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Risk factors of primary liver cancer initiation associated with tumour initiating cell emergence: novel targets for promising preventive therapies

Arthur Brouillet,[1,2] Fouad Lafdil[1,2,3]

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[1]UMR-S955, Université Paris-Est, Créteil, France
[2]U955, Institut National de la Santé et de la Recherche Médicale (INSERM), Créteil, France
[3]Institut Universitaire de France, Paris, France

Correspondence to
Professor Fouad Lafdil;
fouad.lafdil@u-pec.fr

ABSTRACT

Primary liver cancers ranked as the sixth most commonly diagnosed cancers and the third-leading cause of cancer-related death in 2020. Despite encouraging findings on diagnosis and treatments, liver cancer remains a life-threatening disease with a still increasing incidence. Therefore, it is of interest to better characterise and understand the mechanistic process occurring at early steps of carcinogenesis. Inflammatory responses in liver diseases participate in the activation of liver progenitor cells (LPCs) facultative compartment but also to their transformation into cancer stem cells (CSCs) and give rise to primary liver cancer including hepatocellular carcinoma and cholangiocarcinoma. Higher intratumoural heterogeneity has been associated with poorer prognosis and linked to tumour escape from the immune surveillance and to resistance to chemotherapy. A better understanding of the malignant transformation of LPC as tumour initiating cells (ie, CSC) should also provide a potential new therapeutic target for anticancer therapy. In this review, we summarise the recent reports identifying underlying mechanisms by which chronic liver inflammatory responses could trigger the early steps in liver carcinogenesis, notably through the transformation of LPCs into tumour initiating cells.

INTRODUCTION

Primary liver cancers including hepatocellular carcinoma (HCC) and intrahepatic cholangiocarcinoma (CCA) ranked as the sixth most commonly diagnosed cancers and the third-leading cause of cancer-related death in 2020 (Globocan 2020). Among all primary liver cancers, HCC is the most common cancer accounting for more than 80% of cases with a 5-year survival rate of less than 10% in western countries. Despite significant progress in diagnosis and treatments, HCC that is often diagnosed at late stages (70% of cases) remains a life-threatening disease with an increasing incidence. Therefore, a better understanding of the underlying mechanisms triggering the early steps of tumourigenesis represent a great interest to predict and propose more effective therapeutic options for liver cancer prevention.

Numerous studies have classified HCC according to gene expression profiling, immunohistological phenotypes and somatic mutation detection, revealing various patterns of HCC and next-generation sequencing analyses confirmed by high molecular heterogeneity within the same tumour nodule and various clonal evolution.[1–3] The origin of such heterogeneity is still debated. However, clinical and histological studies revealed that 28%–50% of HCC express progenitor/stem cell markers.[4] In healthy livers, the progenitor compartment is composed of resident liver progenitor cells (LPCs) defined as bipotent intrahepatic quiescent cells. They are activated in chronic liver diseases in cases of massive tissue damage or prolonged chronic insult altering the proliferative capacities of remaining healthy hepatocytes, and participate in liver regeneration, fibrogenesis and tissue repair[5] (figure 1). LPC accumulation, known as ductular reaction, is frequently observed in diverse chronic liver diseases, such as in preneoplastic cirrhotic livers with a worse prognosis.[6] In addition, it is admitted that LPCs have the potential to initiate tumours because of their likelihood to transform into cancer stem cells (CSCs) that ultimately lead to the development of heterogeneous lineages of cancer cells.[5 7–9] The underlying mechanisms leading LPCs to become tumour-initiating cells is not yet fully understood, but recent studies reported some promising clues that deserve to be further considered.

Due to its enriched cell composition with high density of immune cells including myeloid immune cells such as resident macrophages called Kupffer cells (KCs), neutrophils or lymphoid cells such as Natural Killer (NK), Natural Killer T (NKT), T and

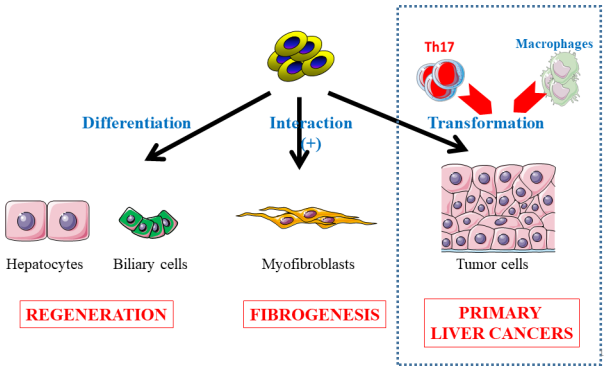

Figure 1 Liver progenitor cell compartment and properties. Liver progenitor cells are bipotent cells involved in: (1) the liver regenerative process by differentiating into hepatocytes or biliary cells; (2) fibrogenesis by tightly interacting with hepatic myofibroblasts and favouring their accumulation; (3) cancer initiation by undergoing a transforming process into cells harbouring a cancer stem cell phenotype in an inflammatory microenvironment shaped by infiltrated-Th17 cells and macrophages.

