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# Limiting tumor seeding as a therapeutic approach for metastatic disease

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ABSTRACT

Here we propose that therapeutic targeting of circulating tumor cells (CTCs), which are widely understood to be the seeds of metastasis, would represent an effective strategy towards limiting numerical expansion of secondary lesions and containing overall tumor burden in cancer patients. However, the molecular mediators of tumor seeding have not been well characterized. This is in part due to the limited number of pre-clinical *in vivo* approaches that appropriately interrogate the mechanisms by which cancer cells home to arresting organs. It is critical that we continue to investigate the mediators of tumor seeding as it is evident that the ability of CTCs to colonize in distant sites is what drives disease progression even after the primary tumor has been ablated by local modalities. In addition to slowing disease progression, containing metastatic spread by impeding tumor cell seeding may also provide a clinical benefit by increasing the duration of the residence of CTCs in systemic circulation thereby increasing their exposure to pharmacological agents commonly used in the treatment of patients such as chemotherapy and immunotherapies. In this review we will examine the current state of knowledge about the mechanisms of tumor cells seeding as well as explore how targeting this stage of metastatic spreading may provide therapeutic benefit to patients with advanced disease.



## 1. Introduction

Metastatic disease is by far the most common cause for the demise of patients affected by solid tumors. Despite a provocative model recently proposed (Narod & Sopik, 2018), it is widely recognized - and supported by exceedingly large evidence - that tumors spread by cancer cells gaining access to the systemic circulation *via* local invasion. Cancer cells departing from primary tumors intravasate into blood vessels or lymphatics, with the latter providing access to loco-regional lymph nodes and then to venous blood *via* the right and left lymphatic ducts (Pereira et al., 2018; Wong & Hynes, 2006). Once in the blood, these Circulating Tumor Cells (CTCs) must avoid cell death by anoikis, acquire immune-resistance, extravasate at the capillary beds of the arresting organs, and subsequently adapt to the new tissue microenvironment as Disseminated Tumor Cells (DTCs) (Shibue & Weinberg, 2011). The tumor cells capable of overcoming this series of hurdles have the opportunity to colonize the new site and expand into a clinically overt metastasis. While CTCs may spread to an array of different tissues, colonization and growth are limiting events and thus it is widely accepted that secondary tumors are spawned by only a minority of DTCs (Labelle & Hynes, 2012; Massagué & Obenauf, 2016).

From a clinical standpoint, the detection of metastatic lesions has been historically interpreted as a critical turning point. At this stage a patient was considered lost to the grip of the disease and only provided palliative treatments; the intent to cure had been abandoned. Even today, with the expanded arsenal of newly developed therapeutics at our disposition, in most instances the goal is an extension of life expectancy on the order of months. There is neither anticipation for a definitive and lasting clinical resolution, nor true commitment to tailored drug development (Steeg, 2016). Understandably, this scenario is an enormous source of frustration for both clinicians and patients. Clinical scenarios involving prostate or breast adenocarcinomas are especially subject to this impasse. In the vast majority of these cases, radical ablation of the neoplastic mass is achieved by local modalities such as surgery and/or radiation therapy, but approximately 30% of cases will eventually develop metastases to which they will inevitably succumb (Brook, Brook, Dharmarajan, Dass, & Chan, 2018; Crawford, Petrylak, & Sartor, 2017).

The pursuit of therapies intended to avert tumor seeding has been traditionally thwarted by the long-held view that, upon diagnosis of metastasis, cancer spreading had already occurred over the course of many years while the primary tumor was clinically undetected. In line with this notion, secondary lesions which have emerged after the eradication of the primary neoplasia are widely perceived as the result of DTCs resuming growth after months or years of proliferative quiescence

*Abbreviations:* COX-2, Cyclooxygenase 2; CTCs, Circulating Tumor Cells; DTCs, Disseminated Tumor Cells; ICAM, IntraCellular Adhesion Molecule; MMP, Matrix Metalloprotease.

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(Morrissey, Vessella, Lange, & Lam, 2016). The triggers for the transition from dormancy to active proliferation are mostly still undefined; however, experimental and clinical evidence indeed supports tumor dormancy as a cause for delayed appearance of metastases (Sosa, Bragado, & Aguirre-Ghiso, 2014). Currently, the primary aim of drug development and clinical efforts is to reduce volume and decelerate growth of detectable tumors, and by the same token also prevent the expansion of smaller and still undetectable malignant foci into larger tumors. However, recent studies have provided convincing genomic evidence that tumor cells may also depart from established metastatic tumors, entering the blood and spreading throughout the body as *secondary* CTCs (Micalizzi, Maheswaran, & Haber, 2017) to seed either new lesions (reseeding) or pre-existing metastatic lesions (cross-seeding). Though we are currently working to improve our understanding of the mechanisms regulating these events, it is easy to envision how reseeding could be responsible for the numerical expansion of the few lesions commonly detected in early metastatic patients, thereby hastening clinical progression towards an unfavorable ending.

The clinical impact of reseeding and cross-seeding is evidenced by the fact that CTCs are detectable in patients presenting with metastatic lesions even after the eradication of their primary tumors. Studies have also shown that CTCs increase in number with clinical progression and that their enumeration can be used as surrogate biomarker for survival (Scher et al., 2015). Thus, the transition from oligometastatic stage, at which many patients are first diagnosed, to a late phase of diffused metastatic disease is likely to occur upon significant contribution from metastasis-to-metastasis seeding. Acknowledging this new model and acting upon it is bound to have significant implications for basic and pre-clinical research and, most importantly, for clinical practice. The development of novel therapeutics capable of successfully attenuating tumor expansion by impairing reseeding will likely cause a paradigm shift in the management and treatment of metastatic patients.

## 2. Mediators of tumor seeding

In order to appropriately discuss the mediators of tumor seeding we must first define the parameters around seeding and how it is delineated from the broader concepts of invasion and migration, both of which relate predominately to the primary tumor. Although it is well understood that tumor cells can acquire characteristics that increase their ability to migrate from the primary site and intravasate into circulation, there is a distinction to be made between the phenotypic changes required for intravasation from the primary site and those required for homing and seeding or extravasation into distant organs. Regrettably, though there are an array of studies which propose potential mediators of 'metastatic seeding', too often this term is used to more generally discuss invasive characteristics of tumor cells which have been assessed exclusively *in vitro*, most notably through 3D cultures, or *in vivo* at the primary site. Neither of these approaches should be considered as effective ways to assess tumor seeding as they fail to take into consideration the mediators present in distant organs and the organ-specific interactions CTCs experience upon arrival to different secondary sites. Along similar lines, studies evaluating distant lesions generated by orthotopically grafted tumors in mice also fail to address mechanisms specific to tumor cell seeding. In studies such as these, molecular mediators that are shown to mitigate or increase metastases could be altering an array of processes such as local invasion, intravasation, viability in circulation, and/or adaptation to the new tissue microenvironment. Each of these aspects, though important to understanding the metastatic cascade, are not necessarily providing conclusive insights on the specific events involved in tumor seeding (extravasation). For this reason, the scope of this section will focus predominately on mediators of seeding that are proposed by studies that utilize *in vivo* experimental approaches capable of evaluating the function of specific molecules during events occurring within hours of the arrival of cancer cells at secondary sites (Labelle & Hynes, 2012). It is also important to note that the most optimal way to assess the seeding of cancer cells to a given secondary site, rather than their ability to escape the primary tumor, is to engraft the cells directly into the blood circulation of the host organism. With these criteria applied, we can appreciate that there is still much to learn about the mechanisms behind tumor seeding at distant organs. The mediators that have been identified, however, can be stratified into two major categories: Cytokines & Chemokines and Cell Adhesion Molecules.

