

Cancer stem cells and immunoresistance: clinical implications and solutions

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Abstract: Tumor cells can be contained, but not eliminated, by traditional cancer therapies. A cell minor subpopulation is able to evade attack from therapies and may have cancer stem cell (CSC) characteristics, including self-renewal, multiple differentiation and tumor initiation (tumor initiating cells, or TICs). Thus, CSCs/TICs, aided by the microenvironment, produce more differentiated, metastatic cancer cells which the immune system detects and interacts with. There are three phases to this process: elimination, equilibrium and escape. In the elimination phase the immune system recognizes and destroys most of the tumor cells. Then the latency phase begins, consisting of equilibrium between immunological elimination and tumor cell growth. Finally, a minor attack-resistant subpopulation escapes and forms a clinically detectable tumor mass. Herein we review current knowledge of immunological characterization of CSCs/TICs. Due to the correlation between CTCs/TICs and drug resistance and metastasis, we also comment on the crucial role of key molecules involved in controlling CSCs/TICs properties; such molecules are essential to detect and destroy CSCs/TICs. Monoclonal antibodies, antibody constructs and vaccines have been designed to act against CSCs/TICs, with demonstrated efficacy in human cancer xenografts and some antitumor activity in human clinical studies. Therefore, therapeutic strategies that selectively target CSCs/TICs warrant further investigation. Better understanding of the interaction between CSCs and tumor immunology may help to identify strategies to eradicate the minor subpopulation that escapes conventional therapy attack, thus providing a solution to the problem of drug resistance and metastasis.

Keywords: Cancer stem cell (CSC); immunoresistance; immunotherapy

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Introduction

Tumor immunosurveillance

Cancer is caused by an accumulation of genetic alterations in cells which drive activation or overexpression of proteins that promote cell cycle arrest and cell survival, while other proteins that promote cell cycle arrest or cell death are inactivated or downregulated (1). In normal circumstances,

most of these lesions are repaired or the mutated cells are eliminated by control mechanisms such as DNA repair enzymes, tumor suppressor genes (2) and the immune system (3). Thus growth of tumor cells is prevented and innate immunity constitutes a first line of defense. Stress induces upregulation of ligands that activate natural killer (NK) cell receptors (4) and other immune stimulatory surface molecules that recognize and eliminate tumor cells.

This response can activate an adaptive immune response against antigens specifically expressed by lysed tumor cells and lead to T cell-dependent tumor control. Key molecules for tumor immunosurveillance are interferon-gamma (5), interleukin-12 (IL-12) (6), perforin (7), TRAIL (8), DR4 and DR5 (9) and the recombination activating genes RAG1 (10), and RAG-2 (5). RAG1 and RAG-2 are required for cell development, as is the T cell receptor (11,12). Loss of any of these molecules results in more frequent or faster spontaneous or carcinogen-induced tumorigenesis. The ability of cells to evade destruction by the immune system is thus recognized as a hallmark of cancer (2).

Cell immune surveillance evasion

The immune system is able to maintain tumor growth in a dormant state for decades without completely eradicating all the malignant cells. Certain factors may reduce the ability of the anti-tumor immune system to detect and eliminate malignant cells: pre-established tolerance resulting from non-recognition of tumor antigens (13), generation of less immunogenic tumor cell subclones and immunosuppressor molecules such as cytokines or hormones that cause NK and T cell suppression in the tumor microenvironment (14). Lower levels of activatory and/or higher levels of inhibitory NK cell receptor ligands may allow some malignant cells to survive (15). Aggressive tumors are often characterized by low levels of classical human leukocyte antigen (HLA) class I molecules. People with immune system deficiencies such as human immunodeficiency virus (HIV) (16), or who have undergone an organ transplant (17), and the very elderly run an increased risk of developing cancer (18).

