

EXPERT OPINION

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Immunotherapy of cancer stem cells in solid tumors: initial findings and future prospective

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Introduction: Conventional chemotherapies seemed to have reached a therapeutic plateau in the treatment of solid tumors and many metastatic diseases are still incurable. Events of chemo-resistance and relapses appear to be sustained by a subset of putative cancer stem cells (CSCs). New anticancer strategies need to face this new challenge exploring their efficacy against CSCs. Immunotherapy has raised enthusiasms in cancer therapy and its potential against CSCs is an intriguing field of research.

Areas covered: In this work we reviewed the immunotherapy approaches directed against CSCs in solid tumors. We schematically divided adaptive immunotherapy strategies, mainly based on dendritic cell-vaccination, and strategies exploiting MHC-unrestricted effectors like natural killer cells, $\gamma\delta$ T lymphocytes and cytokine-induced killer cells. Findings, strength and limitations of these models are discussed and compared highlighting their potential clinical relevance.

Expert opinion: The important biologic role and clinical relevance of CSCs introduced a 'noble target' for immunotherapy and cancer treatments in general. Initial evidences suggest that CSCs may be susceptible to various types of immunotherapy attacks, overcoming their chemo-resistance. Investigation of important safety issues, based on shared features with 'normal' stem cells, along with intriguing synergisms with modulatory agents are open challenges for the next future and effective clinical translation.

Keywords: cancer immunotherapy, cancer stem cells, cell therapy, solid tumors

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1. Introduction

1.1 History of cancer stem cells

Accumulating evidences suggest that a subpopulation of tumor cells with distinct stem-like properties is responsible for tumor initiation, invasive growth and metastasis formation. This population is defined as cancer stem cells (CSCs).

The discovery of human CSCs has important diagnostic and prognostic implications and holds significant promise for the development of novel therapeutic strategies [1].

The concept that cancer might evolve from a small population of cells with stem cells properties was proposed approximately 150 years ago with observations suggesting that tumors arise from embryo-like cells [2,3]. Later on it was formalized by Cohnheim and Durante the 'embryonal rest' theory of cancer, enouncing that adult tissues contain embryonic, generally dormant, remnants that could be activated to become cancer [4].

In 1963, Bruce *et al.* observed that only 1 – 4% of lymphoma cells can form colonies *in vitro* or initiate carcinoma in mouse spleen [5].

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Article highlights.

- Cancer stem cells (CSCs) sustain chemoresistance and relapses of solid tumors.
- Immunotherapy holds potential for effective targeting of CSCs and overcoming their chemoresistance.
- Pre-clinical immunotherapy strategies based on dendritic cell vaccination are capable of eliciting effective immune responses against CSCs *in vitro* and *in vivo*.
- First, clinical trial of therapeutic vaccine against autologous glioblastoma CSCs confirmed the safety and initial activity of this approach.
- Pre-clinical evidences demonstrate the activity of MHC-unrestricted effectors (natural killer cells, $\gamma\delta$ T-lymphocytes, cytokine-induced killer cells) against CSCs of various solid tumors.
- Synergism with chemotherapy and modulator agents like zoledronate may potentiate the immunotherapy effect against CSCs.

This box summarizes key points contained in the article.

CSCs were first described in 1994 by John Dick and colleagues, who identified a subpopulation of leukemic cells with stem-like properties in patients with acute myeloid leukemia [6]. This study provided the paradigm for the general CSC model, followed by demonstration that these cells had the ability to self-renew, proliferate and differentiate [7]. The first report of CSCs in solid cancer came in 2003 from Al-Hajj, who demonstrated the presence of CSCs in breast cancer [8,9]. To date, CSCs have been discovered in a broad spectrum of solid tumors, including lung cancer [10,11], colon cancer [12,13], prostate cancer [14], ovarian cancer [15-17], brain cancer [18,19], melanoma [20-22], skin squamous cell carcinoma [23], head and neck cancer [24] and pancreatic cancer [25].

1.2 Biological features and identification issues

Common features of putative CSCs are considered: i) self-renewal capacity—the CSCs subpopulation can be serially transplanted through multiple generations, indicating the self-renewing capacity; ii) differentiation ability—pluripotent CSCs can generate tumorigenic daughter CSCs, by symmetrical cell division and also generate bulk populations of non-tumorigenic cells by asymmetrical cell division; iii) tumorigenicity—a small subpopulation of CSCs have tumorigenic potential when transplanted into animals; and iv) expression of specific surface markers, by which the CSCs subpopulation could be enriched. CSCs are relatively quiescent with slow proliferation rates [26,27].

Experimental speculations suggest that CSCs may arise from normal stem cells, progenitor cells or more differentiated cells [28]. The biological process underlying the generation of CSCs may include multiple gene mutations as a result of their genomic instability [29] or oncogene-induced plasticity [30]. The genetic and epigenetic instability of these cells may favor the accumulation of mutations that enable the ability of self-renewal and tumorigenicity. Within tumor

microenvironment CSCs may be considered as a functional entity, with the possibility of transition events from differentiated cells into CSC-phenotype upon various stimuli [31,32].

A CSC niche is present in various tumor types by showing a close association between CSCs and stromal cells [33]. An important function of CSC ‘niche’ is to regulate the balance between cellular self-renewal and differentiation. Overall, vascular niche may be considered as a specialized microenvironment that, through paracrine-signaling interactions, control CSC proliferation and fate determination [34].

CSCs can be identified and isolated using different methodologies. They can be isolated by flow cytometry, based on cell surface markers [11-14,35,36], detection of side population (SP) phenotypes by Hoechst 33342 exclusion [37,38] and the ability to grow as floating spheres in serum-free medium [39]. They can be functionally characterized by chemoresistance, multipotency, tumorigenicity, stem gene expression and aldehyde dehydrogenase activity [40-43]. Different cell surface antigens have been identified in several cancers as candidate CSC biomarkers (CD20, CD24, CD34, CD44, CD90, CD117 and CD133) [11-14,35-42]. However, no universal CSC markers exist; CSCs should be seen as a dynamic cell subpopulation, expressing and modulating multiple markers throughout the phases of tumor progression. Molecules reported as CSC markers have an operative value but cannot exclusively define CSCs [44]. Cell populations identified or sorted on the basis of a CSC marker will be enriched in cells with stemness features, but this will not exclude the presence of CSCs in the ‘marker-negative’ cell fraction. Stem cells should be functionally assayed by the ‘stem cell activity’, and the most reliable way seems to replicate the heterogeneity of the tumor in syngeneic or immunodeficient mice by serial transplantations [45]. Relatively to this last issue, it is important to precise that animal models still present important limitations affecting the correct appreciation of CSCs or their frequency. Modifications to xenotransplantation essays were reported to significantly enhance the ability to detect tumorigenic CSCs up to 25% of bulk tumor population, challenging the concept of ‘extremely low frequency’ of CSCs and showing that they can actually be much more common than believed in certain cancers [22].

Functionally, CSCs are radiotherapy and chemotherapy resistant. In contrast to differentiated tumor cells, CSCs are relatively quiescent and have a slow cycling rate. These features protect CSCs against chemotherapeutics that are effective in targeting rapidly dividing cells [46,47]. Their radiation and chemotherapy resistance results from the presence of defense mechanisms, including the expression of ABC transporters and strong responses to DNA damage compared with their differentiated progeny [48-50].

Therefore, the CSC model could help explain essential and poorly understood clinical events, such as therapy resistance, minimal residual disease and tumor recurrence.