### 2.1. Chemokines & cytokines

Successful tumor spreading is facilitated by the dissemination of cancer cells to distant tissues which are conducive to survival and growth of those cells (Obenauf & Massagué, 2015). As such, there have been an array of studies that assess the potential chemoattractant properties that metastatic sites might have towards tumor cells circulating in systemic blood. It is implicit that cytokines and chemokines, which are widely known as being implicated in cellular chemoattraction, would be explored as potential mediators of tumor cell extravasation and seeding. In the context of tumor seeding, Interleukin-8 (IL-8) has been associated specifically with breast cancer cell dissemination (Vazquez Rodriguez, Abrahamsson, Jensen, & Dabrosin, 2018). In this study, investigators observed that breast cancer cells secrete high levels of IL-8, and that production of this cytokine was increased in the presence of breast adipocytes (BAd). Rodriguez and colleagues extended their findings to an *in vivo* study and found that co-injection of breast cancer cells with neutrophils and BAd into zebrafish led to an increase in tumor cell dissemination that was effectively inhibited when anti-IL-8 treatment was used. The zebrafish model, though highly effective in visualizing the dynamics of cellular migration through the entire organism, is limited by fact that it does not fully recapitulate the hemodynamics that cancer cells experience in mammalian circulation. Nevertheless, the involvement of IL-8 in tumor cell seeding has also been proposed by other studies (Bendre et al., 2005; Benoy, 2004; Liang, Hoskins, & Dong, 2010) thereby lending to the possibility that IL-8 may play a role in this process for some cancers. It would be valuable, however, to better understand the mechanism by which IL-8 production in a distant organ might play in the recruitment of CTCs to a secondary site– something these studies only briefly address.

IL-8, along with chemokine IL-6, was also investigated by Kim and colleagues for their role in mediating tumor self-seeding. This group observed that CTCs shed from a tumor mass are able to seed distinctly different tumors. Although this mechanism differs from seeding a tumor-free site, it is still critical to investigate as the seeding of pre-existing metastatic lesions could indeed facilitate disease progression (Kim et al., 2009). In their specific model, IL-8 and IL-6 secreted by pre-existing lesions acted as attractants of CTCs whereas expression and secretion of metalloprotease-1 (MMP1) and actin cytoskeleton factor component Fascin-1 facilitated trans-endothelial migration and seeding to established tumors. It is reasonable to assume that the mechanisms employed by cancer cells to reseed primary lesions or existing metastases may also be involved in the homing of CTCs to tumor-free locations. In fact, the expression of MMP1, as well as MMP2 and COX2, on breast cancer cells has previously been shown to facilitate metastatic extravasation to the lungs even in the absence of a pre-existent tumor (Gupta et al., 2007).

The notion that tissue-specific expression of chemokine/cytokine may influence tumor cell seeding has been previously proposed (Esposito & Kang, 2014). However, as emphasized earlier in this review, most often their involvement has been inferred by detecting and studying tumor cells at a time significantly later than initial arrival in tissues, when additional cell survival events rather than seeding *per se* may likely dictate homing and early colonization. For instance, following several studies implicating the chemokine CXCL12 and its receptor CXCR4 in the distant seeding of prostate and breast tumors (Li et al., 2004; Liang et al., 2005; Sun et al., 2005), more recent work conducted by

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visualizing and enumerating individual cancer cells shortly after their arrival, either in bone or visceral organs, rules out CXCR4 as a culprit in the events leading CTCs migrating from blood into tissues (Price et al., 2016; Qian et al., 2018; Richert et al., 2009). CXCR4 may instead control the egress of cancer cells from the bone marrow back into the blood circulation (Price et al., 2016).

A different scenario is presented by the chemokine receptor CX3CR1, which has only one ligand, the chemokine CX3CL1 (also known as fractalkine) that is abundantly expressed on human bone marrow endothelial cells (Shulby, Dolloff, Stearns, Meucci, & Fatatis, 2004). Interestingly, both breast and prostate cancer cells, which have strong propensity to metastasize to the bone, express high levels of CX3CR1 (Jamieson, Shimizu, D'Ambrosio, Meucci, & Fatatis, 2008; Shulby et al., 2004). Given the peculiar interactions occurring between CX3CR1 and its ligand, which regulate both adhesion and chemoattraction of both normal and tumor cells (Chapman et al., 2000; Shulby et al., 2004) a case for the unique therapeutic potential of targeting this receptor/ligand pair in cancer and metastasis was made (D'Haese, Demir, Friess, & Ceyhan, 2010). After work from our laboratory demonstrated that this was indeed the case with the use of CX3CR1 neutralizing antibodies in experimental models of metastasis (Jamieson-Gladney, Zhang, Fong, Meucci, & Fatatis, 2011), novel antagonists of CX3CR1 were synthesized and provided as additional proof of principle that inhibition of CX3CR1 dramatically mitigates tumor seeding (Qian et al., 2018; Shen et al., 2016).

### 2.2. Cell adhesion molecules

For CTCs to successfully convert into DTCs by egressing the blood *via* chemoattraction (extravasation), they must first find favorable conditions promoting their adhesion to the endothelial cells layering the capillary network of different tissues. While in circulation, CTCs experience an array of shear stress due to hemodynamic forces and the presence of circulating blood and immune cells. Without the participation of adhesion molecules on the cell surfaces, a circulating tumor cell would not have the chance to bind to and extravasate through the endothelium.

Cell adhesion molecules that function specifically during tissue inflammatory response have been implicated in the early colonization of cancer cells during metastasis. It is well established that tumors take advantage of trophic factors released by inflammatory cells (Coussens & Werb, 2002), thus it is logical to conjecture that seeding is mediated by attraction to adhesion molecules induced upon inflammatory conditions. One of the earliest reviews to propose the role of inflammatory mediators in metastasis focused specifically on the role of selectin-carbohydrates such as L-, P-, and *E*-selectin (Bevilacqua & Nelson, 1993; McEver, 1997). Although many of the early studies that explored this notion did not validate their claims in animal models, one study in particular attempted to demonstrate *in vivo* that overexpression of *E*-selectin in the liver could redirect metastatic melanoma cells from colonization of the lungs to that of the liver (Biancone, Araki, Araki, Vassalli, & Stamenkovic, 1996). The results of this work introduced the potential role of E-selectin in tumor seeding. More recently, *E*-selectin has been confirmed to be as integral to the tethering of CTCs to the endothelium (Bendas & Borsig, 2012; Geng, Marshall, & King, 2012; Zarbock, Ley, McEver, & Hidalgo, 2011), a process fundamental to the extravasation and seeding of these cells. Furthermore, pre-clinical studies found the expression of *E*-selectin to be increased in the metastatic foci detected at 10 days post-intracardiac injection of murine breast cancer cells and at 21 days post-injection of human breast cancer cells and thus proposed its role as a potential target for brain metastatic seeding (Soto, Serres, Anthony, & Sibson, 2014). Of note, the aforementioned study by Price and colleagues (Price et al., 2016) demonstrating that CXCR4 does not directly mediate CTCs homing to the skeleton, instead suggests that E-selectin regulates the entry of breast cancer cells colonization to secondary organs such as the bone. Although this study effectively enumerates single cancer cells, it focuses predominately on the mechanisms of tumor cell latency and therefore utilizes end points much longer than needed to draw conclusions about early arrival. Additionally, the study fails to demonstrate E-selectin staining in the blood vessels of animals not prepared for intravital confocal microscopy of their calvaria bone - which poses the question of whether E-selectin expression was caused by inflammatory conditions arising during the surgical preparation.

