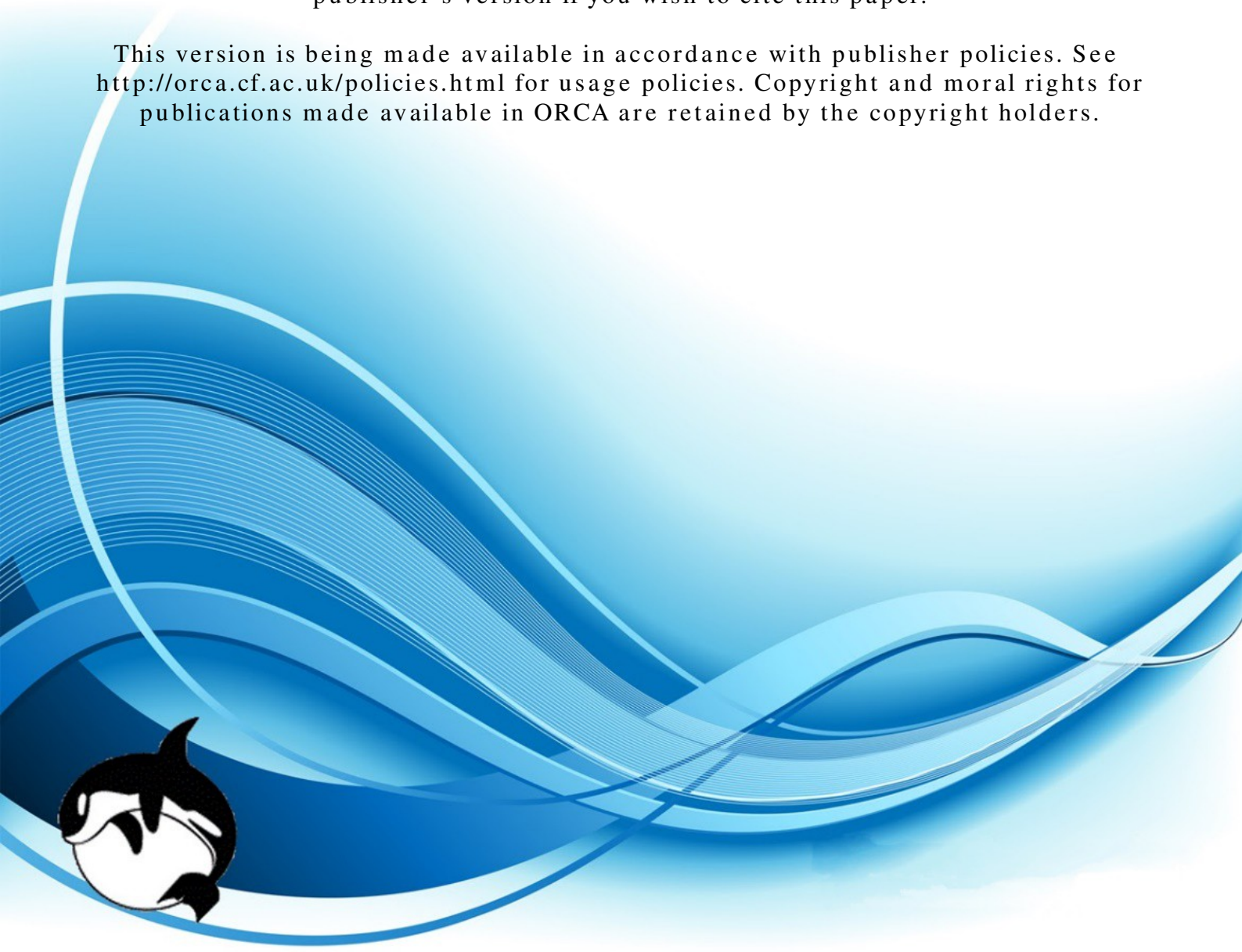

Cancer stem cells as targets for immunotherapy

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Summary

Current cancer therapies target the bulk of the tumour, while a population of highly resistant tumour cells maybe able to repopulate the tumour and metastasise to new sites. Cancer cells with such stem cell-like characteristics can be identified based on their phenotypic and/or functional features which may open up ways for their targeted elimination. In this review we discuss potential off-target effects of inhibiting cancer stem-cell self-renewal pathways on immune cells, and summarise some recent immunological studies specifically targeting cancer stem cells based on their unique antigen expression.

1. Introduction

Cancer stem cells (CSCs), also called stem-like cells or tumour-initiating cells (TICs), are a distinct subpopulation of tumour cells. They may arise in a variable and unpredictable manner due to genetic and epigenetic changes during tumour development (stochastic theory), or they are cells that possess a unique intrinsic ability to initiate tumour growth and self-renewal (hierarchical theory). The existence of CSCs has been postulated for a while before experimental evidence was first provided in 1994. A subpopulation of acute myeloid leukemia cells, with $CD34^{high}$ $CD32^{low}$ phenotype, was shown to be highly capable of engrafting leukemia in SCID mice (1). These cells were called leukemia-initiating cells. Solid tumour TICs were first described in breast cancer ($CD44^{+}CD24^{-/low}/Linage^{-}$ cells) in 2003 (2), followed by further discoveries in a variety of malignancies. As for using the correct designation, CSCs are perceived as immature progenitors of tumour cells, residing at the top of a hierarchical organisation of tumour cell differentiation. On the other hand, the definition of TICs is based on the function of these cells, as they are uniquely capable, at very low cell numbers, to initiate heterogenous, complex tumours in vivo. In this review, we use the combined CSCs/TICs abbreviation, as suggested by Maccalli et al. (3) reflecting the variety of the references.

