are activated by M-CSF[107], prostaglandin F (PGF), prostaglandin $E_2$ (PGE)[79], and vitamin D3. M1 phenotype is characterized by IL-1β, IL-6, IL-12, IL-23, CXCL9, CXCL10, and major MHC molecules[103] expression and M2 phenotype is characterized by IL-10, TGF-β, VEGF-A, MMP-2 and arginase-1[40] expression. M1 macrophages also express surface proteins CD68, CD80 and CD86[104], whereas M2 macrophages express CD163, CD204[99], CD206, and CD301.

angiogenesis factors and proteases[106]. TAM-related subpopulations, such as, TEK tyrosine kinase receptor TIE-2 expressing monocyte subgroup (TEM)[114], myeloid-derived suppressor cells (MDSCs), and myeloid dendritic cells (mDCs) have been associated with a pro-tumorigenic inflammatory microenvironment[115].

Recent evidence shows that TAMs are obligated for tumor cell motility, invasion, and metastasis[116]. Data show that extracellular matrix (ECM) fragments produced by tumors or exosomes establish pre-metastatic niches, which is an appropriate environment for tumor cell survival and expansion induced at distant sites by the tumor[117], to be responsive to the circulating tumor cells by recruiting CD11b and VEGFR1 positive myeloid cells[118,119]. Metastatic cells must resist detachment-induced cell death, also known as anoikis, as they infiltrate into circulation. TAMs can be physically linked to cancer cells via the secretion of cytokines and assist them to travel through circulation[116].

Transcriptional profiling on oligonucleotide arrays enriched TAMs shows overexpression of angiogenic molecules[120]. Angiogenic TAMs, which are characterized by the expression of angiopoietin receptor TIE2[121,122], have an essential role in angiogenesis, regulating the angiogenic switch[123].

TAMs accumulate in tumor necrotic zones, which are characterized by low oxygen levels[124]. It is proposed that TAMs are attracted to hypoxic regions of tumors due to the release of hypoxia-induced chemo-attractants such as VEGF, endothelins, endothelial-monocyte-activating polypeptide II (EMAP2, also known as AIMP1)[125], and hypoxia-inducible factor (HIF-1) dependent factors modulating TAM migration in avascular areas[126].

### Tumor-associated macrophages in cholangiocarcinoma

TAMs are immensely staged in various cancers, and CCA is one of them[18,127-129]. CCA cells and various constituents of the TME can attract TAMs by releasing factors such as monocyte chemo-attractant protein-1 (MCP-1, also known as chemokine ligand-2 or CCL2), macrophage colony-stimulating factor (M-CSF), and vascular endothelial growth factor-A (VEGF-A)[53,130-132]. Recent evidence shows that CCA upregulates TNF-like weak inducer of apoptosis (TWEAK)/fibroblast growth factor-inducible 14 (Fn14), resulting in the induce of MCP-1, CX3CL1, IL-6, IL-8, M-CSF, and GM-CSF secretion through NF-

kB[132,133]. MCP-1 regulates TAM recruitment and is associated with an increase in TAM marker $CD206^+$ and tumor aggressiveness after invading the tumor sites, straying to a pro-tumorigenic phenotype despite the initial role of macrophages[132].

Research reveals that macrophages are likely to display polarization changes during the specific stages of infection. C. sinensis (Clonorchis sinensis infection which is closely associated with CCA formation)[134] infection models in mice showed M1 phenotype polarization at the early stage of infection, followed by an increase in CD47 secretion of CCA cells which indicates blockage of phagocytosis[135], and M2 phenotype polarization at the late stages of cholangiocarcinogenesis, contributing to fibrosis and remodeling of the bile duct[136].

Pre-clinical and clinical data suggest that rich TAM content in TME is linked with poor prognosis[137]. Many studies have found a linkage between the presence of TAMs and their clinical outcomes in CCA[52,138-142]. In a study of 39 patients with intrahepatic cholangiocarcinoma (iCCA), TAM infiltration has shown an association with angiogenesis, regulatory T cell (Treg) infiltration, and poor prognosis, with CCA cells inducing M2-like phenotype via signal transducer and activator of transcription 3 (STAT3) activation[138,139]. In an immunohistochemistry (IHC) analysis of 114 patients with CCA, it has shown that there is a positive interaction between TAMs, Tregs, and tumor-infiltrating neutrophils[52]. An investigation of the mechanism of TAM-derived progression of CCA demonstrated that chronic liver injury results in the inducement of mitochondrial dysfunction, oxidative stress, and recruitment of Kupffer cells[140]. Macrophage deficiency in pre-clinical models showed inhibition of Wnt signaling and limitation in tumor progression with the promotion of apoptosis[141].

As TAM infiltration has been linked to poor patient prognosis, it has been proposed that CCA cells may alter the surrounding stroma to create a tumor-promoting immune niche. Cellular spheroids derived from CCA cells modified macrophages into TAM phenotype which express great invasive capability[142]. TAMs obtained from resected human CCA tissues recited the characteristics of CCA-educated macrophages *in vitro*[142].