Sialyated epitopes, such as Sialyl Lewis-X, are commonly recognized by selectin-carbohydrates and therefore the expression of these epitopes on cancer cells has also been of particular interest in the study of tumor seeding. The neoexpression or overexpression of sialylated epitopes has been implicated as crucial to distinguishing between malignant and benign glycophenotypes (Martinez-Duncker, Salinas-Marin, & Martinez-Duncker, 2011). The overexpression of sialylated tetrasaccharide carbohydrates such as sialyl Lewis X ($SLe^x$) and sialyl Lewis A ($SLe^a$) on CTCs (Gakhar et al., 2013; Martinez-Duncker et al., 2011) supports earlier studies suggesting that, in addition to selectins, these two carbohydrate antigens are responsible for facilitating initial adhesion of CTCs to the vascular endothelium and thereby are integral to the process of extravasation and seeding (Kannagi, 1997; Kannagi, Izawa, Koike, Miyazaki, & Kimura, 2004; Konstantopoulos & Thomas, 2009; Takada et al., 1993). Another such sialyation that has been associated with cancer cells is that of sialic acid (Sia). Studies have shown that Sia expression is a thousand-fold higher on tumor cells as compared to normal cells (Tsoukalas et al., 2018) and in some cancer types its expression correlates with patient prognosis (Cazet et al., 2010; Fernández-Briera, García-Parceiro, Cuevas, & Gil-Martín, 2010). Given the presence of sialylated antigens such as Sia on the cell surface of cancer cells, these antigens have been suspected to play a role in the attachment of CTCs to the endothelium of secondary organs and/or in their ability to seed and extravasate in these sites. Given the ability of Sia and its associated sialoglycans to mediate extravasation it is not surprising that, when applied to an *in vivo* lung metastasis model, treatment with a Sia-blocking glycomimetic significantly reduced early metastatic spread (Büll et al., 2015). In order to assess whether blocking Sia expression in cancer cells has an effect on the early stages of seeding, this study could be expanded to include earlier time points in colonization (*i.e.* 24–48 h). Nevertheless, it is evident from this and other studies that the expression of glycoproteins on the cell surface may have strong implications in mediating tumor cell seeding by interfering with the process of extravasation.

Due to their role in facilitating the attachment of cells to the extracellular matrix as well as their ability to mediate rapid signal transduction responses, integrins are yet another class of cell adhesion molecules that have been implicated in tumor cell progression. The role of integrins, and in particular of αvβ3, in angiogenesis has been established and we direct the readers to excellent reviews addressing it (Avraamides, Garmy-Susini, & Varner, 2008; Duro-Castano, Gallon, Decker, & Vicent, 2017). Here, we will rather focus on the implication of integrins in tumor cell seeding. There are numerous studies which draw correlations between integrin expression on tumor and immune cells and metastatic progression; however, only a handful have appropriately used pre-clinical models to assess tumor cell extravasation and seeding. In the context of breast cancer cell seeding, the expression of β1 integrin has been identified as potentially involved in the arrest of tumor cells. Using intravital imaging of single tumor cells arrested in the vasculature of zebrafish embryos, Stoletov and colleagues (Stoletov et al., 2010) showed that silencing β1 integrin expression in breast cancer cells greatly reduces the ability of these cells to extravasate as early as 24 h post-injection of the cancer cells into circulation. High magnification imaging of the tumor cells arrested in the vasculature showed that knocking down β1 integrin disrupted the adherence of the cancer cells to the vessel endothelium. Another study, which focuses primarily on heterodimeric αβ integrin αvβ3, aptly demonstrates that this integrin is required at the early stages of tumor cell seeding and not critical primary tumor growth (Weber et al., 2016). Although the results of

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this study show a striking increase in the number of tumor cells colonizing the lung when integrin αvβ3 is activated in breast cancer cells as compared to when it is expressed in an inactive form, it is important to note that in this instance seeded tumor cells are quantified by PCR performed on lung homogenates rather than direct enumeration of visualized single cells. Weber and colleagues (Weber et al., 2016) further conclude that the ability of integrin αvβ3 to promote transendothelial migration is dependent on cooperation with platelets, a notion that has been corroborated by several others (Gay & Felding-Habermann, 2011; Tesfamariam, 2016). β1 integrin (Cardones, Murakami, & Hwang, 2003; Kato et al., 2012) and integrin αvβ3 (Pickarski, Gleason, Bednar, & Duong, 2015) as well as others (Huang & Rofstad, 2018) have also demonstrated importance in mediating organ-specific seeding of metastatic melanoma cells. Nevertheless, therapeutic targeting of metastasis-associated integrins in the context of melanoma or any other cancer subtype is not a promising approach due to the extensive involvement of these proteins in normal biological processes (Huang & Rofstad, 2018).

Immune cells often express adhesion molecules which can bind to the integrins expressed on CTCs thereby providing immune-resistance and aiding in transendothelial migration. This mechanism has been especially observed in leukocytes, such as neutrophils, which have been shown to bind CTCs in circulation, facilitate the interaction between CTCs and the endothelium, and thereby promote the arrest of tumor cells into secondary sites (Duda et al., 2010; Stott et al., 2010). This interaction has been suggested to be mediated by intracellular adhesion molecule 1 (ICAM-1), a cell surface glycoprotein expressed on endothelial cells and array of different cancer cells types. ICAM-1 is known to be the counter receptor for $\beta_2$ integrins (CD18) which are abundantly expressed on neutrophils; consequently, some groups have interrogated the interaction between ICAM-1 and CD18 in the context of tumor seeding. In murine models of melanoma and lung carcinoma, CD18 expression on neutrophils has been shown to facilitate transendothelial migration *via* adhesion of these cells to ICAM-1 expressing tumor cells (Huh, Liang, Sharma, Dong, & Robertson, 2010; Spicer et al., 2012). In both cases, however, these studies emphasize that this interaction is likely mediated by inflammation and tumor-derived cytokines such as IL-8, both of which can induce the expression of CD18 on neutrophils.

Another integrin-binding cell adhesion molecule that has more recently been implicated in tumor cell extravasation and seeding is the Cell Adhesion Molecule L1 (L1CAM). L1CAM is a transmembrane glycoprotein that functions as a neuronal cell adhesion molecule. As such, it has been shown to function in the extravasation and seeding of metastatic cell lines with high brain tropism. Specifically, in brain-metastatic lung and breast cancer cell lines, L1CAM is required for disseminated cancer cells to spread over the abluminal surface of the blood vessels to facilitate extravasation as these vessels are encapsulated by collagen and laminin rich basal lamina (Valiente et al., 2014). It was later found that this mechanism is also important for cancer cell colonization to other organs such as lungs and bone (Er et al., 2018). In this study, Er and colleagues propose that this L1CAM mediated tumor cell seeding is due to both the outcompeting of pericytes in the perivascular niches and the activation of mechanotransduction effectors YAP (Yes-associated protein) and MRTF (myocardin-related transcription factor). Of note, this group also found that L1CAM was important of both initial homing and adhesion (2–6 days) as well as post-extravasation (>9 days) survival of the cancer cells.

It is apparent that there are multiple molecular mechanisms working in tandem to mediate not only the adhesion of CTCs to the endothelium, but other important aspects of extravasation such as the interaction between the CTCs and other circulating cells such as platelets and neutrophils. However, prior to considering any of these mediators as targets of therapy, it may seem necessary to evaluate their function in all aspects of the metastatic cascade. As an example, although many generalizations can be made about cell adhesion molecules and tumor progression, there is evidently a balance between the decreased adhesive interactions required for a cancer cell to escape the extracellular matrix and surrounding cells of the primary tumor and the increased adhesive interactions required for extravasation into distant organs (Albelda, 1993). One might then postulate that blocking tumor seeding may, in turn, lead to the retention and further growth of the primary tumor. For most solid tumor subtypes, however, this is not a concern because the majority of tumor seeding and reseeding occurring in patients happens after the removal of the primary tumor.

