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ARTICLE

Signaling Pathways Associated with Cancer Stem Cells Play a Significant Role in Immunotherapy Resistance

Yajuan Zhu[1#] Yuwen Zhou[1#] Yao Xie[2] Pan Song[3] Xuelei Ma[1*]

1. Department of Biotherapy and Cancer Center, State Key Laboratory of Biotherapy, West China Hospital, Sichuan University, Chengdu, Sichuan 610041, China
2. Department of Dermatovenerology, West China Hospital, Sichuan University, Chengdu, Sichuan, 610041, China
3. Department of Urology, Institute of Urology, West China Hospital, Sichuan University, Chengdu, 610041, Sichuan, China

#: These authors contributed equally.

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ABSTRACT

Cancer stem cells (CSCs) are a subpopulation of tumor cells with properties of self-renewal, pluripotency, plasticity, and differentiation, and are associated with various aberrantly stimulated signaling pathways. They are responsible for tumor recurrence, distant metastasis, and drug resistance, thus inducing poor prognosis. Immunotherapy has achieved encouraging results. However, the resistance associated with its clinical application is a persistent problem in clinical and scientific researches. Increasing evidence shows that signaling pathways associated with CSCs mediate immunotherapy resistance. This review highlights the link between them, and focuses on the underlying mechanism so as to provide potential strategies and approaches for the development of new targets against the immune resistance challenge.

1. Introduction

Cancer is considered a heterogeneous disease due to the subsets of cells with distinct phenotypes and functions [1-3]. A small group of cancer cells with stem-like abilities are found in almost all untreated human malignancies. These cells are termed "cancer stem cells" (CSCs) based on their biological similarities with normal stem cells found in the same tissue [1,4]. CSCs were first identified in acute myeloid leukemia (AML), and later were also found in numerous solid tumors, such as breast, thyroid, prostate, brain, lung, colon, melanoma, liver, and stomach cancers [5-15]. CSCs have characteristics of self - renewal, differentiation, quiescence, and potential function to build their heterogeneity and induce cancer growth [16,17].

With the improved detection and treatment of cancer, some primary tumors can be completely cured after surgery. However, patients with advanced, metastatic, and/or recurrent tumors are in need of standard therapies, such as chemotherapy, radiotherapy, and molecular targeted therapy. Mounting studies indicate that these ther-

**Corresponding Author:*
Xuelei Ma,
Department of Biotherapy and Cancer Center, State Key Laboratory of Biotherapy, West China Hospital, Sichuan University, Chengdu, Sichuan, 610041, China;
Email: drmaxuelei@gmail.com

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apies target the relatively differentiated and proliferating cancer cells. While these CSCs are mostly dormant and have been demonstrated to contribute to many clinical therapies, subsequently leading to tumor relapse, metastasis recurrence, and poor prognosis [18,19]. The underlying mechanisms of resistance to therapies by CSCs are explained by the overexpression of anti-apoptotic proteins, augmented DNA-repair capacity, aberrantly stimulated signaling pathways, elevated anti-oxidant proteins, activated epithelial to mesenchymal transition (EMT) program, and adapted metabolism under hypoxia conditions. In addition, the capability of CSCs to evade the immune system make it more difficult to overcome the therapy resistance [4, 20-24].

Recently, immunotherapy has emerged as a promising treatment for cancer patients and regained global attention [25]. Immune checkpoint inhibitors (ICIs) have been approved for the treatment of various aggressive cancers [26-30]. Despite the unprecedented favorable outcome observed with immunotherapies, the response rates remain low, ranging from 15-40% varying from cancer types [31-33]. A majority of the patients do not benefit from the ICIs, mainly because tumors can escape immunosurveillance and elimination by avoiding the detection of the immune system or suppressing immune responses. Like tumor cells, CSCs also have developed diverse strategies to escape the immune protection , including loss of tumor antigen expression, reduce of immune recognition via genetic or nongenetic alterations , enhancement of tolerance to immune cytotoxicity, and promotion of a immunosuppressive microenvironment [34]. Furthermore, previous studies have demonstrated that CSCs are associated with immunotherapy resistance in various cancer types [35,36]. However, the related signaling pathways remain poorly understood. Herein, we summarized the signaling pathways of associated with CSCs with regard to their mechanistic regulation networks and their roles in immunotherapy resistance.

