STEM CELLS FROM HYPE TO HOPE

During the last two decades, stem cells have progressed from merely a concept to a vibrant field of regenerative medicine which is aimed at addressing the root cause of the problem rather than conventional methods of intervention that mostly provide symptomatic relief.

Stem cell therapy either alone or in combination with the other established treatment strategies is a hope for patients who suffer from the "incurable" diseases such as Alzheimer, diabetes, myocardial infarction etc. Besides aspirations in the clinical perspective, stem cells provide excellent in vitro disease models for drug development.

Given the significance of the field, the proposed book will be a compilation of the bench experience of experts from various research labs involved in the cutting edge area of stem cell research.

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STEM CELLS FROM HYPE TO HOPE

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CHAPTER 6

Cancer stem cells and their microenvironment

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ABSTRACT

Cancer Stem Cells (CSCs) are a small population of cells within tumors holding stemness properties that sustain cancer progression, such as enhanced capacities for self-renewal, growing, metastasizing, homing, and reproliferating. CSCs show remarkable organizing capacities as they can educate neighboring cells to provide nutrients and collaborate in the elusion from the immune system, creating an environment favorable for tumor growth. In particular, tumor-specific microenvironments comprise stromal cells, immune cells, networks of cytokines and growth factors, hypoxic regions, and the extracellular matrix. The contribution of the microenvironment in this picture is crucial: it is now accepted that the "cancer" scenario is not simply composed of transformed cells working together in isolated and strictly autonomous machinery. Tumor microenvironment actively collaborates with neoplastic cells at different levels: promoting proliferation while evading growth suppression and immune surveillance, overcoming cell death, modulating cell metabolism, activating angiogenesis and invasion/metastasis programs. Also, the interactions between CSC and microenvironment help in their survival of common anti-cancer therapies thus being partly responsible for disease recurrence. Further studies regarding CSC/microenvironment seem to be promising for new CSC-targeting therapies, which may represent an innovative strategy for the cure of lung cancer.

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KEYWORDS

Cancer; CSCs; Carcinogenesis; Differentiation; Signaling; Stem cell; Tumor.

LIST OF ABBREVIATIONS

APC = Antigen presenting cells
CAFs = Cancer-associated fibroblasts
cCAFs = Circulating CAFs
CCL18 = C-C motif ligand 18
CSCs = Cancer stem cells
CSF-1 = Colony stimulating factor-1
CSF-1R = Colony stimulating factor-1receptor
CTC = Circulating tumor cells
CTLA-4 = Cytotoxic T lymphocyte-associated protein 4
CXCR4 = C-X-C-chemokine receptor 4
ECM = Extracellular matrix
EGF = Epidermal growth factor
EMT = Endothelial-to-Mesenchymal transition
Glu-GNPs = Glucose-coated gold nanoparticles
HA = Hyaluronan
HGF-1 = Hepatocyte growth factor-1
ITH = Inter-tumoral heterogeneity
Mang-NPs = Mangostin-encapsulated Poly (lactic-co-glycolic acid) nanoparticles
miRNAs = MicroRNAs
MMPs = Matrix metalloproteinases
PD-1 = Programmed cell death 1
PDGF = Platelet-derived growth factor
PDGFR = Platelet-derived growth factor receptor
SCC = Squamous cell carcinoma
SDF-1 = Strromal cell-derived factor-1
Shh = Sonic hedgehog
SPARC = Secreted protein acidic and rich in cysteine
TAMs = Tumor associated macrophages
TCR = T-cell receptor
TIL = Tumor infiltrating lymphocytes
TME = Tumor microenvironment
VEGF = Vascular endothelial growth factor

VEGFR1 = Vascular endothelial growth factor receptor-1
VEGFR2 = Vascular endothelial growth factor receptor-2
VM = Vascular mimicry

6.1 INTRODUCTION

6.1.1 Heterogeneity of the Tumor

Given the assortment of cell and tissue types that are known to exist within tumors, recent research has more focused on tumor heterogeneity.[1] Among the early investigators during 1800s, Virchow and Cohnheim postulated the existence of cancer stem cells (CSCs) that arise from what they believed to be "activation of dormant embryonic tissue remnants".[1,2] CSCs are defined as a subpopulation of cancer cells with the capability to auto-regenerate, proliferate and differentiate into multiple cancer cell lineages through symmetric and asymmetric cell division, with tumorigenic potential and specific surface markers.[3–7] The unique characteristics of CSCs include the requirement of a small number of CSCs to initiate new tumor, self-renewal and differentiation potential, possession of specific and distinguishing surface markers that help in their identification and isolation, and resistance to conventional chemotherapy and radiotherapy.[3,4,7,8] Despite these well-known characteristics, the definition of the cellular components of the tumor mass remains contentious.[3]

The differentiation hierarchy that underpins the development of all cellular compartments is indispensable for understanding the origin of tumor cells (Figure-6.1). However, for many tissues, the cellular hierarchies have not been adequately refined thus making it difficult to confirm the cellular origins of cancer.[9] The scientific community has marked the existence of tumor heterogeneity that can be classified as inter-tumoral heterogeneity and intra-tumoral heterogeneity. Inter-tumoral heterogeneity (ITH) is currently defined as multiple interactions as variations between tumors of different tissue and cell types, between tumors of the same tissue type from different patients, and between different tumors within the same individual. On the other hand, intra-tumoral heterogeneity refers to variations observed within a single tumor.[1,10] Different tumor cell populations differ in surface marker expression, genetic or epigenetic changes, genetic stability, resistance or susceptibility to therapy and growth rates.[11]

The inter-tumoral heterogeneity provides the basis of cancer classification into different types and sub-types based on divergence in their histological features, genetic profile, protein signatures, and surface markers expression profile. Many of these variables provide clinically relevant prognostic features and/or predictive

