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Abbreviations: CSC: cancer stem cells; CT: cancer testis; DC: dendritic cells; DNA methyltransferase, DNMT; EMT: epithelial-to-mesenchymal transition; FDA: Food and Drug Administration; head and neck squamous cell carcinomas, HNSCC; immune checkpoint inhibitors,

ICI; major histocompatibility complex class I, MHC I; melanoma-associated antigen gene, MAGE; myeloid-derived suppressor cells, MDSCs; mucin 1, MUC1; The Cancer Genome Atlas, TCGA; tumor growth factor, TGF-β; tumor microenvironment, TME; T-regulatory cells, Tregs

Highlights

- Cancer stem cells (CSC) are involved in tumor resistance, recurrence and metastasis
- CSCs are an obstacle to the success of immune checkpoint inhibitors (ICIs)
- Vaccination is a possible approach to CSC elimination
- Antigens crucial for CSC maintenance and function are ideal targets
- Combining CSC-targeting vaccines, ICIs and other drugs may improve patient outcomes

Abstract

The introduction of immune checkpoint inhibitors (ICIs) has ushered in a new, golden age for cancer immunotherapy. However, their clinical success remains limited in several solid cancer types because of the low intrinsic immunogenicity of tumors and the development of immune escape mechanisms. Cancer stem cells (CSCs), a small population of cancer cells that are responsible for tumor onset, metastatic spread and relapse after treatment, play a pivotal role in resistance to ICIs. The development of novel therapies that can target CSCs would thus improve the outcomes of current immunotherapy regimens. In this light, vaccines that target CSCs are a promising strategy. This paper briefly describes the immunologic properties of CSCs and their antigenic profile, and reviews current preclinical and clinical approaches that combine CSC-targeting vaccines with different synergistic therapies for the development of more effective antineoplastic treatments.

INTRODUCTION

Cancer immunotherapy is revolutionizing the treatment of oncological patients, as immune checkpoint inhibitors (ICIs) ameliorate the prognosis of many patients suffering from tumors such as melanoma, non-small-cell lung cancer, colorectal and renal carcinoma (1). However, ICIs fail to induce clinical responses in a significant proportion of patients, with single-agent response rates of between 10 and 35% (2).

ICI effectiveness is dependent on a pre-existing antitumor immune response, and vaccination is a good strategy with which to induce one (1). Although research on cancer vaccines has led to disappointing results, with many clinical trials failing to improve patient survival (3), and only one FDA-approved vaccine (4), the possible combination of vaccines and ICIs has renewed interest in the topic.

The eradication of a tumor entails the elimination of CSCs, which are a small population of cells with stem-like features that are responsible for tumor initiation and metastasis and, being resistant to current therapies, for its recurrence (5,6). The efficacy of a cancer vaccine might therefore depend on its ability to target CSCs (7). Interestingly, most vaccines tested so far have targeted antigens expressed by differentiated cancer cells, sparing CSCs, which display particular antigenic and immune-modulating properties (8).

1. Cancer stem cell immunology

CSCs are slowly dividing cells endowed with unlimited self-renewal potential that initiate tumors and that, thanks to their high degree of phenotypic plasticity, undergo epithelial-to-mesenchymal transition (EMT) and disseminate to distant organs, generating metastases (5). Being resistant to chemo- and radiotherapy, CSCs are responsible for cancer relapse (9). Moreover, CSCs possess immune-evasive properties that allow them to escape T-cell killing. Indeed, defects in antigen presentation, such as the downregulation of major histocompatibility complex class I (MHC I) and

molecules involved in antigen presentation, are common in CSCs from several cancers, including breast (10), colon (11) and melanoma (12). Moreover, CSCs exert immunosuppressive effects in the tumor microenvironment (TME) through intense cross-talk with immune cells (13). A machine-learning-based analysis of The Cancer Genome Atlas (TCGA) has revealed a negative association between CSC frequency and tumor leukocyte infiltration, which correlates with high tumor grade (14). CSCs impair effector T-cell recruitment and activation by releasing a wide variety of cytokines and growth factors that attract immunosuppressor cells. Indeed, when high amounts of tumor growth factor (TGF)-β are secreted by CSCs (12,15), T-regulatory cells (Tregs), which inhibit effector T-cell proliferation, are activated, and fibrosis, which is a physical barrier that hampers T-lymphocyte infiltration, is promoted (16). Similarly, CSCs inhibit cytotoxic T-cell function and induce the recruitment of myeloid-derived suppressor cells (MDSCs), as well as the polarization of Th2 cells and M2 pro-tumoral macrophages (17-19). Interestingly, the cytokines and growth factors that are released by MDSCs, and other immunosuppressive cells recruited in the TME, support CSC survival and self-renewal, generating a vicious circle that promotes cancer progression (20).

