Universidade de Aveiro Departamento de Química
Ano 2020

MARIA DEL CARMEN MARTÍN SIERRA

AVALIAÇÃO LIPIDÓMICA E DO SISTEMA IMUNE NO CARCINOMA HEPATOCELULAR E NO COLANGIOCARCINOMA

LIPIDOMICS AND IMMUNE SYSTEM EVALUATION IN HEPATOCELLULAR CARCINOMA AND CHOLANGIOCARCINOMA

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Tese apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Doutor em Bioquímica, realizada sob a orientação científica da Professora Doutora Maria do Rosário Gonçalves dos Reis Marques Domingues, Professora Associada com Agregação do Departamento de Química da Universidade de Aveiro, do Professor Doutor Artur Augusto Paiva, coordenador da Unidade de Gestão Operacional de Citometria do Centro Hospitalar e Universitário de Coimbra (CHUC), Professor do Instituto Politecnico de Coimbra, ESTESC-Coimbra Health School, Ciencias Biomedicas Laboratoriais, e da Doutora Paula Margarida dos Santos Laranjeira, Investigadora do CHUC e do Instituto de Investigação Biomédica de Coimbra (iCBR) da Faculdade de Medicina da Universidade de Coimbra.

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Dedicated to my family: to my husband for his untiring support, to my parents for their faith in my work and, to Lila & Turco for their loyalty and for teaching me what is courage.

o júri

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palavras-chave carcinoma hepatocelular, colangiocarcinoma, lipoxidação,sistema imunológico, biomarcadores de prognóstico, imunofenotipagem, lipidómica.

resumo

O cancro do fígado compreende um grupo heterogéneo de tumores malignos com diferentes características histológicas que incluem o carcinoma hepatocelular (HCC) e o colangiocarcinoma (CCA), entre outras neoplasias. O HCC e o CCA apresentam poucas estratégias terapêuticas eficazes, sendo a ablação, resseção cirúrgica e/ou o transplante os únicos tratamentos curativos aplicados até o momento. Além disso, estes carcinomas são caracterizados por uma alta taxa de recorrência após a resseção cirúrgica. Portanto, são necessárias novas estratégias de tratamento e/ou mecanismos para determinar a resposta à terapia, de forma a melhorar o prognóstico dos pacientes com estes tipos de tumores. As abordagens baseadas em imunoterapia representam uma alternativa muito promissora para o cancro do fígado. No entanto, a capacidade de prever e avaliar se os indivíduos estão a responder às terapias imunológicas instituídas é, ainda, um desafio. A presente Tese teve como objetivo principal aprofundar o conhecimento atual sobre a relação entre o cancro e o sistema imune, bem como caracterizar o contexto imune e lipidómico em doentes com cancro do fígado. Para isso, foi realizada uma caracterização funcional e fenotípica das subpopulações do infiltrado leucocitário tumoral (TIL) e das células imunes circulantes, por citometria de fluxo. Além disso, procedeu-se à purificação de populações de células imunes por "cell sorting" para posterior caracterização da expressão de mRNA de mediadores da resposta imune por qRT-PCR. Utilizou-se, ainda, a técnica de ELISA para quantificar mediadores imunes no soro dos pacientes. Por fim, abordagens modernas de lipdómica por LC-MS foram utilizadas para monitorizar as alterações periféricas no fosfolipidoma de doentes com cancro, antes e após a resseção do tumor.

Os resultados obtidos permitiram detetar uma maior infiltração de macrófagos pró-tumorais em HCC de alto grau ou alto risco. Foi ainda observado um estado pró-inflamatório tanto em pacientes com HCC como com CCA, e alterações funcionais em algumas subpopulações de monócitos circulantes e em células dendríticas mielóides, observando-se uma recuperação parcial da função após a resseção do tumor. É ainda de salientar que a expressão de TNFα por monócitos circulantes, bem como os níveis séricos de TNFα, se encontravam associados à presença de tumores de alto grau. A frequência de células Treg circulantes em pacientes com ambos tipos de tumores foi modulada após o período necessário à recuperação da cirurgia de resseção do tumor. A frequência de células Tc17 circulantes e dos linfócitos T CD4 com plasticidade para produzir as citocinas IFNγ e IL-17 diminuiu em doentes com CCA e HCC, sugerindo uma migração específica para o microambiente tumoral. Além disso, as alterações identificadas no fosfolipidoma de pacientes com cancro do fígado foram afetadas pelo procedimento de resseção tumoral. Os resultados obtidos pelos estudos lipidómicos evidenciaram que, após a cirurgia, as alterações observadas no perfil fosfolipídico dos doentes com cancro do fígado são revertidas.

No geral, os estudos apresentados nesta tese demostraram a aplicabilidade da citometria de fluxo para detetar diferenças funcionais e fenotípicas em subpopulações TIL de pacientes com diferentes tipos de cancro (CCA vs. HCC), diferentes graus histopatológicos do tumor e diferentes estratificações de risco, além de monitorizar a competência funcional das células imunes em circulação para avaliar melhor a função imune em doentes com cancro. Por outro lado, a técnica LC-MS de alta resolução mostrou ser uma abordagem promissora para monitorizar alterações periféricas no perfil fosfolipídico de doentes com cancro do fígado, como um meio de avaliar a evolução dos doentes após a cirurgia

keywords hepatocellular carcinoma, cholangiocarcinoma, lipoxidation, immune system, prognosis biomarkers, immunophenotyping, lipidomics.

abstract

Liver cancer comprises a heterogeneous group of malignant tumors with different histological features that include hepatocellular carcinoma (HCC) and cholangiocarcinoma (CCA), among other neoplasms. HCC and CCA present limited options for effective therapeutic strategies, being ablation, surgical resection and/or transplantation the only curative treatments applied so far. In addition, these carcinomas are characterized by a high recurrence rate after surgical resection. Therefore, novel treatment strategies and/or mechanism to determine therapy response are needed to improve the prognosis of HCC and CCA patients. Immunotherapy-based approaches represent an alternative very promising for liver cancer. However, predicting responders and non-responders to immune therapies remains a challenge. The present Thesis had as main objective to advance and deepen the current knowledge on the relationship between cancer and immunity as well as to characterize the immune and the lipidomic context in liver cancer patients. To do so, a deep functional and phenotypical characterization of tumor-infiltrating leukocyte (TIL) subsets and circulating immune cells was performed by multi-color flow cytometry. Purification of immune cell populations by cell sorting was carried out for subsequent analysis of mRNA expression of immune mediators by qRT-PCR. ELISA techniques were used to quantify immune mediators in patients' sera. Additionally, high-resolution LC-MS base lipidomic approach was applied to monitor peripheral changes in the phospholipidome of cancer patients, before and after tumor resection, providing an indirect read of the lipid changes induced by the surgical procedure.

A higher infiltration of protumor macrophages in HCC classified into high-grades or into high-risk was detected. A pro-inflammatory state was observed both in HCC and CCA patients, and functional defects of some circulating monocyte subsets and myeloid dendritic cells were also detected, displaying partial recovery after tumor resection. TNFα mRNA expression by circulating monocytes as well as serum TNFα levels were associated to the presence of high-grade tumors. The frequency of circulating Treg cells in HCC and CCA patients was decreased, being partially recovered after tumor resection. Moreover, the frequency of circulating Tc17 cells as well as CD4 T lymphocytes with the plasticity to produce both IFNγ and IL-17 cytokines were decreased in CCA and HCC patients, suggesting a specific migration to the tumor microenvironment. Furthermore, it was revealed that the alterations identified in the phospholipidome of liver cancer patients were affected by tumor resection procedure. The results obtained showed that the alterations observed in the phospholipidic profile of liver cancer patients were reverted after surgery.

Overall, the studies presented in this Thesis reported the utility of multi-color flow cytometry to detect functional and phenotypic differences in TIL subsets from patients with different types of cancer (CCA vs. HCC), different tumor histopathological grades, and different risk stratification classifications, in addition to monitoring functional competence of circulating immune cells to better evaluate immune dysfunctions in cancer patients. Besides, high-resolution LC-MS was demonstrated as being a usefulness technique to monitor peripheral changes in the phospholipid profile of liver cancer patients that could be used to predict patients' outcomes after surgery.

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Abbreviations

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AC, acid ceramidase;

ACAT1, acetyl-CoA acetyltransferase 1;

ADCC, antibody-dependent cell-mediated cytotoxicity;

AFP, α-fetoprotein;

ALL, acute lymphocytic leukemia;

ASM, acid sphingomyelinase;

B

BCRs, B cell receptors;

Bregs, regulatory B cells;

BTLA, B and T lymphocyte attenuator;

C

CARs, Chimeric Antigen Receptor;

CCA, cholangiocarcinoma;

CDP-DAG, CDP-diacylglycerol;

CERK, ceramide kinase;

CERT, ceramide transfer protein;

CL, cardiolipin;

CLPs, common lymphoid progenitors;

CMPs, common myeloid progenitors;

CRC, Colorectal cancer;

CTL, cytotoxic lymphocytes;

CTLA-4, cytotoxic T lymphocyte protein 4;

D

DAMPs, danger-associated molecular patterns;

DCs, dendritic cells;

CDPs, DC precursors;

E

ECC, extrahepatic cholangiocarcinoma;

ER, endoplasmic reticulum;

F

FA, fatty acid;

FLC, fibrolamellar hepatocellular carcinoma;

FoxP3, forkhead box P3;

G

GC, germinal center;

GCS, glucosylceramide synthase;

G-CSF, granulocyte colony-stimulating factor;

GM-CSF, granulocyte-macrophage colony-stimulating factor;

GPCRs, G-protein coupled receptors;

GPLs, glycerophospholipids;

H

HCC, hepatocellular carcinoma;

HCC-CCA, mixed hepatocellular cholangiocarcinoma;

HLA, human leukocyte antigen;

HSCs, hematopoietic stem cells;

I

IDO, indoleamine-2,3-dioxygenase;

IFNγ, interferon-γ;

Igs, immunoglobulins;

IκB, inhibitor of NF-κβ;

IKK, inhibitor of NF-κβ kinase;

IL, interleukin;

K

KCs, Kupffer cells;

KIRs, killer immunoglobulin-like receptors;

L

LAG-3, lymphocyte activation gene 3 protein;

LCAT, lecithin-cholesterol acyltransferase;

LDL, low-density lipoprotein;

LNs, lymph nodes;

LPA, lysophosphatidic acid;

LPC, lysophosphatidylcholine;

LPS, lipopolysaccharide;

Lyso-PLs, lysophospholipids;

M

MDSC, myeloid-derived suppressor cells;

MHC, major histocompatibility complex;

MoDCs, monocyte-derived DCs;

Mo-Mfs, monocyte-derived macrophages;

MPPs, multipotent progenitors;

MS, mass spectrometry;

N

NCRs, natural cytotoxicity receptors;

NK, natural killer,

NKT, natural killer T cells;

NSCLC, non-small cell lung carcinoma;

NSM, neutral sphingomyelinase 2;

O

OS, overall survival;

P

PAMPs, pathogen-associated molecular patterns;

PC, phosphatidylcholine;

PCs, plasma cells;

PD-1, programmed cell death protein 1;

PDL1, PD-1 ligand 1;

PDAC, pancreatic ductal adenocarcinoma;

pDCs, plasmacytoid DCs;

PE, phosphatidylethanolamine;

PEMT, PE methyltransferase;

PI, phosphatidylinositol;

PIPKIγ, PI4P-5 kinases;

PL, phospholipids;

PLC, phospholipase C;

PLA2, phospholipase A2;

PNETs, pulmonary neuroendocrine tumors;

PMs, pulmonary metastases;

PPARγ, peroxisome proliferator-activated receptor γ;

PRRs, pattern recognition receptors;

PS, phosphatidylserine;

R

RCC, renal cell carcinoma;

RIPK1, serine/threonine-protein kinase 1;

ROS, reactive oxygen species;

S

SCF, stem cell factor;

SIRPα, signal regulatory protein-α;

SIRT, selective internal radiation therapy;

SM, sphingomyelin;

SMS, sphingomyelin synthase;

S1P, sphingosine-1-phosphate;

SPHK1, sphingosine kinase 1;

SPHK2, sphingosine kinase 2;

SPLs, sphingolipids;

T

TAAs, tumor-associated antigens;

TACE, transarterial chemoembolization;

TAMs, tumor-associated macrophages;

TCR, T cell receptor;

TFH, T follicular helper cells;

TGF, transforming growth factor;

Th, T helper;

TIL, tumor-infiltrating leukocyte;

TIM-3, T cell immunoglobulin and mucin-domain containing;

TNFα, tumor necrosis factor-α;

TNM, tumor-node-metastasis;

TRAF2, TNF receptor-associated factor 2;

Tregs, T regulatory cells;

TTR, time to recurrence;

V

VLA4, very late antigen 4.

CHAPTER 1. INTRODUCTION

1. Introduction

1.1. Liver cancer

Liver cancer is the second most common cause of cancer-related death worldwide, presenting a steady increasing incidence and mortality[1,2]. Liver cancer comprises a heterogeneous group of malignant tumors with different histological features that include hepatocellular carcinoma (HCC), cholangiocarcinoma (CCA), mixed hepatocellular cholangiocarcinoma (HCC-CCA), fibrolamellar HCC (FLC), and the pediatric neoplasm hepatoblastoma[3]. Among these, HCC and CCA are the most common primary liver cancers, while the other neoplasms account for less than 1% of cases[3].

HCC represents approximately 90% of all cases of primary liver cancer. The main risk factors associated with HCC include viral hepatitis (B and/or C), alcohol abuse, and nonalcoholic fatty liver disease in patients with metabolic syndrome and diabetes. Other cofactors of HCC development are aflatoxin B1 and tobacco, since it has been reported that they increase the incidence of the disease if other common risk factors are present[4]. HCC treatment strategies consist of curative treatments (ablation, resection, transplantation) or palliative treatment (transarterial chemoembolization (TACE), selective internal radiation therapy (SIRT), multikinase inhibitor sorafenib)[5]. HCC is characterized by a poor prognosis, rapid disease progression, limited options for chemotherapy and a high recurrence rate after surgical resection[6]. Therefore, novel treatment strategies with different mechanisms from those of conventional treatments are needed to improve the prognosis of HCC.

The second most common liver cancer is CCA, a malignancy that originates from biliary epithelia, and presents a high mortality rate[7]. Depending on their site of origin, they have different features and can be classified into intrahepatic, perihilar, and distal CCA[8]. Risk factors for development of intrahepatic CCA include primary sclerosing cholangitis, biliary duct cysts, hepatolithiasis, and parasitic biliary infestation with flukes[9]. More recently, shared risk factors with HCC have also been identified, such as HBV and HCV, particularly for intrahepatic CCAs that develop in cirrhotic liver[10]. During liver development, hepatocytes and cholangiocytes arise from a common progenitor (hepatoblasts). Progenitor cells may give rise to HCCs and CCAs with progenitor-like features. In addition, adult hepatocytes can degenerate into HCC by following sequential dedifferentiation into precursor cells that ultimately can transform into HCC cells with markers of progenitor cells, and can transform into CCA by transdifferentiation into biliary-like cells that at last transform into CCA, whereas adult cholangiocytes, which lack the plasticity and transforming capacity of hepatocytes, can only give rise to CCAs[11–14]. Surgical resection of early CCA tumors is so far the only

effective therapeutic strategy, and recurrence after surgical tumor resection is common[15]. Treatment options for patients with advanced or metastatic CCA are limited with short survival times[9,16]. Systemic conventional chemotherapy with gemcitabine and cisplatin as current standard of care in advanced or metastatic disease has only modest gains[17].

Regardless of the cell type that originate the tumor, the developing tumor also requires a specific microenvironment. The tumor microenvironment plays a vital role in tumor epigenetics, tumor differentiation, immune escape, and metastisation[18] and is especially relevant to liver tumors, 90% of which develop under conditions of chronic inflammation[1]. Prognosis of cancer patients is based on tumor-related factors, as well as on host-related factors, including systemic immune cell activation[19]. Inflammation can contribute to multiple hallmark of cancer by supplying bioactive molecules to the tumor microenvironment, including growth factors that sustain proliferative signaling, survival factors that limit cell death, proangiogenic factors, extracellular matrix-modifying enzymes that facilitate angiogenesis, invasion, and metastasis, and inductive signals that lead to activation of epithelial-to-mesenchymal transition and other hallmark-facilitating programs. Consequently, an altered immune response is an important factor in carcinogenesis[20]. Therefore, the study of the tumor microenvironment, as well as the peripheral immune system homeostasis, will be further described extensively and analyzed in the present thesis.

Additionally, inflammatory cells can release chemicals, notably reactive oxygen species (ROS), that are actively mutagenic for nearby cancer cells, accelerating their genetic evolution toward states of heightened malignancy[21]. In addition, the increase of ROS may lead to a cascade of reactions that ultimately gives rise to protein lipoxidation[22]. Lipoxidation products may have an effect in cancer development through their direct effects on tumor cells, as well as through the alteration of immune components and the consequent modulation of the immune response[23,24]. Due to the importance of this topic, we will further discuss the effect of lipoxidation in cancer and cancer immunity in chapter 2.

1.2. Cancer immunity

Inflammatory responses play decisive roles at different stages of tumor development, including initiation, promotion, malignant conversion, invasion, and metastasis[25]. The different immune cell subsets may have an important role in antitumor immunity, tumor-promoting inflammation or immunosuppression. From the bone marrow, hematopoietic

stem cells (HSCs) give rise to heterogeneous multipotent progenitors (MPPs) generating common myeloid progenitors (CMPs) or common lymphoid progenitors (CLPs) in a colony stimulating factor 1 (CSF-1) dependent manner[26], which give rise through intermediate precursor stages to all forms of circulating blood cells.

1.2.1. Dendritic cells

Dendritic cells (DCs) are a diverse group of specialized antigen-presenting cells with key roles in the initiation and regulation of innate and adaptive immune responses. Distinct DC subpopulations, as categorized by developmental, phenotypical and functional criteria, have been recognized in humans. Classical dendritic cells (cDCs) form a critical interface between innate and adaptive immunity. As myeloid immune cell sentinels, cDCs are specialized in the sensing of pathogen challenges and cancer. cDCs derive from common DC precursors (CDPs) in the bone marrow and comprise two main subsets, the myeloid cDC1 subset and the more heterogeneous myeloid cDC2 subset[27,28]. Plasmacytoid DCs (pDCs) develop from both CDPs and lymphoid progenitors, yielding two functionally distinct pDC subsets[29]. Inflammatory conditions can lead to the CC-chemokine receptor 2 (CCR2)-dependent recruitment of monocytes from the blood that differentiate into monocyte-derived DCs (MoDCs) in peripheral tissues[30,31]. However, MoDCs generated during inflammation are now classified as highly plastic or 'non- classical' monocytes rather than DCs[31]. Generally, functional specialization of DC subsets arises from their expression of different receptors, including pattern recognition receptors (PRRs)[28,30]. cDC1s can induce cellular immunity against intracellular pathogens and tumors due to their efficient processing and cross-presentation of exogenous antigens on major histocompatibility complex (MHC) class I molecules to activate $CD8^+$ T cells, and their ability to prime type 1 T helper cell (Th1 cell) responses[27,30,32,33]. While cDC2s are crucial for inducing $CD4^+$ T cell-mediated immunity in cancer[34] and, depending on the context, human cDC2s can induce the polarization of diverse subsets of $CD4^+$ Th cells and activate $CD8^+$ T cells[32,33,35].

In the tumor microenvironment, DCs acquire, process and present tumor-associated antigens (TAAs) on MHC molecules (signal 1) and provide co-stimulation (signal 2) and soluble factors (signal 3) to shape T cell responses. As $CD8^+$ T cells are often the main effectors of antitumor immunity, promoting cross-presentation of TAAs by DCs is considered fundamental. cDC1s are often associated with superior cross-presentation of antigens, which results in stronger $CD8^+$ T cell immunity. Additionally, cDC1s can support Th1 cell polarization of $CD4^+$ T cells in the tumor microenvironment[36–40].

1.2.2. Monocytes

Monocytes are members of the mononuclear phagocyte system, a family of myeloid cells that also comprises DCs and macrophages. After birth, monocytes derive from hematological precursors in the bone marrow and enter the blood circulation, from which they are recruited into tissues throughout the body. In addition, monocytes have long been known to have the property of maturing into macrophages, and indeed, for decades they were thought to be the main source of tissue macrophages[41]. The heterogeneity of monocytes was first described by Passlick et al.[42], based on the cell surface expression of CD14 (lipopolysaccharide (LPS) receptor) and CD16 (FcγRIII receptor).

Classical monocytes ($CD14^{++}CD16^{-}$) are considered inflammatory cells, which express high levels of CCR2 (chemokine receptor of monocyte chemoattractant protein-1 (MCP-1))[43]. This cell subset comprises about 80–95 % of circulating monocytes in humans, are recruited from the bone marrow and splenic reservoirs to sites of active inflammation and respond to different stimuli generated by damaged and/or infected tissue by producing inflammatory cytokines, such as IL-1, IL-12, and tumor necrosis factor-α (TNFα)[44]. These cells are highly phagocytic and are known to be important scavenger cells.

Intermediate monocytes ($CD14^{++}CD16^{+}$) comprise about 2–8% of circulating monocytes. Their functions include production of ROS and inflammatory mediators (such as TNFα and IL-1β), antigen presentation, participating in the proliferation and stimulation of T cells, inflammatory responses, and angiogenesis. These cells express CCR2 and seem to be highly phagocytic[45–47].

Non-classical monocytes ($CD14^{+}$ $CD16^{++}$) do not express CCR2 and comprise about 2–11% of circulating monocytes. They are mobile in nature and patrol the endothelium in search of injury. They can have pro-inflammatory behavior and secrete inflammatory cytokines in response to infection. These cells are also involved in antigen presentation and T cell stimulation[46–48]. A transition of classical monocytes to non-classical monocytes has been observed, although this does not exclude that some cells in the non-classical monocyte pool might develop without passing through a classical monocyte stage[26]. Phenotypic and functional differences, as well as differentiation potential of these subsets, are listed in Table 1.

Table 1. Classification of monocyte subsets in human.

Monocytes	Phenotype	Functions	Differentiation potential
Classical	$CD14^{++}CD16^{-}$ $CCR2^{++}$	Inflammatory cells that respond to many stimuli originated from damaged/infected tissue and produce inflammatory cytokines[49–52]	Few macrophages and mostly DC[53,54]
Intermediate	$CD14^{++}CD16^{+}$ $CCR2^{+}$	Highly phagocytic cells that produce high levels of ROS and inflammatory mediators[50–52]	Some macrophages and occasional DC[53,54]
Non-classical	$CD14^{+}CD16^{++}$ $CCR2^{-}$	Reparative/patrolling cells that remove debris from vasculature and produce high levels of anti-inflammatory factors[49–52,55]	Mostly macrophages[53,54]

The contribution of monocytes to tumor development is a multifaceted process ranging from the initiation of vessel growth to immune escape and metastasis[26]. Tumors affect myelopoiesis in the bone marrow and induce the expansion of myeloid cells with immunosuppressive activity, both in animal models and in patients[56]. These cells were originally characterized by the expression of CD11b and Gr1 and termed ‘‘myeloid-derived suppressor cells’’ (MDSC). Addition of further cell surface markers unmasked the presence of monocytic and polymorphonuclear MDSC and allowed independent analysis of both subsets[56]. Monocytes infiltrating the tumor tissue under hypoxic conditions and high concentrations of oxidative agents, might acquire a pro-inflammatory signature that dampens lymphocyte activities, survival, and proliferation in general. Classical, intermediate and non-classical monocytes can infiltrate the tumor tissue, and might contribute distinct pro- and anti-inflammatory activities[26]. However, these infiltrating monocytes can further differentiate into tumor-associated macrophages (TAM) which have displayed different roles in cancer progression. In addition, monocytes can promote metastasis by preparing the potential microenvironment and enable the dissemination of metastatic cells[57]. It has been described that classical monocytes are recruited to the metastatic site in a CCL2-CCR2-dependent manner and, once embedded within this niche, these recruited monocyte-derived cells promote the survival and growth of the metastatic tumor[58]. Therefore, classical monocytes have been mostly reported to play pro-tumoral functions once recruited to the tumor microenvironment. On the other hand, non-classical monocytes have independent

mechanisms for infiltration to tumors, and their functions are context-dependent. In this regard, it has been described both pro-tumoral functions, including angiogenesis, as well as anti-tumoral roles, such as NK cell recruitment, for non-classical monocytes[59].

1.2.3. Macrophages

Macrophages are particularly abundant in the liver, and are essential for maintaining tissue homeostasis and ensuring rapid responses to hepatic injury. Specific environmental signals further determine the polarization and function of hepatic macrophages. Macrophages sense injury by recognizing danger-associated molecular patterns (DAMPs) or pathogen-associated molecular patterns (PAMPs) that bind to PRRs and can promote the formation of the inflammasome[60,61]. The hepatic macrophage population in the steady state liver is heterogenic, consisting of both self-sustaining liver-resident Kupffer cells (KCs) and recruited inflammatory monocyte-derived macrophages (Mo-Mfs)[62]. KCs are highly effective phagocytes that recognize, ingest and degrade cellular debris, foreign material or pathogens[63]. They act as critical sentinels that ensure liver homeostasis and eliminate antibodies, debris or dead cells. Additionally, KCs secrete CXC-chemokine ligand 1 (CXCL1), CXCL2 and CXCL8, which attract neutrophils[64], as well as CC-chemokine ligand 1 (CCL1), CCL2, CCL25 and CX3CL1, which promote the infiltration of bone marrow-derived monocytes in the liver[65–68]. Moreover, both KCs and Mo-Mfs are very plastic and can adapt their phenotype according to the signals derived from the hepatic microenvironment, as previously referred[69].

Traditionally, macrophage functions have been assigned as pro-inflammatory or “M1” vs. anti-inflammatory or “M2”[70]. This concept is originally based on cell culture experiments, showing that monocyte-derived macrophages can differentiate towards M1 cells by interferon-γ (IFNγ) or towards M2 cells by IL-4 stimulation, resulting in typical cytokine response profiles. However, this consideration is too simplistic to describe the polarization of liver macrophages, especially in a disease-context[71]. For this reason, instead of the M1/M2 paradigm, the source of macrophages, definition of the activators, and a consensus collection of markers to describe macrophage activation should be reported when referring to a specific condition of macrophage polarization[72].

In liver cancer, dense accumulations of TAMs are regularly observed by immunohistochemistry staining for CD68 or CD14 in resections or explants of patients[73]. These macrophages secrete various cytokines and chemokines, such as IL-1β, IL-6, TNFα, CCL2 and CXCL10, which increase tumor cell proliferation and NF-κβ-mediated protection

from cancer cell apoptosis, as well as pro-angiogenic growth factors as VEGF, PDGF, TGFβ and fibroblast growth factor, which support tumor growth[74–76]. The detection of TAMs as well as macrophage-related biomarkers correlate with HCC progression and poor survival[77–79]. In addition, infiltrating macrophages, as well as KCs, contribute to CCA progression and development. In this regard, infiltrating macrophages produce WNT7B, a ligand of the WNT pathway, whereas activated KCs secrete TNF causing JNK activation and triggering cholangiocyte overgrowth[80–82]. Importantly, macrophages typically express PD-L1, also known as CD274 or B7-H1, by which they suppress the anti-tumor cytotoxic T cell responses[83,84]. Since progression of chronic liver diseases (fibrosis to cirrhosis) and tumor formation usually occur in parallel, it is inherently difficult to dissect if hepatic macrophages provide a tumor-prone inflammatory microenvironment or at which point they respond to tumor-derived signals to actively facilitate HCC growth and spread[71]. Mouse models reveal that macrophages also participate in tumor surveillance. In the absence of chronic liver injury, senescent (pre-malignant) hepatocytes secrete CCL2, which attracts monocyte-derived macrophages[85,86]. The concerted action of $CCR2^+$ macrophages and T cells results in the clearance of senescent hepatocytes, thereby preventing cancer development[86]. On the other hand, the recruitment of $CCR2^+$ macrophages in case of established tumors results in accumulation of immunosuppressive TAMs or MDSC that favor tumor growth by inhibiting $CD8^+$ T cell and NK cell activation[78,85].

1.2.4. Lymphocytes

During lymphocyte development, and the development of many other cell types, binary cell fate decisions ensure that signals driving differentiation generate more than one lineage. Important lymphoid cell fate decisions include commitment to the T or B cell lineages.

B cells

B lineage cells are the central mediators of humoral immunity. The terminal effector cells of this lineage are plasma cells (PCs), which can neutralize pathogens by secreting pathogen-specific antibodies. PCs arise as a consequence of a highly regulated differentiation process that is initiated when B lymphocytes encounter antigens through their B cell receptors (BCRs)[87,88]. The PC secreted antibodies have the ability to specifically bind to the antigen expressed by the pathogen agents and, depending on the antibody isotype, they can impede cell infection by binding and blocking the receptors used by the pathogen to

enter the host cell; they can opsonize the pathogen, recruiting phagocytic cells and facilitating the phagocytic process; they can activate the complement cascade, resulting in the pathogen lysis; and they can promote antibody-dependent cell-mediated cytotoxicity, through binding to CD16 (FcγRIII). B cell activation may occur in a T cell-independent or T cell-dependent manner, and the latter usually presuppose the formation of germinal centers (GC) and the generation of both memory B cells and PCs[89]. However, BCRs can also signal independently of antigen engagement, a process termed tonic signaling, thereby regulating the antigen-independent phase of B cell development[90]. Each B cell produces only one particular structure of the variable (V) regions of the heavy (H) and light (L) chains of Immunoglobulins (Igs) that have been generated by unique somatic rearrangements of the gene segments coding for VH and VL. B cells are different from each other, since each has experienced a unique set of V-gene rearrangements[91]. Several subpopulations of mature B cells with distinct functions and predispositions to differentiate into antibody-secreting cells can be distinguished. Upon encounter with foreign antigen, B cells are activated. T-cell dependent antigen responses often involve the establishment of a GC leading to the production of antibody-secreting cells, but also memory B cells, that drive a superior immune response upon re-exposure to the same antigen. B-cell differentiation in the GC reaction is regulated by an elaborate transcription factor network[92]. Most mature B cells are recirculating cells that home mainly to B cell follicles in secondary lymphoid organs and are known as follicular B cells. Follicles are always adjacent to T cell zones and this arrangement allows activated follicular B cells and activated Th cells to migrate towards each other and interact at the interface between these two areas. However, follicular B cells can also occupy a second niche: they recirculate through the bone marrow where they are positioned in organized aggregates around bone marrow sinusoids and can respond in a T cell-independent manner to blood-borne pathogens[93,94]. Most mature follicular B cells are $IgD^{high}IgM^{low}CD21^{mid}$ cells, and we refer to these cells as follicular type I B cells. A second population of mature follicular B cells are phenotypically $IgD^{high}IgM^{high}CD21^{mid}$ B cells and we refer to them as follicular type II B cells[95]. CD21 (also known as complement receptor type 2) is expressed at lower levels by follicular B cells than by marginal zone B cells. Despite the fact that type I and type II follicular B cells recirculate in an identical manner, they have different requirements for development[96]. Marginal zone B cells are generated as naïve B cells that intrinsically have some properties that resemble those of memory cells, including a pre-activated phenotype and the ability to self-renew and to survive for as long as the life-span of the host[97–99].

Regarding tumor immunity, tumor-infiltrating B cells can provide lymphotoxin, which activates non-canonical and canonical NF-κβ signaling and STAT3 in the remaining cancer cells, resulting in tumor progression[100,101]. Lymphotoxin, however, may also contribute to tumor regression in certain tumor settings[102]. Additionally, there is a B cell subset that produces a variety of immunoregulatory cytokines which is also capable of suppressing the anti-tumor immune response by inhibiting effector cells such as $CD8^+$ T cells and natural killer (NK) cells[103]. These B cells, termed regulatory B cells (Bregs) are a heterogeneous population that suppress inflammatory responses, either directly or indirectly, thus attenuating anti-tumor immune responses[104]. However, B cells can play critical roles in anti-tumor immunity by producing antibodies, acting as antigen presenting cells driving T cell expansion and subsequent memory formation[105], and making inflammatory molecules that regulate other immune cells[102]. In fact, antibodies directed against intracellular tumor antigens are frequently observed in human cancer patients[106]. In addition, B cells also secrete several chemokines and recruit other immune cells to secondary lymphoid organs and effector sites[107–109]. In this regard, there is a need to identify the immunologic conditions that specifically promote the pro-tumorigenic effects of B cells, as well as to identify mediators that enhance the ability of B cells to facilitate anti-tumor immunity.

T cells

T cells are involved in many different types of immune responses that occur in infection, cancer, autoimmune diseases and allergic diseases, and are considered the main component involved in the antitumor immune response. This cell subset experience specialized functional polarization that results in T cell responses that are typical for the disease[110]. T cells are defined by expression of a T cell receptor (TCR) responsible for recognizing antigens presented by the major histocompatibility complex (MHC) family of genes (also called human leukocyte antigen or HLA). These cells develop in the thymus from a common lymphoid progenitor and are classically divided into either $CD8^+$ cytotoxic lymphocytes (CTL) or $CD4^+$ T helper (Th) cells that recognize peptides presented by MHCI or MHCII, respectively. Naïve $CD4^+$ T cells give rise to Th cell subsets with functions that are tailored to their respective roles in host defense. The specification of Th-cell subsets is controlled by networks of lineage-specifying transcription factors, which bind to regulatory elements in genes that encode cytokines and other transcription factors. Th cells were, in the past, divided into IFNγ and TNFα expressing Th1 cells, and in IL-4, IL-5 and IL-13 expressing Th2 cells. Currently, a wide range of additional T cell subsets are recognized,

including T follicular helper cells (TFH), IL-17 expressing Th cells (Th17), and regulatory T cells (Treg)[111]. Paralleling these subtypes in the $CD4^+$ T cell compartment, type 1, type 2, and type 17 $CD8^+$ T cells (Tc1, Tc2, Tc17), as well as regulatory $CD8^+$ T cells, have been described[112–114]. In addition, there also exist two 'innate-like' T cell subsets that can be activated either by cytokines or TCR stimulation: natural killer T (NKT) cells, which recognize lipids and glycolipids, rather than peptides, presented by the non-classical MHC molecule CD1d[115]; and γδ T cells, which are not MHC restricted and recognize a diverse range of molecules, including soluble non-protein antigens[116]. Each of these T lymphocyte subsets presents a different role in tumor development and anti-tumor immunity.

Optimal immune defense against viruses and intracellular bacteria is provided by type 1 immune responses[117], characterized by the production of cytotoxic $CD8^+$ T cells and $CD4^+$ T helper 1 (Th1) cells which are also the principal weapons of immunity against cancer[118]. Shortly after the initiation of strong type 1 immune responses, a range of T cell-inhibitory mechanisms are activated. The efficient attenuation of T cell responses after the initial acute phase of disease is advantageous to avoid extended tissue damage, which can result in immunopathology[119]. However, an efficient anticancer immunity depends on long-term type 1 immune responses. T cell dysfunction occurs because of transcriptional and translational regulation of the various cell populations in the tumor microenvironment. These complex microenvironmental alterations offer therapeutic targets to restore antitumor immunity by disrupting immune-inhibitory mechanisms in the tumor microenvironment.

CD4 T cells

Lineage commitment between $CD4^+$ and $CD8^+$ T lymphocytes occurs during development within the thymus. Further differentiation of the $CD4^+$ lineage, with the exception of T regulatory (Treg) cell development, requires T cell activation through MHCII and co-stimulatory molecules, as well as cytokine-dependent signaling that is responsible for directing the cell towards a particular lineage. IL-12 mediates classical differentiation towards Th1 cells which produce IFNγ, TNF and IL-2; while IL-4 induces differentiation into Th2 lineage[111]. On the other hand, Th17 cells are induced by a combination of IL-6 and transforming growth factor (TGF)-β and mediate their effects through secretion of IL-17A, IL-17F, IL-21 and IL-22[120]. This cell subset is very important in liver cancer since high numbers of IL-17 producing cells in hepatocellular carcinoma (HCC) patients is an indicator of poor prognosis[121]. However, IL-17-dependent inflammation may have both positive and negative effects on tumor growth, depending on the tumor model[122]. A new member of the

Th subset is the IL-9 secreting Th cells, also known as Th9 cells, which impart both protective and harmful effects in our body, and play an important role against tumors[123].

On the other hand, TFH differentiation usually starts with interaction of a naïve $CD4^+$ T cell with a myeloid APC such as a DC. The TFH versus non-TFH cell fate decision is predominantly made within the first two cell divisions[124–126]. TFH cells stay resident in lymph nodes (LNs) and spleen because their purpose is to help B cells[127]. These cells form and initiate B cell germinal center responses to generate high-affinity neutralizing antibodies.

T regulatory (Treg) cells

Treg cells are a subset of $CD4^+$ T lymphocytes essential for maintaining immunological self-tolerance and immune homeostasis, that can be characterized by the presence of CD25, cytotoxic T lymphocyte protein 4 (CTLA-4), and CD62L molecules in their membranes and specifically express the transcription factor forkhead box P3 (Foxp3). Treg cells can develop in the thymus or can be converted in the periphery by exposure to TGF-β[128]. Activated by T cell receptor (TCR) engagement, concurrent with IL-10 and TGF-β signaling, Treg cells inhibit the immune response through various mechanisms including depletion of IL-2 and secretion of immunosuppressive factors, such as TGF-β, IL-10 or adenosine, as well as competition with co-stimulatory CD28 via CTLA-4. Treg-specific CTLA-4 deficiency impairs the suppressive function of Tregs, in particular Treg-mediated down-regulation of CD80 and CD86 expression on dendritic cells[129]. Treg cells may contribute substantially to the suppression of antitumor T cell responses as they frequently accumulate in the tumor microenvironment and can even represent the major population of infiltrating $CD4^+$ T cells[130].

CD8 T cells

During infection, oncologic condition or vaccination, naïve $CD8^+$ T cells engage with antigen-presenting DCs and are presented to their cognate peptide in a MHCI-restricted manner[131]. Upon TCR-mediated recognition of the MHC-peptide complex, antigen-specific CD8 T cells start to proliferate and acquire effector functions, as well as the ability to migrate to sites of infection or tumor sites as differentiated cytotoxic T lymphocytes (CTLs). During this process of T cell priming, newly activated T cells integrate multiple signals in the form of TCR signaling, co-stimulation, cytokine, chemokine, and metabolic signals, all of which can have a major impact on the accumulation, survival, and cell-fate decision of effector T cells[117,132,133]. CTLs kill infected cells (through granzymes and perforin) and secrete cytokines such as IFNγ and TNF. In addition, CXC-chemokine receptor 3 (CXCR3)-

dependent trafficking to virus-infected cells enhances terminal differentiation, probably owing to increased exposure to antigens and type I IFNs[134–136]. Most of the CTLs dies over the following several weeks after activation during the contraction phase of the T cell response. However, a small percentage of effector T cells (5–10%) survives and further develops into functional mature memory $CD8^+$ T cells[117]. These cells are maintained in an antigen-independent, cytokine-dependent manner mainly through the actions of IL-7 and IL-15, which promote memory $CD8^+$ T cell survival and self-renewal[137]. A distinguishing feature of memory $CD8^+$ T cells is their ability to rapidly generate effector functions and to produce a 'burst' of secondary CTLs that can quickly contain a secondary infection.

NK

Natural killer (NK) cells are one of the main components of innate immunity and have an important role in given a prompt response against virus, bacteria, and neoplastic cells by their ability to kill self-altered cells without prior stimulation[138]. Human NK cells are defined as $CD3^-CD56^+$ lymphocytes[139] and can be subdivided into functionally distinct subpopulations based on expression levels of CD56 and CD16[140]. $CD56^{bright}CD16^-$ NK cells (~10% of NK cells in peripheral blood) have a high proliferation potential and the ability to secrete a large amount of cytokines, notably IFNγ in response to IL-12, with limited cytotoxic functions[141], while $CD56^{dim}CD16^+$ NK cells, corresponding to 90–95% of NK cells in peripheral blood, display strong cytolytic activity as well as a significant capacity to secrete cytokines upon triggering of activating receptors[139]. Apart from peripheral blood $CD56^{bright}$ and $CD56^{dim}$ NK cells, there are other subpopulations that can be found in peripheral tissues. Tissue-resident NK cells differ from circulating NK cells and are found not only in secondary lymphoid organs but also in many peripheral tissues including the uterus, lung, and liver where they represent up to 50% of lymphocytes[142–144].

NK cells can efficiently discriminate between transformed or virally-infected cells and normal cells without the need for prior sensitization, and have the capacity to kill abnormal cells before adaptive immunity develops, thereby containing viral replication or tumor development. These cells can clear cellular targets by a number of different mechanisms, including (i) exocytosis of cytotoxic granules containing perforin and granzyme that results in cell lysis, (ii) signaling through Fas ligand or TRAIL death receptors which induces apoptosis, (iii) release of cytokines with potent anti-viral and anti-tumor activities, and (iv) antibody-dependent cellular cytotoxicity (ADCC), triggered through binding of the CD16

receptor on NK cells by the constant (Fc) domain of IgG antibodies. NK cells also play major roles in tuning and controlling adaptive immune response[145].

