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Role of Cancer Stem Cells in Failure of T Cell Immunotherapy

Udit Basak; Sourio Chakraborty; Sumon Mukherjee; Tanya Das*

Bose Institute, P-1/12, Calcutta Improvement Trust Scheme VII M, Kolkata-700054, India.

Abstract

Over the past decade, immunotherapy has emerged as one of the most promising approaches to cancer treatment. Immunotherapy employing Immune-Checkpoint Inhibitors (ICIs), Adoptive Cell Transfer (ACT), Chimeric Antigen Receptor (CAR) - engineered T cells and cancer vaccines, have generated encouraging responses in the treatment of various cancers, and effector anti-tumor T cells are observed as major component in these measures. Despite these noteworthy developments, T cell-based immunotherapy faces challenges, as a small fraction of cancer patients respond to it. Recently developed cancer stem cell (CSC) theory has portrayed CSCs as the major cell-type involved in tumor initiation, progression, drug resistance, relapse, and immune evasion. A recent paper from our laboratory describing that breast CSCs generate immunosuppressive regulatory T (Treg) cells by secreting TGFβ, thereby evading immune-elimination, raises the question of the contribution of CSCs, if any, in immunotherapy failure. In this review, we discuss the significant role of CSCs towards immunotherapy failure. In fact, CSCs modulate anti-tumor T cells of the host to pro-tumor Tregs, inhibit infiltration and functionalities of effector T cells, possess dysregulated antigen presentation, and create an immunosuppressive tumor microEnvironment (TME), thereby signifying their possible contribution in immunotherapy failure.

Interestingly, anti-cancer therapeutic modalities exacerbate CSC levels, and therefore, enhance Tregs and other pro-tumor immune cells to generate an impenetrable immunosuppressive TME for effector T cells. Finally, we discuss about the prospect of combinatorial therapy by employing CSC-targeting agents in combination with immunotherapy, for successful remission of this deadly disease - cancer.

Keywords: Cancer stem cells; CAR-T cell; CD4+ T cell; Check point inhibitors; Immunosuppression; Immunotherapy; Regulatory T cell; T cell; TGFβ.

Abbreviations: ACT: Adoptive T Cell Transfer; APC: Antigen Presenting Cell; CAR-T Cell: Chimeric Antigen Receptor T Cell; CIT: Cancer Immunotherapy; CSC: Cancer Stem Cell; DC: Dendritic Cell; HLA: Human Leukocyte Antigen; ICI: Immune Check-Point Inhibitor; Mab: Monoclonal Antibody; MDSC: Myeloid-Derived Suppressor Cell; MHC: Major Histocompatibility Complex; NK: Natural Killer; NSCC: Non-Stem Cancer Cell; PD1: Programmed Cell Death Protein 1; PDL1: Programmed Cell Death Ligand 1; TAM: Tumor Associated Macrophage; TGFβ: Tumor Growth Factor β; TME: Tumor Microenvironment; Treg: Regulatory T Cell.

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Correspondance: *Tanya Das, ICMR Emeritus Scientist Bose Institute, P-1/12, CIT Scheme VII M, Kolkata-700 054, India.*

Tel: +91-33-2569-3257 & +91-33-2355-3886; Email: tanya@jcbose.ac.in & das_tanya@yahoo.com

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Introduction

Despite numerous modern advances, cancer remains to be a leading cause of mortalities worldwide, estimating upto 9.7 million deaths in 2022, according recent GLOBOCAN statistics [1]. Multiple strategies tackling this challenging disease namely, surgery, chemotherapy, radiotherapy, and immunotherapy [2,3], have also resulted in therapy resistance and failure [4-7]. In this context, mounting evidences confirm a small subset of cancer stem cells (CSCs) within the tumor microenvironment (TME) that plays a crucial role in tumor initiation, metastasis, disease progression, and importantly, drug-resistance, along with immune-evasion [8-12]. A recent study from our laboratory confirms that tumor-initiating CSCs, even when present in low numbers are able to convert effector $CD4^+$ T cells, a crucial player of anti-tumor immunity to regulatory T (Treg) cells, a pro-tumor immune fraction, by secreting TGFβ [13]. These observations substantiated the role of CSCs in immune-editing, specially involving T cells, and hence, in the possible failure of immunotherapy.