The link between stemness and immunosuppression has been further confirmed by a TCGA analysis on 21 solid cancer types, and a negative association between stemness and interferon-α/β signaling, and a striking positive association with several immunosuppressive genes, including *TGFB1* and *CD276* and *CD155*, two inhibitors of T and natural killer cells, were observed (21). Indeed, CSCs also inhibit immune effector cells thanks to the overexpression of several ICs, which, moreover, exert cell-intrinsic pro-tumoral mechanisms, favoring CSC survival and self-renewal (22). For instance, CSCs from melanoma, ovarian, colorectal and breast cancers express high levels of PD-L1 (23,24), which induces the AKT-dependent expression of the stem-cell markers OCT-4A, Nanog and BMI1 (25). Similarly, CD47 is overexpressed on CSCs from liver, pancreatic, breast and other tumors, and, besides inhibiting CSC phagocytosis by macrophages, directly protects CSCs from apoptosis (26). CTLA-4, which promotes melanoma-CSC proliferation and survival, has shown a similar effect (27).

Despite the numerous immune-evasive properties of CSCs, preclinical studies that target them with immunotherapy have provided encouraging results, demonstrating that vaccines based on CSC lysates or dendritic cells (DC) that are loaded with CSCs are more effective than non-CSC-based equivalents in preventing tumor onset (28-34). Clinical trials using CSC-loaded DCs or CSC lysates have been performed on patients affected by different solid cancers, demonstrating that vaccination is safe and effectively induces a specific immune response (35-37). Lasting protection, with about 70% of patients still alive after 20 years, has been reported for AGI-101H, a vaccine that is comprised of irradiated melanoma cell lines engineered with hyper IL-6 (a fusion form of IL-6 and its soluble receptor) that grants it stem-like features (38).

The superiority exhibited by CSC- over non-CSC-based vaccines suggests that the antigenic profile of these two cancer cell populations differs, and that the targeting of CSC antigens is required to achieve durable and effective antitumor responses.

2. Cancer stem cell oncoantigens as targets for vaccination

There is still no clear antigen profile for CSCs. However, it is well known that some antigens are preferentially expressed by CSCs and others by differentiated cancer cells, while a third group is expressed by both CSCs and non-CSCs (5). Tumor cells are characterized by a high degree of plasticity that allows them to dynamically switch between CSCs and differentiated cells (39). It is therefore crucial that vaccines specific for antigens expressed by differentiated cancer cells and overexpressed in CSCs are developed to eliminate both current and *de-novo* generated CSCs for complete tumor eradication (6). Several cancer testis (CT) antigens, such as NY-ESO-1 and some melanoma-associated antigen gene (MAGE) family members, which are expressed by differentiated cells and overexpressed in CSCs, are immunogenic and are good targets for anti-CSC vaccination (40). However, many CSC-specific antigens are self-proteins that are also expressed on normal stem cells, which significantly limits their use as targets for vaccination, as central tolerance would

lead to poor immune responses. Moreover, if vaccination were able to break tolerance, severe side effects and autoimmunity would be induced (41). This occurs for vaccines against stem-cell markers, such as CD44, aldehyde dehydrogenase, and CD133, whose use in patients is limited by safety concerns (42-46).

The recently demonstrated positive association between stemness and cancer-mutational burden in numerous solid tumors (21), has brought forwards the idea of developing neoantigen-based CSC-targeting vaccines. Although this is an interesting strategy, it should be noted that the correlation between tumor mutational load and response to immunotherapy is far from perfect, especially as only a minority of mutations lead to the generation of neoantigens (47). Moreover, mutations often occur in genes that do not play a pivotal role in either carcinogenesis or CSC maintenance, meaning that vaccination against many neoantigens may lead to the expansion of resistant clones that lack their expression, favoring cancer recurrence after initial shrinkage (48). Oncoantigens, i.e. antigens that play a pivotal role in cancer progression and CSC self-renewal, are a valid means with which to overcome this issue (49,50). In particular, oncoantigens expressed on cell surfaces are the most promising for the development of CSC-directed vaccines as they can be targeted by both T and B cell-mediated responses, thus enabling CSC elimination despite their downregulation of MHC I (10,51). Some oncoantigens expressed on CSC plasma membranes are currently giving promising results in preclinical models. For example, one DC-based vaccine targets integrin β4, which is overexpressed in breast and colon cancers and plays a role in CSC self-renewal. The humoral and cellular immune responses elicited by this vaccine are effective in inhibiting tumor growth and spontaneous pulmonary metastases as they kill both CSCs and differentiated cancer cells (52). Cripto-1, a GPI-anchored membrane oncofetal protein that promotes CSC self-renewal, EMT and migration in melanoma and breast cancer, is another promising CSC oncoantigen (53). DNA vaccination against Cripto-1 decreased both tumor growth and lung metastases in preclinical models (53,54). Similarly, the cystine/glutamate antiporter xCT, which is overexpressed in CSCs from different solid tumors and plays a key role in the maintenance of their redox balance and