Unlike other lymphocyte subsets, NK cells lack antigen-specific receptors and lyse target cells following the integration of inhibitory and activating signals. These signals are generated by cell surface molecules, with effector functions, taking place when activating signals overcome the inhibitory ones[146]. The major NK cell receptors, which allow NK cells to discriminate between "self" and a variety of pathological cell states, belong to three main categories: (i) natural cytotoxicity receptors (NCRs) such as NKp46, NKp30, and NKp44, which can bind to several viral or tumor-associated molecules[147,148], (ii) NKG2A/C/E-CD94 heterodimers and NKG2D homodimers, which are c-type lectins binding to the non-classical Human Leukocyte Antigen E (HLA-E) molecule and stress-induced ligands, respectively, and (iii) the killer immunoglobulin-like receptors (KIRs), which primarily recognize HLA class Ia (HLA-Ia) and Ib (HLA-Ib) molecules and related surface molecules[149].

Concerning the NK cell activating receptors, CD16 (FcγRIIIA), is the only receptor capable of triggering resting NK cell activation by its own[150], and it is responsible for ADCC. Of note, CD16-mediated activation triggers the expression of inducible co-receptors in NK cells with stimulatory (such as CD137) or inhibitory (e.g., PD-1, TIGIT) functions, thus becoming a second layer of regulatory checkpoints for NK cell-mediated ADCC activity[151].

Nevertheless, NK cells can also be stimulated by factors secreted by other cells, such as type I IFN, IL-12, IL-15, and IL-18[152]. In fact, IL-12 produced by APC induces NK cell proliferation and IFNγ production, thus leading to DC maturation and contributing to the assembly of a Th1 response[138,152].

The implication of NK cells in defense against cancer, as well as their ability to prevent growth and metastasis of certain tumors *in vivo*, is clearly established[153–155]. For this reason, the development of therapeutic strategies to enhance NK cell activity against tumor cells *in vivo* has become a major field of investigation[140]. The development of NK cell-based cancer immunotherapy is evolving continuously. Therefore, strategies such as unleashing NK cell anti-tumor responses by harnessing surface receptors in combination with cytokines, represent potentially successful immunotherapeutic strategies for cancer treatment.

1.2.5. Neutrophils

In humans, neutrophils are the most abundant immune cell population, representing 50–70% of all leukocytes. More than 10^{11} neutrophils may be produced per day, and tumors can further increase this number[156]. Neutrophils act as the first line of defense against pathogenic agents. These cells are the first ones to arrive to the site of injury, where they have an important role in phagocytosis, pathogen killing (through the production of ROS and anti-bacterial proteins), and secretion of chemotactic factors able to recruit monocytes/macrophages[152].

Depending on the microenvironment, neutrophils can functionally behave as pro-inflammatory cells (producing the pro-inflammatory cytokines TNFα, IL-1β, and IL-12p70), or as immune suppressive and anti-inflammatory cells (associated to the production of IL-1Ra and TGF-β). They also can induce B cell class switching, or assume pro-angiogenic or pro-fibrogenic functions[157].

Maturation of neutrophils depends on different stimulating factors including the granulocyte-macrophage colony-stimulating factor (GM-CSF) and the granulocyte colony-stimulating factor (G-CSF). Neutrophil maturation includes: myeloblast, promyelocyte, myelocyte, metamyelocyte, band neutrophil and, finally, segmented neutrophils stages[56,156,158]. Neutrophil lifespan is altered in cancer and it is associated with maturation, extending from 7 hours in normal conditions to 17 hours in cancer[156,158]. The majority of neutrophils remain in the bone marrow, and are released depending on a series of stimulating factors and cytokines including IL-23, IL-17, G-CSF; and CXC chemokine receptors[159,160].

One major theme that has emerged from the oncology field is that not all tumor-infiltrating neutrophils are equal. Depending on specific tumor-derived factors, neutrophil polarization leads to divergent phenotypes. TGFβ, G-CSF and IFNβ are the best-studied molecules in this process. TGFβ and G-CSF activate a tumor-promoting and metastasis-promoting program, by regulating transcription factors that control the immunosuppressive functions of neutrophils[161–166]. While IFNβ acts as a negative regulator of the pro-tumorigenic phenotype of neutrophils[156]. Cytokine concentration and tumor physiology (such as hypoxia) may also be important for neutrophil polarization, since cytotoxic neutrophils are shaped into cancer-promoting cells as tumors expand and evolve[167]. Neutrophil polarization states have been divided into N1 or N2 categories to mirror the Th1/Th2 nomenclature of T-helper cells and the M1/M2 nomenclature of macrophages[163].

The role of neutrophils in cancer is multifactorial since they can participate in different stages of the oncogenic process including tumor initiation, growth, proliferation or metastatic spreading[156,158]. Nevertheless, the binary N1/N2 classification system is probably an oversimplification of neutrophil polarization for the same reasons that have been given against using M1 and M2 to describe tumor-associated macrophages[168]. Similarly to macrophages, neutrophil polarization probably exists as a spectrum of activation states, rather than only two extremes.

1.2.6. Other leukocyte subsets

Eosinophils, mast cells, and basophils are specialized effector cells of the immune system known to play pivotal roles in defense against parasites and in hypersensitivity type I reactions. These cells present common, but non-redundant roles, in the pathogenesis of allergic diseases as well as in the protection against parasites.

Mast cells take origin from hematopoietic stem cells (HSC) in the bone marrow, circulate through the vascular system as immature progenitors and undergo terminal differentiation in tissues where they are ultimately resident[169]. These cells are tissue-based inflammatory cells that respond to signals of innate and adaptive immunity with immediate and delayed release of inflammatory mediators. Several growth factors, cytokines and extracellular components are involved in mast cell growth and differentiation such as stem cell factor (SCF), also known as Kit ligand (essential for mast cell development and chemotaxis under both physiological and pathological conditions), nerve growth factor, IL-3, IL-4 and IL-9[170,171].

On the other hand, basophils complete their differentiation and maturation usually in the bone marrow and then circulate in the peripheral blood, constituting less than 1% of circulating leukocytes. IL-3 plays a pivotal role by directing granulocyte-monocyte progenitors to differentiate into basophil lineage, especially under pathological conditions[172]. Upon their activation, basophils may leave the bloodstream to enter extravascular (inflamed) tissues[173]. Accordingly, they are classically involved in reactions of atopic dermatitis in the skin and in airway inflammation. Their life span, about 2–3 days, is shorter than that of long-living mast cells, which remain alive in tissues for 2–3 weeks[174,175].

Similarly to basophils, eosinophils differentiate in the bone marrow from IL-5Rα-positive progenitors, and then they migrate into peripheral blood, constituting about 1–6% of circulating leukocytes. The presence of intracellular granules stained with the acidophilic

dye eosin allows their discrimination from other granulocytes such as neutrophils and basophils[171].

Immunoglobulin E (IgE) plays a crucial role in type I hypersensitivity[176] which manifests in various allergic diseases, such as allergic asthma, sinusitis, rhinitis, food allergies, and specific types of chronic urticarial and atopic dermatitis. Nevertheless, IgE production is also involved in the defense against parasites, especially protozoa and helminths. The IgE Cε2-4 constant domain is responsible for the isotype specificity to its high- and low-affinity receptors FcεRI and FcεRII (CD23), respectively[177]. A tetrameric form of FcεRI is expressed by mast cells and basophils. FcεRI also exists in a trimeric complex, in other immune populations, such as subsets of human DC[178], monocytes, neutrophils and eosinophils.

In mast cells and basophils, the activation of FcεRI, triggered by the crosslinking with antigen-specific IgE, results in the release of their granule content composed by inflammatory mediators, which are responsible of early and late-phase anaphylactic reactions. The released mediators comprise histamine, proteases, cytokines and chemokine, which may act locally on other inflammatory cells but also on vessels and smooth muscle to activate protective responses[179,180].

Eosinophil expression of FcεRI is minimal, its aggregation is not associated with cell activation, and it is of unclear functional significance. Nevertheless, eosinophils express a wide array of cell surface molecules, whose aggregation can provoke eosinophils activation, such as receptors for IgG (FcγRII/CD32) and IgA (FcαRI/CD89); complement receptors (CR1/CD35, CR3, and CD88); cytokine receptors (IL-3R, IL-5R, GM-CSF, which promote eosinophil development, along with receptors for IL-1α, IL-2, IL-4, IFN-α, and TNFα); and chemokine receptors (CCR1 and CCR3). Also, they express adhesion molecules (very late antigen 4 (VLA4) and α4β7), sialic acid-binding Ig-like lectins (siglec-8) and toll-like receptors (particularly TLR7/8)[181]. Recent evidences also show that eosinophils can be regulated by IL-33 during inflammatory responses. Additionally, eosinophils express several inhibitory receptors as well[182]. The integration of the positive and inhibitory signals received leads to different secretory pathways: exocytosis [183], compound exocytosis[184], piecemeal degranulation (which is a form of exocytosis involving the fusion of small and rapidly mobilized secretory vesicles with cell membrane[185]) and cytolysis, involving the release of the whole and intact granules following the rupture of cell membrane[186]. The latter two pathways are typically activated into tissues during allergic inflammation[187].

In addition, mast cells, basophils and eosinophils are involved in the orchestration of type 2 immune responses, activated in response to allergens and parasitic infections. Type 2

immune responses are regulated by several cytokines, namely IL-4, IL-5, IL-9 and IL-13 secreted by innate immune cells, including eosinophils. In particular, IL-4 could promote IgE production by B cells in the presence of a specific antigen thus activating allergen specific responses though the link with FcεRI on mast cells and basophils. Thus, these cell types not only participate in the acute phase as effector molecules, but also are able to initiate Th2 polarization of response in the late phases, interacting with adaptive immune cells[188]. Additionally, eosinophil could spread Th2 inflammation through additional mechanisms such as the release of CCL17 and CCL22 and the interaction with DCs[189].

Mast cells, basophils and eosinophils are activated under several pathologic conditions such as allergic reactions, infections, autoimmune disorders and cancer. The outcome of allergic reactions depends on the complex interactions occurring in the inflamed microenvironment between immune cells and structural cells. A similar situation is occurring in the tumor microenvironment, where inflammatory immune cells play a pivotal role[190]. Both mast cells and basophils are known to infiltrate several types of tumors but, due to the wide range of mediators they release, it is difficult to define their specific pro- or anti-tumor activity. Accordingly, mast cells and basophils could modify different aspects of tumor development, acting under tissue remodeling, angiogenesis, and invasive capacity, but also interacting with other innate and adaptive cells[191]. Moreover, their plasticity allows phenotypic changes depending on the composition of the tumor microenvironment. In particular, mast cells could be activated in many ways, besides classical IgE antigen crosslink, by soluble mediators such as SCF or through the interplay with other immune and structural cells. Existing reports sustain a pro-tumorigenic role for eosinophils through the enhancement of tumor angiogenesis and connective tissue formation. In contrast, it is known that eosinophil may mediate anti-tumor effects by direct cytotoxicity or exerting an IL-4 mediated immunomodulatory function[192], which regulates the skewing of the immune response from a Th1 to a Th2 response associated with tumor eradication[192,193]. Nevertheless, eosinophils activated in tumor microenvironment dominated by Th1 factors have been described to favor CD8 cytotoxicity and recruitment, thus promoting tumor rejection[194].

1.2.7. Immune cells in the tumor microenvironment

The biology of tumors is determined not only by the traits of the malignant cells but also by the contributions of the tumor microenvironment to tumorigenesis[20]. The tumor microenvironment comprises various cell types including cancer cells, cancer stem cells,

endothelial cells, pericytes, cancer-associated fibroblasts, and immune cells, as well as extra-cellular components such as cytokines, growth factors, hormones and extracellular matrix, among others that are all nourished by a vascular network.

Regarding immune cells, there are highly diverse tumor-infiltrating leukocyte (TIL) subsets that can operate in conflicting ways: presenting both “anti-tumor” vs. “pro-tumor” roles[122]. The tumor microenvironment may contain innate immune cells (including macrophages, neutrophils, mast cells, MDSC, DC, and NK cells) and adaptive immune cells (T and B lymphocytes), that communicate with each other by means of direct contact or cytokine and chemokine production and act in autocrine and paracrine manners to control and shape tumor growth. The most frequently found immune cells within the tumor microenvironment are TAMs and T cells. TAMs have been associated with the promotion of tumor growth and with angiogenesis, invasion, and metastasis processes[195]. However, the phenotype of infiltrating macrophages determines their function in the tumor niche, promoting different mechanism including antitumor immunity, immune regulation, tumor cell invasion, intravasation, metastasis, or angiogenesis[196]. As previously referred, the phenotype of TAMs can be categorized into two main subpopulations: M1 macrophages, which have been associated with anti-tumor activity, and M2 macrophages, which exert pro-tumor effects[197]. Many studies have been performed to analyze the association of TAM with the pathogenesis of different cancer types or with the response to cancer immunotherapies. Table 2 summarizes some of these studies in which the infiltration of “M2” or pro-tumor macrophages is commonly associated with a bad prognosis.

Table 2. Role of tumor-infiltrating macrophages in different cancer types.

REFERENCES	PRIMARY LIVER TUMORS	SUMMARY
Kang FB. et al. Oncol Rep. 2015[198] Dong P et al. Int J Mol Sci. 2016[199]	**Hepatocellular carcinoma (HCC)**	The B7-H3-mediated STAT3 signaling pathway is an important mechanism for inducing **M2-**type polarization of TAMs, which accelerates **HCC development**. High presence **of CD206^{+} TAMs** were markedly correlated with **aggressive tumor phenotypes**, such as multiple tumor number and advanced tumor-node-metastasis (TNM) stage; and were associated with poor overall survival (OS) and increased time to recurrence (TTR)

REFERENCES	OTHER CANCER TYPES	SUMMARY
Li C. et al. PLoS One. 2015[200] Xiong Y. et al. Cancer Med. 2018[201]	**Colorectal cancer (CRC)**	The frequency infiltrating **macrophages** may serve as a **biomarker** for evaluating the pathogenic degrees of CRC. IL-10$^+$ M2 cells are associated with the development of CRC.
Atanasov G. et al. Oncotarget. 2018[202]	**Pancreatic ductal adenocarcinoma (PDAC)**	Presence of **M2-polarized TAMs** is associated with a **decreased overall and recurrence-free survival** of patients with PDAC.
Sugimoto M. et al. Eur J Cancer. 2014[203]	**Invasive ductal carcinoma of the pancreas**	High accumulation of **M2 macrophages** at extra pancreatic nerve plexus invasion represents an important predictor of **poor prognosis.**
Müller S. et al. Genome Biol. 2017[204]	**Gliomas**	The gene signature of blood-derived **TAM**s, but not microglial TAMs, correlates with significantly **inferior survival** in low-grade glioma.
Madonna G. et al. Oncoimmunology. 2018[205]	**Melanoma**	There was an association between clinical benefit from ipilimumab therapy with the coexistence of **low** densities of **CD8$^+$ T cells** and high densities of **M2 macrophages** (CD163$^+$ PD-L1$^+$ cells) at the periphery of the tumor
Hirayama S. et al. J Thorac Oncol. 2012[206]	**Lung squamous cell carcinoma**	**M2 macrophages**, along with other tumor-promoting stromal cells such as regulatory T cells and endothelial cells, may create **tumor-promoting** microenvironments.

Mature T cells can also exert both anti-tumor and pro-tumor effects, as determined by their effector functions[207,208]. Studies of the role of these cell subpopulations in the tumor progression are summarized in table 3. In general, high infiltration of Th1 cells and CD8$^+$ CTLs in the tumor microenvironment are associated with favorable prognosis while high Treg cells infiltration is usually associated with poor overall survival. However, Th17 cells play a complex and controversial role in tumor immunity presenting a fluctuating identity within the context of cancer, either promoting or suppressing tumor growth, depending on the malignancy, the course of therapeutic intervention, or the stimuli to which these cells are exposed during activation[209].

Table 3. Role of tumor-infiltrating T lymphocytes in different cancer types.

REFERENCES	CANCER	SUMMARY
Wang H. el at. Diagn Pathol. 2018[210]	**Pulmonary neuroendocrine tumors**	Higher **CD8⁺ TILs** within stroma was proved to be an independent prognostic factor for **favorable overall survival** and progression-free survival of PNETs.
Duhen T. et al. Nat Commun. 2018[211]	**Head and neck cancer**	Higher frequencies of $CD103^{+}CD39^{+}$ **CD8 TILs** in patients with head and neck cancer are associated with **better overall survival**.
Bai M. et al. Exp Cell Res. 2017[212]	**Pancreatic cancer**	The disease-free survival of pancreatic cancer patients following tumor resection was positively correlated with the frequencies of circulating and tumor-infiltrating $CD8^{+}CXCR5^{+}$ T cells.
Schweiger T. et al. Clin Exp Metastasis. 2016[213]	**Pulmonary metastases (PMs) from primary colorectal cancer (CRC)**	The **balance of CD8⁺ and FoxP3⁺ T-cells** at the tumor border and in tertiary lymphoid structures provides prognostic information in patients with CRC PM.
Ling A. et al. J Pathol Clin Res. 2015[214] Tosolini M. et al. Cancer Res. 2011	**Colorectal cancer (CRC)**	A high number of infiltrating **Th1** lymphocytes is strongly associated with an improved prognosis in patients with CRC. By hierarchical clustering, patients with high expression of the **Th17** cluster had a poor prognosis, whereas patients with high expression of the **Th1** cluster had prolonged disease-free survival.
Hermans C. et al. PLoS One. 2014[215]	**Ovarian carcinoma**	**FoxP3⁺** cells located within lymphoid aggregates surrounding the tumor were strongly associated with **reduced survival**

		time. Central accumulation of **CD8⁺** effector T cells within the tumor bed shows a **positive effect** on survival.
Munn DH Blood. 2009[216]		**Th17** cells found in ovarian cancers correlate with significantly enhanced survival.
Kondo T. et al. Cancer Sci. 2006[217]	**Renal cell carcinoma (RCC)**	Upregulation of the **Th1**-type immune response in RCC tumors with a favorable prognosis may be mediated by Th1-associated chemokines.

REFERENCES	**PRIMARY LIVER TUMORS**	**SUMMARY**
Gabrielson A. et al. Cancer Immunol Res. 2016[218]	**Hepatocellular carcinoma (HCC)**	**Positive PD-L1** staining was correlated with **high CD3** and **CD8** density and predicted a **lower rate of recurrence**.
Kitano Y. et al. Br J Cancer. 2018[219]	**Extrahepatic cholangiocarcinoma (ECC)**	High tumor associated neutrophils, **low CD8⁺** T cells, and **high Tregs** were significantly associated with **poor overall survival**.

Complementing $CD8^+$ cytotoxic activity, NK cells can efficiently kill transformed cells in the tumor microenvironment. This cell subset expresses a complex repertoire of inhibitory and activating receptors that calibrate their anti-tumor function while ensuring self-tolerance[220,221]. Additionally, NK cells express high levels of low-affinity Fc receptor for IgG (CD16), which allows them to mediate ADCC[222]. It has been described the infiltration of NK cells in various types of tumors showing alterations of their phenotype, as summarized in Table 4.

Table 4. Role of tumor-infiltrating NK cells in different cancer types.

REFERENCES	CANCER	SUMMARY
Platonova S. et al. Cancer Res. 2011[223]	**Non-small cell lung carcinoma**	Intratumoral NK cells exhibited profound **defects** in the ability to activate degranulation and **IFN-γ production**
Ali T.H. et al. Nat Commun. 2014[224]	**Melanoma**	The microenvironment of tumor-infiltrated ipsilateral lymph nodes generates and/or recruits a particularly effective NK cell subset
Mamessier E. et al. J Clin Invest. 2011[225]	**Breast cancer**	Breast tumor progression involves **NK cell dysfunction**. In addition, breast tumors have the ability to model their environment to evade NK cell antitumor immunity.
Muntasell A. et al. Clin Cancer Res. 2019[226]		This study identifies baseline tumor-infiltrating NK cells as an independent **biomarker** with great predictive value for pathologic complete response to anti-HER2 antibody-based treatment.
Pereira M.B. et al. Oncoimmunology. 2018[227]	**Glioblastoma**	It was found a predictive impact on survival with positive role for $CD8^+$ T cells and negative roles for macrophages, MDSC, Tregs, NK and NKT in glioblastoma patients.
REFERENCES	**PRIMARY LIVER TUMORS**	**SUMMARY**
Cai L. et al. Clin Immunol. 2008[228]	**Hepatocellular carcinoma (HCC)**	This study indicates that the frequency of both peripheral blood and liver **$CD56^{dim}$ NK** cells **decreased** and their function with regard to **IFN-γ production** and **cytotoxicity** was **impaired** in HCC patients.

The identification and characterization of tumor-infiltrating leukocyte subsets and their distinct, sometimes antagonistic, functions in the tumor niche allow a better understanding of the interactions between immune components and tumor cells and may serve as a benchmark to design novel therapies that seek to modulate the immune response to slow down progression and/or reduce mortality of cancer. For this reason, we examined the phenotype and clinical relevance of TIL subsets in tumor biopsies from CCA and HCC patients, collected immediately after surgical procedure, and the results obtained will be discussed in chapter 3.

1.2.8. Immune system homeostasis in cancer

Inflammation can contribute to multiple hallmark capabilities of cancer by supplying bioactive molecules to the tumor microenvironment, including growth factors that sustain proliferative signaling, survival factors that limit cell death, proangiogenic factors, extracellular matrix-modifying enzymes that facilitate angiogenesis, invasion, and metastasis, and inductive signals leading to activation of epithelial-to-mesenchymal transition and other hallmark-facilitating programs[20,196,229,230].

It is well established that the immune system plays an active role in oncogenesis. Several types of inflammation, differing by cause, mechanism, outcome, and intensity, can promote cancer development and progression[230]. In fact, it is well described for some types of cancer that chronic inflammation increases cancer risk. As previously mentioned, this is the case of liver cancer, in which Infections with hepatitis B (HBV) or C (HCV) viruses increase the risk of HCC and CCA[4,10]. However, not all chronic inflammatory diseases increase cancer risk, and some of them may even reduce it by the upregulation of tumor suppressor pathways, as happen with psoriasis[231].

A completely different type of inflammation is the one that follows tumor development. Solid tumors trigger an intrinsic inflammatory response that entails the generation of the tumor microenvironment[20]. In addition to cell-autonomous proliferation, certain oncogenes, such as RAS and MYC family members, induce a transcriptional program that leads to the recruitment of leukocytes, expression of tumor-promoting chemokines and cytokines, and the induction of an angiogenic switch[232,233]. Additionally, cancer therapy may also trigger a strong tumor-associated inflammatory response leading to both tumor-promoting functions, like the necrosis that accompanies rapid tumor growth, or inducing an antitumor immune response[230]. Cancer therapies, such as surgery, chemotherapy, and radiation, induce local

or systemic inflammation triggered by tissue injury and cancer cell death. Infection- or stress-sensing pathways are activated after surgical procedures while chemotherapy and radiotherapy kill cancer cells mostly through necrosis, a pro-inflammatory form of cell death[234]. As a consequence of therapy-induced inflammation, inhibition of autophagy in apoptosis-deficient tumors stimulates tumor growth through tumor-associated sterile inflammation and through hypoxia-induced necrosis in the tumor's core[235]. Therefore, the inhibition of therapy-induced inflammation may be an approach to improve the treatment of cancer. Anti-inflammatory drugs have been previously used in cancer prevention and therapy to reduce tumor incidence when used as prophylactics, as well as to slow down progression and reduce mortality when used as therapeutics[236]. Therefore, pre-screening for individuals with high cancer risk that are more likely to benefit from such strategies should greatly improve the efficacy and utility of cancer prevention.

For this reason, we have characterized the peripheral immune response in HCC and CCA patients, before and after surgical procedure, in order to understand the immune status of these patients and to assess the effect of tumor resection in the immune system homeostasis in liver cancer patients. The results of this integrated analysis are described in chapter 4, chapter 5, and chapter 6.

1.3. Immunotherapy approaches in liver cancer

Immunotherapy represents an alternative and a very promising treatment approach that has been successful in many different cancer types. As an inflammation induced cancer, liver cancer represents a very interesting target for immune based approaches[237]. In 2013, cancer immunotherapy was declared as the breakthrough of the year[238], and in the two last years, the American Society of Clinical Oncology has considered immunotherapy the Advance of the Year. In liver cancer, the efficacy and the potential complications associated with immunotherapy may depend on clinical and epidemiological factors. Notably, there is evidence that in other cancer types associated with viral infections, such as head-and-neck cancer, Hodgkin lymphoma, and Merkel cell carcinoma, immune checkpoint inhibitors have shown interesting clinical activity, and that this relationship could be mediated, at least in part, by the presentation of non-self or neo-antigens[239,240]. Immune checkpoint inhibitors have proven safe in patients with HCC and underlying chronic infections, whereas clinical trials of immunotherapy have systematically excluded patients with underlying autoimmune diseases. However, recent evidence suggests that one type of immunotherapy alone is not

effective for the treatment of cancer, particularly solid tumors, so that effective immunotherapy combinations are needed[241].

1.3.1. Checkpoint inhibitors

Among the most promising approaches to activating therapeutic antitumor immunity is the blockade of immune checkpoints. Immune checkpoints are a specific subtype of membrane-bound molecules that are crucial for maintaining self-tolerance and modulating the duration and amplitude of physiological immune responses in peripheral tissues in order to minimize collateral tissue damage[242]. Different cell types involved in the immune response express immune checkpoints, including B and T cells, NK cells, DCs, TAMs, monocytes, and MDSCs. The physiological function of these complexes is to prevent a continuous T cell effector function upon initial stimulation and engagement of antigen-specific T cells. Therefore, most of these molecules prevent uncontrolled T cell responses against infection, in order to limit collateral tissue damage. The immune checkpoints most studied in human cancer are cytotoxic T lymphocyte protein 4 (CTLA-4), programmed cell death protein 1 (PD-1), lymphocyte activation gene 3 protein (LAG-3), B and T lymphocyte attenuator (BTLA), and T cell immunoglobulin and mucin-domain containing (TIM-3)[237]. In the field of primary liver cancer, clinical development has so far focused on CTLA-4 and PD-1/PD-L1 pathways.

CTLA-4 is an essential protein for the activation of $CD4^+$ T cells and the priming phase of the immune response. This protein has great affinity for CD80 and CD86. Thus, it may antagonize the interaction of CD28 with these receptors, resulting in decreased T cell activation upon antigen presentation. Treg cells also express CTLA-4 constitutively. In fact, CTLA-4 is required for Treg cells to exert its suppressive activity[129]. But the role of CTLA-4 is not restricted to the priming phase. Inside the tumor, CTLA-4 promotes immunosuppression by inducing Treg activity and differentiation, as well as up-regulating indoleamine-2,3-dioxygenase (IDO) and IL-10 in DC to suppress T-cell response[243].

PD-1, also known as CD279, is a key factor in the effector phase of the immune response. The major role of PD1 is to limit the activity of T cells in peripheral tissues at the time of an inflammatory response to infection and to limit autoimmunity[244,245]. PD1 expression is induced when T cells become activated[246]. When engaged by one of its ligands, PD1 inhibits kinases that are involved in T cell activation through the phosphatase SHP2[247], although additional signaling pathways are also probably induced. Also, because PD1 engagement

inhibits the T cell antigen receptor (TCR) ‘stop signal’, this pathway could modify the duration of T cell-APC or T cell-tumor cell contact[248]. Similarly to CTLA4, PD1 is highly expressed on Treg cells, where it may enhance their proliferation in the presence of ligand[249]. Since many tumors, including liver tumors, are highly infiltrated with Treg cells that probably further suppress effector immune responses, the blockade of the PD1 pathway may also enhance antitumor immune responses by diminishing the number and/or suppressive activity of intra-tumor Treg cells. The two ligands for PD1 are PD1 ligand 1 (PDL1; also known as B7-H1 or CD274) and PDL2 (also known as B7-DC or CD273)[250,251]. PD1 predominantly regulates effector T cell activity within tissue and tumors, whereas CTLA4 predominantly regulates T cell activation. PD1 is more broadly expressed than CTLA4: it is induced on other activated lymphocyte subsets, including B cells and NK cells[252,253], which limits their lytic activity. Therefore, although PD1 blockade is typically viewed as enhancing the activity of effector T cells in tissues and in the tumor microenvironment, it also probably enhances NK cell activity in tumors and tissues[254]. Additionally, chronic antigen exposure, including chronic viral infection and cancer, can lead to high levels of persistent PD1 expression, which induces a state of exhaustion or anergy among cognate antigen-specific T cells. This state, seems to be partially reversible by PD1-pathway blockade[255]. Finally, although the major role of the PD1 pathway is in limiting immune effector responses in tissues, it can also shift the balance from T cell activation to tolerance at the early stages of T cell responses to antigens within secondary lymphoid tissues[242].

1.3.2. Vaccines

Tumor cells express "altered self" antigens that tend to induce weaker responses than the "foreign" antigens expressed by infectious agents. Therefore, immune stimulants and adjuvant approaches have been explored widely. Vaccine types considered include autologous patient-derived immune cell vaccines, tumor antigen-expressing recombinant virus vaccines, peptide vaccines, DNA vaccines, and heterologous whole-cell vaccines derived from established human tumor cell lines[256].

Peptides are an attractive choice as vaccines due to their potential to directly function as pivotal T cell epitopes[257,258], presenting low toxicity profiles, specificity for their target, low comparative cost, and straightforward chemical synthesis[258,259]. In addition, a single vaccine can incorporate multiple peptide epitopes, increasing the chance of activating multiple T cells and overcoming loss or changes of epitopes during tumor progression which, as result,

avoids tumor escape[257,258]. In fact, using multiple peptides within vaccines has correlated with positive clinical response in several clinical trials[260,261]. Among the TAAs, the immune response to α-fetoprotein (AFP) has been studied deeply since CTL epitopes for AFP were identified at an early stage and AFP is overexpressed in the majority of HCCs[262]. AFP is a glycoprotein physiologically produced by liver cells during fetal development and, also, by malignant liver cells in adults, including HCC patients[263].

Viral vector vaccines (with tumor-associated antigens) are also often designed to co-express costimulatory molecules (for example, the TRICOM vector comprises CD80, CD54 and CD58) or DC activating factors (for example, poly(I:C) or granulocyte-macrophage colony-stimulating factor (GM-CSF)), and recently the FDA granted orphan drug designation to the poly(I:C)-expressing rabies virus-based vaccine YSON-001 for treatment of HCC and pancreatic cancer[264].

Every tumor presents its own unique composition of mutations, with only a small fraction shared between patients. Technological advances in genomics now enable the rapid mapping of the mutations within a genome, the rational selection of vaccine targets, and on-demand production of a therapy customized to a patient's individual tumor. First-in-human clinical trials of personalized cancer vaccines have shown the feasibility, safety, and immunotherapeutic activity of targeting individual tumor mutation signatures. In this regard, vaccinating a patient with individual tumor mutations may become the first truly personalized treatment for cancer[265].

1.3.3. T cell transduced with Chimeric Antigen Receptor (CARs)

Chimeric antigen receptor (CAR)-T cells are T cells modified by viral vectors to express synthetic receptors that redirect polyclonal T cells to tumor surface antigens for subsequent tumor elimination[266]. CAR-T cells are not limited by human leukocyte antigen (HLA) because the antigen recognition site of CAR-T cells consists of monoclonal antibodies that specifically recognize tumor surface antigens.

CARs are composed of an extracellular single-chain variable fragment (scFv) from a specific monoclonal antibody, which serves as the targeting moiety; a transmembrane spacer; and intracellular signaling/activation domain(s). The CAR constructs are transfected into T cells using plasmid transfection, mRNA, or viral vector transduction to direct them toward surface-exposed TAAs.

CAR structure has evolved from an initial composition involving only the CD3ζ signaling domain ("first-generation CAR") to more complex forms in which costimulatory endodomains have been added, giving rise to second-generation and third-generation CARs that have augmented T cell persistence and proliferation[266].

The adoptive transfer of CAR-T cells has demonstrated remarkable success in treating hematologic cancers. CD19-CAR-T cell therapy was reported to be effective in a clinical trial as treatment for recurrent and refractory acute lymphocytic leukemia (ALL)[267]. Moreover, in a global, multi-center trial for recurrent and refractory ALL including 75 patients, 81% achieved remission[268]. Thus, CD19-CAR-T cell therapy may be highly effective, and two products have been approved by the FDA and are used in the clinical practice. Taken into account these favorable results, a growing number of clinical trials have now focused on solid tumors, targeting surface proteins including carcinoembryonic antigen (CEA), the diganglioside GD2, mesothelin, interleukin-13 receptor α (IL-13Rα), human epidermal growth factor receptor 2 (HER2), fibroblast activation protein (FAP), and L1 cell adhesion molecule (L1CAM)[269,270]. Unfortunately, the clinical results in solid tumors have not display the same success than in hematological cancer.

To date, the most positive trials reported have used GD2 CARs to target neuroblastoma (3 of 11 patients with complete remissions)[271], HER2 CARs for sarcoma (4 of 17 patients showing stable disease)[272], and HER1 CARs for lung cancer (2 of 11 patients with partial responses)[273]. The obstacles that reduce the efficacy of CAR-T therapy in solid tumors include a lack of specific tumor antigens, limited trafficking and penetration of CAR-T cells into tumor sites, and the immunosuppressive tumor microenvironment[274]. CAR-T therapy has been tested pre-clinically and clinically in liver cancers. However, to date, no clinical trial of CAR-T therapy against liver cancer has been completed. Therefore, further studies are needed to overcome the obstacles of this therapy.

As an alternative to CAR-T cell therapy to treat solid tumors, T cells genetically engineered with T cell receptors (TCR-T cells) recognize intracellular and cell-surface antigens in the context of major histocompatibility complex (MHC) presentation and, thus, have the potential to access much more target antigens than CAR-T cells, providing great promise in treating solid cancers[275].

1.4. Lipidomics in cancer

The term lipidomics is used to refer to large scale and quantitative analysis of both specific lipid classes as well as individual lipid species within a specific lipid class usually involving mass spectrometry (MS) technologies and methodologies. Lipidomics studies allow to quantitatively and qualitatively analyze multiple lipid classes and species at the same time with high sensitivity, which has led to an explosion in the lipid research field over this time period, allowing a better understanding of several lipid-driven mechanisms and the identification of lipid-based biomarkers in different pathological conditions such as cancer[276]. Lipids possesses many diverse functions on both a cellular and organismal level which include: compartmentalization of cells and organelles through their role in the formation of biological membranes[277]; storage of excess calories, mainly in lipids droplets to provide a reservoir of energy, acting as the primary energy source for high-energy demand organs[278]; regulation of biochemical reactions by influencing the activity of transient and permanent membrane protein interactions[21,278]; or function as a reservoir of bioactive lipids that can be used in signaling following agonist-induced hydrolysis or covalent modifications[279]. In this regard, the study of the lipidome helps in the identification of biochemical anomalies arising from perturbations to the normal homeostasis[280].

In theory there are around 180.000 different lipid species belonging to eight different categories: glycerophospholipids, sterol lipids, sphingolipids, fatty acyls, glycerolipids, prenol lipids, saccharolipids, and polyketides[281–283]. The most abundant lipids species in mammalian cells are glycerophospholipids, sphingolipids and sterol lipids, being glycerophospholipids the most abundant. The distribution of different lipid species in the lipid bilayer is not random and provides the two sides of the plasma membrane with different biophysical properties and influences numerous cellular functions. Alteration of lipid asymmetry plays a prominent role during cell fusion, activation of the coagulation cascade, and recognition and removal of apoptotic cell corpses by macrophages[284]. In fact, the compositions and distributions of membrane lipids are sensitive to cell activity changes. In this regard, it has been shown that immune cells activation leads to the reprogramming of lipid metabolism[285]. In addition, the recruitment of cytosolic proteins to the membrane enables signaling events to be tuned and regulated by local lipid concentrations[286,287]. It has been shown that glycerophospholipids[288], cholesterol[289] and sphingolipids[290] can all regulate membrane protein structure through various types of protein-lipid interactions leading to both activation and inactivation of immune cells.

There is a large body of evidence that certain lipid species, in addition to their diagnostic/prognostic roles, also play causative roles in cancer. Eicosanoids and specialized pro-resolving lipid mediators have been linked to inflammation[291–295] associated with cancer development and progression[296–298], as well as with tumor growth and maintenance in different types of cancer[297,299]. Therefore, disrupting lipid metabolic pathways to unbalance lipid homeostasis, through the targeting of enzymes, receptors or bioactive lipids, may induce tumor regression and inhibit metastatic spread. These effects may be achieved by fundamental changes in lipid raft composition; or sustained endoplasmic reticulum stress-induced unfolded protein response, both leading to cancer cell death; or disruption of the lipid-mediated crosstalk between stromal and tumor cells[297].

Moreover, considerable efforts have been made to identify reliable biomarkers to enable the early detection of cancer, and to provide new insights into cancer pathogenesis[300]. Cellular metabolites constantly undergo flux and thus, many of them can be detected in serum or other body fluids. Since the lipid profiles of body fluids reflect the general condition of the whole organism, qualitative and quantitative assessment of lipids in blood and other body fluids could reveal novel biomarkers for the early detection and prognosis of cancer[301], in addition to be used as sensitive markers of patient metabolic status[302]. In fact, different studies have demonstrated the association of cancer with changes in specific lipids levels in body fluids like plasma, serum, and urine[303–309], presenting promising applications to help in the non-invasive diagnosis of cancer. Additionally, it has been reported that prolonged disorder in lipid homeostasis may lead to different human diseases, including cancer[301].

Ubiquitous to all tissues, phospholipids (PLs) are essential components of cell membranes consisting of a hydrophilic head group and a hydrophobic tail that gives PLs their amphiphilic properties. The most abundant PL in mammalian cells is phosphatidylcholine (PC), comprising 40–50% of total PLs. A variety of other PL, including phosphatidylethanolamine (PE), phosphatidylserine (PS), sphingomyelin (SM), cardiolipin (CL), as well as phosphatidylinositol (PI) and its phosphorylated derivatives, are also important membrane constituents[310]. In addition to the PLs, other lipids such as cholesterol and glycosphingolipids are components of mammalian cell membranes. PE, PI, PS and PC can be classified as glycerophospholipids (GPLs), since they share a common structure consisting of two fatty acid (FA) molecules esterified in the sn-1 and sn-2 positions of the glycerol moiety. This portion of the molecule contributes to its hydrophobicity. The sn-3 position consists of a phosphate group with a hydrophilic residue that contributes hydrophilicity. Lysophospholipids (Lyso-PLs) refer to phospholipids whose fatty acid chain

has been removed from either the sn-1 or sn-2 position, including the well-characterized lysophosphatidic acid (LPA) and lysophosphatidylcholine (LPC). They play a major role as extracellular mediators by activating G-protein coupled receptors (GPCRs) and stimulating diverse cellular responses from their signaling pathways[311]. Sphingolipids (SPLs) contain the long-chain amino alcohol sphingosine (instead of glycerol) esterified to a fatty acid and a phosphate group. SM is the most representative SPL, which consists of sphingosine and bares a choline molecule[312]. CL, also known as diphosphatidylglycerol, which is required for normal functioning of mitochondria, is present only in mitochondria, particularly in the inner membranes[310].

It has been shown that PL have an essential role in cell transformation and tumor progression[313–315]. In this regard, abnormal PL metabolism has been detected in cells, animal models of cancer, as well as in the tumors of cancer patients[316]. In fact, the reprogramming of energy metabolism has been included among the principal hallmarks of cancer[20]. One of the most important organs in energy metabolism is the liver, since most plasma apolipoproteins, endogenous lipids and lipoproteins are synthesized in this organ[317,318]. In fact, liver ensures the homeostasis of lipid and lipoprotein metabolism[319], but hepatic cellular damage and/or oncogenic condition impair these processes, leading to alterations in plasma lipids and lipoproteins composition.

In this regard, changes in circulating lipid profile and in lipid metabolism have been addressed for liver cancer patients[320–322]. In serum, some plasmalogens species such as PEp (36:4) and (40:6) showed a fair capability to discriminate HCC patients from healthy controls, and were significantly associated with HCC tumor grades, and thus have been suggested as potential diagnostic and prognostic biomarkers of HCC[322]. Moreover, plasma concentrations of some LPC species were reduced in HCC patients[320] while some PC species such as PC(32:1) and PC(32:0) were found elevated[323]. These studies pointed out the potential of some PLs in predicting HCC patient mortality[323].

Abnormal choline metabolism has been identified in multiple cancers. The molecular causes of abnormal choline metabolism are changes in choline kinase-α, ethanolamine kinase-α, phosphatidylcholine-specific phospholipase C and D and glycerophosphocholine phosphodiesterases, as well as several choline transporters. Enzymes mediating the abnormal choline metabolism are being explored as targets for cancer therapy. PC synthesis is increased in response to fatty acid and fatty acid-derived substrates, which is frequently observed in cancer cells. However, it has also been observed a compensatory increase in PC degradation. On the other hand, phospholipase A2 (PLA2) enzymes are a

diverse class of esterases that preferentially cleave GPLs at the sn-2 position, resulting in the liberation of a fatty acid and a lysophospholipid, thus contributing to the production of bioactive lipid mediators in inflammatory cells[324]. Secretory PLA2 isoforms are commonly overexpressed during bouts of inflammation, in inflammation associated diseases and in cancer. In fact, it appears that some isoforms of PLA2 can have an oncogenic role by stimulating tumor cell growth and angiogenesis[325,326].

