Circulating Tumor Cells in Metastatic Cascade

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Circulating tumor cells (CTCs) are a heterogeneous population of tumor cells that have shed from a tumor into the lymphatics and vasculature, ultimately disseminating into blood circulation. Circulating tumor cells are a key player in cancer metastasis, a multi-step and complex process that involves (1) local invasion of primary tumor cells into adjacent tissue; (2) intravasation (trans-endothelial migration into nearby blood vessels); (3) circulation (transient travel and survival in the circulatory system as CTCs); (4) extravasation; (5) colonization. To successfully metastasize, CTCs must evade immune surveillance at every step once they leave the immunosuppressive tumor microenvironment. On the other hand, immune cells can promote or inhibit tumorigenesis, depending on the cell type and context.

Keywords: circulating tumor cells (CTCs) ; metastasis cascade ; immune cells

1. Intravasation

Intravasation is the process by which cancer cells shed from primary or metastatic deposits and traverse the endothelium to enter the bloodstream, thus, forming Circulating tumor cells (CTCs) and pioneering the metastatic cascade. Immune cells that interact with CTCs during the initial stages of metastasis include neutrophils, natural killer (NK) cells, monocytes, macrophages, and T lymphocytes.

1.1. Neutrophils

The recruitment of neutrophils by primary tumor is implicated in the elevated rate of intravasation in vivo [1]. In breast cancer patients, as well as in mouse models, neutrophils facilitate the intravasation of tumor cells by forming CTC-neutrophil clusters, which express higher levels of positive regulators of cell cycle and DNA replication programs than CTCs alone, leading to increased metastatic propensity and poor prognosis [2]. Activated neutrophils secrete neutrophil extracellular traps (NETs), which contain factors such as matrix-metalloprotein-9 (MMP-9), neutrophil elastase (NE), and cathepsin G (CG) that promote extracellular matrix (ECM) degradation and cellular aggregation. Evidently, these factors can promote CTC migration and invasion while enhancing proliferation and anti-apoptosis traits of CTCs at the same time [3]. Accordingly, transcriptomic analysis on peripheral blood leukocytes from treatment-naïve renal cell carcinoma patients indicated that NET formation, as indicated by elevated expression of NET formation regulators, promotes CTC viability [4][5]. Another study also demonstrated that neutrophil activation in the blood correlates with CTC survival [5]. In early-stage breast cancer patients, increased levels of NETs in the blood have been proposed as a biomarker that specifically predict the chronic risk of liver metastases [4].

1.2. NK Cells

A study by Santos et al. demonstrated a close link between the number of CTCs and the cytotoxic activity of NK cells in the blood of breast, colorectal, and prostate cancer patients [6]. The NK cells from patients with a high number of CTCs showed diminished cytotoxicity, as compared to those isolated from patients with a low number of CTCs. This is consistent with an earlier study, where mice with low NK cell activity showed enhanced blood-borne tumor cell survival and increased incidence of metastasis [7][8].

1.3. Monocytes and Macrophages

Monocytes and macrophages have been reported to aid in intravasation via myriad mechanisms. A three-cell complex, termed “tumor microenvironment of metastasis doorway”, composed of a perivascular macrophage, a tumor cell, and an endothelial cell, is found to act as a gateway for tumor cell hematogenous dissemination. Intravital high-resolution microscopy revealed that perivascular macrophages promote transient vascular permeability by interacting with endothelial cells, via VEGFA signaling, and consequently facilitate the intravasation of tumor cells [9][10][11]. In addition, tumor- or CTC-educated macrophages influence almost all the steps of the metastatic cascade, such as accelerating invasion, intravasation, survival in the circulation, tumor cell arrest, extravasation, as well as durable growth at distant

sites [12][13]. Solid tumor-based studies suggested that CTCs intravasate into the circulatory system along with TAMs [14][15]. Additional clinical evidence of the continued interaction of macrophages with tumor cells was found in the blood circulation of cancer patients. Despite the rarity of cell types, and the shear stresses within the vascular circulation, circulating CAMLs and macrophages with pro-angiogenic capacity have been found to migrate through the circulation, attached to CTCs in 10% of late-stage patients with breast, pancreatic, or prostate cancers [16]. Together, these lines of evidence demonstrated a critical role of macrophages in metastatic intravasation.

