NARRATIVE REVIEWS

The Role of Myeloid Cells on the Development of Hepatic Metastases in Gastrointestinal Cancer

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The development of hepatic metastases is the leading cause of mortality in gastrointestinal (GI) cancers and substantial research efforts have been focused on elucidating the intricate mechanisms by which tumor cells successfully migrate to, invade, and ultimately colonize the liver parenchyma. Recent evidence has shown that perturbations in myeloid biology occur early in cancer development, characterized by the initial expansion of specific innate immune populations that promote tumor growth and facilitate metastases. This review summarizes the pathophysiology underlying the proliferation of myeloid cells that occurs with incipient neoplasia and explores the role of innate immune-host interactions, specifically granulocytes and neutrophil extracellular traps, in promoting hepatic colonization by tumor cells through the formation of the "premetastatic niche". We further summarize the role of additional myeloid subpopulations such as monocytes and macrophages, dendritic cells, platelets, and eosinophils on promoting disease metastases in GI cancers. Lastly, we describe burgeoning therapeutic approaches aimed at targeting specific myeloid populations to reduce liver metastases and highlight the inherent challenges that exist in studying the efficacy of these treatments in preclinical models. As the inception and outgrowth of liver metastases are primary drivers of prognosis in GI malignancies; further research into the complex mechanisms involved in this critical process is urgently needed.

Keywords: Tumor microenvironment; Hepatic metastases; Granulocytes; Neutrophil extracellular traps (NETs); Colorectal cancer; Pancreatic cancer; Premetastatic niche

Introduction

The liver is a highly vascular organ tasked with homeostatic functions such as detoxification of the blood, protein biosynthesis, and various metabolic processes essential to digestion and nutrient derivation. As such, the liver serves as a reservoir and the first site of venous return from the gastrointestinal (GI) tract, deriving much of its oxygen and nutrient supply from this source. This robust vascularity makes it a common site of cancer metastasis from the GI tract and other solid organ malignancies such as melanoma, lung, breast, and renal carcinomas. Among GI tract cancers, colorectal cancer (CRC) and pancreatic ductal adenocarcinoma (PDAC) have the highest rates of hepatic metastases, and the presence of liver lesions at diagnosis are major predictors of overall survival in these malignancies. In CRC, 15%–25% of patients will present with synchronous hepatic metastases at diagnosis and another 25% of patients will be expected to develop metachronous liver disease following diagnosis.[1–3] The burden of cancer metastases is even more pronounced in PDAC, where only 15%–20% of patients are considered candidates for surgical resection at diagnosis due to widespread metastatic disease.[4] The liver remains the primary site of PDAC metastases, with 50% of patients having liver lesions evident at the time of diagnosis.[5]

Research into the properties that underlie the propensity for both CRC and PDAC tumors to seed the liver has revealed numerous mechanisms for this organotropism. In this review, we discuss the role of the myeloid cells in promoting liver metastases. We explore recent insights into formation of the "premetastatic niche" – an encompassing term that describes systemic changes induced by GI cancers that make the liver receptive to cancer metastases before their arrival through remodeling of the local immune and stromal environment. The role of innate immunity, and specifically the role of granulocyte-derived neutrophil extracellular traps (NETs), in this process is also described. Additionally, we highlight the pleiotropic role of additional myeloid subsets (monocytes and macrophages, dendritic cells, platelets, and eosinophils) in this process. Therapeutic approaches and preclinical models used to study these phenomena are also summarized.

Cancer Cell Uptake in the Liver – First Contact

Oncogenic transformation alone does not ensure that tumor cells can invade and metastasize, demonstrating that

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tumorigenesis and metastasis are distinctly separate phenotypes in cancer cells.[6] Cancer metastases proceeds in an orderly fashion that is reproducible even among diverse malignancies: local invasion and violation of the basement membrane, intravasation into systemic vasculature, transit and survival within the bloodstream, extravasation at the target tissue, and eventual colonization and tumor expansion[7,8] (Figure). Research efforts have predominately focused on delineating the various mechanisms involved in local invasion into the vasculature through diverse cellular processes such as epithelial-to-mesenchymal transition (EMT) and the induction of tumor-associated angiogenesis, as these intuitively have been considered the most critical processes to metastatic disease development.[9,10] However, emerging clinical evidence has demonstrated that disseminated tumor cells and circulating tumor DNA are present in the early stages of disease development in many patients who do not develop metastatic cancer, thereby suggesting invasiveness alone is insufficient for metastasis to occur.[11,12] Recent investigations into the mechanisms of how circulating tumor cells specifically target, invade, and colonize target tissues have been increasingly helpful in our understanding of GI hepatic metastases.