Important research efforts are currently directed at visualizing CSCs within cell cultures and *in vivo*.

Several studies have used dyes to differentiate between SP and non-SP cells (e.g., Hoechst 33342, Calcein, Vybrant1 DyeCycle™ Violet) including assays for determining ABCG2 inhibitors [51-54].

Another interesting methodology is based on PKH26 labeling of CSCs, demonstrating their asymmetric division as the dye irreversibly binds to the lipid bilayers on cell membranes and is equally distributed among daughter cells during each subsequent cell division [55].

An appealing approach involves the research and analysis of peculiar genes or molecular pathways reported to associate with stemness features. CSCs are characterized by high activity of the Notch, Hedgehog and Wnt pathways, and express high levels of Oct4 [56-58]. Moreover, the expression of the Oct4 gene seems to be reliably associated with putative CSCs of various histotypes [59-61]. Gene transfer strategies, based on plasmid or lentiviral vectors encoding the enhanced green fluorescence protein under control of the Oct4 or Nanog promoter, have been used to transduce bulk tumor cells and visualize CSCs exploiting their exclusive ability to activate stem cell-specific promoters [62-64].

None of the methods mentioned above have resulted conclusive to isolate solid tumor CSCs, highlighting the imperative to delineate more specific markers or to use combinatorial methodologies that may account for the functional definition of CSCs.

2. Targeting CSCs by immunotherapy approaches

Conventional chemotherapies seemed to have reached a therapeutic plateau in the treatment of solid tumors and many metastatic diseases are still incurable. Events of chemoresistance and relapses appear to be sustained by a small subset of putative CSCs.

New anticancer strategies need to face this new challenge exploring their efficacy in killing CSCs. From this angle the setting of solid tumors is probably more challenging than hematological malignancies. Direct targeting of CSCs might be problematic because normal and cancer SCs share many characteristics [65] with important safety implications. Similarities between normal stem cells and CSCs include self-renewal ability, production of differentiated progeny, common surface markers, oncogenes, signaling pathways and the strict functional interaction with stem cell niche [31-66]. Systemic treatments targeting these shared pathways may consequently harm normal stem cells with potential long-term negative effects, very difficult to be appreciated and quantified by current pre-clinical models [65].

Immunotherapy has recently raised enthusiasms in cancer therapy, and its potential against CSCs is an intriguing field of research. Immune effectors may succeed in overcoming some main chemoresistance mechanisms adopted by CSCs, like their proliferative quiescence or efflux pumps.

Several immunotherapy approaches are conceivable, either targeting precise MHC-restricted antigens or alternatively exploiting MHC-independent mechanisms. Pre-clinical activity against putative CSCs has been recently advocated in various tumor settings, using adaptive immunotherapy approaches or effectors belonging to the innate immunity like natural killer (NK) cells and $\gamma\delta$ T-lymphocytes or also with *ex vivo* expanded cytokine-induced killer (CIK) cells. The pre-clinical nature of these models presents limitations but provides crucial proof of concept that set the basis for further investigations and dedicated experimental clinical trials. Crucial issues under investigation are the expression and modulation by CSCs of MHC-restricted antigens and membrane expression of MHC-unrestricted target molecules. Main pre-clinical cell-immunotherapy approaches against CSCs in solid tumors are summarized in Figure 1.

Within this review we will focus on vaccines and adoptive immunotherapy approaches against CSCs in solid tumor models. We will discuss the initial results and their clinical prospective, schematically dividing approaches exploiting MHC-restricted cellular components of the adaptive immunity and strategies based on MHC-independent mechanisms for tumor killing.

2.1 MHC-restricted adaptive immunotherapy approaches

In the last years, few but important pre-clinical works explored the possibility to elicit, mostly by dendritic cell (DC)-based vaccination, adaptive immune responses against putative CSC in solid tumors. Relevant topics faced by these researchers were the presence of functional MHC molecules on CSCs along with tumor-associated antigens (TAA). Most of the findings derive from prostate cancer and malignant glioblastoma models providing important proofs of concept and relevant clinical speculations.

2.1.1 The prostate cancer model

The expression of both class I and class II MHC molecules, along with TAA, was demonstrated in CSCs generated from transgenic adenocarcinoma of the mouse prostate (TRAMP) mice, which spontaneously develop prostate cancer resembling the human pathology [67]. On these bases, prostate CSCs were effectively targeted by cytotoxic T lymphocytes (CTLs) *in vitro* and *in vivo* and an effective immune response could be elicited vaccinating mice with CSC-pulsed DC that induced cancer regression. Indeed, the same group reported that, along with TAA, prostate CSCs also express ligands recognized by NK and lymphokine-activated killer cells allowing effective recognition and killing by the innate immune system [68]. The same TRAMP model provided experimental evidence of protective immunity by vaccination with DC loaded with DNA encoding for the prostate stem cell antigen (PSCA) [69,70]. PSCA is considered a marker and potential suitable target antigen for prostate CSCs, upregulated also in other types of cancer; it has been associated with clinical