Although different studies have been conducted to determine the PL profile in liver cancer patients, no study has been made to monitor these alterations after curative treatments. For this reason, we have studied the molecular phospholipidome of serum from patients with liver cancer before and after tumor resection to monitor the effect that surgical resection causes in the altered lipid metabolism. The results of this study will be further discussed in chapter 7.

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2. Objectives

The overall objective of the present Thesis was to better understand the relationship among cancer and immunity as well as to characterize the immune and the lipidomic context in liver cancer patients. The main goals of this thesis were:

- To establish the relationship among lipoxidation and cancer immunity (chapter 2).
- To evaluate the functional and phenotypic differences in TIL cell sub-populations between CCA and HCC, as well as among different histopathological grades and tumor aggressiveness degrees (chapter 3).
- To characterize circulating myeloid dendritic cells (mDCs) and their precursor cells, FcεRI^{+} monocytes, in HCC and CCA patients (chapter 4).
- To characterize the immune response in HCC and CCA patients, before and after surgical procedure, in order to understand the immune status of these patients and to assess the effect of tumor resection in the immune system homeostasis (chapter 5 and chapter 6).
- To characterize the PL profile in HCC and CCA patients, before and after tumor resection, to monitor the effect that surgical resection causes on the altered lipid metabolism of patients with liver cancer (chapter 7).

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CHAPTER 2. LIPOXIDATION AND CANCER IMMUNITY

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Review article

Lipoxidation and cancer immunity

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ABSTRACT

Lipoxidation is a well-known reaction between electrophilic carbonyl species, formed during oxidation of lipids, and specific proteins that, in most cases, causes an alteration in proteins function. This can occur under physiological conditions but, in many cases, it has been associated to pathological process, including cancer. Lipoxidation may have an effect in cancer development through their effects in tumour cells, as well as through the alteration of immune components and the consequent modulation of the immune response. The formation of protein adducts affects different proteins in cancer, triggering different mechanism, such as proliferation, cell differentiation and apoptosis, among others, altering cancer progression. The divergent results obtained documented that the formation of lipoxidation adducts can have either anti-carcinogenic or pro-carcinogenic effects, depending on the cell type affected and the specific adduct formed. Moreover, lipoxidation adducts may alter the immune response, consequently causing either positive or negative alterations in cancer progression. Therefore, in this review, we summarize the effects of lipoxidation adducts in cancer cells and immune components and their consequences in the evolution of different types of cancer.

1. Introduction

Oxidative stress is usually associated with an increase of reactive oxygen species (ROS), or a decrease on the antioxidant defences which, in turn, can favour the peroxidation of the polyunsaturated fatty acids (PUFAs) in membrane lipid bilayers, leading eventually to the formation of highly reactive aldehydes [1]. These electrophilic reactive aldehydes can spread from the site of origin and react with major biomolecules, like proteins, even at distant sites [2], causing a lipoxidation process. Lipoxidation is a well-known reaction between electrophilic carbonyl lipids species formed during oxidation of lipids and specific proteins [3].

Lipid oxidation products may accumulate and covalently modify proteins, driving not only to physiological but also to pathological process through altering protein structure and function or changing signalling pathways. This has an effect in different pathologies such as cancer, in which lipid oxidation products may influence cancer progression either directly, through the modulation of cancer cells behaviour, or through the modulation of the immune response (Fig. 1) [4].

The biological effects of reactive lipid carbonyl species generated by lipid peroxidation process are modulated by their local concentration and availability, which depends on the initial lipid targeted by peroxidation, as well as on the presence of cellular detoxifying and conjugating systems, and the cell ability to degrade modified proteins [5].

Abbreviations: ACR, acrolein; ADCC, antibody-dependent cellular cytotoxicity; AKR, aldo-keto reductases; AP-1, activator protein-1; ARE, antioxidant response element; ASK1, apoptosis signal regulating kinase; COX-2, cyclooxygenase-2; CTLs, cytotoxic T lymphocytes; cyPG, cyclopentenone prostaglandins; 15d-PGJ_2, 15-deoxyΔ^{12-14} PGJ_2; EGFR, epidermal growth factor receptor; ERK, extracellular signal-regulated kinase; PDGFR, platelet-derived growth factor receptor; GCL, glutamate cysteine ligase; GSH, glutathione; GST, glutathione S-transferasaes; 4-HHE, 4-hydroxy-hexenal; HNE, 4-hydroxy-2-nonenal; hPGD2s, hematopoietic prostaglandin D_2 synthase; IκB, inhibitor of kappaB; IKK, IκB kinase; iNOS, inducible nitric oxide synthase; JNKs, c-Jun N-terminal kinase; Keap1, Kelch-like ECH associating protein 1; LKB1, liver kinase B1; MAPK, mitogen-activated protein kinase; MDA, malondialdehyde; MSA, mouse serum albumin; NFκB, nuclear factor-κB; NK, natural killer; PGA1, prostaglandin A1; PGA2, prostaglandin A2; PGD_2, prostaglandin D_2; Pin1, peptidylprolyl cis/trans-isomerase A1; PKB, protein kinase B; PPARs, peroxisome proliferator activated receptors; PUFAs, polyunsaturated fatty acids; RAGE, receptor for advanced glycation end products; RNS, reactive nitrogen species; ROS, reactive oxygen species; Th, T helper; TKRs, tyrosine kinase receptors; Tregs, $Foxp3^+$ regulatory T cells; XIAP, X-linked inhibitor of apoptosis protein

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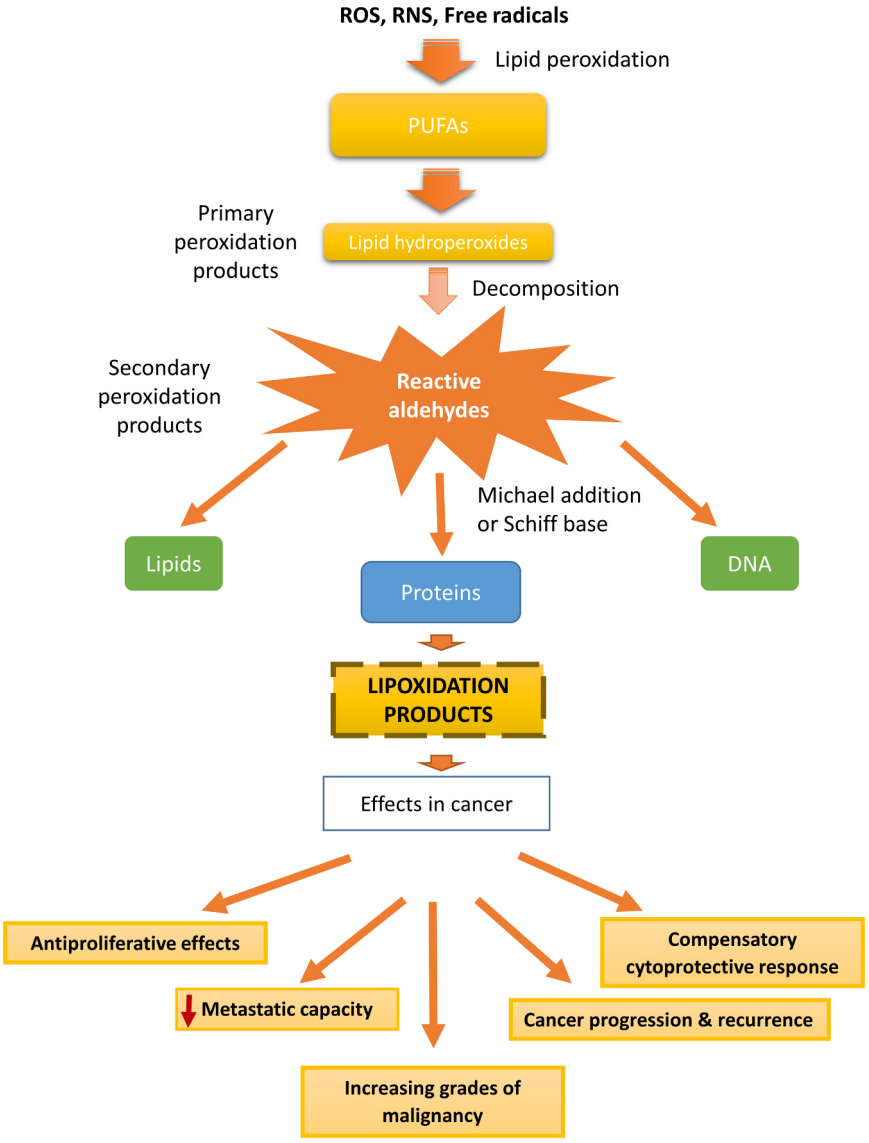

Fig. 1. Diagram illustrating the formation of lipoxidation adducts and their possible effects on the progression of cancer.

Also, quite important as well, depending on the type of protein modified, different effects can occur in the physiologic or the pathophysiologic signalling [6].

Oxidative modified molecules, including lipoxidation adducts, are also reported to have a significant role in the modulation of inflammation and immune response. They can induce adaptive immunity and have been implicated in the pathogenesis of various diseases [7]. In fact, it has been reported that the covalent reaction of electrophilic aldehydic products with proteins might lead to the formation of immunogenic biomolecules [8], and these lipoxidation products may alter the cellular signalling in the immune response in several pathologies, including cancer [9]. Moreover, it is well established that the immune system plays a very important role in cancer progression. In this regard, several studies in the past few years have demonstrated a dual role of leukocytes themselves contributing to either "pro-tumour" microenvironment or to "anti-tumour" microenvironment [10].

In this review, we will discuss and summarize the most recent advances in lipoxidation formation and its influence on the pathophysiology of cancer. We will also highlight the effect of lipoxidation in tumour and immune cells during cancer progression.

2. Chemistry of lipoxidation adducts and its relevance in disease pathophysiology

The unsaturated fatty acid are main targets of oxygen radicals leading to the formation of primary peroxidation products. These oxidized lipids can be decomposed to form secondary peroxidation products (carbonyl-based derivatives), and can react by addition reactions of the carbonyl groups (electrophiles) with amino and thiol groups (nucleophiles), leading to the formation of lipid-protein adducts or lipoxidation products [11] (Fig. 1). The aldehydes and other electrophilic carbonyl species generated will depend on the initial PUFA targeted by peroxidation. In this sense, the peroxidation of n-3 PUFAs (α-linolenic acid and docosahexaenoic acid) generates mainly 4-hydroxy-hexenal (4-HHE), while the peroxidation of n-6 PUFAs, such as linoleic acid and arachidonic acid, generates mostly 4-hydroxy-2-nonenal (HNE), which is the most intensively studied electrophilic reactive aldehyde [12–14]. The type of adducts that can be generated depends on the reactivity of the oxidized lipid species. In addition, the reaction of these compounds with a protein can take place by two principal reactions: (i) the addition of the aldehydic group to an amino group of the protein (e.g. lysine) forming a Schiff´s base adduct by loss of water and (ii) by a Michael addition to a nucleophile by the active C=C double bond [3,9]. While

Schiff base formation is reversible, Michael adducts are quite stable, thus the formation of the latter seems to be preferred *in vivo*. It is also important to consider that lipoxidation depends on the balance of the rate of formation of the lipid oxidation product, its reactivity, and the rate of detoxification by enzymes such as glutathione peroxidases [15], glutathione S-transferasaes (GST) [16], or aldo-keto reductases (AKR) [17].

Lipoxidation can occur in healthy individuals [18,19], since protein modification by reactive electrophilic species not only may inhibit protein function, but also, in a small number of cases, may cause a gain of function, even leading to beneficial effects [20–22].

Nevertheless, the importance of lipoxidation and its pathophysiological relevance have been broadly discussed in several works [14,23–26]. In fact, the measurement of global protein adducts, such as HNE-protein adducts, is commonly used as a biomarker of inflammation/oxidative stress/lipid peroxidation under various pathological conditions [27]. The accumulation of lipid peroxidation products, and therefore of lipoxidation adducts, has been involved in ageing and in well-defined diseases of liver, kidney, neurological and cardiovascular systems, endocrine and metabolic disorders, diabetes and its complications, and other oxidative stress related pathologies [28].

Furthermore, lipoxidation is highly associated with chronic degenerative diseases such as cancer. These topics will be discussed in the following section.

3. Lipoxidation in cancer: Effect on tumour and immune cells

Carcinogenesis and cancer therapies are strongly influenced by oxidative stress and by lipid peroxidation [28] and, consequently, by lipoxidation adducts. The most reported reactive carbonyl products formed during lipid peroxidation are malondialdehyde (MDA), acrolein (ACR), 4-hydroxy-hexenal (4-HHE) and 4-hydroxy-2-nonenal (HNE) [29], and several studies reported the formation of protein adducts with several proteins in different types of cancer [30–33]. In fact, the greater reactivity of HNE, one of the major products of lipid peroxidation, with proteins, gave rise to the assumption that HNE has a cytotoxic and carcinogenic effect through the modulation of proteins involved in DNA repair [34]. Moreover, other works demonstrated that oxidative stress and electrophilic lipid peroxidation products, such as HNE, also play important roles in the induction of cell cycle arrest, differentiation process, and apoptosis in cancer cells [35]. However, some studies show controversial results regarding the influence of HNE, or HNE-adducts in different types of human cancers [36–39], and the pattern of HNE histological appearance has been shown to be dependent on the histological origin of cancer [40].

Likewise, cancer cells are sensitive to lipid oxidation products since these products act as second toxic messengers of free radicals, as well as signalling molecules and growth regulating factors that influence important processes for cancer progression such as proliferation, differentiation and apoptosis [28]. But there are discrepancies in the appearance of lipoxidation adducts in distinct cancer types. For example, in hepatoma cells, it was shown that the majority of HNE was converted to the HNE-GSH conjugate, which was rapidly and efficiently exported from the cell [41]. However, in astrocytic and ependymal glial tumours, HNE-protein adducts were detected in mitotic, necrotic and apoptotic cells, and were associated with increasing grades of malignancy [42].

The disparity observed in the formation of lipoxidation adducts in various tumours may be explained by: a) the different membrane composition of lipids in different cancer cell types, as well as different cholesterol/PUFAs ratios, which determine different tendencies to form lipid peroxidation products and, therefore, different electrophilic lipids and, thus, different lipoxidation adducts [43]; b) the higher expression of detoxification enzymes and antioxidant proteins observed in some tumour cells, what results in a more efficient and rapid metabolism of lipid peroxidation products [44]; c) the different effects, either physiological or pathological, triggered by some lipid peroxidation products, that act through the antioxidant response element (ARE) to induce the expression of key metabolizing enzymes, such as GST [45], influencing on Keap1–Nrf2–ARE pathway [46,47]; d) the local of formation and e) the targeted protein or enzyme that are adducted to the electrophilic lipid.

3.1. Effect of lipoxidation in tumour cells

As it was mentioned above, the level of oxidative stress and, consequently, the level of lipoxidation products vary among cancer types in relation with cell type. In liver cancer, it was found lower levels of lipid peroxidation products in hepatoma cells when compared to normal liver cells [48,49], probably leading to lower levels of lipoxidation products, what can be explained, in part, by the observed increase in the activity of enzymes metabolizing toxic aldehydes during rat liver carcinogenesis [50], thus rendering the cancer cells more protected against the cytotoxic effect of lipoxidation products.

Several enzymes involved in tumour resistance due to their ability to metabolize electrophilic lipids are, at the same time, targets for lipoxidation themselves. This is the case of AKR that catalyse the reduction of ketones and aldehydes [51] or GST enzymes that are involved in drug detoxification [3]. AKR1B10, a member of AKR family, is overexpressed in several types of tumours, and it may contribute to tumorigenesis through various mechanisms, in addition to be involved in chemoresistance [52,53]. This protein is a selective target for lipoxidation and inhibition by A-class cyclopentenone prostaglandins (cyPG) and it has been demonstrated that low concentrations of prostaglandin A1 (PGA1) potentiate the intracellular accumulation and G2/M cell cycle arresting effect of the topoisomerase inhibitor doxorubicin in A549 lung cancer cells [54,55]. Due to their electrophilic nature, cyPG may form Michael adducts with GSH both enzymatically, through the action of GSTs, and non-enzymatically [56,57]. Likewise, it has been found HNE adducts with GST detected by immunoprecipitation of GST followed by Western blot analysis using anti-HNE antibody [58]. On the top of that, GSTP1-1, a very important enzyme in tumour chemoresistance, can be covalently bound by various electrophilic lipids, including PGA1 and PGA2, causing its inactivation [22,59,60]. Hence, lipoxidation of GSTP1-1 could help to overcome the resistance of certain tumour cells to chemotherapy or radiation [55,61].

On the other hand, lipoxidation adducts were found in renal [62], and colon cancer cells [63], as well as in astrocytic and ependymal glial tumours, in which the incidence of HNE-positive tumour cells increased with increasing grades of malignancy [42]. Although the amount of lipoxidation products in cancer cells, like HNE-protein adducts, has been often assayed as a means of assessing the level of oxidative stress, only in some cases the identification and the consequences of HNE-protein adduct formation on cancer cell growth or behaviour have been reported [14].

We have summarized the effect of HNE-protein adducts in distinct cancer cell lines, such as human epidermoid carcinoma, leukemic cells, adenocarcinoma human alveolar basal epithelial, breast cancer cells, or colon cancer cells, reported by different studies [64–71], in Fig. 2. Both endogenous and exogenous HNE lead to lipoxidation adducts with many diverse proteins such as epidermal growth factor receptor (EGFR), α-enolase, peptidylprolyl cis/trans-isomerase A1 (Pin1), liver kinase B1 (LKB1), IκB kinase (IKK), or glutamate cysteine ligase (GCL), triggering different effects very important in avoiding cancer progression, such as suppression of cell growth, reduction of metastatic capacity or anti-proliferative effects, but also in other cases triggering effects that favour cancer progression, as the modulation of tumour microenvironment to become more pro-tumorigenic or the cytoprotective response in cancer cells (Fig. 2).

Moreover, other studies have shown that the formation of HNE protein adducts in renal and colon cancer tissues has been related to the growth and progression of kidney and colon cancer [30], although the progression of colon cancer results in loss of lipoxidation adducts in the

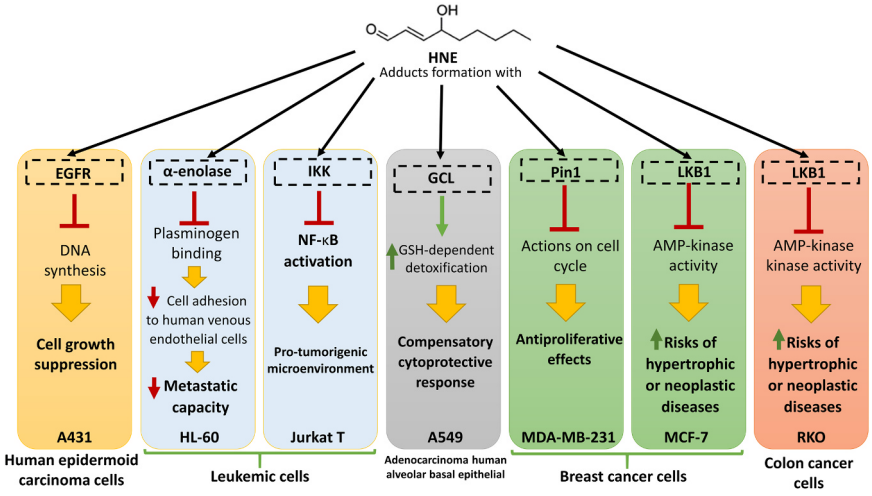

Fig. 2. Summary of the possible effects of HNE-protein adducts on different proteins and different cancer cell lines.

malignant tissue and increase of reactive aldehydes in the surrounding area [31]. In accordance with these results, a different study in prostate cancer showed that ACR protein adducts could be associated with tumour progression and recurrence [32]. Moreover, tumour tissues in lung cancer showed lower antioxidant capacity than healthy tissues, which was accompanied by lower levels of fatty acids and higher levels of reactive aldehydes detected in the necrotic and stromal cells in these tumours, thus favouring the formation of lipoxidation products like the HNE-His protein adducts observed in necrotic lung cancer tissues [33].

Protein adducts are also involved in the inactivation of the proteasome [72], which is responsible for the intracellular degradation of proteins, whether they are damaged or no longer required for cellular processes [73]. Proteasome is then essential for many cellular pathways, including cell cycle, regulation of gene expression and resistance to oxidative stress. Therefore, protein lipoxidation adducts could alter carcinogenesis through their effect in the inactivation of the proteasome since cross-linked proteins are able to inhibit the proteasome, and further impair cellular protein turnover [74]. In fact, there are some studies showing that proteasome inhibitors induce apoptosis in leukemic cell lines, turning the proteasome into one of the possible targets with potential for therapeutic agents against cancer [75–77].

It is important to remark that, in several cases, the progression of malignancy is accompanied by reductions of oxidative stress, due to the upregulation of antioxidant capacity [78], and the induction of the Nfr2/Keap1 pathway, which negatively regulates the HNE intracellular concentration [79]. This also matches with the results showing that the adaptation to intrinsic oxidative stress in cancer cells can confer drug resistance. Thus, anticancer drugs and radiotherapy can induce oxidative stress and trigger cancer cells to undergo apoptosis, however some cancer cells escape from this process through the adaptation to intrinsic oxidative stress [34]. On the other hand, despite the reduction of intrinsic oxidative stress, the level of lipoxidation products in cancer cells may increase, due to the inflammatory response present in the tissues surrounding cancer lesions [14].

Transcription factors of the peroxisome proliferator activated receptors (PPARs) family play a key role both in tumour biology and in immune function [80]. The mechanisms reported so far suggest that each PPAR isotype is associated with pathways that relate to carcinogenesis due to direct effect in the cancer cells themselves, since they are involved in the control of cell proliferation, cell differentiation and apoptosis [81,82]. But in addition to these functions, PPARs may act on the tumour environment by regulating inflammatory processes [83–85]. This family of nuclear receptors is also a target of lipoxidation processes. It has been demonstrated that 15-deoxyΔ^{12-14} PGJ_2 (15d-PGJ_2) binds covalently to a cysteine residue located in the PPARγ ligand binding pocket [86–88]. Further on, it was shown that 15d-PGJ_2 activates PPARδ's transcriptional activity through formation of a covalent adduct between its endocyclic enone at C_9 and Cys249 in the receptor's ligand-binding domain [89]. In addition, HNE has been reported as an endogenous ligand for PPARβ/δ that causes its activation [90].

The divergent results obtained documented that the formation of lipoxidation adducts can have either anti-carcinogenic or pro-carcinogenic effects, depending on the cell type affected and the specific adduct formed [14]. The abundance of a protein, as well as the high reactivity and accessibility of some nucleophilic sites, may determine if a protein becomes, or not, a lipoxidation target [91,92]. Moreover, depending on the nature/structure of the lipid oxidation product, which could have different structural features and, as well, different reactivity, it may lead to the formation of different types of lipoxidation adducts and thus to different functional consequences in the targeted protein [22,93,94]. In fact, it has been shown that biotinylated cyPG mimic many of the effects of cyPG in cellular models, including inhibition of inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2), and induction of HO-1 and Hsp70 expression, but they are unable to elicit PPAR activation *in vitro* or in intact cells [95,96]. Hence, by adding a bulky moiety to the carboxyl group of cyPG, it may be possible to dissociate some biological actions [97]. More studies are needed to disclose these effects depending on the type of cancer, their stage, the implicated targeted protein, or the reactive species involved.

3.2. *Effect of lipoxidation on immune cells and their correlation with cancer*

Chronic inflammatory processes induce oxidative/nitrosative stress and, as consequence, lipid peroxidation products and lipoxidation processes. In addition, it has been described that different chronic inflammatory conditions pre-dispose susceptible cells to malignant transformation and cancer progression [28], so that it has been estimated that chronic infection and associated inflammation contribute to about one in four of all cancer cases worldwide [98].

ROS, reactive nitrogen species (RNS) and lipid peroxidation products can modulate signalling molecules [99] and alter functions of proteins involved in inflammation and carcinogenesis [100], such as the nuclear transcription factor NFκB or stress response enzymes, namely iNOS and COX-2 [101,102]. Furthermore, it has been reported that non-enzymatic oxidative modification of proteins, including lipoxidation, renders proteins immunogenic and leads to the generation of antibodies against oxidatively modified proteins [8,103].

In fact, aldehydes exert a dual effect on inflammatory signalling, mainly depending on the concentration levels. On the one hand, at low concentrations, HNE activates PKCβ-signalling, inducing the

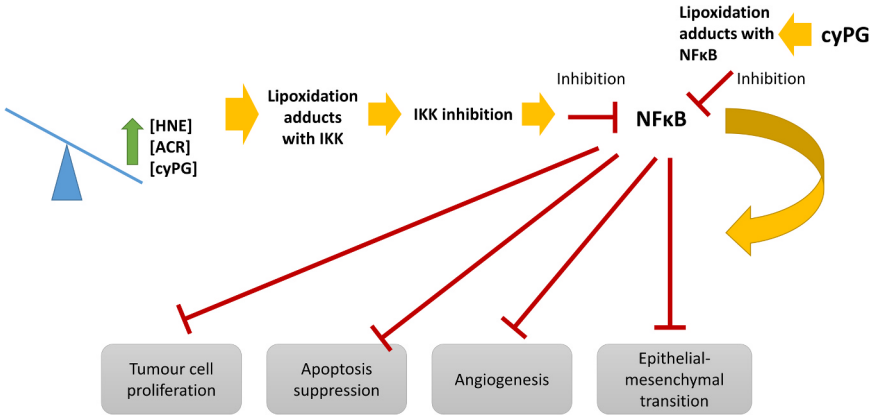

Fig. 3. Effects of NFκB inhibition mediated by lipoxidation adducts. High concentration of aldehydes, such as HNE or acrolein, or high concentration of cyclopentenone prostaglandins (cyPG) inhibits IKK activity through the formation of lipoxidation products. IKK inhibition results in the suppression of NFκB activity, hindering the effects triggered by NFkB, such as tumour cells proliferation, suppression of apoptosis, angiogenesis and epithelial-mesenchymal transition, which facilitates distant metastasis. Moreover, cyPG can directly modify NFκB subunits leading to NFκB inhibition, and therefore, the suppression of NFkB effects.

production and secretion of CCL2 (MCP-1) by macrophages [104]. On the other hand, high concentrations of reactive aldehydes, such as HNE or ACR, inhibit the activation of NFκB, either via a direct inhibitory effect on proteasome, or via inhibition of the phosphorylation of inhibitor of kappaB (IκB) and its subsequent proteolysis [105], or a modification of IκB kinase (IKK) β -sub-unit by aldehydes [106] that has also been found to be a target of cyPG (Fig. 3) [107]. Moreover, 4-HHE activates the IKK, via the IKK/NFκB inducing kinase (NIK) pathway, through the increase in the activity of p38 MAPK and ERK1/2 kinase, resulting in NFκB activation [108]. In contrast, it has been described that cyPG can directly modify NFκB subunits p65 and p50, leading to NFκB inhibition by blocking its ability to bind DNA, studied by immunohistochemistry and Western blot analysis (Fig. 3) [109,110]. Moreover, it has been proposed a role for 15d-PGJ_2 in the control of lymphocyte proliferation and activation through mechanisms relying on NFκB inhibition, studied in knockout mice for hematopoietic prostaglandin D_2 synthase (hPGD2s), which metabolizes cyclooxygenase (COX)-derived PGH_2 to PGD_2 and 15d-PGJ_2 [111]. Furthermore, it was shown that 15d-PGJ_2 controlled the balance of pro- vs. anti-inflammatory cytokines regulating leukocyte influx and macrophage efflux through draining lymphatics [112]. This is very important for cancer progression since NF-κB activation promotes the accumulation of pro-inflammatory cytokines at the tumour site, contributing to the pro-tumorigenic microenvironment. The activation of this transcription factor has been associated with tumour cells proliferation, suppression of apoptosis, angiogenesis and epithelial-mesenchymal transition, which facilitates distant metastasis [113].

Additionally, it has been demonstrated that PPAR-α ligands and PPAR-γ ligands (15d-PGJ_2) inhibit cell growth and induce monocytic differentiation in human promyelocytic leukemia cells (HL-60 cells), and HNE, which alone induces granulocytic-like differentiation of HL-60 cells, potentiates the monocytic differentiation induced by 15d-PGJ_2. Moreover, HNE treatment significantly inhibits U937 (human histiocytic lymphoma) cell growth and potentiates the inhibition of cell growth in PPAR-γ ligand-treated cells [68]. And, in addition, it has been reported that HNE can form adducts with cysteine residues in the extracellular domain of TLR4 peptides, demonstrated by LC–MS/MS analysis, inhibiting its activation [114]. Hence, the formation of lipoxidation adducts with HNE can differentially regulate the activation of TLR4 and subsequently provoking an effect in the immune response.

It has been shown that both MDA-adducted mouse serum albumin (MSA) and HNE–MSA were able to significantly promote $CD4^+$ T cell proliferation, leading to the hypothesis that lipoxidation adducts, could serve as an immunological trigger in the activation of $CD4^+$ T cells. Moreover, it has been suggested that lipid peroxidation derived aldehydes preferentially promote Th1 differentiation, analysed by flow cytometry and ELISA in splenic lymphocytes from trichloroethene-treated mice [115]. In that sense, we could consider lipoxidation adducts a positive factor since Th1 cells have been associated with the promotion of anti-tumour responses: Th1 cells enhance the cytotoxic functions of NK and $CD8^+$ cells, upregulate MHC Class I expression in tumour cells, and support $CD8^+$ cell proliferation through the secretion of IL-2 [116].

Regarding monocytes function, it has been suggested that synthetic MDA-Lys, used as a prototype of advanced lipoxidation end products, can promote monocyte activation and vascular complications via the induction of inflammatory pathways and networks. In a candidate gene profiling approach, MDA-Lys increased the expression of key NFκB-dependent genes, such as MCP-1, iNOS, RAGE, IP-10, CCR-2, IL-6, IL-8, and COX-2 that are associated with monocyte activation. Antibody array profiling revealed that MDA-Lys can upregulate the chemokines CCL11 (eotaxin), TNFSF14, and CCL18. In addition, key factors that were noted to be induced by MDA-Lys, such as MCP-1, eotaxin, IL-6, IL-8, β1- and β2-integrins, and COX-2, are associated with monocyte activation, adhesion, and migration [117].

Neutrophils mediate key components of the cellular immune response which involves cellular adhesion, migration, phagocytosis and degradation and turnover of phagocytic metabolites [118]. It has been demonstrated, by mass spectrometry analyses, the existence of lipoxidation adducts of HNE with proteins involved in key pathways of neutrophil oxidative burst, phagocytosis, redox homeostasis and glucose metabolism. The same study also confirmed the formation of neutrophil protein-HNE adducts using candidate proteins found to be modified, by mass spectrometry. Taken together, these data suggest that HNE induces a pleiotropic mechanism to inhibit neutrophil function [119].

In addition, it has been reported that HNE seems to be an important cell growth regulating factor, acting as a signalling molecule interacting with the growth regulating effects of various cytokines [120–123]. HNE, as a second messenger of ROS, activates activator protein 1 (AP-1) that promotes TGFβ synthesis and fibrogenesis. Hence, HNE could, at the same time, support fibrogenesis and inhibit the cancer growth.

The regulation of the immune system is very important in determining cancer progression [10]. Therefore, lipoxidation products may have an effect in cancer development by affecting immune components and modulating the immune response.

3.3. Overview of tumour immunology at tumour microenvironment and its relation with reactive aldehydes and lipoxidation

There are few studies on the role of lipoxidation adducts with respect to tumour immunology, but considering what is known about lipid peroxidation products, their influence in immunology, as described above, and the influence of immune microenvironment in tumour progression [10,124–126], altogether it suggests that lipoxidation is a very important process in this field. Moreover, recent studies have revealed that immune cells possess distinct metabolic characteristics that influence their immunological functions [127]. For example,

macrophage polarization is related to distinct metabolic characteristics pertaining to lipid metabolism, among others [128]. In this sense, it has been found that genes involved in glycolysis and phospholipid metabolism, differentially expressed between M1 and M2 macrophages, are major distinguishing features of inflammatory (M1) macrophages [128].

Clinically manifest neoplasms can develop when tumour cells are able to escape immunosurveillance [129,130]. In addition, the efficacy of most chemotherapeutic and radiotherapeutic agents commonly employed in the clinic, critically depends on the activation or reactivation of tumour-targeting immune responses [131–133].

Tumour-infiltrating leukocyte subsets can play strikingly antagonistic functions. One of the key features of inflammation is the functional phenotype of macrophages that depend on the activating stimuli in their microenvironment. Macrophages are prototypical $O_2^{\cdot-}$, H_2O_2, and NO producing cells, and oxidants represent one of the most potent weapons of activated macrophages in the fight against cancer cells [134,135]. In addition, it is known that the increase of oxidant is associated with higher formation of lipid peroxidation products and, therefore, this could lead to a higher presence of lipoxidation adducts [136]. Moreover, it has been reported that macrophages, when stimulated, can produce HNE through COX-2 [124]. The inhibition of COX-2 in murine macrophages was associated with a decrease in HNE production following *E. faecalis* infection ($P < 0.001$). In the same study, using IL-10-knockout mice colonized by *E. faecalis,* it was observed increased levels of COX-2 expression in colonic macrophages in association with HNE-protein lipoxidation adducts [124].

Natural killer (NK) cells and $CD8^+$ cytotoxic T lymphocytes (CTLs) provide highly complementary anti-tumour strategies. Oxidants have a dual role in the regulation of CTLs and NK cell function. It has been observed that the most potent caspase inhibitor, X-linked inhibitor of apoptosis protein (XIAP), confers resistance to antibody-dependent cellular cytotoxicity (ADCC). Thus, XIAP is a critical modulator of ADCC responsiveness [137]. In this sense, strategies have been proposed to reduce the oxidative stress to enhance the ability of CTLs to kill tumour cells. However, activated CTLs may partly adapt to the oxidative stress in the tumour microenvironment by upregulating antioxidant proteins as demonstrated with IL-2-activated NK cells [138] and as was described above.

On the other hand, Th17 cells have been associated with poor prognosis in some type of cancers and its pro-tumour functions have been tightly linked to angiogenesis and promotion of tumour vascularization. Nevertheless, the role of Th17 cells is much more controversial due to its association with better overall survival in ovarian cancer and in esophageal squamous cell carcinoma [10]. In this sense, lipid peroxidation products may also have an influence since it has been reported that aldehydes, such as MDA, transcriptionally upregulate the expression of IL-17E in lymphocytes and alter lymphocyte differentiation towards the pathogenic Th17 subset [68]. Finally, $Foxp3^+$ regulatory T (Treg) cells accumulation in the tumour microenvironment is considered a bad prognosis factor [10]. This population can also be influenced by lipoxidation effects, as it was observed in atherosclerotic lesions of a mice model, in which there was an inhibition in the generation of Treg cells induced by MDA-laminin adduct [126].

In sum, the modulation of immune components in the tumour microenvironment has a very relevant effect over the development of tumours as well as over the type of patient's response to a specific treatment, and lipoxidation products may have a very important role in this modulation. In this regard, the combination of conventional therapeutics with ROS modulators may increase specific tumour cytotoxicity.

3.4. Molecular targets and signalling properties of lipoxidation

Lipoxidation adducts may alter progressively the structure and function of circulating and tissular proteins, with consequences on the inflammatory status, cell proliferation and viability, thus influencing cancer development [5]. Studies of proteins modified by reactive aldehydes indicated hundreds of molecular targets [8,139,140], therefore, we will highlight in this section targeted protein involved in cell proliferation, apoptosis, and some protein kinases.

3.4.1. Modification of tyrosine kinase receptors

It has been previously reported that HNE present in oxLDLs or exogenously added induces both modification and dysfunction of tyrosine kinase receptors (TKRs), such as epidermal growth factor receptor (EGFR) and platelet-derived growth factor receptor (PDGFR), involving lipoxidation adducts, which triggers TKR autophosphorylation and the activation of the downstream signalling pathways, extracellular signal-regulated kinase (ERK)1/2 phosphorylation and cell cycle progression [141,142]. However, high concentrations of HNE inhibit cell proliferation mediated by EGFR and PDGFR involving the formation of HNE and ACR adducts with PDGFRβ [64,143]. Thus, it has been suggested that HNE and others electrophilic lipids may potentially disturb PDGFR-mediated responses such as proliferation and cell migration [144].

3.4.2. Apoptosis signalling and other protein kinases

In human myeloid HL-60 cells, HNE adducts were shown to be correlated with the induction of apoptosis, the activation of c-Jun N-terminal kinase (JNK) and caspase 3, and they have been associated with the activation of caspases 3, 8, and 9 in embryonic fibroblasts isolated from mice [145,146]. Moreover, HNE induce the expression of antioxidant genes such as heme-oxygenase and thioredoxine-1 via the activation of the mitogen-activated protein kinase (MAPK) pathway and the transcription factor Nrf2 [147,148]. Thioredoxin and thioredoxin reductase are involved in the maintenance of various proteins in a reduced state required for their normal function, and they are also targets of lipoxidation by 15d-PGJ_2, what results in their inactivation [149]. Modified thioredoxin reductase may mediate the conformational disruption of p53 and PG-induced apoptosis via activation of caspase 3 [150]. Moreover, in Jurkat cells, it was reported that both Fas and Daxx proteins are targets of lipoxidation by HNE. Fas adducts promote proapoptotic signalling via ASK1, JNK, and caspase 3. While Daxx lipoxidation promotes its export from the nucleus to the cytosol, where it interacts with Fas to self-limit the extent of apoptosis by inhibiting the downstream proapoptotic signalling [151]. In addition, the proapoptotic protein BAX is a direct target of lipoxidation by PGA2, triggering a conformational change that leads to BAX activation and induction of apoptosis [152]. Different studies reported the direct modification and inactivation of the phosphoinositide-3-phosphatase and tumour suppressor PTEN by several reactive aldehydes and ketones, such as ACR, HNE and α,β-enones such as PGA2, Δ12-PGJ2 and 15d-PGJ_2, with ensuing activation of PKB/Akt kinase, phosphorylation of Akt substrates, increased cell proliferation, and increased nuclear β-catenin signalling [153–155]. This combined and sustained inactivation of tumour suppressors could contribute significantly to inflammation-associated tumorigenesis [153]. Additionally, it has been observed that cyPG and cyclopentenone isoprostanes target the oncogenic H-Ras proteins. Whereas 15d-PGJ_2 and Δ12-PGJ_2 preferentially target the C-terminal region, PGA1 and 8-iso-PGA1 bind mainly to cysteine 118, located in the GTP-binding motif what has been correlated with H-Ras activation [156]. In human hepatic stellate cells, the p46 and p54 isoforms of JNKs were identified as HNE targets and were activated by this aldehyde. This leads to JNK nuclear translocation as well as to c-jun and AP-1 induction [157]. Furthermore, it has been shown that 15d-PGJ_2 can covalently modify c-Jun at cysteine 269, which is located in the c-Jun DNA binding domain, and directly inhibit the DNA binding activity of AP-1, both *in vitro* and in intact cells [59,158].

4. Concluding remarks and future perspectives

Many of the previously described studies provide emerging molecular evidence of the importance of lipoxidation in carcinogenesis, where inflammation represents one of the fundamental links. There is a great complexity in the possible roles of lipoxidation products in cancer pathology. It has been reported contradictory results in which lipoxidation products seem to be toxic for tumour cells [159] but also, other studies report an association with the increase of the level of malignancy in tumours [31]. Therefore, lipoxidation products can have a crucial role not only in carcinogenesis but also in the host defence against cancer, through their effects in tumour cells and through their interactions with immune components.

Future studies will be necessary to distinguish physiologic and pathologic roles of lipoxidation processes occurring during carcinogenesis, with particular attention to the pro-oxidant anticancer agents and the drug-resistant mechanisms, which could be modulated to obtain a better response to cancer therapy [34].

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Conflicts of interest

The authors have no competing financial interests to declare.