B lymphocytes, the liver is considered as an immunological organ. During chronic liver diseases whatever the aetiology, the liver is frequently subjected to a continuous regenerative process occurring in a particularly active inflammatory context.

Increasing evidence suggests that LPC expansion is carried out within a particular microenvironment requiring cellular interactions with non-parenchymal and immune cells. Chronic non-resolving inflammation and tissue damage participate not only in the activation of LPC compartment, but also regulate several mechanisms leading to their transformation into CSCs, including metabolic and epigenetic reprograming pathways.

In this review, we propose to summarise the latest findings that identified molecular inflammatory mechanisms that trigger the LPC growth, accumulation and their transformation into CSCs considered actively participating in the rise of primary liver cancers. These recent findings will allow to open discussion and thought for novel therapeutic strategies aiming at preventing liver cancer initiation.

LPC COMPARTMENT ACTIVATION AS A RISK FACTOR FOR TRIGGERING EARLY STEPS OF LIVER CARCINOGENESIS

HCC and CCA have been considered as independent tumours that originate from distinct parenchymal cells (ie, hepatocytes vs biliary cells respectively). However, accumulating evidence suggests that both types of primary liver cancer could also originate from a common tumour-initiating cell population. Converging data demonstrated that CSCs give rise to primary liver cancers including HCC or CCA[10] and increasing evidence indicates that CSC are mainly responsible for tumour aggressiveness, relapse, metastasis and therapeutic failures to conventional anticancer treatments.[11] As LPCs and CSCs were found in already established HCC[4] and CCA,[12] LPCs were defined as tumour initiating cells that ultimately evolve into heterogeneous lineages of cancer cells addressing tumour heterogeneity and plasticity in primary liver cancer.[9 13] A non-resolving inflammatory process observed in various chronic liver diseases could not only participate in the activation of LPC facultative compartment, but also to their transformation into CSC.[8 14] This small subset of cancer cells acquiring robust stem cell properties within tumours could originate from a 'dedifferentiation' of non-tumour LPCs considered as normal intrahepatic cells in the liver. Another point of view explaining the accumulation of CSC is to consider that differentiation of LPC into mature parenchymal cells is impaired.[8 9 13–15] The underlying mechanisms leading to such CSC accumulation involve regulatory signalling pathways that induce or maintain the stemness properties in the CSC niche surrounded by stroma cells and immune cells.[16 17]

IMPACT OF SUSTAINED LIVER INFLAMMATORY RESPONSE ON LPC TRANSFORMATION INTO CSC

Chronic inflammation is known to actively participate in liver carcinogenesis by favouring tumour initiation and progression.[16–18] Unambiguously, immune regulation plays a key role in primary liver cancers in all chronic liver disease.[19] Several cytokines exert their potential mutagenic effect by the induction of genomic instability through the production of highly reactive molecules that can damage DNA.[20]

Impact of liver macrophages on LPC activation and transformation into CSCs

During the liver inflammatory response, both innate and adaptive immune cells are critical for activation of the LPC compartment. KC depletion reduced LPC proliferation in mice fed with Choline-Deficient and Ethionine supplemented (CDE) diet[21] or in 2-acetylaminofluorene (AAF)/Partial Hepatectomy (PH) rat model,[22] which was associated with reduced recruitment of circulating monocytes and liver regeneration. Moreover, the proinflammatory cytokines produced by KC such as Interleukin (IL)-6 and Tumour Necrosis Factor (TNF)-α or TNF-like weak inducer of apoptosis were found to support LPC proliferation.[23] Long-term treatment of rat LPC WB-F344 cells by TNF-α (but not IL-6) promotes spheroid formation in vitro and tumour after subcutaneous inoculation into immunodeficient NOD/SCID mice. Aberrant expression of LPC markers such as Alpha-fetoprotein (AFP), Cytokeratin (CK)-19 and Ovalbumin (OV) 6 was detected in tumours from TNFα-treated WB-F344 cells revealing their malignant transformation.[8] Interestingly, chronic TNF-α exposure was also able to trigger chromosomal instability due to an aberrant expression of ubiquitin D and checkpoint kinase 2. Incorporation of the KrasG12D mutation into murine LPC increased their proliferative capacity and their capability to form colonies in vitro, revealing a mechanism of LPC transformation by horizontal transfer of oncogene.[24]