metabolism (55), is a good candidate for anti-cancer vaccination. Indeed, immunotargeting xCT using DNA-, viral vector- or virus-like particle-based vaccines induced a strong antibody response and, in some cases, a cytotoxic T-cell response that significantly protected mice from breast-cancer growth and metastasis (56-59).

EMT-associated altered glycosylation is another mechanism of oncoantigen generation, in addition to protein overexpression, in CSCs (60). Clinical trials with vaccines that target some aberrantly glycosylated CSC oncoantigens, such as mucin 1 (MUC1), are currently ongoing (61). Although a lack of response was observed in patients with a history of premalignant lesions and who displayed elevated levels of circulating MDSCs, encouraging results were obtained in less immunosuppressed patients (62).

3. Combination therapies

The results obtained from the MUC1-vaccine trials suggest that, like all monotherapies, single immunotherapy is insufficient for cancer treatment, and that combinatorial approaches are needed (**Figure 1**). Several studies on preclinical models of melanoma, breast, colon and head and neck squamous cell carcinomas (HNSCC) have shown that combining CSC-targeting vaccines with ICIs induces increased activation of tumor-specific $CD8^+$ T cells, decreased CSC frequency and better tumor-progression control than single treatments, paving the way for clinical experimentation (33,52,63,64).

Besides the use of ICIs, strategies to revert TME immunosuppressive activity may improve the efficacy of anti-CSC vaccination. Tadalafil is an inhibitor of phosphodiesterase-5, which alters the TME by reducing the number of MDSCs and Tregs and thus promotes tumor immunity, and is a possible candidate (65). Interim results from a phase I clinical trial (NCT02544880) in patients with primary HNSCC treated with tadalafil and a MUC1 vaccine indicate that the combination therapy was well tolerated and able to decrease the number of PD-$L1^+$ macrophages and increase that of

activated tumor-infiltrating T cells. However, PD-L1 upregulation was observed on tumor cells, suggesting that a combination of more than two therapies may be needed (62).

The administration of multiple vaccines that target oncoantigens that are involved in different cell processes is a good strategy with which to attack cancer on multiple fronts. We have recently demonstrated that a combination of two vaccines that target HER2 and xCT exerts a synergistic effect in preclinical models of $HER2^+$ breast cancer, with HER2 immunotargeting slowing primary-tumor growth and xCT immunotargeting mainly affecting CSCs and inhibiting metastases (66). Combining CSC-targeting vaccines with non-CSC-targeted treatments is an additional approach that can potentially induce tumor shrinkage and prevent metastasis and recurrence. CSC-vaccination may be successfully combined with chemo- and radiotherapy or with oncolytic viruses, which target differentiated cancer cells and further activate the immune response through the induction of immunogenic cell death, inducing the release of other tumor antigens (67-69). A phase I clinical trial (NCT00179309) has demonstrated that the combination of a MUC1-targeting vaccine and chemotherapy significantly improved progression-free survival, compared to chemotherapy alone, in breast-cancer patients (70).

Combining CSC immunotargeting with epigenetic drugs, which augment CSC immunogenicity by upregulating MHC and antigen-processing machinery (71), and decrease MDSC and Treg numbers (72), is a further way to improve treatment efficacy. Histone deacetylases and DNA methyltransferase (DNMT) inhibitors are currently used in clinical trials as they hinder tumor-cell growth and induce cell differentiation (73). Clinical trials performed on patients with ovarian (NCT01673217) and pediatric brain tumors (NCT02332889, NCT01241162) that were treated with the DNMT inhibitor decitabine, in association with a NY-ESO-1 peptide vaccine or a DC-based vaccine that targeted MAGE-A1, MAGE-A3 and NY-ESO-1, demonstrated that these regimens are safe (74,75). In 6 out of 10 patients treated with decitabine plus the NY-ESO-1 peptide, specific antibodies and T-cell responses either led to disease stabilization or partial clinical response (74).

