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Liver tropism in cancer - the hepatic metastatic niche

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Abstract:

The liver is the largest organ in the human body and is prone for cancer metastasis. Although the metastatic pattern can differ depending on the cancer type, the liver is the organ to which cancer cells most frequently metastasize for the majority of prevalent malignancies. The liver is unique in several aspects: the vascular structure is highly permeable and has unparalleled dual blood connectivity, and the hepatic tissue microenvironment presents a natural soil for the seeding of disseminated tumour cells. While 70 % of the liver is composed of the parenchymal hepatocytes, the remaining 30 % is composed of non-parenchymal cells including Kupffer cells, liver sinusoidal endothelial cells, and hepatic stellate cells. Recent discoveries demonstrate that both, the parenchymal and the non-parenchymal cells can modulate each step of the hepatic metastatic cascade, including the initial seeding and colonisation as well as the decision to undergo dormancy versus outgrowth. Thus, a better understanding of the molecular mechanisms orchestrating the formation of a hospitable hepatic metastatic niche and the identification of the drivers supporting this process is critical for the development of better therapies to stop or at least decrease liver metastasis. The focus of this perspective is on the bidirectional interactions between the disseminated cancer cells and the unique hepatic metastatic niche.

Introduction:

Metastasis is the spreading of cancer cells from the primary tumour site to secondary distant sites in the human body, and it is estimated that metastasis accounts for about 90 % of cancer related deaths (Chaffer and Weinberg 2011). The liver is a highly metastasis-permissive organ and the majority of the most common solid cancers, namely lung, pancreas, breast, colorectal, prostate, gastric, oesophagus, cervix uteri, thyroid, and bladder cancer frequently metastases to the liver (Budczies et al. 2015). As a result, the liver represents the organ with the highest metastatic incidence (Hess et al. 2006). In fact, liver metastases are even more common than primary liver tumours (Bosch et al. 2004). It is estimated that 30-70 % of cancer patients die with liver metastasis (Pickren et al. 1982) and most patients with liver metastasis will die of their disease (Gilbert et al. 1982; Clark et al. 2016).

Clinical observations show that different cancer types display dramatic variations in their metastatic pattern. Some tumours mainly disseminate to only one organ (e.g. ocular melanoma to liver, prostate

to bones), whereas others metastasise to multiple organs (e.g. skin melanoma, breast and lung cancer) (Vanharanta and Massague 2013). The cancers with high hepatic metastatic prevalence include uveal melanoma (>90 %) (Amaro et al. 2017) and gastrointestinal cancers, namely pancreas (75-80%) (Ryan et al. 2014) and colorectal (50 %) (Chow and Chok 2019).

The concept of organ selectivity of metastases, or tissue tropism, was first introduced in 1889 by the English surgeon Stephen Paget (Paget 1989). He proposed that tumour cells have an affinity to certain organs, where they seed into a friendly “soil”, which facilitates their initial survival and their later outgrowth. Since this original work, significant advances have been made in our understanding of both the cell-autonomous mechanisms that drive metastasis, and alterations in the “soil” at the secondary site that allow efficient metastatic colonization and outgrowth leading to clinically relevant metastatic lesions (Minn et al. 2005; Lu et al. 2011; Qian et al. 2011; Sevenich et al. 2014; Kitamura et al. 2015; Nielsen et al. 2016; Roe et al. 2017; Quaranta et al. 2018). Systemic effects from the primary tumour that occur before metastasis have also been shown to affect the tropism and efficiency of disseminated cancer cells to colonise the secondary site (Kaplan et al. 2005; Hoshino et al. 2015). This pre-condition of the future metastatic niche, known as the pre-metastatic niche, has also been described for liver and enhances the engraftment, survival, and outgrowth of arrested disseminated tumour cells (DTCs) (Costa-Silva et al. 2015; Lee et al. 2019).

In general, the metastatic colonisation of the liver is divided into five phases: (1) Adhesion and arrest phase within the sinusoidal lumen, (2) extravasation phase into the Space of Disse, (3) hepatic niche activation phase, (4) latency and resistance, (5) outgrowth phase (expansion of metastasis). The first four phases do not require angiogenesis and likely are the entirety of the process for dormant metastases; these could remain as such for years to decades or proceed due to unknown stimuli to emergent masses as noted in the last phase. In addition, the pre-metastatic phase could set the stage for efficient liver colonization by DTCs.

This perspective aims to discuss our emerging understanding of the bidirectional interactions between the disseminated tumour cells and the hepatic metastatic niche, and to highlight potential therapeutic opportunities to develop better treatments for liver metastasis.

Main text:

Liver architecture and hemodynamic flow:

The liver is the largest organ in the human body and is composed of smaller functional units called lobules. The main components of each lobule are the parenchymal hepatocytes and they are aligned in a sheet like structure. These structures are surrounded by branches of the hepatic artery, portal vein, and bile duct, which build together the portal triad. The liver is the only organ that has a dual blood connectivity receiving blood from both the hepatic artery and the portal vein. The hepatic artery provides the oxygenated blood and contributes to approximately 25 % of the blood influx. The portal vein brings nutrient-rich blood from visceral circulation, which is connected to the intestine, pancreas, spleen and contributes to approximately 75 % of the blood supply to the liver (**Figure 1A**). At the cellular level, the liver is composed of 70 % parenchymal hepatocytes and cholangiocytes, and 30 % non-parenchymal cells. Parenchymal cells are responsible for the metabolic, detoxification, and glandular functions. The non-parenchymal cells are represented by a mixture of highly specialised cells, including Kupffer cells (KC), liver sinusoidal endothelial cells (LSEC), and hepatic stellate cells (HSC). (Kmiec 2001; Heymann and Tacke 2016). Finally, the liver can be functionally further classified into zones (I, II, II), depending on the level of oxygen, with zone I being proximate to the portal triad and thus the most oxygenated, and zone III being located close to the central vein and thus the most hypoxic (Kmiec 2001) (**Figure 1B**).

The liver has several unique features which are necessary for its normal physiological functions, but make the liver intrinsically susceptible for blood borne metastasis: i) the liver is a highly vascularised organ, ii) has an exceptional low blood flow rate, and iii) the LSEC are highly fenestrated and lack a sub-endothelial basement membrane making them the most permeable endothelial cells of the mammalian body (Poisson et al. 2017).. These organ-specific features not only facilitate the exchange of larger molecules necessary for the blood detoxification, as part of the livers' homeostatic function, but also allow the extravasation of DTC, as shown in quantitative cell-tracking studies in mice (Chambers et al. 2002).

While the close proximity and direct connection of the gastrointestinal track to the liver might in part explain the high hepatic metastatic prevalence of gastrointestinal carcinomas, including pancreas and colorectal cancer (Ryan et al. 2014; Chow and Chok 2019). it does not explain the high hepatic

metastatic rate for other cancer types such as breast, lung, and uveal melanoma. Thus, there is an emerging interest to better understand the cellular and molecular processes responsible for the high hepatic metastatic rate.