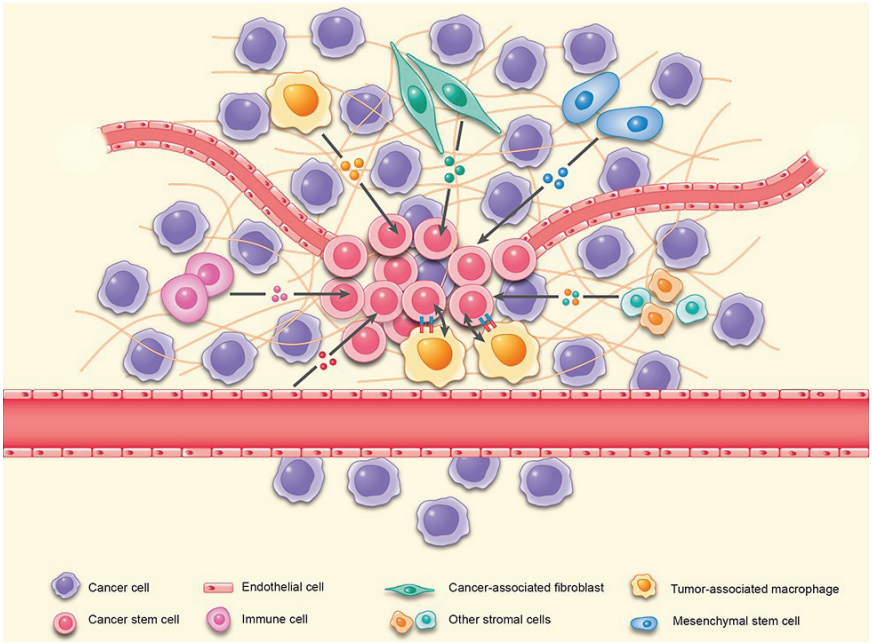

Figure-6.1. The cellular components of the tumor microenvironment.

information.[12] On the other hand, intra-tumoral heterogeneity complicates cancer prognosis and treatment.[13] Currently, clinical evaluation of tumor heterogeneity is an emerging issue to improve clinical oncology while intra-tumor heterogeneity is closely related to cancer progression, resistance to therapy, and the probability of recurrence.[14] It is inter-connected with complex molecular mechanisms including spatial and temporal phenomena, which are often peculiar for every single patient.[14]

Because of ITH in the primary tumors as well as metastases, and due to the wide clinical heterogeneity amongst cancer patients, it is imperative to apply clinical research methods directly to patients' material in contemporary clinical practice to ensure specific, optimal and effective treatment.[14] For any tumor type, only few molecular biomarkers are being currently employed for diagnosis and an only minor part of the available treatment targets is being exploited. It is now anticipated that clinical research, directly performed on cancer patients, will be increasingly diffused as a requirement to obtain more efficient and personalized tumor therapy protocols.[15] Moreover, phase III clinical trials in oncology have recently encountered wide criticisms,[16–18] because of the long duration required, the high cost, and the less than expected results.

The concept of CSCs has been included in the definition of the intra-tumor heterogeneity which is postulated to develop over time as CSCs divide and

differentiate asymmetrically.[19,20] The loss of normal cellular controls allows the development and propagation of genetic or epigenetic alterations that give the cells novel properties associated with metastasis, self-renewal, treatment resistance, and recurrence.[19,21] As the presence of multiple clonal sub-populations within the same tumor imparts divergent cell phenotypes, characterized by obvious growth advantage or treatment resistance, a substantial therapeutic challenge exists, as only some cells within a tumor would be affected by any one treatment.[22] The first clear evidence to support a role for CSCs activity in intact tumors was provided by three independent studies carried out using experimental brain, skin and intestinal tumor mouse models.[23] Using the genetically engineered lineage-tracing experiments, these studies provided clear evidence that CSCs arise *de novo* and contribute to the tumor growth.[24–26] These studies resolve the debate on whether CSCs do exist or are merely a xenotransplantation artifact. Nevertheless, the key question remains whether targeting of CSCs alone would be sufficient or whether non-CSCs could take their place after de-differentiation. Unfortunately, the efficacy of CSCs targeting and the capacity to revert to the CSC state has been difficult to study due to the limited characterization of CSC-specific markers. Several markers including CD133, CD44, CD166, CD24, and ALDH1 activity, have proven useful for prospective isolation of CSCs in multiple solid tumors.[27] However, CSC-specific marker expression profile differs between tumor types. For instance, while CD133 has been used as a marker to identify CSCs in glioblastoma[28] and CRC,[23] it is not a reliable marker in breast cancer where CD44+ CD24– is commonly used to enrich CSCs.[29] CSC-specific marker expression also varies between cancer sub-types and even, between patients in the same subtype.[30] For instance, CD44high-CD24low fails to efficiently enrich CSCs in triple-negative breast cancer[31] and CD133 has been debated in colon cancer. Furthermore, the lack of consistency has generated confusion in the identification of CSCs and questioned the importance of CSC-specific markers.[32–34] These observations indicate that the phenotype of CSCs is not as well defined as would be required for optimal detection in clinical material.

CSCs also exhibit several genetic and cellular adaptations that confer resistance towards classical therapeutic strategies. These include relative dormancy/slow cell cycle kinetics, efficient DNA repair, high expression of multidrug-resistance-type membrane transporters, and resistance to apoptosis. Cancer often acquires resistance to chemotherapy or radiotherapy after non-lethal exposure.[35,36] This process most likely represents the natural selection of resistant CSCs. Radiotherapy and most types of chemotherapy protocols exert their antineoplastic function by disrupting cancer cell DNA integrity and hence, it is possible that the oncogenic

resistance of CSCs results from increased expression of DNA integrity-maintenance systems.[36] Besides, the elevated expression of drug efflux pumps may promote oncogenic resistance against chemotherapeutic agents.[37,38] Logically, combination of therapeutic regimens targeting both tumor cells as well as CSCs could be a more effective strategy to improve long-term prognosis.[39]

There are two models about the origin, maintenance, progression, and heterogeneity of tumors.[12,13,39] These models include the stochastic or clonal evolution (CE) model, and the hierarchy or CSC model.[12,13,39] According to the stochastic model, malignancy constitutes a homogeneous population of cells which generates their heterogeneity in response to some unique combinations of endogenous and exogenous factors.[40] While endogenously, these would include gene dosage effects, transcriptional and translational control mechanisms, exogenously it includes cytokine concentrations, cell–cell interactions and niche environment (Figure-6.2).[40]

On the contrary, the hierarchy model predicts malignancy in a manner analogous to the normal tissue hierarchy wherein cancer/tissue stem cells are able to produce identical daughter stem cells with self-renewal capacity, and committed progenitor daughter cells with limited, although potentially still significant, potential to divide.[40,21] The limitation of the stochastic model is that it is based on