Conclusions

Although ICIs have founded a new golden age for cancer immunotherapy, not all tumors are immunogenic, and several resistance mechanisms hinder ICI efficacy. As CSCs are an obstacle to ICI success, vaccines directed towards CSC oncoantigens may improve immunotherapy efficacy. Deeper genomic, biological and immunological characterization of CSCs, and their crosstalk with the immune system, is crucial if we are to address the difficulties associated with CSC heterogeneity and plasticity, and will lay the foundations for the development of novel combination therapies for the eradication of cancer.

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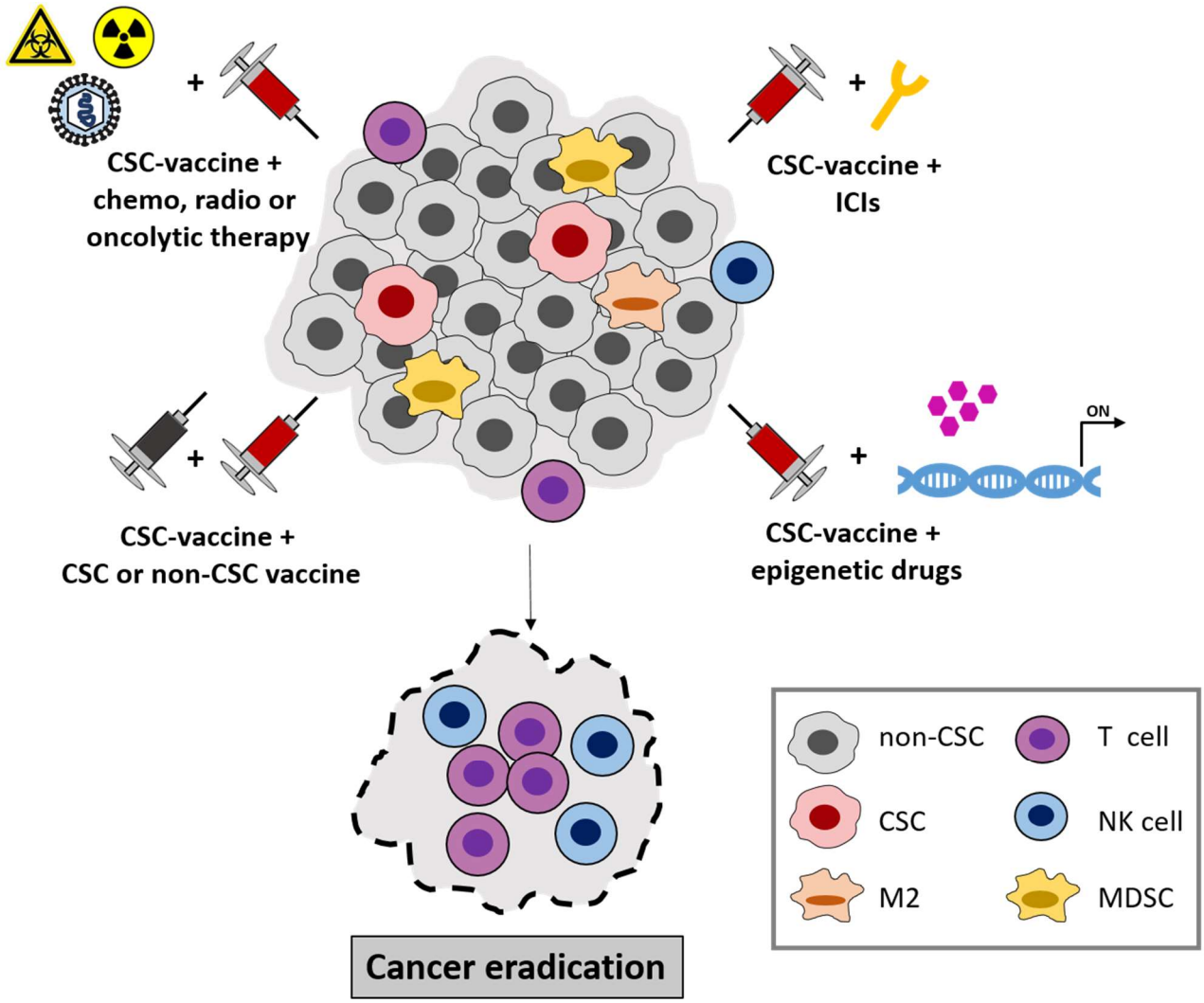

Figure 1. Combination therapies based on anti-CSC vaccination. Schematic representation of the effects exerted by combining CSC-oncoantigen-directed vaccines with chemotherapy, radiotherapy and oncolytic therapies, ICIs, vaccines to other oncoantigens and epigenetic drugs. All these combination therapies have the potential to induce the elimination of both CSCs and differentiated cancer cells, finally leading to cancer eradication.