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Immunotherapy of Cancer Stem Cells in Solid Tumors: Initial findings and future prospective

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1. Introduction

History of CSCs

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 about 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].

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 (AML) [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- 16- 17], brain cancer [18- 19], melanoma [20- 21- 22], skin squamous cell carcinoma (SCC) [23], head and neck cancer [24], pancreatic cancer[25].

Biologic features and identification issues

Common features of putative CSCs are considered: (a) self-renewal capacity—the CSCs subpopulation can be serially transplanted through multiple generations, indicating the self-renewing capacity; (b) 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; (c)

tumorigenicity — a small subpopulation of CSCs have tumorigenic potential when transplanted into animals; and (d) 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 biologic process underlying the generation of CSCs may include multiple gene mutations as 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 chemo-resistance, multipotency, tumorigenicity, stem gene expression and aldehyde dehydrogenase (ALDH) activity [40- 41- 42- 43]. Different cell surface antigens have been identified in several cancers as candidate CSC biomarkers (CD20, CD24, CD34, CD44, CD90, CD117, and CD133) [11- 12- 13- 14- 35- 36- 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- 49- 50]. Therefore the CSC model could help explaining 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 employed dyes to differentiate between SP and non-SP cells (e.g. Hoechst 33342, Calcein, Vybrant1 DyeCycleTM Violet) including assays for determining ABCG2 inhibitors [51- 52- 53- 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- 57- 58]. Moreover, the expression of the Oct4 gene seems to be reliably associated with putative CSCs of various histotypes [59- 60- 61]. Gene transfer strategies, based on plasmid or lentiviral vectors encoding the enhanced Green Fluorescence Protein (eGFP) 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- 63- 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 chemo-resistance 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 then hematologic malignancies. Direct targeting of CSCs might be problematic since 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 preclinical 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 chemo-resistance 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. Preclinical 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 γδ T lymphocytes or also with ex-vivo expanded Cytokine-induced killer (CIK) cells. The preclinical 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 preclinical 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 preclinical 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 researches 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.

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 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 (LAK) 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 stages and metastasis of prostate cancer along with progression from pre-malignant 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 mechanist 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.

The Glioblastoma model

Similar to prostate cancer, important preclinical 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 co-stimulatory 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 co-stimulation 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 preclinical models are not enough representative of human settings. The promising preclinical 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 7

patients, against CSCs lysates and interestingly also against hTERT or Survivin peptides in vitro, while only 1 patient had a positive delayed-type hypersensitivity(DTH) reaction. The discrepancy between *in vitro* and *in vivo* responses could be attributed to the relative low lymphocytes 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 7 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 mm^3 (363–1,526 mm^3). Subsequently Magnetic Resonance Imaging (MRI) 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 days 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 preclinical data suggest that adaptive 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 toward specific immune response with a central inhibitory role attributed to IL4 production at CSCs level [77- 78]. Interestingly the low immunogenicity of glioblastoma CSCs could be rescued by demetilating agents or IFN-γ, opening to investigations of potential synergic strategies.

Even if these data may appear in contrast with the positive preclinical findings described so far, they express the complexity of the issue and difficulties in finding representative and reliable preclinical 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 IL4, 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, leaded to the sensitization and synergism with chemotherapeutic agents *in vitro* and *in vivo* [79].

Possible role of monoclonal antibodies.

Even if not included in the topic of this review, it is important to mention that immunotherapy strategies based on monoclonal antibodies (mAb) against CSCs are currently explored and may positively synergize with cell therapy or conventional chemotherapy approaches in the next future[80]. Targets of mAbs may be antigens associated with putative CSCs like CD133[81] and EpCAM/ESA[82- 83- 84] but also indirect approaches are possible with anti-angiogenic or anti-stroma mAbs interfering with the establishment of a supportive CSC niche[85- 86- 87]. A promising development is the exploitation of bi-specific 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

Natural Killer cells.