While traditional cancer therapies, such as radio- or chemotherapy, may eliminate the bulk of the tumours, treatment resistance in CSCs/TICs is thought to be responsible for relapse. In order to prevent or significantly delay relapse, these cells should be specifically targeted and eliminated. There are numerous ongoing trials, targeting CSCs/TICs (for latest review see (4)), however, in order

to design efficient novel treatment approaches, we need a better understanding of the biology of these cells. In this review we summarise the current state of knowledge about the feasibility of immune targeting CSCs/TICs in solid tumours. We also point out how some of the biological targeting of CSCs/TICs may act as a double edge sword by also affecting immune responses.

2. Developmental pathways in CSC/TIC signalling

Developmental pathways play important roles in normal stem cell function. The core stem cell pathways, Notch, Wnt/β-catenin, Hedgehog (discussed in more detail below) and some other crucial pathways, such as JAK/STAT, PI3K/PTEN and NFκB, promote cell proliferation and the formation of CSC-like colonies (5, 6). They are frequently altered in cancers, such as become deregulated or persistently activated and suggested to be responsible for CSC/TIC regulation (7-10).

Notch – Notch ligands, located in the plasma membrane of adjacent cells are trans-activated, triggering the transcription of Notch target genes, such as the HES (hairy and enhancer of split)-related family, c-myc, PI3K, AKT, NF-κB, PPAR, CyclinD1, p21 and p27. The activation of these downstream targets regulates cell fate leading to differentiation, cell-cycle progression and survival, depending on the particular signalling context. In stem-like cells Notch may delay differentiation and promote cell survival (11, 12). There are numerous ongoing phase I and II clinical trials in cancer with a range of targets and mechanisms investigating the usefulness of Notch targeting, alone or in combination with other therapies (11). However, Notch signalling has also been linked to peripheral T cell maturation into effector cells, such as developing cytotoxic T cell function or cytokine production (13). T cell activity has been shown to be impaired by Notch-inhibition with a γ-secretase inhibitor (14).

Wnt – two pathways have been identified: the canonical one is β-catenin dependent and involved in cell fate determination. The non-canonical pathway is β-catenin independent and involved in cell movement and polarity. Wnt signalling is initiated by soluble ligands released by neighbouring cells. In cancer, gain of function and loss of function mutations, regulation by methylation and histone modification of this pathway have all been observed (9, 15, 16). Its inhibition in CSCs is a main area of cancer therapy research (17). However, the Wnt/β-catenin pathway is also a key regulator of T cell development and activation. Wnt/β-catenin signalling is crucial for CD8+ memory T cell development (18), while agonists of the pathway improve immunotherapy outcomes (19).

The ***Hedgehog*** (Hh) signalling pathway has been implicated in tissue homeostasis and repair and epithelial to mesenchymal transition (EMT) in normal tissues. In cancer, aberrant signalling such as overexpression of its ligands, loss of function of the receptor and dysregulation of transcription factors promote tumorigenesis and tumour progression. Hh signalling can also be canonical and non-canonical, and triggered by a variety of factors in the tumour microenvironment, such as TGF-β, TNF-α, and IL-6. Inhibition of Hh signalling is

also undergoing intense investigations for cancer treatment (20). Hh signalling is relevant in immune cell development and function, although its effect on peripheral T cell function is controversial (21-24). As it is also involved in myeloid derived suppressor cell (MDSC) function (25), Hh inhibitors may deliver additional benefits.

As there is a considerable overlap between these pathways, single targeting is unlikely to achieve a physiologically relevant level of inhibition. Furthermore, the fact that they are also involved in normal tissue homeostasis and development, including immune cell behaviour and peripheral effector function, makes their targeting a difficult challenge.

3. Identification and isolation of CSCs/TICs

(a) Surface marker-based identification

CSCs/TICs are typically isolated based on their expression of proteins shared in common with healthy stem cells. The markers most commonly used across solid tumours to identify CSCs/TICs are CD133, CD44, IL-6R, CD24, EpCAM, Lgr5, CD166 and CD29, alone or in combinations. The use of these markers is relatively conserved across the spectrum of solid cancers. However, there are technical considerations which may give rise to false positives or inconsistencies in the results including subjectivity in flow cytometry gating, the use of cell lines vs. primary cells, confirmation of function in clonogenic cultures and animal models. For some of these markers there is evidence for direct stem cell-like function, while recently the validity of some, as bona fide CSC/TIC markers, has been called into question, as discussed later. A few common markers are discussed below.