Colonization of the liver begins as disseminated cancer cells are mechanically trapped within terminal sinusoidal capillaries within the hepatic microvasculature. The shear stress from the sequential change in caliber of these vessels results in the deformation and destruction of many of the circulating cancer cells. Therefore, very few cells can successfully traverse the hepatic sinusoid to the central vein to implant in the liver parenchyma or travel outward to the lung or other distant organs.[13] Experiments by Ishii and colleagues utilizing *in vivo* fluorescence videomicroscopy have elegantly demonstrated this phenomenon using dual-labeled rhodamine B isothiocyanate-dextran and calcein AM CX-1 human CRC cells in nude mice. Tumor cell injection resulted in a substantial reduction in viable cells arrested within the portal venule or hepatic sinusoid. Only a small fraction (0.5%) of cells could traverse the hepatic sinusoidal endothelial cell barrier and subsequently implant and successfully establish metastatic tumors.[14] Interestingly, further studies have demonstrated that those surviving cells are also not necessarily destined to form gross metastases. Luzzi et al showed that only a small minority of injected B16F1 melanoma cells result in micrometastases and even fewer go on to form macroscopic lesions, with a substantial portion of injected cells lying dormant within the liver vasculature.[15] These findings highlight an important physiologic principle and help explain why the presence of circulating cells does not always result in metastatic colonization: *the development of metastases is a very inefficient process*.

Transendothelial migration is the next essential phase of metastasis in those cells that manage to survive in the liver microvasculature. Extravasation of tumor cells is largely mediated by the expression of specific adhesion molecules

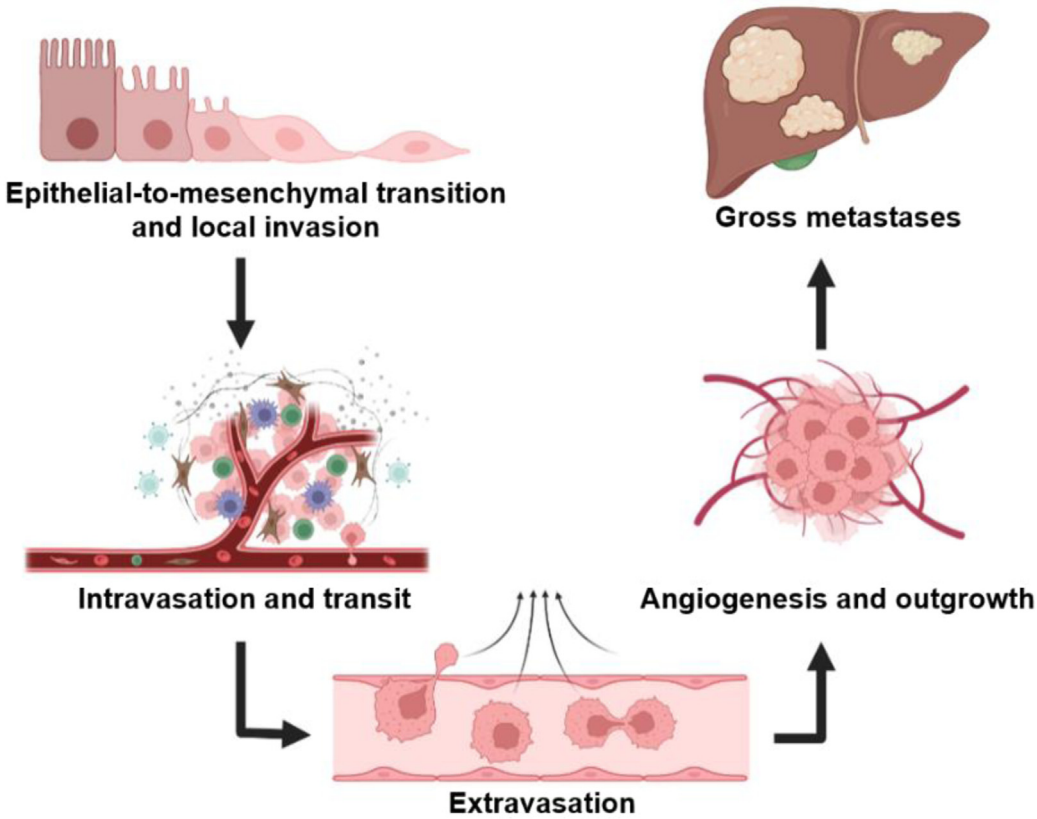

Figure. Classical steps in tumor metastases.