## An insight into immunotherapeutic strategies in Cholangiocarcinoma

### *Immune checkpoint inhibitors in Cholangiocarcinoma*

Immune checkpoints are receptors expressed by immune cells that are crucial for maintaining immune balance and regulating the activity of T cells. In the TME, immune checkpoints can become overexpressed leading to a state of T cell exhaustion, characterized by impaired effector function reducing cytotoxicity and cytokine production[143].

Immune checkpoint inhibitors (ICIs) are designed to disrupt the signaling pathways mediated by immune checkpoints, thereby facilitating enhanced activation of the immune system within the tumor microenvironment[144]. Currently, the role of immunotherapy in the management of CCA is delineated based on two separate situations[145]. The first scenario evolves around the identification of specific molecular alterations in the tumor, allowing for the selection of patients who would benefit from targeted treatment. In this context, pembrolizumab, an immune ICI, has demonstrated efficacy in patients with dMMR, MSI-H, and a high TMB, leading to its approval for these specific subsets of CCA patients[146-148].

The second involves the application of immunotherapy in unselected populations of patients with advanced-stage CCA. Initially, clinical trials focused on utilizing monotherapy with ICIs. However, this approach yielded limited effectiveness in patients[149].

Consequently, alternative strategies have been pursued, primarily involving the combination of ICIs with cytotoxic chemotherapy. The positive outcomes observed from combining ICIs with cytotoxic chemotherapy have led to the approval of a specific regimen by the U.S. Food and Drug Administration (FDA) in 2022[150].

Moreover, the latest version of the National Comprehensive Cancer Network (NCCN) guidelines for hepatobiliary cancer endorses this combination as the preferred first-line treatment option for advanced-stage CCA[151]. These regulatory approvals and guidelines reflect the growing evidence supporting the efficacy of combining ICIs with cytotoxic chemotherapy in advanced-stage CCA. The gemcitabine, cisplatin, and durvalumab regimen represents a significant advancement in the treatment landscape, providing a potential treatment option that has demonstrated favorable outcomes in this patient population.

*Chimeric antigen receptor in cholangiocarcinoma*

Genetically engineered T cells represent a promising and potent therapeutic approach with the potential for curative responses in cancer patients. Chimeric antigen receptor (CAR)-T cell therapies have shown significant success in the treatment of hematological malignancies. However, their efficacy against solid tumors, such as solid cancers, has been comparatively limited[152]. Various strategies are being explored to target frequently expressed antigens in CCA. One approach involves the use of EGFR-specific CAR-engineered autologous CAR-T cells, which have been tested in a study involving 19 CCA patients[153]. Additionally, CAR-T cells targeting antigens such as MUC1 and CD133 have been developed and have shown promising efficacy in early *in vitro* studies for potential therapeutic use in CCA patients[154,155]. Further research and development are required to improve the efficacy of these immune cells against solid tumors and broaden their application to a broader spectrum of malignancies.

*Novel approach to immunotherapy*

Recent advances in the field of CCA treatment have emphasized the significant role of TAMs and granulocytic MDSCs[156].

TAMs are considered the primary source of programmed death-ligand 1 (PD-L1) expression in CCA. However, blocking PD-L1$^+$ TAMs alone did not result in a reduction in tumor progression. This lack of response was attributed to the compensatory emergence of granulocytic MDSCs. These cells play a role in suppressing the immune response and promoting tumor growth. A dual approach targeting both TAMs and granulocytic MDSCs has shown promise in enhancing the response to immune checkpoint inhibitors in mice[157]. Another study has introduced a novel approach using different mouse models of CCA, which demonstrated that activating macrophages and dendritic cells through CD40 signaling significantly improves the response to anti-PD1 therapy in iCCA.

To further enhance the therapeutic response, a combination treatment involving anti-PD1, anti-CD40, gemcitabine, and cisplatin was employed. Remarkably, this combination led to a significant increase in mouse survival[158].

The recent studies highlighted the crucial significance of gaining a deeper understanding of TAMs within the TME to enhance the effectiveness of immunotherapies.

## CONCLUSION

The ongoing investigation into distinct immune subpopulations and their potential role in future therapies is progressively advancing, although many aspects still lack clarity. This can be attributed to the intricate

interplay among different subpopulations within the TME. In order to advance the development of innovative therapies, it is essential to gain a comprehensive understanding of the specific characteristics and functions of immune cells in CCA. We will direct our attention towards future research and investigations that explore the interaction between macrophages and dendritic cells, as these cells play a crucial role in suppressing the immune response and promoting tumor growth. This interaction suggests that targeting these cells could hold promise as a viable approach for treatment[158].

Considering the substantial impact of immune cells in shaping the characteristics of the TME, the regulation of immune cell effects in CCA presents a promising strategy for suppressing tumor progression[159].

## DECLARATIONS

### Authors' contributions

Manuscript writing: Lodetti Zangrandi G, Tirpanlar D
Critical revision of the manuscript: Lleo A, Soldani C, Raggi C, Pastore M

### Availability of data and materials

Not applicable.

### Financial support and sponsorship

None.

### Conflicts of interest

All authors declared that there are no conflicts of interest.

### Ethical approval and consent to participate

Not applicable.

### Consent for publication

Not applicable.



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