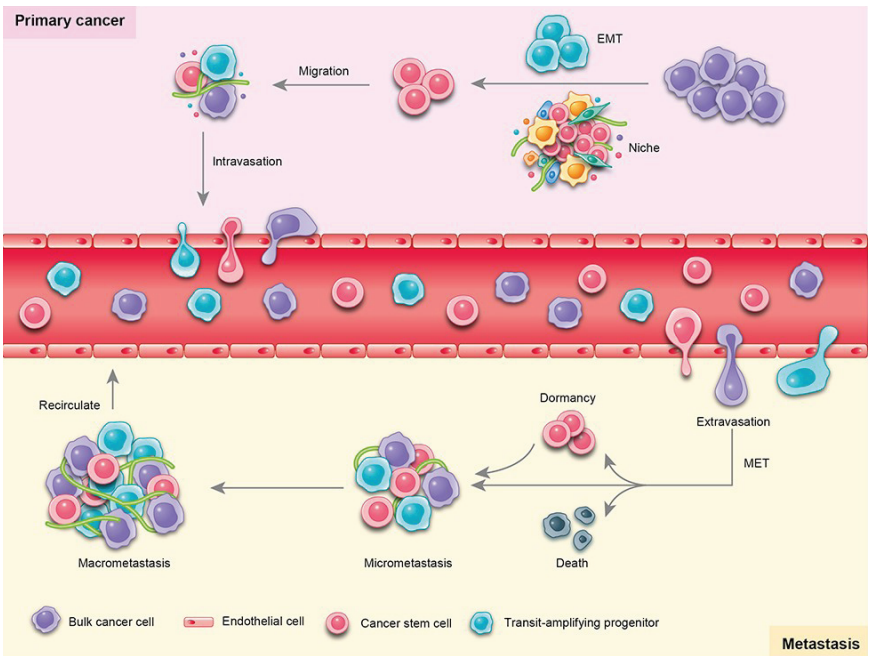

Figure-6.2.

the unpredictable capacity to understand whether stemness is found truly within each population, or whether the cells first undergo a process of de-differentiation to a more tissue-specific stem cell-like phenotype before re-acquiring stemness in the process (Figure-6.3).[1,24,40] There is convincing evidence in the published data that cancer cells, as well as stem cells, are subject to clonal evolution during which new clones continuously develop with new genetic, and potentially epigenetic, changes.[21,24,40] Environmental factors result in constantly adapting cancer cell populations with altered characteristics in terms of rate of proliferation, metastatic potential, and drug resistance.[42] These processes could be accommodated by the CSC model as well as the hierarchical and stochastic models of heterogeneity.[1,21,40] Nevertheless, a scientifically sound and globally accepted definition regarding the CSC model is still warranted and necessitates future research to clarify the origin and the development of CSCs.

6.1.2. Role of CSCs in Carcinogenesis

Cancer is a group of diseases involving abnormal cell growth with the potential to invade or spread to other parts of the body with a high mortality rate and without sustainable treatment.[43] Peter Nowell first described the concept of clonal cell cancer evolution in 1976.[44] It has been applied to try to understand tumor growth, aggressiveness, and resistance to treatment, migration, proliferation, and mediatization. Aberrant cell division initiates cancer that also gains the augmentation ability throughout the body by some complex biochemical and signalling processes. To elucidate cancer progression, the CSC model gives a comprehensive proposal about cancer development and progression.[43] Nevertheless, much about cancer is yet to be understood. We cannot solely focus on tumor heterogeneity but also that the tumor grows up in a complex ecosystem, with many cell types such as endothelial, hematopoietic, stromal and other types that can influence the tumor main driver pathway to survival.[11,45,46] Genetic diversity, tumor micro-environment and epigenetics are coming together and influence the concept of maintenance of stem cell state.[11] This revolutionary idea changed the historical concept that tumor cells harbor stem cells, and with these active pro-normal stem cells, are rare intra-organ cells with the capacity of self-renewal, which can generate all kind of different cells that make up an organ and lead to organogenesis.[3] On the other hand, CSCs are rare intra-tumoral cells, a sub-population of cancer cells with un-bridled renewal capacity; they generate phenotypically diverse tumor cell lineages thus leading to tumorigenesis. These cells are considered highly malignant, fundamental for the growth of neoplasia, for recurrence, for drug resistance and metastasis.[47] Many

signalling pathways have been shown to get dysregulated in CSCs.[48] The most well-studied and established pathways include Wnt/β-catenin, Hedgehog (Shh), Notch, and JAK/STAT3 pathways.

6.1.2.1 *Wnt/β-catenin*

The Wnt family of proteins transduce signals through the Frizzled (FZD) andLRP5/6 receptors to the Wnt/β-catenin and Wnt/STOP (stabilization of proteins) signalling cascades, also known as the canonical Wnt signalling cascade, and through the FZD and/or ROR1/ROR2/RYK receptors to the Wnt/PCP (planar cell polarity), Wnt/RTK (receptor tyrosine kinase) and Wnt/Ca^{2+} signalling cascades (also known as the non-canonical Wnt signalling cascades).[49–55] The canonical Wnt/β-catenin signalling cascade is involved in self-renewal of stem cells and proliferation or differentiation of progenitor cells,[56,57] whereas non-canonical Wnt signalling cascades are involved in the maintenance of stem cells, directional cell movement or inhibition of the canonical Wnt signalling cascade.[58–61] Both canonical and non-canonical Wnt signalling cascades are instrumental in the development and evolution of CSCs. Wnt activating mutations occur early during colon tumorigenesis whereas the progression of the disease is often accompanied by other genetic alterations, most commonly seen in *KRAS*, *BRAF*, *TP53,* and *SMAD4*.[34] Although these alterations are recurrently described as driver mutations in various cancers, it is still unknown which of these are required to maintain established tumors and whether interfering with Wnt signalling might be a viable therapeutic target in the background of additional drivers.

6.1.2.2 *Sonic Hegdehog/GLI (Shh/GLI)*

The Sonic Hegdehog/GLI (Shh/GLI) pathway has been extensively studied for its role in both developmental biology as well as cancer biology.[62] The Shh pathway is involved mainly in pattern formation during early embryonic development, while in latter stages its function in stem/progenitor cell proliferation becomes increasingly relevant.[62] During postnatal development and in adult tissues, Shh/GLI promotes cell homeostasis by actively regulating gene transcription, recapitulating the function observed during normal tissue growth. The fundamental importance of Shh/GLI in tumor growth and cancer evolution and insights into a novel mechanism of Shh action in cancer through autophagy modulation in cancer stem cells have been previously described.[62,63] In a recent study focussed on autophagy it was observed that the disruption of autophagy accelerates tumor progression in both cancer cells and the stroma that harbors tumorigenesis.[63]

6.1.2.3 *Notch*

CSCs self-renew and generate more differentiated cancer progenitor cells upon replication which possesses the capacity to dedifferentiate and acquire a stem-like phenotype by following a series of signalling pathways, molecular circuitries and epigenetic modifications.[64] Notch is one of the highly conserved pivotal signalling pathways that regulate cell proliferation, maintenance of stemness, cell fate specification, differentiation, and homeostasis of multicellular organism in general.[65] Notch also plays a key role in embryonic vasculature development.[66] Given its significance in various cellular processes, Notch signalling is one of the most activated pathways in cancer cells and their metastasis. Taking into consideration the critical participation of Notch pathway in both CSCs self-renewal and tumor angiogenesis, it has been extensively studied as a target to eliminate CSCs. The inhibition of Notch signalling has been reported as an emerging therapeutic strategy to cure cancer and eliminate CSCs.[67]