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CHAPTER 3. FUNCTIONAL AND PHENOTYPIC CHARACTERIZATION OF TUMOR-INFILTRATING LEUKOCYTE SUBSETS AND THEIR CONTRIBUTION TO THE PATHOGENESIS OF HEPATOCELLULAR CARCINOMA AND CHOLANGIOCARCINOMA

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Functional and Phenotypic Characterization of Tumor-Infiltrating Leukocyte Subsets and Their Contribution to the Pathogenesis of Hepatocellular Carcinoma and Cholangiocarcinoma

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Abstract

Hepatocellular carcinoma (HCC) and cholangiocarcinoma (CCA) represent the most common primary liver malignancies whose outcome is influenced by the immune response. In the present study, we evaluated the tumor-infiltrating leukocyte (TIL) populations in 21 HCC patients and 8 CCA patients by flow cytometry immediately after the surgical procedure. Moreover, $CD4^+$ T cells, $CD8^+$ T cells, monocytes, and macrophages were purified by cell sorting for further analysis of gene expression by quantitative reverse-transcription polymerase chain reaction. Regarding tumor-infiltrating macrophages, we observed a significantly higher expression of markers associated with M2 phenotype and a higher expression of PD-L1 in patients with HCC in comparison to CCA. In addition, for HCC patients, we found a significant increase in the expression of CD200R in macrophages from tumors that were in grade G3-G4 as compared to tumors in grade G1-G2. Besides, a significantly higher frequency of tumor-infiltrating

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lymphocytes, $CD8^+CD56^+$ T cells, and natural killer cells was detected in HCC biopsies in comparison to CCA. In summary, this study has revealed functional and phenotypic differences in TIL cell subpopulations between CCA and HCC, as well as among different histopathological grades and tumor aggressiveness degrees, and it has provided evidence to better understand the tumor immune microenvironment of CCA and HCC.

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Introduction

Hepatocellular carcinoma (HCC) is the most frequent type of liver cancer and presents high morbidity and mortality rates [1,2]. It has a poor prognosis, generally due to its late presentation and, thus, late diagnostic. Cholangiocarcinoma (CCA), a malignancy that originates from biliary epithelia, is an aggressive cancer with high mortality rates [3] and, along with HCC, represents a major primary liver cancer. CCA is difficult to diagnose due to its silent and nonspecific clinical features, and in most cases, the symptoms occur when the tumor has reached an advanced stage [4]. For patients with advanced disease, current systemic treatment options provide only limited therapeutic benefit for a subset of patients, and novel therapeutic options to treat these carcinomas are needed [5]. The tumor microenvironment plays a vital role in tumor epigenetics, tumor differentiation, immune escape, and metastasis [6]. Accordingly, an increasing body of evidence supports the utility of immunotherapy as antitumor treatment, including active vaccination, adoptive cell transfer therapy, and immune checkpoint blockade [7]. There are many clinical trials to assess these therapies, and the results have demonstrated a definite clinical application value [8]. Therefore, an evaluation of the tumor microenvironment may facilitate and prioritize development of new immunotherapy strategies for HCC and CCA patients. Previous clinical studies have found that the composition of tumor-infiltrating leukocytes (TILs) may correlate with prognosis in cancer patients [9–11]. In HCC, most studies indicate that high levels of $CD8^+$ and $CD3^+$ TILs have a better prognostic value on overall survival, while high levels of $FOXP3^+$ TILs have been associated with a worse prognosis [7]. In addition, high presence of $CD206^+$ tumor-associated macrophages (TAMs) was markedly correlated with aggressive tumor phenotypes, such as multiple tumor number and advanced TNM stage, as well as being associated with poor overall survival in HCC patients [12]. In the same way, low *versus* high $CD206^+$ TAMs density has been associated with better overall survival in HCC, suggesting that the characterization of $CD206^+$ tumor-infiltrating macrophages could be considered to improve the risk stratification system. In CCA, high tumor-associated neutrophils, low $CD8^+$ T cell, and high Treg populations, as well as the enrichment of $CD163^+$ TAMs, have been correlated with poor prognosis and have been associated with worse overall survival in human extrahepatic CCA patients with surgical resection [13]. Moreover, high levels of TAMs in the tumor invasive front or absence of histologic tumor necrosis has been associated with a significantly improved recurrence-free and overall survival of patients with intrahepatic CCA [14].

In this study, we have functional and phenotypically characterized, by flow cytometry and by quantitative reverse-transcription polymerase chain reaction (qRT-PCR) expression analysis, the TIL populations of a group of patients with HCC and CCA immediately after surgical resection, determining its association with tumor grades and aggressiveness, in order to detect prognosis biomarkers as well as identify new molecular targets for the development of new immunotherapy strategies in neoadjuvant, adjuvant, or palliative setting.

Materials and Methods

Patients

Twenty-one patients with HCC (3 women and 18 men; average age: 62.2 ± 14.5 years) and 8 patients with CCA (5 women and 3 men; average age: 61.0 ± 14.7 years) were included in this study. The clinical background of the patients included in this study is summarized in Table 1. Patients were classified according to the eighth TNM classification, and tumors were categorized depending on their histopathological grading. Moreover, for purposes of classification, HCC patients were dichotomized into low risk and high risk, taking into consideration the pathological features described in the literature as predictors of worst outcome and poor prognosis. Tumors were considered of high risk if one or more of the following features were present: microvascular invasion [15]; stage III or superior in the American Joint Committee for Cancer Classification [16]; tumor grade equal or superior than G3, as defined by Edmonson [17,18]; and cytokeratin 19 expression [19].

Samples were collected immediately after surgical procedure, and no patients received any antitumor therapy or medication prior to surgery. Cells from biopsies were mechanically extracted from tumors

Table 1. Clinical Data from HCC and CCA Patients Enrolled in This Study

		CCA ($n = 8$)	HCC ($n = 21$)
Age		61 ± 15	62 ± 15
Sex	Female	5 (63%)	3 (14%)
	Male	3 (38%)	18 (86%)
TNM	Stage I	1 (13%)	3 (14%)
	Stage II	4 (50%)	16 (76%)
	Stage IIIA	0 (0%)	1 (5%)
	Stage IV	3 (38%)	1 (5%)
Histologic grade (G)	G1	2 (25%)	2 (9%)
	G2	3 (38%)	12 (57%)
	G3	3 (38%)	6 (29%)
	G4	0 (0%)	1 (5%)
Risk stratification	Low	2 (25%)	10 (48%)
	High	6 (75%)	11 (52%)
HBsAg	Positive	0 (0%)	1 (5%)
HCV	Positive	0 (0%)	6 (29%)
Cirrhosis		0 (0%)	16 (76%)
Relapse		3 (38%)	1 (5%)
Death		3 (38%)	2 (9%)
Liver transplant		0 (0%)	7 (33%)

Number (*N*) and percentage (%) of cases are indicated.

using syringes and needles in phosphate-buffered saline (PBS; Gibco, Life Technologies, Carlsbad, CA) and immediately processed.

Study Approval

The experimental protocols were approved by the Ethical Committee of the Faculty of Medicine, University of Coimbra, Coimbra, Portugal (CE-136/2016). All procedures performed involving human participants were in accordance with the ethical standards of Ethical Committee of the Faculty of Medicine, University of Coimbra, Coimbra, Portugal (CE-136/2016), and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. All participants gave their signed informed consent before entering in the study.

Immunophenotypic Study of Tumor Cells and Tumor-Infiltrating Leukocytes

Cells from the biopsies were mechanically extracted in PBS and aliquoted in different tubes for immunophenotyping.

For the immunophenotypic study of tumor cells, monocytes, and macrophages, 250 µl of biopsy sample was stained with the monoclonal antibodies (mAbs) described in Supplementary Table S1 (tube 1) and incubated for 10 minutes at room temperature (RT) in the dark. After this incubation period, samples were incubated with 2 ml of FACS Lysing solution [Becton Dickinson Biosciences (BD), San Jose, CA] for 10 min in the dark at RT and then centrifuged for 5 minutes at 540*g*. The supernatant was discarded, and the cell pellet was washed twice in 1 ml of PBS, resuspended in 0.5 ml of PBS, and immediately acquired.

Macrophages and monocytes were identified and distinguished based on CD14 and CD45 positivity and their typical forward scatter (FSC) and side scatter (SSC) light dispersion properties, whereas tumor cells were identified by the absence of expression of CD45, the expression of cytokeratin 18 and specific FSC and SSC light dispersion properties (Figure 1*A*). The expression of membranous markers such as CD200R, CD80, CD206, CD163, and PD-L1 (CD274) on monocytes and macrophages infiltrating the tumor was assed. Moreover, the expression of membranous PD-L1 (CD274) on tumor cells was also assessed by flow cytometry.

To evaluate cytokine production by T cells infiltrating the tumor, lymphocytes were stimulated by adding phorbol myristate acetate (PMA; 0.25 µg/ml, Sigma-Aldrich, Saint Louis, MO) and ionomycin (1 µg/ml, Boehringer Mannheim, Germany) to 250 µl of sample previously diluted 1:1 (v/v) in RPMI 1640 complete culture medium (Invitrogen, Life Technologies, Carlsbad, CA). Brefeldin-A (10 µg/ml, Sigma-Aldrich) was added to prevent the release of the newly synthesized cytokines. All samples were then incubated in a sterile environment and 5% CO_2 humid atmosphere, at 37°C, for 4 hours.

Immunophenotypic analysis of tumor-infiltrating T cells and natural killer (NK), cultured in the presence of PMA plus ionomycin, was performed by using a seven-color mAb combination, detailed in Supplementary Table S1 (tube 2). In short, cells were stained with the mAbs for surface proteins antigens (CD45, CD4, CD56, CD3, and CD8) and, after an incubation period of 10 minutes in the dark at RT, washed with PBS. For intracellular staining, Fix&Perm (Caltag, Hamburg, Germany) reagent was used in accordance with the instructions of the manufacturer, and samples were stained with the mAbs for IFN-γ and IL-17 (Supplementary Table S1, tube 2). After washing twice with PBS, the cell pellet was resuspended in 500 µl of PBS and immediately acquired.

T cells were identified on the basis of CD3 and CD45 positivity and intermediate FSC and SSC properties. Within this cell population, $CD4^+$ and $CD8^+$ T-cell subsets (phenotypically

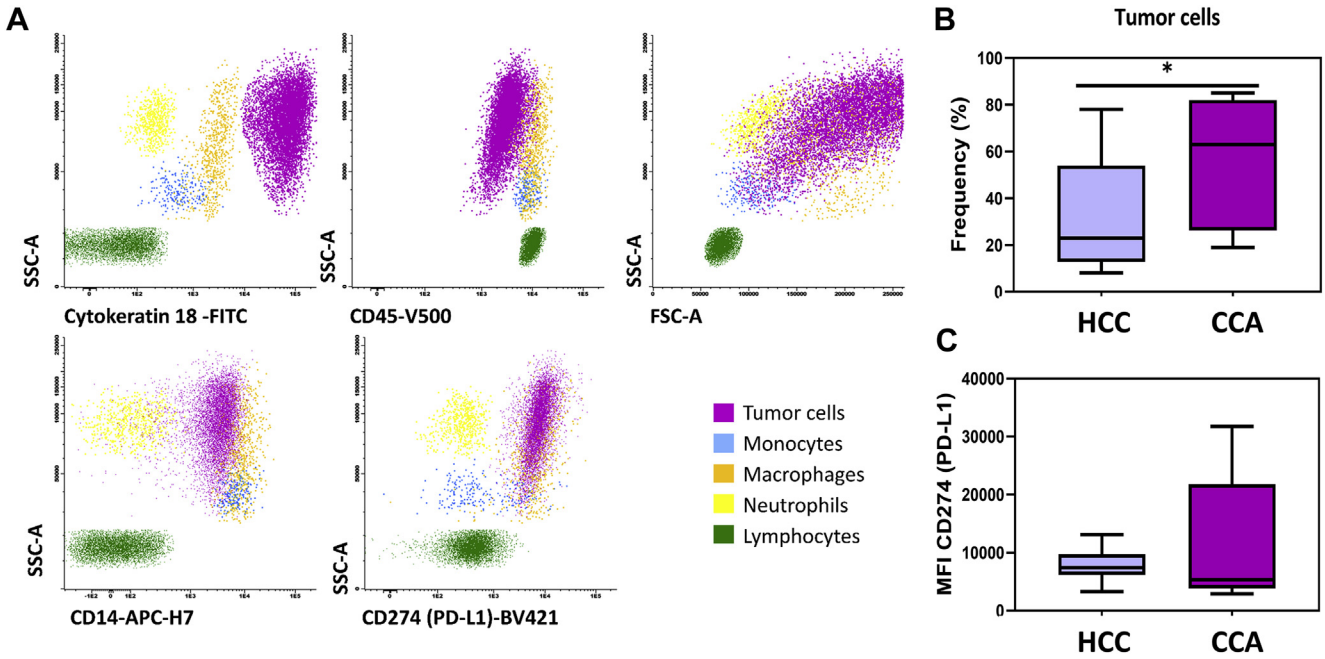

Figure 1. Phenotypic characterization of tumor biopsies from HCC and CCA patients. (A) Bivariate dot plot histograms illustrating the identification of tumor cells (purple events), monocytes (blue events), macrophages (orange events), neutrophils (yellow events), and lymphocytes (green events) infiltrating tumor biopsies. (B) Boxplot with the frequency of tumor cells identified in each group of patients (HCC and CCA) and (C) boxplot with the expression levels of CD274 (PD-L1) in tumor cells, measured as MFI. The results are given by median with interquartile range. Statistical significant differences were considered when $P < .05$; *between the groups indicated in the figure.

Table 2. Frequency (%) of Tumor-Infiltrating Leukocyte Subsets in HCC and CCA BIOPSIES, Classified in Different Tumor Grades and Risk Stratification Categories

%			Monocytes	Macrophages	Lymphocytes	Neutrophils
			Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD
	CCA (global mean)	($n = 8$)	**1.44 ± 1.29**	**2.07 ± 1.83**	**7.7 ± 5.6**[a]	**14.8 ± 16.7**
CCA	Risk stratification	Low	0.40 ± 0.42	2.25 ± 2.90	8.0 ± 8.5	6.8 ± 2.5
		High	1.97 ± 1.28	1.98 ± 1.65	7.5 ± 5.4	18.9 ± 20.0
	Grades	G1-G2	1.17 ± 1.16	2.08 ± 1.81	5.8 ± 5.5	19.8 ± 18.8
		G3-G4	2.00 ± 1.84	2.05 ± 2.62	11.4 ± 5.2	4.9 ± 5.9
	HCC (global mean)	($n = 21$)	**2.56 ± 3.16**	**1.24 ± 1.23**	**33.1 ± 20.8**[a]	**7.2 ± 5.7**
HCC	Risk stratification	Low	3.52 ± 4.16	1.36 ± 1.36	30.4 ± 21.9	5.5 ± 4.4
		High	1.49 ± 0.67	1.08 ± 1.13	36.1 ± 20.7	8.9 ± 6.6
	Grades	G1-G2	2.75 ± 3.74	1.08 ± 1.24	28.7 ± 19.9	7.4 ± 6.6
		G3-G4	2.10 ± 0.94	1.83 ± 1.22	43.6 ± 21.2	6.7 ± 3.4

Independent-samples Mann-Whitney *U* test and Kruskal-Wallis multiple comparison tests were performed with a significance level of .05 ($P < .05$). The results are given as mean ± SD. Global mean indicates mean values in CCA and HCC groups without discrimination among tumor grades or stages.

[a] CCA vs. HCC.

characterized as $CD3^+CD4^+CD8^-$ and $CD3^+CD4^-CD8^+$, respectively) were identified. NK cells were identified on the basis of CD45 and CD56 positivity, absence of CD3 expression, and intermediate FSC and SSC properties.

Flow Cytometry Data Acquisition and Analysis

Data acquisition was performed in a FACSCanto II flow cytometer (BD) and analyzed with Infinicyt 1.8 software (Cytognos SL, Salamanca, Spain).

Cell Purification by Fluorescence-Activated Cell Sorting

Macrophages, monocytes, and $CD4^+$ and $CD8^+$ T-cell populations from the biopsies were purified by fluorescence-activated cell sorting (FACS) using a FACSAria III flow cytometer (BD) according to their typical phenotype. Thus, the seven-color mAb combination used (Supplementary Table S1, tube 3) allowed the identification of $CD4^+$ T cells ($CD3^+CD4^+CD8^-$) and $CD8^+$ T cells ($CD3^+CD4^-CD8^+$). The remaining mAbs found in the panel were used to identify macrophages and monocytes ($CD14^+$ $CD45^+CD33^+$). Cell suspensions were centrifuged for 5 minutes at 300*g*, and the pellet was resuspended in 350 μl of RLT Lysis Buffer (Qiagen, Hilden, Germany).

The purified cell populations were subsequently stored at −80°C for the quantification of mRNA expression.

Evaluation of mRNA Expression by qRT-PCR

Total RNA was extracted and purified with RNeasy Micro Kit (Qiagen) according to the manufacturer's instructions. Reverse transcription was performed with SensiScript Reverse Transcription Kit (Qiagen) according to supplier's instructions and with Random Hexamer Primer (Thermo Fisher Scientific, San Jose, CA). Relative quantification of gene expression was performed in a QuantStudio (Thermo Fisher Scientific) by a real-time qRT-PCR. qRT-PCR was done with PowerUp SYBR Green Master Mix (Thermo Fisher Scientific) using optimized primers for IFN-γ, IL-17, FOXP3, IL-10, TNFα, and endogenous control glyceraldehyde 3-phosphate dehydrogenase (Qiagen) according to the manufacturer's instructions.

Statistical Analysis

For all variables under study, their mean values, standard deviation, median, and range were calculated. To determine the statistical significance of the differences observed between groups, the nonparametric Mann-Whitney comparison test was performed using the Statistical Package for Social Sciences software (SPSS, version 19, IBM, Armonk, NY). Statistically significant differences were considered when $P < .05$. The Spearman rank test was used to evaluate the correlation among variables.

Results

Phenotypic Characterization of Biopsies' Populations

From the data obtained by flow cytometry immunophenotyping, we observed a significantly higher frequency of tumor cells (cytokeratin18$^+$ CD45$^-$) in the analyzed biopsies from CCA patients in comparison to HCC (Figure 1*B*). In both groups of cancer patients, tumor cells were positive for PD-L1 (CD274) and presented no significant differences among PD-L1 protein expression [measured as mean fluorescence intensity (MFI)] (Figure 1*C*).

Regarding tumor-infiltrating major leukocyte subsets, we observed a higher frequency of lymphocytes infiltrating HCC tumors in comparison to CCA (global mean), while no significant differences were observed among the different risk stratification categories or tumor grades either in HCC or in CCA (Table 2).

Immunophenotyping of Tumor-Infiltrating Lymphocytes

As shown in Table 3, the frequency of NK cells infiltrating HCC tumors was significantly higher compared to the frequency of NK-infiltrating CCA tumor biopsies. Moreover, we observed that the frequency of $CD8^+$ T cells with NK activity ($CD56^+$) infiltrating HCC tumor biopsies was significantly higher in comparison to CCA (Table 3). In addition, the expression of IL-17 by $CD8^+$ T cells, measured as MFI, was significantly higher in HCC tumors as compared to CCA. However, no significant differences were observed among the different risk stratification categories or tumor grades for tumor-infiltrating lymphocyte subsets in HCC patients (Table 4). Due to the small number of cases presenting CCA, we did not subdivide this group of patients into other categories.

Additionally, $CD4^+$ T cells infiltrating tumor biopsies presented higher expression levels of IFNγ mRNA, measured by qRT-PCR, in comparison with FOXP3 and IL-17 expression levels. Moreover, in HCC patients, a nonsignificant decrease was observed in the expression levels of IFN-γ mRNA, expressed by both $CD4^+$ and $CD8^+$ T cells infiltrating tumor biopsies, in comparison to CCA infiltrating $CD4^+$ and $CD8^+$ T cells (Figure 2).

Immunophenotyping of Tumor-Infiltrating Monocytes and Macrophages

Macrophages and monocytes were identified and distinguished based on CD14 and CD45 positivity and their typical FSC and SSC

Table 3. Frequency (%) of Tumor-Infiltrating Lymphocyte Subsets in HCC and CCA Biopsies

		CCA $n = 8$	HCC $n = 21$	Statistical Significance
		Mean ± SD	Mean ± SD	*P* Value
%LyT		10.8 ± 8.7	16.7 ± 12.7	.353
	% LyT CD4	49 ± 11	57 ± 20	.312
	% IFNγ	46.3 ± 22.3	60.9 ± 26.8	.282
	MFI IFNγ	6067 ± 1883	8999 ± 4937	.179
	% IL17	3.08 ± 1.81	2.47 ± 1.62	.521
	MFI IL17	283 ± 84	387 ± 150	.152
	%IFNγ$^+$IL17$^+$	1.42 ± 0.86	1.53 ± 0.80	.750
	% LyT CD8	36 ± 11	26 ± 13	.207
	% IFNγ	62 ± 23	66 ± 25	.701
	MFI IFNγ	3914 ± 1355	4706 ± 2151	.639
	% IL17	1.13 ± 1.63	1.06 ± 1.24	.831
	MFI IL17	223 ± 42	355 ± 123	**.045**
	%CD56$^+$	8.5 ± 6.6	21.7 ± 14.2	**.041**
	%IFNγ$^+$IL17$^+$	0.36 ± 0.38	1.01 ± 1.07	.313
% NK		0.60 ± 0.37	2.79 ± 3.82	**.041**
	% IFNγ	41 ± 15	50 ± 25	.579
	MFI IFNγ	2049 ± 679	2456 ± 866	.282
	% CD8$^+$	5.2 ± 5.1	2.6 ± 2.6	.494

Protein expression levels of IFN-γ and IL-17 (measured as MFI) are also indicated. Independent-samples Mann-Whitney *U* test was performed with a significance level of .05 ($P < .05$). The results are given as mean ± SD.

light dispersion properties, as indicated in Figure 3*A*. After characterization of the populations of interest, we observed a significantly higher expression of CD206 and CD163 in HCC-infiltrating macrophages in comparison to CCA (Figure 3*B*). Additionally, a nonsignificant higher expression of PD-L1 (CD274), CD200R, and CD80 markers in HCC-infiltrating macrophages in comparison to CCA was observed (Figure 3*B*), while no significant differences were found for tumor-infiltrating monocytes among cancer patient groups. After the purification of macrophages and monocytes by cell sorting, we measured the mRNA levels of IL-10 and TNFα by qRT-PCR. The expression levels of IL-10 mRNA among tumor-infiltrating macrophages/monocytes were higher in the HCC group when compared to the CCA group, without reaching statistical significance (Figure 3*C*). On the other hand, no differences were observed on TNFα mRNA expression among macrophages/monocytes infiltrating CCA or HCC tumor biopsies (Figure 3*C*).

For HCC patients, we observed higher expression levels (MFI) of markers such as PD-L1 (CD274), CD200R, CD80, CD163, and CD206 in macrophages infiltrating tumors that were in grade G3 or G4 ($n = 7$) in comparison with tumors that were in grade G1 or G2 ($n = 14$) (Figure 4*A*). In fact, a significant increase in the expression levels of CD200R (MFI) in macrophages from tumors that were in grade G3 or G4, in comparison to tumors that were in grade G1 or G2, was observed (Figure 4*A*). In the same line, an increase of CD163 and CD80 expression (MFI) in both monocytes and macrophages infiltrating tumors that were in grade G3 or G4 in comparison to tumors that were in grade G1 or G2 was detected, although without reaching statistical significance (Figure 4*A*). When comparing tumors classified into different risk stratification, we observed significantly higher expression levels of PD-L1 (CD274) by macrophages infiltrating tumors that were considered with a high degree of aggressiveness in comparison to macrophages infiltrating tumors classified as low risk (Figure 4*A*). However, a nonsignificant decrease was detected in the expression of CD206 by tumor-infiltrating macrophages in high-risk tumors as compared to low risk, and no differences were observed on the other markers when comparing MFIs.

Furthermore, we observed a higher IL-10 mRNA expression among macrophages/monocytes infiltrating tumors that were in grade G3 or G4 in comparison to tumors that were in G1 or G2 in HCC patients, and minor differences in the case of TNFα mRNA expression, which was higher in macrophages infiltrating HCC tumors that were in grade G3 or G4 in comparison to tumors in G1 or G2 (Figure 4*B*). Regarding the dichotomized classification into low risk and high risk based on the aggressiveness of the tumor, we observed a significant increase in IL-10 mRNA expression among

Table 4. Frequency (%) of Tumor-Infiltrating Lymphocyte Subsets in HCC Biopsies, Classified into Tumors That Were in Grade G1 or G2 (G1-G2) and Tumors That Were in Grade G3 or G4 (G3-G4), and Dichotomized into Low Risk and High Risk.

			Hepatocellular carcinoma					
			Histologic grade			Stratification risk		
			G1-G2	G3-G4	p-value	Low	High	p-value
			Mean ± SD	Mean ± SD	Sig.	Mean ± SD	Mean ± SD	Sig.
%LyT			14.8 ± 12.6	20.0 ± 13.6	0.3710	18 ± 14	25 ± 19	0.6889
	% LyT CD4		52 ± 20	66 ± 19	0.1645	45 ± 22	60 ± 18	0.2721
		% IFNγ	58.2 ± 28.6	65.2 ± 26.1	0.6064	55 ± 17	50 ± 37	0.8981
		MFI IFNγ	9306 ± 5885	8507 ± 3466	0.6993	9535 ± 5151	7676 ± 4155	0.6993
		% IL17	1.85 ± 1.12	3.45 ± 1.93	1	2.86 ± 3.01	1.31 ± 1.63	0.2977
		MFI IL17	376 ± 118	404 ± 206	0.3543	367 ± 98	439 ± 180	0.5237
		%IFNγ$^+$IL17$^+$	1.35 ± 0.75	1.78 ± 0.88	0.5237	1.89 ± 2.42	1.00 ± 1.27	0.5035
	% LyT CD8		28 ± 12	22 ± 16	0.8591	25 ± 14	25 ± 16	0.9546
		% IFNγ	63 ± 29	71 ± 19	0.7972	58 ± 18	54 ± 32	0.7972
		MFI IFNγ	5081 ± 2561	4105 ± 1292	0.6993	5233 ± 2401	3926 ± 1366	0.2977
		% IL17	0.99 ± 1.39	1.17 ± 1.09	0.8329	1.00 ± 1.13	0.56 ± 1.03	0.3543
		MFI IL17	325 ± 81	405 ± 184	0.5476	338 ± 67	433 ± 184	0.7143
		%IFNγ$^+$IL17$^+$	1.07 ± 1.28	0.93 ± 0.81	0.4762	0.70 ± 0.68	0.54 ± 0.83	0.5167
		%CD56$^+$	23.5 ± 12.2	18.5 ± 18.4	0.6787	22 ± 9	22 ± 23	0.5287
% NK			3.72 ± 4.54	1.12 ± 0.76	0.2544	2.83 ± 3.61	2.82 ± 4.51	0.6070
		% IFNγ	52 ± 22	48 ± 32	1	54 ± 21	45 ± 35	0.8981
		MFI IFNγ	2586 ± 640	2247 ± 1203	0.3636	2699 ± 892	2265 ± 1186	0.4376
		% CD8$^+$	2.0 ± 1.5	3.7 ± 4.0	0.4396	6.2 ± 6.1	4.7 ± 7.5	0.3880

Protein expression levels of IFN-γ and IL-17 (measured as MFI) are also indicated. Independent-samples Mann-Whitney *U* test was performed to compare G1-G2 vs. G3-G4 and low- vs. high-classification groups with a significance level of .05 ($P < .05$). The results are given as mean ± SD.

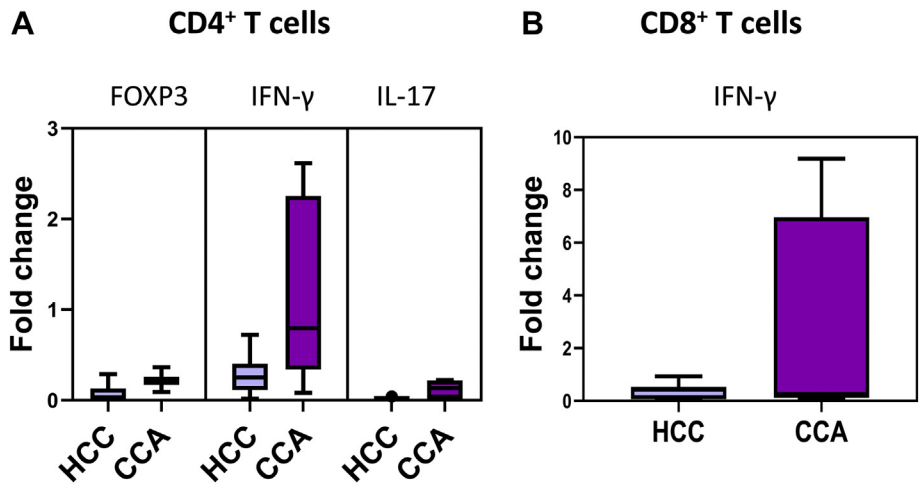

Figure 2. Functional characterization of tumor-infiltrating T lymphocytes. (A) Boxplots with the mRNA expression levels of FOXP3, IFNγ, and IL-17 by tumor-infiltrating $CD4^+$ T cells (purified by cell sorting) in HCC and CCA patients. (B) Boxplot with the mRNA expression levels of IFNγ by tumor-infiltrating $CD8^+$ T cells (purified by cell sorting) in HCC and CCA patients.

macrophages/monocytes infiltrating HCC tumors that were considered to have a high degree of aggressiveness as compared to those with a low degree of aggressiveness or low risk (Figure 4*B*). In contrast, regarding TNFα mRNA expression, we observed slightly decreased levels of expression of TNFα mRNA among macrophages/monocytes infiltrating HCC tumors classified as high risk or high degree of aggressiveness as compared to low risk (Figure 4*B*). Moreover, comparing HCC macrophages that presented amplification for IL-10 mRNA expression by qPCR ($IL\text{-}10^+$) and those who did not ($IL\text{-}10^-$), we observed elevated levels of expression of markers associated with protumor macrophages such as CD200R, CD206, and CD163, measured as MFI, in $IL\text{-}10^+$ macrophages in comparison to $IL\text{-}10^-$ macrophages (Figure 5*A*). In addition, we observed a positive correlation between IL-10 mRNA expression in macrophages and the expression of CD163 in macrophages infiltrating HCC tumors, assessed by Spearman's rank correlation (rho = 0.786), with $P = .001$ (Figure 5*B*). Moreover, a positive correlation among CD274 (PD-L1) expression (MFI) and CD200R expression (MFI) in HCC tumor-infiltrating macrophages was observed assessed by Spearman's rank correlation (rho = 0.615), with $P = .033$ (Figure 5*B*).

Discussion

To evaluate the potential of TIL subsets in the pathogenic progression of CCA and HCC, we examined the phenotype and clinical relevance of TIL subsets in tumor biopsies from CCA and HCC patients, collected immediately after surgical procedure and classified into different histopathological grades and aggressiveness degree (risk stratification), by multicolor flow cytometry and qRT-PCR. Despite the great variability detected among different individuals, we have observed different types of common patterns associated with the pathogenesis of these tumors.

Regarding tumor-infiltrating monocytes and macrophages in HCC, we have not detected differences in the total frequency of infiltrating monocytes and macrophages in tumors classified within different grades or risk stratification. However, after the phenotypic and functional characterization, interesting findings came out. In general, the phenotype of TAMs can be categorized into two subpopulations, and each of them has diverse effects on the tumor microenvironment: M1 macrophages, which have been associated with antitumor activity, and M2 macrophages, which exert protumor effects [20,21]. In our study, we have observed higher expression levels of markers associated to M2 phenotype (CD206 and CD163), together with a higher expression of PD-L1 (CD274) in macrophages infiltrating HCC tumors that were in grade G3 or G4 compared to the expression levels of macrophages infiltrating HCC tumors in grade G1 or G2. Moreover, taking into account the dichotomized classification into low risk and high risk based on the aggressiveness of the tumor, we have observed significantly higher expression levels of PD-L1 by macrophages infiltrating tumors that were considered to have a high degree of aggressiveness or high risk as compared to macrophages infiltrating tumors classified as low risk. Furthermore, we observed as a highlighted result that the marker CD200R was significantly increased in macrophages infiltrating HCC tumors in grade G3 or G4 compared to the expression levels of macrophages infiltrating HCC tumors in grade G1 or G2. In addition, we identified a positive correlation among CD274 (PD-L1) expression and CD200R expression, measured as MFI, in HCC tumor-infiltrating macrophages, assessed by Spearman's rank correlation. CD200 is a cell membrane protein that interacts with CD200 receptor (CD200R) of myeloid lineage cells. During tumor initiation and progression, CD200-positive tumor cells can interact with macrophages through CD200-CD200R complex, leading to different mechanism such as the inhibition of immune cells, the modulation of cytokine profiles from Th1 to Th2, the differentiation of T cells into regulatory T cells, and the facilitation of anti-inflammatory IL-10 and TGF-β production [22–24]. Our results are in line with previous studies which had already suggested a promising role of CD200R as a prognostic marker in predicting elevated recurrence and reduced survival, and a potential therapeutic target in treating HCC [23]. Regarding the functional characterization of HCC-infiltrating macrophages, macrophages infiltrating HCC tumors that were in grade G3 or G4 presented higher expression levels of IL-10 mRNA when compared to tumors in G1 or G2, without reaching statistical significance. Accordingly, in HCC tumors classified as high risk or high degree of aggressiveness, we observed a significantly higher expression of IL-10 mRNA by infiltrating macrophages when compared to macrophages infiltrating tumors classified as low risk or low degree of aggressiveness, supporting the idea that M2 macrophages possess an

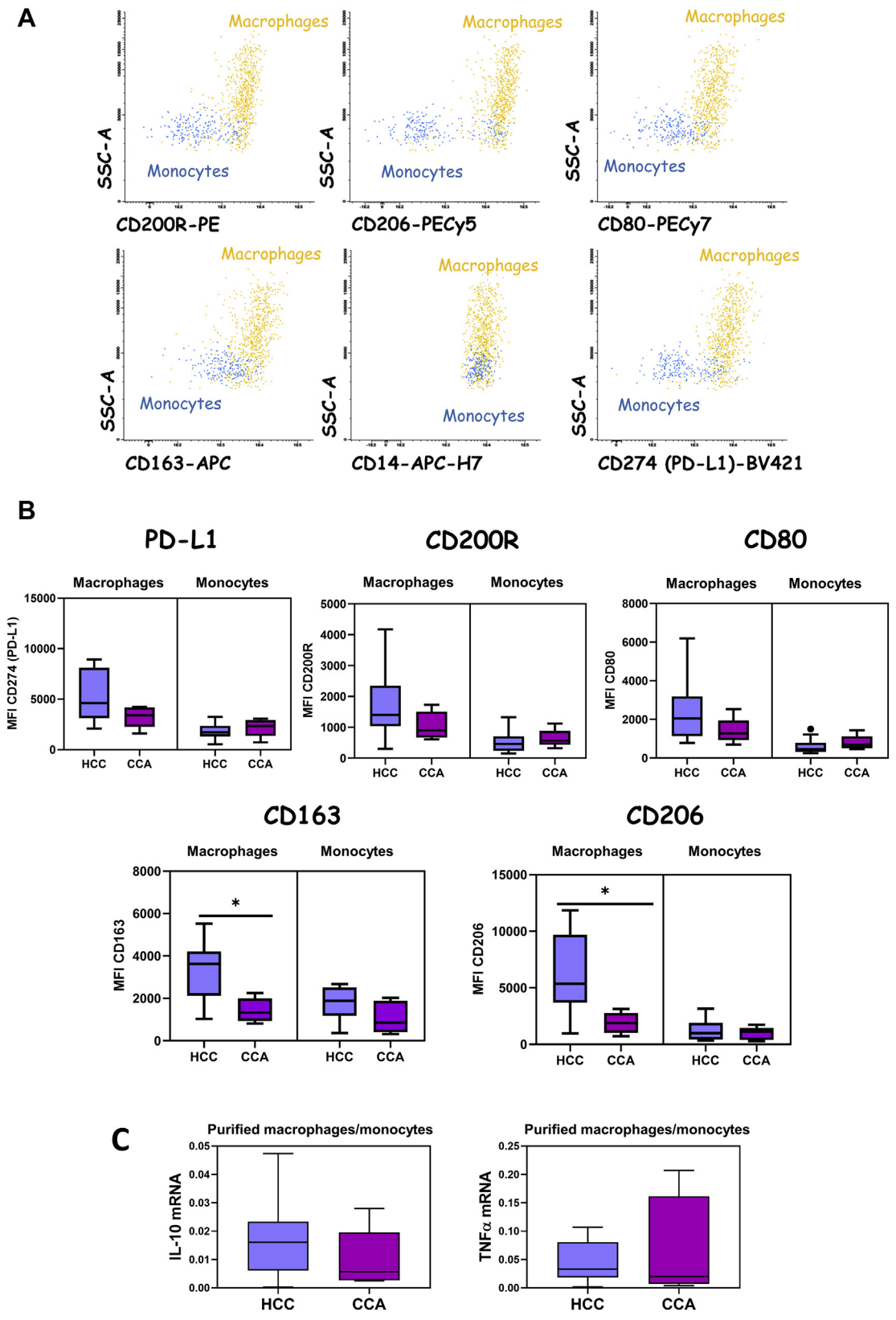

Figure 3. Phenotypic characterization of tumor-infiltrating monocytes and macrophages in HCC and CCA patients. (A) Bivariate dot plot histograms illustrating the phenotypic characterization of monocytes (blue events) and macrophages (orange events) infiltrating tumor biopsies. (B) Comparison among HCC and CCA tumor-infiltrating macrophages and monocytes expression levels of CD274 (PD-L1), CD200R, CD206, CD80, and CD163 markers, measured as MFI. The results are given by median with interquartile range. Statistically significant differences were considered when $P < .05$; *between the groups indicated in the figure. (C) Boxplots with the mRNA expression levels of IL-10 and TNFα by tumor-infiltrating macrophages (purified by cell sorting) in HCC and CCA. The results are given as median with interquartile range.

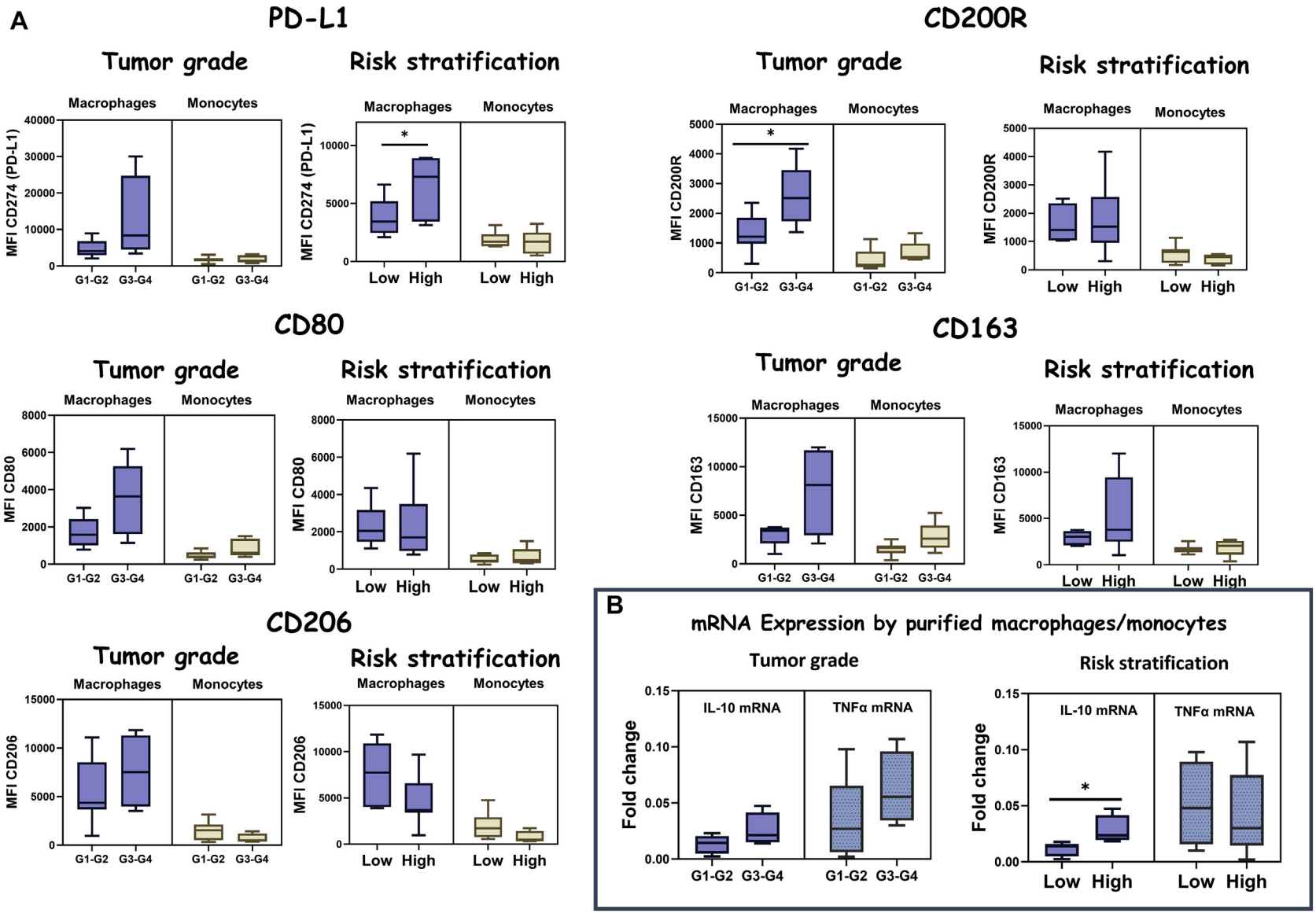

Figure 4. Phenotypic characterization of tumor-infiltrating monocytes and macrophages in HCC tumors. (A) Comparison of tumor-infiltrating macrophages and monocytes expression levels of CD274 (PD-L1), CD200R, CD80, CD206, and CD163 markers, measured as MFI, among HCC tumors that were in grade G3 or G4 and tumors that were in grade G1 or G2 (Tumor grade) and comparing tumors that were considered as low risk or low degree of aggressiveness (low) with tumors that were considered as high risk or high degree of aggressiveness (high) (risk stratification). (B, left) Boxplots with the mRNA expression levels of IL-10 and TNFα among purified macrophages/monocytes from HCC biopsies comparing tumors that were in grade G1 or G2 with tumors that were in grade G3 or G4. (Right) Boxplots with the mRNA expression levels of IL-10 and TNFα among purified macrophages/monocytes from HCC biopsies comparing tumors that were considered as low risk or low degree of aggressiveness (low) with tumors that were considered as high risk or high degree of aggressiveness (high).