Metastatic steps to the liver:

The metastatic cascade:

The final step of cancer progression is the development of distant metastatic tumours, also known as secondary tumour sites. During the metastatic cascade neoplastic cells need to undergo a series of steps prior they are able to generate clinically detectable metastases. To start the metastatic cascade, at the primary site, cancer cells must invade from the confined primary tumour into the adjacent parenchyma and must intravasate into the circulation. Once tumour cells are in the circulation they must survive until they reach a potential secondary site. Survival of tumour cells in the circulating blood depends on their interaction with platelets and platelet-derived factors such as transforming growth factor beta (TGFβ) and fibrin that promote a mesenchymal phenotype in the DTCs and protect them from natural killer (NK) cell-mediated elimination (Palumbo et al. 2005; Labelle et al. 2011). In breast cancer, DTCs have also been found within the blood stream in association with neutrophils, which enhanced cell cycle progression of DTCs and increased their metastasis to the lungs (Szczerba et al. 2019). Whether a similar survival mechanism exists for DTCs targeting the liver is currently unknown.

The main rate-limiting step for metastasis formation occurs during the colonization of distant organs (Vanharanta and Massague 2013). DTC reaching the new microenvironment of the distant organ are vulnerable to immune surveillance and host-tissue defence. In the liver, initial immune surveillance is mediated by tissue resident KCs and NK cells (Heymann and Tacke 2016). In general, upon entering the liver via either the hepatic artery or the portal vein, DTCs become arrested and trapped in the sinusoidal capillaries of the liver whereby LSEC play a key function (phase 1). At this stage, DTCs are either able to extravasate or they die. Upon arrest, DTCs access the perisinusoidal space (Space of Disse) by endothelial transmigration (phase 2). The upregulation of cell adhesion molecules by LSEC promotes the arrest, retention, and trans-endothelial migration of DTCs. Even after successful extravasation, the vast majority of cancer cells die, but a minority of these cells may remain in the

pre-angiogenic phases in dormancy (phase 3 and 4) or start their metastatic expansion (phase 5) (**Figure 2**) (Massague and Obenauf 2016). Noteworthy, the colonisation of the hostage environment represents a bottleneck in the metastatic cascade and can be critically facilitated by a fine-tuned bidirectional interaction between cancer cells and the hepatic microenvironment. The hepatic metastatic niche can facilitate the metastatic colonization in many different ways, including protecting of tumour cells from immune surveillance, providing growth and survival signals, and promoting the formation of intra-tumoral stroma and blood vessels as described in more detail in the following sections.

The pre-metastatic niche:

A growing body of research has demonstrated that organs of future metastasis are selectively and actively modified by the primary tumour before metastatic spread occurs. Thus, tumours can induce the formation of a susceptible metastatic microenvironment before their arrival at these sites. These microenvironments are termed pre-metastatic niches (PMN) (Kaplan et al. 2005; Hoshino et al. 2015). Although the dependency of metastasis on these PMN formation remains controversial and difficult to verify in patients, numerous pre-clinical studies have identified various molecular and cellular changes that occur in the PMN, including the liver, to support future metastatic tumour growth (Psaila and Lyden 2009). Tumour-secrete factors and tumour-shed extracellular vehicles, called exosomes, have been identified to orchestrate step by step the formation of the PMN. Enhanced vascular leakage is the earliest event in this sequence, followed by the recruitment of bone marrow derived cells and the local activation of resident stroma cells, such as fibroblasts, which all aim to better attract, arrest, and retain DTCs (Joyce and Pollard 2009; Becker et al. 2016). Tumour derived tissue metallopeptidase 1 (TIMP1) has been linked to PMN formation in the liver in colorectal cancer (CRC). CRC patients showed increased TIMP1 levels, which correlated with liver metastasis. TIMP-1 led to increased stromal derived factor (SDF) 1α levels, which in turn promoted recruitment of neutrophils to the liver (Seubert et al. 2015). VEGF-A has been identified as another CRC derived factor linked to hepatic PMN formation. VEGF-A expressed by CRC induces the secretion of CXCL-1 in macrophages, which lead to the accumulation of $CXCR2^{+}$ MDSC in the liver and the formation of a hepatic PMN (Wang et al. 2017). S100 family proteins A8, A9, and P were also identified to drive liver PMN formation in pre-

clinical CRC models (Zhang et al. 2013; Weidle et al. 2015). More recently, the role of exosomes in hepatic PMN formation attracted major attention. Macrophage migration inhibitory factor -1 (MIF-1) was identified as main cargo of PDAC-derived exosomes, able to induce a hepatic PMN. MIF containing exosomes taken up by KCs, upregulated their expression of TGFβ which led to the activation of resident HST. Activated HST formed a fibronectin-rich niche, thereby facilitating the adhesion of DTCs and the infiltration of bone marrow derived cells via their fibronectin-binding surface receptors α4β7 and α4β1, respectively (Costa-Silva et al. 2015). Interestingly, high plasma exosomal MIF-1 levels were also detected in early stage PDAC patients, suggesting that a PMN could be formed at very early stages of PDAC development. Indeed, an early metastatic spreading during tumour progression has been reported in a genetically engineered mouse model of pancreatic cancer (Rhim et al. 2012). The molecular characterisation of pancreatic cancer derived-exosomes found in the circulation of PDAC patients and tumour bearing mice also provides an explanation of how cancers determine organ tropism. Intergrin αvβ5 expression on PDAC-derived exosomes was identified as a key adhesion receptor that specifically binds to KC and is necessary for the update of exosomes into KC, leading to the subsequent formation of the hepatic PMN (Hoshino et al. 2015). In colorectal cancer, microRNA-21-5p-rich exosomes released by tumour cells induce the formation of a pro-inflammatory pre-metastatic niche in the liver by binding to TLR7 on macrophages leading to the release of interleukin 6 (IL-6) (Shao et al. 2018). While proteoglycan Glypican-1 positive exosomes have been described to specifically accumulate in PDAC patients, their role in hepatic PMN formation has not been reported (Melo et al. 2015). Likewise, the contribution of circulating tumour cells, present early in tumour development and after resection of the primary tumour, to hepatic PMN formation remains unknown (Bork et al. 2015; Tsai et al. 2016).