## 3. Metastatic seeding in the absence of primary tumor

The prognostic value of CTCs for several types of solid tumors has been established (Dawood et al., 2008; Scher et al., 2015) and has relevance at all stages of disease progression. For patients with localized or locally advanced tumors (stages I-III), the detection of cancer cells in blood circulation indicates neoplastic spreading and the increased likelihood of additional colonization and growth at distant sites. Accordingly, the efficacy of adjuvant therapies is frequently monitored by the enumeration of CTCs, a standard application for a personalized use of 'liquid biopsy' (Bidard, Proudhon, & Pierga, 2016; Toss, Mu, Fernandez, & Cristofanilli, 2014). The enumeration of CTCs to determine therapy efficacy is also relevant in patients with metastatic disease (stage IV). At this stage, cancer cells in the systemic circulation are detected more frequently than in patients with localized tumors (Bidard et al., 2016; Maestro et al., 2009; Tsai et al., 2016). This increase in CTCs observed in patients presenting with overt metastatic disease results from both the multiplicity of lesions and the increased tumor burden. Furthermore, the correlation between late stage metastatic disease and CTC number is supported by the evidence that patients with oligometastatic disease commonly have lower numbers of CTCs than patients with numerous lesions (Boffa, Guo, Molinaro, Finan, & Detterbeck, 2010).

In these latter stages of disease when primary solid malignancy is absent, the CTCs detected in peripheral blood must be departing from secondary tumors; based on their established role in tumor spreading we should then expect that in this scenario, CTCs have the potential to generate metastases similarly as if they were mobilized from a primary tumor. In fact, the process of generating metastases in the absence of primary tumor (reseeding from pre-existent metastases) has the potential to be even more efficient than initial mobilization from the primary tumor. This outcome is highly probable not only because the percentage of apoptotic CTCs in metastatic patients is lower than in early neoplastic stages (Kallergi et al., 2013) but also in light of the fact that metastases plausibly harbor more aggressive clones, endowed with the appropriate adaptive features to successfully colonize the same organ from which they are departing and possibly additional ones (Chaffer & Weinberg, 2011). Despite these considerations, there has been substantial hesitation in recognizing the potential role of CTCs in worsening the clinical progression of patients with metastatic disease (van der Toom, Verdone, & Pienta, 2016). This reluctance is likely due to a variety of factors, including our inability to track the transfer of cancer cells from an existing metastasis to another site of colonization, a limited knowledge about timing and dynamics of cancer cells recirculation kinetics, and the uncertainty about CTC viability and tumor-initiating potential. However, groundbreaking evidence supporting the occurrence of metastasis-to-metastasis spreading has been provided by two studies in prostate cancer patients (Gundem et al., 2015; Hong et al., 2015). In both of these studies, somatic mutations that were not detected in the primary tumors were shared by distinct metastases, thus revealing complex patterns of tumor spreading and hinting to a cooperativity between heterogenous cancer cells populations and the host tissues as underpinning the acquisition of novel and increasingly malignant features. Notwithstanding the possible caveat of polyclonal metastases generated by spreading of sub-clones not detected in the primary tumor, the seeding of new metastases by clonal populations with superior survival

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advantage and spreading from existing secondary lesions appears the most likely scenario.

These crucial findings should lead to an unprecedent paradigm shift in treatment; one which supports the concept that impairment of metastasis-to-metastasis seeding would contain the number of disseminated tumors, thereby reducing both overall tumor burden and the likelihood of organ failure. Furthermore, fewer lesions emerging over time would restrict the fraction of highly proliferating cancer cells that normally populate small malignant foci and possess higher propensity to acquire drug resistance through mutational escape than their quiescent and less metabolically active counterparts populating larger tumors (Raguz & Yagüe, 2008; Swierniak, Kimmel, & Smieja, 2009). Finally, clones with higher metastatic potential cross-seeding to established lesions are likely to cooperate with less aggressive clones, thereby enhancing their malignant features and bestowing them with additional organ-tropism either directly or *via* microenvironment remodeling (R. Axelrod, Axelrod, & Pienta, 2006; Bidard, Pierga, Vincent-Salomon, & Poupon, 2008; Zhou, Neelakantan, & Ford, 2017).

The prospect of decelerating clinical progression by directly impairing tumor seeding would be most relevant for the subset of patients in which the clinical transition into metastatic stage – months or years after ablation of their primary tumor – is announced by a very limited number of secondary lesions, a condition defined as oligometastatic cancer (Palma et al., 2014). Although the criteria used to define this clinical entity and its management are still matter of debate (Foster, Weichselbaum, & Pitroda, 2018; Reyes & Pienta, 2015), there are few doubts that oligometastatic cancer is seen as a valuable opportunity to treat individual lesions by either surgical or radiotherapy approaches, with radical intent and avoiding systemic therapies (Lancia et al., 2019; Treasure, 2012). Accumulating evidence suggests that this strategy enables a better 5-year survivorship (Kaneda & Saito, 2015), but it might be arduous to uphold that patients are rendered truly disease-free. Asynchronously growing tumor deposits, not yet detectable by imaging modalities, could be mobilizing cancer cells thereby perpetuating the disease unless appropriate treatment aimed to counteract this process were adopted. Indeed, the appeal of localized intervention not combined with cytotoxic or targeted therapies is obvious, particularly for elderly patients. However, for younger cancer patients with longer life-expectancy the outcome over time might be less favorable, with recurrences emerging after an apparent tumor-free period of variable length. For these particular cases, while also preserving the intent of avoiding therapies targeting tumor cell viability and the associated side effects, novel therapeutics impairing CTC seeding lacking direct cytotoxic effects could prove of significant therapeutic value.

In spite of the recently acquired genomic evidence mentioned above, it is widely perceived that true commitment to pursue drug discovery and development avenues, and eventually conceive therapeutic strategies aimed to counteract metastasis-to-metastasis spreading, requires additional conceptual and experimental support. For instance, the tumorigenic potential of CTCs has been questioned or proved hard to demonstrate (Carvalho et al., 2013; van der Toom et al., 2016). Furthermore, the true clinical benefit that could derive from targeting this specific cellular compartment in metastatic patients is still undefined. It is our opinion that an appropriate use of pre-clinical animal models is invaluable to systematically dissect the mechanisms for CTCs mobilization and reseeding, to recognize the molecule mediators involved in this process, and to define its effect on disease progression, as this is expected to pave the way to establishing innovative modalities by which metastatic reseeding can be hampered.

Over the past years, many groups have contributed invaluable knowledge towards this goal. Several studies were aimed to assess whether CTCs harvested from cancer patients could generate tumors. Despite some lack of success (Carvalho et al., 2013), most attempts have shown that human CTCs are tumorigenic when xenografted either directly into the bone, subcutaneously, or intra-venously in immunocompromised mice (Aceto et al., 2014; Baccelli et al., 2013; Hodgkinson et al., 2014; Rossi et al., 2014). Similar results were obtained by others, which reported that brain metastasis could be generated by human breast CTCs expressing a specific genomic signature (Zhang et al., 2013). Each of these studies thus support the notion that the blood of cancer patients harbors sub-populations of metastasis initiating cells (MICs) that can be identified by both specific cell-surface antigens and expression of markers for cell stemness. These findings are fully in line with what it is known concerning tumor-initiating potential, which is bestowed only to a sub-clonal population of cancer cells in primary tumors (Qureshi-Baig, Ullmann, Haan, & Letellier, 2017), as suggested by the observation that CTCs isolated from breast cancer patients proliferate *in vitro* best as tumor spheres, which are originated by cancer stem cells (Yu et al., 2014).