2. The Related Signaling Pathways of CSCs Implicated in Immunotherapy Resistance

Several cellular signaling pathways, such as Notch, Hedgehog (Hh), Transforming growth factor-beta (TGF-β), WNT/β-catenin, EGFR, NF-κB, HIF-1α, MAPK, PTEN/PI3K, and JAK/STAT [37-39], have been described to play a vital role in the induction and maintenance of stemness in CSCs. Among these, TGF-β, WNT/β-catenin, Hippo, HIF-1α, and Hh pathways are associated with immunotherapy resistance (Figure 1).

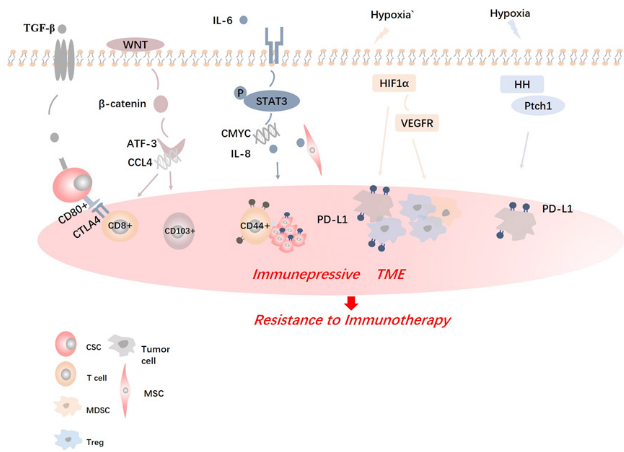

Figure 1. Signaling Pathways of Cancer Stem Cells in Resistance to Immunotherapy

Note: Collectively, TGF-β, WNT/β-catenin, Hippo, HIF-1α, and Hh pathways are associated with immunotherapy resistance.

2.1 TGF-β-responding CSCs Via CD80 Activation are Responsible for Immunotherapy Resistance

TGF-β signaling plays a dominant role in mediating EMT in CSCs [40-43]. It becomes phosphorylated upon binding to the TGF-β receptor. Subsequently, SMAD2/SMAD3 is activated and composes into a complex with SMAD4. This complex translocates to the nucleus as a transcription factor, leading to the expression of target genes implicated in stemness and invasion property of cancer cells [44]. The TGF-β signal can also remodel the tumor microenvironment (TME) by inhibiting T cell differentiation and activity, thus resulting in poor prognosis [45,46].

Two studies have identified the TGF-β signaling is a determining factor of T cell rejection and poor response to ICIs [45,47]. Furthermore, in mouse models, promising preclinical evidence showed that the combination of TGF-β inhibitors and ICIs can facilitate T cell infiltration into the tumor center, extensively promoting anti-tumor immunity [48]. A similar model was designed for squamous cell carcinoma. It revealed that the CSCs equipped with the surface CD80 not only have the power to resist immunotherapy by stimulating direct dampening of cytotoxic T lymphocyte (CTL) activity but also accelerate tumor growth. In contrast, the loss of CD80 can restore CTL proliferation to a greater extent than ICIs, making CSCs vulnerable and diminishing the immune-related tumor relapse. This is because CD80 is only activated in TGF-β-responding CSCs, and its expression could be influenced by TGF-β signaling. The single-cell RNA sequencing (RNA-seq) of TGF-β-responding CSCs shows that they are superior at resisting CTL responses and constitute the root of tumor recurrence [49]. The role of TGF-β responding CSCs in assisting cancer

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immune escapes has also been demonstrated in bladder and colon cancer after conventional PD-L1 immunotherapy [47,48,50]. These results indicate that the combination of TGF-β inhibitors and ICIs might be effective in targeting the CSCs to overcome immunotherapy resistance.