on the surface of sinusoidal endothelial cells that promote their escape from the vasculature. These diverse membrane receptors, such as E-selectin and vascular cell adhesion molecule-1, play important roles in normal immune cell chemotaxis in response to inflammatory signals derived from neighboring cells.[16,17] Circulating cancer cells can coopt these mechanisms through the release of soluble cytokines and chemokines, resulting in robust expression of receptors and ligands on endothelial cells that facilitate diapedesis of tumor cells into the hepatic parenchyma.[18,19] Huang and colleagues demonstrated that IL-35 derived from tumor cells promotes the expression of intercellular cell adhesion molecule-1 on endothelial cells within the sinusoids of the liver, enabling the engraftment of PDAC metastasis in mice.[20] Similarly, Khatib et al demonstrated in murine metastatic models of CRC and lung cancer that invading cancer cells induce tumor necrosis factor (TNF)-α production by Kupffer cells (KCs) within the perisinusoidal space, resulting in elevated expression levels of E and P-selectins, vascular cell adhesion molecule-1, and intercellular cell adhesion molecule-1 on endothelial cells within the liver.[21] The induction of receptor-ligand complexes on endothelial cells can also be induced by local cytokine-mediated interactions secondarily orchestrated by innate immune cells that are lured into the microenvironment by tumor cells.[22] Thus, the intersection between inflammation, immune remodeling, and endothelial cell activation appears to be a key event for tumor cells to successfully escape the vasculature and colonize the liver. However, those tumor cells that undergo successful transendothelial migration into the perisinusoidal space of Disse still face a hostile environment that prevent their outgrowth, including detection and destruction by KCs and other resident innate immune cells within the liver.[23] With such significant challenges to their survival, tumor cells have evolved clever mechanisms to enhance their efficiency of uptake at target organ sites by priming tissues for their arrival – a concept referred to as the formation of the premetastatic niche.

The Premetastatic Niche and the Role of Myeloid Cells in Metastasis

In his work, *Distribution of secondary growths of cancer of the breast*, surgeon Stephen Paget detailed metastatic disease patterns among over 700 women with breast cancer and observed that highly vascular organs such as the spleen were often spared from metastatic disease outgrowth.[24,25] Aptly named the "Seed and Soil" hypothesis, this theory implied for the first time that specific tumor cells have specific affinity for select organs, a concept referred to as *organotropism*.[25,26] These findings were further supported by a series of autopsy experiments by the physician scientist Leonard Weiss, who noted that observed patterns of metastases in a variety of solid organ tumors could not be explained by blood flow dynamics alone in clinical cases.[27] Specific to CRC, Schluter et al showed that colon cancer cells preferentially bind to the vascular endothelium of the liver and the lung, demonstrating that organ-specific metastases are a fundamental property of specific tumor cells.[28] To explain these poignant findings, researchers then embarked on mechanistic studies to delineate how target organs are prepared to receive tumor cell implants.

A landmark study by Kaplan and associates in 2005 demonstrated that tumors prime distant organs for uptake of distant metastases, appropriately termed the "premetastatic niche". Interestingly, further studies proved this phenomenon is largely mediated by immune remodeling at target locations, thus implicating myeloid cells as critical components in the pathogenesis of distant metastatic disease. Mice injected with either Lewis lung carcinoma or B16 melanoma cells demonstrated an increase in bone marrow-derived cells within premetastatic sites that preceded tumor cell seeding of these organs. Accordingly, researchers found that ablation of specific vascular endothelial growth factor 1 positive progenitor bone marrow-derived cells blocked organ-specific metastases, demonstrating these cells are essential for metastatic disease development and play an invaluable role in the phenomenon of organotropism.[29] Further studies have subsequently expanded our understanding of the immune remodeling underlying the premetastatic niche in the context of liver metastases in both CRC and PDAC. Indeed, immature myeloid populations known as myeloid-derived suppressor cells (MDSCs) have been shown to infiltrate early into the premetastatic niche and establish a microenvironment favorable to the outgrowth of metastases. Among soluble factors in MDSC recruitment, tumor cell-derived vascular endothelial growth factor A is a key activator of tissue-resident macrophages to produce the chemokine C-X-C motif chemokine ligand 1. C-X-C motif chemokine ligand 1 is a potent chemoattractant of immature C-X-C motif chemokine receptor 2 ($CXCR2^+$) MDSCs and drives their recruitment into the liver, resulting in enhanced metastatic disease engraftment in CRC.[30] These findings were additionally described by Steele et al. in PDAC, who demonstrated that genetic ablation of CXCR2 or $Ly6G^+$ depletion in mice potently suppressed metastases, further suggesting the important role of neutrophil/granulocytic precursors in the establishment of liver metastases.[31] Similarly, tissue inhibitor of metalloproteinases-1 has been shown to expand granulocyte recruitment and lead to the formation of the premetastatic niche. Seubert and colleagues demonstrated that tissue inhibitor of metalloproteinases-1 acts on hepatic stellate cells to release C-X-C motif chemokine receptor ligand 12/stromal-derived factor-1, a potent chemoattractant of neutrophils, and disruption of this signaling axis suppressed granulocyte infiltration and reduced metastasis *in vivo*.[32] Proinflammatory TNF signaling has also been shown to be a critical mediator of liver metastasis in CRC and lung models of hepatic metastasis by facilitating the uptake of $CD11b^+Gr1^+$ MDSCs into the liver, thus further linking the innate immune system as an essential component of liver metastases and the establishment of the premetastatic niche.[33]