Furthermore, in the context of an already established tumour immune environment, tumour-associated macrophages (TAMs) produce cytokines which induce CSC accumulation,[25] such as IL-6 and TNF-α through the STAT3 signalling pathway[8] or by inducing Epithelio-Mesenchymal Transition (EMT) and stemness features via the Wnt/β-catenin pathway.[26]

Involvement of lymphocytes on LPC-driven transformation into CSCs

Activation of LPC compartment was drastically weakened in mice lacking T cells[27] and T-cell-mediated hepatitis induced by concanavalin A triggers NK cell-sensitive LPC expansion following partial hepatectomy.[28] Moreover, other inflammatory-related proteins including lymphotoxin β, interferon-γ, IL-22, galectin-3 promote liver regeneration from LPCs.[23 29 30] In patients with various chronic liver diseases, LPC accumulation correlates with the recruitment of IL-17 producing cells and the severity of ductular reaction.[31] Among the key players in modulating liver inflammation, T helper-(Th)17 lymphocytes have been implicated in several types of liver diseases and the proinflammatory IL-17 cytokine involved in the crosstalk between innate and adaptive immunity.[32] The key role of IL-17 in LPC activation was evidenced in an experimental model of mice fed with a CDE diet, showing that IL-17 is responsible for macrophage-induced IL-27 expression that favours LPC differentiation into hepatocytes.[31] These results highlight collaborative work between IL-17 and IL-27 that is required to properly achieve liver regeneration from LPC.

In the context of inflammation-induced tumourigenesis, IL-17 is a crucial cytokine produced by immune cells, especially activated T-helper 17 cells, which contribute to the initiation and progression of several cancers through activation of stem/progenitor compartment in gastrointestinal,[33] skin,[34] ovarian,[35] pancreatic,[36] breast[37] and prostate[38] cancers. In the liver, the proinflammatory IL-17 cytokine drives progression of steatohepatitis,[39] hepatic fibrosis and LPC expansion[31] which are a known risk factor of HCC development.[40 41] The tumour-promoting inflammatory impact of IL-17 was recently confirmed on a cohort of 404 patients with cirrhosis, where plasma IL-17 and AFP combination effectively predicts imminent HCC occurrence within a year.[42] Recently, IL-17-producing cells were localised within ductular reaction close to LPC and CSC in human preneoplastic cirrhotic livers from diverse aetiologies.[43] The direct effect of IL-17 on LPC transformation was evidenced in vitro with long-term IL-17 stimulation of murine and human LPCs, which promotes expression of CSC (such as Cd133, EpCAM) and tumour (alpha-fetoprotein and glypican-3) markers and acquired self-renewal capacity highlighted by spheroid formation. These data demonstrated that chronic exposure to IL-17 induces the conversion of LPCs into cells acquiring CSC phenotype. The potential tumourigenic effect of IL-17 was confirmed in vivo by increased expansion of the tumour mass after a subcutaneous engraftment of IL17-pretreated LPCs into immunodeficient NOD/SCID mice, while no significant cell proliferation was observed in control mice engrafted with non-pretreated LPC. Interestingly when compared with untreated LPC, tumours from IL-17-pretreated LPC exhibited an aggressive phenotype with histopathological features of mixed HCC and CCA phenotype, in accordance with malignant transformation of bipotential LPC.

Gamma delta (γδ) T-cells represent a major T-cell population IL-17 participate in IL-17 production[23] have been reported to promote tissue repair by interacting with tissue-resident stem cells and participate in HCC.[44] Moreover, lymphatic endothelial cells could secrete IL-17 to potentiate the self-renewal and tumourigenesis properties of CSCs by upregulating programmed cell death-ligand 1 (PD-L1) expression. Then, IL-17 may participate in hepatocarcinogenesis by driving the transformation of LPCs into CSCs and favouring their ability to escape from antitumour immune response.