CD133

CD133 (Prominin-1) is a five-transmembrane glycoprotein used to identify CSCs/TICs in prostate, pancreatic, colon and liver cancers and glioblastoma (5). Although the precise function of CD133 has not been elucidated, it is known to bind cholesterol and is localised in protrusions of the membrane e.g. in villi and cilia. Despite its initial acceptance as a CSC/TIC marker, in some instances cells expressing this marker have not demonstrated exclusive tumour initiating ability (26, 27). CD133 is also present in a number of adult tissues including the kidneys, pancreas and colon (28, 29) and it is used as a marker for haematopoietic stem cells. Thus it is important to acknowledge that it is not a universal CSC marker nor is it a cancer cell specific antigen.

Some of the inconsistencies observed in the application of CD133 as a CSC/TIC marker may be associated with its pattern of expression and the antibodies used to detect it (30). The most commonly used antibodies for CD133 detection are mouse monoclonal antibodies ‘CD133/1’ and ‘CD133/2’, which detect the epitopes AC133 and AC141 respectively. These epitopes are distinct of each other and both are glycosylated. Different glycosylation status of CD133 across different tissues may give rise to false negatives. Glycosylation status is also suggested to change as a result of differentiation in some lineages (31, 32),

although this may be advantageous in the specific detection of early progenitor cells. However, a number of studies have shown that AC133 epitope expression (as detected by the CD133/1 antibody) doesn't correlate with CD133 protein or mRNA levels (32). The functional outcome of the loss of this epitope upon differentiation is unclear.

CD44

CD44 is used to identify CSCs/TICs in breast, prostate, colon, head and neck and pancreatic cancer. CD44 is a transmembrane glycoprotein that functions as a receptor for hyaluronic acid. It has a multitude of physiological and pathological functions including adhesion and migration, proliferation, growth and survival. However, CD44 is widely expressed in healthy tissues and in multiple cell types in the cancer microenvironment, making it difficult to apply as a specific CSC/TIC marker. CD44 is subject to alternate splicing and it has been suggested that CD44 splice variants 'CD44v' specifically identify cells with greater tumorigenic potential compared to cells expressing 'CD44s', the standard isoform (33, 34). Additionally, certain splice variants have been suggested to have pathological functions in colon and pancreatic cancer (35) and have prognostic utility in other cancers including NSCLC, AML and gastric cancer (34). $CD44^{high}$ cells were shown to be less immunogenic than $CD44^{low}$ tumour cells in head and neck cancer, partially via enhanced expression of the programme cell death ligand -1 (PD-L1) (36). A Phase I trial of a humanised IgG1 antibody, targeting the extracellular hyaluronic acid binding domain of all CD44 isoforms (37) in heavily pretreated cancer patients, proved to be safe but had only modest clinical effects. On the other hand however, CD44 also regulates Th1 cell survival, memory function (38), T cell IL-17 and IFNγ production thus its targeting may impair anti-tumour immune responses (39).

IL-6R

IL-6 has been shown to enhance stemness markers (Notch, Lgr5 and Oct-4) in colon cancer (40) and the survival and tumorigenicity of cancer stem cells identified as $ALDH^{high}/CD44^{high}$ in head and neck carcinoma (41). Inhibition of IL-6 signalling with an IL-6R inhibitor antibody (tocilizumab) prevents human CSC-mediated tumour initiation (41). Similar observations were made in an NSCLC cell line, where inhibition of IL-6 or IL-6R, separately or in combination, significantly inhibited CSC proliferation and growth (42). In colon cancer, blocking IL-6 receptor with a monoclonal antibody reduced spheroid formation, stem cell-related gene expression and enhanced resistance to chemotherapy by 5-fluorouracil (40). However, IL-6R also plays an important role in naïve and central memory T cells, regulating their survival, proliferation and effector function while also blocking Treg function (43).

Further markers

There is a huge array of further markers that have been used alone or in combination in a variety of cancers to identify CSCs/TICs. They include CD24, EpCAM, Lgr5, CD90, CD117, CD166, CD29, CD177, ESA, AFP, Nestin, CXCR4, and SSEA4. However, the plasticity of these markers means that they can be upregulated on cells originally not expressing them (44). Their heterogeneous

expression throughout the tumour tissue (45) has also been observed, making their isolation more difficult due to being a moving target. From the point of view of our review, some of these markers, when unique or overexpressed, can serve as CSC/TIC-associated antigens for T cell recognition, as discussed later.

(b) Metabolism-based identification

Aldehyde dehydrogenase

The aldehyde dehydrogenase (ALDH) family consists of 19 genes (in humans) that express enzymes which catalyse the oxidation of aldehyde (46). Aldehyde oxidation is required to metabolise many physiological substrates, such as vitamins, lipids and amino acids (46). These enzymes also catabolise aldehydes derived from pharmacological substrates; overall they have a protective detoxifying effect. High ALDH activity is associated with both normal stem cells and CSCs. ALDH activity has been used to identify CSCs in breast, colon, head and neck, pancreatic and liver cancer. It is measured using the flow cytometry-based ALDEFLUOR assay. An advantage this assay has over phenotypic detection methods is the direct readout of a fluorescent signal, based on enzyme activity. This may be less susceptible to inconsistencies encountered by antibody-based staining, such as epitope downregulation or masking, or expression of splice variants. The ALDEFLUOR assay mainly measures the activity of the ALDH1 family, as the inhibitor DEAB used in the assay is a specific inhibitor of ALDH1 (47). However, this may mean that the frequency of ALDH high cells is underestimated in tissues in which the predominant ALDH isozyme is not of the ALDH1 family.