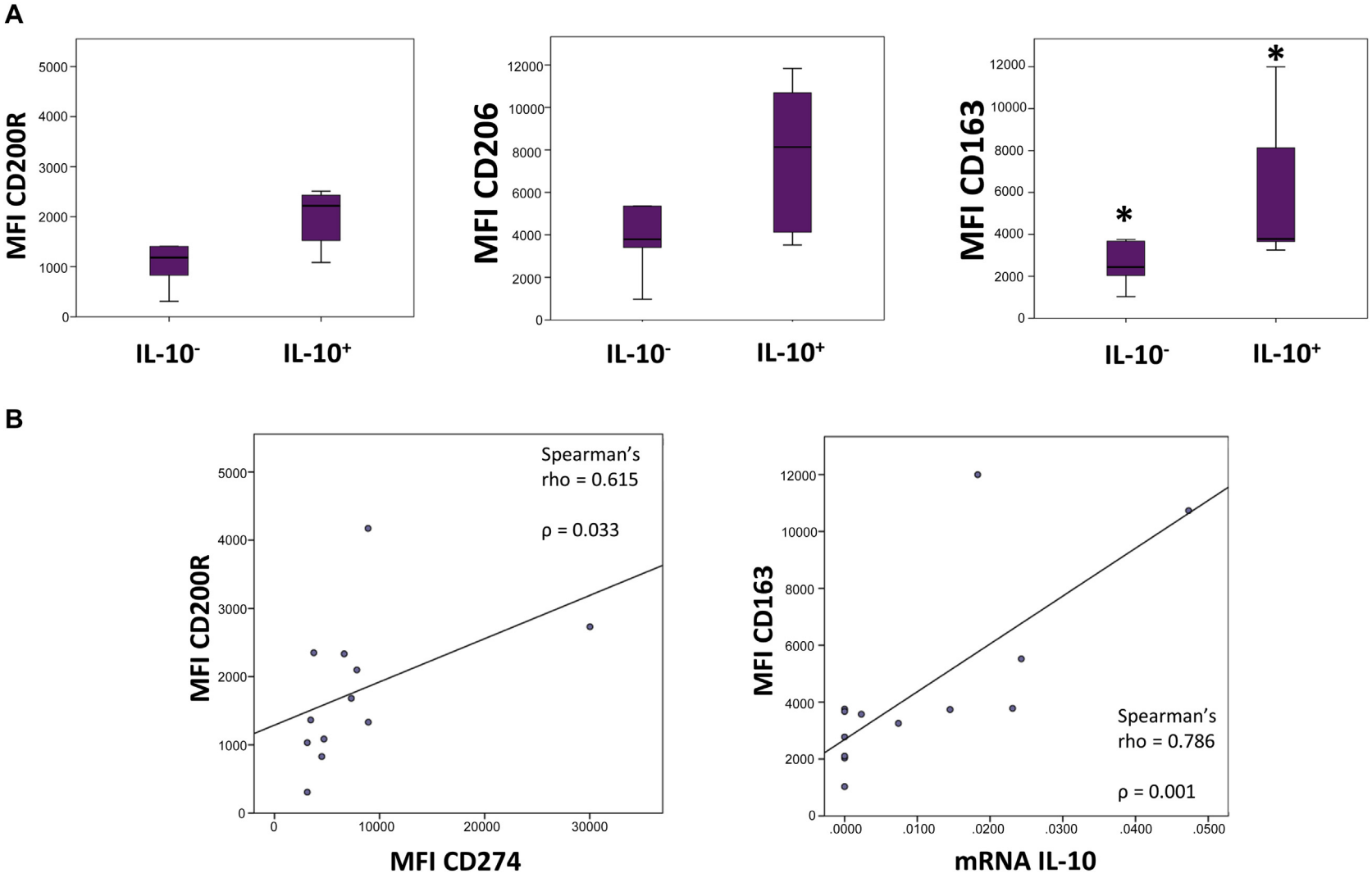

Figure 5. Tumor-infiltrating macrophages in HCC. (A) Expression levels of CD200R, CD206, and CD163 markers, measured as MFI, by HCC tumor-infiltrating macrophages that presented amplification for IL-10 mRNA expression by qRT-PCR (IL-10^{+}) in comparison to those that did not (IL-10^{-}). The results are given by median with interquartile range. (B, left) Positive correlation among CD274 (PD-L1) MFI and CD200R MFI in HCC tumor-infiltrating macrophages (rho = 0.615) as assessed by Spearman's rank correlation, with $P = .033$. (Right) Positive correlation between CD163 expression (MFI) and IL-10 mRNA expression by HCC tumor-infiltrating macrophages (rho = 0.786) as assessed by Spearman's rank correlation, with $P = .001$. Statistical significant differences were considered when $P < .05$; *between the groups indicated in the figure.

important role in advanced HCC tumors. Despite the fact that immunophenotyping did not allow a strict classification into M1 or M2 macrophages, we observed that HCC macrophages that presented amplification for IL-10 mRNA expression by qPCR ($IL\text{-}10^+$) exhibited a higher expression of markers associated with protumor macrophages such as CD200R, CD206, and CD163 when compared to those who did not present amplification for IL-10 mRNA ($IL\text{-}10^-$). Moreover, we found a positive correlation between CD163 protein expression, measured as MFI, and IL-10 mRNA expression for HCC-infiltrating macrophages. However, for HCC, TNFα expression seems to be increased in HCC tumors that were in grade G3 or G4 in comparison to tumors in grade G1 or G2. Therefore, our data suggest that TNFα expression levels cannot be used as an indicator of M1 phenotype. In fact, it has been previously reported that individual TAMs frequently coexpressed canonical markers of both M1 and M2 activation as IL-10 and TNFα [25]. Three distinctive HCC subtypes with immunocompetent, immunodeficient, and immunosuppressive features have been previously defined by multiomics approaches. Our results suggest that HCC tumors that were in grade G3 or G4 displayed a phenotype that could be classified into HCC subtype 3 (immunosuppressive subtype), characterized by normal lymphocyte infiltration, macrophages with higher expression levels of PD-L1, and increased frequencies of immunosuppressive cells [26]. In this type of scenario, immune checkpoint inhibitors therapy may be a reasonable approach for patients under this classification.

Regarding tumor-infiltrating monocytes and macrophages in CCA, we have not detected differences in the total frequency of infiltrating monocytes and macrophages in different grades or risk groups. Due to the small number of cases presenting CCA, we did not subdivide this group of patients, but we compared the results obtained with the HCC patients' group. In this regard, we have observed significantly higher expression of markers associated to M2 phenotype (CD206 and CD163), together with a higher expression of PD-L1 (CD274), in patients with HCC in comparison to CCA patients. Moreover, macrophages from HCC biopsies presented higher expression of IL-10 mRNA when compared to CCA tumor-infiltrating macrophages, indicating a higher infiltration of protumor macrophages in HCC patients in comparison to CCA. In contrast, we did not observe differences in the levels of TNFα mRNA expression by macrophages between the two groups of cancer patients.

Regarding tumor-infiltrating lymphocytes, we have detected a higher frequency of lymphocytes infiltrating HCC tumor biopsies in comparison to CCA. Moreover, among lymphocyte subsets, we have observed a higher frequency of NK cells as well as $CD8^+$ T cells with NK activity ($CD56^+$) infiltrating HCC tumors in comparison to CCA tumor biopsies. A successful antitumor immune response requires recruitment of specific T cells followed by recognition of tumor antigens and of NK cells, and the generation of antitumor cytotoxicity. In this sense, it has been previously reported that HCC tumors contained significant numbers of both T cells and NK cells at various stages (II/III/IV) [27]. While several reports demonstrated the inefficacy of NK cells in controlling tumor growth and invasion, NK cells' role in the prevention of metastasis has been described in different types of cancer, and a higher number of tumor-infiltrating NK cells have been associated with a better prognosis [28,29]. Therefore, this higher infiltration of NK cells and $CD8^+$ $CD56^+$ T cells in HCC tumors could contribute to the better prognosis of HCC patients in comparison to CCA [19]. Interestingly, $CD4^+$ T cells infiltrating tumor biopsies presented higher expression levels of IFNγ mRNA, measured by qRT-PCR, in comparison with FOXP3 and IL-17 mRNA expression levels in both HCC and CCA. Moreover, in HCC patients, a nonsignificant decrease in the expression levels of IFN-γ mRNA, expressed by both $CD4^+$, and $CD8^+$ T cells infiltrating tumor biopsies, in comparison to CCA infiltrating $CD4^+$ and $CD8^+$ T cells was observed, without reaching statistical significance.

Finally, we have detected a higher frequency of tumor cells in CCA tumor biopsies in comparison to HCC. In addition, in both groups of patients, tumor cells presented high expression levels of PD-L1 (CD274). It is well established that PD-L1 binding to PD-1 inhibits cytotoxic T-cell activity and promotes other immunoinhibitory effects [30]. Moreover, it has been previously reported that PD-L1 is implicated in immune suppression in HCC by its presence in tumors and adjacent tissue, and high PD-L1 expression in HCC has been positively correlated with reduced overall survival [31]. Consequently, these results suggest that inhibiting PD-L1/PD-1 interaction may increase the antitumor immunity in both HCC and CCA.

Previous works already detected functional impairments in different immune cell subsets in HCC and CCA patients [32,33]. Therefore, the study of the phenotype and functions of immune cell subsets in the tumor microenvironment may help to understand the complex processes that drive to tumor progression and may work for identifying new therapeutic targets for further studies.

Conclusions

To conclude, we highlight several potential implications. First, the confirmation of PD-L1 expression in HCC and CCA tumor cells as well as in HCC tumor-infiltrating macrophages, supporting the continued development of immune checkpoint inhibitors targeting the PD-1/PD-L1 pathway in both HCC and CCA. Second, the reported expression levels of CD200R in HCC infiltrating macrophages may serve as a benchmark to evaluate novel therapies that seek to target CD200/CD200R axis. Third, we have observed a significantly higher expression of markers associated with protumor macrophages in HCC tumors that were classified into high grades or into high-risk tumors, suggesting an important role of these macrophages in tumor progression. And, therefore, future therapies should consider protumor macrophages as one of the main targets for the development of new combined immunotherapies.

Moreover, this work has demonstrated the utility of multicolor flow cytometry of freshly processed tumor samples to reveal functional and phenotypic differences in TIL cell subpopulations among different cancer patients (CCA vs. HCC), different histopathological grades, and a new risk stratification classification for HCC patients, in addition to providing evidence to better understand the tumor immune microenvironment on both carcinomas. Future work is needed to evaluate its relevance in the design of treatment strategies and in the optimal selection of patients for immunotherapy.

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Author Contributions

C. M. S. processed the samples; performed the cell sorting, the flow cytometry and the molecular biology analyses; analyzed the results; and was a major contributor in the writing of the manuscript. R. M., A. M. A., R. C. O., J. G. T., M. F. B., and E. F. provided the biological samples, performed patients' selection, revised the clinical data, and reviewed the manuscript. P. L. supervised the data analysis, helped in sample processing, and reviewed the manuscript. M. C. supervised the molecular biology analyses. M. R. D. supervised the work and reviewed the manuscript. Finally, A. P. conceived the main idea of the work, interpreted the results, and reviewed the manuscript. C. M. S. is a PhD student in the Biochemistry program at University of Aveiro, and this work is part of her doctoral thesis. All authors read and approved the final manuscript.

Competing Interests

The author(s) declare no competing interests.

Data Availability

The authors declare that the main data supporting the results of the present study are available within the article and its Supplementary Information files. Extra data are available from the corresponding author upon request.

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Functional and phenotypic characterization of tumor-infiltrating leukocyte subsets and their contribution to the pathogenesis of hepatocellular carcinoma and cholangiocarcinoma

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Supplementary information

Table S1. Panel of monoclonal antibody reagents (with clones and commercial source) used for the immunophenotypic characterization and for cell purification by fluorescence-activated cell sorting of tumor cells and immune cells.

Tube	BV421 V450	V500	FITC	PE	PECy5	PECy7	APC	APCH7
1	**CD274** 29E.2A3 Biolegend	**CD45** 2D1 BD	**Cytokeratin 18** Ks18.04 Cytogonos	**CD200R** OX-108 Biolegend	**CD206** 15-2 Biolegend	**CD80** 2D10 Biolegend	**CD163** GH/61 Biolegend	**CD14** MφP9 BD
2	**CD4** RPA-T4 BD	**CD45** 2D1 BD	**cyIFNγ** 4S.B3 BD Pharmingen	**cyIL-17** SCPL1362 BD Pharmingen		**CD56** N901 Beckman Coulter	**CD3** SK7 BD	**CD8** SK1 BD
3	**CD4** RPA-T4 BD	**CD45** 2D1 BD	**Cytokeratin 18** Ks18.04 Cytogonos		**CD3** SK7 BD	**CD8** SFCI21Thy2D3 Beckman Coulter	**CD33** P67.6 BD	**CD14** MφP9 BD

Commercial sources: Biolegend (San Diego, CA, USA), BD Pharmingen (San Diego, CA, USA), BD (Becton Dickinson Biosciences, San Jose, CA, USA), Beckman Coulter (Miami, FL, USA), Cytogonos (Salamnaca, Spain). APC, allophycocyanin; APCH7, allophycocyanin-hilite 7; FITC, fluorescein isothiocyanate; PE, phycoerythrin; PECy5, phycoerythrin-cyanine 5; PECy7, phycoerythrin-cyanine 7.

CHAPTER 4. FUNCTIONAL IMPAIRMENT OF CIRCULATING FCεRI^{+} MONOCYTES AND MYELOID DENDRITIC CELLS IN HEPATOCELLULAR CARCINOMA AND CHOLANGIOCARCINOMA PATIENTS

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Brief Communication

Functional Impairment of Circulating FcεRI$^+$ Monocytes and Myeloid Dendritic Cells in Hepatocellular Carcinoma and Cholangiocarcinoma Patients

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Background: Hepatocellular carcinoma (HCC) and cholangiocarcinoma (CCA) represent the most common primary liver malignancies whose outcome is influenced by the immune response.

Methods: In this study, we have functionally characterized, by flow cytometry, circulating myeloid dendritic cells (mDCs) and FcεRI$^+$ monocytes in a group of healthy individuals ($n = 10$) and in a group of patients with HCC ($n = 19$) and CCA ($n = 8$), at the time point of the surgical resection (T0) and once the patient had recovered from surgery (T1). Moreover, we proceeded to a more in depth phenotypic characterization of the FcεRI$^+$ monocyte subpopulation.

Results: A significant decrease in the frequency of TNFα producing FcεRI$^+$ monocytes and mDCs in HCC and CCA patients when compared to the group of healthy individuals was observed, and a close association between FcεRI$^+$ monocytes and mDCs dysfunction was identified. In addition, the phenotypic characteristics of FcεRI$^+$ monocytes from healthy individuals strongly suggest that this population drives to mDCs, which matches with the fact that both populations are functionally affected.

Conclusions: The frequency and the function of circulating mDCs and FcεRI$^+$ monocytes are affected in both HCC and CCA patients, and FcεRI$^+$ monocytes could represent those fated to become mDCs. © 2019 International Clinical Cytometry Society

Key terms: myeloid dendritic cells; monocytes; TNFα; hepatocellular carcinoma; cholangiocarcinoma

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Additional supporting information may be found online in the Supporting Information section at the end of the article.

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BACKGROUND

Hepatocellular carcinoma (HCC) and cholangiocarcinoma (CCA) are the most common primary liver malignancies presenting high morbidity and mortality rates (1), associated with poor prognosis, generally due to its late presentation (2). It has been reported that dendritic cells (DCs) are critical for the induction of an antitumor immune response (3). However, in vitro propagated DCs from HCC patients display a decreased ability to produce IL-12 (4), HCC tumor-derived alpha-fetoprotein impairs both the differentiation and T-cell stimulatory activity of human DCs (5). In the same line, tumor immune evasion is associated with the defective DC function in cancer patients as a result of decreased numbers of competent DCs and accumulation of immature cells (6,7).

High affinity receptor FcεRI has a role in mediating inflammatory signaling and enhancing T-cell immunity (8). This receptor is homogeneously and constitutively expressed by the majority of human DC subsets (9), and it is expressed in a subset of monocytes, characterized as a $CD2^+$ population (10), that rapidly obtains DC-like features in culture (11,12).

In this study, we have characterized circulating mDCs and $FcεRI^+$ monocytes in peripheral blood (PB) from patients with HCC, CCA, and in a group of healthy individuals, identifying a functional impairment both in mDCs and in $FcεRI^+$ monocytes.

METHODS

Participants

A total of 19 patients with HCC (3 women and 16 men; average age: 62.0 ± 14.8 years) and 8 patients with CCA (5 women and 3 men; average age: 61.0 ± 14.7 years) were included in this study. Regarding the TNM classification, 1 CCA and 2 HCC patients were in stage I, 4 CCA and 14 HCC patients were in stage II, 1 HCC patient was in stage IIIA, and 3 CCA patients and 1 HCC patient were in stage IV. A group of 10 healthy individuals was included in the study as a control group (7 women and 3 men; average age: 51.6 ± 5.6 years). PB samples were collected at the time point of the surgical resection, just before the beginning of the surgical intervention (T0), and once the patient was completely recovered from the surgery (generally one month after the surgery) (T1). No patients took medication prior to surgery or at T1. Nevertheless, six HCC patients underwent liver transplantation and took tacrolimus just after the surgery. Tacrolimus target T lymphocytes (13) but do not affect monocyte function (14) and has no influence in the maturation of DCs (15).

Phenotypic Characterization of Peripheral Blood $FcεRI^+$ Monocytes and mDCs

For the identification of $FcεRI^+$ monocytes and mDCs, 250 μL of PB were stained with CD45-V500-C (clone 2D1, Becton Dickinson Biosciences (BD), San Jose, CA), anti-IgE-PE (clone BE5, EXBIO Praha, Vestec, Czech Republic), HLA-DR-V450 (clone L243, BD), CD16-PE-Cy7 (clone 3G8, BD), CD33-APC (clone P67.6, BD), and CD14-APC-H7 (clone MϕP9, BD). Furthermore, for the characterization of the populations above mentioned, samples were stained with CD1c-BV421 (clone F10/21A3, BD), HLA-DR-PerCP-Cy5.5 (clone L243, BD), CD35-FITC (clone E11, BD), CD11c-PerCP-Cy5.5 (clone B-ly6, BD), CD305-PE (clone DX26, BD), CD11b- APC (clone D12, BD), CD33-PerCP-Cy5.5 (clone P67.6, BD), CD2-FITC (clone RPA-2.10, BD), anti-IgE-FITC (clone BE5, EXBIO Praha), and CD13-PE (clone WM15, BD), using a lyse and wash procedure, as previously described (16). An isotype control was performed for CD2-FITC and CD1c-BV421 (Supporting Information).

TNFα Expression in Peripheral Blood $FcεRI^+$ Monocytes and mDCs after in vitro Stimulation

LPS (100 ng/mL, Sigma-Aldrich, St. Louis, MO), IFNγ (100 U/ml, Promega, Madison), and Brefeldin-A (10 μg/mL, Sigma-Aldrich) were added to 500 μL of PB sample diluted 1:1 (v/v) in RPMI 1640 complete culture medium (Invitrogen, Life Technologies, Carlsbad, CA). All samples were then incubated in a 5% CO_2 humid atmosphere, at 37°C, for 6 h. For all samples, a tube was included without any stimulating agent in order to evaluate the basal production of TNFα.

Each cultured sample was aliquoted (300 μL) into one tube and stained with CD16-PE-Cy7 (clone 3G8, BD), anti-IgE- FITC (clone BE5, EXBIO Praha), HLA-DR-V450 (clone L243, BD), CD45-V500-C (clone 2D1, BD), CD14-APC-H7 (clone MϕP9, BD), and CD33- APC (clone P67.6, BD) for 15 min in the dark at RT. For intracellular staining, Fix&Perm (GAS002, Life Technologies, Frederick) reagent was used in parallel with TNFα- PE (clone MAb11, BD).

Flow Cytometry Data Acquisition and Analysis

Data acquisition was performed in a FACSCanto™ II flow cytometer (BD) and analyzed with Infinicyt™ 1.8 software (Cytognos SL, Salamanca, Spain).

Statistical Analysis

To determine the statistical significance of the differences observed between groups, the nonparametric Mann–Whitney test was performed using the Statistical Package for Social Sciences software (SPSS, version 20, IBM, Armonk, NY). Statistically significant differences were considered when $P < 0.05$. The Spearman rank test was used to evaluate the correlation among variables.

RESULTS

Phenotypic Characterization of Peripheral Blood $FcεRI^+$ Monocytes and mDCs

The strategy used for the identification and characterization of $FcεRI^+$ monocytes and mDCs is represented in Figure 1A: in the first gate, the cell population positive for IgE bound to its receptor was selected; within this cell

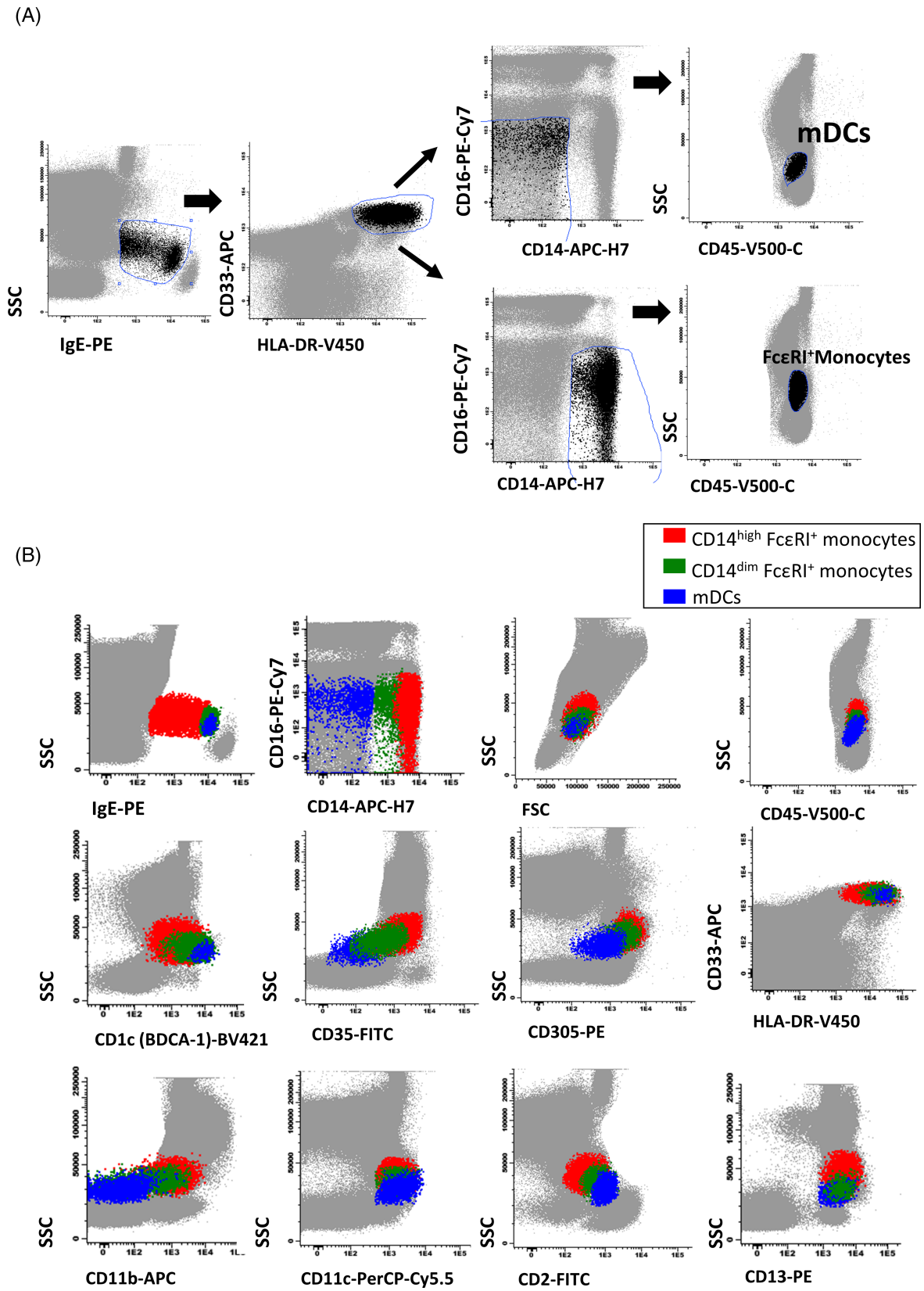

FIG. 1. Legend on next page.

population, those events positive for both HLA-DR and CD33 were further selected. FcεRI$^+$ HLA-DR$^+$ CD33$^+$ cells include both mDCs (corresponding to CD16$^-$ CD14$^-$ events) and FcεRI$^+$ monocytes (CD14$^+$ events).

Within FcεRI$^+$ monocytes, two subpopulations were distinguished based on the expression levels of IgE bound to FcεRI$^+$ receptor and the expression levels of CD14: *CD14^{bigh} FcεRI$^+$ monocytes* (CD14 mean fluorescence intensity (MFI) = 5633) and *CD14^{dim} FcεRI$^+$ monocytes* (CD14 MFI = 2749). *CD14^{bigh} FcεRI$^+$ monocytes* are phenotypically characterized as CD14^{high}, HLA-DR ^{high}, CD33 ^{high}, negative for CD16, with similar SSC properties and CD45 expression than mDCs, and lower expression of IgE bound to high affinity FcεRI receptor compared to mDCs and CD14^{dim} FcεRI$^+$ monocytes (Fig. 1B). Lower expression levels of CD1c and CD2, similar expression levels of CD13 and CD11c, and higher expression of CD35, CD305, and CD11b were exhibited by this population, in comparison to CD14^{dim} FcεRI$^+$ monocytes and mDCs (Fig. 1B). CD35 expression appears in the later stages of monocytic maturation (17,18). CD35 is differentially expressed between monocyte subsets and mDCs, being highly expressed in classical monocytes, intermediately expressed in CD14^{dim} FcεRI$^+$ monocytes, presenting lower expression in mDCs. The expression of CD305 (LAIR-1) on monocytes and monocyte-derived DCs is distinctly and reversibly controlled by myeloid cytokines, and it has been associated with in vitro differentiation of CD14$^+$ monocytes to DCs (19–21). Our results demonstrate that CD305 is highly expressed in FcεRI$^+$ CD14^{high} monocytes, less expressed in CD14^{dim} FcεRI$^+$ monocytes, being mDCs the population that exhibits the lowest expression.

CD14^{dim} FcεRI$^+$ monocytes are phenotypically characterized as CD14^{dim}, HLA-DR ^{high}, CD33 ^{high}, negative for CD16, with similar SSC properties and similar expression of CD45 and IgE bound to high affinity FcεRI receptor than mDCs (Fig. 1B). When compared to mDCs and CD14^{high} FcεRI$^+$ monocytes, CD14^{dim} FcεRI$^+$ monocytes exhibit an intermediate expression of CD1c and CD2, similar levels of CD13 and CD11c, and decreasing expression of CD35, CD305, and CD11b (Fig. 1B). *mDCs* were phenotypically identified as negative for CD14 and CD16, presenting a characteristic FSC/SSC light dispersion properties together with high expression of HLA-DR, CD33, and IgE bound to high affinity receptor FcεRI (Fig. 1B). mDCs display higher expression levels of CD1c and CD2 than FcεRI$^+$ monocyte populations, similar expression levels of CD13 and CD11c, decreasing expression of CD305 and CD35 are negative for CD11b (Fig. 1B). The detailed phenotypic characterization of these three populations allows the observation of a clear differentiation path from CD14^{high} FcεRI$^+$ monocytes to CD14^{dim} FcεRI$^+$ monocytes and, finally, to mDCs (Fig. 1B).

Frequency and Absolute Value of Peripheral Blood FcεRI$^+$ Monocytes and mDCs in CCA and HCC Patients

CCA and HCC patients displayed a decrease in the frequency and absolute numbers of peripheral blood FcεRI$^+$ monocytes and mDCs, both at T0 and T1, compared to the control group. The frequency of mDCs was significantly decreased in both groups of patients when compared to the healthy group, both at T0 and T1, and the absolute counts of mDCs was significantly decreased at T0 and T1 in CCA patients and at T0 in HCC patients. A nonsignificant decrease in FcεRI$^+$ monocytes, both in frequency and absolute numbers, was verified in comparison to the healthy group (Table 1).

Functional Characterization of Peripheral Blood FcεRI$^+$ Monocytes and mDCs in CCA and HCC Patients

Regarding TNFα production (Fig. 2), a statistically significant decrease in the frequency of TNFα producing FcεRI$^+$ monocytes and TNFα producing mDCs, for both groups of patients at T0, was observed when compared to the control group. A frequency of 61.3% ± 41.0 and 70.7% ± 26.7 of TNFα-producing FcεRI$^+$ monocytes was found for CCA and HCC patients, respectively, while the healthy group presented a frequency of 97.1% ± 5.80 (Table 1). Of note, the frequency of TNFα-producing classical monocytes in the healthy group was 98.1% ± 1.7 (unpublished data). Interestingly, a partial recovery of TNFα-producing FcεRI$^+$ monocytes was observed at T1, especially in CCA patients, without reaching the values of the control group. On the contrary, we did not observe significant differences in the amount of TNFα expressed *per* cell (MFI) among the different groups (Table 1). Noteworthy, the decrease identified in the frequency of TNFα-producing cells affected evenly both cell subsets, as there is a positive correlation between the percentages of TNFα-producing FcεRI$^+$ monocytes and TNFα-producing mDCs in the PB of cancer patients (Fig. 2C), with a correlation coefficient of 0.789 in the Spearman rank test, significant at the 0.01 level.

DISCUSSION

DCs are considered the most effective antigen-presenting cells for activating naïve T-cells (22), and the interaction among DCs and tumor cells may profoundly dictate the outcome of a neoplasm (23). In fact, DC infiltration in HCC lesions has been associated with a better prognosis in resected patients (23), and it has been reported that the inhibition of DC maturation in HCC may be a critical feature of tumor escape (24).

Therefore, to contribute to a better understanding of the role of fully differentiated mDCs as well as FcεRI$^+$

FIG. 1. Phenotypic characteristics of peripheral blood FcεRI$^+$ monocytes and myeloid dendritic cells (mDCs). (A) Bivariate dot plot histograms illustrating the gating strategy for the identification of mDCs (up) and FcεRI$^+$ monocyte subpopulations (down) from peripheral blood samples. (B) Bivariate dot plot histograms illustrating the phenotypic characterization of the identified populations from peripheral blood samples. CD14^{high} FcεRI$^+$ monocytes are represented as red events, CD14^{dim} FcεRI$^+$ monocytes are represented as green events, and mDCs are represented as blue events. [Color figure can be viewed at wileyonlinelibrary.com]

Table 1
Frequency among total leukocytes (%) and absolute numbers ([], number of cells/μL) of peripheral blood FcεRI⁺ monocytes and myeloid dendritic cells (mDCs) in cholangiocarcinoma (CCA) and hepatocellular carcinoma (HCC) patients, at the time of surgery (T0) and once the patients were recovered from surgery (T1), and in healthy individuals (HG); frequency (%) of tumor necrosis factor (TNF)-α-producing cells and amount of TNFα expressed per cell, measured as mean fluorescence intensity (MFI), within each cell population

	Cholangiocarcinoma (*N* = 8)		Hepatocellular carcinoma (*N* = 19)		HG (*N* = 10)
	Mean ± SD T0	Mean ± SD T1	Mean ± SD T0	Mean ± SD T1	Mean ± SD
% FcεRI⁺ monocytes	0.12 ± 0.23	0.13 ± 0.23	0.12 ± 0.22	0.11 ± 0.14	0.20 ± 0.21
FcεRI⁺ monocytes [number of cells/μL]	4.80 ± 10.1	4.71 ± 8.30	10.25 ± 15.9	8.95 ± 11.3	14.2 ± 15.2
%TNFα producing FcεRI⁺ monocytes	61.3 ± 41.0[a]	84.7 ± 14.8[a]	70.7 ± 26.7[a]	78.7 ± 15.1[a]	97.1 ± 5.80
MFI TNFα FcεRI⁺ monocytes	11,202 ± 9795	5580 ± 2204	6614 ± 7325	5293 ± 4087	5254 ± 3465
% mDCs	0.06 ± 0.06[a]	0.07 ± 0.02[a]	0.07 ± 0.05[a]	0.10 ± 0.07[a]	0.16 ± 0.06
mDCs [number of cells/μL]	3.24 ± 2.83[a]	3.40 ± 1.44[a]	5.66 ± 5.07[a]	6.53 ± 5.80	9.69 ± 3.38
%TNFα producing mDCs	37.4 ± 30.8[a]	48.5 ± 23.1	43.7 ± 26.7[a]	47.0 ± 23.7[a]	75.1 ± 8.10
MFI TNFα mDCs	6730 ± 7521	2529 ± 985	2902 ± 2215	2917 ± 2594	2127 ± 1212

Independent-samples Mann–Whitney U test was performed with a significance level of 0.05 ($P < 0.05$).
[a]versus CONTROL;
[b]versus CCA. The results are given by mean ± standard deviation.

monocytes, in CCA and HCC cancers, we have functionally characterized these populations in PB from patients with HCC, CCA, and in a group of healthy individuals.

We have observed a decrease in the frequency and the absolute numbers of circulating FcεRI⁺ monocytes and mDCs, both at T0 and T1, in CCA and HCC patients

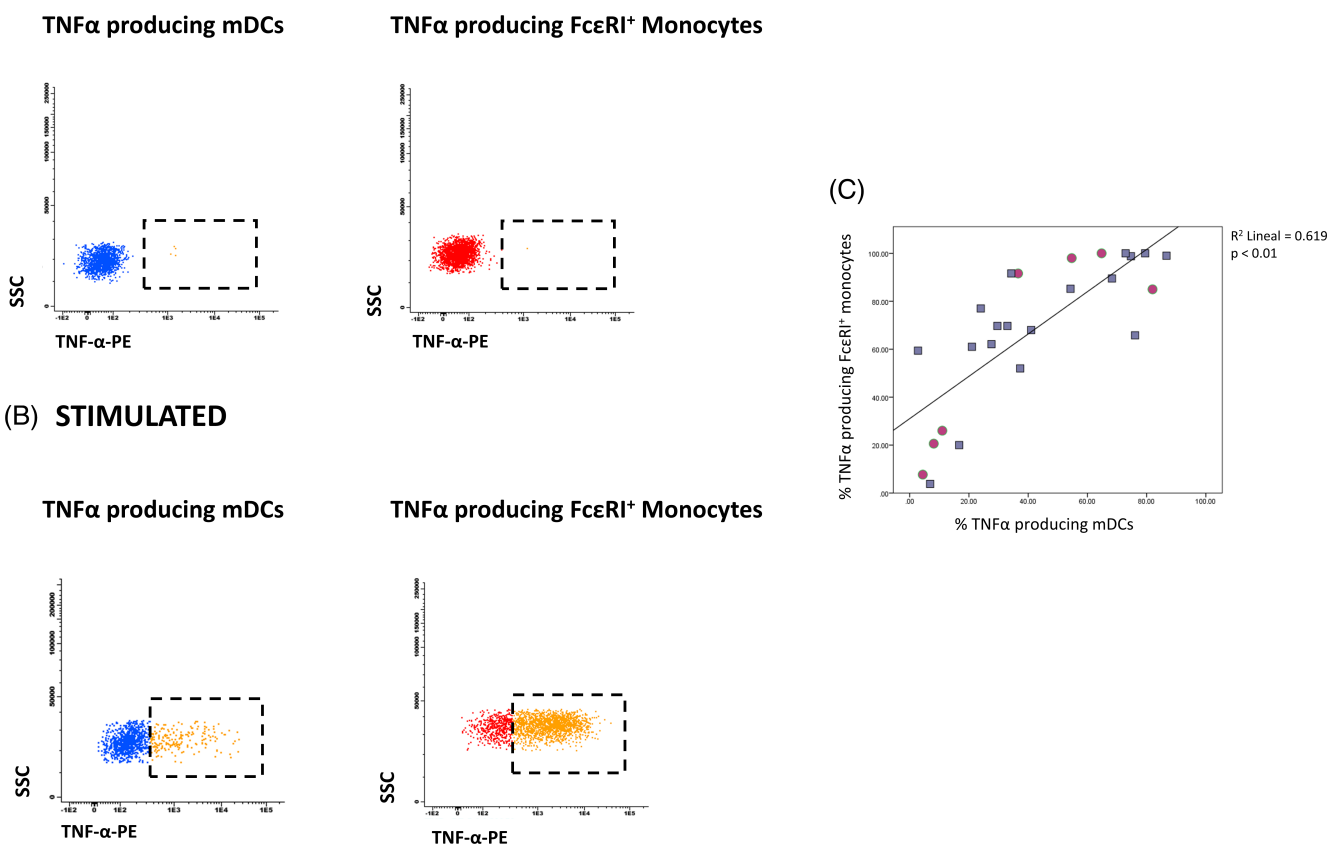

FIG. 2. TNF-α production by mDCs and FcεRI⁺ monocytes. Bivariate dot plot histograms illustrating TNF-α production (indicated with dashed rectangle) by mDCs (left) and FcεRI⁺ monocytes (right) in basal condition (without stimulation agents) (A), and after stimulation with LPS and IFN-γ (B). Correlation between the frequency of TNFα producing mDCs and TNFα producing FcεRI⁺ monocytes in hepatocellular carcinoma (purple squares) and cholangiocarcinoma patients (pink circles), confirmed by the Spearman test (C). [Color figure can be viewed at wileyonlinelibrary.com]

compared to the control group. Significant differences were found in the frequency and absolute numbers of mDCs, in CCA patients, at T0 and T1, and in HCC at T0. Moreover, regarding the functional characterization of these cells, despite the high variability observed, a significant decrease in the frequency of TNFα-producing FcεRI$^+$ monocytes associated to a significant decrease in TNFα-producing mDCs was observed in both groups of cancer patients, at T0, when compared to the control group. This significant decrease was maintained for FcεRI$^+$ monocytes at T1 in both groups of patients and for mDCs at T1 in HCC patients. The positive correlation verified in TNFα production dysfunction (Fig. 2C) suggests that FcεRI$^+$ monocytes may represent those fated to become mDCs, what is further supported by our results on the phenotypic characterization of this cell population (Fig. 1B), particularly by the expression kinetics of CD14, HLA-DR, CD1c (BDCA-1), CD2, CD35, CD305, and CD11b, that display a phenotypic continuum from circulating FcεRI$^+$ monocytes to circulating mDCs. However, in differentiation experiments, it was reported that both culture-derived and primary CD1c$^+$ preconventional DCs purified from peripheral blood acquire CD14 expression in culture (25). In addition, Meyerson et al. reported that CD1c myeloid DCs (mDCs) were noted to have partial expression of CD14 (26). Therefore, the classification of CD14^{dim} FcεRI$^+$ CD1c$^+$ cells into mDCs or monocytes subsets is controversial and still under debate. Further studies in transcriptomics or/and at single cell level would be needed to clarify this classification. Nevertheless, it seems, from the phenotypic point of view, that there is a differentiation pathway from CD14^{high} FcεRI$^+$ cells to CD14^{dim} FcεRI$^+$ cells and finally to mDCs.

In conclusion, a functional defect in circulating mDCs and FcεRI$^+$ monocytes from HCC and CCA patients was identified. In addition, we have thoroughly characterized the phenotype of FcεRI$^+$ monocytes in healthy individuals, and our data strongly suggest that FcεRI$^+$ monocytes could differentiate into mDCs.