6.1.2.4 *JAK/STAT3 pathways*

STAT3 is an important regulator of cell proliferation and survival; it has a major role in the maintenance of stem cells and their differentiation and is involved in the cancerous potential of many cell types. STAT3 acts through regulation of oncogenes and tumor suppressor genes, as well as influencing tumor microenvironments.[68–72] It exerts various but sometimes contrasting functions in the normal as well as transformed cells. As STAT3 expression and activation are regulated by multiple signals and it has a role in many signalling pathways, STAT3 is considered as a flexible and adaptable regulator of cell function in different types of cells under different conditions and regulate gene expression either directly or indirectly through interaction with other transcription factors.[73]

Many novel small molecules are now being developed and tested in clinical trials to block the above-mentioned signalling pathways, which otherwise become dysregulated in CSCs. Some of these small molecules block the self-renewal and induction of apoptosis in CSCs.[45] Although not recognized as kinase inhibitors, they act by inhibiting the Wnt/β-catenin pathway, STAT3 pathway, NOTCH pathway and the Shh pathway. The STAT3 pathway is critical for the self-renewal and survival of CSCs in various neoplasms. Inhibition of STAT3 pathway inhibits cell proliferation *in vitro* and reduces tumor growth *in vivo*.[74,75] CSCs are also involved in tumor relapse and mediastization possibly due to mutations or epigenetic modifications in the daughter CSCs that exhibit more aggressive growth to become the driver of tumor formation thereafter.[76]

Genetic signatures in CSCs are thought to predict tumor recurrence and metastases, providing support for the concept that CSCs are the metastatic precursors.[77] For example, expression of the CSCs marker CD133 in glioblastoma and lung adenocarcinomas has been correlated with both the expression of cell proliferation marker Ki67 and poorer clinical outcomes.[78,79] CD133 expression has also been correlated with patient survival in high-grade oligodendroglial tumors,[80] rectal cancer,[81] gastric adenocarcinomas,[82] and non-small cell lung cancer.[83] Additionally, in patients with colorectal carcinoma, combined expression of CD133, CD44, and CD166 successfully identify the patients at low-, intermediate-, and high-risk of recurrence and metastasis.[84] Likewise, methylation of Wnt target-gene promoter is a strong predictor of colorectal cancer recurrence thus suggesting that CSC gene signatures, rather than reflecting CSC number, reflect the differentiation status of the malignant tissue and the risk for dissemination.[85] One of the key steps in the metastatic cascade is the migration of tumor cells to the distant tissues and organs away from the primary tumor that is facilitated by CSCs migration. The emigrational potential of cells is a physiological process in development, and tumor cells appear to capitalize on these physiologic mechanisms. Most adult tissues maintain some aspect of this emigrational potential primarily through epithelial to mesenchymal transition (EMT)-like process during wound healing, tissue regeneration, and organ fibrosis. It has been hypothesized that CSCs may also activate their migration through the process of EMT (Figure-6.2).

During the final stages of cell division, each daughter cell must lose contact with each other to generate independent progeny. The final step in this process occurs within a tube or bridge that is connecting the two daughter cells while a protein structure called the mid-body is essential for the process of separating the two cells. Cancer cells accumulate mid-body derivatives, which enhance the tumorigenicity of cancer cells.[86] Moreover, several microRNAs (miRNAs) also participate in the activation of CSC-like activities.[87]

6.2 DEFINITION AND ROLE OF THE TUMOR MICROENVIRONMENT

Although researchers now have a general understanding of most characteristics of cancer,[88] the characteristics promoting cancer formation remain less well-understood. After the 'ecological therapy' strategy was widely employed,[88] much effort has been devoted to determining how cellular and non-cellular components of the tumoral niche help the tumors to acquire these characters. These cellular and non-cellular components of the tumoral niche comprise the tumor microenvironment (TME).[88–90] It is well accepted that the TME[91,92] comprising of stromal fibroblasts,

inflammatory/immune cells, neuronal cells, the vasculature, and the extracellular matrix, etc. influences tumorigenesis, but the potential impact of TME on the origin of cancer cells has only come to light recently (Figure-6.1).[93] Strikingly, inflammation can alter the fate of the cells that are normally refractory to cellular transformation and convert them into stem-like cells capable of tumor initiation. Tumor microenvironment or niche remains a major factor that extrinsically influences the tumor heterogeneity (Figure-6.2). Tumor niche comprises of various cell types, i.e., stromal cells, immune cells, endothelial cells, and cancer cells per se, as well as connective tissue components, growth factors, and cytokines that play an essential role in CSC maintenance/enrichment, preservation of the phenotypic plasticity, immune-surveillance, differentiation/dedifferentiation, angiogenesis activation and invasion/metastasis.[94–96] CSCs reside in the tumor niche which not only provides the much needed physical support for CSCs but also fundamentally influences their functionality. A tumor can locally and metastatically colonize at suitable sites with a central role for CSCs in these processes.

6.2.1 Niche Components that Contribute to the Stemness of CSCs

Using a cell-lineage-tracing approach, Tammela *et al.* [96] found that lung tumors i.e., adenocarcinomas, populate tumor cells that produce a mix of two cell types: tumor cells and (non-tumor) support cells that constitute the tumor niche.[101] The niche cell population derived from the tumor cells expresses the enzyme porcupine that contributes to maturation of Wnt signalling protein in the endoplasmic reticulum which is secreted from the cell. The binding of Wnt protein with its receptor on a tumor cell activates the downstream signalling to drive tumor growth.[97] Lim *et al.*[98] investigated a different type of lung tumor and reported that the niche cells can also support tumor growth through the secretion of a protein that activates the Notch signalling pathway in the tumor cells.[98]

6.2.1.1 *Endothelial cells*

The vascular endothelium is a dynamic cellular "organ" that controls the passage of nutrients into the tissues, maintains the flow of blood, and regulates the trafficking of leukocytes.[99] In tumors, various factors i.e., hypoxia and chronic exposure to growth factor stimulation, result in endothelial dysfunction. Tumor-associated endothelial cells play a key role in the cancer process. On the one hand, they form tumor-associated (angiogenic) vascular structures through sprouting of the locally pre-existing blood vessels or *via* recruitment of bone marrow-derived endothelial progenitor cells, to provide nutritional support to the growing tumor (Figure-6.2).[100]