THE ROLE OF NON-CODING RNAs IN CONTROLLING LPC TRANSFORMATION INTO CSCs

Small non-coding RNAs including micro-RNAs (miRNAs) are actively involved in both physiological and pathophysiological processes in the liver. In chronic liver diseases, the alteration of miRNAs expression is associated with metabolic syndrome, liver injury and fibrogenesis, and to tumourigenesis, making theses miRNAs attractive targets for the diagnosis and treatment of liver diseases.[45] A dysregulated expression of numerous miRNAs has been identified as key component in the carcinogenic process. Indeed, they harbour a high capacity of regulating many genes involved in tumour cell expansion, and regulating immune response.[46] Recent studies also reported miRNA as potential biomarkers.[47] Among the downregulated miRNAs in HCC, miR-122 which is the most abundant miRNA in the normal liver is downregulated in almost 50% of diagnosed HCC patients.[48 49]

Impact of the inflammatory response on epigenetic cell reprograming

The role of IL-17 in driving the tumour-initiating abilities of CSC includes epigenetic alteration that was identified in carcinogenesis.[50] Interestingly in IL-17-treated LPC, expression of miR-122 which accounts for 70% of the liver's total miRNAs that was considered as a tumour suppressor miRNA,[51] was sharply decreased in experiments performed in vitro and in vivo. Recently, long-term exposure to IL-17 cytokine has been shown to strongly downregulate miR-122 expression in LPCs allowing their dedifferentiation and transformation into CSCs with increased risk of primary liver cancer.[43] In addition, transfection of miR-122 mimic into LPC was sufficient to abolish their acquired self-renewal

capacities by long-term-IL-17 pretreatment. The mechanism by which IL-17 reduces miR-122 levels could be the consequence of the STAT3-mediated inhibition of Hepatocyte Nuclear Factor (HNF) 4-α expression[31] that is involved in the transcriptional regulation of mir-122.[52]

Non-coding RNA transfer through exosomes as a significant mean of cell communication controlling HCC development

Exosomes are micro or nano extracellular vesicles that originate from cell membranes and able to influence the proliferative and migration rates of cancer cells. Depending on their cargo, exosomes can either suppress or promote tumour cell progression. They can act as means of communication between cells by transferring their contain in miRNAs, long non-coding RNAs (lncRNAs) or circular RNAs (circRNAs) and modulate other molecular signalling pathways such as PTEN and PI3K/Akt in cancer.

Exosomal miRNA has been described reflecting the miRNA expressed by tumour cells and for this reason, it has been considered as plasma biomarkers for cancers. In the liver, various elevated levels of exosomal miRNA were positively or negatively associated with HCC progression, including miR-122.[53 54] Numerous studies revealed that exosomal miRNA participate in hepatocarcinogenesis by regulating cell proliferation, immune response and EMT.[55] For instance, HCC cell-derived exosomes containing miR-21 lead to tumour cell expansion through the inhibition of PTENp1 and PTEN expression.[56] MiR-142 and miR-223 expressed in macrophages were found to be transferred by exosomes in HCC cells and affected posttranscriptional regulation of proteins leading to the inhibition of the tumour cell growth.[57] In a different way of communication, tumour-derived exosomes were shown to possibly activate Dendritic Cells (DCs), or to induce the proliferation of immature T cells, and their differentiation into an antigen-specific cytotoxic T lymphocytes (CTLs) phenotype, that ultimately allow increasing the antitumour immune response.[58 59] In ectopic and orthotopic mouse models of HCC growth, it has been reported that treatment with dendritic cells pulsed with tumour cell-derived exosomes was able to trigger a strong immune response characterised by an increased number of T cells and in interferon gamma (IFN-γ) production, and in parallel, by a decrease in IL-10 and Transforming Growth Factor (TGF)-β, resulting in a significant decrease in tumour growth.[60]

Extracellular vesicles coming from neutrophils were shown to transfer their contain to hepatocytes. For instance, miR-223 were reported to prevent Non-Alcoholic Fatty Liver Diseases (NAFLD) and Non-Alcoholic Steatohepatitis (NASH) progression via hepatocyte uptake of neutrophil-derived extracellular vesicles that consequently exerted its anti-inflammatory and antifibrotic properties,[61] making it a potential therapeutic target for NASH and liver cancer treatments. Recently, it was demonstrated in two models of inflammation-associated HCC that miR-223 attenuates hypoxia-induced tumour immunosuppression and angiogenesis in HCC via the inhibition of hypoxia-inducible factor 1α. Then, gene delivery of miR-223 via adenovirus in hepatocytes may improve the efficacy of the current therapy for HCC with PD-1/PD-L1 inhibitors combined with antiangiogenic agents.[62]