Besides immunosurveillance, macrophages are also involved in the establishment of the premetastatic niche, particularly in the lungs. Using an intravital two-photon lung imaging system, CTCs lodged in the capillaries of the lungs were found to shed microparticles into the vasculature, driven by high shear forces within seconds of arrival. Following CTC entry, myeloid cells, such as monocytes, macrophages, neutrophils and dendritic cells (DCs), ingest these large microparticles. Of note, tumor-ingesting macrophages display an activated phenotype and pro-metastatic function, while lung-resident conventional dendritic cells showed anti-metastasis effects [17]. These findings demonstrated the complex interplay between CTCs and various immune cells.

1.4. T Cells

There are limited investigations into the role of $CD4^+$ helper T cells and $CD8^+$ cytotoxic T cells in the immune surveillance of CTCs. As such, receptor activator of nuclear kappa-B ligand (RANKL) expressed by tumor-infiltrating $CD4^+$ T cells are thought to stimulate RANK, expressed on CTC from breast cancer, to enhanced intravasation [18][19]. In a more recent study, $CD4^+$ T cells were found to be unexpectedly involved in vasculature and immune reprogramming, thereby contributing to impeding cancer cell intravasation [20]. In that study, $CD4^+$ T cell deficiency resulted in reduced immune responses, lower expression of good-prognosis angiogenesis genes (GPAGs), and higher expression of poor-prognosis angiogenesis genes (PPAGs). Loss of $CD4^+$ T cells also altered pathways and genes that regulate vessel normalization. Two studies have illustrated the safeguarding role of $CD8^+$ T cells in intravasation. Increased CTCs were found in CD8 knockout mice with breast cancer, demonstrating that loss of cytotoxic T cells promoted CTCs survival, as well as increased intravasation [20][21]. However, the mechanisms by which interaction of CTCs and T lymphocytes contribute to intravasation have not been fully clarified. Therefore, understanding the role of different T cell subsets in different stages of metastasis cascade is necessary to validate the above findings.

2. CTC Survival in Circulation

Most CTCs undergo apoptosis, owing to physical stress, through shear and tear in the circulation and anoikis [22]. Further, immune cytotoxicity eliminates the majority of CTCs in the blood [23], and deprivation of growth and survival factors outside the tumor niche also contribute to CTC death [24]. Despite these harsh survival conditions, CTCs can survive in the blood and contribute to metastasis to secondary sites by mechanisms such as cellular mutation, cytokine and growth factor stimulation, and interactions with surrounding cell types [25][26].

The small number of CTCs that survive are able to hijack immune cells and improve their survival odds. These resistant CTCs gain enhanced metastatic seeding capability by utilizing immune cells to amplify certain traits, such as migration ability and invasiveness, as well as to modify and adapt to unfavorable circumstances. Some of the major immune cell types that interact with CTCs in circulation include neutrophils, monocytes/macrophages, lymphocytes, and NK cells [25][27].

2.1. Neutrophils

CTC–neutrophil clusters are one of the common CTC clusters found in blood. As first responders, neutrophils are attracted to primary tumors and CTCs that produce granulocyte colony stimulating factor (G-CSF) and other neutrophil stimulating cytokines [2][28]. Initially, neutrophils induce inflammation and release hydrogen peroxide that are cytotoxic against CTCs [29]. However, as the cancer progresses, immunosuppressive TGFβ1 that originates from the primary tumor microenvironment, and platelets, can induce N2 neutrophil polarization, which aids in CTC survival and epithelial–mesenchymal transition (EMT) [30][31][32]. Additionally, CTCs can develop resistance towards neutrophil-mediated cell death, through mutation in the TLE1 gene that promotes NFkB-mediated cancer progression [33]. Neutrophils in CTC clusters are found to express adhesion factors, such as VCAM-1 and intracellular adhesion molecule 1 receptor (ICAM-1), which likely explains how neutrophils form close “piggyback” adhesions with CTCs [34][35]. This close interaction between CTCs and neutrophil enhances the activation of proliferative pathways in CTCs via crosstalk of cytokines, such as IL-6 and IL-1β, from neutrophils [28]. In addition to the pro-survival effects mentioned previously, NETs can serve as a protective cloak that bind to CTCs, via β1-integrin, to protect them from shear stress and immune cytotoxicity in the blood [36].