One of the most investigated tumor setting is that of colorectal cancer with experimental evidences focused on the activity of NK cells and γδ lymphocytes against putative CSCs.

NK cells represent about 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- 91- 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 to 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-meditated 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, while colorectal CSCs were susceptible to fresh resting NK cells, Glioblastoma and melanoma CSCs were only killed upon NK activation with IL2 or IL15. Furthermore DNAM-1 ligands were reported to be recognized by NK cells on glioblastoma CSCs while, 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 histologic 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 preclinical 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.

γδ T lymphocytes. Recently interesting reports highlighted the potential immunotherapy activity of γδ T lymphocytes capable of targeting CSCs in preclinical colorectal cancer models [98- 99]. As two main subsets of γδ T lymphocytes are usually distinct in humans, depending on the subtype of δ chain, those with Vδ2 chain paired to the Vγ9 type (Vγ9Vδ2 T cells) have been explored in these studies against CSCs. Vγ9Vδ2 T cells are mainly found in peripheral blood and secondary lymphoid organs, recognize non-peptidic 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- 102- 103]. Colorectal putative CSCs were actually spontaneously resistant to the cytotoxic activity of Vγ9Vδ2 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 Vγ9Vδ2 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 zoledronc acid determines increased production and expression of phosphoantigens recognized by the TCR of Vγ9Vδ2 T lymphocytes.

These observation are intriguing and with prospective clinical relevance. They demonstrated the possibility of new therapeutic approaches based on Vγ9Vδ2 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 chemo-resistance 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 toward M1 phenotype within tumor associated macrophages[106- 107].

Cytokine-induced killer cells. Recently another MHC-unrestricted immunotherapy strategy is reporting very interesting results in the field of solid tumor exploiting cytokine-induced killer (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 preclinical 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 preclinical 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 to involve 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 appear 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 Vγ9Vδ2 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. Conclusions

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 biologic 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 chemo-resistance 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, have been reported with both MHC-restricted and unrestricted strategies. The first, almost exclusively based on DC-loaded vaccination and the second, employing at least three different types of immune effectors like NK, γδ T lymphocytes and CIK cells. Reviewing the preclinical 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 preclinical works, it is likely to provide appropriate antigen-presentation and co-stimulation signals capable to revert or overcome 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 doses 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 pro-inflammatory 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, chemochine receptors or signaling pathways involved in the mesenchymal-epithelial transition events (e.g. Wnt, TGFβ)[79- 85- 86- 87- 115- 116- 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 the type of immunotherapy approach, a better investigation of CSC targeting and its translation into clinical applications faces common issues like the a) identification of reliable and representative preclinical models and b) safety concerns.

a) Definition of reliable preclinical models, representative of realistic tumor biology scenarios is very important for accurate investigations and to obtain clinical 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- 63- 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 patients' favor.

b) Safety of immunotherapy approaches is of course a major issue in clinical perspective and preclinical 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 non-tumor 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 preclinical 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 (CAR) gene-modified T cells [118- 119- 120], where there is not a preclinical model capable to appropriately test the potential toxicity.