Studies have shown that there are specific signaling events mediated by tumor-derived soluble factors that act on both the bone marrow and/or directly within the liver parenchyma to alter the immunophenotype of tumor-bearing animals and promote the uptake of cancer cells from circulation.[34] These signaling molecules are either directly secreted from tumor cells or released in small, endosomal-derived vesicles known as tumor-derived exosomes. Exosomes are secreted from tumor cells into the extracellular space and contain diverse biomolecules such as lipids, nucleic acids, and proteins, including cytokines.[35] Tumor-derived exosomes play a critical role in the recruitment of immunosuppressive myeloid populations, regulatory B-cells, and inhibition of natural killer (NK) cell function and dendritic cell (DC) maturation, promoting an immunosuppressive microenvironment that favors tumor cell uptake and growth in target organs.[36] Tumor-derived exosomes are critically important in establishing the premetastatic niche.[37] Shao and colleagues demonstrated that exosomes derived from CRC cells carried miR-21, a microRNA that binds to TLR7 on the surface of KCs within the liver. This ligand-receptor interaction promoted polarization of macrophages to a proinflammatory M1-like phenotype characterized by the secretion of IL-6 and was essential in the development of organotropic liver metastases.[38] Similarly, tumor-derived exosomes were found to be vital to hepatic metastases in PDAC through modulation of resident KCs. Costa-Silva and colleagues demonstrated that tumor-derived exosomes provoked secretion of TGF-β from KCs via exosome-derived macrophage migration inhibitory factor, leading to coordinated activation of neighboring hepatic stellate cells. In turn, these changes enhanced recruitment of bone marrow-derived macrophages and neutrophils into tumors, thus establishing the premetastatic niche and promoting subsequent liver metastases.[39] Given these properties, many experimental therapeutics are being designed against tumor-derived exosomes to suppress the distinct immunophenotype of the premetastatic niche and curtail metastasis in GI cancer.[40]

Granulocytes and the Formation of Neutrophil Extracellular Traps

Given the dependency of myeloid cells on cancer metastases, further insights have mechanistically defined the role of neutrophils and granulocytic precursors in metastatic disease formation through the development of NETs. As one of the most abundant innate immune cell populations, neutrophils are tasked with a crucial role in the protection against foreign invaders within the body. NETs, web-like structures released from neutrophils in response to microbial invasion, consist of multiple effector proteins arranged on a chromatin scaffold. These extracellular DNA structures act as traps to immobilize bacteria, fungi, viruses, and parasites and facilitate their destruction.[41] NETs can be formed through either direct lysis of neutrophils or through non-lytic pathways in a process termed "NETosis" that is triggered by a diverse group of upstream mediators.[42] Cancer cells have coopted NETosis for use in nearly all phases of metastatic disease development. NETs facilitate metastases by promoting EMT in tumor cells, establishing the premetastatic niche within distant tissues, trapping circulating tumor cells, anchoring them for target organ engraftment, and even promoting disease outgrowth at distant sites.[43] In a study by Cools-Lartigue et al, NETs were shown to be induced in a cecal puncture and ligation model, leading to enhanced deposition of tumor cells within the liver due to capture of circulating tumor cells within the hepatic vasculature.[44] Tohme et al similarly showed that surgical stress in an ischemia/reperfusion hepatic model results in NET formation within the liver, thereby enhancing CRC cell uptake and metastatic disease development.[45] A study by Pieterese and colleague showed that NETs compromise vascular integrity at target organs via degradation of VE-cadherin, further implicating these DNA-protein complexes in cancer metastasis.[46] Using transgenic murine knockout models or inhibition of CEACAM1, a surface protein present predominately on neutrophils, Rayes et al demonstrated that this protein is essential for cancer cell adhesion and metastases associated with NETosis, providing further mechanistic insight into the intricate interactions between granulocytes and tumor cell dynamics at target organs.[47] Additionally, Yang and colleagues showed that extracellular DNA from NETs functions to physically attract tumor cells to target organs, not simply act as a sticky "trap" which embeds circulating tumor cells. This tumor-myeloid crosstalk proceeded through the surface receptor CCDC25 on primary cancer cells, which both senses and activates downstream signaling in malignant cells to promote promotility pathways and thus directly influence metastases.[48]