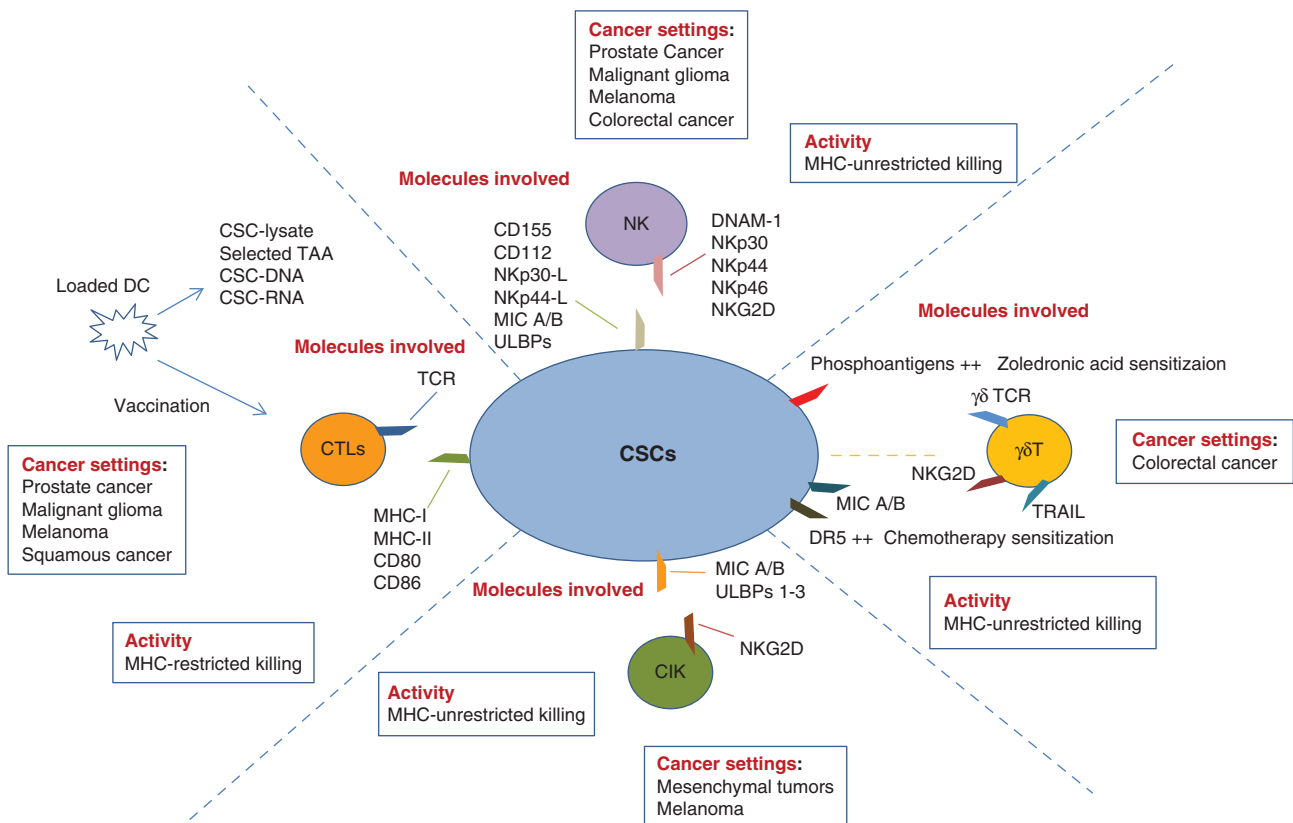


Figure 1. Pre-clinical immunotherapy models to kill CSCs. Solid tumors CSCs may be attacked by both MHC restricted and independent strategies. The figure reports a summary of the main immunotherapy approaches that have so far produced pre-clinical evidences of CSCs targeting in solid tumors.

CSCs: Cancer stem cells.

stages and metastasis of prostate cancer along with progression from premalignant to malignant lesions [70,71]. The protective immunity following PSCA-loaded DC vaccination was associated with prostate infiltration by CD4 and CD8 lymphocytes and upregulation of MHC molecules, providing mechanistic support to the reported protective effect [69].

These works represent important evidences that prostate CSCs are potential source of immunogenic TAA that can be exploited to redirect CTL-mediated immune responses. The reported susceptibility to both adaptive and innate immunity by prostate CSCs is in apparent contrast with the natural history of this cancer in patients, where the immune system does not seem to be ultimately capable of arresting the disease. This paradox, common to other immunotherapy models, highlights the existence in human patients of immunosuppressive and escapes mechanisms that prevent the effective elimination of CSCs.

2.1.2 The glioblastoma model

Similar to prostate cancer, important pre-clinical murine models recently reported the possibility to target CSCs of malignant gliomas by the adaptive immune system. Neurospheres enriched in CSCs were exploited to load DC and subsequently obtain a striking curative effect vaccinating

the animals; the effect was associated with infiltration by CD4 and CD8 T cells [72]. Interestingly *ex vivo* culturing of neurospheres seemed to increase the membrane expression of HLA class II and costimulatory molecules (CD80; CD86) on CSCs. This observation may have important implications in clinical perspective as a main hurdle of vaccine strategies is the lack of proper costimulation signals to overcome classic immune-escape mechanisms. In line with these findings, another research group confirmed that high-grade glioma CSCs were capable of antigen processing and presentation, expressed functional MHC class I and ICAM-1 molecules and were susceptible to CTLs-mediated lysis *in vitro* and *in vivo* [73]. These achievements were generated within murine models; it was subsequently demonstrated that human glioblastoma CSCs followed a similar pattern, being a valuable source of TAA capable of triggering effective CTL-mediated immune responses upon DC vaccination [74]. These findings are of course very important as it may be speculated that entirely murine pre-clinical models are not enough representative of human settings. The promising pre-clinical findings recently sustained a first clinical trial where patients were vaccinated against glioblastoma CSCs [75]. Seven patients, following standard radio-

chemotherapy treatment, were intradermal vaccinated with DC loaded with RNA extracted from autologous neurospheres. The procedure resulted completely safe without any significant adverse event. The vaccination induced *in vitro* detectable immune responses in all seven patients, against CSCs lysates and interestingly also against hTERT or survivin peptides *in vitro*, whereas only one patient had a positive delayed-type hypersensitivity reaction. The discrepancy between *in vitro* and *in vivo* responses could be attributed to the relative low-lymphocyte count of the patients due to temozolamide treatment following the vaccination course. Even if clinical activity was not the aim of this trial, the study provides interesting observations. Five out of seven patients had recurrent disease before immunotherapy treatment and their tumor grew in size during the first phase of vaccination reaching a maximum size of 805 mm³ (363 – 1526 mm³). Subsequently magnetic resonance imaging (MRI) reported tumor dimensions to decrease down to a minimum of 209 mm³ (9 – 452 mm³) after 448 days (342 – 568 days). Furthermore, considering a historical group of matched controls, vaccinated patients had prolonged progression free survival (694 vs 236 days, *p* = 0.0018). Even if not conclusive, these first clinical data support further randomized study and are important to confirm the feasibility and potential therapeutic effect of targeting CSCs.

Besides prostate cancer and malignant gliomas, few pre-clinical data suggest that adoptive immunotherapy may be active also in other tumor settings. Interesting data were reported vaccinating immunocompetent mice with DC loaded with CSC lysates from melanoma and squamous cancer cell lines. Protective immunity developed following vaccination and CSC specific antibody and CTLs were described [76].

Other investigations described low immunogenicity of CSCs in glioblastoma and colorectal cancer cell lines, with reduced expression of MHC class I and II molecules, deficient antigen-processing machinery and even inhibitory activity towards specific immune response with a central inhibitory role attributed to IL-4 production at CSCs level [77,78]. Interestingly the low immunogenicity of glioblastoma CSCs could be rescued by demethylating agents or IFN- γ , opening to investigations of potential synergic strategies.

Even if these data may appear in contrast with the positive pre-clinical findings described so far, they express the complexity of the issue and difficulties in finding representative and reliable pre-clinical models using cancer cell lines. It could be speculated that vaccinations using DC played a crucial role in overcoming the spontaneous low immunogenicity of CSC, providing proper costimulation signals and appropriate antigen presentation. The improved identification of immune-inhibitory and antiapoptotic elements surrounding CSCs, like IL-4, opens the field to new experimental strategies aiming at contrasting their escape and protective activity. In colorectal CSCs, the inhibition of IL-4 signaling by neutralizing antibody or IL-4 receptor α antagonist led to the

sensitization and synergism with chemotherapeutic agents *in vitro* and *in vivo* [79].

2.1.3 Possible role of mAbs

Even if not included in the topic of this review, it is important to mention that immunotherapy strategies based on mAbs against CSCs are currently explored and may positively synergize with cell therapy or conventional chemotherapy approaches in the near future [80]. Targets of mAbs may be antigens associated with putative CSCs like CD133 [81] and EpCAM/ESA [82-84] but also indirect approaches are possible with antiangiogenic or antistroma mAbs interfering with the establishment of a supportive CSC niche [85-87]. A promising development is the exploitation of bispecific antibodies recognizing both CSC markers and tumor antigens, with the final result of increasing the specificity of CSC targeting [80]. As recent example it was reported the effective targeting of glioblastoma CSCs using a bispecific antibody against a variant form of EGF receptor (EGFRvIII) and CD133. Anti-EGFRvIII/CD133 mAb reduced the tumorigenicity of glioblastoma cells better than any reagent directed against a single epitope [88]. This proof of principle could be extended to other CSC marker and TAA combinations. We remand to dedicated reviews and original works for extensive and deeper description of the potential role of mAb against CSCs.

2.2 MHC-unrestricted adoptive immunotherapy approaches

2.2.1 NK cells

One of the most investigated tumor settings is that of colorectal cancer with experimental evidences focused on the activity of NK cells and $\gamma\delta$ lymphocytes against putative CSCs.