It is thereby apparent that human CTCs can spawn tumors when xenografted in mice; but how can we assess the ability of cancer cells to re-circulate from existing lesions and initiate additional tumors? Recent genomic studies on the human specimens mentioned above have directly addressed this crucial point. However, the development of novel treatment strategies aimed to counteract tumor reseeding requires clinically-relevant experimental models that reliably replicate the temporal and cellular dynamics of human metastasis. Dissemination to distant organs, from tumors grafted either *via* orthotopic or subcutaneous injections, is routinely observed in a variety of animal models for adenocarcinomas of the breast, prostate, and other solid tumors (Bos, Nguyen, & Massagué, 2010; Cifuentes, Valenzuela, Contreras, & Castellón, 2015; Wright et al., 2016). However, late stages of the metastatic cascade such as tumor seeding, initial colonization of skeleton and soft-tissue organs, and eventually progression into detectable neoplastic lesions can be effectively reproduced by delivering cancer cells in the arterial blood circulation *via* intracardiac injection (Eckhardt, 2012). This approach is particularly relevant when the goal of the study is to investigate reseeding in the absence of any growing tumor aiming to replicate human primary neoplastic disease. When appropriately executed, intracardiac injection will disperse cancer cells in any organ of the receiving mouse, while avoiding any spillage of the tumor cell suspension into the thoracic cavity. Appropriate processing of animal tissues allows visualization and enumeration of DTCs, particularly when tumor cells are engineered to stably express fluorescent proteins, thus permitting the assessment of the effects exerted on tumor seeding by genetic manipulations or pharmacological treatments (Naumov et al., 1999; Shen et al., 2016; Steinbauer et al., 2003; Yang et al., 1999). Engineering tumor cells to express fluorescent proteins in combination with luciferases, which are capable of generating bioluminescence signals detectable by dedicated instruments and in live animals, allows the identification of initial tumor foci generated by the DTCs and also monitoring over time their growth in different tissues (Guise et al., 1996; Shahriari et al., 2017; Wetterwald et al., 2002; Wurth et al., 2015)

Using this approach, our group produced compelling evidence that mobilization of cancer cells from existing disseminated tumors causes an increase in additional tumors detected over time in mice grafted with human breast cancer cells (Fig. 1). The forced dislodgement of tumor cells from their metastatic niches was achieved in this particular case by administration of AMD-3100 (Plerixafor, Mozobil), an antagonist of the chemokine receptor CXCR4 (Hatse, Princen, Bridger, De Clercq, & Schols, 2002), which has been shown to mobilize hematopoietic progenitors both in humans (Dar et al., 2005; Uy, Rettig, & Cashen, 2008) and mice (Broxmeyer et al., 2005) as well as cancer cells (Domanska et al., 2014) from the bone marrow. These results were further validated by the finding that cancer cells forcefully mobilized by targeting CXCR4 retained metastasis initiation potential, as shown by collecting murine 4 T-1 breast cancer cells as CTCs from mice with disseminated tumors and immediately re-injecting them in tumor-free animals (Qian et al., 2018).

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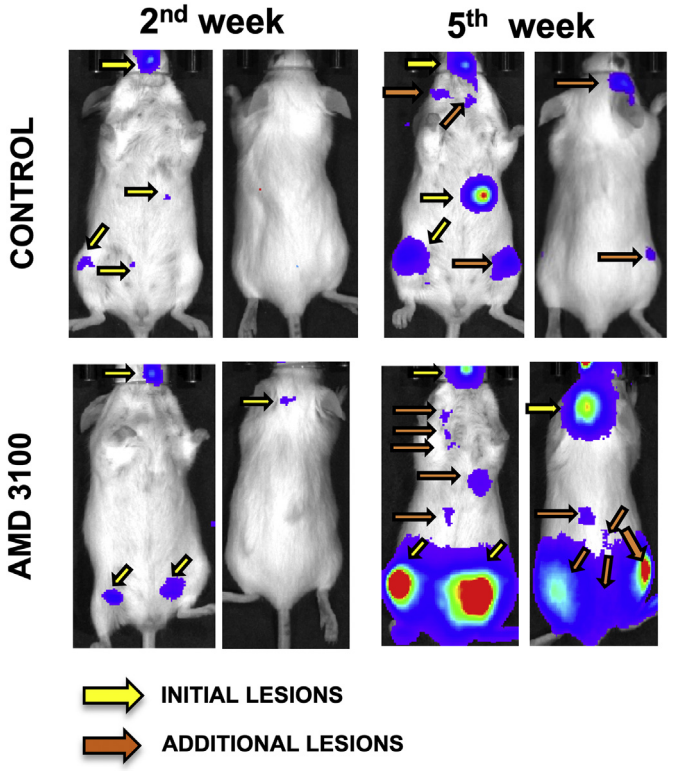


**Fig. 1.** Cancer cells mobilized from disseminated tumors reseed additional lesions. Mice harboring disseminated tumors generated by 4 T-1 murine breast cancer cells and reproducing early metastatic disease were treated with the CXCR4 antagonist AMD-3100, which dislodged cancer cells from the existing tumors and doubled the number of additional lesions as compared to control animals, (Control 11 mice/group, Treated 7 mice/group; ***P = .0002, One-way Anova with Dunnett's post-test).
Reproduced with permission from (Qian et al., 2018).

Based on this accumulated evidence and exploitation of the animal models described above, it is now possible to envision innovative means to thwart the clinical progression of metastatic disease.

## 4. Impeding seeding to contain metastatic expansion: A multi-pronged therapeutic strategy

### *4.1. Elimination of CTCs*

Once the notion that CTCs can *de facto* seed metastatic lesions also when departing from disseminated tumors was recognized, attempts were then made to provide *proof of principle* that the elimination of CTCs could produce clinical benefits. A recent example is the killing of cancer cells in the blood by photodynamic activation of photosensitizers such as rose bengal, achieved by energy transfer from green fluorescent protein (GFP) illuminated by a blue laser (Kim, Yoo, Jeong, & Choi, 2018). Mice were irradiated with a 473-nm wavelength laser at the femoral vein, surgically exposed by a skin flap, immediately after being inoculated with non-small cell lung cancer cells *via* the tail vein. The results from this study showed that treated animals had a reduction in CTC number as expressed by decreased colonies generated in a clonogenic assay performed with blood collected 15 min after tumor cell injection. This inferred decrease in CTC number resultant from the treatment led to a concurrent decrease in the number of tumor nodules in the lungs and ultimately increased survival in treated animals. On the same line, Choi and collaborators had previously developed a novel approach based on dual-wavelength acoustic flow cytography coupled with nanosecond laser therapy and reported the specific killing of melanoma CTCs in animals (He et al., 2016). Despite the application of techniques unlikely to be rapidly adopted in the clinic as well as the inferred CTCs enumeration, these findings offer compelling evidence that targeting cancer cells while in transit through the blood circulation is a promising strategy.

### *4.2. Blocking extravasation*

An alternative approach to the direct elimination of CTCs would be one that interferes with initial steps of tumor seeding by targeting the molecules and mechanisms mediating the extravasation of CTCs into surrounding tissues, a process that corresponds with the conversion of CTCs to DTCs. While the importance of this event in metastasis has been long recognized, efforts to hinder it with the intent of containing metastatic dissemination have been very scarce. However, a study by Sipkins and coworkers (Price et al., 2016) reported that the glycomimetic E-selectin binding inhibitor GMI-1271, administered to mice grafted with human breast cancer cells *via* the intracardiac route, significantly reduced the number of CTCs homing to the perisinusoidal areas of the calvarial bone marrow. These regions were identified as niches harboring predominantly dormant DTCs, implying that targeting E-selectin binding by specific ligands expressed on cancer cells would prevent the formation of reservoirs of dormant DTCs that could eventually migrate to different areas within the bone marrow and resume growth.