Therapeutic modalities aimed at targeting NETosis and neutrophil migration have been explored including the use of recombinant DNAse (degrading the core component of NETs – chromatin) or therapies directed at myeloperoxidase or neutrophil elastase, neutrophil-derived factors essential for NET production. How effectively these compounds are able to suppress NET formation and prevent liver metastasis in the clinical arena is unknown, but preclinical data are compelling.[43]

Monocytes and Macrophages

Monocyte and macrophages play a crucial role in normal liver homeostasis. Hepatic stellate macrophages, commonly referred to as KCs, line the endothelium of the liver sinusoids and are responsible for diverse tasks such as clearance of bacterial pathogens, detoxification, and hemoglobin metabolism among many others. Additionally, KCs reshape the immune microenvironment by their production of various pro and anti-inflammatory cytokines and can promote parenchymal remodeling and fibrosis through these mechanisms.[49] Data show the dichotomous role of KCs in both preventing and promoting GI metastases. Antitumor functions of KCs are highlighted in a study by Deng et al.

through expression of ID3, a transcriptional repressor that not only plays a role in differentiation of embryonic macrophage precursors into KCs. Interestingly, continued expression of ID3 in adult KCs attenuates their surface level of the receptor Sirpa, leading to increased phagocytosis of tumor cells, as well as recruitment of NK and T cells to the peritumoral niche in the liver. This antitumoral function was replicated in liver metastases across mouse models featuring various cancer cell lines, including PDAC and CRC.[50] Thomas et al. also confirmed the important effects KCs have on the T cells of the peritumoral niche, demonstrating that pathogen-associated molecular patterns (PAMPs) induce T cell-mediated antitumor immunity through effects on KCs within the liver to suppress PDAC metastases.[51] These data highlight that KC may play a critical role in suppressing GI metastasis.

However, conflicting data indicates that KC may function to promote hepatic metastases in certain circumstances and their pleiotropic function may vary depending on the stage of tumor development. In a murine model of CRC metastases, KC depletion before tumor induction resulted in an increased tumor burden in the liver, whereas KC depletion at a late stage of exponential tumor growth resulted in a decreased tumor load. This suggests KC antitumor function may be restricted only to seeding and the initial stages of metastases.[52] This is supported by the aforementioned studies by Khatib et al., which demonstrated that KCs are responsible for increased expression of TNF-α in response to metastatic CRC cancer cells, which then triggers expression of cell adhesion molecules in sinusoidal endothelial cells, facilitating cancer cell invasion and seeding.[21,53] Other studies have shown select macrophages may continue to play a role in facilitating later stages of metastasis. Sathe et al. focused on single-cell RNA sequencing and immune cell deconvolution of liver metastasis in microsatellite stable CRC patients and found that macrophages in the tumor microenvironment had an altered gene-expression signature that included expression of *SPP1*, *APOE*, *TREM2*, and *CD9* – genes which promote inflammatory fibrosis as well as increased lipid metabolism. They further demonstrated that these reprogrammed macrophages communicate via ligand-receptor interactions with fibroblasts, and subsequently engage with T cells to promote an immunosuppressed landscape in the liver that may promote tumor outgrowth.[54] Yu et al. similarly established that non-resident monocyte-derived macrophages in the liver are responsible for intratumoral T cell depletion and immune deserts postestablishment of metastasis. Their group showed that it in the setting of a murine model of CRC metastatic to the liver, monocyte-derived macrophages at the metastatic site induce T cell apoptosis via the Fas-FasL pathway, resulting in decreased $CD8^+$ T cell function in the metastatic niche as well as systemic T cell depletion.[55] These studies provide mechanistic insight into how macrophages may diminish T cell mediated adaptive immunity within established metastatic tumor in the liver and highlight the dichotomous roles of monocytes in the progression of established GI tumors.