NK cells represent approximately 10% of circulating lymphocytes and are the main effectors of the innate immune system involved in the first response against pathogens and tumor immunosurveillance [89]. The antitumor activity of NK cells is mediated by a complex of activating receptors such as NKG2D, NKp30, NKp44, DNAM-1, FAS-L, as well as the inhibitory killer immunoglobulin-like receptors (KIR) activated by 'missing-self mechanisms' [90-92].

NK cells were reported to preferentially recognize and kill colorectal CSCs based on their increased expression of ligands for natural cytotoxicity receptors (NKp30; NKp44) and the relative downregulation of MHC class I compared with differentiated tumor cells [93]. These findings offer intriguing speculations to the observed correlation between NK infiltration and prognosis in colorectal cancer patients [94]. Other experimental evidences supported the susceptibility of putative CSCs to NK-mediated killing [95]. Human glioblastoma stem-like cells were shown to be killed by both autologous and allogeneic NK cells, and similar findings were also reported against melanoma cells lines [96,97]. However, although colorectal CSCs were susceptible to fresh resting NK cells, glioblastoma and melanoma CSCs were only killed upon NK activation with IL-2 or IL-15. Furthermore, DNAM-1 ligands were reported to

be recognized by NK cells on glioblastoma CSCs, whereas, as said above, NKp30 and NKp44 ligands seemed to mediate colorectal CSC killing. These differences could be simply due to the experimental model or may reflect the different histological derivation of the tumors, epithelial for colorectal cancer and ectodermic for glioblastoma and melanoma. As already mentioned in the previous paragraph, also murine prostate cancer in the TRAMP model was shown as suitable NK target based on membrane expression of NK activating ligands [68]. All these pre-clinical findings support the potential role of NK cells as valuable immunotherapy in the attempt to target CSCs. Further investigations should clarify important issues like possible synergism with chemotherapy or the role of KIR mismatches, still unclear in the setting of solid tumors.

2.2.2 $\gamma\delta$ T-lymphocytes

Recently, interesting reports highlighted the potential immunotherapy activity of $\gamma\delta$ T-lymphocytes capable of targeting CSCs in pre-clinical colorectal cancer models [98,99]. As two main subsets of $\gamma\delta$ T-lymphocytes are usually distinct in humans, depending on the subtype of δ chain, those with V δ 2 chain paired to the V γ 9 type (V γ 9V δ 2 T cells) have been explored in these studies against CSCs. V γ 9V δ 2 T cells are mainly found in peripheral blood and secondary lymphoid organs, recognize nonpeptidic antigens in MHC-unrestricted manner. Their antitumor activity may be exerted either directly, through Fas/FasL, TNF/TNF-R and TRAIL/TRAIL-R pathways [100], or indirectly, based on cross-talking with other effectors of the immune system by secretion of Th1- and Th2-type cytokines [101-103]. Colorectal putative CSCs were actually spontaneously resistant to the cytotoxic activity of V γ 9V δ 2 T cells but were effectively sensitized and killed following exposure to either low doses of chemotherapy (5FU and doxorubicin) or zoledronic acid [98,99]. Chemotherapy was shown to induce upregulation of DR5 receptor leading to TRAIL-mediated killing of CSCs, following their recognition through the NKG2D receptor on V γ 9V δ 2 T cells. The mechanism for the effect observed with zoledronic acid appeared to be different, with cytotoxicity operated by the granzyme/perforin pathway following TCR-mediated recognition. Possible explanation of this effect, even if not completely clear, is that zoledronic acid determines increased production and expression of phosphoantigens recognized by the TCR of V γ 9V δ 2 T-lymphocytes.

These observations are intriguing and with prospective clinical relevance. They demonstrated the possibility of new therapeutic approaches based on V γ 9V δ 2 T cells that could be either endogenously activated or adoptively infused, and at the same time highlighted the importance of synergism with other drugs. These studies sustain the concept that combinatorial strategies should be designed not necessarily to research 'more killing' but a 'better killing' instead, capable to involve tumor roots responsible for chemoresistance and relapses. Doses of chemotherapy drugs with sensitizing purposes are often significantly lower than those used in conventional therapeutic protocols, with a consequent favorable

toxicity profile that could favor its translation into experimental trials. The findings with zoledronic acid confirm the great immunomodulatory potential already known for this compound [104,105]. Many immunotherapy strategies might in theory benefit from its activity, as it was demonstrated to facilitate antigen presentation by DC, enhance NK activity and induce a favorable switch towards M1 phenotype within tumor-associated macrophages [106,107].

2.2.3 CIK cells

Recently, another MHC-unrestricted immunotherapy strategy had reported very interesting results in the field of solid tumor exploiting CIK cells as antitumor effectors [108]. CIK cells are *ex vivo* expanded T-lymphocytes with mixed T-NK lymphocytes [109,110]. Their mechanism of tumor killing is MHC-independent, mainly based on interaction of NKG2D receptor with stress-inducible molecules on tumor targets (MIC A/B; ULBPs) but also other receptors like DNAM-1, NKp30, LFA-1 may be involved [111,112].

Two recent works reported the pre-clinical activity of CIK cells against autologous melanoma and mesenchymal tumors, capable of involving putative CSCs [63,64]. An important aspect of both these works is the use of autologous pre-clinical models based on spontaneous human tumors. CIK cells efficiently killed *in vitro* autologous melanoma, bone and soft tissue sarcoma cells with putative stemness features. Ligands recognized by NKG2D receptor (MIC A/B; ULBPs) were equally expressed on both CSCs and their differentiated counterpart. The findings were confirmed *in vivo* where the antitumor effect of CIK cells appeared capable of involving putative CSCs within a sarcoma xenograft model into immunocompromised mice. These initial data call for further investigations but introduce CIK cells as an alternative or additional immunotherapy tool that appears promising for next exploration in clinical trials.

Considering that receptors and molecules involved in tumor recognition and killing by CIK cells are in part overlapping those of NK and V γ 9V δ 2 T cells, it could be imagined that also CIK cells might benefit from tumor-sensitizing strategies that should be investigated. Furthermore, the great *ex vivo* expansibility and cost-effectiveness of CIK cells production are favorable features that may facilitate their clinical translation.

3. Conclusion

In the last years, growing enthusiasms have surrounded research efforts for designing and clinical translation of new immunotherapy strategies as treatment of solid tumors. Evidences on the important biological role and clinical relevance of putative CSCs have introduced a 'noble target' for immunotherapy and cancer treatments in general. Initial reports seem to sustain that CSCs display a certain degree of resistance to conventional chemotherapy agents but may be susceptible to various types of immunotherapy attacks, either

based on DC vaccination or exploiting MHC-unrestricted effectors.

Investigation of important safety issues, based on shared features with 'normal' stem cells, along with intriguing synergisms with chemotherapy and modulatory agents are open challenges for the next future and effective clinical translations.

4. Expert opinion

Current evidences sustain the hypothesis that successful treatments for current incurable tumors rely on the eradication of putative CSCs, considered responsible for chemoresistance and disease relapses. Immunotherapy strategies may have the potential to meet this challenge and a new research field is now open in this direction, with particular attention to synergism strategies with other conventional therapies. Indirect speculations may be derived by the recent positive results obtained in the clinic with antagonists of immune-modulatory checkpoints against chemoresistant tumors, showing that the immune system may be activated against targets that may include chemoresistant CSC subsets [113,114].