Caution is warranted when trying new immunotherapies in patients, considering low doses 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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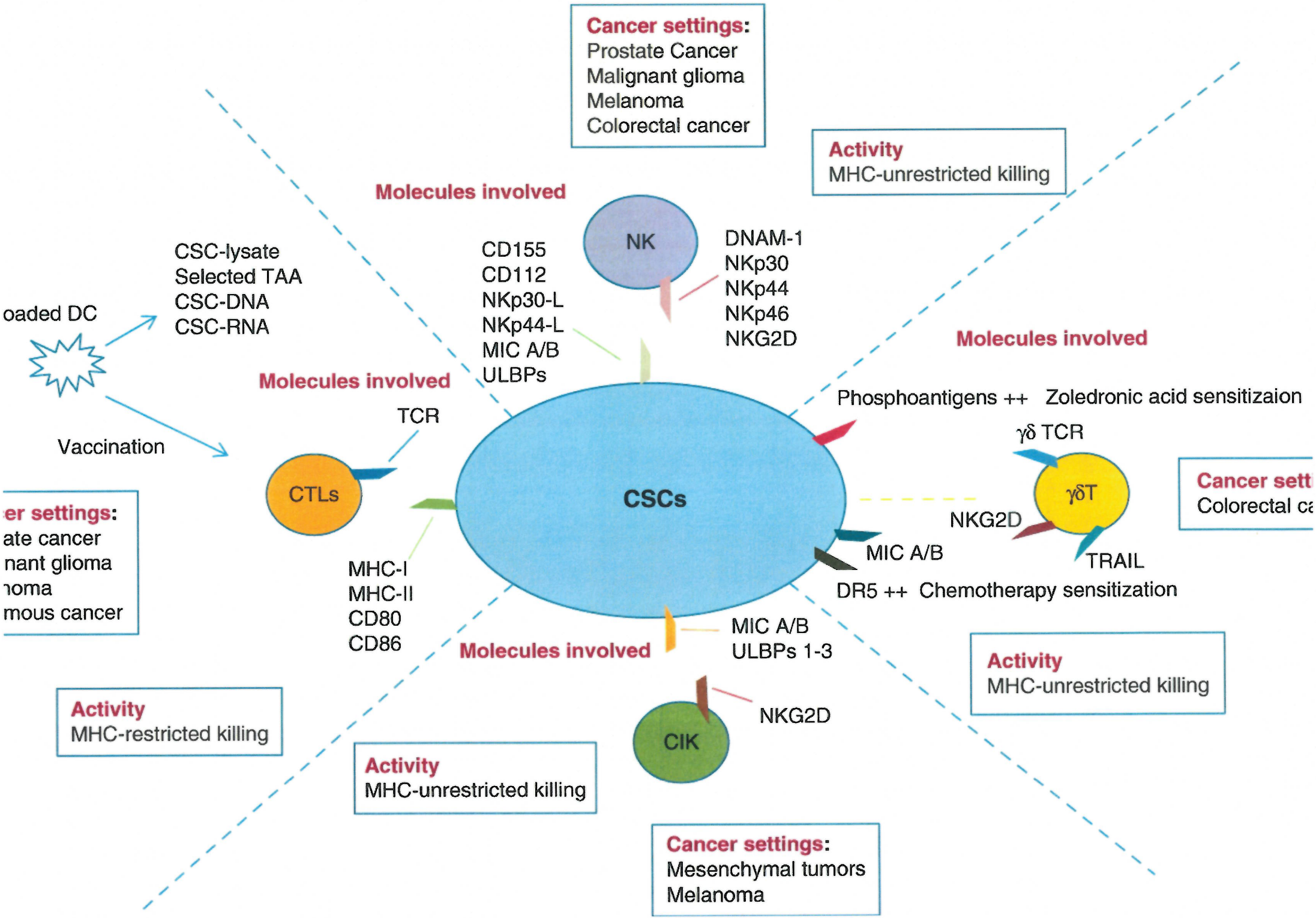
Cancer settings:
Prostate Cancer
Malignant glioma
Melanoma
Colorectal cancer
Activity
MHC-unrestricted killing
Molecules involved
NK
DNAM-1
NKp30
NKp44
NKp46
NKG2D
CD155
CD112
NKp30-L
NKp44-L
MIC A/B
ULBPs
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TCR
CTLs
CSCs
Molecules involved
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γδ TCR
γδT
NKG2D
TRAIL
MIC A/B
DR5 ++ Chemotherapy sensitization
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nant glioma
ıoma
mous cancer
MHC-I
MHC-II
CD80
CD86
Molecules involved
MIC A/B
ULBPs 1-3
Activity
MHC-unrestricted killing
Activity
MHC-restricted killing
CIK
NKG2D
Activity
MHC-unrestricted killing
Cancer settings:
Mesenchymal tumors
Melanoma