Cancer immunotherapy (CIT) potentiates the patient's immune system to fight cancer. CIT has been documented to be effective in improving patient survival and is less toxic than conventional treatments [14]. Types of CIT techniques where evidently, T cells are found to play an active role [14,15] include Immune-Checkpoint Inhibitors (ICI), Adoptive Cell Transfer (ACT), Chimeric Antigen Receptor (CAR) T cell therapy, and cancer vaccines. However, only 20-40 percent of patients are observed to respond to immunotherapy [16]. Resistance to immunotherapy can be a consequence of dysfunction or exhaustion of T cells, as well as, the escalation of immunosuppressive, low T cell-infiltrating TME [14,17].

In this review, we discuss about different T cell-mediated immunotherapy techniques devised against cancer. Further, we highlight the probable causes underlying immunotherapy failure, wherein CSCs play a major role mainly by (1) generating Treg cells, (2) manipulating antigen presentation, and (3) increasing their own pool during therapies, all of which eventually result in immunosuppressive TME. Hence, a combinatorial application of CSC-targeting agent(s) and immunotherapy which might derive positive clinical outcomes, has also been discussed.

CIT involving T cells

Different modalities of CIT that activate the anti-tumor immune subsets, mainly, $CD8^+$ and $CD4^+$ T cells, are in use for cancer remission [18]. Interestingly, several studies demonstrate $CD8^+$ T cells rely on $CD4^+$ T cells to carry out their effector functions [19]. The activity, proliferation, and recruitment of $CD8^+$ T cells to the tumor site are enhanced by $CD4^+$ T cells through IL2 [20]. Therefore, effector $CD4^+$ T cells maintains a holistic approach towards CIT. Some of the main immunotherapy avenues harnessing T cells are discussed below.

Immune-Checkpoint Inhibitors (ICI)

Immune-checkpoints, PD1 and CTLA4 are co-inhibitory receptors on the T cell surface that regulate T cell response. However, tumor cells make use of these inhibitory molecules to promote tumor tolerance and exhaustion in T cells [21]. Hence, ICIs like anti-CTLA4, anti-PD1, and anti-PDL1 can bind to these receptors, thus reinvigorating the immune response against cancer cells [21,22]. In multiple clinical trials, usage of ipilimumab (anti-CTLA4) and Pembrolizumab (anti-PD1) resulted in increased circulating $CD4^+$ T cells and Th1 cytokines, as well as a positive outcome in patient survival [14,23]. Other clinical trials also depicted that pembrolizumab enhances overall survival if used alone, or with chemotherapy, over standard treatments [21]. Several other immune-checkpoints like, Lymphocyte Activating Gene-3 (LAG3) and T cell Immunoglobulin and ITIM domain (TIGIT) expressed on T cells, have also been the subjects of research and antibody-mediated treatment options in several cancers [24].

Adoptive cell transfer (ACT)

This method uses autologous T cells isolated from cancer patients, then identifying and isolating those with anti-tumor function, followed by expanding them *ex vivo* and introducing back to the patients [25]. This mechanism has been observed to be the most effective one in metastatic melanoma patients where tumor regression was found in almost 50 percent of the patients [25]. Moreover, administration of T cell- activating IL2 enhanced the effectiveness of these effector cells in mice model [26]. The combinatorial $CD8^+$ and $CD4^+$ T cell transfer in mouse metastasis models also showed promising outcomes [27]. In fact, owing to the lower infiltrating status of $CD8^+$ T cells, $CD4^+$ T cells were seen to infiltrate the tumor sites where they then stimulated tumor antigen specific-$CD8^+$ T cells [27]. In metastatic epithelial cancer, Tran *et al.* [28], showed disease regression in patients when more than 95 percent of pure $CD4^+$ T helper 1 cells were utilized.