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Functional impairment of circulating FcεRI^{+} monocytes and myeloid dendritic cells in hepatocellular carcinoma and cholangiocarcinoma patients

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Supporting Information

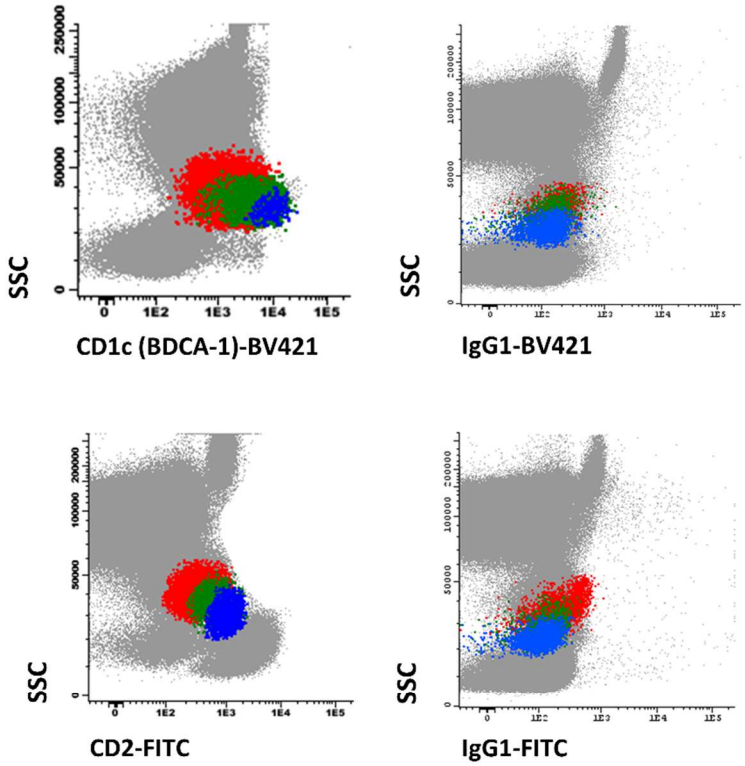
Methods

For the isotype control of CD1c-BV421 (Mouse IgG_1, κ), 250μL of PB were stained with CD45-V500-C (clone 2D1, Becton Dickinson Biosciences (BD), San Jose, CA, USA), anti-IgE-PE (clone BE5, EXBIO Praha, Vestec, Czech Republic), HLA-DR- PerCP-Cy5.5 (clone L243, BD), CD16-PE-Cy7 (clone 3G8, BD), CD33-APC (clone P67.6, BD) and CD14-APC-H7 (clone MφP9, BD), for the identification of the population of interest and BV421 Mouse IgG1, k Isotype Control (clone X40, BD) was also added. Then the protocol was followed as described in the main manuscript.

For the isotype control of CD2-FITC (Mouse BALB/c IgG_1, κ), 250μL of PB were stained with CD45-V500-C (clone 2D1, BD), anti-IgE-PE (clone BE5, EXBIO), HLA-DR-V450 (clone L243, BD), CD16-PE-Cy7 (clone 3G8, BD), CD33-APC (clone P67.6, BD) and CD14-APC-H7 (clone MφP9, BD), for the identification of the population of interest and FITC Mouse IgG1, κ Isotype Control (clone MOPC-21, BD) was also added. Then the protocol was followed as described in the main manuscript.

Figure's legend

Figure S1. Negative control for CD2-FITC and CD1c-BV421 staining. The bivariate dot plot histograms on the left illustrate the expression levels for CD1c-BV421 (up) and CD2-FITC (down) for the populations identified in this study: **$CD14^{high}$ $FcεRI^{+}$ monocytes** (red events), **$CD14^{dim}$ $FcεRI^{+}$ monocytes** (green events) and **mDCs** (blue events). The bivariate dot plot histograms on the right display the isotype control for these fluorochromes. The table presents CD2 and CD1c mean fluorescence intensity (MFI) for each identified cell population as well as isotypes MFI values.



	MFI CD2	MFI CD1c	MFI IgG1-FITC	MFI IgG1-BV421
FcERI^{+} monocytes CD14^{high}	364	1924	112	175
FcERI^{+} monocytes CD14^{dim}	787	5871	44	145
mDCs	1021	9562	35	127

CHAPTER 5. ELEVATED SOLUBLE TNFα LEVELS AND UPREGULATED TNFα mRNA EXPRESSION IN PURIFIED PERIPHERAL BLOOD MONOCYTE SUBSETS ASSOCIATED WITH HIGH-GRADE HEPATOCELLULAR CARCINOMA

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Elevated soluble TNFα levels and upregulated TNFα mRNA expression in purified peripheral blood monocyte subsets associated with high-grade hepatocellular carcinoma

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Abstract

Chronic inflammation plays an important role in the initiation and progression of various cancers, including liver cancer. The current study focuses on the characterization of the peripheral immune response in hepatocellular carcinoma (HCC) and cholangiocarcinoma (CCA) patients, before and after surgical procedure, in order to assess the effect of tumor resection in the immune system homeostasis and to determine possible prognostic factors associated with high-grade tumors. We developed a whole-blood assay to monitor immune alterations and functional competence of peripheral monocytes in a group of 10 healthy individuals (HG), in 20 HCC patients and 8 CCA patients, by multi-color flow cytometry, qRT-PCR, and ELISA techniques. The qRT-PCR analysis showed an upregulation of TNFα expression by classical and intermediate monocytes purified from HCC patients presenting tumors in grade G3-G4 as compared to G1-G2 HCC patients. Moreover, ELISA assay confirmed elevated serum levels of TNFα in G3-G4 compared to G1-G2 HCC patients. A significant decrease of circulating non-classical monocytes was detected in both CCA and HCC patients before and after surgical procedure. In addition, a functional defect in circulating classical and intermediate monocytes was observed in both groups of cancer patients when compared to the HG, with partial recovery after the surgical intervention. This integrated analysis permitted the identification of altered functional competence of monocyte subsets in CCA and HCC patients. In addition, our results point to a potential role of TNFα as a prognostic peripheral biomarker in HCC patients, indicating the presence of high-grade tumors that should be further validated.

Keywords: Flow cytometry, qRT-PCR, hepatocellular carcinoma, cholangiocarcinoma, circulating monocytes, TNFα.

Abbreviations[1]

[1] CCA, cholangiocarcinoma; FACS, fluorescence-activated cell sorting; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; HCC, hepatocellular carcinoma; HG, control group or healthy group; LARC, liver activation regulated chemokine; mAbs, monoclonal antibodies; MIP3α, macrophage inflammatory protein-3α; NK, Natural Killer; PB, peripheral blood; PBS, phosphate-buffered saline; PMA, phorbol myristate acetate.

Introduction

Liver cancer is the second most common cause of cancer-related death worldwide, with a steady increasing incidence and mortality [1] and, therefore, a major public health challenge. It is considered an immunogenic cancer because 90% of cases develop under conditions of chronic inflammation [1,2]. This inflammation leads to tumor development and is associated to higher tumor immunogenicity. For this reason, immunotherapeutic approaches may be suitable therapeutic strategies for this type of cancer [3]. Hepatocellular carcinoma (HCC) is the most frequent liver cancer and is associated to high morbidity and mortality rates [1,4]. It has poor prognosis, generally due to its late presentation and thus, late diagnostic. Cholangiocarcinoma (CCA), a malignancy that originates from biliary epithelia, is an aggressive cancer with a high mortality rate [5] and, along with HCC, represents a major primary liver cancer [2]. CCA is difficult to diagnose due to its silent and nonspecific clinical features and, in most cases, the symptoms occur when the tumor has reached an advanced stage [6]. For patients with advanced disease, there are only limited therapeutic treatment options that provide limited benefits for a subset of patients. Therefore, novel therapeutic options are needed [7]. Partial surgical resection and liver transplantation are potentially curative treatments in selected patients with HCC and CCA [8,9]. Unfortunately, a high postoperative tumor recurrence rate significantly decreases long-term survival outcomes [9].

Prognosis of cancer patients is based on tumor-related factors as well as on host-related factors, including systemic immune cell activation [10]. Given the difficulties in acquiring liver tissues, circulating biomarkers as well as comprehensive studies monitoring the peripheral immune system homeostasis, are needed. In fact, it has been demonstrated that immune monitoring of peripheral blood (PB) cells might lead to the identification of biomarkers, which could serve to predict prognosis and/or therapy response [11]. Due to its immunogenic origin, further knowledge of the changes on immune cell populations may help define the potential of the combination of therapies, such as adoptive immunotherapy and/or checkpoint inhibition [12]. Moreover, monitoring of the peripheral immune response after surgical resection will provide information to advance our understanding of the mechanisms underlying the clinical response to surgical resection and enable us to determine the prognosis of patients after surgery.

In the present study, we have characterized, by multi-color flow cytometry, the frequency, composition and activity of circulating monocyte subsets in PB samples from HCC and CCA patients, in order to understand the immune status of these patients, and to obtain evidence

about the changes in the proportion or functions of these populations, for application in the clinical diagnosis and future development of treatments to HCC and CCA.

Patients and methods

Patients and healthy individuals

A total of 20 patients with HCC (3 women and 17 men; average age: 62.2 ± 14.5 years) and 8 patients with CCA (5 women and 3 men; average age: 61.0 ± 14.7 years) were included in this study. PB samples were collected at the time point of the surgical resection, just before the beginning of the surgical intervention (T0), and once the patient was completely recovered from the surgery (generally one month after the surgery) (T1). In addition, a group of 10 age- and sex-matched healthy individuals was included in the study as a control group (HG). No patients took any anti-tumor therapy or medication prior to surgery nor at T1. Nevertheless, 7 HCC patients underwent liver transplantation and took tacrolimus just after the surgical procedure. Tacrolimus target T lymphocytes [13,14] but do not affect monocyte function [15].

The clinical background of the patients included in this study is summarized in Table 1. Patients were classified according to the 8th TNM classification and tumors were categorized depending on their histopathological grading.

Study Approval

The experimental protocols were approved by the Ethical Committee of the Faculty of Medicine, University of Coimbra, Coimbra, Portugal (CE-136/2016). All procedures performed involving human participants were in accordance with the ethical standards of Ethical Committee of the Faculty of Medicine, University of Coimbra, Coimbra, Portugal (CE-136/2016), and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. All participants gave their signed informed consent before entering in the study.

In vitro stimulation of cytokine production by circulating monocytes

To evaluate the TNFα production by classical, intermediate and non-classical monocytes, PB samples were collected from participants and healthy individuals into lithium heparin

(Becton Dickinson Biosciences, BD, San Jose, CA, USA) and stimulated with lipopolysaccharide (LPS, 100 ng/mL, Sigma-Aldrich, St. Louis, MO, USA) plus interferon gamma (IFNγ, 100 U/ml, Promega, Madison, WI, USA) as previously described in Martín-Sierra et al. 2019 [16].

Immunophenotypic analysis was performed by using a seven-color monoclonal antibodies (mAbs) combination, detailed in Supplementary Table S1 (tube 1). Each cultured sample was aliquoted (300 µL) into one tube and stained with the mAbs for surface proteins antigens (CD45, HLA-DR, IgE, CD16, CD33 and CD14). For intracellular staining, Fix&Perm (Life Technologies, Frederick, MD, USA) reagent was used in parallel with TNFα mAb. After washing twice with phosphate-buffered saline (PBS; Gibco, Life Technologies, Carlsbad, CA), the cell pellet was resuspended in 500 µL of PBS and immediately acquired.

Flow cytometry data acquisition and analysis

Data acquisition was performed in a FACSCanto II flow cytometer (BD) and analyzed with Infinicyt 1.8 software (Cytognos SL, Salamanca, Spain).

Cell purification by fluorescence-activated cell sorting

Classical ($CD14^{++}CD16^{-}$), intermediate ($CD14^{++}CD16^{+}$) and non-classical ($CD14^{+}CD16^{++}$) monocyte subpopulations from PB were purified by fluorescence-activated cell sorting (FACS), using a FACSAria III flow cytometer (BD) according to their typical phenotype. Thus, the four-color mAbs combination used (Supplementary Table S1, tube 2) allowed the identification of each monocyte subset.

For subsequent mRNA expression analysis, purified cell populations were centrifuged for 5 min at 300 g, the cell pellet was resuspended in 350 µL of RLT Lysis Buffer (Qiagen, Hilden, Germany), and stored at -80°C.

RNA isolation and quantitative real-time reverse transcriptase-polymerase chain reaction (qRT-PCR)

Total RNA was extracted and purified with RNeasy™Micro Kit (Qiagen) according to the manufacturer's instructions. Reverse transcription was performed with SensiScript Reverse Transcription Kit (Qiagen) according to supplier's instructions and with Random Hexamer

Primer (Thermo Fisher Scientific, San Jose, USA). Relative quantification of gene expression was performed in a QuantStudio (Thermo Fisher Scientific) by a real time (qRT)-PCR reaction. qRT-PCR was done with PowerUp™ SYBR™ Green Master Mix (Thermo Fisher Scientific), using optimized primers for TNFα, CX3CR1 and endogenous control glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (Qiagen), according to the manufacturer's instructions.

Assessment of cytokine and chemokine serum concentrations

PB samples were collected from patients and healthy individuals into VACUETTE Serum Gel tubes (Greiner-Bio-One, Kremsmünster, Austria) and were centrifuged for 10 minutes at 2000 g. Then, serum samples were subdivided into small aliquots to be stored at -80°C until tested for cytokine and chemokines levels. ELISA kits were used to determine monocyte chemotactic protein-1 (MCP-1 or CCL2, Thermo Scientific), MIP-3a (CCL20, Thermo Scientific), IP-10 (CXCL10, Thermo Scientific) and TNFα (Thermo Scientific) serum levels, according to the manufacturer's instructions.

Statistical analysis

For all variables under study, their mean values, standard deviation, median and range, were calculated. To determine the statistical significance of the differences observed between groups, the non-parametric Mann-Whitney and Kruskal-Wallis multiple comparison tests were performed using the Statistical Package for Social Sciences software (SPSS, version 25, IBM, Armonk, NY, USA). The non-parametric Wilcoxon signed-rank test of non-independent data was performed to compare T1 vs T0. Statistically significant differences were considered when $p < 0.05$. GraphPad Prism software was used to create the graphics.

Results

Alterations of peripheral blood leukocyte subsets in CCA and HCC patients

From the data obtained by flow cytometry immunophenotyping, CCA and HCC patients displayed a decrease in the frequency and absolute numbers of PB monocytes, both at T0 and T1, compared to the HG. Regarding monocyte subsets, CCA patients displayed a

significant decrease in the absolute numbers of PB classical, intermediate and non-classical monocytes, both at T0 and T1, when compared to the HG, and a significant decrease in the relative frequency of non-classical monocytes (within circulating monocytes) at T0 in comparison to the HG, that was partially restored at T1. HCC patients presented a significant decrease in the relative frequency and absolute numbers of circulating non-classical monocytes, both at T0 and T1, when compared to the HG, and a significant increase in the relative frequency of classical monocytes, both at T0 and T1, as compared to the HG. Interestingly, the relative frequency of intermediate monocytes presenting high-level surface expression of HLA-DR [17] ($HLADR^{++}$) was significantly decreased in HCC patients at T0 in comparison to the HG (Table 2).

Functional alterations of peripheral blood monocyte subsets in CCA and HCC patients

The different leukocyte subpopulations were identified and distinguished based on CD45 positivity, their typical forward scatter (FSC) and side scatter (SSC) light dispersion properties, and their positivity for specific membrane markers as shown in Figure 1A. Regarding TNFα production after stimulation (Figure 1B), a statistically significant decrease in the frequency of TNFα producing classical monocytes, for CCA (74% ± 29) and HCC (85% ± 20) at T0, was observed when compared to the HG (98% ± 2). HCC patients displayed a significant decrease in the frequency of TNFα producing classical monocytes at T1 (90% ± 16) as compared to the HG (98% ± 2), while no differences were found for CCA patients at T1. A statistically significant decrease in the frequency of TNFα producing intermediate monocytes with mid-level surface expression of HLA-DR ($HLADR^{+}$) was observed for CCA patients (87% ± 20) vs. HG (99% ± 1). Additionally, both CCA (93% ± 10) and HCC (97% ± 5) patients displayed a statistically significant decrease in the frequency of TNFα producing intermediate monocytes with high-level surface expression of HLA-DR ($HLADR^{++}$) at T0 in comparison to the HG (100% ± 0).

We did not observe significant differences when comparing patients presenting tumors classified as G1-G2 to patients presenting tumors classified as G3-G4 (data not shown).

After the purification of monocyte subpopulations by cell sorting, the mRNA levels of CX3CR1 and TNFα were measured by qRT-PCR. The expression levels of CX3CR1 mRNA among classical monocytes were significantly decreased in both groups of cancer patients at T0 when compared to the HG, with partial recovery at T1 in both groups of cancer patients (Figure 2A). A significant increase in the expression levels of CX3CR1 mRNA among

intermediate monocytes was detected in HCC patients at T1. Moreover, the expression levels of CX3CR1 mRNA by non-classical monocytes were significantly decreased in HCC patients at T0 when compared to the HG, with a recovery at T1. The same pattern was observed in CCA patients but without reaching statistical significance.

On the other hand, no differences were observed on TNFα mRNA expression among classical and non-classical monocytes, in CCA or HCC patients, when compared to the HG. Nevertheless, CCA patients displayed a significant increase of TNFα mRNA in intermediate monocytes at T0 in comparison to the HG, while at T1, TNFα mRNA levels approach to those observed in HG (Figure 2A).

Taking into account the histopathological grade of the tumors, HCC patients presenting tumors classified as grade G3-G4 displayed significantly higher levels of TNFα mRNA in classical and intermediate monocytes at T0 in comparison to HCC patients with G1-G2 tumors; non-classical monocytes displayed the same tendency, without reaching statistical significance (Figure 2B). The same pattern was observed in CCA patients but without reaching statistical significance (Figure 2B).

Analysis of chemokines and cytokines serum levels in CCA and HCC patients

With regard to the chemokines analyzed by ELISA, elevated serum levels of CCL20 were found in both CCA and HCC patients at T0, compared to the HG. While CCA patients showed a partial recovery at T1, HCC patients' CCL20 levels remained high at T1 (Figure 3A). Circulating levels of CXCL10 were also increased in HCC patients, at T0, in comparison to the HG, followed by a partial recovery at T1. Conversely, no significant differences were observed for CCL2 among the studied groups (Figure 3A).

Concerning serum levels of TNFα, CCA patients displayed a significant increase of TNFα at T0, in comparison to the HG, partially restored at T1, while no significant differences were observed for HCC patients (Figure 3A).

Notably, serum levels of TNFα were significantly higher in G3-G4 compared to G1-G2 HCC patients (at T0). A similar pattern was observed in CCA patients without reaching statistical significance (Figure 3B).

Discussion

Increasing evidence suggests that chronic inflammation plays a central role in the initiation and progression of various cancers, displaying a very important role in liver cancer [18]. In addition, inflammation can also be orchestrated by the tumor itself through the secretion of factors that recruit inflammatory cells to the tumor microenvironment [19]. Therefore, targeting inflammation may represent a promising avenue for the treatment of liver cancer, such as HCC or CCA. The current study focuses on the characterization of the peripheral blood monocyte subsets in HCC and CCA patients, before and after surgical procedure, in order to understand the immune status of these patients, to assess the effect of tumor resection in the immune system homeostasis, and to identify alterations associated to patients presenting high-grade tumors for further investigations to provide novel prognostic factors in these carcinomas.

Inflammatory factors affect nearly all the stages of tumor development and the effectiveness of the applied therapy. TNFα is considered one of the most important inflammatory mediators of the cancer-associated inflammatory networks. In this regard, preclinical studies in breast cancer have suggested that TNFα promotes tumor growth *in vivo* and could be considered a therapeutic target [20]. In the present study, we pointed out an upregulation of TNFα mRNA expression in peripheral classical, intermediate and non-classical monocytes purified from HCC patients presenting tumors classified as grade G3-G4, as compared to G1-G2 HCC patients. Moreover, in line with these results, we detected a significant increase of TNFα serum levels in HCC patients with tumors in grade G3-G4 as compared to grade G1-G2 HCC. In this regard, further studies will be needed to validate the potential use of TNFα serum levels as a prognostic factor or indicator of histopathological grading. Supporting this idea, it has been reported that aberrant elevated TNFα levels might promote not only tumor growth, but also invasion and poor prognosis [21]. Additionally, a previous work in a rat liver cancer model demonstrated that TNFα inhibition and deletion could decrease tumor incidence and showed that clinically TNFα expression was correlated to hepatic progenitor cells activation and HCC recurrences [22].

Regarding the characterization of circulating monocytes, we observed a significant decrease in the frequency and absolute counts of PB monocytes, both in CCA and HCC patients, at T0, as compared to the HG. Interestingly, values continued to be diminished comparatively to the HG after surgical procedure (at T1). Peripheral blood monocytes constantly enter the liver to replenish hepatic macrophages and dendritic cells, playing important roles in the pathogenesis of inflammatory disorders [23]. Our previous findings

had already shown altered frequencies and altered absolute numbers of circulating FcεRI^{+} monocytes and myeloid dendritic cells in CCA and HCC patients, as well as functional defects in both cell subsets [16]. Thus, we wondered whether other monocyte subsets might be affected in these patients. The results of the present study extend our previous findings, showing alterations on monocytes function, as well as a significant decrease in the relative frequency and absolute numbers of circulating non-classical monocytes both in CCA and HCC patients. These values remained diminished as compared to the HG at T1, excepting the frequency of non-classical monocytes in CCA patients, that was partially restored after tumor resection. Non-classical monocytes “patrol” the vasculature via a mechanism that requires the fractalkine receptor CX3CR1, covering apoptotic endothelial cells and sensing danger signals coming from the tissue [24]. A previous work has shown that this population of monocytes reduces tumor metastasis by recruiting NK cells [25]. Therefore, we hypothesized that the observed decrease in this monocyte subset may be having a role in cancer progression. Additionally, it has been suggested that non-classical monocytes are crucial regulators in the pathogenesis of chronic liver disease in humans, by the secretion of abundant cytokines, perpetuating chronic inflammatory processes within the liver and by directly activating hepatic stellate cells that, in turn, can secrete multiple chemokines for monocyte recruitment into the liver [26]. Therefore, indicating that the modulation of monocyte-subset recruitment into the liver may represent possible novel approaches for interventions targeting pro-inflammatory actions of monocyte subsets in liver cancer.

When evaluating monocyte function, we observed, at T0, a significant decline in TNFα production by classical and HLADR^{++} intermediate monocyte subsets, under LPS plus IFNγ stimulation, in both CCA and HCC patients as compared to the HG, whereas no significant differences were detected for non-classical monocytes. TNFα production by HLADR^{+} intermediate monocyte was also affected in CCA patients before tumor resection, but not in HCC patients. The frequencies of TNFα-producing cells seem to be restored after tumor resection, excepting classical monocyte subsets that displayed a significant decrease in TNFα production in HCC patients at T1. This data indicate a functional defect in classical and intermediate monocytes in both, CCA and HCC patients that it is partially restored for intermediate monocytes at T1. In line with these results, previous studies in other carcinomas have reported a diminished percentage of TNFα-producing CD14^{+} cells in PB of lung adenocarcinoma patients, showing that malignant cells inhibited the capability of monocytes to produce TNFα [27]. Thus, the decreased TNFα expression, observed in classical and intermediate monocytes, may influence tumor progression and relapse, since an altered response of innate immune cells might underscore a reduced capacity to mount

an efficient antitumor immune response [28]. Monocytes can differentiate into a variety of macrophage or dendritic cell subtypes that can either activate or inhibit the immune response [29], and it has been shown that CX3CR1 is rapidly downregulated during monocytes differentiation [30]. In relation with this process, classical and non-classical monocytes purified from CCA and HCC patients presented a downregulation of the mRNA expression of the fractalkine receptor, CX3CR1, at T0, when compared to the HG, with partial recovery at T1, indicating a possible differentiation process in these monocyte subsets occurring before surgical procedure. Additionally, CX3CR1 expression could participate in promoting cell-to-cell interactions with an inflamed endothelium, as well as increasing monocytes survival [30]. We also detected, in intermediate monocytes from CCA patients, an upregulation of TNFα mRNA expression at T0 that was restored after surgical procedure, which is further supported by the results obtained from the measurement of serum TNFα levels, in which we detected significantly elevated TNFα serum levels in CCA patients at T0, that were restored at T1. Indicating an important role of peripheral TNFα in CCA associated inflammatory response.

In addition, we detected elevated circulating levels of CXCL10 in HCC patients, before surgery, that were only partially restored at T1. CXCR3 is the receptor for CXCL10 (also known as interferon-induced protein-10, IP-10), a member of the CXC chemokine family with pro-inflammatory and anti-angiogenic properties [31] that has been associated with a variety of human diseases, including tumor development, metastasis, and dissemination. More importantly, CXCL10 has been identified as a major biological marker mediating disease severity and may be used as a prognostic indicator for various diseases [32], thus, its possible role as disease severity biomarker for liver cancers should be further evaluated. Moreover, circulating levels of CCL20 in HCC patients before and after tumor resection were significantly increased, in comparison to the HG, as well as in CCA patients at T0, which presented a partial recovery after tumor resection. CCL20, alternatively called liver activation regulated chemokine (LARC) or macrophage inflammatory protein-3α (MIP3α), is a strong chemoattractant for leukocytes that express its sole receptor CCR6 [33]. The CCL20/CCR6 axis has been shown in many studies to contribute to the initiation and progression of HCC by mediating the migration of circulating T cells into the tumor microenvironment [34]. Thus, the observed increase in the circulating levels of CCL20 indicates a probable migration of T cells to the tumor microenvironment of these carcinomas.

The detected increase in the circulating levels of CCL20, CXCL10, and TNFα suggests a pro-inflammatory state in HCC and CCA patients and an activate state for monocyte subsets although classical and intermediate monocyte subsets displayed a functional defect at T0 and, therefore, a limited capacity to respond under further stimulation processes.

Conclusions

This integrated analysis permitted the identification of different immune background in CCA and HCC patients, as well as altered functional competence of circulating monocytes in these carcinomas. Intriguingly, most of these alterations appear to be restored in CCA patients after tumor resection, however, most of them remained in HCC patients after surgical procedure. Moreover, this work has demonstrated that flow cytometry serves as a powerful analytical platform for the rapid characterization of individual cells within heterogenic cell populations and it may help in monitoring functional competence of immune cell populations to better evaluate immune dysfunctions in cancer patients. This approach could be applied in clinical routine to evaluate innate and adaptive immunity and to monitor responses to different treatments. In addition, the identified immune alterations should be further studied to consider clinically relevant therapeutic targets. A better understanding of the potential of inflammation and innate immunity to inhibit tumor progression should lead to the development of new and improved immunomodulatory approaches for the treatment of liver carcinomas. Notably, our results point out a potential role for TNFα as a prognostic factor in HCC patients indicating the presence of high-grade tumors. However, further studies should be conducted with larger sample sizes to validate the findings we have presented and correlate these findings with post-surgery outcomes after long-term follow-up.

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Author Contributions

CMS processed the samples, performed the cell sorting, the flow cytometry, the ELISA assays and the molecular biology analyses, analyzed the results and was a major contributor in the writing of the manuscript. RM, AMA, RCO, JGT, MFB and EF provided the biological samples, performed patients' selection, revised the clinical data and reviewed the manuscript. PL supervised the data analysis, help in sample processing and reviewed the manuscript. MC supervised the molecular biology analyses. MRD supervised the work and reviewed the manuscript. Finally, AP conceived the main idea of the work, interpreted the results and reviewed the manuscript. CMS is a PhD student in the Biochemistry program at University of Aveiro and this work is part of her doctoral thesis. All authors read and approved the final manuscript.

Competing Interest

The author(s) declare no competing interests.

Data Availability

The authors declare that the main data supporting the results of the present study are available within the article and its Supplementary Information files. Extra data are available from the corresponding author upon request.

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Figure legends

Figure 1. Phenotypic and functional characterization of circulating monocyte subsets. (**A)** Bivariate dot plot histograms illustrating the identification of circulating leukocyte subsets: eosinophils (light pink events), neutrophils (yellow events), classical monocytes (blue events), intermediate monocytes (light green events), non-classical monocytes (orange events), myeloid dendritic cells (mDCs, light blue events), lymphocytes (purple events), and basophils (green events), and an example bivariate dot plot histogram illustrating TNFα production (indicated with dashed rectangle) by classical monocytes after stimulation with LPS and IFN-γ. (**B)** Boxplots with the frequency of classical monocytes, intermediate monocytes with mid-level surface expression of HLA-DR ($HLADR^{+}$), intermediate monocytes presenting high-level surface expression of HLA-DR ($HLADR^{++}$) and non-classical monocytes producing TNFα, after stimulation with LPS plus IFNγ, in cholangiocarcinoma (CCA) and hepatocellular carcinoma (HCC) patients, both at T0 and T1, and in the healthy group (HG). Statistically significant differences were considered when $p < 0.05$; * between the groups indicated in the figure and the HG.

Figure 2. Gene expression in purified classical, intermediate and non-classical monocyte subsets. (**A)** Boxplots with the mRNA expression levels of CX3CR1 and TNFα in classical, intermediate and non-classical monocytes purified from CCA and HCC patients, at T0 and T1, and from the HG. Statistically significant differences were considered when $p < 0.05$; * between the groups indicated in the figure and the HG; # between T0 and T1. (**B)** Boxplots with the mRNA expression levels of TNFα in classical, intermediate and non-classical monocytes purified from CCA and HCC patients, at T0, comparing G1-G2 *versus* G3-G4 tumor histopathological grades. Statistically significant differences were considered when $p < 0.05$; * between the groups indicated in the figure.

Figure 3. Quantification of the serum levels of CCL2, CCL20, CXCL10 and TNFα. (**A)** Boxplots with serum levels of CCL2, CCL20, CXCL10 and TNFα, measured by ELISA in serum samples from cholangiocarcinoma (CCA) and hepatocellular carcinoma (HCC) patients, at T0 and T1, and from the healthy group (HG). Statistically significant differences were considered when $p < 0.05$; *between the groups indicated in the figure and the HG; #between CCA and HCC at the same time point. **(B)** Boxplots with serum levels of TNFα, measured in CCA and HCC patients, at T0, comparing G1-G2 *versus* G3-G4 tumor

histopathological grades. Statistically significant differences were considered when $p < 0.05$; * between the groups indicated in the figure.

Tables

Table 1. Clinical data from hepatocellular carcinoma (HCC) and cholangiocarcinoma (CCA) patients enrolled in this study. Number of patients and frequencies (%) are indicated.

		CCA N = 8	**HCC N = 20**
TNM	Stage I	1 (13%)	3 (15%)
	Stage II	4 (50%)	15 (75%)
	Stage IIIA	0 (0%)	1 (5%)
	Stage IV	3 (38%)	1 (5%)
Histologic grade (G)	G1	2 (25%)	2 (10%)
	G2	3 (38%)	11 (55%)
	G3	3 (38%)	6 (30%)
	G4	0 (0%)	1 (5%)
HBsAg	Positive	0 (0%)	1 (5%)
HCV	Positive	0 (0%)	6 (30%)
Vascular microinvasion	Positive	-	8 (40%)
Cirrhosis		0 (0%)	15 (75%)
Relapse		3 (38%)	1 (5%)
Death		3 (38%)	2 (10%)
Liver Transplant		0 (0%)	7 (35%)

Table 2. Frequency (%) and absolute numbers (cells/ µL) of peripheral blood monocyte subsets in cholangiocarcinoma (CCA) and hepatocellular carcinoma (HCC) patients, both at the time of surgical procedure (T0) and once the patients were recovered from surgery (T1), and in healthy individuals (HG). Frequencies of monocyte subsets are related to the total of monocytes.

	CCA N = 8		**HCC N = 20**		**HG N = 10**
	T0	**T1**	**T0**	**T1**	
% Monocytes (in whole blood)	**3,0 ± 2,0[a,b]**	**5,3 ± 2,0[a]**	**4,8 ± 2,3[a,b]**	**5,4± 2,4[a]**	8,4 ± 1,7
Monocytes/µL	**220 ± 140[a]**	**229 ± 62[a]**	**415 ± 364[a]**	377 ± 207	516 ± 134
% Classical	88 ± 8	84 ± 6	**85 ± 13[a]**	**88 ± 7[a]**	80 ± 8
Classical monocytes/µL	**156 ± 95[a,b]**	**179 ± 66[a]**	381 ± 312[c]	317 ± 182	416 ± 131
% Intermediate	5,8 ± 5,2	8,0 ± 3,5	6,4 ± 9,5	6,0 ± 3,9	5,4 ± 2,1
Intermediate monocytes/µL	**13 ± 14[a]**	**13,8 ± 3,4[a]**	32,6 ± 52,2	27,5 ± 25,7	26,0 ± 11,3
%HLADR^{+}	40 ± 20	46 ± 12	**46 ± 14[a]**	46 ± 19	34 ± 12
%HLADR^{++}	60 ± 20	54 ± 12	**54 ± 14[a]**	54 ± 19	66 ± 12
% Non-Classical	**4,7 ± 2,7[a]**	6,5 ± 5,4	**5,2 ± 5,3[a]**	**4,7 ± 3,6[a]**	11,6 ± 6,7
Non-classical monocytes/µL	**10 ± 9[a]**	**11,1 ± 9,4[a]**	**20 ± 19[a]**	**18,7± 17,9[a]**	56,3 ± 20,7

Independent-samples Mann-Whitney U test was performed to compare: each group of patients vs healthy group (a); CCA vs HCC (b), The non-parametric Wilcoxon signed-rank test of non-independent data was performed to compare T1 vs T0 (c), all of them with a significance level of 0.05 ($p<0.05$). The results are given by mean ± standard deviation.

Figures

Figure 1

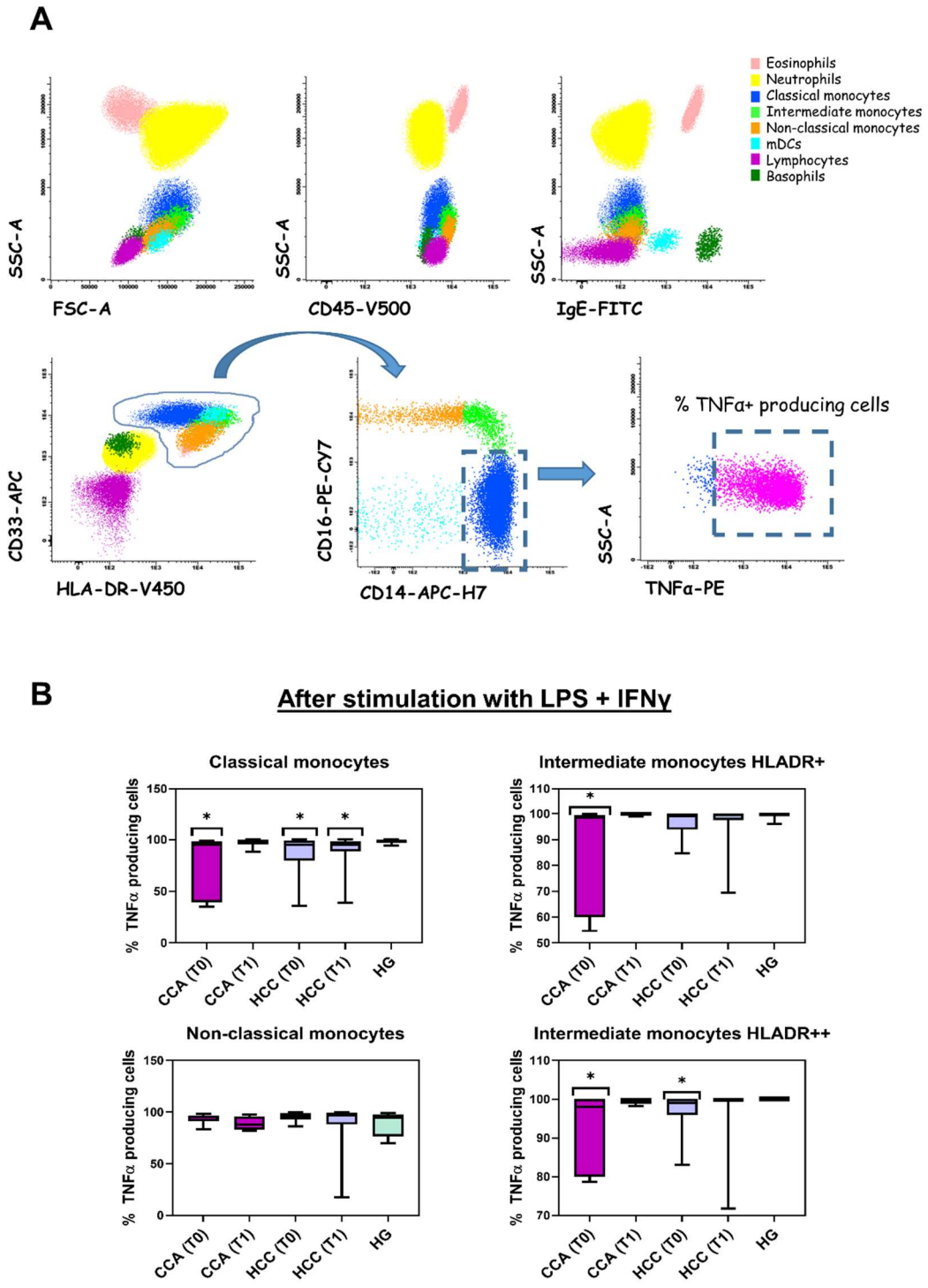


Figure 2

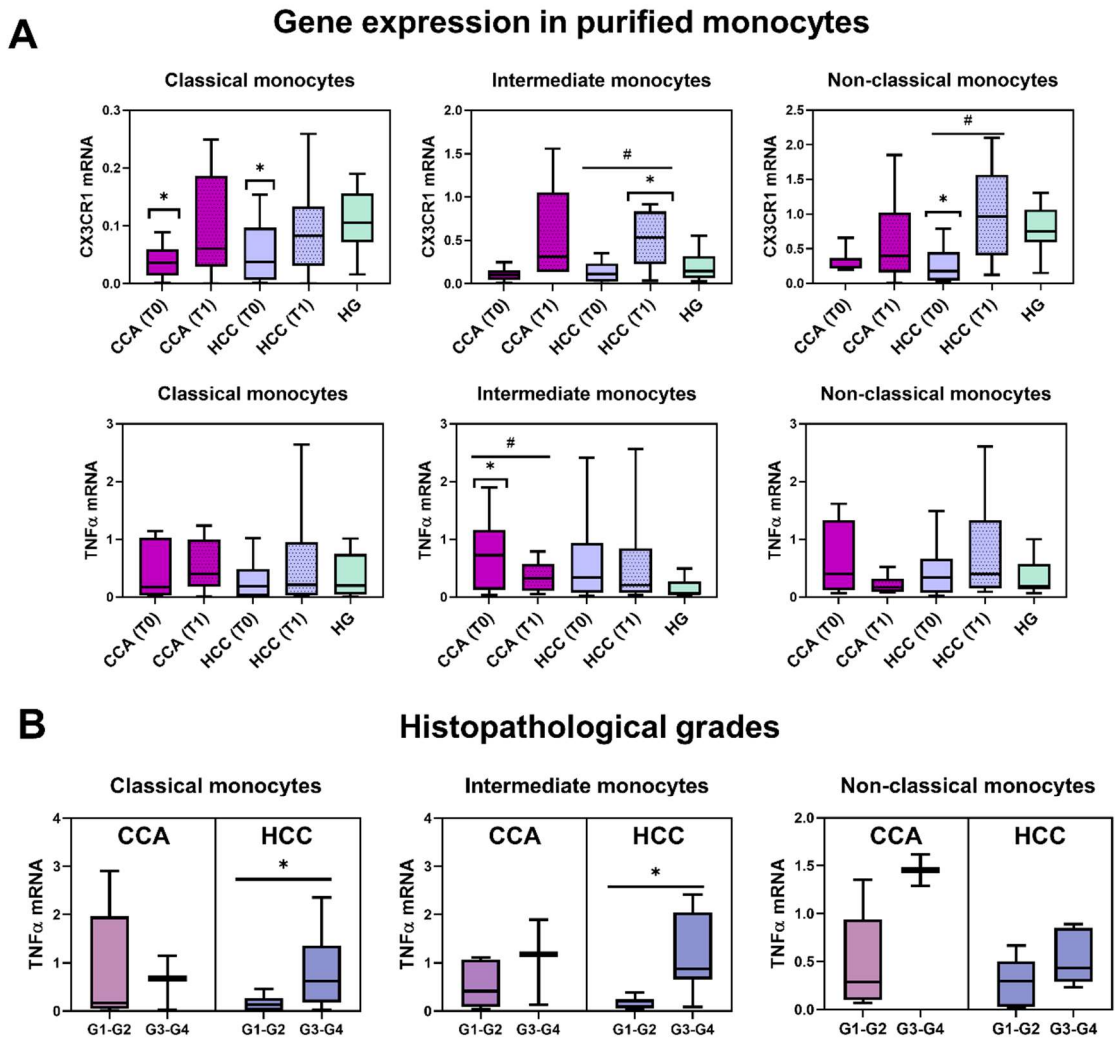
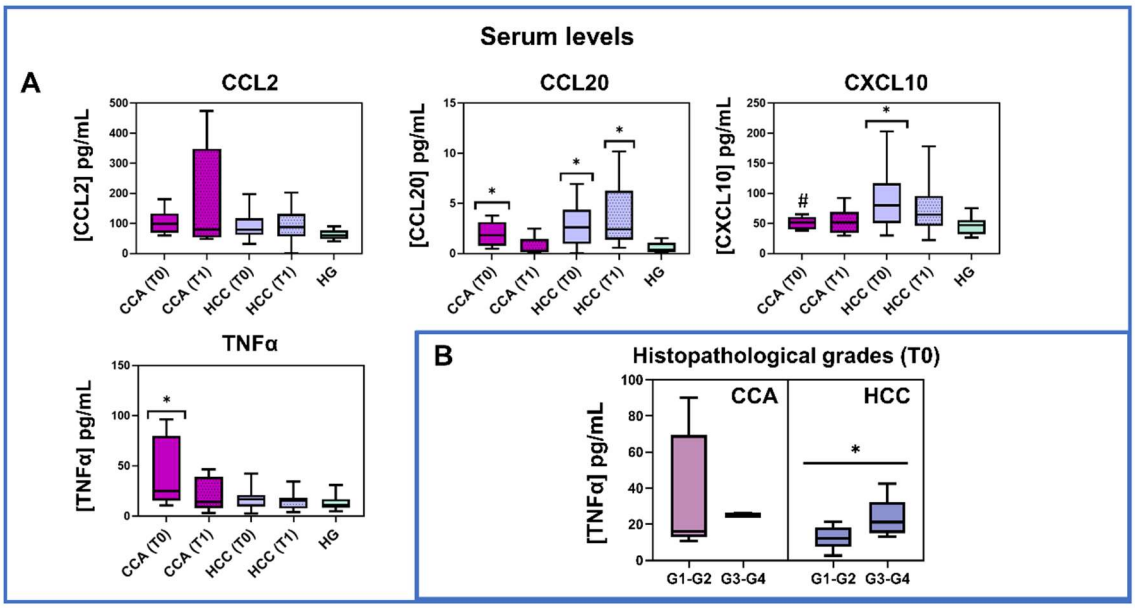

A
Gene expression in purified monocytes
Classical monocytes
Intermediate monocytes
Non-classical monocytes
CX3CR1 mRNA
TNFα mRNA
CCA (T0)
CCA (T1)
HCC (T0)
HCC (T1)
HG
B
Histopathological grades
CCA
HCC
G1-G2
G3-G4

Figure 3.