ALDH activity has been used to identify CSCs in a number of cancer types. In prostate cancer, $ALDH^{high}$ population frequency varies from 1.2%-8.3% in the classic prostate cancer cell lines (DU145, PC3, LNCaP and 22RV.1) while cell lines derived from metastatic prostate cancer cells had even higher $ALDH^{high}$ frequency; up to 30% (48, 49). $ALDH^{high}$ PCa CSCs have demonstrated greater clonogenicity and migration *in vitro*, expression of stemness-associated genes, *in vivo* tumour initiation (49-51) and induction of metastasis (48). ALDH activity has been correlated with the expression of other CSC or clinical markers; CD44, EpCaM and integrin expression were significantly higher in $ALDH^{high}$ compared to $ALDH^{low}$ cells in one report (48), while CD44 and α_2-integrin levels didn't differ in another PCa cell line (50). CD133 was not detectable in both of these studies; in primary samples it was detected in freshly isolated cells but was greatly reduced upon passaging of the cells (52). In ovarian cancer, both ALDH activity and CD133 expression were detected in primary specimens. Upon passage of the primary cells, CD133 expression was reduced but it could be rescued by 'CSC' culture conditions i.e. sphere culture and serum free conditions (53). $ALDH^{high}$ ovarian cancer cells, which range in frequency from 0.1 to 7.9% in cell lines and 1-7% in primary samples, demonstrated tumorigenicity *in vivo* (53, 54). High ALDH activity has been used to identify both stem and cancer stem cells in breast cancer with frequencies of 8% and 4% $ALDH^{high}$ cells respectively(55). $ALDH^{high}$ $CD44^{+}$ $CD24^{-}$ and $ALDH^{high}$ $CD44^{+}$ $CD133^{+}$ breast cancer cells demonstrated greater tumorigenicity than the corresponding low/

negative populations (56). Conflicting evidence exists for the utility of ALDH activity as a CSC marker in lung cancer (57). In one study, both $ALDH^{high}$ and $ALDH^{low}$ cells (from a single cell line) were capable of initiating tumours *in vivo* (58) while in another, $ALDH^{high}$ but not $ALDH^{low}$ cells from two different cell lines demonstrated tumorigenicity *in vivo* (59). One further study using 8 lung cancer cell lines identified STAT3 signalling as a mediator of ALDH3A1 activity and demonstrated tumorigenicity in two cell lines (60). This suggests that variability in cell lines could lead to rejection of a potentially applicable CSC marker and highlights the importance of testing in multiple cell lines/ primary tissues.

ALDH expression has also been correlated with clinical outcome in a number of cancers. High ALDH expression (as well as the presence of other CSC markers) correlates with poor prognosis in pancreatic cancer (61) and both in serous (n=62) and clear cell (n=37) ovarian carcinoma cases (54) and in 112 serous carcinoma cases in a further study (62). In prostate cancer, ALDH expression correlates with more advanced stage, compared to localised cancer and BPH (63), while there seems to be no consensus on the utility of ALDH activity for the selection of tumorigenic melanoma cells (64).

ALDH has been shown to contribute to resistance mechanisms to radiation therapy and chemotherapy, thus a different, more specific, therapeutic approach must be undertaken. Some groups have investigated chemical inhibitors (47); such drug development requires the design of highly specific inhibitors as the widespread expression of ALDH in healthy stem cells may result in off-target effects. It is also possible that functional redundancy within the large isozyme family could compensate for inhibition of one ALDH target. In the immunotherapy setting, $ALDH^{high}$ CSC-loaded dendritic cells have been used successfully in two *in vivo* melanoma models (65, 66). ALDH activity has been demonstrated in the regulatory T cell immune subset in the transplantation setting (67); thus ALDH targeting may also have an anti-tumour effect via affecting the Treg subset.

Glycolytic activity. CSCs/TICs have fewer mitochondria thus they are more glycolytic than differentiated tumour cells, as shown in melanoma, breast, lung and liver carcinomas (68-71). This can be detected by reduced mitochondrial activity, perinuclear mitochondrial distribution, lower intracellular concentrations of ROS and ATP and lower amounts of mitochondrial DNA in CSCs/TICs (72).

CSC/TIC inhibitors targeting either self-renewal pathways, surface markers or enzymes have potential off-target effects on other cell types. Figure 1 illustrates the potential side effects of some of these inhibitors on immune cells.