Targeting of CSCs, within solid tumor models, has been reported with both MHC-restricted and -unrestricted strategies. The first almost exclusively based on DC-loaded vaccination, and the second using at least three different types of immune effectors like NK, $\gamma\delta$ T-lymphocytes and CIK cells. Reviewing the pre-clinical information so far available, it seems that putative CSCs can be source of TAA capable of eliciting effective MHC-restricted responses. It is possible that CSCs are per se low immunogenic by means of downregulation of MHC molecules or production of inhibitor mediators like IL-4. The DC vaccination approach, largely used in pre-clinical works, is likely to provide appropriate antigen presentation and costimulation signals capable of reverting or overcoming the low immunogenicity of CSCs and appear to be a good candidate strategy for clinical trials.

Targeting precise TAA may present restrictions to limited HLA-haplotypes, selecting the patients that could benefit in theory from the approach. This limitation is in part reduced using whole irradiated CSCs, whole DNA or RNA as antigen source.

On the contrary, MHC-unrestricted effectors do not require haplotype restrictions and potentially all patients may benefit from their activity. Furthermore, part of the recognized MHC-unrestricted ligands is potentially inducible by various agents with the opportunity to design strategies that exploit the sensitizing effect of low-dose chemotherapies or other modulators.

Both MHC-restricted and -unrestricted immunotherapy approaches are not mutually exclusive, An appealing future development could see their potential combination for a wider tumor killing; furthermore, elements of the innate immunity may exert a proinflammatory action, facilitating release of tumor antigens that could in turn potentiate and

sustain adaptive responses. Open challenges are the comprehension and exploitation of complex physical and functional interactions that CSCs might have within their 'niche'. Intrinsic and extrinsic factors within tumor microenvironment regulate CSC homeostasis and may represent novel therapeutic targets. Examples of these approaches are inhibition and targeting of key cytokines, angiogenic factors, integrins, chemokine receptors or signaling pathways involved in the mesenchymal-epithelial transition events (e.g., Wnt, TGF- β) [79-117]. These indirect approaches may interfere with the 'source' of new CSCs, disrupting the conversion process taking place at the microenvironment level, and offering intriguing synergisms with other strategies directly killing CSCs.

Regardless of the type of immunotherapy approach, a better investigation of CSC targeting and its translation into clinical applications faces common issues like the: i) identification of reliable and representative pre-clinical models; and ii) safety concerns.

i) Definition of reliable pre-clinical models, representative of realistic tumor biology scenarios, is very important for accurate investigations and to obtain clinically relevant information.

The common use of commercially available tumor cell lines may determine allogeneic mismatches with immune effectors that could in part lead to misinterpreted results. Furthermore, allogeneic tumor cell lines often have been obtained many years ago with the possible occurrence of important genetic-shift events.

Important improvements in this direction are given by recent efforts to set autologous models with *in vitro* targets and *in vivo* xenografts obtained by fresh or short-term cultured tumor samples, with immune effectors generated from the same patient. The natural CSC niche is likely to provide protection from immunoediting and immunosurveillance, a difficult scenario to reproduce using *ex vivo* isolated putative CSCs. Interesting improvements may derive from strategies that aim to visualize and track CSCs directly within their tumor stroma [62-64]. Direct implication of these considerations is that immunotherapies directed against CSCs might benefit and should be explored in synergism with strategies modulating the activity of tumor microenvironment in the patient's favor.

ii) Safety of immunotherapy approaches is of course a major issue in clinical perspective and pre-clinical animal models may only partially be indicative of this aspect. For MHC-restricted approaches like vaccinations, even when HLA transgenic mice are used, they cannot fully predict potential undesired 'autoimmune' reactions based on the complex and species-specific machinery underlying processing and presentation of TAA to the adaptive immune system. One major concern in targeting CSCs is the possibility of cross-reaction against nontumor somatic stem cells. In the first clinical trial, targeting glioblastoma CSCs, the authors looked at this aspect monitoring hematopoietic stem

cell-derived lineages, as well as symptoms from organs highly dependent on stem cells for cellular turnover, such as skin and the gastrointestinal tract without evidences of toxicities [75].

Reviewing the pre-clinical models tested so far, it seems that in general all immunotherapy approaches against CSCs present an optimal safety profile. It is however important to note that certain undesired and serious events could not be predicted, for example, if based on massive cytokine storm, cross-reactions with molecules expressed in normal human tissues or expression of low levels of the TAA in healthy organs. Examples of this type have recently been reported with the lethal toxicity associated with chimeric antigen receptor gene-modified T cells [118-120], where there is not a pre-clinical model capable of appropriately testing the potential toxicity.

Caution is warranted when trying new immunotherapies in patients, considering low-dose escalation if the adoptive infusion of lymphocytes is planned.

It is possible to imagine that narrowing the target, with hypothetical CSC-specific effectors, might eventually bring to a risk reduction for these kinds of problems, producing 'less' but 'more precise' 'tumor killing'.

In the last years, cancer immunotherapy stepped from old time promise to real clinical applications. Revitalized research efforts are now sharpening the antitumor immunotherapy

activity against cancer cells with stemness features responsible for tumor recurrence and drug resistance. These approaches are hoped to be soon incorporated into clinical trials, introducing relevant clinical goals and opening new scenarios of synergism with conventional therapies that could significantly enhance the overall anticancer therapeutic potential.

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Declaration of interest

The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.

Bibliography

Papers of special note have been highlighted as either of interest (●) or of considerable interest (●●) to readers.

1. Visvader JE, Lindeman GJ. Cancer stem cells in solid tumours: accumulating evidence and unresolved questions. *Nat Rev Cancer* 2008;8:755-68
2. Virchow R. Cellular-Pathologie. *Arch Pathol Anat Physiol Klin Med.* 1855;8:3-39
3. Cohnheim J. Congenitales, quergestreiftes Muskelsarkom Der Nieren. *Virchows Arch* 1875;65:64
4. Durante F. Nesso fisio-pathologico tra la struttura dei nei materni e la genesi di alcuni tumori maligni. *Arch Memori Ed Osservazioni Di Chirurgia Practica* 1874;11:217-26
5. Bruce WR, Van der Gaag H. A quantitative assay for the number of murine lymphoma cells capable of proliferation in vivo. *Nature* 1963;199:79-80
6. Lapidot T, Sirard C, Vormoor J, et al. A cell initiating human acute myeloid leukaemia after transplantation into scid mice. *Nature* 1994;367(6464):645-8
- **First description of cancer stem cells (CSCs) in acute myeloid leukemia.**
7. Bonnet D, Dick JE. Human acute myeloid leukemia is organized as a hierarchy that originates from a primitive hematopoietic cell. *Nat Med* 1997;3(7):730-7
8. Al-Hajj M, Wicha MS, Benito-Hernandez A, et al. Prospective identification of tumorigenic breast cancer cells. *Proc Natl Acad Sci USA* 2003;100(7):3983-8
- **First description of CSCs in a solid tumor model of breast cancer.**
9. Ponti D, Costa A, Zaffaroni N, et al. Isolation and in vitro propagation of tumorigenic breast cancer cells with stem/progenitor cell properties. *Cancer Res* 2005;65(13):5506-11
10. Kim CF, Jackson EL, Woolfenden AE, et al. Identification of bronchioalveolar stem cells in normal lung and lung cancer. *Cell* 2005;121(6):823-35
11. Eramo A, Lotti F, Sette G, et al. Identification and expansion of the tumorigenic lung cancer stem cell population. *Cell Death Differ* 2008;15(3):504-14
12. O'Brien CA, Pollett A, Gallinger S, Dick JE. A human colon cancer cell capable of initiating tumour growth in immunodeficient mice. *Nature* 2007;445(7123):106-10
13. Ricci-Vitiani L, Lombardi DG, Pilozzi E, et al. Identification and expansion of human colon-cancer-initiating cells. *Nature* 2007;445(7123):111-15
14. Collins AT, Berry PA, Hyde C, et al. Prospective identification of tumorigenic prostate cancer stem cells. *Cancer Res* 2005;65(23):10946-51
15. Szotek PP, Pieretti-Vanmarcke R, Masiakos PT, et al. Ovarian cancer side population defines cells with stem cell-like characteristics and mullerian inhibiting substance responsiveness. *Proc Natl Acad Sci USA* 2006;103(30):11154-9
16. Zhang S, Balch C, Chan MW, et al. Identification and characterization of ovarian cancer-initiating cells from primary human tumors. *Cancer Res* 2008;68(11):4311-20
17. Curley MD, Therrien VA, Cummings CL, et al. Cd133 expression defines a tumor initiating cell population in primary human ovarian cancer. *Stem Cells* 2009;27(12):2875-83
18. Piccirillo SG, Reynolds BA, Zanetti N, et al. Bone morphogenetic proteins inhibit the tumorigenic potential of