On the other hand, they are at the interface between circulating blood cells, tumor cells and the extracellular matrix, thereby playing a central role in various functions including controlling leukocyte recruitment, tumor cell behavior, and metastasis. Hypoxia is a critical parameter modulating the tumor microenvironment and endothelial/tumor cell interactions through stimulating tumor cells to produce pro-angiogenic factors and factors supporting the migratory activity of tumor cells, thus promoting metastasis.[100]

It was noticed a long time ago that tumor blood vessels were morphologically deviating from the normal structure.[101] Three-dimensional scanning electron microscopy of vascular plaster casts showed networks of tortuous endothelium that was missing the normal hierarchical arrangement of artery-arteriole capillary.[101] Poor tumor vessel stability is caused by defects in the pericytes, which are in lower abundance and are loosely attached compared to normal vessels, thus effecting the vascular stability and hence the blood flow.[101,102] This is evident from the observations that some tumor vessels remain un-perfused whereas the others are perfused but may have blood flowing in reverse directions. For example, vascular endothelial growth factor (VEGF) and some of the pro-inflammatory chemokines are also immune modulators, which increase angiogenesis and lead to immune suppression.[103] Amongst these pro-angiogenic factors, VEGF, one of the main angiogenic modulators, also plays a critical role in the control of immune tolerance. Albini *et al.* have discussed the regulation of angiogenesis by innate immune cells in the tumor microenvironment, specific features, and roles of major players: macrophages, neutrophils, myeloid-derived suppressor and dendritic cells, mast cells, γδT cells, innate lymphoid cells, and natural killer cells.[103] Anti-VEGF or anti-inflammatory drugs could balance out an immunosuppressive microenvironment into an immune-permissive one. Anti-VEGF, as well as anti-inflammatory drugs, could, therefore, represent partners for combinations with immune checkpoint inhibitors, enhancing the effects of immune therapy.[103]

6.2.1.2 *Extracellular matrix (ECM)*

The extracellular matrix (ECM) is composed of various proteins including collagen, proteoglycans, laminin, and fibronectin. Even amongst these ECM components, some subtypes that further specify their properties and functions.[104] The function of ECM may be best described in the context of embryonic development. The development of a mammalian embryo to a fully developed organism is a well-orchestrated and meticulously controlled process. It is tightly regulated in terms of the spatiotemporal composition, amount, and characteristics of the ECM. Several

studies have shown that mutated ECM components lead to birth defects or even embryonic lethality in some cases, which emphasizes its role in development.[105,106] The geometry, rigidity, and other physical properties of the ECM are sensed by the cells and ultimately direct their adherence, proliferation, migration and differentiation, thus culminating into the complex spatial and structural arrangements they form in the tissues. The ECM influences the migration track and the rate of migrating cells through its topography, composition, and physical properties. The alignment of the underlying ECM directs cell migration and proliferation. The traditional perspective of cancer has shifted to reflect the important role of the ECM in regulating cell proliferation, migration, and apoptosis. As the tumor cells proliferate, the surrounding ECM undergoes significant architectural changes in a dynamic interplay between the microenvironment and resident cells. These changes, including increased secretion of fibronectin and collagens I, III, and IV, show that tumor progression necessitates an uninterrupted and close interaction between the ECM and the tumor cells.[107] Increased deposition of ECM proteins promotes tumor progression by interfering with cell–cell adhesion, cell polarity, and ultimately amplifying growth factor signalling.[108]

6.2.1.3 *Cancer-associated fibroblasts (CAFs)*

Cancer-associated fibroblasts (CAFs) constitute a primary source of the fibrotic ECM. CAFs organize collagen fibrils which undergo biomechanical alterations to provide pathways for the invading tumor cells either under the guidance of CAFs or following their EMT.[109] The increased hyaluronan (HA) metabolism in the tumor microenvironment instructs the cancer cells to initiate and disseminate multiple functions. The key effects of HA reviewed here include its role in activating CAFs during the pre-malignant and malignant stroma and facilitate invasion by promoting motility of both CAFs and tumor cells, thus enabling their invasion to the nearby tissues. The circulating CAFs (cCAFs) also form heterotypic clusters with circulating tumor cells (CTC), which are considered to be precursors of metastatic colonies.[109]

Clinically, CAF-like fibroblast-induced stromal ECM changes precede the process of tumor formation and these early changes in ECM have prognostic significance that permits risk stratification. For example, high mammographic density is a strong risk factor in breast cancer.[110–112] The important clinical features of this condition, which precede the subsequent detectable tumor formation, include adipocyte loss and high ECM production. For example, this condition has been linked to the loss of expression of the mesenchymal differentiation regulator

CD36 in the stromal fibroblasts, which phenocopies the clinical features of high mammographic density breast tissue.[113–115]

A number of studies using mouse models also predict that elevated extracellular matrix component HA production, primarily by fibroblasts, pre-disposes epithelial cells to tumor initiation. Examples include evidence that an HA-rich stroma precedes increased mammary tumor formation in transgenic mice expressing both MMTV-driven HAS2 and a c-neu proto-oncogene.[116] CAFs play a significant role in tumor dissemination by inducing an invasive phenotype in the tumor cells via promoting motile phenotypes and remodeling in the ECM. Invasion is achieved in part by CAF-driven EMT and consequent cell migration that is driven by factors such as TGF-β, HGF-1, and CXCL12/SDF-1α.[116] Paladin-expressing CAF create "tunnels" in the ECM, which cancer cells migrate through.[110] Under CAF guidance, tumor cells also migrate and invade as groups in the absence of apparent EMT. This collective migration and invasion is driven by heterotypic E-cadherin/N-cadherin interactions between tumor cells and CAFs that result in a mechanically active adhesion.[111]

6.2.1.4 *Tumor-associated macrophages*

The tumor microenvironment is a complex assembly of a genetically heterogeneous population of cancer cells supported in the sustenance of their biological activity by different cell types that constitute the local environment.[116] Tumor-associated macrophages (TAMs) are one of the most abundant immune cells in the tumor microenvironment of solid tumors.[117,118] TAMs are one of the most abundant immune cells in the microenvironment of solid tumors and their presence correlates well with reduced survival in most cancers. They are present during all stages of tumor progression and stimulate angiogenesis, tumor cell invasion, and intravasation at the primary site.[116] At the metastatic site, macrophages and monocytes prepare for the arrival of disseminated run-away tumor cells from their primary location and support extravasation and survival by inhibiting their immune-mediated clearance or by directly engaging with tumor cells to activate pro-survival signalling pathways. Moreover, macrophages also promote the growth of the disseminated tumor cells at the metastatic site by organizing and supporting the formation of a supportive metastatic niche. Various researchers have independently reported a strong correlation between the density of macrophages and poor survival in carcinomas of pancreas and breast, lung, cervix, the bladder, and Hodgkin's lymphoma.[119–123] The expression of colony-stimulating factor-1 (CSF-1), the major lineage regulator for macrophages, or its receptor CSF-1R correlates with poor survival in liver, breast

and pancreatic cancer,[124,125] respectively. A macrophage transcriptional signature in patients with breast cancer has been reported as a predictor of poor prognosis and reduced survival in the patients.[126,127] They are also involved in the recurrence and mediatization for their several pro-tumorigenic functions that have important roles in cancer development and progression, such as the ability to express and secrete cytokines and induce tumor angiogenesis.[128]