2.2 Tumor-intrinsic Active WNT/β-catenin Signaling Results in T-cell Exclusion

WNT signaling plays a substantial role in keeping CSCs in a undifferentiated and self - renewal state; therefore, the activated WNT signaling is associated with cancer occurrence [16]. In colon cancer, WNT/β - catenin can be activated by protein - 4 (AP4), thereby increasing the number of CSCs and modulating their homeostasis [51]. In lung cancer, β - catenin signaling contributes to the maintenance of CSC phenotype, and stemness [52,53]. The activation of WNT signaling via the hepatocyte growth factor (HGF) promotes the transition of cancer cells into CSCs [54, 55].

The role of WNT signaling in immune escape has recently been discovered. The molecular analysis of human metastatic melanoma samples shows that the activated WNT signaling is correlated with T-cell exclusion [56]. Similarly, β-catenin appears to inhibit CTL activation [57]. Mechanistically, previous reports have indicated that CCL4 can induce T-cell infiltration [58,59]. Meanwhile, the WNT/β-catenin signaling suppressed the CCL4 gene expression via ATF3-dependent transcriptional expression, resulting in immune evasion [60]. In a melanoma mouse model with constitutively high β-catenin activity, the failure of T-cell initiation against tumor antigens is mainly attributed to the decreased infiltration of $CD103^+$ dendritic cells [61]. The restoration of dendritic cell recruitment into the tumor via injection can enhance anti-PD-L1/CTLA4 therapy. Moreover, the upregulation of IL-12 by β-catenin signaling can also modulate and impair the dendritic cell function [60]. Similarly, in colon cancer, the inhibition of β-catenin activity of increases CD8+ T cells and $CD103^+$ levels in tumor area. β-catenin signal may mediate immunotherapy resistance of colon cancer [62]. Collectively, the manipulation of Wnt/β-catenin signaling pathway combined with ICIs might represent a novel therapy for cancer, further studies investigating the interaction between tumor intrinsic WNT/β-catenin signaling and immunotherapy are expected.

2.3 STAT3 Signaling-mediated IL-8 Derived from Gastric Cancer Mesenchymal Stem Cells (GCMSCs) Increases PD-L1 Expression to Resist CD8+T Cell Cytotoxicity

Signal transducers and activators of transcription (STAT) factors and the receptor-associated JAK kinases, are the downstream effectors of both extrinsic and intrinsic signals [63,64]. Tyrosine-phosphorylated (YP)-STATs compose into an active dimer and control target genes expression in the nucleus [65]. Excessive activation of STAT3 was reported to play many roles in cancer cells, including the promotion of cancer cell survival, proliferation and tumor angiogenesis, down-modulation of anti-tumor immune responses, enhancement of tumor recurrence and metastasis by inducing EMT, and increasing the number of CSCs. Finally, STAT3 activity can induce CSC features in solid tumors [66-68]. Therefore, STAT3 is regarded as an oncogene and a target for anti-cancer treatments