- human brain tumour-initiating cells. *Nature* 2006;444(7120):761-5
19. Singh SK, Hawkins C, Clarke ID, et al. Identification of human brain tumour initiating cells. *Nature* 2004;432(7015):396-401
 20. Fang D, Nguyen TK, Leishear K, et al. A tumorigenic subpopulation with stem cell properties in melanomas. *Cancer Res* 2005;65(20):9328-37
 21. La Porta C. Cancer stem cells: lessons from melanoma. *Stem Cell Rev* 2009;5(1):61-5
 22. Quintana E, Shackleton M, Sabel MS, et al. Efficient tumour formation by single human melanoma cells. *Nature* 2008;456(7222):593-8
 23. Malanchi I, Peinado H, Kassen D, et al. Cutaneous cancer stem cell maintenance is dependent on beta-catenin signalling. *Nature* 2008;452(7187):650-3
 24. Prince ME, Sivanandan R, Kaczorowski A, et al. Identification of a subpopulation of cells with cancer stem cell properties in head and neck squamous cell carcinoma. *Proc Natl Acad Sci USA* 2007;104(3):973-8
 25. Li C, Heidt DG, Dalerba P, et al. Identification of pancreatic cancer stem cells. *Cancer Res* 2007;67(3):1030-7
 26. Wilson A, Laurenti E, Oser G, et al. Hematopoietic stem cells reversibly switch from dormancy to self-renewal during homeostasis and repair. *Cell* 2008;135(6):1118-29
 27. Fuchs E. The tortoise and the hair: slow-cycling cells in the stem cell race. *Cell* 2009;137(5):811-19
 28. Clarke MF, Dick JE, Dirks PB, et al. Cancer stem cells—perspectives on current status and future directions: aac workshop on cancer stem cells. *Cancer Res* 2006;66(19):9339-44
 29. Li L, Borodyansky L, Yang Y. Genomic instability en route to and from cancer stem cells. *Cell Cycle* 2009;8(7):1000-2
 30. Rapp UR, Ceteci F, Schreck R. Oncogene-induced plasticity and cancer stem cells. *Cell Cycle* 2008;7(1):45-51
 31. Reya T, Morrison SJ, Clarke MF, Weissman IL. Stem cells, cancer, and cancer stem cells. *Nature* 2001;414(6859):105-11
 32. Alison MR, Lim SM, Nicholson LJ. Cancer stem cells: problems for therapy? *J Pathol* 2011;223(2):147-61
 33. Calabrese C, Poppleton H, Kocak M, et al. A perivascular niche for brain tumor stem cells. *Cancer Cell* 2007;11(1):69-82
 34. Li L, Neaves WB. Normal stem cells and cancer stem cells: the niche matters. *Cancer Res* 2006;66(9):4553-7
 35. Takaishi S, Okumura T, Tu S, et al. Identification of gastric cancer stem cells using the cell surface marker Cd44. *Stem Cells* 2009;27(5):1006-20
 36. Tirino V, Desiderio V, d'Aquino R, et al. Detection and characterization of Cd133+ cancer stem cells in human solid tumours. *PLoS One* 2008;3(10):e3469
 37. Pearce DJ, Bonnet D. The combined use of hoechst efflux ability and aldehyde dehydrogenase activity to identify murine and human hematopoietic stem cells. *Exp Hematol* 2007;35(9):1437-46
 38. Rybak AP, He L, Kapoor A, et al. Characterization of sphere-propagating cells with stem-like properties from Du145 prostate cancer cells. *Biochim Biophys Acta* 2011;1813(5):683-94
 39. Dean M, Fojo T, Bates S. Tumour stem cells and drug resistance. *Nat Rev Cancer* 2005;5(4):275-84
 40. Suvà ML, Riggi N, Stehle JC, et al. Identification of cancer stem cells in Ewing's sarcoma. *Cancer Res* 2009;69(5):1776-81
 41. Rosen JM, Jordan CT. The increasing complexity of the cancer stem cell paradigm. *Science* 2009;324(5935):1670-3
 42. Tirino V, Desiderio V, Paino F, et al. Cancer stem cells in solid tumors: an overview and new approaches for their isolation and characterization. *FASEB J* 2013;27(1):13-24
 43. Awad O, Yustein JT, Shah P, et al. High Aldh activity identifies chemotherapy-resistant ewing's sarcoma stem cells that retain sensitivity to Ews-Flil1 inhibition. *PLoS One* 2010;5(11):e13943
 44. Hu Y, Yu X, Liu S. Cancer stem cells: a shifting subpopulation of cells with stemness? *Med Hypotheses* 2013;80(5):649-55
 45. La Porta CA. Thoughts about cancer stem cells in solid tumors. *World J Stem Cells* 2012;4(3):17-20
 46. Graham SM, Jørgensen HG, Allan E, et al. Primitive, quiescent, Philadelphia-positive stem cells from patients with chronic myeloid leukemia are insensitive to Stt571 in vitro. *Blood* 2002;99(1):319-25
 47. Januchowski R, Wojtowicz K, Zabel M. The role of aldehyde dehydrogenase (Aldh) in cancer drug resistance. *Biomed Pharmacother* 2013;67(7):669-80
 48. Mohrin M, Bourke E, Alexander D, et al. Hematopoietic stem cell quiescence promotes error-prone DNA repair and mutagenesis. *Cell Stem Cell* 2010;7(2):174-85
 49. Viale A, De Franco F, Orleth A, et al. Cell-cycle restriction limits DNA damage and maintains self-renewal of leukaemia stem cells. *Nature* 2009;457(7225):51-6
 50. Hirschmann-Jax C, Foster AE, Wulf GG, et al. A distinct side population of cells with high drug efflux capacity in human tumor cells. *Proc Natl Acad Sci USA* 2004;101(39):14228-33
 51. Zhou S, Schuetz JD, Bunting KD, et al. The Abc transporter Bcrp1/Abcg2 is expressed in a wide variety of stem cells and is a molecular determinant of the side-population phenotype. *Nat Med* 2001;7(9):1028-34
 52. Allen JE, Hart LS, Dicker DT, et al. Visualization and enrichment of live putative cancer stem cell populations following P53 inactivation or Bax deletion using non-toxic fluorescent dyes. *Cancer Biol Ther* 2009;8(22):2194-205
 53. Braut-Boucher F, Pichon J, Rat P, et al. A non-isotopic, highly sensitive, fluorimetric, cell-cell adhesion microplate assay using calcein am-labeled lymphocytes. *J Immunol Methods* 1995;178(1):41-51
 54. Telford WG, Bradford J, Godfrey W, et al. Side population analysis using a violet-excited cell-permeable Dna binding dye. *Stem Cells* 2007;25(4):1029-36
 55. Richichi C, Brescia P, Alberizzi V, et al. Marker-independent method for isolating slow-dividing cancer stem cells in human glioblastoma. *Neoplasia* 2013;15(7):840-7
 56. Salcido CD, Larochelle A, Taylor BJ, et al. Molecular characterisation of side population cells with cancer stem cell-like characteristics in small-cell lung cancer. *Br J Cancer* 2010;102(11):1636-44
 57. Sullivan JP, Spinola M, Dodge M, et al. Aldehyde dehydrogenase activity selects