While elucidating the underlying molecular mechanism, it has been reported that the release of CSF by the tumor cells induces EGF expression in TAMs.[120] This autocrine loop leads to the co-migration of tumor cells and TAMs towards the blood vessels where TAMs produce VEGF-A to promote increased vessel permeability. Additionally, TAMs-derived molecules such as secreted protein acidic and rich in cysteine (SPARC; a multifaceted matricellular protein), C-C motif ligand 18 (CCL18), and proteases promote increased tumor cell invasion and migration. At the metastatic site, tumor cell-derived CCL2 recruits inflammatory monocytes to the metastatic site, where they differentiate into metastasis-associated macrophages (MAPs) that produce VEGF-A and cathepsin S to promote cancer cell extravasation. MAPs promote survival at the metastatic site through the expression of integrin $\alpha 4$ that engages VCAM1 on the tumor cells at the metastatic site, which increases tumor cell survival through PI3K/Akt signalling.[120] MAPs also bind with fibrin complexes on the tumor cell-associated platelets, which increase tumor cell survival in the initial phase of metastatic colonization. MAPs promote metastatic niche formation and release granulin that activates HSTC to produce ECM molecules, such as collagen and periostin, which enhances the colony formation abilities of cancer cells in the metastatic niche.[120]

6.3 CSCs AND MICROENVIRONMENT INTERACTION

The modulation of CSCs activities by the tumor microenvironment is still poorly understood. CSCs and tumor microenvironment mutually interact in a unique manner depending on the tumor microenvironment cells (endothelial, epithelial, extracellular matrix (EMT), stromal and macrophage) which respond to signals from the CSC or vice versa.

6.3.1 The Endothelial Compartment

The endothelial cell compartment is considered a key player in supporting CSC-phenotype.[129] It is well established now that endothelial cells maintain stem-like cells and their activities in tumors, exerting their functions by secreting growth factors, such as epidermal growth factor (EGF), that induces EMT and stem cell

features in tumors, as previously described for human head and neck squamous cell carcinoma (SCC). Endothelial cells also promote cancer cell conversion towards the endothelial phenotype. Moreover, it has been reported that EGF inhibition in the endothelial cells rendered the *in vivo* xenograft-derived tumors less invasive and contained a lower proportion of $ALDH^{+}CD44^{+}$ CSCs.[129] Different conditions, i.e., hypoxia and neo-angiogenesis, are the leading causes that confer on cancer cells the ability to behave like endothelial cells as a consequence to promote their adoption of CSC-phenotype. About these conditions:

(a) Hypoxia promotes aerobic glycolysis in the tumor cells in order to survive in the oxygen-free environment, thus contributing to tumor growth and metastasis. Hypoxia-inducible factor (HIF) is the main tissue controller of oxygen homeostasis.[130] Besides hypoxia, changes in pH can also regulate stem cell behavior by modulating their metabolic status and promoting metabolic re-configuration of cancer cells towards glycolysis, induction of the EMT phenotype (including C-X-C-chemokine receptor 4 (CXCR4), Snail and Twist gene expression), increasing in the number and renewal potential of CSCs, as well as induction of pluripotency-associated transcription factors i.e., Oct-3/4, Nanog and Sox-2.[118] This scenario indicates that "stemness" is more a cellular state than a cancer cell characteristic, modulated by the microenvironment.
(b) Neo-angiogenesis ensures the much-needed nutrient and oxygen supply that is essential for cancer cell survival, growth, and dissemination.[131,132] Tumor cells develop their vasculature through different mechanisms, including formation of new vessels from pre-existing ones, simulation of the vasculature through vasculogenic mimicry and recruitment of endothelial progenitor cells. The vasculogenic mimicry (VM) was first reported in melanomas and referred to *de novo* formation of tubular structures that were perfused by plasma and red blood cells.[133] With increasing knowledge of CSCs-phenotypes and functions, there is mounting evidence which supports the notion that CSCs are involved in VM as promoters of tumor vascularization.[134,135] This could be justified by the CSCs' lineage plasticity in generating tumor.

6.3.2 The Extracellular Matrix

The EMT is a potent driving factor in tumor initiation and progression.[136,137] EMT and CSCs have an inherent relation[138] that has been implied in the metastasis of human tumors.[139,140] This interplay contributes to the mechanism through which CSCs reside in a tissue dormant for years, and later primes tumor recurrence or metastasis in cancer patients (Figure-6.3). There is a mechanism which induces

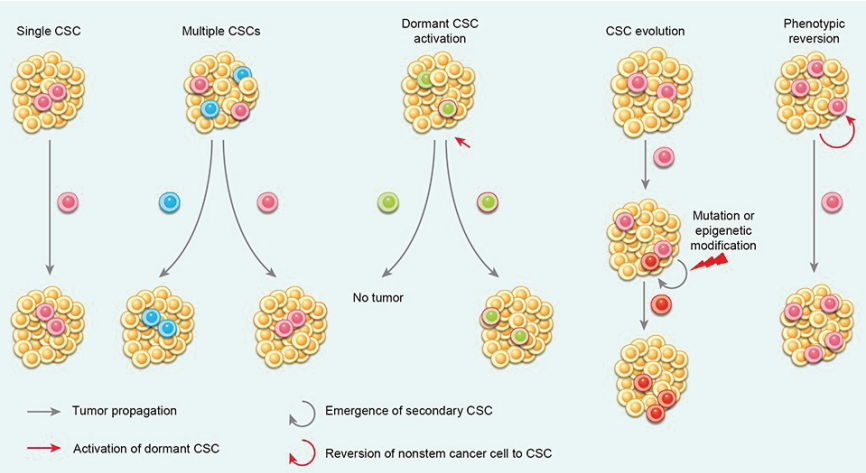

Figure-6.3.

the cancer cells to not only lose their cell–cell adhesions and exhibit elevated motility and invasion but also to gain increased resistance to apoptosis, elevated endurance to chemotherapeutic intervention and develop stem-cell like properties through EMT.[107]