Dendritic Cells and Adaptive Immune Crosstalk

Dendritic cells are antigen presenting cells that act as the bridge between innate and adaptive immune cells. As the liver receives the blood from the GI system via the portal vein, resident dendritic cells are exposed to a multitude of toxins, dietary pathogens, and commensal microbes. They are responsible for processing these substances and presenting antigens to T cells in draining lymph nodes for subsequent activation of the adaptive immune response. While dendritic cells have been shown to be scarce in the malignant tumor microenvironment, they hold the dual capacity for promoting either immunity or tolerance in the setting of cancer by inducing activation of effector T cells vs T cell anergy.[56] Kenkel et al. found that a discrete subset of dendritic cells has the capacity to create an immunosuppressed, protumor microenvironment in the liver in PDAC. Using an immunocompetent murine model, they observed $CD11b^+$ dendritic cells accumulating at areas of early liver metastasis as a response to tumor-derived factors, creating a subsequent increase in regulatory T cells and inactivation of $CD8^+$ T cells.[57] This crosstalk between the innate immune system and regulatory T cells has proven to be play a role in metastatic CRC as well, as a study analyzing blood samples from metastatic CRC patients with liver metastasis also found that patients with higher levels of regulatory T cell mediated immunosuppression prior to resection of metastatic lesions were more likely to experience recurrence of disease.[58]

In contrast, success of immune checkpoint blockade therapy in combatting metastatic GI cancers in the liver has been shown to rely on increased dendritic cell activity. A study by Ho et al. explored mechanisms to explain why immunotherapy efficacy is decreased in metastatic microsatellite stable CRCs and found that a lack of dendritic cells within the metastatic tumor was a significant contributing factor. Implementation of treatments that enhanced dendritic cell invasion into the liver metastasis in combination with immune checkpoint blockade therapy resulted in increased expression of effector T cells and improved survival outcomes in their preclinical model.[59] Dendritic cells levels have also been shown to correlate with the timing of development of liver metastases, with higher levels of mature dendritic cells being found in tissue of metachronous CRC liver metastasis rather than synchronous occurrence.[60] Overall, as with other myeloid subsets, future dendritic-targeted therapies must take into account the pleomorphic effects of this population in tumorogenesis. Additionally, the heterogenous states of dendritic cell maturation and differentiation that occur in cancer likely explain these diverse phenotypes and are a burgeoning area of interest in immunology, particularly in mediating the response to tumor vaccines and checkpoint inhibition.

Other Myeloid Subsets

Additional myeloid cells contribute to metastasis and formation of the premetastatic niche of the liver. Elevated

platelet count has been positively associated with increased risk of CRC metastasis and decreased survival outcomes, which may be partly attributed to their ability to create a supportive premetastatic niche.[61] Data have shown that platelets are involved in the success of NETs in promoting seeding at metastatic sites. Ren et al. used a murine hepatic ischemia/reperfusion model to show that activated platelets increased platelet-CRC tumor cell aggregates and facilitates capture of these aggregates by NETs at distant metastatic sites.[62] Although their study focused on formation of distant metastasis in the lung, it provides a clear mechanism by which activated platelets and NETs work together to form micrometastasis in CRC. In addition to enhancing capture of circulating tumor cells by NETs, platelets have been implicated in promoting EMT in cancer cells, priming them for metastasis. Labelle et al. found that platelet derived TGF-β, as well as interaction of platelets and CRC tumor cells, resulted in transformation of cancer cells to a mesenchymal-like phenotype and increased lung metastasis in a murine model.[49] Additionally, platelets aid tumor cells in evading immune surveillance in the premetastatic niche. Cell-interaction analysis and functional studies involving circulating tumor cells from the portal vein of metastatic PDAC patients demonstrated that these tumor cells evade natural killer cell cytotoxicity through platelet derived upregulation of HLA-E. Subsequent overexpression of HLA-E was able to inhibit NK cells from attacking tumor cells, highlighting their immunosuppressive function.[63]

Although further data is needed to establish the role eosinophils play in tumorogenesis, evidence suggests that their activity is an integral part of the tumor microenvironment as well. A study evaluating the histopathology of CRC patients found that an increase of eosinophils at the tumor border was associated with a significantly decreased rate of metastasis, suggesting eosinophils in the primary tumor influence disease outgrowth.[64] Although data exploring eosinophils in GI cancers is limited, studies in breast cancer have found eosinophils decrease pulmonary metastatic growth. Grisaru-tal et al. studied the lung biopsies of metastatic breast patients and found that eosinophils are recruited to areas of lung metastasis via tumor secreted factors and promoted infiltration of $CD8^+$ and $CD4^+$ T cells.[65] An additional study evaluating a eosinophil function with a transgenic mouse model of breast cancer found that eosinophils were able to reduce pulmonary metastasis by decreasing early lung colonization by metastatic tumor cells.[66] Investigations into these findings in the context of GI cancer will be important to explore in future studies to discern the role of eosinophils in curtailing hepatic metastases.