2.2. Monocytes and Macrophages

Monocytes are another abundant circulating immune cell type found in CTC clusters. They patrol within the blood circulation, where they eliminate cellular debris and are a significant mediator of the inflammatory processes. Monocytes can present themselves with a diverse subset of phenotypes and functions, depending on the external stimuli in their immediate environment [37]. Shibuya et al. demonstrated that tissue repair promoting $Ym1^+$ $Ly6C^{hi}$ monocytes promoted CTC-mediated lung metastasis in the presence of systemic inflammation [38], highlighting an important role for immunosuppressive monocytes in supporting CTC metastasis. Monocytes can mature into macrophages that acquire the ability to phagocytose larger targets, including CTCs. In fact, macrophages can detect and distinguish foreign cells from self-cells through CD47, a cell surface glycoprotein that serves as a “do not eat me” signal. Furthermore, CTCs and secondary metastases are found to express high levels of CD47, suggesting that they acquire better survival advantages in blood circulation and secondary growth sites, likely by evading immunogenic responses [39].

Aside from evading phagocytosis, CTCs also take advantage of macrophages in a direct manner, through cytokine crosstalk. Tumor cells release colony stimulating factor 1 (CSF1) that recruits macrophages and polarizes them towards tissue repair-promoting M2 TAMs, which, in turn, produce growth factors like epidermal growth factor (EGF) that further stimulates tumor cells to produce CSF-1, thus, forming a positive feedback loop [40][41].

2.3. T Cells

Effector $CD4^+$ and $CD8^+$ T lymphocytes exert hostile responses toward CTCs in the circulation. Further, PD-L1 expression on T cells has been found to correlate with increased CTC survival in metastatic genitourinary cancer and advanced non-small cell lung cancer (NSCLC), along with lower $CD4^+$ and $CD8^+$ T cell numbers [42][43]. It is suggested that $PDL1^+$ CTCs are able to suppress T cell activation and, thereby, dampen the immune response against CTCs in the blood [44]. In another study, CTCs expressing elevated levels of T cell programmed cell death Fas ligand were detected in breast cancer patients, causing increased apoptosis of T cells in circulation [45]. These observations indicate that CTCs may depend on T cell deactivation and apoptosis to prolong their survival in circulation, as supported by their negative correlation with $CD4^+$ and $CD8^+$ T cell numbers and shorter overall patient survival [46].

Regulatory T cells (Tregs) are an immunosuppressive T lymphocyte subset that negatively regulate cytotoxic T lymphocyte activation [47]. The CTCs preferentially recruit circulating Tregs through the release of chemokines, such as CCL5, CCL22, CCL17, CXCL9 and CXCL10 [48]. Subsequently, circulating Tregs are activated by tumor-derived suppressive factors (TDSFs), including IL-10 and TGFβ1, to promote an immunosuppressive environment that limits T effector functions and contributes to CTC survival [49][50]. In addition, the RANKL, secreted by Tregs, can help promote tumor migration in $RANK^+$ CTCs [18][51]. Evidently, CTC counts are positively correlated to circulating Tregs in various cancers, such as inflammatory breast cancer, hepatocellular carcinoma (HCC), and NSCLC [46][52][53].

2.4. NK Cells

Similar to T lymphocytes, NK cells play a pivotal role in restricting CTC survival and metastasis through direct interception in circulation. The NK cells can initiate the indirect killing of CTCs through the secretion of the tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) that binds to apoptotic receptors on tumor cells [54][55]. Resistant CTCs are able to mitigate this by downregulating their death receptor 5 (DR5) expression on the cell surface [56]. The secretion of granzyme and perforin from NK cells represents another form of cytotoxicity towards CTCs that is deemed more effective, capable of eliminating up to 80% of CTCs and slowing down metastatic spread [57]. Low NK cell numbers are indicated to be an independent risk factor for CTCs and their progression [46]. In recent studies, CTC clusters were shown to evade NK cytotoxicity much more effectively than single CTCs [58]. Notably, NK cell-mediated cytotoxicity is suggested to be more potent towards CTCs with partial mesenchymal-like traits, though further insight is still required to comprehend the dynamic interaction between CTCs and NK cells from the blood to metastatic colonization [59].