During the progression of tumors, the ECM becomes more disorganized due to the influence of local modulators, thus tipping the ECM as a master regulator in tumor progression through providing sustained proliferative signals, evading growth suppressors, resisting cell death, enabling replicative immortality, inducing angiogenesis and promoting invasion and metastasis. The increased expression and activity of matrix metalloproteinases (MMPs) and collagen cross-linkers are also preponderant for the modulation of ECM within the TME and are generally responsible factors in the poor prognosis.[140,141] Indeed, MMPs are major players in cell invasion, since they are responsible for proteolysis and detachment of tumor cells from the ECM, resulting in CSCs formation and metastasis.[142]

6.3.3 Cancer-Associated Fibroblasts

CAFs in the stroma are influential in reverting differentiated cells towards a de-differentiated phenotype.[143] CAFs support multiple aspects of cancer progression including tumor initiation, invasion, and metastasis. The first evidence in this regard was published by Nai *et al.* who reported that CSCs are one of the key

sources of CAFs in the tumor niche.[144] This has been proposed as one of the primary mechanisms in generating CSCs.[15] Recent studies indicate that CAFs have substantial clinical implications in terms of disease staging and cancer recurrence. However, CAFs have not been fully characterized due to several limitations. The first limitation is the uncertainty regarding the origin of CAFs.[144] CAFs have been reported to originate from epithelial cells, mesenchymal stem cells, adipocytes, resident fibroblasts, and bone marrow stem cells.[119] The divergent sources of CAFs account for their broad range of characteristics and molecular markers. Secondly, it is difficult to isolate and maintain CAFs which significantly hampers their characterization. Notably, the microenvironment that supports the growth of CAFs is similar to the microenvironment that supports the viability of CSCs. Recent studies suggest that several types of stromal cells in the niche play a pivotal role in maintenance of the very small population of CSCs which are responsible for cancer recurrence and chemotherapeutic drug resistance.[144,145] However, it remains unclear whether CSCs directly support tumor maintenance and survival by generating CAFs.

First described by Otto Warburg in 1956,[146] metabolic reprogramming in cancer cells involved a shift in energy metabolism away from an oxidative cycle to a glycolytic one — even under aerobic conditions — subsequently termed the "Warburg effect" or "aerobic glycolysis". In this respect, CAFs exert a metabolic reprogramming of cancer cells by inducing a reverse Warburg phenotype.[146] Spreading of tumor from local to distant sites necessitates a supportive and accommodating environment for the disseminating cancer cells. The so-called "metastatic niche" may also be a native stem cell niche of the distant organ, enhancing stem cell properties while repressing differentiation.[146] Overall, the role of CAFs and CSCs regarding the metastatic progression of the tumor has not been fully demarcated. In this regard, CAFs contribute to the metabolic reprogramming of cancer cells by inducing a reverse Warburg phenotype.[144–147]

6.3.4 Tumor-Associated Macrophages (TAMs)

Like normal stem cells, CSCs exist in a cellular niche comprised of numerous cell types including TAMs which provide a unique microenvironment to protect and promote CSC functions.[107] TAMs provide pivotal signals to promote CSCs survival, self-renewal, maintenance, and migratory ability, and in turn, CSCs deliver tumor-promoting cues to TAMs that further enhance tumorigenesis. Studies during the last decade have primarily focused on understanding the molecular mediators of

CSCs and TAMs, and recent advances have begun to elucidate the complex cross-talk that occurs between the two cell types.[107] Another area of intense investigation has been to understand the role of inflammatory cells in the CSCs niche. The tumor microenvironment is characterized by chronic inflammation which favors tumor formation by stimulating cell proliferation, activating CSCs, and promoting metastasis.[147,148] In this regard, TAMs lead the tumor inflammatory response TAMs.[149,150] A correlation between high numbers of TAMs and rapid disease progression has been established with poor patient outcome[39,151]; however, this paradoxical phenotype has been explained only recently. While TAMs in the pre-invasive niche contribute to oncogenic transformation and survival, a growing body of evidence suggests that they are critical for the self-renewal and maintenance of CSCs in the established tumors. STAT3 and NFκB are the key regulators of these processes. Once infiltrated into tumors, TAMs contribute to chronic inflammation by secreting inflammatory cytokines i.e., IL-1β, IL-6, and IL-8 (CXCL8).[119,152] In addition to mediating CSCs' self-renewal and expansion, TAMs are also responsible for the maintenance of the CSCs niche. A recent study by Lu *et al.* demonstrated juxtacrine signaling by TAMs and tumor-associated monocytes with mouse mammary CSCs to support the maintenance of a stem-like state.[153]

While numerous studies have demonstrated that TAMs directly regulate CSCs' self-renewal and maintenance, there is a growing body of research that suggests that CSCs, in turn, recruit macrophages to solid tumors and enhance a pro-tumor phenotype in the TAMs. Zhou *et al.* have reported that the ECM protein periostin is preferentially expressed on $CD133^{+}CD15^{+}$ glioma stem cells and recruits macrophages through integrin $\alpha v\beta 3$ from the peripheral blood to the brain. Deletion of periostin in glioma stem cells decreases M2 TAM density, reduces tumor growth, and consequently increases survival of the glioblastoma xenografts.[154–156]

6.4 FUTURE PERSPECTIVES

Chemotherapy and radiotherapy, either alone or combined with surgery,[157] mainly target the fast-cycling cells. The role of CSCs in tumor formation and progression is being highlighted, however it is still difficult to synthesize CSCs-targeting drugs due to lack of their specific surface markers that could be exploited as targets. Since the discovery of several important mutations that contribute to carcinogenesis, i.e., EGF receptor, p53, and c-Myc, they have been extensively studied as targets for the development of more selective drugs for cancer therapy. Despite the effectiveness of these drugs, multidrug resistance (MDR) is on the rise which often results in tumor relapse.[158] To be therapeutically effective, an anti-cancer agent should be uniformly distributed

throughout the tumor circulation, across the vessel wall, and pass through the ECM. On the other hand, tumors create multiple obstacles to drug transport mechanisms, hence, the requirements for effective drug delivery may vary considerably.[158] Expression and proteomic profiling of the individual cell types constituting the cancer microenvironment represent important advances.[158,159] Besides targeting the surviving CSCs, the contemporary oncology research is now mainly focused on innovative therapy targeting both CSCs, TME as well as tumor microenvironment.[158–160]