Chimeric antigen receptor (CAR) T cell therapy

CAR-T cell therapy, another facet of T cell transfer where engineered T cells, better suited for recognizing tumor antigens and causing lymphocyte stimulation, are used. These engineered T cells present themselves as attractive subject for new generation-immunotherapy [14,29]. CARs are chimeric proteins composed of antigen binding domains, which are selected single-chain fragment variable from antibodies and one or more intracellular signaling domains of T cell receptor along with additional costimulatory domains [30]. CARs address the drawbacks of previously engineered and modified T Cell Receptor (TCR) by overcoming the need for Major Histocompatibility Complex (MHC) expression, MHC identity, and co-stimulation. This constitutes a fundamental advantage over immune evasion in cancer, as tumor cells often down-regulate MHC molecules [31]. However, CAR still requires the presence of extracellular targets on cancer cell surface [31]. CAR therapies targeting CD19 have been shown to achieve complete remission in 70 to 90 percent of patients with relapsed and refractory pre-B cell acute lymphoblastic leukemia [32], and demonstrated remarkable responses in B cell lymphomas [32]. In fact, only a small dose of CAR-T cells has been found to be effective in eradication of disease load [32]. Interestingly, $CD4^+$ T cells were as effective as $CD8^+$ T cells towards implementing cytotoxicity, and showed lower exhaustion compared to $CD8^+$ T cells in CD19 targeted-CAR therapy in mice models for lymphoblastic leukemia [14,32].

Therapeutic cancer vaccination

Much like the vaccination mechanisms eliciting immune responses, cancer antigens or personalized neoantigens, after immunization, are processed by Antigen Presenting Cells (APCs) and express them on MHC I/II complexes, which in turn, prime and

activate T cell response. This further propagates and leads to T cell infiltration to tumor site [33]. Peptide vaccine against HPV-16 positive intraepithelial vulvar neoplasia resulted in high IFNγ responses by $CD4^+$ and $CD8^+$ T cells [34]. Moreover, $CD4^+$ T cells were observed to have higher IFNγ expression in patients demonstrating complete response [34]. A long lasting immune response with robust $CD4^+$ T cell levels was observed in a phase I/II clinical trials with prostate cancer patients when treated with peptide vaccine targeting RhoC [35].

Limitations and failure of cancer T cell immunotherapy

Although these varied immunotherapy strategies have proven effective in multiple cancers and generated durable response, they have often resulted to therapy- resistance and failure [5,36]. For example, T cell dysfunction as a consequence of chronic antigen stimulation causes loss of its effector and anti-tumor functionalities [14]. On the other hand, an immunosuppressive tumor milieu, characterized by high levels of cancer-aiding factors like TGFβ, VEGF, and IL8, metabolites like, Indoleamine 2, 3-Dioxygenase (IDO), low antigenicity, lack of immune 'call signal' chemokines, dysregulated signaling pathways, and most importantly, the presence of tumor-promoting immune subsets, results in exhaustion and lower infiltration of active effector T cells [5]. In this context, the infiltration of Tregs and conversion of anti-tumor T cells to Treg cells play quite a significant role in defining immunotherapy resistance [17,37]. Tumor-promoting Tregs play a crucial role in suppressing the body's immune function against tumors, while also aiding in Epithelial-Mesenchymal Transition (EMT), angiogenesis, and invasion [13,37]. Presence of increased Tregs and their infiltration at the tumor site leads to a negative clinical prognosis [38].

As directed by our previous report and other concurrent findings [13,39], the marked role of CSCs towards shifting the anti-tumor T cell population to pro-tumor Tregs is well understood. Consequently, immunotherapy strategies discussed above, aimed at enhancing effector T cell numbers and their activity, although portray an interesting prospect, might be facing the hurdle of therapy resistance mainly conferred by the interplay of Treg cells and CSCs [13,37].

Cancer stem cells

Other than tumor cells, the TME consists of immune cells, the extracellular matrix, exosomes, cytokines, and cell-secreted cytokines [40]. CSCs are a small subset of tumor cells that have been identified as the primary cause of cancer initiation, along with treatment failures, cancer recurrence, tumor growth, invasion, and metastasis, as supported by increasing evidences [8,41]. These tiny sub-population of cancer cells can even escape the stress signals such as radiotherapy, chemotherapy drugs by utilizing different modalities to hide in the TME [8,41]. Most importantly, during the early stages of tumor development, tumor initiating-CSCs must avoid immune- surveillance and immune-editing to survive in the TME for propagating and populating the entire tumor mass [8,43,44]. CSCs accomplish this difficult task by evading identification and restricting anti-tumor actions by the immune subsets, or by regulating several TME compartments, including immune and non-immune ones, in their favor [8,41]. These immune-evading properties of CSCs support their possible seminal roles in CIT failure.

Role of CSCs in T cell-immunotherapy failure

Effector T cells primed and induced by CIT, fail to furnish their 'original' anti-tumor function, mainly due to the following reasons.