To further expand on these concepts and test their potential applicability to the clinical setting, we recently conducted pre-clinical studies in which the seeding of breast CTCs was shown to be inhibited by our novel, small-molecule antagonists of the chemokine receptor CX3CR1 (Issued U.S. patents 8,435,993; 9,375,474 and 9,856,260). As mentioned above, we have previously demonstrated the crucial role of CX3CR1 in tumor seeding and reported on the ability of the JMS-17-2 compound (Fig. 2A) to dramatically reduce the number of breast DTCs detected in the skeleton and lungs (Shen et al., 2016). The impairment of tumor seeding had a direct impact on the number of disseminated tumors observed in animals over time following intracardiac grafting, thereby indicating a role of CX3CR1 antagonism in containing metastasis-to-metastasis events. Further evidence in support of this concept was provided by a successive study (Qian et al., 2018), which reported a decrease in tumor cells reseeding bone and soft-tissues in animals treated with the improved CX3CR1 antagonist FX-68. Next, it was demonstrated for the first time that CTCs can indeed be retained in the blood circulation by preventing their seeding. For these studies, cancer cells were mobilized in the blood using AMD-3100 in animals harboring metastatic lesion and treated with FX-68 to target CX3CR1. In FX-68 untreated animals, the number of CTCs peaked following CXCR4 targeting – as a result of forced mobilization – and then progressively declined until reaching the pre-mobilization, steady-state levels after 48 h. However, the treatment with FX-68 dramatically increased the number of CTCs in blood following their forced mobilization (Fig. 2B), due to impairment of their reseeding caused by interference with CX3CR1. Remarkably, this tactic worked also in containing the number of additional lesions caused by the AMD-3100 mobilized cancer cells (Fig. 2C).

### *4.3. Mobilizing dormant cancer cells*

Based on the accumulated evidence, Sipkins and coworkers also proposed a strategy to relocate dormant cancer cells to the peripheral circulation by targeting CXCR4, with the intent of increasing the effectiveness of adjuvant treatments. It is our view that this approach would also be justified for active micrometastases and larger solid tumors which are notoriously difficult to be uniformly engaged by therapeutics. This challenge is especially difficult to surmount when attempting to treat tumors harbored in the bone marrow, which offers a protective environment to DTCs due to its anatomical topography, vascular architecture, and trophic support provided by local soluble factors and extracellular matrix components. (Meads, Hazlehurst, & Dalton, 2008; Nair,

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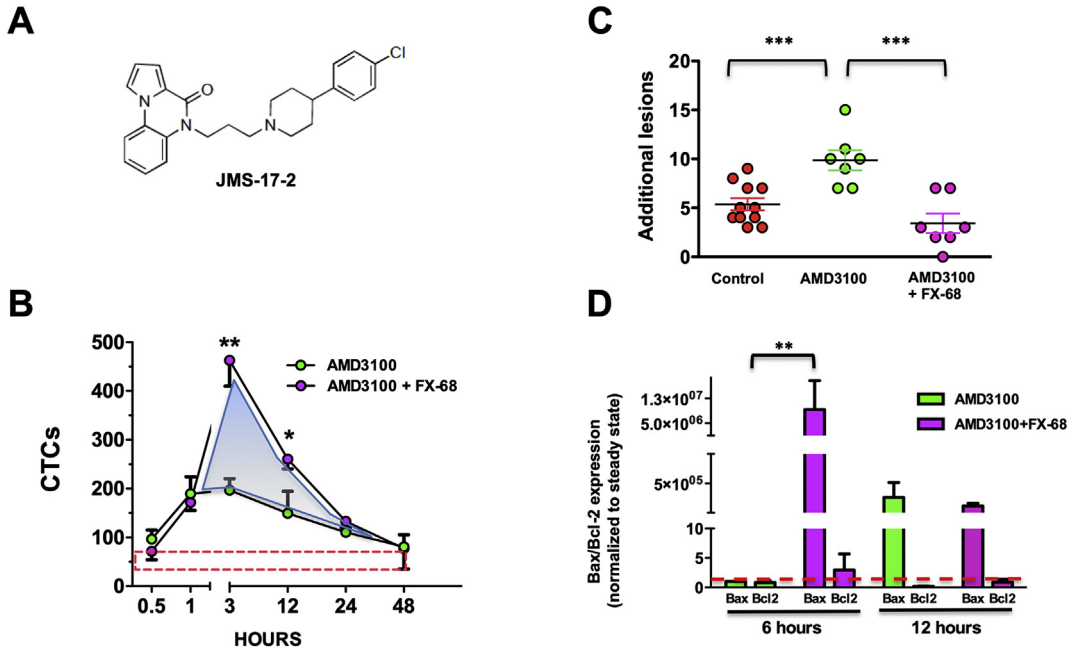


**Fig. 2.** Impeding reseeding by targeting CX3CR1 prolongs the time CTCs spend in circulation and promotes cell death. (A) The chemical structure of JMS-17-2, a novel, potent and selective small-molecule antagonist of CX3CR1. (B) CTCs were enumerated at different time points following administration of AMD-3100 alone or combined with the improved CX3CR1 antagonist FX-68 to mice harboring disseminated tumors. The area under the curve was measured as 485 for AMD-3100 alone and 852 for AMD3100 + FX-68, which equates to a 75% increase induced by the CX3CR1 antagonist (shaded area). The red-dotted box indicates the numerical range of CTCs detected at steady-state, *i.e* in the absence of any treatment (3 mice/group; *P = .04 paired Student's t-test; **P = .02 One-way Anova with Dunnett's post-test. (C) The combination of FX-68 with AMD-3100 fully blunted the increase in additional lesions caused by the administration of AMD-3100 alone (refer also to Fig. 1) (Control 11 mice, Treated 7 mice/group; ***P = .0002, One-way Anova with Dunnett's post-test). (D) CTCs collected upon treatment with AMD-3100 alone or AMD-3100 + FX-68 were collected at 6 h and 12 h (refer also to B) and levels of Bax and Bcl2 transcripts were measured by qRT-PCR as indication of the extent of apoptotic cells for each time-point and treatment. Bax expression was found to be dramatically increased at 6 h when the reseeding of CTCs was impaired by FX-68; at 12 h the apoptotic fractions were comparable between CTCs mobilized by AMD-3100 in the presence or absence of FX-68. All results were normalized to Bax and Bcl2 expression measured in CTCs collected at steady state (red dotted line) **P = .01 One-way Anova with Dunnett's post-test.
Reproduced with permission from (Shen et al., 2016) and (Qian et al., 2018).

Tolentino, & Hazlehurst, 2010; Patel, Dave, Murthy, Helmy, & Rameshwar, 2010).

Indeed, we found that retaining CTCs in circulation also negatively affects their viability, suggesting that prolonging the time spent in the blood upon inhibition of reseeding is sufficient to force a significant fraction of cancer cells into apoptosis by lack of substrate attachment, or anoikis (Gilmore, 2005), an outcome we observed in our studies (Fig. 2D). It is widely recognized that resistance to anoikis is a crucial feature of cancer cells with metastatic potential (Kim, Koo, Sung, Yun, & Kim, 2012); thus, one could theorize that while selected cancer cells forcefully mobilized by CXCR4 antagonism would have spontaneously re-entered the circulation anyway and were then equipped to resist anoikis, other cells were unfit for the task and their coerced re-entry combined with CX3CR1 inhibition propelled them to their apoptotic demise. To further speculate, using this approach to deplete this latter contingent of malignant phenotypes may be more effective than attempting to therapeutically target tumor cells retained within the protective bone marrow microenvironment. Taken together, these findings provide strong backing to the idea that effective means to keep CTCs in the systemic blood will reduce metastasis-to-metastasis seeding and also, upon their induced mobilization from the bone marrow, potentially dispose of cancer cells that would otherwise be impervious to treatments.