The hepatic metastatic niche:

Role of tissue resident cells

Liver sinusoidal endothelial cells (LSEC):

DTCs entering through the blood circulation first encounter the LSEC, which cover the luminal side of the sinusoids. LSEC are a heterogeneous cell population (Strauss et al. 2017) that, similarly to the

KC, function as scavengers, clearing macromolecular waste molecules from the circulation. LSEC express different scavenger receptors, including stabilin 1 and 2 allowing a high endocytic capacity (Sorensen et al. 2012). LSEC can regulate the arrest and adhesion of DTCs by expressing of cell adhesion molecules, including E-selectin, vascular cell adhesion molecule 1(VCAM-1), and intercellular cell adhesion molecule 1 (ICAM-1). The induction of cell adhesion molecules on LSECs can be triggered by an inflammatory response mediated by KC and NK cells in response to the arriving DTCs. Although, this immune response was initially thought to be a tumoricidal response, the inflammatory response and the activation of LSEC facilitates cancer cells adhesion and endothelial transmigration into the pre-sinusoidal space, where cancer cells are protected from KC and NK cells (Glinskii et al. 2005). In pancreatic cancer, IL-35 is highly expressed by cancer cells and induces ICAM-1 expression on LSEC, thereby increasing adhesion of DTC to the endothelial wall and enhancing liver metastasis (Huang et al. 2017). Inhibition of integrin $\beta 2$ expression, a ligand of ICAM-1, on the C26 CRC cell line led to reduced retention of DTCs in the liver in a pre-clinical mouse model of colon cancer (Benedicto et al. 2017), while blockade of adhesion molecules or inhibition of the inflammatory TNFα/TNFR2 signalling axis reduced CRC liver metastasis (Khatib et al. 2002; Yoshimoto et al. 2012; Ham et al. 2015). LSEC-mediated activation of Notch signalling also increased metastasis of melanoma and colorectal cancer cells to the liver. In this study, reduced liver metastasis was linked to a reduction of the cell adhesion molecule ICAM-1 expressed by LSEC, resulting in impaired adhesion and retention of tumour cells in sinusoids. Similarly, anti-ICAM-1 antibody treatment significantly reduced tumour cell adhesion to LSEC under normal Notch expression levels suggesting that Notch signalling controls liver metastasis via modulation of adhesion molecule expression on LSEC (Wohlfeil et al. 2019).

The interaction of DTCs with LSECs not only facilitates the extravasation of DTCs into a more protected environment, but also triggers the activation of signalling pathways in cancer cells enhancing their survival and growth potential. Namely, the interaction of ligands sLewA, sLewX, and CD44 isoforms expressed on cancer cells with E-selectin expressed on inflamed LSEC enhances liver metastasis in CRC (Witz 2008; Elliott et al. 2014) and induces the release of the pro-inflammatory signal factor high mobility group box 1 (HMBG1), thereby further fuelling the expression of adhesion molecules on LSEC (Aychek et al. 2008).

LSEC also secrete factors that enhance the metastatic potential of cancer cells. LSEC-derived fibronectin and macrophage migration inhibitory factor (MIF) induced EMT in CRC cells resulting in increased invasion and migration of CRC cells into the liver parenchyma (Ou et al. 2014; Hu et al. 2015).

LSEC are a key component of tumour angiogenesis, which is a critical step in tumour expansion. While LSEC under homeostatic conditions are fenestrated, fibrosis and the growth of neoplastic cells in the liver can lead to LSEC trans-differentiation with loss of LSEC markers and sinusoidal fenestrae, a process known as capillarization, during which LSECs lose their protective properties and promote angiogenesis and vasoconstriction (Ding et al. 2014; DeLeve 2015). In contrast, non-angiogenic dependent expansion of metastatic lesions in the liver has also been described (Vermeulen et al. 2001; Stessels et al. 2004). In the liver, tumours cells have been shown to hijack the existing dense vascular network by migrating along LSECs instead of inducing angiogenesis. This mechanism is called vessel co-option and in patients with colorectal cancer liver metastases, vessel co-option is associated with poor response to the anti-angiogenic agent bevacizumab (Frentzas et al. 2016)

Taken together, LSEC can play multiple roles in liver metastasis. While they present a natural barrier for DTCs to access the liver parenchyma, in response to local inflammation, activated LSEC can help DTCs enter the liver. The increased expression of adhesion molecules on LSEC not only helps the DTCs to arrest in the sinusoids, but can also trigger pro-metastatic functions in cancer cells upon ligation of cancer-cell specific receptors. Co-option of the dense LSEC network in the liver further promotes metastatic tumour growth by providing tumours with access to a pre-existing blood supply system.

Kupffer cells:

The liver parenchyma is rich in cells of the innate immune system, potentially posing an obstacle to cancer cells. Particularly macrophages are highly abundant. In rodent livers, every 100 hepatocytes are accompanied by 10-20 macrophages (Lopez et al. 2011). These constitutively resident hepatic macrophages are known as Kupffer cells (KC) and . . are seeded along the LESC (Tacke and Zimmermann 2014). KC originate from fetal liver-derived erythromyeloid progenitors and they rely on

self-renewal rather than on infiltrating monocytes for their maintenance (Gomez Perdiguero et al. 2015). Since in human and mouse the expression of KC surface markers largely overlaps with monocyte-derived macrophages and other phagocytes, a combination of several surface markers is needed to identify KCs. Murine KC stain positive for $F4/80^{+}$, $CD11b^{+/-low}$, $CD68^{+}$, and C-type lectin domain family 4 member F (CLEC4F), while they are negative for CX3CR1 due to their non-monocytic origin (Heymann et al. 2015). Human KC are less well characterised and are commonly identified by $CD14^{+}$, $CD68^{+}$, $TLR4^{+}$, $CX3CR1^{neg}$ expression (Krenkel and Tacke 2017).

KCs are highly specialized macrophages and act as a prime sensor for immune surveillance during homeostasis and disease. The constant low level exposure to bacterial components such as lipopolysaccharide (LPS) renders the cells refractory to LPS stimulation. The tolerogenic potential of KCs in homeostasis is reflected by their constitutive ability to secrete IL-10 upon LPS stimulation, as well as by expression of the T-cell inhibitory molecule programmed cell death 1 ligand (PD-L1) and the induction of regulatory T (Treg) cells under homeostatic conditions *in vivo* (Heymann et al. 2015). In their interaction with invading cancer cells, KCs can play diverse and opposing roles that depend on several factors including the stage of the metastatic process, tumour antigen load, and interactions with other immune cells. KCs can exert cytotoxic activity towards DTCs by releasing oxygen metabolites, phagocyte tumour cells, release cytotoxic cytokines, and secrete proteases (Gardner et al. 1991; Wang et al. 2000; Seki et al. 2011). DTC adhesion to KCs can induce a rapid phagocytosis of tumour cells and their removal from the liver (Bayon et al. 1996). This anti-tumoral activity of KCs may require the recruitment of other inflammatory cells, such as NK cells, which promote the tumoricidal effect of KCs by secreting inflammatory factors such as granulocyte macrophage colony stimulating factor (GM-CSF) and interferon gamma (IFNγ) (Timmers et al. 2004). KC can decrease hepatic metastatic tumour burden of CRC during early stages and this was associated with increased TNFα and IL-1β levels (Khatib et al. 2005; Matsumura et al. 2014). Extensive phagocytosis and elimination of tumour cells is attributed to the first 24 h of tumour cell entry, suggesting that the tumoricidial activity of KC is limited to the early events of metastatic colonization of the liver (Matsumura et al. 2014). Cancer cells, which survive the initial insult of KC can later benefit from the KC tumour promoting functions. The switch is determined predominantly by tumour cell burden. KCs exhibit a capacity for immune-surveillance when tumour cell numbers are low. However, KCs switch

to promote liver colonisation and metastatic progression when their phagocytic capacity is overwhelmed due to excessive number of DTCs invading the liver (Bayon et al. 1996).