Treg generation

Our previous study reported a strong correlation between CSCs with Tregs in tumor [13]. In fact, our report showed that very low number of CSCs could successfully convert infiltrating $CD4^+$ T lymphocytes to Treg cells in a contact-independent manner. Moreover, these CSCs not only escaped chemotherapy, but also generated more Treg cells. Our exploration further reported that CSCs generated Treg cells from effector T cells by secreting immune-suppressive, Treg-polarizing cytokine TGFβ, which was further exacerbated by chemotherapy [13]. Similarly, other studies corroborated that under hypoxic effects, glioblastoma CSCs inhibited T cell functions, induced $FOXP3^+$ Tregs and generated immunosuppressive TME [43]. Additionally, SOX-expressing CSCs release CCL1, which attracts Tregs to the TME, the Tregs in turn produce TGFβ and IL17, augmenting self-renewal, cancer stemness, and EMT towards tumor development and invasion [44]. Reportedly, STAT3 signaling protects gastric CSCs from T cell-mediated death and differentiates uncommitted $CD4^+$T cells into Tregs [45]. CSCs also influence the Th17/Treg balance by altering the levels of IL6, IL8, and CCL5 in the TME [46]. TGFβ further enables CD80 ligand-expressing CSCs to interact with Tregs and CTLA4, resulting in diminished cytotoxic T cell activity and immunotherapy resistance [47]. Therefore, these findings strongly nudge towards the impact of CSCs in promotion of Tregs.

Conversely, Tregs release VEGF, which promotes angiogenesis and thereby aids in CSC survival, stemness modulation, and their self-renewal in hypoxic condition [48]. Treg-derived cyclooxygenase 2 (COX2) inhibits effector T cells *via* PEG-E2- dependent mechanism [47]. The release of TGFβ, IL10, and IL35 by Tregs hinders T cell proliferation and effector functions, including IFNγ generation [13,49] and granzyme and perforin-mediated killing, thus, contributing to immunotherapy failure [50] (Figure 1). There, therefore, exists a deadly liaison between CSCs and Treg cells in TME that might affect the fate of CIT.

Antigen presentation

The immune system targets cancer cells through antigen processing and presentation [51]. APCs detect cancer cell-associated antigens and display them on their surfaces in order to be recognized by T cells [51]. Unfortunately, CSCs avoid the immune system by down-regulating MHC expression or inhibiting the expression of antigens identified by immune cells [51,52]. Head and neck cancer $CD44^+$ CSCs have been documented to downregulate Human Leukocyte Antigen-A2 (HLA-A2), HLA class II, and TAP2 expression [53]. Whereas, melanoma CSCs apart from expressing low MHCI and MHCII, circumvent tumor immune surveillance by inhibiting transcription of melanoma-associated antigens as well [52].

Also, PDL1 and PDL2, which are overexpressed in CSCs, inhibit T cell activity and allow immunological escape [41,52] (Figure 1). In addition, interaction of PDL1 and PDL2 with $PD1^+$ T cells releases IL10 from tumor cells, which then suppresses effector T cells [54], thereby, dampening the functions of T cells in multiple ways. By activating PI3K/AKT and mTOR signaling pathways, PDL1 also

maintains CSC pluripotency [55]. Moreover, CSCs show heightened expression of CD47, which interferes with the phagocytic activity of tumor-associated macrophages (TAMs), hence, limit their role as APCs, and consequently, T cell priming and activation [8].

Cancer therapies augment CSC population and further add to immunosuppressive TME

Multiple treatment regimens, such as, chemotherapy, radiotherapy, and even, immunotherapy enhance CSC nature and its pool [10,58-60]. Previous report from our laboratory demonstrated that chemotherapy not only failed to induce apoptosis in CSCs, it also enhanced the CSC pool via de-differentiation of Non-Stem Cancer Cells (NSCCs)[10]. CSCs also showed radio-resistance while radiotherapy promoted EMT and generation of CSCs [56].