Serum levels
A
CCL2
[CCL2] pg/mL
CCL20
[CCL20] pg/mL
CXCL10
[CXCL10] pg/mL
TNFα
[TNFα] pg/mL
CCA (T0)
CCA (T1)
HCC (T0)
HCC (T1)
HG
B
Histopathological grades (T0)
CCA
HCC
G1-G2
G3-G4

Elevated soluble TNFα levels and upregulated TNFα mRNA expression in purified peripheral blood monocyte subsets associated with high-grade hepatocellular carcinoma

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Supplementary material

Table S1. Panel of mAb reagents (with clones and commercial source) used for the immunophenotypic characterization and for cell purification by fluorescence-activated cell sorting of tumor cells and immune cells.

Tube	V450	V500	FITC	PE	PE-Cy7	APC	APC-H7
	HLA-DR	**CD45**	**IgE**	**TNFα**	**CD16**	**CD33**	**CD14**
1	L243	2D1	BE5	MAb11	3G8	P67.6	MφP9
	BD	BD	EXBIO Praha	BD	BD	BD	BD
	HLA-DR	**CD45**			**CD16**		**CD14**
2	L243	2D1			3G8		MφP9
	BD	BD			BD		BD

Commercial sources: BD (Becton Dickinson Biosciences, San Jose, CA, USA), EXBIO Praha (Vestec, Czech Republic). APC, allophycocyanin; APC-H7, allophycocyanin-hilite 7; FITC, fluorescein isothiocyanate; PE, phycoerythrin; PE-Cy7, phycoerythrin-cyanine 7.

CHAPTER 6. TUMOR RESECTION IN HEPATIC CARCINOMAS RESTORES CIRCULATING T REGULATORY CELLS FREQUENCY

This Chapter has been submitted as follows:

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Tumor resection in hepatic carcinomas restores circulating T regulatory cells frequency

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Abstract

Hepatocellular carcinoma (HCC), along with cholangiocarcinoma (CCA), represents a major primary liver cancer. The liver is considered as an immunogenic organ, and it is one of the most vital organs in the human body. T regulatory (Treg) cells play an important role in liver cancers through the immunosuppression of protective immune responses. The current study focuses on the characterization of circulating Treg cells in HCC and CCA patients, before and after surgical procedure, in order to understand the effect of tumor resection in the peripheral T cells homeostasis. We developed a whole-blood assay to monitor immune alterations and functional competence of peripheral lymphocytes, mainly focusing on Treg cells in a group of 10 healthy individuals, in 20 HCC patients and 8 CCA patients, before and one month after surgical procedure, by multi-color flow cytometry, cell sorting, and qRT-PCR. We did not detect any functional impairment on circulating Treg cells neither in CCA nor in HCC patients. However, this analysis permitted the identification of decreased frequencies of circulating Treg cells in HCC and CCA patients before tumor resection. In addition, we observed that the frequency of peripheral Treg was restored after tumor resection, indicating mechanisms of immune modulation induced by tumor resection that should be further studied.

Keywords: Flow cytometry; qRT-PCR; hepatocellular carcinoma; cholangiocarcinoma; Treg cells.

Abbreviations[2]

[2] CCA, cholangiocarcinoma; FACS, fluorescence-activated cell sorting; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; HCC, hepatocellular carcinoma; HG, control group or healthy group; LARC, liver activation regulated chemokine; mAbs, monoclonal antibodies; MIP3α, macrophage inflammatory protein-3α; NK, Natural Killer; PB, peripheral blood; PBS, phosphate-buffered saline; PMA, phorbol myristate acetate.

Introduction

One of the most vital organs in the human body is the liver which is considered as an immunogenic organ [1]. Hepatocellular carcinoma (HCC), the most frequent liver cancer, is one of the most prevalent cancers worldwide and it is still considered the second leading cause of cancer- related death worldwide [2,3]. Cholangiocarcinoma (CCA), the second most common liver cancer, is an aggressive cancer that originates from biliary epithelia presenting a high mortality rate [4,5]. The only effective approaches for patients with HCC or CCA are tumor resection or liver transplantation, therefore, there is a need for novel therapeutic options, such as immunotherapies [6]. The tumor-mediated immunosuppression or the direct suppression of effector cells is one of the most important mechanisms proposed for an attenuated immune response against tumors [7]. The local lymphocyte population in the liver is enriched in natural killer (NK) and NKT cells, which might have essential roles in the recruitment of circulating T cells [8]. Marked infiltration of T regulatory (Treg) cells, which usually suppress the inflammation resulting from innate and adaptive immunity, has been observed in the livers from patients with HCC, and the number of intra-tumor Treg cells is increased compared with the peri-tumor regions and the periphery [1]. Treg cells are a subgroup of $CD4^+$ T cells characterized by the expression of the interleukin 2 receptor α chain (CD25), the intracellular expression of FoxP3 and the low expression or absence of alpha chain of the IL-7R (CD127) [9]. This cell subset suppresses activity and proliferation of both $CD4^+CD25^-$ and $CD8^+$ T cells [10]. There is accumulating clinical pieces of evidence that Treg cells play an important role in liver cancers such as HCC, through the inhibition of protective non-specific and tumor-specific immune responses [11]. Considering the importance of Treg cells in inhibiting an effective antitumor immune response, we have studied the presence and the function of this cell subset in peripheral blood (PB) of HCC and CCA patients before and after tumor resection and, in a group of age and sex-matched healthy adults (HG), in order to understand the immune status of these patients, and to obtain evidence about the changes in the proportions or functions of this population after tumor resection. Monitoring of the peripheral immune response after surgical resection will provide information to

advance our understanding of the mechanisms underlying the clinical response to surgical resection in these carcinomas.

Patients and methods

Patients and healthy individuals

A total of 20 patients with HCC (3 women and 17 men; average age: 62.2 ± 14.5 years) and 8 patients with CCA (5 women and 3 men; average age: 61.0 ± 14.7 years) were included in this study. PB samples were collected at the time point of the surgical resection, just before the beginning of the surgical intervention (T0), and once the patient was completely recovered from the surgery (generally one month after the surgery) (T1). A group of 10 age- and sex-matched healthy individuals was included in the study as a control group (HG). No patients took any anti-tumor therapy or medication prior to surgery nor at T1. Nevertheless, 7 HCC patients underwent liver transplantation and took tacrolimus just after the surgical procedure. Tacrolimus target T lymphocytes [12,13], therefore, we have excluded HCC patients that underwent liver transplantation from the comprehensive analysis at T1.

The clinical background of the patients included in this study is summarized in Table 1.

The study protocol was approved by the Ethical Committee from the Faculty of Medicine, University of Coimbra, (Coimbra, Portugal). Informed consent was obtained from all individual participants included in the study.

Circulating Treg cells characterization

Circulating Treg cells were characterized without any stimulation. PB samples were collected from participants and healthy individuals into K3-EDTA tubes (BD) and stained by using a five-color mAbs combination, detailed in Supplementary Table S1 (tube 1). For the immunophenotyping, 300

μL of PB were incubated with the mAbs indicated in Supplementary Table S2 (tube 1) for 10 minutes in the dark at RT. Then, samples were incubated with 2 mL of FACS Lysing solution (BD) for 10 min in the dark at RT, and then centrifuged for 5 min at 540g. The supernatant was discarded, and the cell pellet was washed in 1 mL of PBS, resuspended in 500 μL of PBS and immediately acquired.

Flow cytometry data acquisition and analysis

Data acquisition was performed in a FACSCanto II flow cytometer (BD) and analyzed with Infinicyt 1.8 software (Cytognos SL, Salamanca, Spain).

Cell purification by fluorescence-activated cell sorting

Treg cell population from PB was purified by fluorescence-activated cell sorting (FACS), using a FACSAria III flow cytometer (BD) according to its typical phenotype. Thus, the five-color mAbs combination used (Supplementary Table S1, tube 1) allowed the identification of Tregs ($CD3^{+}CD4^{+}CD25^{+}CD127^{low}$).

For subsequent mRNA expression analysis, purified cells were centrifuged for 5 min at 300 g, the cell pellet was resuspended in 350 μL of RLT Lysis Buffer (Qiagen, Hilden, Germany), and stored at -80°C.

RNA isolation and quantitative real-time reverse transcriptase-polymerase chain reaction (qRT-PCR)

Total RNA was extracted and purified with RNeasy™Micro Kit (Qiagen) according to the manufacturer's instructions. Reverse transcription was performed with SensiScript Reverse Transcription Kit (Qiagen) according to supplier's instructions and with Random Hexamer Primer

(Thermo Fisher Scientific, San Jose, USA). Relative quantification of gene expression was performed in a QuantStudio (Thermo Fisher Scientific) by a real time (qRT)-PCR reaction. qRT-PCR was done with PowerUp™ SYBR™ Green Master Mix (Thermo Fisher Scientific), using optimized primers for TGFβ, FOXP3, IL-10 and endogenous control glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (Qiagen), according to the manufacturer's instructions.

Statistical analysis

For all variables under study, their mean values, standard deviation, median and range, were calculated. To determine the statistical significance of the differences observed between groups, the non-parametric Mann-Whitney and Kruskal-Wallis multiple comparison tests were performed using the Statistical Package for Social Sciences software (SPSS, version 25, IBM, Armonk, NY, USA). The non-parametric Wilcoxon signed-rank test of non-independent data were performed to compare T1 vs T0. Statistically significant differences were considered when $p < 0.05$. GraphPad Prism software was used to create the graphics.

Results

Phenotypic characterization of peripheral blood Treg cells

From the data obtained by flow cytometry immunophenotyping, circulating Treg subset was identified as displayed in Fig.1A for all individuals included in this study.

Alterations of peripheral blood Treg cells in CCA and HCC patients

We did not identify significant differences for circulating T cell subset neither for $CD4^+$ T cells nor $CD8^+$ T cells among the groups under study (Supplementary Table S2). The frequency of circulating

Tregs was significantly reduced in CCA and HCC patients at T0 in comparison to the HG (Fig.1B), but those values were restored for both CCA and HCC patients at T1 (Fig. 1B). In addition, the absolute numbers of peripheral Treg cells were significantly decreased for CCA patients at T0, as compared to the HG. While HCC patients presented the same pattern without reaching statistical significance. After tumor resection, we observed a significant recovery in the absolute counts of Treg cells for HCC patients (Fig. 1B). Regarding the functional characterization of Tregs, we did not observe any significant difference among the groups under study for IL-10, TGFβ and FOXP3 mRNA expression (Fig. 1C).

Discussion

The current study focuses on the characterization of peripheral Treg cells in HCC and CCA patients, before and after surgical procedure, in order to understand the immune status of these patients and to assess the effect of tumor resection on the T cell immune homeostasis.

We found that both CCA and HCC patients displayed a significant decrease in the frequency of circulating Treg cells, before surgical procedure, as compared to the HG, with a partial recovery of some CCA patients and, a total recovery of HCC patients after tumor resection. In addition, absolute numbers of Treg cells were decreased in HCC patients and significantly decreased in CCA patients when compared to the HG, at T0, being these values significantly increased for HCC patients after tumor resection. Previous studies in HCC found increased numbers of $CD4^+CD25^+$ regulatory T cells in their peripheral blood [14]. However, this apparent discrepancy could be explained by the different approach used for the identification of Treg cells, since in the abovementioned study all $CD4^+CD25^+$ T cells were considered Tregs. However, the $CD4^+CD25^+$ T cell subset includes both Treg cells (those presenting low expression levels or absence of CD127 and high expression levels of FoxP3, identified as Tregs in our study) and other T cells that are non-Tregs. The observed decrease in circulating Treg cells in CCA and HCC patients at T0 indicates a probable migration of this T cell

population to the tumor microenvironment. This is further supported by the observed increase in the circulating levels of CCL20 (unpublished results) and by numerous studies that have detected infiltration of Treg cells in the tumor microenvironment of HCC and CCA patients [15–17]. In this regard, the detection of high levels of intra-tumor Treg cells could be considered a promising prognostic factor in patients with hepatic carcinomas [18]. However, no previous publications have evaluated the effect of tumor resection on circulating Treg cells. Here, we first detected the recovery of Treg cells in circulation after surgical resection on HCC and CCA patients, suggesting a regulation of immune system homeostasis induced by tumor resection.

In addition, we analyzed Foxp3, IL-10, and TGFβ mRNA expression in purified Treg cells from HCC and CCA patients, as well as from healthy donors, by qRT-PCR. Foxp3 is an important transcription factor essential for immunosuppressive Treg differentiation [19]. We found elevated Foxp3 mRNA expression in all Treg cell subsets purified by cell sorting and no differences among the biological groups under study, confirming the T regulatory phenotype of the purified cell populations. Treg cells can exert their immunosuppressive function by diverse mechanisms targeting specific effector pathways, including contact-dependent mechanisms, by producing immunomodulatory cytokines, such as IL-10 and TGF-β, as well as through metabolic perturbation of target cells [20]. However, we did not detect significant alterations when evaluating Treg cells function, indicating there are not functional defects in this cell subset, neither in HCC nor in CCA patients, when IL-10 and TGFβ mRNA expression is concerned.

Regarding circulating lymphocytes, a great heterogeneity among the frequencies of T lymphocyte subsets present in different cancer patients was observed. Notably, we detected a decrease in the frequency of CD4 T lymphocytes with the plasticity to produce both IFNγ and IL-17 cytokines, together with a decrease in the frequency of Tc17 cells in CCA and HCC patients. In this regard, we hypothesized that the observed decrease in these cell subsets could be due to a specific migration to the tumor microenvironment which is also supported by the elevated circulating levels of CCL20 in HCC and CCA patients (unpublished results).

A better understanding of the potential of inflammation to react and inhibit tumor progression should lead to the development of new and improved immunomodulatory approaches for the treatment of liver carcinomas. Since tumor resection and liver transplant are the most applied treatments for liver carcinomas [21,22], it is important to assess the effect of these therapies on the patient immune system homeostasis.

Conclusions

In conclusion, our results have demonstrated that surgical resection partially restores immune system homeostasis in CCA and HCC patients. However, further studies with larger sample sizes and after long-term follow-up are needed to validate the findings presented here.

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Declaration of Competing Interest

The authors declare that there is no conflict of interests regarding the publication of this paper.

Authors contribution

C.M.S. processed the samples, performed the cell sorting, the flow cytometry and the molecular biology analyses, analyzed the results and was a major contributor in the writing of the manuscript. R.M., A.M.A., R.C.O., J.G.T., M.F.B. and E.F. provided the biological samples, performed patients' selection, revised the clinical data and reviewed the manuscript. P.L. supervised the data analysis, help in the study design in sample processing and reviewed the manuscript. M.C. supervised the molecular biology analyses. M.R.D. supervised the work and reviewed the manuscript. A.P. conceived the main idea of the work, interpreted the results and reviewed the manuscript. C.M.S. is a PhD student in the Biochemistry program at the University of Aveiro and this work is part of her doctoral thesis. All authors read and approved the final manuscript.

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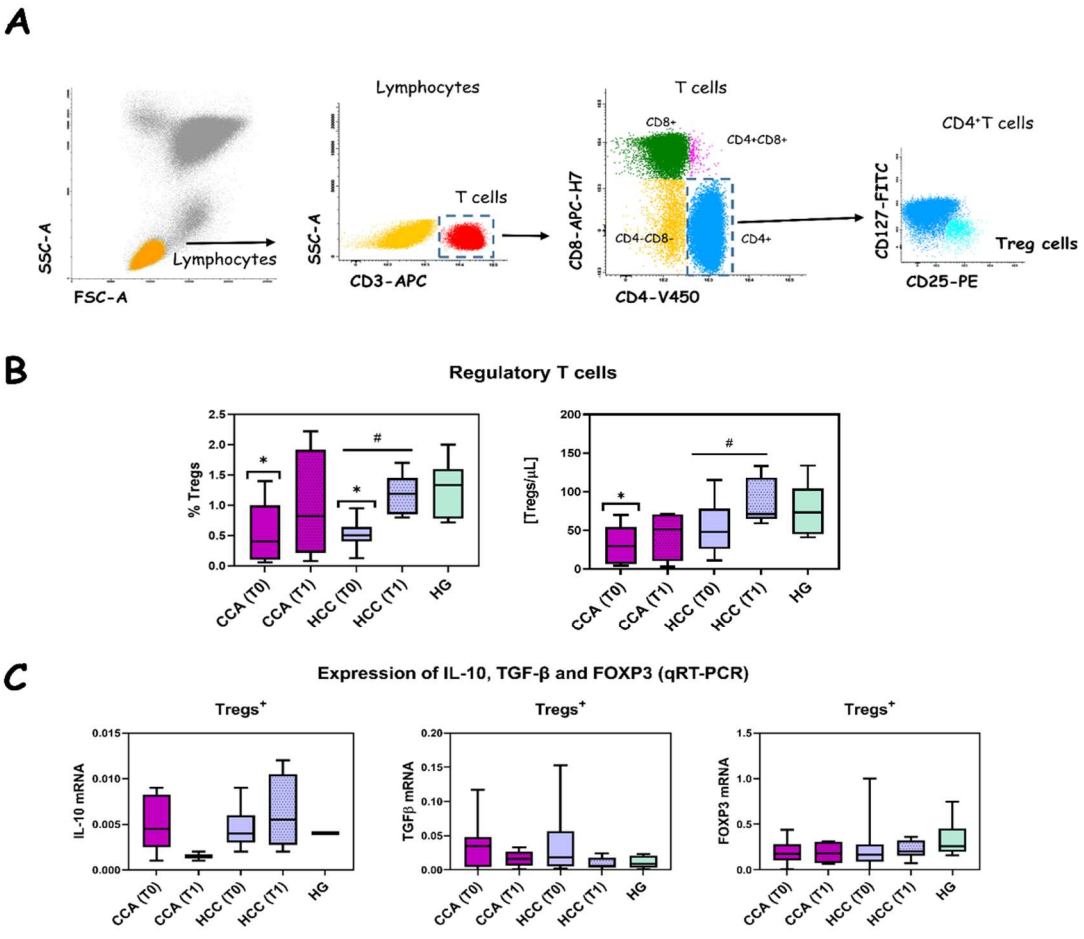
Figure legends

Fig.1 Characterization of circulating Tregs. **(A)** Bivariate dot plot histograms illustrating the identification of circulating lymphocytes (orange events), T lymphocytes (red events), $CD4^+$ T cells (blue events) and Treg cells (light blue events) by flow cytometry. **(B)** Boxplots with the frequency (%) and absolute numbers (cells/µL) of circulating T regulatory cells (Tregs) in cholangiocarcinoma (CCA) and hepatocellular carcinoma (HCC) patients, at T0 and T1, and in the healthy group (HG). **(C)** Boxplots with the mRNA expression levels of IL-10, TGFβ and FOXP3 among Tregs purified from CCA and HCC patients, at T0 and T1, and from the HG. Statistically significant differences were considered when $p < 0.05$; *between the groups indicated in the figure and the HG; #between T0 and T1.

Figures



Tables

Table 1. Clinical data from hepatocellular carcinoma (HCC) and cholangiocarcinoma (CCA) patients enrolled in this study. Number of patients and frequencies (%) are indicated.

		CCA N = 8	**HCC N = 20**
TNM	Stage I	1 (13%)	3 (15%)
	Stage II	4 (50%)	15 (75%)
	Stage IIIA	0 (0%)	1 (5%)
	Stage IV	3 (38%)	1 (5%)
Histologic grade (G)	G1	2 (25%)	2 (10%)
	G2	3 (38%)	11 (55%)
	G3	3 (38%)	6 (30%)
	G4	0 (0%)	1 (5%)
HBsAg	Positive	0 (0%)	1 (5%)
HCV	Positive	0 (0%)	6 (30%)
Vascular microinvasion	Positive	-	8 (40%)
Cirrhosis		0 (0%)	15 (75%)
Relapse		3 (38%)	1 (5%)
Death		3 (38%)	2 (10%)
Liver Transplant		0 (0%)	7 (35%)

Tumor resection in hepatic carcinomas restores circulating T regulatory cells frequency

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Supplementary material

Methods

In vitro stimulation of cytokine production by circulating T lymphocytes and NK cells

To evaluate cytokine production by circulating T cells and natural killer (NK) cells, lymphocytes were stimulated by adding phorbol myristate acetate (PMA: 0.25 μg/mL, Sigma-Aldrich) and ionomycin (1 μg/mL, Boehringer Mannheim, Germany) to 250 μL of PB previously diluted 1:1 (v/v) in RPMI 1640 complete culture medium (Invitrogen). Brefeldin-A (10 μg/mL, Sigma-Aldrich) was also added to prevent the release of the newly synthesized cytokines. All samples were then incubated in a sterile environment and 5% CO_2 humid atmosphere, at 37°C, for 4 hours.

Immunophenotypic analysis of circulating T and NK cells, cultured in the presence of PMA plus ionomycin, was performed by using a seven-color mAbs combination, detailed in Supplementary Table S1 (tube 2). Cells were stained with the mAbs for surface proteins antigens (CD45, CD4, CD56, CD3, and CD8) and, after an incubation period of 10 minutes at room temperature (RT) in the dark, washed with PBS. For intracellular staining, Fix&Perm (Life Technologies) reagent was used, in accordance with the instructions of the manufacturer and samples were stained with the mAbs for IFN-γ and IL-17 (Supplementary Table S1, tube 2). After washing twice with PBS, the cell pellet was resuspended in 500 μL of PBS and immediately acquired.

T cells were identified based on CD3 and CD45 positivity and intermediate FSC and SSC properties. Within this cell population, $CD4^+$, $CD8^+$ and $CD4^+CD8^+$ T cell subsets (phenotypically characterized as $CD3^+CD4^+CD8^-$, $CD3^+CD4^-CD8^+$ and $CD3^+CD4^+CD8^+$, respectively) were identified. NK cells were identified on the basis of CD45 and CD56 positivity, absence of CD3 expression and intermediate FSC and SSC properties.

Results

Alterations of peripheral blood lymphocyte subsets in CCA and HCC patients

Regarding T lymphocyte subsets, the frequency of $IFN\gamma^{+}IL\text{-}17^{+}$ CD4 T cells was significantly decreased in CCA patients at T0 comparatively to the HG. Moreover, the frequency of $IL\text{-}17^{+}$ CD8 T cells was significantly decreased in CCA patients, both at T0 and T1, comparatively to the HG. HCC patients also showed lower levels of circulating $IFN\gamma^{+}IL\text{-}17^{+}$ CD4 T cells and $IL\text{-}17^{+}$ CD8 T cells, both at T0 and T1, without reaching statistical significance (Supplementary Table S2).

Finally, regarding NK characterization, we detected an increased relative frequency of peripheral $CD56^{BRIGHT}CD8^{+}$ NK cells in both CCA and HCC patients, only reaching statistical significance for HCC patients at T1 (Supplementary Table S3).

Tables

Table S1. Panel of mAb reagents (with clones and commercial source) used for the immunophenotypic characterization and for cell purification by fluorescence-activated cell sorting of tumor cells and immune cells.

Tube	V450	V500	FITC	PE	PE-Cy7	APC	APC-H7
1	**CD4** RPA-T4 BD		**CD127** HIL-7R-M21 BD Pharmingen	**CD25** B1.49.9 Beckman Coulter		**CD3** SK7 BD	**CD8** SK1 BD
2	**CD4** RPA-T4 BD	**CD45** 2D1 BD	**cyIFNγ** 4S.B3 BD Pharmingen	**cyIL-17** SCPL1362 BD Pharmingen	**CD56** N901 Beckman Coulter	**CD3** SK7 BD	**CD8** SK1 BD

Commercial sources: BD (Becton Dickinson Biosciences, San Jose, CA, USA), BD Pharmingen (San Diego, CA, USA), Beckman Coulter (Miami, FL, USA). APC, allophycocyanin; APC-H7, allophycocyanin-hilite 7; FITC, fluorescein isothiocyanate; PB, pacific blue; PE, phycoerythrin; PE-Cy7, phycoerythrin-cyanine 7.

Table S2. Frequency (%) of peripheral blood lymphocyte subsets present in cholangiocarcinoma (CCA) patients and hepatocellular carcinoma (HCC) patients at the time of surgical procedure (T0) and once the patients were recovered from surgery (T1), and in a group of healthy individuals (HG). Absolute numbers ([], number of cells/µL) of peripheral blood lymphocytes and T lymphocytes, as well as protein expression levels of IFN-γ and IL-17 (measured as mean fluorescence intensity, MFI), are also indicated.

		CCA N = 8		**HCC N = 20**		**HG N = 10**
		T0	T1	T0	T1	Mean ± SD
% Lymphocytes		33 ± 14	37 ± 12	29 ± 14	28 ± 9	42 ± 8
[Lymphocytes/µL]		**1903 ± 585[a]**	1253 ± 127	2730 ± 2798	2220 ± 904	2392 ± 500
% T lymphocytes		22 ± 12	27 ± 11	20 ± 9	21 ± 9	29 ± 8
[T Lymphocytes/µL]		1305 ± 520	1281 ± 882	1619 ± 977	1631 ± 802	1539 ± 395
% $CD4^+$		57 ± 19	59 ± 16	61 ± 16	63 ± 15	66 ± 10
	% $IFN\gamma^+$	15 ± 9	19 ± 6	23 ± 15	20 ± 14	19 ± 8
	MFI IFNγ	6667 ± 2294	8862 ± 2543	6887 ± 3206	7286 ± 3823	8531 ± 1983
	% $IL\text{-}17^+$	0.90 ± 0.51	1.45 ± 0.94	1.42 ± 1.15	1.14 ± 0.82[b]	1.63 ± 1.45
	MFI IL-17	557 ± 110	619 ± 131	621 ± 173	514 ± 159	534 ± 124
	% $IFN\gamma^+IL\text{-}17^+$	**0.11 ± 0.14[a]**	0.15 ± 0.11	0.21 ± 0.28	0.16 ± 0.11[b]	0.29 ± 0.22
% $CD8^+$		39 ± 20	37 ± 17	32 ± 17	32 ± 16	28 ± 8
	% $IFN\gamma^+$	43 ± 23	59 ± 19	52 ± 26	47 ± 35	47 ± 13
	MFI IFNγ	5512 ± 2714	7189 ± 2313	5280 ± 3061	5487 ± 2886	6630 ± 1836
	% $IL\text{-}17^+$	**0.12 ± 0.17[a]**	**0.08 ± 0.04[a]**	0.15 ± 0.19	0.17 ± 0.15[b]	0.20 ± 0.12
	MFI IL-17	365 ± 162	540 ± 211	470 ± 152	385 ± 118	438 ± 190
	% $IFN\gamma^+IL\text{-}17^+$	0.08 ± 0.15	0.03 ± 0.02	0.08 ± 0.013	0.06 ± 0.06	0.09 ± 0.06
	%$CD56^+$	11.6 ± 6.7	9 ± 5	16 ± 14	17 ± 14	10 ± 9
$CD4^+$ $CD8^+$		1.5 ± 0.8	1.2 ± 0.26	2.2 ± 1.0	1.5 ± 2.1	1.0 ± 0.8
	% $IFN\gamma^+$	35 ± 25	44 ± 15	44 ± 29	32 ± 19	41 ± 16
	MFI IFNγ	5545 ± 2485	6513 ± 2684	6049 ± 3258	5660 ± 3172	6941 ± 1858
	%$CD56^+$	13 ± 14	12 ± 6	14 ± 11	11 ± 8	12 ± 9

Independent-samples Mann-Whitney U test was performed to compare: each group of patients vs healthy group (a) with a significance level of 0.05 (p<0.05). The non-parametric Wilcoxon signed-rank test of non-independent data was performed to compare T1 vs T0 (b) with a significance level of 0.05 (p<0.05). The results are given by mean ± standard deviation (SD).

Table S3. Frequency (%) of peripheral blood natural killer (NK) cell subsets present in cholangiocarcinoma (CCA) patients and hepatocellular carcinoma (HCC) patients at the time of surgical procedure (T0) and once the patients were recovered from surgery (T1), and in a group of healthy individuals (HG). Protein expression levels of IFN-γ and IL-17 (measured as mean fluorescence intensity, MFI), are also indicated.

	CCA N = 8		**HCC N = 20**		**HG N = 10**
	T0	**T1**	**T0**	**T1**	**Mean ± SD**
% NK (Leukocytes)	2.6 ± 2.6	1.7 ± 0.9	3.2 ± 2.3	3.0 ± 1.7	2.3 ± 1.0
% $CD56^{DIM}$	82 ± 13	83 ± 12	91 ± 6	92 ± 4	88 ± 5
% IFNγ+	30 ± 27	54 ± 26	39 ± 29	35 ± 27	42 ± 25
MFI IFNγ+	1705 ± 766	2063 ± 888	1704 ± 761	1694 ± 583	1913 ± 604
% $CD8^+$	4.1 ± 3.7	6.4 ± 4.0	3.4 ± 3.4	3.1 ± 3.8	2.3 ± 2.2
$HLADR^+$	2.3 ± 1.6	2.1 ± 1.9	1.2 ± 1.5	0.9 ± 0.8	2.0 ± 1.8
%$CD56^{BRIGHT}$	17 ± 13	17 ± 13	8.7 ± 5.3	7.8 ± 4.4	12 ± 5
% IFNγ+	26 ± 29	34 ± 21	38 ± 30	23 ± 21	28 ± 14
MFI IFNγ+	2157 ± 952	1892 ± 997	1916 ± 1054	1805 ± 711	1939 ± 604
% $CD8^+$	5.6 ± 10	4.0 ± 2.9	3.8 ± 4.1	5.8 ± 5.7[a]	1.3 ± 1.3

Independent-samples Mann-Whitney U test was performed to compare: each group of patients vs the healthy group (a) with a significance level of 0.05 (p<0.05). Moreover, the non-parametric Wilcoxon signed-rank test of non-independent data was performed to compare T1 vs T0 (b) with a significance level of 0.05 (p<0.05). The results are given by mean ± standard deviation (SD).

CHAPTER 7. TUMOR RESECTION INDUCES ALTERATIONS ON SERUM PHOSPHOLIPIDOME OF LIVER CANCER PATIENTS

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Tumor resection induces alterations on serum phospholipidome of liver cancer patients

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Abstract

Hepatocellular carcinoma (HCC) and cholangiocarcinoma (CCA) are the most common primary malignant liver tumors. Since the liver plays a key role in lipid metabolism, the study of serum phospholipid (PL) profiles may provide a better understanding of alterations in hepatic lipid metabolism. In this study, we used a high-resolution HILIC-LC-MS lipidomic approach to establish the serum phospholipidome profile of patients with liver cancer before (T0) and after tumor resection (T1) and a control group of healthy individuals (CT). After the analysis of PL profiles, we observed that the phospholipidome of patients with liver cancer was significantly modified after the tumor resection procedure. We observed an upregulation of some phosphatidylcholine (PtdCho) species, namely PtdCho(36:6), PtdCho(42:6), PtdCho(38:5), PtdCho(36:5), PtdCho(38:6) and phosphatidylcholine plasmalogens (PlsCho) and/or alkyl-acyl-phosphocholine (PakCho) in patients with liver cancer at T0 compared to the CT group, and a downregulation after tumor resection (T1) when compared to T0. These results show that LC-MS can detect different serum PL profiles in patients with liver cancer, before and after tumor resection, by defining a specific PL fingerprint that was used to determine the effect of tumor and tumor resection on lipid metabolism.

Introduction

The liver is one of the most important organs of energy metabolism. In fact, most plasma apolipoproteins, endogenous lipids and lipoproteins are synthesized in the liver (Bell 1979; Tietge et al. 1998), and their synthesis depends on the integrity of liver cell functions (Eisenberg and Levy 1975; Nguyen et al. 2008). In the case of hepatic cell damage and oncogenic conditions, the homeostasis of lipid and lipoprotein metabolism is impaired, resulting in alterations in the plasma composition of lipids and lipoproteins (Sherlock 1995; Jiang et al. 2006).

Liver cancer comprises a heterogeneous group of malignant tumors with different histological features, including hepatocellular carcinoma (HCC), the most common type of liver cancer, and cholangiocarcinoma (CCA), the second most common liver cancer (Sia et al. 2017). In some patients with HCC and CCA, partial surgical resection and liver transplantation are potentially curative treatments (Lubezky et al. 2015; Zhang et al. 2018). Unfortunately, a high rate of postoperative tumor recurrence significantly reduces long-term survival (Zhang et al. 2018). Liver function monitoring, such as the detection of peripheral indirect markers that have been altered as a reflection of liver dysfunction, could establish new biomarkers to predict prognosis and therapeutic response in liver cancer patients. However, although changes in lipid profile and metabolism have barely been studied in HCC and CCA (Patterson et al. 2011; Muir et al. 2013; Prachayakul et al. 2016; Kwee et al. 2017; Nakagawa et al. 2018), no studies were conducted to monitor these alterations after tumor resection.

In this study, we used a high-resolution HILIC-LC-MS lipidomic approach to profile the molecular phospholipidome of serum from patients with liver cancer before and after tumor resection and a control group of healthy individuals. Determination of a phospholipid (PL) mass fingerprint could be used to determine changes in the PL profiles of patients with liver cancer, as well as to monitor the effect of surgical resection on lipid metabolism.

Materials and Methods

Participants and sample collection

A total of 7 patients with liver cancer (1 female and 6 males, mean age: 61.7 ± 12.4) were included in this study, 4 patients with CCA and 3 patients with HCC. Peripheral blood (PB) samples were collected from tubes containing coagulants for serum collection, at the time of surgical resection (T0), and once the patient was completely recovered from the surgery (T1). In addition, a group of 5 healthy individuals, matched by sex and age, was included in

the study as a control group (CT). No patient took medication before surgery, nor at T1. Serum was collected by centrifugation of PB at 2000xg for 10 min, recovering the supernatant in a new tube which was stored at -80 °C until its subsequent use in lipidomics.

All procedures were in accordance with the ethical standards of the Ethics Committee of the Faculty of Medicine of the University of Coimbra, Coimbra, Portugal (CE-136/2016), and the Helsinki Declaration of 1964 and subsequent amendments or comparable ethical standards. All participants gave their signed informed consent before entering the study.

PL extraction and quantification

Total PL extracts from patient and control serum samples were obtained using solid phase extraction (SPE) with HybridSPE-phospholipid columns (Sigma-Aldrich, SUPELCO, Bellefonte, PA, USA; U) previously used in our laboratory (Pinto et al. 2014; Anjos et al. 2019). To each 100 µL of sample, 900 µL of acetonitrile:formic acid (99:1, v/v) were added, followed by 30 s of vortex and centrifugation at 2000 rpm for 5 minutes for protein precipitation. The supernatant was transferred to the HybridSPE column, which was previously conditioned with 1 mL of acetonitrile. Then, 1 mL of acetonitrile:formic acid (99:1, v/v) and 1 mL of acetonitrile were added to wash the column. PL were eluted from the column by adding 2 mL of acetonitrile with 5% aqueous ammonia solution. The collected PL extracts were dried under a nitrogen stream and kept at -80ºC prior to LC-MS analysis.

Quantification of total PL content was performed by phosphorous measurement as previously used in our lab (Sousa et al. 2016). Firstly, 10 µL of the total PL extract previously dissolved in chloroform (300 µL) were transferred into clean glass tubes and the solvent was completely evaporated under a nitrogen stream. Next, 125 µL of 70% perchloric acid was added, and the tubes were placed in a heated block at 180°C for 45 min, for PL degradation. After cooling, 825 µL of water, 125 µL of 2.5% molybdate solution, and 125 µL of 10% ascorbic acid solution were added to each tube. The mixture was stirred on a vortex mixer after each addition. The tubes were placed in a boiling water bath for 10 min. After this period, the absorbance of all samples was read at 797 nm. The standards from 0.1 to 2 µg of phosphorus (P) were performed diluting the stock solution of sodium dihydrogen phosphate dihydrated ($NaH_2PO_4 \cdot 2H_2O$) (100 µg/mL of P) in water and perchloric acid, without performing the digestion step. The amount of PL was calculated after multiplying the amount of phosphate by 25.

High resolution LC-MS

The PL were separated by hydrophilic interaction liquid chromatography (HILIC-LC-MS) using a high-performance LC-system (HPLC) (Thermo scientific Accela ™) with an on-line autosampler coupled to the Q-Exactive® hybrid quadrupole Orbitrap® mass spectrometer (Thermo Fisher Scientific, Bremen, Germany), as previously used in our laboratory (Colombo et al. 2018; Anjos et al. 2019). Data acquisition was performed using the Xcalibur data system (V3.3, Thermo Fisher Scientific, USA). For quality control, we have checked the retention time and the peak areas of the peaks corresponding to each internal standard added for each sample. In addition, the mixture of internal standards in the same concentration as the one used for sample analysis was analyzed in the LC-MS before and after the analysis of the patients' samples. Both allowed confirming data reproducibility.

Data pre-processing and statistical analysis

Integration of the PL peaks and the assignments were performed using MZmine 2.30 (Pluskal et al. 2010). When processing raw data acquired in full MS mode, all peaks of less than 1×10^4 raw intensity were excluded. For all assignments, peaks less that 5 ppm of the exact mass of lipids were considered.

To normalize the data, the peak areas of the extracted ion chromatograms (XIC) of the PL precursors of each class, were divided by the total ion current (Wong et al. 2005). The whole list of the identified PL species can be found in Supplementary Table S2, the total chain length (C) and degree of unsaturation (N) are indicated as composition C: N.

Multivariate and univariate analyzes were performed using Rstudio version 1.1.4 (version R 3.4.2 downloaded from https://www.R-project.org) with the packages RFmarkerDetector, FactoMineR, Factoextra and Ropls.

Results and Discussion

Extracts of PL obtained by SPE were quantified by phosphorus assays to determine the total content of PL recovered from each sample (Supplementary Tables S1). The total amount of PL obtained was significantly lower in the T0 liver cancer samples compared to the control (CT) group ($p < 0.01$), which is consistent with previous findings in which plasma concentrations of different lipids and lipoproteins have been found to be slightly to

significantly decreased in patients with liver cancer (Jiang et al. 2006). Since the liver plays a vital role in the production and catabolism of plasma lipoproteins and apolipoproteins, under hepatic cancer conditions, these functions may be altered leading to lower production of plasma lipids. After surgery (T1), the amount of PL increased to a value similar to that of the control samples (Fig.1). No significant difference was observed in the total amount of PL between CT and T1, suggesting a trend toward recovery of liver function after surgery, since the liver is one of the most important organs responsible for producing lipids (Nguyen et al. 2008).

The phospholipidome profile of the serum samples was identified by high resolution HILIC-LC-MS. A total of 160 ions attributed to molecular species of PL have been identified, belonging to 5 different classes: phosphatidylcholine (PtdCho or PC, 70); phosphatidylethanolamine (EtnGpl or PE, 35); phosphatidylinositol (PtdIns or PI, 9); lyso-PC (LPC, 26); phosphatidylserine (PtdSer or PS, 1); and sphingomyelin (CerPCho or SM, 19). The list of the 160 identified and quantified PL species can be found in Supplementary Tables S2.