KC can increase the adhesion of DTCs to the endothelium by inducing the expression of vascular endothelial cell adhesion molecules, (Khatib et al. 1999; Khatib et al. 2002). Moreover, KC can produce a plethora of factors including IL-6, hepatocyte growth factor (HGF), VEGF and matrix metalloproteinases (MMPs) 9 and 14 that can accelerate tumour invasion into and within the parenchymal space as well as promote tumour cell proliferation and angiogenesis, thereby enhancing liver metastasis.

Hepatic stellate cells:

In normal liver, hepatic stellate cells maintain a quiescent, non-proliferative phenotype and are located in the peri-sinusoidal space (Space of Disse), interposed between the basolateral surface of hepatocytes and LSEC. The storage of retinyl esters in cytoplasmic lipid droplets is this cell type is the most distinctive feature and enables their isolation by density gradient centrifugation (Friedman and Roll 1987). HSC can be further characterised by the expression of platelet derived growth factors receptor (PDGFR)-β, enzyme lecithin retinol acyltransferase (LRAT), the cytoskeletal proteins desmin and glial fibrillary acidic protein (GFAP) (Mederacke et al. 2013; Mederacke et al. 2015; Tsuchida and Friedman 2017). In response to inflammatory stimuli, triggered by liver damage or the presence of neoplastic cells, quiescent HSC transdifferentiate from vitamin A-storing cells to myofibroblasts, which are proliferative, migratory, and contractile. In addition, activated HSC secrete a variety of chemokines and cytokines, which can shape the immune response and the tumour microenvironment. HSC are characterised by enhanced ECM production, which makes them a major driver for liver fibrosis and cancer-associated desmoplasia (Tsuchida and Friedman 2017).

HSC have been shown to promote the formation of the pre-metastatic hepatic niche as introduced before (Kaplan et al. 2006). Lyden and colleagues demonstrated that in pancreatic cancer models, resident KC are initially activated by PDAC- derived exosome released by the primary tumour. These exosomes contain MIF, and are taken up by KCs, triggering their activation. Activated KCs release the HSC-activating factor TGFβ which leads to the transactivation of HSC and increased deposition of fibronectin at the future metastatic site. Lyden and colleagues further showed that a FN-rich hepatic

niche facilitates the recruitment of bone-marrow derived myeloid cells. Most likely, extracellular deposited FN within the perisinusoidal space serves as a docking site for circulating myeloid immune cells, which characteristically express high levels of FN –binding integrins, including $\alpha4\beta1$ and $\alpha v\beta3$ (Costa-Silva et al. 2015).

Tissue fibrosis can enhance cancer metastasis by creating a growth-permissive fibrotic microenvironment capable of supporting metastatic growth by enhancing tumour cell survival. Treatment of mice with the fibrosis inducing chemical dimethylnitrosamine (DMN) increased hepatic metastatic frequency of orthotopically implanted 4T1 breast cancer carcinoma cells. The enhanced hepatic spreading was linked to the presence of lysyl oxidase (LOX), secreted by activated (αSMA+) HSCs (Cox et al. 2013). LOX is an extracellular amine oxidase whose primary function is to post-translationally modify collagens and elastins, which is a critical step in organ fibrosis and in the formation of a desmoplastic tumour stroma (Barker et al. 2012).

In pancreatic cancer metastasis, the transactivation of resident HSC by immune cells has been identified to be critical for efficient hepatic metastatic outgrowth. Upon initial seeding of the liver by disseminated pancreatic cancer cells, bone marrow derived inflammatory monocytes are rapidly recruited to the metastatic niche, where they differentiate into metastasis associated macrophages (MAMs). The accumulation of inflammatory monocytes and MAMs preceded the aberrant activation of HSC and the genetic and/or pharmacological targeting of MAMs abolished the formation of a desmoplastic hepatic niche (Nielsen et al. 2016). MAMs promote the activation of resident HSC by secreting progranulin, a glycoprotein and a potent activator of fibroblasts (He et al. 2003; Elkabets et al. 2011). Activated HSC secrete a plethora of ECM proteins, including periostin, which enhances the metastatic outgrowth of disseminated PDAC and CRC cells by increasing cell survival via the AKT pathway (Bao et al. 2004; Nielsen et al. 2016).

A proangiogenic role of HSC was also described. In a metastatic B16 melanoma model, activated HSC secrete VEGF-A and Angiopoietin 1 to initiate angiogenesis (Olaso et al. 2003; Taura et al. 2008; Copple et al. 2011). In hepatic CRC metastasis, TIMP-1 is highly expressed in HCS which are in close proximity to $CD34^+$ endothelial cells, suggesting a vascular remodelling function of HSC (Illemann et al. 2016). Isolated HSC secrete laminin and show enhanced endothelial cell network formation in matrigel assays. Co-injection of HSC together with colorectal cancer cells enhanced the

metastatic process to the liver by supporting angiogenesis (Eveno et al. 2015). Activated HSCs can also directly promote tumour growth by secreting HGF and TGFβ (Thompson et al. 2015). Less well understood is the function of HSCs in controlling the immune response in liver metastasis. Skin cancer associated fibroblasts regulate the recruitment of immune cells and their functions by releasing cytokines/chemokines (Erez et al. 2010). Activated HSC secrete a plethora of cytokines and chemokines with well-known effects on immune cell recruitment and functions (Nielsen et al. 2016), suggesting that HSC also shape the immune response during liver metastasis. In primary HCC, HSC derived factors promote immune suppression by promoting the expansion of immunosuppressive regulatory T cells (Tregs) and the induction of myeloid derived suppressor cells (Zhao et al. 2012; Xu et al. 2016). However, whether or how HSC influence immune cell recruitment during liver metastasis requires further investigation. Myofibroblasts have also been linked to the adaptive immune response, particularly regulating T cell infiltration and survival. In pancreatic cancer and melanoma models, cancer associated fibroblasts at the primary tumour site have been shown to impair $CD8^+$ T cell infiltration (Kraman et al. 2010; Feig et al. 2013) or induce their depletion (Lakins et al. 2018), although the exact mechanism(s) by which pancreatic tumours prevent $CD8^+$ T cell recruitment is not fully understood yet. In regard to the hepatic niche, in liver biopsies of metastatic PDAC patients, $CD8^+$ T cells were found in αSMA^+ myofibroblast-rich regions. In a metastatic mouse model of PDAC, activation of HSC led to $CD8^+$ T cell exclusion and resistance to immune checkpoint therapy (αPD-1), which could be reversed by reducing the fibrotic stroma (Quaranta et al. 2018). Taken together, these results suggest that liver fibrosis driven by HSC enhances hepatic metastasis growth and further showcases a bidirectional crosstalk between immune cells and HSC in the hepatic metastatic niche.