In a cohort of 112 HCC patients compared with healthy individuals, 31 miRNAs, 4 lncRNAs and 2 circRNAs were identified in exosomes and are considered as biomarkers to predict HCC prognosis.[63] In a TCGA analysis from 371 HCC samples, 5 exosome-related lncRNA were associated with poor outcome and also related to overexpressed immune checkpoints.[64] This lncRNA-exosomal signature may help to propose an adapted and personalised immunotherapy for each HCC patient. Interestingly, one of these lncRNA, muskelin 1 antisense RNA (MKLN1-AS), was shown to promote tumour growth[65] or drive EMT of liver cancer cells.[66]

Then, altered expression of non-coding RNAs are being investigated as biomarkers for early diagnosis and prognosis of primary liver cancers. Their implication on intercellular communication via extracellular vesicles between parenchymal and LPC with immune cells and liver CSC niche reveals their usefulness as therapeutic targets in liver carcinogenesis process.[45] Accumulating data have demonstrated that various miRNAs/lncRNAs/circRNAs regulate activation of LPC compartment[67–69] and are essential for maintaining CSC properties in liver cancer.[70 71] Although the involvement of non-coding RNAs is well described in liver injury and liver fibrosis, the exact role of miRNAs/lncRNAs/circRNAs in the initiation and promotion of hepatocarcinogenesis has rarely been explored, notably tumours resulting in malignant transformation of LPCs, including the capacity of progenitor cell dedifferentiation to form CSCs (figure 2).

PROMISING THERAPEUTIC STRATEGIES FOR LIVER CANCER PREVENTION

In terms of therapy research aiming at preventing liver cancer occurrence, maintaining LPC compartment integrity by limiting their activation/transformation could be considered as attractive therapeutic strategy. In addition to the inflammatory microenvironment related to the chronic liver diseases, the exosome-mediated bidirectional communication between tumour cells and their microenvironment brings novel insight to decipher the underlying molecular mechanisms involved in early steps of liver cancer development.

Targeting immunomodulatory components

In that context, recent studies evidenced the tumourigenic potential of proinflammatory cytokines including IL-6, TNF-α and IL-17 as presented above (figure 2). Therefore, targeting and inhibiting the release or action

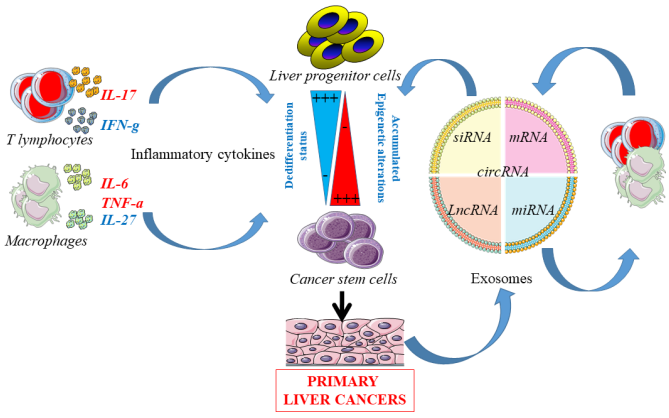

Figure 2 Transforming process of liver progenitor cells (LPCs) into cancer stem cells (CSCs). In a chronic inflammatory context, epigenetic changes occur in LPCs: (1) through a sustained stimulation by cytokines that either promotes dedifferentiation and transformation of LPCs to CSCs (IL-17, TNF-α and IL-6), or in contrast avoiding their transformation (IFN-γ, IL-27); (2) by hosting small non-coding RNAs transferred by exosomes coming from already established tumour cells or immune cells composing the microenvironment with a possible bidirectional communication. circRNA, circular RNA; IFN, interferon; IL, interleukin; LncRNA, long non-coding RNA; miRNA, microRNA; mRNA, messenger RNA; TNF, tumour necrosis factor; siRNA, small interference RNA.

of such cytokines could be considered to prevent LPC acquirement of tumour-initiating phenotype.