- for lung adenocarcinoma stem cells dependent on notch signaling. *Cancer Res* 2010;70(23):9937-48
58. Vermeulen L, de Sousa e Melo F, Richel DJ, Medema JP. The developing cancer stem-cell model: clinical challenges and opportunities. *Lancet Oncol* 2012;13(2):e83-9
59. Kumar SM, Liu S, Lu H, et al. Acquired cancer stem cell phenotypes through Oct4-mediated dedifferentiation. *Oncogene* 2012;31(47):4898-911
60. Levings PP, McGarry SV, Currie TP, et al. Expression of an exogenous human Oct-4 promoter identifies tumor-initiating cells in osteosarcoma. *Cancer Res* 2009;69(14):5648-55
61. Chiou SH, Wang ML, Chou YT, et al. Coexpression of Oct4 and nanog enhances malignancy in lung adenocarcinoma by inducing cancer stem cell-like properties and epithelial-mesenchymal transdifferentiation. *Cancer Res* 2010;70(24):10433-44
62. Gerrard L, Zhao D, Clark AJ, Cui W. Stably transfected human embryonic stem cell clones express Oct4-specific green fluorescent protein and maintain self-renewal and pluripotency. *Stem Cells* 2005;23(1):124-33
63. Gammaitoni L, Giraudo L, Leuci V, et al. Effective activity of cytokine-induced killer cells against autologous metastatic melanoma including cells with stemness features. *Clin Cancer Res* 2013;19(16):4347-58
- **First report on preclinical activity of cytokine-induced killer cells against autologous melanoma CSCs.**
64. Sangiolo D, Mesiano G, Gammaitoni L, et al. Cytokine-Induced killer cells eradicate bone and soft-tissue sarcomas. *Cancer Res* 2014;74(1):119-29
65. Francipane MG, Chandler J, Lagasse E. Cancer stem cells: a moving target. *Curr Pathobiol Rep* 2013;1(2):111-18
66. Ashley N. Regulation of intestinal cancer stem cells. *Cancer Lett* 2013;338(1):120-6
67. Greenberg NM, DeMayo F, Finegold MJ, et al. Prostate cancer in a transgenic mouse. *Proc Natl Acad Sci USA* 1995;92(8):3439-43
68. Jachetti E, Mazzoleni S, Gritti M, et al. Prostate cancer stem cells are targets of both innate and adaptive immunity and elicit tumor-specific immune responses. *Oncoimmunology* 2013;2(5):e24520
- **Preclinical evidences are provided about effective targeting of prostate cancer stem cells by both innate and adaptive immune responses.**
69. Garcia-Hernandez MeL, Gray A, Hubby B, et al. Prostate stem cell antigen vaccination induces a long-term protective immune response against prostate cancer in the absence of autoimmunity. *Cancer Res* 2008;68(3):861-9
70. Gu Z, Thomas G, Yamashiro J, et al. Prostate stem cell antigen (PscA) expression increases with high gleason score, advanced stage and bone metastasis in prostate cancer. *Oncogene* 2000;19(10):1288-96
71. Bahrenberg G, Brauers A, Joost HG, Jakse G. Reduced expression of PscA, a member of the Ly-6 family of cell surface antigens, in bladder, esophagus, and stomach tumors. *Biochem Biophys Res Commun* 2000;275(3):783-8
72. Pellegatta S, Poliani PL, Corno D, et al. Neurospheres enriched in cancer stem-like cells are highly effective in eliciting a dendritic cell-mediated immune response against malignant gliomas. *Cancer Res* 2006;66(21):10247-52
73. Brown CE, Starr R, Martinez C, et al. Recognition and killing of brain tumor stem-like initiating cells by Cd8+ cytolytic T cells. *Cancer Res* 2009;69(23):8886-93
74. Xu Q, Liu G, Yuan X, et al. Antigen-specific T-cell response from dendritic cell vaccination using cancer stem-like cell-associated antigens. *Stem Cells* 2009;27(8):1734-40
75. Vik-Mo EO, Nyakas M, Mikkelsen BV, et al. Therapeutic vaccination against autologous cancer stem cells with Mrna-transfected dendritic cells in patients with glioblastoma. *Cancer Immunol Immunother* 2013;62(9):1499-509
- **First clinical trial reporting data on therapeutic vaccination against autologous glioblastoma CSCs.**
76. Ning N, Pan Q, Zheng F, et al. Cancer stem cell vaccination confers significant antitumor immunity. *Cancer Res* 2012;72(7):1853-64
77. Di Tomaso T, Mazzoleni S, Wang E, et al. Immunobiological characterization of cancer stem cells isolated from glioblastoma patients. *Clin Cancer Res* 2010;16(3):800-13
78. Volonté A, Di Tomaso T, Spinelli M, et al. Cancer-initiating cells from colorectal cancer patients escape from T cell-mediated immunosurveillance in vitro through membrane-bound IL-4. *J Immunol* 2014;192(1):523-32
79. Francipane MG, Alea MP, Lombardo Y, et al. Crucial role of interleukin-4 in the survival of colon cancer stem cells. *Cancer Res* 2008;68(11):4022-5
80. Deonarain MP, Kousparou CA, Epenetos AA. Antibodies targeting cancer stem cells: a new paradigm in immunotherapy? *MAbs* 2009;1(1):12-25
81. Smith LM, Nesterova A, Ryan MC, et al. Cd133/prominin-1 is a potential therapeutic target for antibody-drug conjugates in hepatocellular and gastric cancers. *Br J Cancer* 2008;99(1):100-9
82. Goel S, Bauer RJ, Desai K, et al. Pharmacokinetic and safety study of subcutaneously administered weekly Ing-1, a human engineered monoclonal antibody targeting human epcam, in patients with advanced solid tumors. *Ann Oncol* 2007;18(10):1704-7
83. Naundorf S, Preithner S, Mayer P, et al. In vitro and in vivo activity of Mt201, a fully human monoclonal antibody for pancarcinoma treatment. *Int J Cancer* 2002;100(1):101-10
84. Oberneder R, Weckermann D, Ebner B, et al. A phase I study with adecatumumab, a human antibody directed against epithelial cell adhesion molecule, in hormone refractory prostate cancer patients. *Eur J Cancer* 2006;42(15):2530-8
85. Folkins C, Man S, Xu P, et al. Anticancer therapies combining antiangiogenic and tumor cell cytotoxic effects reduce the tumor stem-like cell fraction in glioma xenograft tumors. *Cancer Res* 2007;67(8):3560-4
86. Ottaiano A, di Palma A, Napolitano M, et al. Inhibitory effects of anti-Cxcr4 antibodies on human colon cancer cells. *Cancer Immunol Immunother* 2005;54(8):781-91
87. Sneddon JB, Werb Z. Location, location, location: the cancer stem cell niche. *Cell Stem Cell* 2007;1(6):607-11
88. Emler DR, Gupta P, Holgado-Madruga M, et al. Targeting a glioblastoma cancer stem-cell population