Hepatocytes:

The role of the parenchymal hepatocytes in liver metastasis is less well understood. In CRC, the interaction of tumour cells with hepatocytes increased their metastatic potential. Integrin αv, α6, and β1, desmosomes, as well as osteopontin enhanced the interaction of cancer cells with the hepatocyte ECM (Shimizu et al. 2000; Mook et al. 2008; Huang et al. 2012).The interaction of CRC with ECM induced the induction of gene signatures related to tumour cell survival and stemness, suggesting that tumour cells – hepatocyte ECM interaction promotes the adaptation of DTCs to the hepatic niche.

Disseminated CRC expressing Fas ligand (FasL) induce apoptosis of Fas receptor bearing hepatocytes upon arrival in the liver. The induction of hepatocyte apoptosis creates a niche in the liver where blood borne DTC can settle and propagate (Zvibel et al. 2013).

Hepatocytes release several growth factors, including insulin-like growth factor 1 (IGF-1) and inhibition of IGF-1 reduces liver metastasis of colon and lung carcinoma cells (Wang et al. 2015). Overexpression of the Ron receptor, a member of the Met family of surface receptor tyrosine kinases, has been reported in several human cancers including breast, pancreas, colon, liver and bladder. Ron is activated by the hepatocyte growth factor-like protein/macrophage stimulating-protein (HGFL) which is primarily secreted by hepatocytes. Activation of RON leads to the induction of signalling pathways related to cellular growth, motility, invasion and metastasis (Wagh et al. 2008). Hepatocyte derived Heregulin (HRG) phosphorylates erbB3 and erbB2 in CRC cells, promoting intergrin $\alpha v\beta 5$ depended migration. Depletion of integrin αv or erbB3 reduced liver metastasis of CRC cells (Yoshioka et al. 2010).

Parenchymal hepatocytes promote hepatic metastasis for different cancer types, including pancreatic and colorectal carcinomas, by coordinating the formation of a pro-metastatic niche. In response to primary tumour derived factors, particularly IL-6, hepatocytes upregulate the release of serum amyloid A1 and A2 (SAAs), which increases myeloid cell recruitment and liver fibrosis. The secretion of SAAs by hepatocytes occurs in response to IL-6 stimulation and is STAT3 dependent. IL-6 is mainly produced by non-malignant stroma cells at the distant primary tumour site, including αSMA+ myofibroblasts, but not at the metastatic site, thereby controlling hepatocyte activation in a systemic manner. The identified IL-6 – STAT3 - SAA signalling axis is responsible for the formation of a pro-metastatic niche in the liver, but not in the lung (Lee et al. 2019). Thus, this study provides an example of how parenchymal cells might steer metastatic organ tropism.

Role of recruited inflammatory cells:

Monocyte-derived macrophages:

In addition to the presence of tissue resident KCs, liver metastasis is accompanied by the accumulation of blood-derived myeloid cells, including monocytes and neutrophils. Monocytes in the

circulation can be differentiated into two subsets based on cell surface expression of different markers. Inflammatory monocytes are characterised by Ly6C^{high} CX3CR1^{mid} CCR2^{+} CD62L^{+} CD43^{low} expression, whereas patrolling monocytes are characterised by Ly6C^{low} CX3CR1^{high} CCR2^{-} CD62L^{-} CD43^{high} expression (Geissmann et al. 2010). The recruitment of inflammatory monocytes is necessary for efficient hepatic metastasis for several cancer types with liver tropism. In colorectal and lewis lung carcinoma models, inflammatory monocyte accumulation is mediated by the CCL2/CCR2 axis. Tumour-secreted CCL2 attracts CCR2+ myeloid cells to the liver and genetic and/or pharmacological ablation of CCL2/CCR2 signalling reduced myeloid cells recruitment and metastatic tumour burden. In pancreatic cancer, inhibition of CCR2 reduced primary tumour formation and metastatic spreading to the liver (Mitchem et al. 2013).

Upon infiltration of the hepatic niche, monocytes differentiate into monocyte-derived macrophages (Nielsen et al. 2016). Macrophages are highly plastic and depending on their activation status, macrophages can execute tumoricidal or protumorigenic functions (Biswas et al. 2013; Mantovani et al. 2017). . Upon infiltration of the hepatic niche, monocytes differentiate into monocyte-derived macrophages (Nielsen et al. 2016). MAMs play a significant role in pancreatic cancer metastasis. During metastatic tumour growth in pancreatic cancer, MAMs rapidly accumulate at the metastatic site and represent the most abundant immune cell population. Bone-marrow chimera studies revealed that MAMs mainly originated from bone marrow derived monocytes (Nielsen et al. 2016). Genetic inhibition of MAM accumulation by depleting PI3Kγ reduced MAM accumulation and PDAC metastasis. Similarly, chemical depletion of macrophages by clodronate containing liposomes or pharmacological targeting of macrophages by anti-CSF-1/anti-CSF-1R ablated MAM numbers and impaired hepatic metastatic growth of disseminated PDAC cells (Nielsen et al. 2016; Quaranta et al. 2018). In regard of MAM activation, smaller, micro- metastatic lesions were rich in macrophage expressing pro-inflammatory markers including *Cox*, *Nos2*, and *Mhc class II*, while established, large metastatic lesions were highly infiltrated by immunosuppressive macrophages, expressing elevated levels of *Arginase, Tgfb and Il10*. The phenotype of macrophage polarization directly affected the activation state of metastasis infiltrating CD8^{+} T cells. Increased abundance of immune-suppressive MAMs reduced CD8^{+} T cell activation, while MAM-targeted therapies restored cytotoxic CD8^{+} T cell functions (Quaranta et al. 2018). A clinical study demonstrated that high numbers of circulating inflammatory monocytes correlates with shortened survival in pancreatic cancer, a disease which

primarily metastasises to the liver (Sanford et al. 2013). In a pre-clinical colorectal cancer model, the genetic depletion of the N-myc downstream-regulated gene 2 (NDRG2) reduced metastatic tumour burden, which correlated with an increased percentage of M1-like MAMs within the hepatic metastatic niche. Mechanistically, NDRG2-deficiency induced nuclear factor (NF)-κB pathway activation in macrophages, thereby promoting the expression of M1-like inflammatory cytokines including *Il12*, *Il1b*, and *Tnfa* (Li et al. 2018).