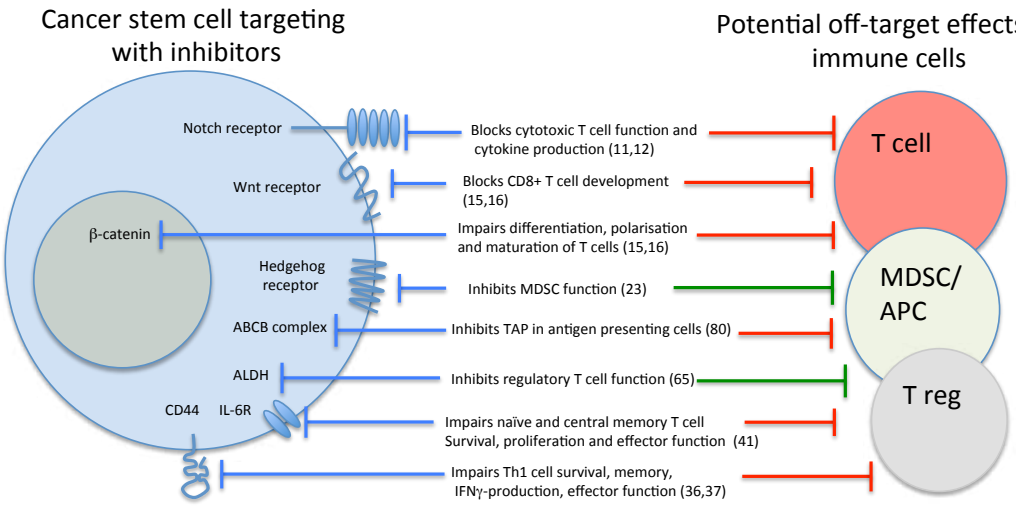

Figure 1. Targeting stem cell pathways, functions or markers may have off-target effects on immune cells. The red blocking signs on the right represent negative effects by inhibiting immune responses, while the green ones indicate positive effects by blocking immunosuppressive cells.

(c) Function-based identification

Slow cell division of CSCs/TICs that exist mostly in G0 phase can be detected by their characteristic of retaining dyes that normally become diluted during proliferation, such as PKH, BrdU or CFSE. Dye-retaining cells that give progeny to xenotransplants have been observed in glioma, melanoma, breast and pancreatic cancer (73-77).

Enhanced drug resistance and detoxifying pathways: the ATP-binding cassette (ABC) transporter family of proteins, especially ABCB1, ABCC1, ABCG2, and ABCB5, are active in cancer stem cells, but switched off during differentiation (78). These transporter proteins are extremely efficient in pumping out complex molecules from the cytoplasm, thus protecting the cells from exogenous toxins, including numerous chemotherapeutic drugs. They have a wide range of substrates, such as peptides, lipids, hydrophobic drugs, polysaccharides and proteins (79). Their targeting with specific inhibitors is one of the active areas of drug development in cancer (80). As CSCs/TICs also exclude hydrophobic Hoescht dyes via this mechanism, they can be identified by forming a side population (SP) based on low dye levels (81). However, ABCB proteins, such as TAP, also have an important role in intracellular peptide trafficking across membranes, with crucial involvement in major histocompatibility complex (MHC) class I antigen presentation and dendritic cell function (82). Thus, the off-target effect of tumour ABCB targeting may be deleterious for generating efficient anti-tumour T cell responses.

Enhanced resistance to radiotherapy. It has been speculated that the failure of radiation therapy correlates with the survival of at least some of the CSCs/TICs in the tumour, although the underlying protective mechanisms are incompletely understood. Radiation causes direct damage to the DNA, such as single or double strand breaks or acts indirectly via reactive oxygen species (ROS). The

consequence of radiation damage can be temporary or permanent cell cycle arrest, mainly via checkpoint kinase activity (Chk-1 and Chk-2), which allows time for DNA damage repair (DDR) to take place. If the damage is irreparable, DDR mediates senescence or cell death (83). ROS mediates oxidative stress, which, if beyond the capacity of the cell's antioxidant defence mechanism, may also lead to cell death. Both DDR and the ROS scavenging system have been reported to be highly efficient in CSCs/TICs in numerous solid cancers (84), contributing to their intrinsic radioresistance. Extrinsic radioresistance may be supported by the localisation of CSCs. It has been suggested that CSCs/TICs residing in hypoxic areas are more resistant to radiation-induced damage than cells in normoxic areas. Hypoxia-inducing factors HIF1α and HIF2α may lead to the activation of the Notch, Wnt and Hedgehog signalling pathways, which are essential for CSC/TIC maintenance. There is clinical evidence that radiotherapy is generally less successful in CSC-rich laryngeal cancer where CSCs were identified as a residing subset within the $CD44^{high}$ population (85).

However, the effects of hypoxia are far from inhibitory on immune cell function. Activated dendritic cells become glycolytic and their long-term survival is regulated by HIF1α (86). Effector T cells are also resistant to hypoxia – even more, their cytolytic machinery is turned on by HIF2α (87). As effector T cells also display glycolytic characteristics, they can exert their effector function even in the depth of tissues with poor vasculature, maybe where CSCs/TICs reside. Furthermore, we have shown that low dose radiation spares effector and memory T cells as compared to naïve T cells (88). Thus, combination of radiation with T cell targeting of stem cells sounds like a two-pronged attack with potential synergistic effects.

High dose radiation alone may eliminate CSCs/TICs, as early prostate and lung cancers can be cured by radiotherapy alone. It may partly happen via generating immunogenic cell death, which then initiates tumour antigen uptake and antigen cross-presentation by dendritic cells (89). One could speculate whether radiation-induced CSC death is also immunogenic and weather radiation would generate CSC/TIC-specific effector and memory T cells, significantly contributing to an abscopal effect and subsequent protection from relapse. Despite its obvious clinical importance, this question has not been studied before.