In fact, immunotherapy has also been observed to increase CSC nature in breast cancer [58]. Since CSCs and Tregs have a strong association, increase in CSC population during different treatment modalities, invariably amounts to increase in Treg numbers [13]. Other components of the TME, such as TAMs, being promoted by CSCs, also end up aiding Treg cells [8,59]. Myeloid- Derived Suppressor Cells (MDSCs) are recruited by TGFβ-activated $CD133^+$ CSCs in melanoma [47]. Incidentally, MDSCs are reported to stimulate Tregs and TAMs [60]. Again, Tregs, TAMs, and MDSCs promote CSC status in tumor [41,47], thereby, maintaining an overall crosstalk, leading to immunosuppressive TME and impaired infiltration of anti-tumor T cells [61] (Figure 1).

Therefore, challenges for immunotherapy measures like, ACT and vaccines, which aim at increasing active T cells, eventually may end up being ineffective due to the T cell to Treg cell converting potential of CSCs [13]. As discussed above, additional therapies may worsen the situation, accounting for enhanced levels of CSCs and pro- tumor subsets, resulting in 'cold' tumor status and immunotherapy failure [13,41,61]. Hence, it was not unexpected when poor efficacy, clinical outcomes, and exhaustion of T cells were seen as limitations of ACT and cancer vaccines [33,62]. Notably, TGFβ- responsive CSCs were observed to be ACT resistant [63]. Other than high immune- checkpoint expression, CSCs demonstrate intrinsic and extrinsic resistance factors, which are hindrance to ICI therapy [36]. Supporting this notion, a study revealed a positive correlation of CSC marker with genes associated with ICI-immunotherapy resistance [64]. Additionally, similar to ACT, CAR-T faces challenges owing to low T- cell infiltration and low cancer antigen expression [52,65] (Figure 2).

Possible remedy

In essence, combinatorial therapy involving sensitization and elimination of CSCs followed by conventional therapies might sustain an anti-tumor TME. However, therapeutic stress induces NSCCs to de-differentiate into CSCs [10], and again establish an immunosuppressive environment. We found that pre-treating CSCs with aspirin sensitizes them to chemo-treatment [10]. By down-regulating WNT pathway with paclitaxel, CSC content and tumor development were considerably decreased [66,67]. Likewise, Metformin has been reported to alter epigenetic landscape of CSCs, rendering them vulnerable to chemotherapy [68].

Monoclonal antibodies (mAbs) have recently been proved effective and garnered acceptance in cancer therapy. Multiple clinical trials have employed mAbs to target CSC-associated biomar-

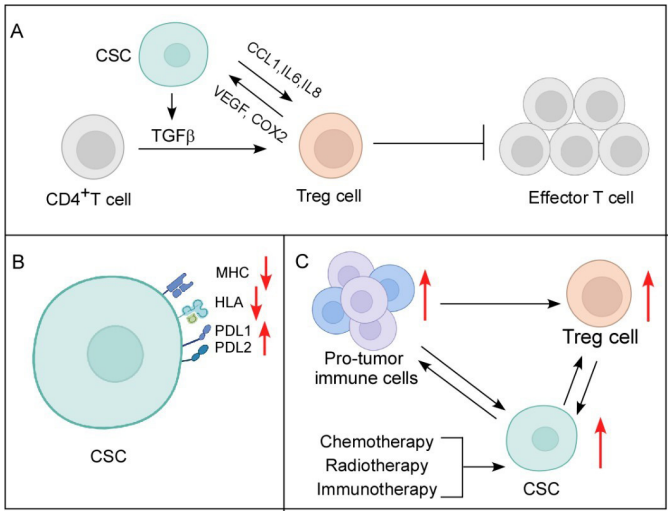

Figure 1: Mechanisms of CSCs to escape anti-tumor T cells and promote immunosuppressive microenvironment. **(A)** CSCs directly converts $CD4^+$ T cells to immunosuppressive Tregs by secreting immunomodulatory cytokines or chemokines or soluble factors and thereby, interfere effector T cell functions. **(B)** CSCs escape recognition by immune cells by downregulating MHC and HLA expression, and inhibit anti-tumor T cells by overexpressing checkpoint regulators, PDL1 and PDL2. **(C)** Anti-cancer therapies increase the number of putative CSCs, which in turn convert effector T cells into immunosuppressive Tregs and increase the number of other pro-tumor immune cell subsets, which are in reciprocal feedback loop to enrich one another and thereby, sustain an immunosuppressive milieu.