### *4.4. Improving drug exposure in the blood*

An intriguing corollary to the strategy depicted above, is that blocking tumor reseeding has the potential to extend the time that CTCs are exposed to therapeutics. Most chemotherapeutics show variable bioavailability in different tissues and, for the majority of drugs, fully engaging cancer cells growing in malignant nodules, particularly when harbored in certain organs, is often problematic. On the other hand, cancer cells are fully exposed to drugs when circulating in the blood and, if sufficient time is spent in this compartment, they should be more vulnerable to therapeutic targeting than when located in solid tumors. We recently validated this concept in pre-clinical studies, in which CTCs were retained in circulation by FX-68 administration prior to treatment with Doxorubicin. When compared to animals that received Doxorubicin alone, 50% more CTCs were found positive to drug incorporation, an effect that was not observed when FX-68 was administered 3 h after doxorubicin treatment, a condition that failed to block reseeding (Fig. 3).

A caveat to this reasoning would be that in the majority of patients the systemic circulation likely harbors MICs - initiators of metastasis endowed with stem cell properties, resistance to conventional chemotherapeutics, and the ability to evade apoptosis/anoikis (Oskarsson, Batlle, & Massagué, 2014). Thus, prolonging the time of circulation in blood might increase the susceptibility of the bulk of CTCs to chemotherapy, possibly reducing total tumor load, while still failing to significantly deplete the much smaller MICs pool. However, we have recently associated CX3CR1 expression with tumor-initiation features of breast and prostate adenocarcinoma cells (unpublished), following initial evidence that the minority of cancer cells that, despite the administration of a CX3CR1 antagonist were still able to seed the skeleton of mice, did not generate tumors (Shen et al., 2016). Should these findings be confirmed, they could reveal that MICs depend on CX3CR1 expression for reseeding and would be kept in circulation if exposed to an antagonist of this receptor, without opportunity to start new lesions. Furthermore, the defiance of apoptotic death that characterizes/MICs could be successfully circumvented in the near future by upcoming new approaches (Jaworska & Szliszka, 2017; Talukdar et al., 2018; Wang, Du, & Liu, 2017). A schematic representation of the main concepts expressed so far in this section is shown in Fig. 4.

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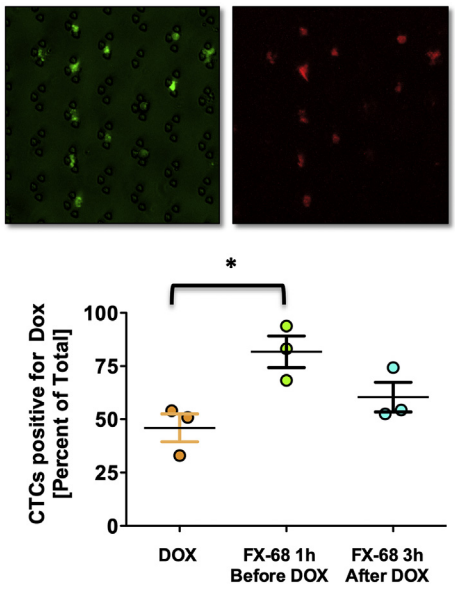


**Fig. 3.** Obstructing the reseeding of CTCs improves drug exposure in blood. Retaining CTCs in the blood by administering FX-68 increased the exposure to Doxorubicin, as measured by the percentage of cells showing red fluorescence emitted by the drug. Yellow arrows show two cancer cells that did not incorporate Doxorubicin (3 mice/group; *P = .03, One-way Anova with Dunnett's post-test.
Reproduced with permission from (Qian et al., 2018).

Integration of inhibitors of tumor cell seeding into the current standard of care therapies may also be have benefit beyond the context of chemotherapy. Immune therapy, as an example, has emerged as a leader in treatment for an array of different cancer types and might also have a synergistic effect with blockade of tumor seeding.

It is now widely recognized that malignant phenotypes can effectively escape the controlling activities of the immune system by engaging specific immune-checkpoints expressed mainly on T lymphocytes. While new checkpoints are being continuously identified and validated (Burugu, Dancsok, & Nielsen, 2018), programmed death-ligand 1 (PD-L1) is one of the earliest to be characterized and most effectively pursued (Constantinidou, Alifieris, & Trafalis, 2018). Therapeutics preventing PD-L1 expressed by cancer cells from inactivating T lymphocytes provide a dramatic boost to the immune-response (Leone, Poggiana, & Zamarchi, 2018).

There is evidence that common targets of immunotherapies such as PD-L1 are present on the CTCs of many disease types including breast, bladder, and non-small cell lung cancer (Anantharaman et al., 2016; Mazel et al., 2015; Nicolazzo et al., 2016). Interestingly, PD-L1 was found frequently expressed in HER-2 positive breast CTCs (Mazel et al., 2015); in non-small cell lung patients, CTCs were found positive to PD-L1 more frequently than tumor tissues (Guibert et al., 2018). Most of these studies have suggested the potential of using PD-L1 expression on CTCs as a predictive prognostic factor of disease progression; however, this application has been quite challenging as in some cases PD-L1 expression is abundant on CTCs, thereby making it difficult to identify a cohort that is distinctly negative for PD-L1 expression (Nicolazzo et al., 2016). Additionally, PD-L1 is a major target in immune-oncology as evidenced by the large suite of FDA approved PD-L/PD-L1 inhibitors. To date, there is a lack of clear evidence that PD-L1 expressing CTCs experience target engagement with any of these inhibitors in circulation; however, it is conceivable that PD-L/PD-L1 inhibitors may be able to target CTCs as well as established metastases. In such an instance, combining these inhibitors with an agent that blocks tumor seeding would increase exposure of CTCs to this therapy and thereby increase efficacy.

Although the direct effect of immune therapies on CTCs requires further exploration, some immune therapies have been shown to have indirect effects on cancer cells in circulation. Gül and colleagues were among the first to propose a mechanism by which monoclonal antibody (mAb) immunotherapy can have a detrimental effect on CTCs. Specifically, they found that treatment with TA99 mAb promoted the phagocytosis of CTCs by macrophages and the Kupffer-mediated arrest of tumor cells in the liver (Gül et al., 2014). These effects were demonstrated using a metastatic melanoma cell line (B16F10) because they are the only syngeneic murine solid tumor cell line for which here exists a