To reduce the dimensionality of the data and to visualize the clustering of the samples, we performed a principal component analysis (PCA) on the phospholipidomic dataset. The PCA bi-plot for principal components 1 and 2 showed that the eigenvalues of the two first principal components represented 64.8% of the total variance (PC1 41.1%; PC2 23.7%) of the observations (Fig. 2). The PCA bi-plot shows segregation of the T0 and T1 groups along the second dimension, whereas, in the CT group, this dimension is influenced by the values of the means within the cohort. These results suggest that T0 group is more related to the CT than to the T1 group. Species and coefficients (loadings) of component 2 of the PCA (>1 % contribution) are shown in Supplementary Table S3.

The major 16 estimated coefficients (loadings) of component 2 of the PCA (>1.8% contribution) are shown in Supplementary Table S4 and, in the box plots in Fig.3, displaying a slight increase in the relative content of these PtdCho and PlsCho species in patients with liver cancer at T0 compared to the CT group (Fig.3), being many of these abundances (T0) significantly decreased at T1. As it can be observed, the main contributors in the component 2 were the following 16 species: PlsCho(38:5)/PakCho(38:6) *m/z 792.592*, PlsCho(38:4)/PakCho(38:5) *m/z 794.606*, PlsCho(40:4)/PakCho(40:5) *m/z 822.637*, PlsCho(40:5)/PakCho(40:6) *m/z 820.621*, PlsCho(36:4)/PakCho(36:5) *m/z 766.575*, PlsCho(42:4)/PakCho(42:5) *m/z 850.669*, PtdCho(36:6) *m/z 778.537*, PlsCho(42:6) *m/z 846.638*, PtdCho(42:6) *m/z 862.632*, PlsCho(44:4)/PakCho(44:5) *m/z 878.705*,

PlsCho(42:5)/PakCho(42:6) *m/z 848.655*, PtdCho(38:5) *m/z 808.585*, PtdCho(36:5) *m/z 780.550*, PlsCho(46:6)/PakCho(46:7) *m/z 902.702*, PlsCho(44:6)/PakCho(44:7) *m/z 874.670* and PtdCho(38:6) *m/z 806.570*. Among these 16 species, 11 were phosphatidylcholine plasmalogens (PlsCho) and 5 were PtdCho with long chain FA and polyunsaturated FA (PUFA).

Univariate analysis (non-parametric ANOVA-type statistic with repeated measurements approximation calculated for time effects) showed a significant decrease in the relative content of circulating PtdCho species with PUFA, as well as several phosphatidylcholine plasmalogens (PlsCho) species from patients with liver cancer after tumor resection (T1), when compared to T0 (Fig.4). The phosphatidylcholine species with the most significant differences are shown in Fig.4. Similarly, as observed from PCA analysis, among the 15 species more discriminant, there are 9 phosphatidylcholine plasmalogens (PlsCho) showing decreased relative content at T1 as compared to T0.

The most abundant PL class in mammalian cells is PtdCho, comprising 40-50% of the total number of PL. In plasma, specifically, phosphatidylcholine plasmalogens represent 5% of total PtdCho (Otoki et al. 2017), therefore these are minor components of the total PtdCho species. However, they showed the most significant differences in the phospholipidomic profile, displaying an upregulation in liver cancer patients at T0 compared to the CT group, and a downregulation after surgery (T1) when compared to T0. Many studies observed altered plasmalogen production in colorectal, gastric, pancreatic and esophageal cancer patients, detected in tissue, serum and plasma (Messias et al. 2018). Although the role of plasmalogens has not yet been fully elucidated, studies suggest that they are involved in cell differentiation and signaling pathways and are thought to function as endogenous antioxidants (Messias et al. 2018). Therefore, our results suggest that there is an upregulation of the production of phosphatidylcholine plasmalogens (PlsCho) in liver cancer patients at T0, probably due to an increase in oxidative stress generated by the oncogenic condition and the need to enhance antioxidant defense mechanisms (Winyard et al. 2005; Wu et al. 2019), while at T1 there is a lower production of these species indicating a decreased condition of oxidative stress. In addition, increased levels of plasmalogens in plasma were reported in gastric carcinoma patients (Yang et al. 2015) and breast cancer (Chen et al. 2016).

Increase of some PtdCho species in plasma was previously observed in liver cancer patients such as PtdCho(32:1) (Cotte et al. 2019). In our study, we detected elevated relative content of PtdCho with PUFA both by univariate and multivariate analysis in liver

cancer patients at T0 that was reverted after surgical procedure (T1) and could be related to mechanisms in response to the inflammatory state of liver cancer patients (Llovet et al. 2016), since long chain n-3 PUFA are considered anti-inflammatory due to their multiple effects on inflammation pathways, such as interference with the NF-κβ signaling system (Mullen et al. 2010) or the inhibition of tumor necrosis factor alpha (Calder and Yaqoob 2009).

The strength of these findings is limited by the small number of patients included in this study. However, this is a pilot study that indicates the feasibility to perform a larger study since interesting findings and patterns have come out despite the small number of patients analyzed. In general, our results indicate aberrant lipid metabolism in patients with liver cancer, which can be detected by minimally invasive procedure through serum phospholipidome analysis. We also observed that resection of the tumor alters the PL profile of liver cancer patients by reducing the relative content of circulating PtdCho species, particularly phosphatidylcholine plasmalogens (PlsCho) species and PtdCho species containing long-chain FA and PUFA, suggesting that alterations in PL profiles induced by hepatic carcinomas are reverted by surgical resection. In addition, this study supports the idea that lipidomics is a very useful tool to monitor the effect of surgical resection on the altered lipid metabolism of liver cancer patients.

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Figure legends

Fig. 1 Total phospholipid (PL) content recovered by SPE from serum samples from liver cancer patients before tumor resection (T0), after tumor resection (T1) and from healthy individuals (CT), expressed in µg of PL/100µL of serum sample.

Fig. 2 Principal Component Analysis (PCA) score plot of the serum phospholipid profiles obtained from liver cancer patients before surgical resection (T0), after surgical resection (T1) and healthy individuals (Control).

Fig. 3 Box plots of the Principal Component Analysis (PCA) from the most significant loading values to discriminate among groups: liver cancer patients before surgical resection (T0), after surgical resection (T1) and healthy individuals (Control, CT).

Fig. 4 Box plots of the most significant species showing discrimination between liver cancer patients before surgical resection (T0), and after surgical resection (T1) when applying the non-parametric ANOVA-type statistic with repeated measurements approximation calculated for testing group and time effects. All species displayed showed significant differences ($p<0.05$) after Benjamini–Hochberg correction for the false discovery rate (FDR).

Figures

Figure 1

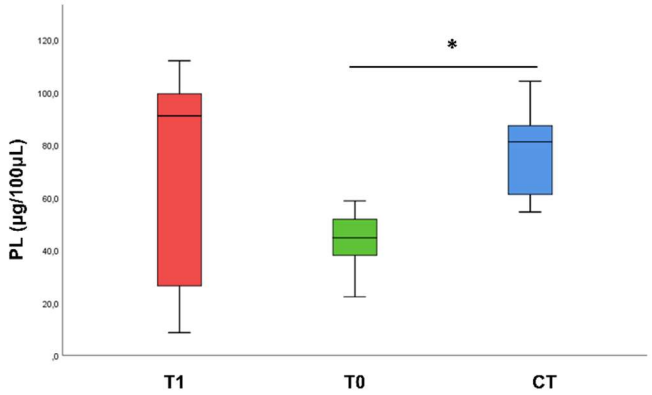


Figure 2

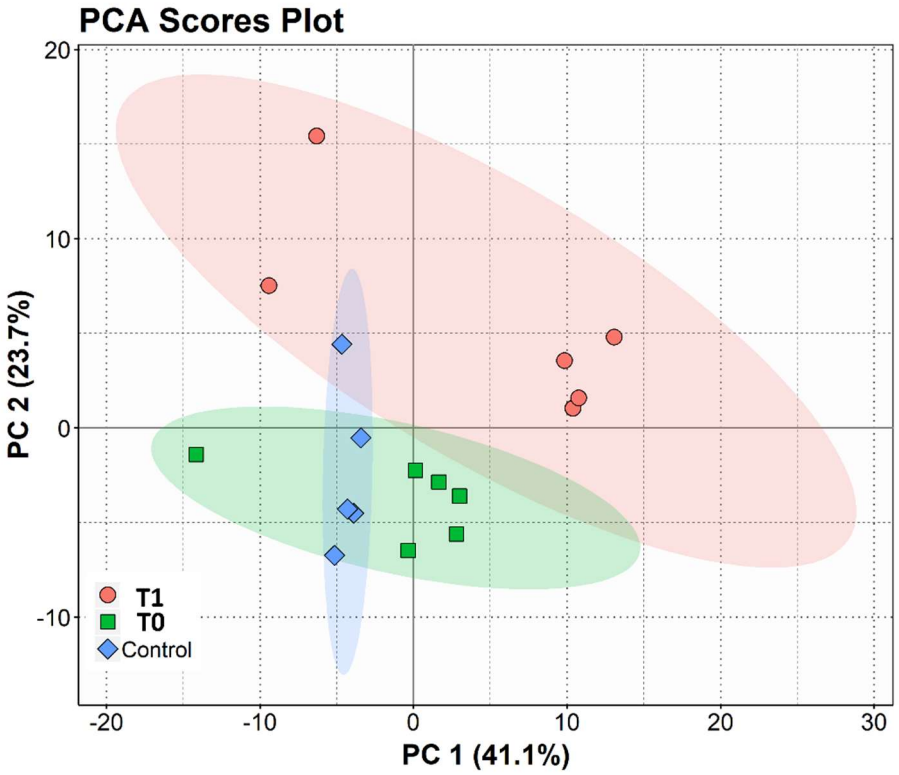


Figure 3

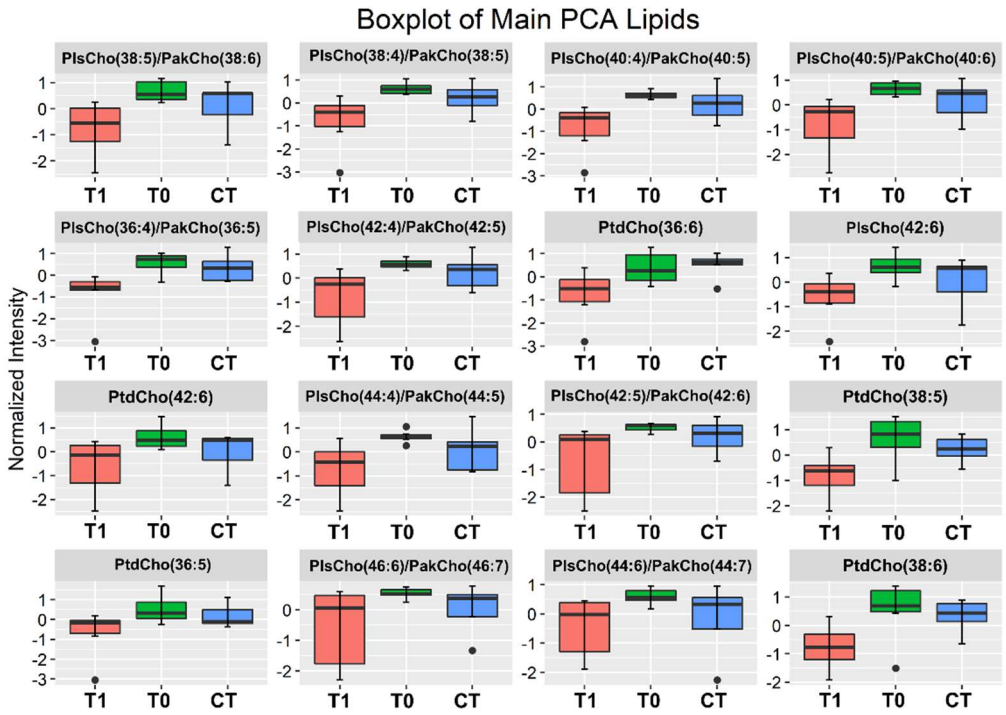
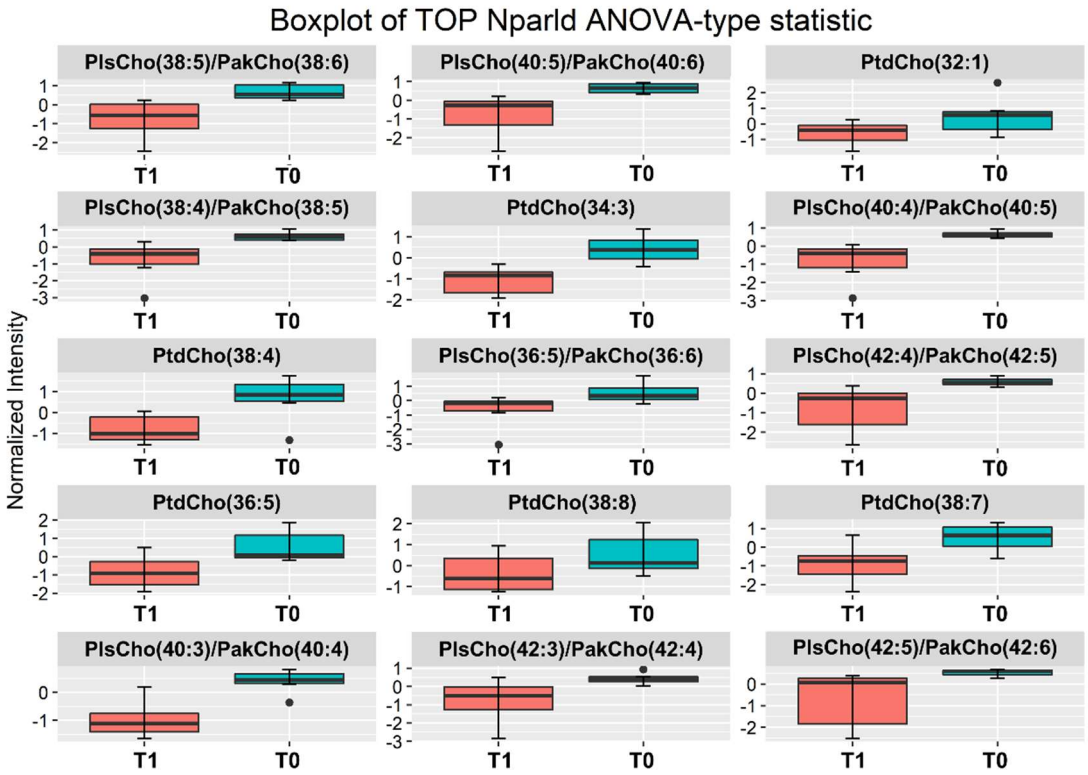


Figure 4



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Tumor resection induces alterations on serum phospholipidome of liver cancer patients

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Supplementary Material

Tables

Supplementary Table S1. Phospholipid quantification by phosphorous measurement in the extract obtained from serum samples of patients with liver cancer before surgical resection (T0) and after surgical resection (T1) and of healthy individuals (Control).

Clinical group	Sample code	Phospholipid amount [µg]	Mean ± SD
Control	CTL1	61.18	77.7 ± 20
	CTL2	87.34	
	CTL3	81.22	
	CTL4	104.19	
	CTL5	54.54	
T0	CCA_ 3	36.95	43.6 ± 12.2 [a]
	CCA_ 4	39.07	
	CCA_ 5	44.65	
	CCA_10	22.24	
	HCC_ 2	50.30	
	HCC_11	58.73	
	HCC_13	53.20	
T1	CCA_ 3	96.16	66.2 ± 43.6
	CCA_ 4	102.69	
	CCA_ 5	111.99	
	CCA_10	91.03	
	HCC_ 2	8.65	
	HCC_11	25.50	
	HCC_13	27.42	

Independent-samples Mann-Whitney U test was performed with a significance level of 0.05 (p<0.01). [a] vs. CONTROL

Supplementary Table S2. Positive and negative ion mode MS-based identification of the phospholipid molecular species that were quantified in the present study.

PC, $[M+H]^+$					
Molecular Species (C:N)	***m/z* observed**	**Error (ppm)**	**Molecular Species (C:N)**	***m/z* observed**	***Error (ppm)***
PC(30:0)	706,540	1,415	PC(44:11)	880,587	1,136
PC(30:1)	704,522	-1,419	PC(44:5)	892,681	1,120
PC(32:0)	734,571	1,361	PC(44:6)	890,663	-1,123
PC(32:1)	732,552	-2,730	PC(44:7)	888,648	0,000
PC(32:2)	730,539	0,000	PC(44:9)	884,616	-1,130
PC(34:1)	760,584	-2,630	PC(46:7)	916,679	-1,091
PC(34:2)	758,570	0,000	PC(46:8)	914,663	-1,093
PC(34:3)	756,555	1,322	PC(O-30:0)	692,559	0,000
PC(34:4)	754,540	1,325	PC(O-32:0)	720,591	0,000
PC(36:2)	786,601	0,000	PC(P-32:0)/PC(O-32:1)	718,575	0,000
PC(36:3)	784,586	0,000	PC(P-34:0)/PC(O-34:1)	746,606	0,000
PC(36:4)	782,569	-1,278	PC(P-34:2)/PC(O-34:3)	742,575	0,000
PC(36:5)	780,550	-5,125	PC(P-34:3)/PC(O-34:4)	740,559	0,000
PC(36:6)	778,537	-2,569	PC(P-36:2)/PC(O-36:3)	770,605	-1,298
PC(38:3)	812,616	-1,231	PC(P-36:3)/PC(O-36:4)	768,591	0,000
PC(38:4)	810,602	1,234	PC(P-36:4)/PC(O-36:5)	766,575	0,000
PC(38:5)	808,585	-1,237	PC(P-38:2)/PC(O-38:3)	798,635	-3,756
PC(38:6)	806,570	0,000	PC(P-38:3)/PC(O-38:4)	796,620	-2,511
PC(38:7)	804,552	-2,486	PC(P-38:4)/PC(O-38:5)	794,606	0,000
PC(38:8)	802,537	-2,492	PC(P-38:5)/PC(O-38:6)	792,592	1,262
PC(40:10)	826,536	-3,630	PC(P-40:3)/PC(O-40:4)	824,652	-1,213
PC(40:4)	838,631	-2,385	PC(P-40:4)/PC(O-40:5)	822,637	-1,216
PC(40:5)	836,616	-1,195	PC(P-40:5)/PC(O-40:6)	820,621	-1,219
PC(40:6)	834,602	1,198	PC(P-42:3)/PC(O-42:4)	852,681	-4,691
PC(40:7)	832,585	-1,201	PC(P-42:4)/PC(O-42:5)	850,669	-1,176
PC(40:8)	830,567	-3,612	PC(P-42:5)/PC(O-42:6)	848,655	2,357
PC(40:9)	828,552	-2,414	PC(P-44:4)/PC(O-44:5)	878,705	4,690
PC(42:10)	854,571	1,170	PC(P-44:5)/PC(O-44:6)	876,686	1,141
PC(42:11)	852,555	1,173	PC(P-32:1)/PC(O-32:2)	716,559	0,000
PC(42:5)	864,648	0,000	PC(P-36:5)/PC(O-36:6)	764,559	0,000
PC(42:6)	862,632	-1,159	PC(P-38:6)/PC(O-38:7)	790,576	1,265
PC(42:7)	860,616	-1,162	PC(P-40:6)/PC(O-40:7)	818,606	0,000
PC(42:8)	858,598	-3,494	PC(P-42:6)/PC(O-42:7)	846,638	0,000
PC(42:9)	856,585	-1,167	PC(P-44:6)/PC(O-44:7)	874,670	1,143
PC(44:10)	882,601	0,000	PC(P-46:6)/PC(O-46:7)	902,702	4,755
LPC, $[M+H]^+$					
Molecular Species (C:N)	***m/z* observed**	**Error (ppm)**	**Molecular Species (C:N)**	***m/z* observed**	***Error (ppm)***
LPC(14:0)	468,310	2,135	LPC(20:5)	542,323	-3,688
LPC(16:0)	496,340	0,000	LPC(22:1)	578,419	0,000
LPC(16:1)	494,325	0,000	LPC(22:4)	572,371	-1,747

LPC(18:0)	524,373	1,907	LPC(22:5)	570,355	-1,753
LPC(18:1)	522,357	1,914	LPC(22:6)	568,340	0,000
LPC(18:2)	520,341	1,922	LPC(24:0)	608,465	-1,643
LPC(18:3)	518,323	-3,859	LPC(24:1)	606,450	1,649
LPC(18:4)	516,307	-3,874	LPC(P-16:0)/(O-16:1)	480,347	4,164
LPC(20:0)	552,403	0,000	LPC(O-18:0)	510,394	3,919
LPC(20:1)	550,388	1,817	LPC(P-18:0)/(O-18:1)	508,377	0,000
LPC(20:2)	548,372	0,000	LPC(O-20:0)	538,421	-4,572
LPC(20:3)	546,356	0,000	LPC(P-18:1)	506,361	0,000
LPC(20:4)	544,340	0,000	LPC(P-20:0)/(O-20:1)	536,408	0,000

SM, $[M+H]^+$

Molecular Species (C:N)	*m/z* observed	Error (ppm)	Molecular Species (C:N)	*m/z* observed	*Error (ppm)*
SM(d30:1)	647,514	1,544	SM(d36:3)	727,575	0,000
SM(d32:0)	677,560	0,000	SM(d38:1)	759,638	0,000
SM(d32:1)	675,545	1,480	SM(d38:2)	757,623	1,320
SM(d32:2)	673,530	2,969	SM(d38:3)	755,606	-1,323
SM(d34:0)	705,592	1,417	SM(d40:1)	787,668	-1,270
SM(d34:1)	703,575	0,000	SM(d40:2)	785,654	0,000
SM(d34:2)	701,560	0,000	SM(d40:3)	783,639	1,276
SM(d36:0)	733,621	-1,363	SM(d42:2)	813,685	0,000
SM(d36:1)	731,607	0,000	SM(d42:3)	811,670	1,232
SM(d36:2)	729,593	2,741			

PE, $[M+H]^+$

Molecular Species (C:N)	*m/z* observed	Error (ppm)	Molecular Species (C:N)	*m/z* observed	*Error (ppm)*
PE(30:3)	658,443	-3,037	PE(P-34:2)/PE(O-34:3)	700,528	0,000
PE(34:1)	718,537	-2,783	PE(P-36:2)/PE(O-36:3)	728,559	0,000
PE(34:3)	714,508	1,400	PE(P-36:3)/PE(O-36:4)	726,544	0,000
PE(36:4)	740,523	0,000	PE(P-38:5)/PE(O-38:6)	750,542	-2,665
PE(38:4)	768,554	0,000	PE(P-40:4)/PE(O-40:5)	780,589	-2,562
PE(38:6)	764,524	1,308	PE(P-40:5)/PE(O-40:6)	778,574	-1,284
PE(40:10)	784,492	0,000	PE(P-40:7)/PE(O-40:8)	774,544	0,000
PE(40:5)	794,569	-1,259	PE(P-36:4)/PE(O-36:5)	724,529	1,380
PE(40:6)	792,554	0,000	PE(P-38:7)/PE(O-38:8)	746,511	-2,679
PE(40:8)	788,521	-2,536	PE(P-40:9)/PE(O-40:10)	770,511	-2,596
PE(40:9)	786,506	-1,271	PE(P-40:6)/PE(O-40:7)	776,560	1,288
PE(42:10)	812,521	-2,461	PE(P-40:8)/PE(O-40:9)	772,526	-2,589

PE, $[M-H]^-$

Molecular Species (C:N)	*m/z* observed	Error (ppm)	Molecular Species (C:N)	*m/z* observed	*Error (ppm)*
PE(38:3)	768,552	-2,602	PE(P-36:5)/PE(O-36:7)	720,499	2,776
PE(38:5)	764,521	-2,616	PE(P-38:3)/PE(O-38:4)	752,557	-2,658
PE(38:7)	760,492	0,000	PE(P-38:4)/PE(O-38:5)	750,546	2,665
PE(40:7)	788,522	-1,268	PE(P-38:6)/PE(O-38:7)	746,514	1,340
PE(P-34:1)/PE(O-34:2)	700,530	2,855	PE(P-44:11)/PE(O-4:12)	820,527	-1,219
PE(P-36:1)/PE(O-36:2)	728,558	-1,373			

PI, $[M-H]^-$					
Molecular Species (C:N)	***m/z* observed**	**Error (ppm)**	**Molecular Species (C:N)**	***m/z* observed**	***Error (ppm)***
PI(34:1)	835,532	-2,035	PI(38:3)	887,565	0,000
PI(34:2)	833,520	2,399	PI(38:4)	885,552	3,049
PI(36:2)	861,551	1,973	PI(38:5)	883,531	-3,056
PI(36:3)	859,533	-0,814	PI(42:0)	949,673	-1,579
PI(36:4)	857,520	2,332			
PS, $[M-H]^-$					
Molecular Species (C:N)	***m/z* observed**	**Error (ppm)**	**Molecular Species (C:N)**	***m/z* observed**	***Error (ppm)***
PS(30:2)	702,438	4,840			

Supplementary Table S3. Species and coefficients (loadings) of component 2 of the PCA (>1 % contribution).

Species	Coefficients
PC(34:3)	1.813047
PC(34:4)	1.043337
PC(36:4)	1.651624
PC(36:5)	1.548506
PC(36:6)	2.415614
PC(38:4)	1.218919
PC(38:5)	2.188402
PC(38:6)	1.883577
PC(38:7)	1.787786
PC(40:4)	1.062347
PC(40:5)	1.365479
PC(40:6)	1.248729
PC(40:7)	1.76442
PC(42:5)	1.144752
PC(42:6)	2.405604
PC(O-34:3)/PC(P-34:2)	1.202562
PC(O-36:3)/PC(P-36:2)	1.579671
PC(O-36:4)/PC(P-36:3)	1.858467
PC(O-36:5)/PC(P-36:4)	2.594962
PC(O-38:3)/PC(P-38:2)	1.164491
PC(O-38:4)/PC(P-38:3)	1.857409
PC(O-38:5)/PC(P-38:4)	2.726655
PC(O-38:6)/PC(P-38:5)	2.730782
PC(O-40:4)/PC(P-40:3)	1.396942
PC(O-40:5)/PC(P-40:4)	2.717331
PC(O-40:6)/PC(P-40:5)	2.692873
PC(O-42:4)/PC(P-42:3)	1.52998
PC(O-42:5)/PC(P-42:4)	2.431357
PC(O-42:6)/PC(P-42:5)	2.237967
PC(O-44:5)/PC(P-44:4)	2.270008
PC(O-44:6)/PC(P-44:5)	1.749561
PC(P-36:5)	2.100468
PC(P-42:6)	2.412643
PC(P-44:6)	1.936846
PC(P-46:6)	2.073612
PE(O-40:10)/PE(P-40:9)	1.225178
PE(O-40:9)/PE(P-40:8)	1.555277
PE(O-38:6)/PE(P-38:5)	1.142085
PE(P-40:7)	1.251283

Supplementary Table S4. The major 16 species and estimated coefficients (loadings) of component 2 of the PCA (>1.8% contribution).

Species	Coefficients
PC(O-38:6)/PC(P-38:5)	2.730782
PC(O-38:5)/PC(P-38:4)	2.726655
PC(O-40:5)/PC(P-40:4)	2.717331
PC(O-40:6)/PC(P-40:5)	2.692873
PC(O-36:5)/PC(P-36:4)	2.594962
PC(O-42:5)/PC(P-42:4)	2.431357
PC(36:6)	2.415614
PC(P-42:6)	2.412643
PC(42:6)	2.405604
PC(O-44:5)/PC(P-44:4)	2.270008
PC(O-42:6)/PC(P-42:5)	2.237967
PC(38:5)	2.188402
PC(P-36:5)	2.100468
PC(P-46:6)	2.073612
PC(P-44:6)	1.936846
PC(38:6)	1.883577

CHAPTER 8. DISCUSSION AND CONCLUDING REMARKS

Discussion and concluding remarks

The present Thesis has advanced the current understanding on liver cancer progression and tumor immunity and addressing the putative role of lipids and lipoxidation products. It has contributed to a better comprehension of the role of tumor-infiltrating leukocyte (TIL) subsets in the pathogenic progression of hepatocellular carcinoma (HCC) and cholangiocarcinoma (CCA), as well as the systemic responses to surgical resection occurring in liver cancer patients, by applying multi-color flow cytometry immunophenotyping, molecular biology techniques, and lipidomic analytical approaches. In addition, it allowed to identify functional defects on the peripheral immune response of CCA and HCC patients.

In Chapter 2, the literature gathered until now on lipoxidation products, formed in oxidative conditions, and their effects on tumor cells, as well as the alteration of immune components and the consequent modulation of the immune response, was comprehensively reviewed. Many studies provide emerging molecular evidence of the importance of lipoxidation in carcinogenesis, reporting contradictory results in which lipoxidation products seem to have a crucial role in carcinogenesis but also in the host defense against cancer, through their effects on tumor cells as well as through their interactions with immune components. The formation of lipoxidation adducts may trigger different mechanisms to avoid cancer progression, such as suppression of cell growth, reduction of metastatic capacity or anti-proliferative effects. But also, in other cases, it may trigger effects that favor cancer development, as the modulation of tumor microenvironment to become more pro-tumorigenic or the cytoprotective response in cancer cells. The divergent results described, documented that the formation of lipoxidation adducts can have either anti-carcinogenic or pro-carcinogenic effects, depending on the cell type affected and the specific adduct formed. Furthermore, lipoxidation adducts can lead to many different effects on the immune components such as the regulation of TLR4, the promotion of $CD4^+$ T cell proliferation, the induction of monocyte activation, as well as the inhibition of neutrophil function or the inhibition of Treg cells generation. Additionally, it has been demonstrated that high concentrations of reactive aldehydes, such as 4-hydroxy-2-nonenal (HNE) or acrolein (ACR), leads to lipoxidation products that can directly or indirectly inhibit the activation of NFκβ and, therefore, inhibit the mechanisms triggered by this transcription factor such as tumor cells proliferation, suppression of apoptosis, angiogenesis and epithelial-mesenchymal transition, which facilitates distant metastasis. Moreover, it has been proposed a role for

the prostaglandin 15d-PGJ2 in the control of lymphocyte proliferation and activation through mechanisms relying on NFκβ inhibition. Therefore, lipoxidation products may alter cancer development by altering tumor cells' behavior as well as by modulating the immune response.

Focusing on the molecular characterization of TIL subsets in hepatic carcinomas, Chapter 3 has provided evidence to better understand the tumor immune microenvironment of CCA and HCC patients. Different types of common patterns associated with the pathogenesis of these tumors were detected after phenotypical and functional characterization of tumor cells and immune cell populations by multi-color flow cytometry and qRT-PCR. Therapeutically, PD-1/PD-L1 blockade has been shown to suppress HCC tumor growth in pre-clinical models. Moreover, several clinical trials of anti-PD-1/anti-PD-L1 immunotherapies are currently underway[1]. In this regard, this study has confirmed the expression of PD-L1 by HCC and CCA tumor cells, as well as by HCC tumor-infiltrating macrophages, supporting the continued development of immune checkpoint inhibitors targeting the PD-1/PD-L1 pathway in both HCC and CCA. The tumor microenvironment in liver cancer patients is characterized by functionally distinct macrophage populations that can alter tumor progression. The reported expression levels of CD200R detected in macrophages infiltrating high-grade tumors, added to its positive correlation to PD-L1 expression levels, may serve as a benchmark to evaluate novel therapies that seek to target CD200/CD200R axis in HCC. In fact, a study on the inhibitory CD200:CD200 receptor axis has previously suggested a promising role of CD200R as a prognostic marker in predicting elevated recurrence and reduced survival in HCC[2]. Therefore, further studies should be conducted in the development of immunotherapies targeting this pathway for HCC patients with tumors classified in grade G3 or G4. In addition, significant higher expression levels of markers associated with protumor macrophages, such as PD-L1, CD206, and CD163, in HCC tumors that were classified into high-grades or into high-risk tumors were detected, reinforcing the importance of these macrophages in tumor progression. Therefore, functional and phenotypic differences in TIL subpopulations between CCA and HCC, as well as among different histopathological grades and tumor aggressiveness degrees, were revealed.

HCC and CCA present limited options for effective therapeutic strategies, being ablation, surgical resection and/or transplantation the only curative treatments applied so far. These carcinomas are characterized by high recurrence rate after surgical resection[3–5]. The eventual resistance to cancer therapies remains a major challenge in clinical oncology. The majority of studies devoted to investigating the basis of resistance to cancer therapy have focused on tumor-related changes and tumor aggressiveness.

However, over the last decade, a growing body of evidence demonstrated that cancer therapy can induce host-mediated local and systemic responses, many of which shift the delicate balance within the tumor microenvironment, ultimately facilitating or supporting tumor progression[6]. Therefore, novel treatment strategies with different mechanisms from those of conventional therapies are needed to improve the prognosis of HCC and CCA. Immunotherapy-based approaches represent a very promising alternative for liver cancer; these approaches have been successful in many different cancer types and could be used to mitigate the host response to conventional cancer therapies[7]. However, predicting responders and non-responders to immune therapies remains a challenge, despite the active research in this field[1]. In this regard, the monitoring of circulating immune cells might lead to the identification of biomarkers, which could serve to predict prognosis and/or therapy response. For this purpose, chapter 4, chapter 5 and chapter 6 have contributed to better understand the immune status of HCC and CCA patients and to assess the effect of tumor resection in the immune system homeostasis. In addition, chapter 4 revealed the phenotypical characterization of a population of monocytes that express the high-affinity receptor FcεRI, which display phenotypic and functional similarities with circulating myeloid dendritic cells (mDCs), suggesting that this population of monocytes could be the circulating precursor of mDCs. Regarding the characterization of myeloid cells in liver cancer patients, the frequency and the function of circulating mDCs and FcεRI^{+} monocytes are affected in both HCC and CCA patients, before and after surgical procedure. The frequency of circulating non-classical monocytes is decreased in both CCA and HCC patients, before and after surgery. Moreover, a functional defect in circulating classical and intermediate monocytes was disclosed with partial recovery after the surgical intervention. Overall, in chapter 4 and chapter 5 we have shown the detection of altered functional competence of mDCs and FcεRI^{+}, classical, and intermediate monocyte subsets in CCA and HCC patients and, thus, a limited capacity to respond under further stimulation processes. In addition, a pro-inflammatory state was observed in HCC and CCA patients that presented a partial recovery after surgical procedure. Inflammatory factors affect nearly all the stages of tumor development and the effectiveness of the therapy applied[8]. TNFα is considered one of the most important inflammatory mediators of the cancer-associated inflammatory networks[9]. Interestingly, chapter 5 points out a potential role for TNFα as a prognostic factor in HCC patients, indicating the presence of high-grade tumors. In this regard, further studies will be needed to validate the potential use of TNFα serum levels, or TNFα mRNA expression by circulating monocytes, as a prognostic factor or indicator of high histopathological grading.

Regarding lymphoid cells characterization, a great heterogeneity among different individuals was detected making it difficult to identify behavioral patterns among the different populations under study. Notably, decreased circulating levels of Treg cells were observed in both HCC and CCA patients, with a partial recovery observed in some CCA patients and a total recovery in HCC patients after tumor resection, suggesting that tumor resection partially restores immune system homeostasis in CCA and HCC patients. Notably, we detected a decrease in the frequency of CD4 T lymphocytes with the plasticity to produce both IFNγ and IL-17 cytokines, together with a decrease in the frequency of Tc17 cells in CCA and HCC patients. In this regard, we hypothesized that the observed decrease in these cell populations could be due to a specific migration to the tumor microenvironment which is also supported by the elevated circulating levels of CCL20 in HCC and CCA patients and by the detection of these subsets in the tumor microenvironment. Therefore, these results support the evaluation of novel therapies that seek to target IL-17 producing cells in CCA and HCC patient[10–12].

Additionally, under hepatic cellular damage and/or oncogenic conditions, the homeostasis of lipid and lipoprotein metabolism is impaired leading to alterations on plasma lipid and lipoprotein composition[13]. Taking this into account, the study of the circulating phospholipidome provides an indirect read of the lipid changes in the liver. Concerning the lipidomic characterization, in chapter 7, it was shown that the alterations identified in the serum phospholipidome of liver cancer patients, seems to be reverted after tumor resection procedure. These results indicate that the use of high-resolution HILIC-LC-MS lipidomics approaches to detect different serum phospholipids (PL) profiles in patients with liver cancer, allow to define a specific PL fingerprint that could be used to determine the effect that tumor resection causes in the altered lipid metabolism and that could be used to predict the outcome after tumor resection procedure.

In conclusion, this work has demonstrated the utility of multi-color flow cytometry of freshly processed samples to reveal functional and phenotypic differences in TIL subsets among different cancer patients (CCA vs. HCC), different tumor histopathological grades, and different risk stratification classifications, in addition to monitor functional competence of circulating immune cells to better evaluate immune dysfunctions in cancer patients, as well as demonstrating the use of high-resolution LC-MS to monitor peripheral changes in the phospholipidome of cancer patients, before and after tumor resection, providing an indirect read of the lipid changes induced by surgical procedure.

Final Conclusions

I. The expression of PD-L1 by HCC and CCA tumor cells, as well as by HCC tumor-infiltrating macrophages, was confirmed in tumors classified in different histopathological grades.

II. A significantly higher expression of markers associated with protumor macrophages, such as PD-L1, CD200R, CD206, and CD163, was detected in HCC tumors that were classified into high-grades or into high-risk tumors.

III. A peripheral pro-inflammatory state was observed in HCC and CCA patients and functional defects of some circulating monocyte subsets and mDCs were detected, displaying partial recovery after tumor resection.

IV. A potential role of TNFα as a prognostic peripheral biomarker in HCC patients, indicating the presence of high-grade tumors, was revealed.

V. Despite the great heterogeneity observed in the immune response involving lymphoid cells, a modulation of circulating Treg cells induced by tumor resection was observed in HCC and CCA patients.

VI. The alterations identified by high-resolution HILIC-LC-MS in the phospholipidomic profile of liver cancer patients were partially reverted after tumor resection procedure.

Future perspectives

We are moving towards a new era of anticancer treatment for hepatic carcinomas, where immunotherapy-based strategies will soon become a cornerstone, both as monotherapy and in combination with conventional surgical therapies. The contribution of immune checkpoint inhibition to immunomodulation is clear, yet some of the side effects are associated with a robust increase in cytokines and growth factors supporting autoimmune responses[14]. In the era of precision medicine, characterizing host effects may help to predict the therapeutic outcome of patients as well as to identify appropriate treatments that could be administered for a patient, based on the factors and cells that are altered in response to the initial therapy[15]. As suggested by the present Thesis, the continued development of immune checkpoint inhibitors targeting the PD-1/PD-L1 pathway alone or in combination with other immune checkpoint inhibitors, such as CD200, are very promising approaches for the treatment of hepatic carcinomas, especially HCC. Moreover, future studies with larger sample sizes to better characterize tumor-infiltrating macrophages in HCC may lead to the identification of a specific population of protumor macrophages that could be used as prognostic factor.

Several studies have demonstrated that the collateral effects of therapy on host cells induce local and systemic changes in the treated host that can promote tumor aggressiveness, thereby partially or completely negating the intended therapeutic activity[16].The results presented in this Thesis have outlined a pro-inflammatory state in HCC and CCA patients subjected to be modulated by the host response to surgical therapy. In this regard, an increasing body of evidence provides rationale for the utility of peripheral blood tests to predict cancer patient prognosis and treatment effectiveness[17]. Therefore, future studies should be conducted to validate the utility of multi-color flow cytometry to monitor immune competence in response to anticancer treatments to help in the selection of patients susceptible to respond to a specific therapy, as well as to determine new targets for the development of new immunotherapy strategies. Future panels could include more cell markers, such as CCR10, CCR5, CCR6, CXCR3, CCR4 and CXCR5, in order to characterize more T cell subpopulations and determine their functional plasticity in cancer patients[18,19]. In addition, the present work has set up the basis for a potential role of TNFα as a prognostic peripheral biomarker in HCC patients, indicating the presence of high-grade tumors that should be further validated. The implementation of this type of biomarkers in the clinical routine would be very beneficial since it is low cost in comparison to more invasive traditional diagnostic and staging tests,

such as tumor size, histological grade, vascular invasion or lymph node metastases, that would require surgery or expensive imaging techniques, and could be performed on a regular basis, with minimal risk to the patient.

Finally, many directions could be taken in the area of lipidomics applied to the oncology field. One of the main clinical applications of lipidomics is the monitoring of therapeutic responses and the potential side effects of existing treatments, as well as the evaluation of newly developed ones[20]. The symbiosis of lipid profiling and multivariate statistics in a lipidomics approach can help in the discovering of potential biomarkers, allowing a better understanding of cancer pathology, and drug/therapy response monitoring. The use of peripheral PL fingerprint profiling as an analytical tool to indirectly determine lipid changes in the liver in response to the oncologic condition, as well as in response to therapy, is presented as a very promising avenue that should be deeply investigated. Indeed, further studies with larger sample sizes and after long-term follow-up are needed to validate the findings presented in this Thesis.

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