4. Immunological characteristics of CIC/CSC

Immunosuppression

Proliferative T cell responses and IL-2 production were inhibited by CSCs/TICs in gliobastoma (90) and melanoma (91) in vitro. Treg frequencies were also increased in melanoma (91) but not in glioblastoma CSC–T cell co-cultures (90). Secretion of TGFβ, IL-10, IL-4 and IL-13 by CSCs/TICs has been shown to have immunosuppressive effects on NK cells, T cells and antigen presenting cells (90, 92, 93). Cell surface molecules, such as CD200, expressed on CSCs/TICs (94), can also dampen immune responses. CD200 overexpression has been associated with the suppression of Th1 responses, decreased neutrophil infiltration and increased IL-10 production induced by the tumour, as shown in a breast

carcinoma model (95). PD-L1 is often overexpressed on tumour cells, with a function of promoting tumour glycolysis. Upregulation on CSCs/TICs is probably tumour type or localisation dependent, as e.g. hypoxia is one of the triggers that can upregulate PD-L1 (96). High expression on CSCs/TICs has been reported on head and neck carcinoma (36), on CD133+ colorectal (97) and gastric (98), but not on melanoma CSCs/TICs (91).

Immune resistance

Some extent of MHC Class I downregulation has been shown on glioblastoma CSCs/TICs compared to non-CSCs. Nevertheless, these CSCs/TICs were still able to induce autologous T cell responses in vitro. Furthermore, IFNγ-treatment enhanced the susceptibility of CSCs to T cell-mediated immune responses (90).

Cancer vaccines, that generate T cell- and antibody-responses against tumour-associated antigens (TAA), work well in pre-clinical preventative models, however, the results obtained with therapeutic vaccines in the clinic are rather disappointing. Focusing on CSCs, it is feasible that stem-like cells are inherently resistant to T cell attack, although lack of vaccine specificity for CSC/TIC-antigens is also a possibility. Development of stem-like features, such as Nanog in surviving tumour cells following vaccination has been observed (99). Resistance to T cell killing was successfully abolished by silencing Nanog in these cells. This work suggests that stem-like features may develop in a population of cells under immunological pressure, however, it does not show inherent stem cell resistance.

The argument that cancer stem-like cells may not be inherently resistant to immune attack is also supported by using purified CSCs/TICs as a vaccine (66). This treatment generated T cells in immunocompetent mice that were highly efficient at killing CSCs/TICs and provided greater protection in the D5 melanoma model against pulmonary metastasis than vaccination with unseparated cells. As a further example to prove T cell susceptibility, CD33+ brain tumour CSCs/TICs, transfected with the pp65 antigen of human cytomegalovirus, were efficiently killed by virus-specific memory T cells (100), indicating the feasibility of CSC/TIC targeting by T cells.

5. Potential immune targeting of CSCs/TICs

a) Antigen non-specific immune targeting

NK cells

As MHC Class I molecule expression is often lower on CSCs/TICs than on the bulk of tumour cells, CSCs/TICs are more likely to be susceptible to NK cell-mediated killing. However, they also often lack NK-activating ligands, such as NKG2D, as shown in brain and breast cancers (101, 102). On the other hand, glioma, oral squamous cell carcinoma and colorectal cancer CSCs/TICs (103-105) have been reported to express various ligands for NK cells (most frequently PVR which is

recognised by DNAM-1) and are highly susceptible to NK cell killing. However, while cytokine-activated NK cells have efficient CSC/TIC killing ability, freshly isolated NK cells from the same patient do not kill CSCs (103). This points towards microenvironmental regulation of NK cell activity in cancer patients.

γδ T cells

γδ T cells also exhibit MHC-unrestricted lysis of targets, including tumour cells. Vγ9Vδ2 T cells can be activated with phosphoantigens or aminobisphosphonates and have been shown to efficiently kill CSCs/TICs in colon, ovarian and breast cancer models, especially after treatment with Zoledronate (106-108). They have also been observed infiltrating the tumour tissue, emphasising their physiological relevance.

b) Antigen-specific targeting by T cells

Solid cancer cells and their CSCs/TICs express HLA Class I but not HLA Class II molecules. Their efficient targeting by CD8+ T cells depends on a sufficient level of HLA Class-I molecule expression and intact antigen presenting machinery in these cells. CSC/TIC resistance or susceptibility to T cell killing has both been reported, depending on tumour type, origin of cells and culture conditions. Tissue-derived CSCs/TICs from colon cancer were shown to express lower levels of MHC Class I molecules than non stem-like cells (105).

Some TAA, such as MUC-1 or CEP55, are expressed equally in both CSCs/TICs and non-CSCs/TICs (109, 110), while others, such as the olfactory receptor family 7 subfamily C member 1 (OR7C1) are dominantly expressed in CSCs/TICs (111). The former TAA are classified as shared antigens and the latter are functionally linked to cancer stemness because their expression ceases after CSC differentiation into non-CSCs. T-cell responses to shared TAA could achieve temporary tumour-control but it may lead to tumor escape by inducing loss of antigens that are not necessary for cellular fitness (immunoediting) (112). Multiple antigen targeting, incorporating those TAA that are specifically expressed in CSCs/TICs is much more likely to result in therapeutic success in clinical settings (113), as indicated in Figure 2. Although it may be crucial to have high frequencies of CSC/TIC-specific T cells present in the tumour tissue, breaking the localized immunosuppressive milieu is likely be helped by activated infiltrating T cells of multiple specificity.