Neutrophils:

Neutrophils are part of the innate immune response and are also rapidly recruited to site of tumour formation, including the liver. Similar to macrophages, neutrophils can acquire opposing roles in cancer depending on the environmental context (Fridlender et al. 2009; Shaul and Fridlender 2017). Neutrophils can release cytolytic factors and produce high levels of TNFα, FasL, reactive oxygen species (ROS), thereby executing tumorical activity. In contrast, it has been demonstrated that, particularly in cancer, neutrophils can acquire an immunosuppressive phenotype and express high levels of arginase, MMP-9, and VEGF-A (Fridlender et al. 2009). In mice, independent of their phenotype, neutrophils are identified by their expression of CD11b and Ly6G. The same combination of markers is also used to identify granulocytic myeloid derived suppressor cells (gMDSC), which are defined by their potent immunosuppressive activity (Talmadge and Gabrilovich 2013; Coffelt et al. 2016). It has recently become apparent that neutrophils represent a heterogeneous population of cells with significant functional plasticity and that immune suppressive functions associated to MDSC might have been carried out by immunosuppressive neutrophils sharing the same surface markers and vice versa.

Several factors have been reported to promote the recruitment and accumulation of neutrophils/MDSC to metastatic liver tumours including CXCL1, CXCL2, CXCL5, and SDF-1α (also known as CXCL12) which bind to their cognate receptors CXCR2 or CXCR4 expressed on neutrophils (Zhao et al. 2013; Seubert et al. 2015; Steele et al. 2016). Immunosuppressive functions of Neutrophils/MDSC promote liver metastasis. In a pancreatic cancer model, primary and hepatic metastatic tumours are infiltrated by Ly6G+ neutrophils. Neutrophil depletion resulted in increased T cell infiltrating into the pancreas and the liver. Inhibition of neutrophil recruitment by blocking CXCR2 ablated hepatic metastatic spreading (Steele et al. 2016). This study suggests that

immunosuppressive neutrophils play an important role in metastatic spreading of pancreatic cancer to the liver, at least during the initial steps of the pre- and/or early metastatic niche formation. In CRC models, neoplastic cell-derived VEGF-A induced the secretion of CXCL1, a CXCR2 ligand for neutrophils/MDSC at the primary tumour site. Elevated CXCL1 levels promoted the accumulation of neutrophils/MDSCin the premetastatic niche that ultimately promoted liver metastasis (Wang et al. 2017). Increased circulating MDSC levels correlate with advanced clinical cancer stage and metastatic tumour burden. Although the analysis included a limited number (n=56) patients with different types of cancer (colon, pancreas, gastric, breast among others), patients with extensive metastatic involvement tended to have the highest number of circulating MDSC, suggesting their important role in promoting metastasis (Diaz-Montero et al. 2009).

Neutrophils promote hepatic metastasis independent of their immunosuppressive capacity. In a murine lung carcinoma model, neutrophils promote cancer cell adhesion within the liver sinusoids by providing a docking site for cancer cells to arrest. Intravital microscopy revealed that DTC adhere directly on top of neutrophils arrested on LSEC within the liver sinusoids. Cancer cell –neutrophil interaction was dependent on CD11b expression by neutrophils and ICAM by cancer cells (Spicer et al. 2012). Moreover, neutrophils can arrest DTC in the liver by neutrophil extracellular trap (NET) formation. NETs are extracellular structures composed of chromatin coated with histones, proteases and granular and cytosolic proteins that help catch and kill microorganisms. In the presence of circulating tumour cells, NET formation in the liver sinusoids results in increased retention of DTC and enhanced tumour cells adhesion, proliferation, migration, and increased liver metastasis (Cools-Lartigue et al. 2013; Tohme et al. 2016).

Tumour promoting functions of neutrophils have been attributed to the release of extracellular matrix-degrading proteinases, including MMP-8, MMP-9, elastases and cathepsin G, thereby increasing tumour invasion (Sionov et al. 2015), and by promoting angiogenesis through the release of VEGF and Bv8 (Coffelt et al. 2016). However, these studies focused on the TME at the primary tumour site and pulmonary metastatic site, and to understand whether these mechanisms also play a role in the formation of an hepatic metastatic niche requires further investigation (Jablonska et al. 2017).

Role of liver infiltrating lymphocytes:

$CD8^+$ T cells and NK cells are the main effector cells of the immune system that kill cancer cells. $CD8^+$ cytotoxic T cells are part of the adaptive immune system and recognise tumour antigens presented in the context of MHC class I and deliver cytolytic factors including granzymes, perforin and FasL (Hadrup et al. 2013). $CD8^+$ T cells also release the pro-apoptotic cytokines IFNγ and TNFα to suppress tumour growth (Barth et al. 1991; Detjen et al. 2001). NK cells belong to the innate immune system and do not need MHC class I mediated antigen presentation to recognise and kill cancer cells. Similar to $CD8^+$ T cells, NK deliver cytotoxic hits to cancer cells through the release of perforin and granzymes (Lowry and Zehring 2017). The inhibition and evasion of a specific tumoricidal T cell and/or NK cell mediated immune response is critical for neoplastic cells to survive and grow in the liver. In contrast, the infiltration of other lymphocytes, namely regulatory T cells which suppress effector T cells favours liver metastasis. Tregs are a subset of immunosuppressive $CD4^+$ T cells and are defined by their expression of the FoxP3 transcription factor. Although immunosuppressive Tregs have a critical role during tumour development and in response to therapy (Takeuchi and Nishikawa 2016; Oweida et al. 2018; Shabaneh et al. 2018), their role in the formation of a hepatic metastatic niche is less well understood. Efficient liver metastasis of colon and lung carcinoma correlates with not only the increase of MDSC, but also with the recruitment and accumulation of CD4+FoxP3+ Tregs. In this report, the recruitment of Tregs was dependent on TNFR2 signalling, since TNFR2 depleted animals showed a marked decrease in Treg accumulation (Ham et al. 2015). In a retrospective analysis for FoxP3+ Treg and CD8+ cytotoxic T cell infiltration, using tissue sections from resected colorectal cancer liver metastases, a high ratio of FoxP3+: CD8+ cells correlated with shorter overall survival of patients after surgery (Katz et al. 2013), suggesting a potential pro-metastatic role for Tregs at the hepatic niche.