The activation of STAT3 signal is involved in the modulation of PD-L1 expression [69,70]. IL-8 derived from the GCMSCs induces PD-L1 expression in gastric cancer (GC) cells [71]. In contrast, IL-8 inhibition weakened the protective effects of GCMSCs on GC cells against CD8+ T cell cytotoxicity. The inhibition of IL-8 derived from GCMSCs may suggest a potential strategy to sensitize PD-L1 antibody therapy in GC. In addition, the combinative blockade of multiple cytokines with ICIs in the future may have the potential to overcome the immunotherapy resistance induced by the high expression of PD-L1. Furthermore, CD44+ cells are also found to have an EMT property and are less immunogenic. CD44+ cells were observed to have a high inducible expression of PD-L1 and associated with the phosphorylation of STAT3. Therefore, CD44+ cells are characterized with drug immunotherapy resistance. Inhibition of STAT3 could decrease the expression of PD-L1 on CD44+ cells and selectively enhance the immune responses [72]. Interestingly, subsets of CSCs with an EMT phenotype are low immunogenicity due to elevated PD-L1 expression, driven by the constitutive phosphorylation of STAT3 [72,73]. Considering these evidences, STAT3 expression may decrease the therapeutic efficacy of ICIs, and the combination of immunotherapy with STAT3 inhibitors may be a promising strategy to effectively suppress malignant tumors. Further investigation of the specific function of STAT3-regulated PD-L1 expression on the surface of cancer cell and CD44+ cells will be required to fully understand the intriguing link between immune escape and signaling pathways associated with CSCs.

2.4 HIF Signaling Drives the Expression of PD-L1 and Induces the Immunosuppressive Tumor Microenvironment

Hypoxia is one of the most common features of the TME driving the aggressiveness of tumors [74]. Hypoxic remodeling is mostly regulated by hypoxia-inducible

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factors (HIFs) [75]. Three HIF-α family proteins are described in humans: HIF-1α, -2α, and -3α. Among these, HIF-1α expression up-regulation is well understood and found in many tumors, such as prostate cancer, breast cancer, colon cancer, and hemangioblastoma [76]. Activated HIF pathway can initiate genes associated with vasculogenesis, drug resistance, glucose metabolism, immune escape, and metastasis [75,77], resulting in the reduced overall survival of patients in various cancers [75]. Consistently, the inhibition of HIF-1α can reduce the CSC numbers and suppress drug resistance in various cancer types, such as glioma, hematological cancers, and breast cancer [78-80].

EMT is widely known to induce stem-like properties in cancer cells [81]. The HIF-1 signaling pathway is crucial for the modulation and maintenance of CSCs and the EMT phenotype [82]. In thyroid and prostate cancer, HIF-1α-mediated EMT can increase stem-like cells [83,84]. In tumor tissues, the hypoxic or necrotic area of is considered a niche of CSCs. HIF-1 regulates CSC-signature genes, such as *CD44, CD133, OCT4, SOX-2, NANOG*, and *MYC*, that are increased in the CSCs of this niche. In pancreatic cancer, gastric cancer, and neuroblastoma, the discontinuous hypoxia upregulates HIF-1α, enhancing stem-like characteristics of theses cancer cells [85-87]. HIF-1 also plays an important role in promoting mammary tumor growth and metastasis by direct regulation of CSCs [87]. These studies highlight the vital role of HIF-1 in accelerating tumorigenesis, metastasis, and drug resistance because of CSC sustenance.

HIF-1α has been demonstrated to regulate PD-L1 expression on both tumor cells and myeloid-derived suppressor cells (MDSCs), leading to immune evasion [88]. HIF-1α also increases the secretion of vascular endothelial growth factor A (VEGEFA), thus promoting the recruitment of MDSCs and Tregs to the TME [89]. Furthermore, HIF-1α promotes the shedding of NKG2D ligands, causing tumor immune evasion from natural killer cells [90]. Owing to the complex regulatory network of HIF-1, designing specific and ideal inhibitors remains a challenge. Although several HIF-1α inhibitors have been studied and reported, so far none of them has been approved for clinical use [91]. Despite the incomplete success of direct HIF-1α antagonists, several other drugs, such as heat shock protein 90 (HSP90) inhibitors, are shown to have the potential to indirectly inhibit HIF-1α [92]. Anthracycline agents, including doxorubicin and daunorubicin can inhibit HIF-1α by suppressing the binding of HIF-1α to DNA [93]. Overall, given the role of HIF-1α in the immunosuppressive TME, HIF-1α inhibitors may hold promise for improving the efficiency of combined immunotherapy.