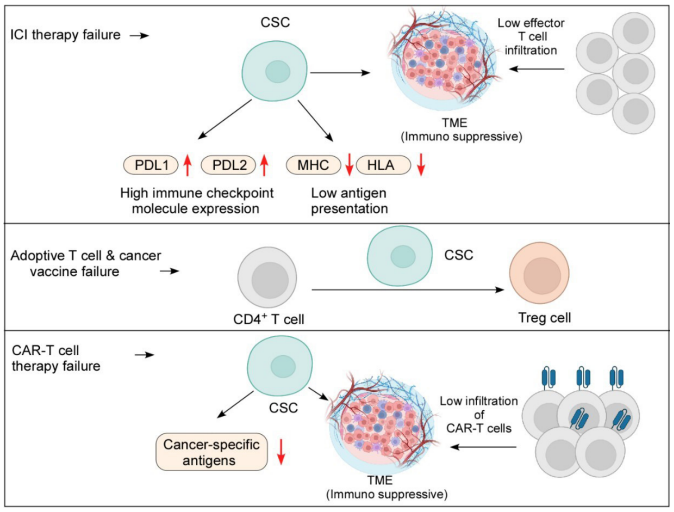

Figure 2: Role of CSCs towards resistance to different T cell-immunotherapeutic modalities. CSCs foster resistance to immune-checkpoint therapy by expressing high levels of PDL1 and PDL2, as well as, altering antigen presentation pathways by downregulating MHC or HLA expression, and maintaining an immunosuppressive microenvironment that interferes with T cell infiltration **(Upper panel)**. CSCs interfere with adoptive T cell transfer treatment or T cell vaccine therapy by converting effector T cells into immunosuppressive Treg cells **(middle panel)**. CSCs also contribute to the failure of CAR-T cell therapy as they downregulate expression of cancer-specific antigens identified by CAR- T cells and create an immunosuppressive microenvironment, which inhibits CAR-T cell infiltration to the tumor milieu **(lower panel)**.

kers. For example, CD20, a common CSC marker was targeted by rituximab which effected favourable response rate in lymphoma [69]. CSC marker, EpCAM was used as a target by employing Adecatumumab, that demonstrated remarkable therapeutic potential in hormone-resistant breast cancer patients [70].

Recently, CAR-T cells targeting CSC-specific antigens have been tested in cancer models. CAR-T cells against EpCAM antigens have successfully eliminated CSCs in PC3M and PC3 prostate cancer cell line models [71]. CSCs were selectively killed by adoptively transplanted cytotoxic T cells specific for ASB4 antigen in colon cancer [72]. Adoptively transferring CAR natural killer (NK) cells have also shown to eliminate CSCs with high efficiency [47].

Dendritic Cell (DC)-based vaccinations are found to be effective against CSCs. DCs pulsed with cancer cell lines or CSC-lysates were applied as vaccines to test therapeutic effectiveness. In the malignant melanoma model, CSC-lysate-pulsed DCs decreased tumor development and prolonged life in vaccinated mice by inducing IFNγ and IL4 production [73,74]. DCs charged with Panc-1 CSC-lysates stimulated INFγ and IL2 production, leading to lymphocyte infiltration in pancreatic cancer [75]. DCs with NANOG peptides generated highly specific anti-tumor T cell responses against ovarian cancer CSCs [76].

However, detail studies are required to authentically identify the possible agents/molecules/processes that will successfully kill/sensitize CSCs, thereby, will pave the way for successful immunotherapy in combinatorial treatment.

Concluding remarks

Above discussion highlights different T cell based-immunotherapy modalities available, as well as, their limitations. We have further elaborated that CSCs play a crucial part in immunotherapy-resistance by generating or recruiting Tregs, limiting effector T cell activity, presenting lower antigens, expressing high immune check-points and creating a cumulatively interacting immunosuppressive TME, thereby impairing anti- tumor T cell infiltration. To overcome such obstacles, potent therapies targeting CSC-associated antigens, alone or in conjunction with CSC-supportive TME, must be considered to be used prior to immunotherapy. As CSCs share tumor-initiating and immune evasion features, immunotherapies tailored against CSC-specific neoantigens and stemness transcription factors, are of utmost need of present day. However, ample research involving CSC-targeted CAR-T/ DC/NK cell cancer therapies are required to ensure clinical safety and efficacy, assuring their use in future interventions for ensuring overall success of CIT.

Declarations

Conflicts of interest: The authors have no competing interests to declare that are relevant to the content of this article.

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