For instance, in a murine HCC xenograft model, the use of anti-IL-6 tocilizumab antibody showed reduced HCC growth and prevented the occurrence of CSCs.[25]

In same lines of evidence, clinically TNF-α expression was correlated to LPCs activation and HCC recurrence. In an experimental model of TNF-α-inhibition or deletion, LPC activation and proliferation were inhibited.[72] At last, preclinical benefits of anti-TNF-α treatments have also been reported for multiple types of cancers, including HCC.[73]

In addition, in a murine model of HCC developed on a fibrotic context, recapitulating the clinical and histological features reported in human, IL-17-deficiency or anti-IL-17 therapy was shown to reduce liver tumour growth by repressing LPC transformation into CSCs.[43] Interestingly, several ongoing clinical trials using anti-IL17 or anti-TNF-α therapies to treat autoimmune diseases including psoriasis,[74] showed a well-tolerated treatment suggesting their potential use for other diseases to prevent or cure HCC.

Targeting exosomal transfer

Exosomes not only are considered as biomarkers for HCC but also could constitute a new target for HCC treatment.[56] Numerous studies revealed that HCC-derived exosomes elicit HCC initiation and progression.[55] Then, inhibiting the release of exosomal oncomiR or increasing the effect of tumour suppressor miRNA may constitute a novel strategy to treat HCC initiation and also progression. For example, blockade of miR-23a-3p in HCC cells prevent their exosomal release and upregulation of PD-L1 expression in macrophages, which plays an important role in tumour cell escape from antitumour immunity.[75] Similarly, targeting exosomal miRNA released from HCC cells which stimulate angiogenesis[76] or favouring tumour immune escape[77] may provide new therapeutic tools in HCC treatment.

Origin of exosomes from other cell types could carry and effectively deliver therapeutic miRNAs. For example, mesenchymal stem cells (MSCs) are known to produce large amounts of exosomes and their transfection with miR-122 can effectively package this liver-specific miRNA in released exosomes. Consequently, the transfer of miR-122 from adipose tissue-derived MSCs into recipient HCC cells was successfully observed, rendering cancer cells sensitive to chemotherapeutic agents. Additionally, intratumour injection of miR122-exosomes significantly increased the antitumour efficacy of sorafenib on HCC in vivo.[78] Similar result was obtained with lentivirus-mediated transfection of miR-199a by increasing HCC sensitivity to doxorubicin in vitro and in vivo through inhibiting the mTOR pathway.[79] Among stromal cells, cancer-associated fibroblasts (CAF) can also transfer miRNA to HCC and it was demonstrated that miR-320a[80] and miR-150–3p[81] could function as an antitumour miRNA. Then, transfer of these CAF-derived tumour suppressor miRNA might be a potential treatment option to inhibit HCC progression. Similarly, delivery of miR-214 which function as a tumour suppressor in HCC by human endothelial cell-derived exosomes, in combination with anticancer drugs (sorafenib and oxaliplatin) reduces the viability and invasion of HCC cells compared with monotherapy.[82] On the contrary, transfer of oncomiR 1228-3p from CAF-derived exosomes into HCC cells increases their resistance to sorafenib,[83] requiring to propose a targeting of this exosomal miRNA. Thus, exosomes considered as biomarkers for HCC could be used as an efficient tool to target and control the tumour cell development. Taken together, these recent studies in early steps of tumourigenesis, cell-derived exosomes may become an important tool for not only early cancer diagnosis but also useful for therapeutic drug delivery.

CONCLUSION

Primary liver cancers develop mainly in an inflammatory context evidenced in virtually all chronic liver diseases. Despite significant advances in liver cancer diagnosis and therapies, the current anticancer treatments remain poorly effective in advanced stages of the disease over the past decade. Chronic non-resolving inflammation drives malignant initiation, tumour growth by increasing cancer stemness (self-renewal, EMT, chromosomal instability, immune escape), cancer metastasis and recurrence. Implication of LPCs in carcinogenic processes may enlighten the plasticity of these cells with high capacity to self-renew and their putative malignant features responsible for

genetic heterogeneity observed in tumour development. Dissecting and evaluating the contribution of stromal and immune cells allows understanding the intricated crosstalk between these cells localised in CSC niche, which control the LPC transformation into CSCs and giving rise to HCC and CCA. In this inflammatory context, Th17 cells secreting IL-17 are crucial components among infiltrating immune cells that drives transformation of LPC into CSC. Therefore, treatment aiming at neutralising IL-17 production in combination with other therapeutic strategies (targeting angiogenesis, immune escape, metastasis) may constitute a novel strategy for CSC eradication and could prevent liver cancer initiation from LPC origin.

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