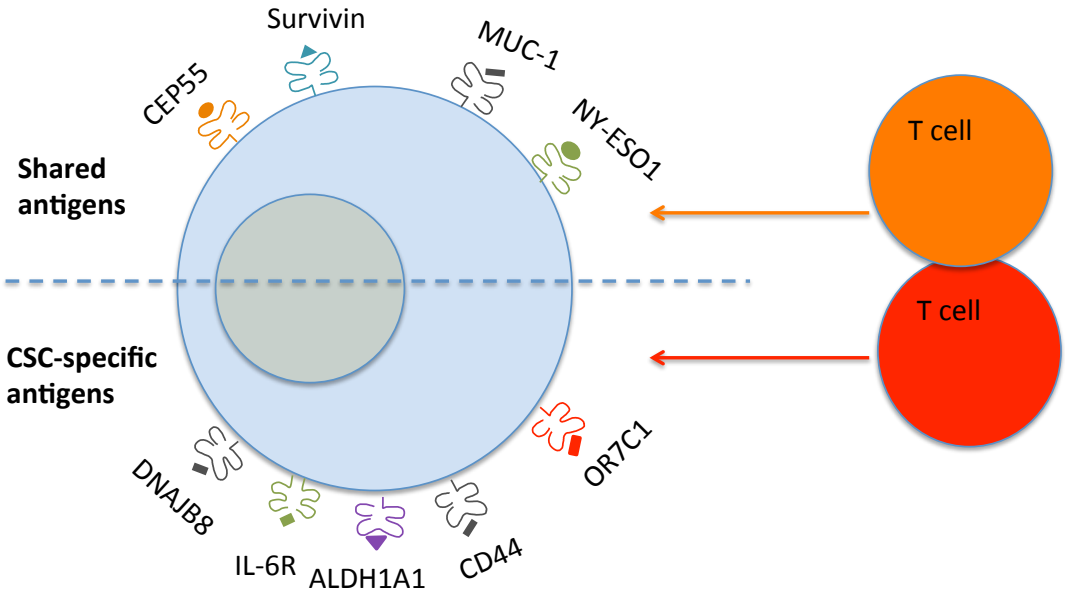

Figure 2. Simultaneous targeting of shared and CSC/TIC-specific antigens by antigen-specific T cells can eliminate CSC/TIC-like cells and differentiated tumour cells alike.

The main types of TAA, expressed in CSCs/TICs and can be targeted by T cells, are summarised below.

Cancer/testis (CT) antigens

CT antigens are expressed only in germ cells, however, they have been shown to be re-expressed in some malignancies. This cancer-specific expression makes these, and the onco-fetal antigen group (see next), therapeutically highly relevant compared to other types of TAA that are discussed later. Germ cells do not express MHC Class-I molecules, thus deletional tolerance may not occur during the negative selection phase of T cell development against these antigens. CT antigens have oncogenic functions, such as supporting tumour growth, enhancing treatment resistance and facilitating metastasis (114). There are more than 100 gene families of CT antigens listed on the Ludwig Institute of Cancer Research's website (http://www.cta.lncc.br/), such as MAGE, BAGE, GAGE, XAGE, SPANX, NY-ESO1, etc. (115). Because of their unique expression pattern, they often serve as TAA for immunotherapy trials. Interestingly, a transcriptome analysis of side population (SP) vs. main population (MP) cells from colon, breast and lung cancer cell lines revealed that 18/74 of these antigens are preferentially expressed on CSCs/TICs (116). Novel CT antigens have recently been identified by transcriptome analysis, such as the DNAJ Hsp40 homolog, subfamily B, member 8 (DNAJB8), in SP cells of renal cancer cells (117). OLF7C1 is also a novel cancer-testis antigen, observed on SP cells of colorectal cancer. It has been successfully targeted by HLA A24-restricted T cells (111). Another CT antigen, the brother of the regulator of the imprinted site (BORIS), subfamily 6, was found preferentially expressed on cervical cancer CSCs/TICs. It has a role in maintaining CSC function and serves as a target for BORIS-specific cytotoxic T cells (118).

Oncofetal antigens

These antigens are typically only expressed during embryonic development in fetal tissues, but similarly to CT antigens, they can be re-expressed in some cancers. A typical example, carcino-embryonic antigen (CEA) is expressed in numerous solid cancers but not associated with stem-like cells. Other antigens, such as stage specific embryonic antigen 3 and Globo-H are expressed on stem-like and non stem-like breast cancer cells at similar rates (119), while 5T4 is preferentially expressed on CSC-like cells in lung cancer and head and neck cancer (120) (121). 5T4 expression, in both malignancies, is associated with poor prognosis. Xenograft models indicated that the CSC-fraction can be reduced, tumour progression halted and local recurrence inhibited by treatment with an antibody-drug conjugate targeting 5T4 (120, 121).