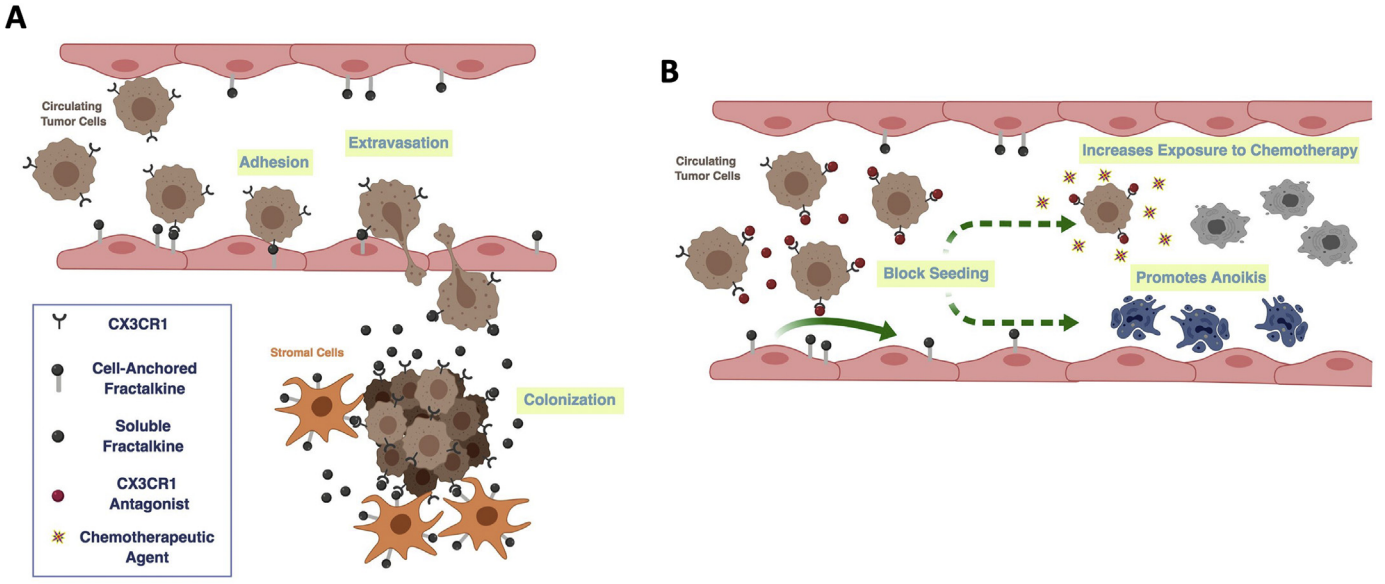


**Fig. 4.** Rationale for therapeutic targeting of tumor seeding to prevent of metastasis. (A) CTCs utilize the adhesive molecular interactions between CX3CR1 and the cell-anchored form of Fractalkine to facilitate extravasation through the endothelium. After successful extravasation, newly seeded cancer cells migrate in response to the chemoattractant gradient established by the soluble Fractalkine released from cells of the surrounding stroma. Given a conducive microenvironment, the DTCs can proliferate and form metastases in the secondary organ. (B) Administering a CX3CR1 antagonist blocks the initial CX3CR1-fractalkine interaction thereby preventing extravasation. Blocking of tumor cell seeding has a dual benefit. Failed extravasation will cause CTCs to be retained in circulation, which consequentially increases their exposure to chemotherapeutic agents and improves clinical outcome. In addition, CTCs with chemoresistant phenotypes will have extended retention in circulation and eventually undergo programed cell death due to their prolonged detachment from the extracellular matrix (anoikis). Schematic created with BioRender graphics web application.

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specific mAb. However, the authors emphasize the importance of these results to other types of cancers such as colorectal cancer (CRC) which commonly metastasizes to the liver. Thus, it is possible that preoperative mAb immunotherapy should be considered for patients undergoing resection for primary CRC as treatment of this kind would promote the elimination of CTCs by Kupffer cells thereby preventing post-surgical metastases (Gül et al., 2014). One could imagine, however, that maximum efficacy of this approach could be achieved by combining preoperative mAb immunotherapy with blockade of tumor cell seeding. Because Kupffer cells reside in the liver sinusoids, they are the first line of defense against tumor cells entering the liver (Paschos, Majeed, & Bird, 2010). For this reason, incorporating treatment with an antagonist against a target that mediates tumor cell seeding would trap CTCs in the blood and thereby increase their exposure to the Kupffer cells, and consequentially increase the incidence of their phagocytosis and clearance from the blood. Although this example is specific to CRC, as more is learned about the indirect effect of immunotherapies on CTCs of different cancer types, it is undeniable that there may be benefit of combining these drugs with pharmaceuticals that target tumor seeding.

Additionally, novel immune-cell targeted therapeutic approaches aimed specifically at targeting and depleting CTCs are being conceived. One such proposed therapy utilizes neutrophil-mediated drug delivery (NM-NP) (Kang et al., 2017), by which neutrophil membranes are coated with poly(lactic-*co*-glycolic acid) nanoparticles that can be loaded with an array of anti-cancer agents. When loaded with second-generation proteasome inhibitor carfilzomib, NM-NP's were successfully able to neutralize and deplete CTCs thereby preventing early metastases and formation of the pre-metastatic niche (Kang et al., 2017). This study, though promising, has limitations as the effect of carfilzomib loaded NM-NP's (NM-NP-CFZ) was not assessed on CTCs coming directly from circulation of an animal model but rather on blood spiked with cancer cells. However, given the striking reduction in early metastases (observed at 7, 14, and 21 days post inoculation), it is reasonable to assume that NM-NP-CFZs would be able to selectively target CTCs in the blood. With the recently emerged evidence supporting the notion that inhibitors of tumor seeding can increasing the exposure of CTCs to cancer targeting agents, future studies exploring the application of NM-NPs would greatly benefit from combination with an anti-seeding agent.

### *4.5. Limiting drug resistance*

Another potentially impactful outcome deriving from limiting CTC reseeding would be the mitigation of acquired drug resistance, which regularly arises in patients treated with chemotherapeutic regimens and often also during targeted therapies. Drug resistance can be caused by a gamut of factors, including lack of optimal drug penetration in solid tumors (Minchinton & Tannock, 2006; Trédan, Galmarini, Patel, & Tannock, 2007) and other events often implemented by a supportive tumor-associated stroma (Sebens & Schafer, 2012). However, changes in uptake, metabolism, and export of drugs as well as alterations of the molecular targets against which therapies are directed, are the results of epigenetic changes and genetic mutations occurring in cancer cells as a consequence of intrinsic genetic instability (Gerlinger et al., 2014) and follow a clonal evolution paradigm (Friedman, 2016). The likelihood that one or more randomly occurring mutation confers a survival advantage to tumor cells under selective pressure from cytotoxic or targeted therapeutics, is dependent on the rounds of clonal expansion occurring within a tumor (Nowell, 1976). In other words, the fraction of cells that resist to a specific treatment increases with the number of cell divisions (Iwasa, Nowak, & Michor, 2006) and tumors expanding rapidly due to higher rates of cell proliferation are more likely to experience mutations, which may, over a short period of time, make them impervious to therapeutics. It is understood that smaller tumors grow at a faster pace than larger tumors, following a *Gompertzian growth curve* that was first applied to cancer cells by A.K. Laird (Laird, 1964). This algorithm later provided the foundation for the Norton-Simon hypothesis, from which modern chemotherapeutic regimens are currently designed (Simon & Norton, 2006). Given these principles, drug-resistant variants are expected to arise much more frequently in small neoplastic foci, such as those resulting from individual DTCs colonizing new tissue ecosystems, than in larger lesions. In line with this model, preventing the reseeding of CTCs should drastically restrain the DTC-derived highly-proliferating foci from supplying drug-resistant clones that eventually render metastatic disease incurable.

## 5. Conclusions

The fact that solid tumors spread by exploiting the systemic circulation has been long recognized (Talmadge & Fidler, 2010) and the development of different platforms to attain the daunting tasks of identifying and collecting CTCs from blood has both provided the scientific community with unique research tools and allowed the invaluable prognostic and diagnostic contribution of liquid biopsy to the clinical settings (Alix-Panabières, Bartkowiak, & Pantel, 2016; Toss et al., 2014; Woo & Yu, 2018). These advancements – and the recent recognition that CTCs recirculate following the ablation of a primary neoplasia – have heightened the attention towards effective means of interrupting the continuous seeding of new lesions in patients with either initial or established metastatic disease. The long reigning concept that *the horse is out of the barn* is fading and there is confidence that counteracting tumor seeding as a way of decelerating disease progression will finally loose its aura of a mostly futile endeavor.

With a better understanding of the dynamics and mechanistic foundation of CTCs recirculation, and the development of new therapeutics tailored to oppose tumor seeding, we are poised for the dawning of entirely new strategies for the management of patients with advanced tumors.

## Conflict of interest statement

The authors declare that there are no conflicts of interest.

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