2.5 Hedgehog Signaling Regulates the PD-L1 Expression under Hypoxic Conditions

Hh is a conserved signaling pathway in the development of intercellular communication. Three ligands, including Sonic hedgehog (SHH), Indian hedgehog (IHH), and Desert hedgehog (DHH) can activate Hh signaling [94]. The primary receptor for these ligands is Patched-1 (Ptch1). Without the ligand, Ptch1 suppresses smoothened (Smo), but upon the binding of ligand, Ptch1 inhibition is released and Smo is activated. Subsequently, Smo stimulates the glioma-associated oncogene (Gli) transcription factors Gli1, Gli2, and Gli3 [95]. Gli1 activates the target genes related to tumorigenesis as well as angiogenesis factor genes [96].

Hh signaling is aberrant in various types of cancers and contributes to cancer initiation, proliferation, progression, and invasion [97]. In pancreatic CSCs, SHH and other HH signaling components are expressed more than in normal pancreatic stem cells or pancreatic ductal epithelial cells [98]. In addition, Gli-independent Hedgehog signaling is observed in CSCs-enriched cancer and required for CSC survival. Thus, the dysfunction of HH signaling is considered one of the key events in CSCs origin.

Previous researches have demonstrated that Hh signaling promotes cell cycle-dependent tumor growth and invasion by improving the metalloproteinase expression [99,100]. Therefore, hedgehog inhibitors (HHIs) are used for therapy. However, HHIs do not meet the anticipated outcome. To clarify the cause, HH signaling itself should be considered, it is complex and plays a role not only in tumor development but also drug resistance. Of these, the mutation of signaling components is responsible for the non-effectiveness of HHIs. Interestingly, recent studies show that Hh signaling may modulate PD-L1 expression under hypoxic conditions. Additionally, Hh inactivation and/or the blockade of PD-L1 increases the anti-tumor activity of lymphocytes [101]. These results indicate that the action of Hh signaling may contribute to the ICIs resistance via PD-L1 expression and inhibition of the lymphocyte anti-tumor activity. The combination of ICIs and new generation HHIs in the future may shed insights into overcoming the development of resistance.

3. Summary

The different signaling pathways associated with CSCs may play a vital role in the immune resistance. The specific mechanisms inducing the immune resistance include—the recruitment of immunosuppressive cells, especially MDSCs and Treg cells, to the TME; enhancement of CSC properties, especially the EMT; the regulation of PD-L1

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expression on the tumor or CSC surface to inhibit CD8+ T cell cytotoxicity and even the direct loss of CD8+ T cells (Figure 2). Of note, hypoxia can directly induce PD-L1 expression in cancer cells; meanwhile, HIF-1α and HH signaling can be directly activated by hypoxia, thus contributing to the immune resistance. Moreover, these possible mechanisms may function together as a network rather than in isolation. However, to tackle the problem of immune resistance, considerable research efforts are needed to gain an accurate understanding of the underlying mechanisms.

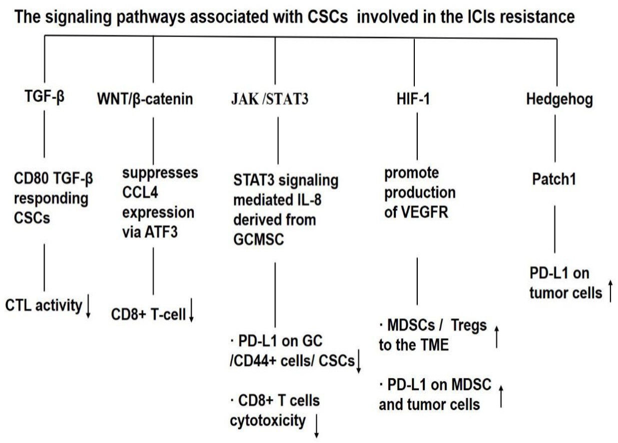

Figure 2. The Schematic Diagram for Signaling Pathways Associated with Cancer Stem Cells in Immunotherapy Resistance

Conflicts of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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