3. Extravasation and Colonization

As CTCs roam in the bloodstream, a small percentage of these cells will get arrested in tight capillaries, which may be a prerequisite for extravasation [60]. The CTC clusters have better odds at initiating this process due to the large size that can get embedded in tight spaces for longer periods [61]. The cancer infiltration process can be promoted through several factors, including CTC expression of surface receptors and integrins for attachment, mechanical and physical pressure, chemotactic gradient from certain secondary sites, and with aid from immune cells in clusters [62].

Upon arrest, leukocytes in CTC clusters interact with endothelial cells lining the vasculature that are essential for endothelial attachment. During this process, tumor cells secrete factors such as IL-8 to promote the leukocyte expression of adhesion receptors like β2-integrin, that can bind directly to ICAM-1 and E-selectin, present on endothelial cells [63]. For instance, tumor cells that are entrapped within NETs from neutrophils act as passengers, whereby neutrophils that express CD11a (LFA1) and CD11b (Mac-1) are able to interact with ICAM-1 on endothelial cells [64]. At the same time, tumor cells produce factors that upregulate these adhesion receptors and promote migration potential in neutrophils by delaying apoptosis [65]. Monocytes and TAMs, on the other hand, secrete cytokine and chemotactic factors, such as VEGF, TGFβ1, and CCL2, to increase vessel permeability and to destroy endothelial tight junctions, thereby mediating trans-endothelial migration [66][67]. Tumor cells are also capable of following the “microtracks” generated by macrophages, as they cross the endothelial border [68].

Tumor cells that infiltrated secondary sites, hereby termed disseminated tumor cells (DTCs), have to overcome immune surveillance at the secondary site and undergo mesenchymal–epithelial transition (MET) to gain the capacity to colonize upon the local niche. In this regard, immune surveillance at different organs poses different threats, depending on the local immune composition. In breast cancer/prostate cancer-mediated bone metastases, tumor cells actively modulate the local immune niche by secreting extracellular vesicles (EVs) and factors, including VEGF, IL-6, and IL-8, that promote osteoclast differentiation and activate osteoblasts to support osteoclast activities, which causes osteolysis. Subsequently, these activated bone cells also secrete tumor growth promoting factors like TGFβ1, to advocate tumor growth and, ultimately, form a positive feedback loop [69]. These interactions enable tumor cells to undergo “osteomimicry”, where they express bone-related genes to adapt and expand upon bone sites [70]. T cells and NK cells play a prominent role in eliminating DTCs in metastatic niches [71]. In lung metastasis, tumors modify pre-metastatic niche by recruiting neutrophils to the lungs via immune–cancer crosstalk [72]. These neutrophils suppress T cell cytotoxicity via inducible nitric oxide synthase (iNOS) expression [73]. Furthermore, DTCs with altered antigen presentation characteristics are capable of minimizing T cell and NK cell cytotoxicity. In such cases, PD-L1 expression and downregulation of major histocompatibility complex I (MHC I) are modifications that allow tumors to evade NK and T cell-mediated killing, though NK cells may still recognize tumor cells with abnormally low MHC I levels [74][75]. Additionally, DTCs may recruit immunosuppressive myeloid cells to suppress NK cell activity [76]. For DTCs to fully integrate into the local niche, MET process is necessary for tumors to revert into epithelial forms. Moreover, TAMs secrete IL-35 that facilitates MET in tumor cells, through the activation of JAK2–STAT6-GATA3 signaling [77]. In the late stage of metastasis, DTCs that survive and have undergone EMT expand to form overt metastases upon acquiring sufficient growth signals in a favorable condition.

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