Cancer cell intrinsic genetic programs driving liver metastasis:

Unlike the deep understanding of mutational mechanisms that initiate cancer progression, the genetic basis for liver metastasis is less well understood. General metastasis promoting transcriptional programs regulate pathways including self-renewal and EMT (Kong et al. 2011; Neureiter et al. 2014; Krebs et al. 2017). A few studies have focused on cell intrinsic programs that specifically drive liver metastasis. Pro-metastatic programs driving liver metastasis have been identified in cancer from the gastrointestinal tract. In CRC, miR-551a and miR-483 suppress liver colonization and metastasis by

inhibiting creatine kinase, brain type (CKB) in CRC cells. The release of CKB by metastasised cancer cells generate phosphocreatine in the extracellular space, which was imported back into CRC cells and used for ATP generation and thereby enhanced metastatic survival (Loo et al. 2015). In pancreatic cancer, the expression of the pioneer factor FOX1 increases anchorage-independent growth of PDAC cells in vitro, and invasion and liver metastasis in vivo (Roe et al. 2017). Claudin-2 has been shown to mediate breast cancer liver metastasis and was identified to be specifically expressed by liver-metastatic breast cancers cells compared to populations derived from bone or lung metastasis. The extracellular loop of claudin-2 mediated tumour-hepatocyte interaction and thereby increased metastatic tropism to the liver (Tabaries et al. 2012).

Concluding remarks

The here discussed papers, and many other reports which due to word limitation we could unfortunately not cite, provide evidence that the hepatic metastatic niche is critical for promoting liver metastases and that inhibition of key effector proteins and/or cells within the hepatic niche could open new avenues for better therapies against liver metastasis. Inhibition of the various pro-survival cues provided by the hepatic environment could improve the response to anti-cancer treatments. However,a major challenge that requires further investigation is that the type of interactions DTC establish with the different components of the hepatic niche constantly change during the metastatic steps. This might explain why targeting specific cellular interactions and effectors in the TME have markedly reduced, but not yet completely eradicated metastatic disease progression.

Although it has been demonstrated that stromal cells promote liver metastasis, an additional layer of complexity that also deserves further investigation is their potential heterogeneity and their distinct contribution to the formation (or inhibition) of a hepatic niche. However, the recent technical advances in single cell analysis now allows to deconvolute the complex structure of the hepatic niche to a single cell level and such studies will most likely shed light on the cellular heterogeneity of hepatic metastatic lesions .

The cellular and molecular structure of the hepatic metastatic niche is not only forged by DTC, but is also influenced by cues from the primary tumour site. The importance of the cross-talk between the

primary and secondary tumour site has been clearly demonstrated by the importance of the pre-metastatic niche formation in the metastatic process. However, whether and how the genomic profile of cancer cells orchestrates the formation of a cancer cell "personalised" hepatic niche is a question that remains to be addressed. Thus, to better understand how the hepatic niche supports metastasis, future studies should use pre-clinical (mouse) models that faithfully recapitulate the primary-secondary tumour interaction, and also provide the diversity of all stromal/immune partners. Based on the overall literature herein presented, we would like to reinforce the importance of further investigating the hepatic niche and help translate the exciting pre-clinical observations into the clinics.

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Figure legends

Figure 1. Anatomy of the liver and hemodynamic flow.

A. The liver is the largest organ of the human body and is the only organ which is connected to two blood circulation systems. Disseminated tumour cells (DTCs) in the arterial and visceral circulation are drained to the liver by the hepatic artery and the portal vein, respectively. Gastrointestinal cancers (pancreas, colon, stomach) are directly connected to the visceral circulation and show high liver tropism for metastases.

B. General microanatomy of the liver showing the location of the portal triads (consisting of the hepatic artery (HA), portal vein (PV), bile duct (BD)), the central vein (CV) and the direction of the blood flow across the three different zones (I, II, III). Owing to extensive branching of portal vessels into liver sinusoids, and the accompanying increase of vascularization, the hepatic microcirculation is characterised by low pressure and slow blood flow.

Figure 2. Tumour cell interactions with parenchymal and non-parenchymal cells during liver colonisation by disseminated tumour cells.

The colonization of the liver is a multistep process including arrest (1), extravasation (2), supportive niche formation (3), latency and resistance (4), and finally outgrowth (5). Major intercellular interactions and factors involved in this process are depicted. Primary tumours release factors involved in the generation of a pre-metastatic niche. Upon entry of disseminated tumour cells (DTC) into the sinusoidal vessels, DTCs first interact with LSEC and KC. Arrest of DTC is increased by cell adhesion molecules expressed by inflamed LSEC and via neutrophil interaction. Depending on the activation state, KCs release tumoricidal factors, suppress cytotoxic CD8+ T cells, or secrete growth and survival factors for DTCs. Upon extravasation, tumour secreted FasL induces apoptosis of hepatocytes, which facilitates colonisation of the parenchyma. Metastasis associated macrophages (MAMs), mainly monocyte derived, rapidly accumulate in high numbers and MAM-released factors, including PGRN, CXCL1, MMPs, TGFβ, and VEGF-A promote the generation of a hospitable hepatic niche. Key events are activation of hepatic stellate cells (HSC), recruitment of immunosuppressive neutrophils, and remodelling of extracellular matrix (ECM). Hepatocyte derived factors such as IGF1, HGFL, and SAAs contribute to the generation of a supportive niche. The supportive niche also protects neoplastic cells during potential latency and against anti-cancer therapies. Metastatic expansion and outgrowth requires the formation of new blood vessels (angiogenesis), sustained suppression of an anti-tumoral immune response, and continuous ECM remodelling.

ARG= Arginase; ANGPT1= Angiopoietin 1; FN= Fibronectin, HGF= Hepatocyte growth factor; HMGB1 = High mobility group box 1; IGF1 = Insulin like growth factor 1; IL= Interleukin; LOX= Lysil oxidase; MIF= Macrophage migration inhibitory factor; MMP= Matrix metalloproteinase, PD-L1= Programmed death ligand 1; PGRN = Progranulin; ROS = Reactive oxygen species; SAA= serum

amyloid A1/2; TGFβ = Transforming growth factor beta; TNFα= Tumour necrosis factor alpha; VEGF-A= Vascular endothelial growth factor A.