Overexpressed antigens

This group of antigens has recently been reviewed by Hirohashi et al. (122) and includes apoptosis-resistance genes, such as survivin, proto-oncogenes, such as HER-2, CEP55, SOX-2 and COA-1 (90) and stress-response related genes, such as heat shock protein (Hsp) or HOX genes (123). Some of the overexpressed antigens are general stem cell- and not exclusively cancer stem cell-specific. In prostate cancer, Numb has been identified as a potential target for controlling tumourigenesis (124). Numb is lost in differentiating tumor cells, due to exaggerated ubiquitination and subsequent proteasomal degradation, but its expression is maintained in stem-like cells. Its close homologue, Numb-like (NumbL) is able to regulate the CSC/TIC pool by inhibiting the Notch pathway (125). Numb-1- and Notch-specific T cells eliminated luminal CSC/TIC-like cells in a breast cancer model (126). In a gynecological cancer (endometrioid adenocarcinoma), $ALDH^{high}$ cells preferentially expressed Hsp27, the overexpression of which is associated with poor prognosis (127). MAPK13, PTTG1IP, CAPN1 and UBQLN2 were also found highly expressed in these cells compared with that in $ALDH^{low}$ cells (128). SOX2, a transcription factor that regulates the Wnt/β-catenine pathway, is amplified in numerous solid tumours and expressed predominantly in CSCs/TICs (129, 130). Interestingly, class I histone deacetylase (HDAC) inhibitors increase the frequency of SP cells with CSC/TIC markers, due to de-differentiation of cancer cells. HOXA5 was shown to be the main transcription factor responsible for de-differentiation and induction of SOX2 in lung cancer cells (131).

Differentiation antigens

Many differentiation antigens are expressed in primary cancer cells as well as in CSC, such as MUC-1 in breast cancer, tyrosinase and gp100 in melanoma. The human telomerase reverse transcriptase (hTERT) is also a target in primary cancer cells and in stem cells. T cells have been generated against an HLA-A3-restricted epitope: KLFGVLRLK (132). Successful targeting of these antigens has the potential of eliminating CSCs and non-CSCs alike.

Neoantigens

Although CT and onco-fetal antigens can also be considered as neoantigens, this category typically includes mutated tumour antigens. The mutations may generate entirely new T cell epitopes for which high affinity T cell receptors have not been deleted during development and thus these T cells can be efficiently expanded. High mutation index has been indicated to be the underlying mechanism behind successful immune checkpoint inhibitor therapy (133). Although it is yet unclear weather the mutation index of CSCs is different from that of primary cancer cells, there is data available that neoantigens are present in colorectal cancer both in CSCs and non-CSCs in a manner that is targetable with T cells (134). Identifying CSC-specific neoantigens may lead to further breakthroughs in cancer immunotherapy.

6. Immune targeting CSCs/TICs – clinical applications

Antigen-specific immunological targeting of CSCs/TICs requires either the generation of primary T cell responses, reactivation of memory responses or the adoptive transfer of engineered antigen-specific T cells into the host.

Generation of T cell responses:
Most clinical trials, listed on the http://clinicaltrials.gov website that use immunotherapy and target cancer stem cells are based on isolating CSCs/TICs from solid tumours and loading them onto dendritic cells, which are then used as a cancer vaccine. The approach is based on pre-clinical data showing the success of this approach in immunocompetent mice (66). Trials are listed in pancreatic, nasopharyngeal, colorectal, ovarian, lung, liver and brain tumours. One trial studied the outcome of vaccination with DC, transfected with hTert and survivin as amplified ovarian CSC mRNA (NCT01334047). The stem like cell-associated antigen(s), serving as a vaccine in these trials, are undetermined and likely to be individual patient-specific. The outcome of these trials is not yet publicly available, however, as with non CSCs-DC vaccines, it is unlikely that any monotherapy alone will be hugely effective. The explosion of approved immunotherapies with checkpoint inhibitors opens up the way for designing combination therapies. However, a shift in treatment design must be considered: vaccines or redirected T cells should be targeting multiple antigens, including CSC-specific ones; this treatment then should be combined either with appropriate chemotherapy based on patient stratification data, or high dose radiation and/or immune checkpoint inhibitor treatments in order to provide the best chance for generating robust and long-lasting T cell responses leading to tumour rejection.

T cell transfer:
The success of CAR T cells in treating hematological malignancies (https://www.fda.gov/NewsEvents/Newsroom/PressAnnouncements/ucm574058.htm) generated interest in using the approach against solid tumours and CSCs as well. CAR T cells express a chimeric antigen receptor (CAR) which consists of an extracellular binding domain of a single-chain fragment of the antibody variable

region (scFv) providing antigen-specificity, and the intracellular signalling domains of CD3-zeta chain. The receptor can be coupled with co-stimulatory molecules, such as CD28 and CD137. Cell surface antigens, expressed on CSCs, such as CD44, CD133, aldehyde dehydrogenases (ALDH) and EpCAM can be targeted this way. Preclinical work using these CAR T cells in glioblastoma, prostate cancer and gynecological tumours have been encouraging (reviewed by Guo (135)). Clinical trials are currently ongoing against EGFR and CD133 (NCT01869166 and NCT02541370).

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