Animal Models of Gastrointestinal Liver Metastases: Inherent Strengths and Challenges to Studying Host-Immune Interactions *In Vivo*

Delineating the intricate mechanistic underpinnings of GI liver metastases from patient samples is challenging. Therefore, the use of preclinical mouse models has proven essential in our understanding the inciting events and progression of hepatic metastases. The ideal mouse model is one that is 1) immunocompetent, 2) spontaneously develops colon or pancreatic tumors that reproducibly metastasize, and 3) recapitulate the immense genetic and transcriptional diversity seen in human patients. Unfortunately, a paradigm that flawlessly exemplifies these traits does not exist, but there are a number of preclinical mouse models to individually address each of these characteristics. The strength and weaknesses of each model should be known to investigators to ensure they are appropriately used in addressing a particular clinical question.

In orthotopic liver metastases models, tumors cells can be injected either into the portal vein, spleen, or into target organ (pancreas, cecum, etc.) and allowed to spontaneously expand and metastasize to the liver. This can be performed in immunocompetent mice using syngeneic tumor cell lines that are derived from murine tumors in the same genetic background. The advantage of the former is that the immune system is intact, allowing precise delineation of the tumor-stromal-immune interactions that evolve with the establishment of metastatic disease. Given the importance of the immune system in hepatic metastasis, this model has been critical in advancing our understanding of these intricate mechanisms.[67] A major shortcoming of these models is their inability to recreate the significant heterogeneity of human disease in regard to driver mutations and transcription and epigenetic diversity.[68] Often, tumor cells utilized in these experiments are isolated from genetically-engineered mouse models (GEMMs) driven by anywhere from 1 to 2 transgenes that favor oncogenic transformation *in vivo*. Certain murine cell lines (IE CMT-93) have been isolated from carcinogen-induced mouse models of cancer, but the degree of genomic similarity of these models to spontaneous human cancer remains poorly characterized. Strategies to employ conditional knockout of proteins important in DNA damage repair into existing constructs containing canonical genomic drivers of GI cancer may present an interesting avenue to circumvent this limitation.[69] Alternatively, nonobese diabetic-severe combined immunodeficiency or athymic nude mice can be utilized to examine human tumor cell lines and their ability to metastasize and colonize the liver *in vivo* without concern for tumor rejection in these models. This enables researchers to recapitulate the genetic and phenotypic diversity of human disease by using such models as patient-derived xenografts or immortalized human cell lines – often which contain hundreds of driver mutations – to examine the stepwise progression and therapeutic response in mice.[70] However, these models are limited by the lack of intact immune system and specifically cannot be relied upon to examine immunomodulatory therapies in cancer metastases, a rapidly growing field with tremendous therapeutic potential. Regardless, an important phase in any of these experimental models is generation of cell lines that reproducibly metastasize when implanted. Often, this will require *in vivo* selection, where implantation occurs in a specific site and

clones are then isolated from the liver in mice where metastases develop. This can often be an efficient process and *in vivo* selection allows for the isolation and reinjection of cells to generate immunoedited clones with a propensity for metastasizing to the liver.[71,72] An important and often overlooked step in this strategy is the isolation and purification of single-cell clones after each *in vivo* isolation using limited cell dilution, as this eliminates contamination from non-tumor cell constituents and ensures monoclonal expansion for other downstream applications.