- defined by Egf receptor variant Iii. *Cancer Res* 2014;74(4):1238-49
89. Farag SS, Caligiuri MA. Human natural killer cell development and biology. *Blood Rev* 2006;20(3):123-37
 90. Caligiuri MA. Human natural killer cells. *Blood* 2008;112(3):461-9
 91. Moretta L, Moretta A. Unravelling natural killer cell function: triggering and inhibitory human Nk receptors. *EMBO J* 2004;23(2):255-9
 92. Lanier LL. Up on the tightrope: natural killer cell activation and inhibition. *Nat Immunol* 2008;9(5):495-502
 93. Talerico R, Todaro M, Di Franco S, et al. Human Nk cells selective targeting of colon cancer-initiating cells: a role for natural cytotoxicity receptors and Mhc class I molecules. *J Immunol* 2013;190(5):2381-90
 94. Sandel MH, Speetjens FM, Menon AG, et al. Natural killer cells infiltrating colorectal cancer and Mhc class I expression. *Mol Immunol* 2005;42(4):541-6
 95. Jewett A, Tseng HC, Arasteh A, et al. Natural killer cells preferentially target cancer stem cells; role of monocytes in protection against Nk cell mediated lysis of cancer stem cells. *Curr Drug Deliv* 2012;9(1):5-16
 96. Castriconi R, Daga A, Dondero A, et al. Nk cells recognize and kill human glioblastoma cells with stem cell-like properties. *J Immunol* 2009;182(6):3530-9
 97. Pietra G, Manzini C, Vitale M, et al. Natural killer cells kill human melanoma cells with characteristics of cancer stem cells. *Int Immunol* 2009;21(7):793-801
 98. Todaro M, D'Asaro M, Caccamo N, et al. Efficient killing of human colon cancer stem cells by gammadelta T lymphocytes. *J Immunol* 2009;182(11):7287-96
 - **Preclinical report of potential activity of Gammadelta T Lymphocytes against CSCs in colorectal cancer, highlighting the potential synergism with zoledronate.**
 99. Todaro M, Orlando V, Cicero G, et al. Chemotherapy sensitizes colon cancer initiating cells to Vγ9vδ2 T cell-mediated cytotoxicity. *PLoS One* 2013;8(6):e65145
 - **Interesting data on the potential synergism of chemotherapy and immunotherapy to target CSCs in colorectal cancer.**
 100. Constant P, Davodeau F, Peyrat MA, et al. Stimulation of human gamma delta T cells by nonpeptidic mycobacterial ligands. *Science* 1994;264(5156):267-70
 101. Vermijlen D, Ellis P, Langford C, et al. Distinct cytokine-driven responses of activated blood gammadelta T cells: insights into unconventional T cell pleiotropy. *J Immunol* 2007;178(7):4304-14
 102. Wesch D, Glatzel A, Kabelitz D. Differentiation of resting human peripheral blood gamma delta T cells toward Th1- or Th2-phenotype. *Cell Immunol* 2001;212(2):110-17
 103. Caccamo N, La Mendola C, Orlando V, et al. Differentiation, phenotype, and function of interleukin-17-producing human Vgamma9vdelta2 T cells. *Blood* 2011;118(1):129-38
 104. Fiore F, Castella B, Nuschak B, et al. Enhanced ability of dendritic cells to stimulate innate and adaptive immunity on short-term incubation with zoledronic acid. *Blood* 2007;110(3):921-7
 105. Hamilton E, Clay TM, Blackwell KL. New perspectives on zoledronic acid in breast cancer: potential augmentation of anticancer immune response. *Cancer Invest* 2011;29(8):533-41
 106. Nussbaumer O, Gruenbacher G, Gander H, Thurnher M. Dc-like cell-dependent activation of human natural killer cells by the bisphosphonate zoledronic acid is regulated by gammaelta T lymphocytes. *Blood* 2011;118(10):2743-51
 107. Coscia M, Quaglino E, Iezzi M, et al. Zoledronic acid repolarizes tumour-associated macrophages and inhibits mammary carcinogenesis by targeting the mevalonate pathway. *J Cell Mol Med* 2010;14(12):2803-15
 108. Mesiano G, Todorovic M, Gammaitoni L, et al. Cytokine-induced killer (Cik) cells as feasible and effective adoptive immunotherapy for the treatment of solid tumors. *Expert Opin Biol Ther* 2012;12(6):673-84
 109. Schmidt-Wolf IG, Lefterova P, Mehta BA, et al. Phenotypic characterization and identification of effector cells involved in tumor cell recognition of cytokine-induced killer cells. *Exp Hematol* 1993;21(13):1673-9
 110. Lu PH, Negrin RS. A novel population of expanded human Cd3+Cd56+ cells derived from T cells with potent in vivo antitumor activity in mice with severe combined immunodeficiency. *J Immunol* 1994;153(4):1687-96
 111. Baker J, Verneris MR, Ito M, et al. Expansion of cytolytic Cd8(+) natural killer T cells with limited capacity for graft-versus-host disease induction due to interferon gamma production. *Blood* 2001;97(10):2923-31
 112. Verneris MR, Karami M, Baker J, et al. Role of Nkg2d signaling in the cytotoxicity of activated and expanded Cd8+ T cells. *Blood* 2004;103(8):3065-72
 113. Topalian SL, Hodi FS, Brahmer JR, et al. Safety, activity, and immune correlates of anti-Pd-1 antibody in cancer. *N Engl J Med* 2012;366(26):2443-54
 114. Wolchok JD, Neyns B, Linette G, et al. Ipilimumab monotherapy in patients with pretreated advanced melanoma: a randomised, double-blind, multicentre, phase 2, dose-ranging study. *Lancet Oncol* 2010;11(2):155-64
 115. Rizzo P, Osipo C, Foreman K, et al. Rational targeting of notch signaling in cancer. *Oncogene* 2008;27(38):5124-31
 116. de Sousa EM, Vermeulen L, Richel D, Medema JP. Targeting Wnt signaling in colon cancer stem cells. *Clin Cancer Res* 2011;17(4):647-53
 117. Korkaya H, Kim GI, Davis A, et al. Activation of an Il6 inflammatory loop mediates trastuzumab resistance in Her2+ breast cancer by expanding the cancer stem cell population. *Mol Cell* 2012;47(4):570-84
 118. Morgan RA, Yang JC, Kitano M, et al. Case report of a serious adverse event following the administration of T cells transduced with a chimeric antigen receptor recognizing Erbb2. *Mol Ther* 2010;18(4):843-51
 119. Leuci V, Mesiano G, Gammaitoni L, et al. Genetically redirected T lymphocytes for adoptive immunotherapy of solid tumors. *Curr Gene Ther* 2014;14(1):52-62
 120. Linette GP, Stadtmayer EA, Maus MV, et al. Cardiovascular toxicity and titin cross-reactivity of affinity-enhanced T cells in myeloma and melanoma. *Blood* 2013;122(6):863-71

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