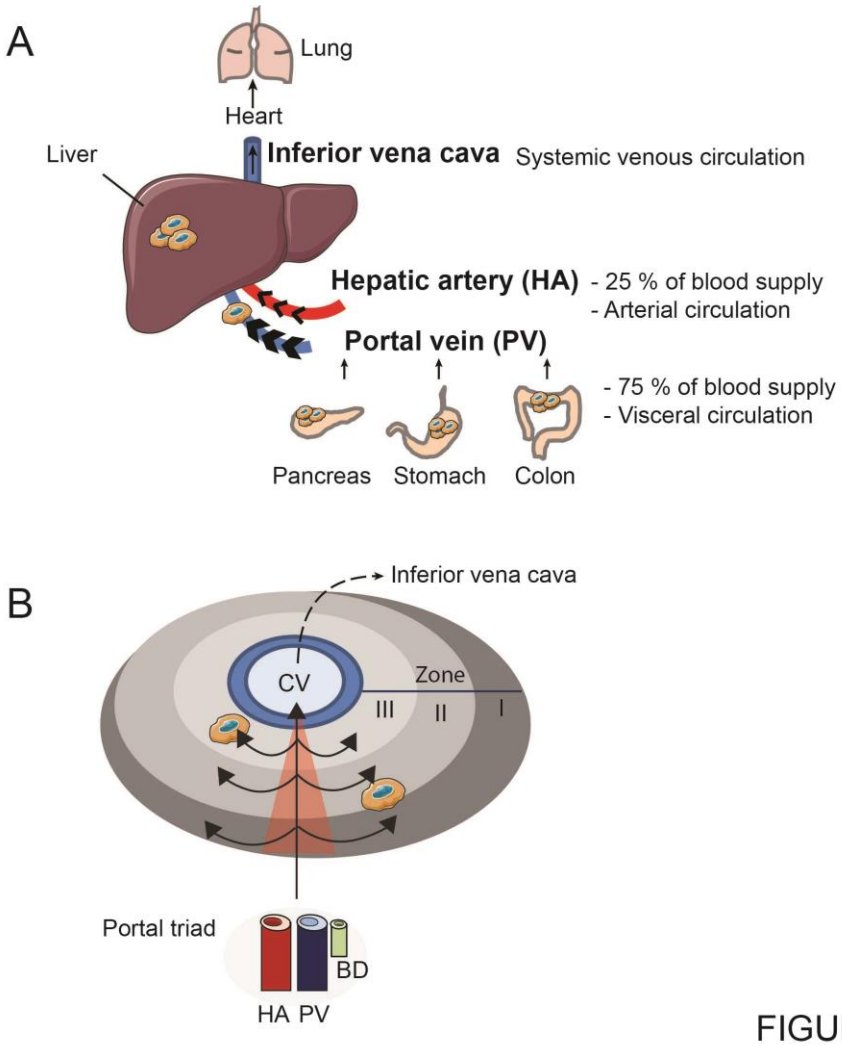

FIGURE 1

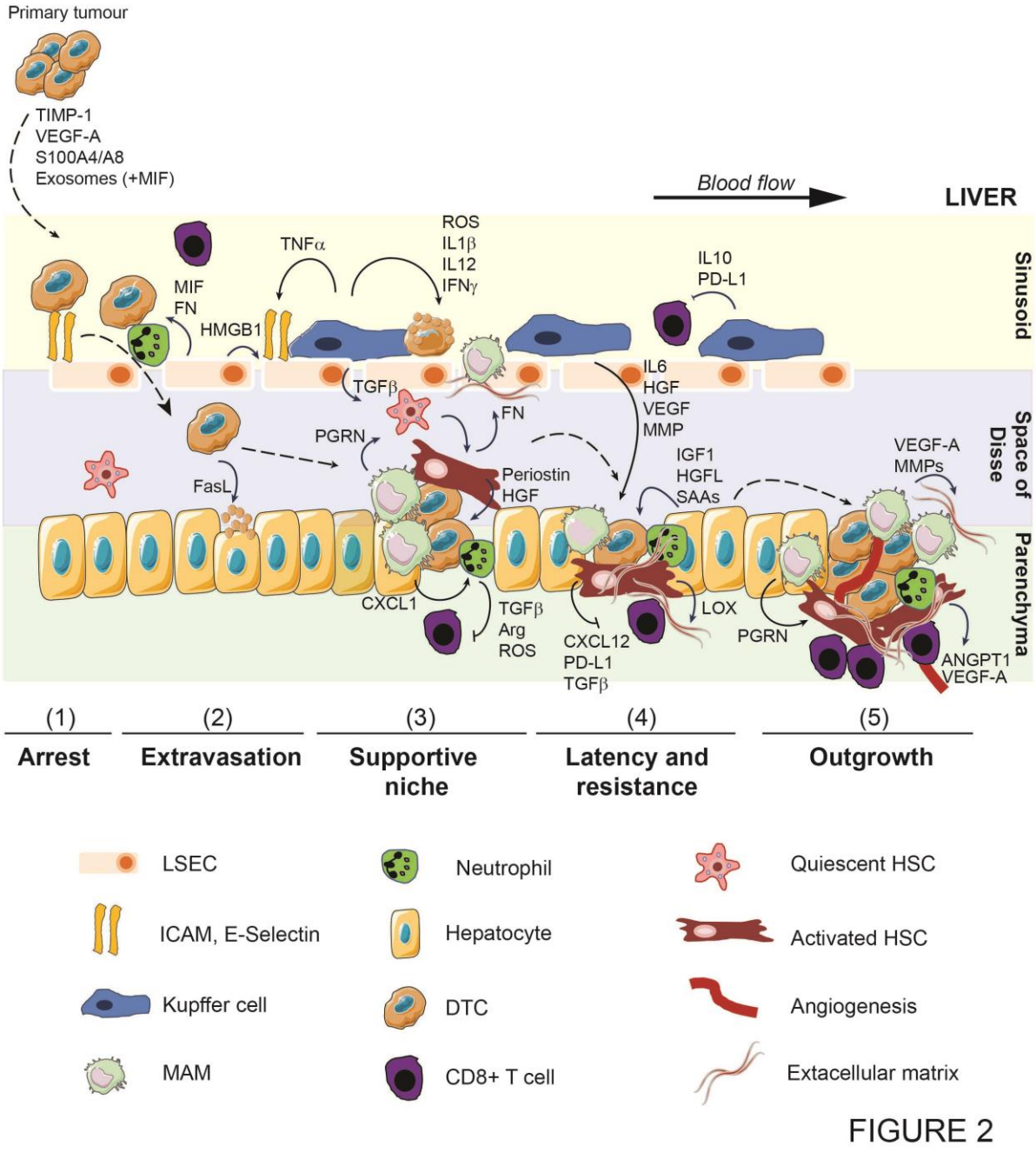
Primary tumour
TIMP-1
VEGF-A
S100A4/A8
Exosomes (+MIF)
Blood flow
LIVER
Sinusoid
Space of Disse
Parenchyma
MIF
FN
HMGB1
TNFα
ROS
IL1β
IL12
IFNγ
IL10
PD-L1
TGFβ
FN
IL6
HGF
VEGF
MMP
PGRN
FasL
Periostin
HGF
IGF1
HGFL
SAAs
VEGF-A
MMPs
CXCL1
TGFβ
Arg
ROS
CXCL12
PD-L1
TGFβ
LOX
PGRN
ANGPT1
VEGF-A
(1)
Arrest
(2)
Extravasation
(3)
Supportive niche
(4)
Latency and resistance
(5)
Outgrowth
LSEC
ICAM, E-Selectin
Kupffer cell
MAM
Neutrophil
Hepatocyte
DTC
CD8+ T cell
Quiescent HSC
Activated HSC
Angiogenesis
Extacellular matrix

FIGURE 2