GEMMs have emerged that allow for the study of spontaneous liver metastases without the need for surgical implantation. These models are important as metastasis occurs in the natural progression of disease and obviates the stresses of surgery which elicit a potent systemic inflammatory response and may unduly influence tumor growth. In PDAC, activating *KRAS* mutations are found in over 90% of tumors and similarly *TP53* alterations are present in a majority of cases.[73] Therefore, widespread use of the transgenic *LSL-Kras$^{G12D/+}$;LSL-Trp53$^{R172H/+}$;Pdx-1-Cre* (KPC) mouse model derived by Hingorani and colleagues in 2005 has been the mainstay of preclinical PDAC research.[74] KPC mice contain an activating *Kras^{G12D}* mutation under the control of a pancreas-specific Cre recombinase along with the R172H point mutation in *Tp53*, with corresponds to the orthologous R175H mutation in humans. Metastatic disease spread to the liver occurs within over 60% of mice in this model.[75] Interestingly, a recent study by Maddipati et al has shown that there is significant heterogeneity that occurs between primary tumors and liver metastases in KPC mice and demonstrates how ongoing clonal evolution underlies the development of metastasis in these GEMM mice, similar to what is observed in human disease.[76] Similarly in colon cancer, *Kras*-driven tumorigenesis has been used to generate murine models of spontaneous liver metastases. A significant barrier to using GEMMs to study hepatic metastases in CRC is that overwhelming disease burden from primary tumors often leads to mouse death prior to the formation of gross metastatic disease. In the traditional *Apc* multiple intestinal neoplasia (*Apc^{Min}*) model, transgenic mice which contain a truncating mutation at codon 850 of the murine *Apc* gene, widespread polyposis of the small bowel occurs with only scarce lesions in the colon.[77,78] Synergistic *Apc* mutations with *Smad4* or *Tgbr2* loss have been shown to enhance invasiveness and tumorigenesis, but there is a scarcity of models that procedure spontaneous colorectal liver metastases in mice.[79,80] Work by Hung and colleagues circumvented these issues through development of a mouse containing homozygous floxed *Apc* gene crossed with an LSL-*Kras* mutant construct. By use of a Cre recombinase-expressing adenovirus, locally invasive colon tumors could reproducibly be formed and 20% of mice demonstrated spontaneous liver metastases by 24 weeks following adenovirus exposure.[81] Boutin et al demonstrated using *Villin-Cre^{ERT2}* mice containing floxed *Apc$^{fl/fl}$* and *Trp53$^{fl/fl}$* constructs combined with a Tet-inducible *Kras^{G12D}* allele that local enema with tamoxifen induced colonic tumors with a 25% disease metastasis rate in mice, further demonstrating that a spontaneous syngeneic model of colorectal liver metastases that does not require surgery is feasible in mice.[82] An elegant study by Roper et al showed that orthotopic injection of syngeneic *Apc$^{fl/fl}$;Kras$^{LSL-G12D/+}$;Trp53$^{fl/fl}$* colon organoids into murine colons could also produce spontaneous metastases in 33% of mice.[83] A methodology utilizing dextran sodium sulfate (DSS) to induce mucosal injury combined with *Apc^{mut}/Kras^{G12D}/p53^{mut}* organoid transplantation was similarly shown by O'Rourke and colleagues to induce liver metastases in mice.[84] Mouse tumor organoids have also been used in sentinel works by Kasahima et al and Tauriello et al to reproducibly establish liver metastases with genomic drivers that closely genocopy human disease, thus further expanding the repertoire of immunocompetent murine models available to study CRC liver metastases.[85–87]

Utilization of these mouse models to explore mechanisms of liver metastases in GI cancers is essential, as they are typically low-cost, high yield, and in many cases can recapitulate the core genetic drivers of gut malignancies. As evidence mounts for the importance of immune cells and tumor-stromal interactions in the initiation and outgrowth of metastases, the authors believe the use of immunocompetent murine models is essential to accurately study these processes to maximize translational benefit. However, no true murine model can reliably and practically recapitulate the genetic diversity and environmental exposures that duly influence GI cancers in humans and research into this area is thus urgently needed.

Conclusion and Future Directions

The initiation of liver metastases in GI cancers involves a complex, coordinated series of events dependent on localized changes to the sinusoidal vasculature, the recruitment and activation of diverse myeloid and immune populations, and the systemic inflammatory changes induced by malignancy to drive tumor cell uptake and survival within the hepatic parenchyma. Although our knowledge of these mechanisms has greatly evolved, there is much to be explored about this important event in end-stage cancer progression. A major limitation to the study of liver metastases, and particularly the premetastatic niche, is the lack of transgenic mice models available to study this phenomenon. The development of a spontaneous, reproducible murine model of hepatic metastasis and dormancy that recapitulated the genomic and immunologic alterations in human metastatic liver disease would greatly enable the development of novel therapeutics available to treat and prevent metastases. Further elucidating the key genomic and transcriptional drivers that underlie the substantial heterogeneity in the frequency and severity of cancer metastasis from patient to patient will also be critical to advance our understanding of GI liver metastases.

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