6.4.1 Targeting CSCs

Genetic variability and genomic instability of the CSCs are the primary hurdles in the development of CSC-specific drugs. Currently, efforts are underway in targeting CSCs' surface markers. One of the most established and commonly used CSC biomarkers is CD44 that has been targeted using anti-CD44 specific antibody to successfully eradicate acute myeloid leukemia.[161,163] Similarly, CD133, a transmembrane glycoprotein well-known in several tumors such as glioblastoma, hepatocellular and colon cancers, has been targeted using anti-CD133 antibody conjugated with a potent cytotoxic drug, monomethyl auristatin. This antibody-drug conjugate was efficiently internalized, co-localized with the lysosome and showed high effectiveness.[163]

Besides antibody-based drug targeting, nanoparticles are being used as an interesting strategy to target CSCs with minimal damage to surrounding normal cells. In this regard, construction of glucose-coated gold nanoparticles (Glu-GNPs) has been shown to facilitate the entry of GNP into leukemia stem cells overexpressing CD44 (TH1-P) with promise.[164] Similarly, mangostin-encapsulated Poly (lactic-co-glycolic acid) nanoparticles (Mang-NPs) have successfully downregulated the known stemness genes c-Myc, Nanog and Oct4, besides abrogation of two CSC-specific markers, i.e., CD24 and CD133, and blocking Shh pathway.[165] Another example of nanoparticle therapy is represented by salinomycin and paclitaxel that are also used to eradicate breast cancer cells including CD44 breast CSCs.[129] Another unraveled aspect is the targeting of CSCs' mitochondrial biogenesis due to their strict dependence on mitochondrial activity. For this reason, specific antibiotics that inhibit mitochondrial biogenesis were studied.[166] An example that supported this new therapeutic approach is doxycycline with positive results in cancer patients,[167] and by metformin which seems to eliminate CSCs. The combination of these two drugs seems to enhance anti- tumor activity. In summary, as the CSCs are more resistant to conventional cancer therapies than non-CSCs their elimination is crucial in treating malignant diseases.[168]

6.4.2 Targeting Tumor Microenvironment

The TMEs are instrumental in mediating the resistance of CSCs to anti-cancer therapies. CSCs in glioblastoma, which are inherently radio-resistant, are "protected" from conventional therapies by factors within the vascular niche, thus enabling CSCs to cause tumor relapse. Hence, treatments that disrupt the aberrant vascular environment may be active against glioblastoma. Various clinical trials of the anti-angiogenic drugs such as bevacizumab[169] and cediranib (AZD2171) have achieved encouraging results in patients with glioblastoma.[170] Moreover, the CSCs associated with the stromal cells are near blood vessels forming a niche characterized by severe hypoxia and increased angiogenesis.[79,83] These aspects of the tumor microenvironment have been explored as possible pharmaceutical targets to eliminate CSCs.[171] The pathways involved in angiogenesis provide a crucial target of cancer therapy. A plethora of anti-angiogenic agents have been developed and tested in preclinical experiments.[163] For example, bevacizumab, a humanized monoclonal antibody that sequesters vascular endothelial growth factor (VEGF) to impair VEGF signaling was approved for the treatment of metastatic colorectal cancer and other metastatic cancers, including non-squamous NSCLC and cervical cancer.[164,165] Another strategy to inhibit tumor angiogenesis is the use of tyrosine kinase inhibitors, such as sorafenib, an inhibitor of VEGFR-1, -2, -3 and PDGFR-b, and sunitinib that blocks VEGFR-2 and PDGFR phosphorylation. The former was used for the treatment of metastatic renal cell carcinoma and un-resectable hepatocellular carcinoma, and the second treatment for gastrointestinal tumor and metastatic renal cell carcinoma.[171] Although anti-angiogenic therapy may potentially have clinical implications, the increase of oral somministration (OS) is insufficient. This is probably due to acquired resistance, the increment of tumor hypoxia and the diminished delivery of chemotherapeutic agents.[172–174]

Concerning the tumor microenvironment ECM, several drugs targeting matrix metallopeptidases (MMPs) have been developed. For example, cyclinide (also known as CMT-3 and COL-3) is an MMP inhibitor that went through several clinical trials for advanced carcinomas (Clinical trials NCT00004147, NCT00003721, NCT00001683, and NCT00020683). The new frontier of cancer treatment is aimed at strengthening the immune system's defense against cancer cells. Targeting T-regulatory lymphocytes (Tregs) directly in the TME has been proposed as another method to re-establish the anti-tumoral immune response. Tregs cells in tumors are immune-enriched for the cell surface markers CTLA-4 and OX-40, to deplete Tregs from the TME. By directly injecting mouse tumors with anti-CTLA-4 and anti-OX-40 antibodies to deplete Tregs, along with the TLR9-activating agonist CpG to trigger the innate immune response, the authors

showed the establishment of a systemic antitumor immune response capable of eradicating disseminated disease in mice. Furthermore, this treatment modality was effective against established lymphoma in the central nervous system, which is traditionally considered to be a sanctuary for tumor cells in the face of systemic therapies. This study suggested that antibody therapy could be used to target tumor infiltrating lymphocytes (TILs) locally, thereby inducing an effective systemic immune response. This pioneering therapy has not been tested sufficiently in the clinical setting. More importantly, Tregs produced immunoregulatory factors which might include TGF-β, IL-10, and IL-35. Of these, TGF-β seems a particularly desirable target due to its roles in promoting metastasis and tumor stroma formation, besides its potent inhibitory effects.

Several molecules based on the inhibition of immune checkpoints have been approved by the FDA since 2011. The most promising of these therapies have been represented by the antibodies targeting the cytotoxic T lymphocyte-associated protein 4 (CTLA-4) or the programmed cell death 1 (PD-1) pathway, administered as single or in combination therapy. To induce antitumor responses, T-cells are initially activated in the lymph node in two subsequent steps which are: the engagement of T-cell receptor (TCR) with a tumor antigen MHC complex on antigen-presenting cells (APCs) and the binding of CD28 to the costimulatory molecule B7. Following T-cell activation, CTLA-4 translocates from the intracellular compartment to the cells' surface to compete with the costimulatory molecules, causing the inhibition of T-cell proliferation. The blockade of this essential immune checkpoint with monoclonal antibodies enables T-cells to active, expand and reach the tumor burden, where they can find the cognate antigen presented by cancer cells. These mechanisms are generally implemented to impede the overstimulation of the immune system. Nevertheless, in the context of cancer, they become detrimental to cancer cell elimination. Hence, an immune checkpoint blockade may be exploited to potentiate an anti-tumor immune response.[175–178]

In summary, future therapies need to be optimized for improving their effects inside the tumor microenvironment, efficiently accessing CSCs, with the result to reduce side effects in patients. Gaining a better understanding of the relationship between TME and CSCs at each stage of tumor development and progression, we may discover new approaches to interfere with the TME-CSC cross talk.

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