Cancer stem cells (CSCs) hypothesis

Tumors are composed of heterogeneous cell subpopulations, defined by two different theories: the stochastic or clonal evolution model, and the hierarchical or CSC model. These theories appear to be mutually exclusive but new data suggest that neither should be discounted (19). In the stochastic model, all tumor cells are biologically equivalent, with a similar capacity for self-renewal and formation of new tumor cells. Cell heterogeneity arises from subclonal differences resulting from genetic and/or epigenetic changes during cancer development. In the hierarchical model, only a cell subpopulation—also known as tumor initiating cells (TICs) (20)—is able to initiate tumor growth. The hierarchical hypothesis defines

CSCs as a minority cell tumor subpopulation endowed with properties such as self-renewal, differentiation and multi-potency. CSC-like properties may also be a function of cell type origin, signals from the stromal microenvironment, accumulated somatic mutations and stage of malignant progression (21). These cells display resistance to chemotherapy (22), radiotherapy (23) and immunotherapy (24) and are TICs (4).

Several mechanisms, such as quiescence, are involved in chemoresistance (22). Certain drug-resistant proteins also make stem cells more resistant to toxins that kill their terminally differentiated counterparts (25). For example, resistance is dependent on IL-4 signaling, since up-regulation of IL-4 may result in resistance to apoptosis (26). In addition, CSCs/TICs that have undergone an epithelial-mesenchymal transition (EMT) appear to be more resistant to chemotherapy (27). An increase in aldehyde dehydrogenase (ALDH) activity in these cells seems able to mediate resistance to some chemotherapeutic agents (28). B-cell lymphoma-2 (BCL-2) protein and its family members (29) also constitute another mechanism of chemoresistance. Therefore, CSCs/TICs possess different mechanisms of resistance to several therapies.

Exact characterization of markers that allow identification of CSCs/TICs in different tumors is still not possible, since no markers have been reported as being unique to CSCs/TICs. Markers such as CD166 have been defined for several tumors. For example, CD166 is a marker of CSCs/TICs in non-small cell lung cancer (NSCLC) (30). The diversity of markers associated with CSCs/TICs may be due to the existence within the tumor tissue of different subpopulations endowed with stem cell features but also with distinct biological properties (31) reflecting differences in patients' genetic backgrounds and intra-and/or inter-cancer heterogeneity of the primary tumor (32).

CSCs/TICs and the immune system

Immune system and elimination of CSCs/TICs

The process by which the immune system detects and interacts with tumor cells, both before and after clinical detection of the tumor, is known as tumor immunoediting. This process has three phases: elimination, equilibrium and escape (33). In the elimination phase, the innate and the adaptive immune system recognize and destroy most of the tumor cells. However, some malignant cells escape and a latency phase begins, consisting of equilibrium

between immunological elimination and growth of tumor cells that may persist for months, years or decades (34). During this period, the cells suffer genetic and epigenetic changes and some generate new immunogenic peptides, enabling the tumor to eliminate these cells. However, some of these changes generate a poorly immunogenic stem cell subpopulation that circumvents immune recognition and also these cells may manipulate the immune system to promote their own growth (35). However, the lack of a favorable microenvironment, and a low rate of cell division, still prevents the formation of a tumor mass (36). Finally, the less immunogenic CSCs/TICs, and the more aggressive clones, are able to form a clinically detectable tumor mass and initiate the escape phase. The reasons for this are as follows: (I) CSCs/TICs can produce immunosuppressive molecules that attenuate the immune system (34); (II) CSCs/TICs recruit cells that suppress the immune system (37); (III) immunology tolerance due to loss of tumor antigen expression, loss of antigen processing and presentation machinery, down-regulation major histocompatibility complex (MHC) class-I (MHC I) expression, and inhibition of co-stimulatory or MHC II molecule expression on antigen presenting cells (APCs) due to genetic alterations (38). Also, the immune system may be weakened by illness, aging or therapeutic immunosuppression. Certain signaling pathways, such as Notch, Wnt and Hedgehog, are able to promote CSC/TIC escape (39).

Immunological characteristics of CSCs/TICs

The capacity of CSCs/TICs to present tumor antigens to T cells for immune recognition or to elicit immune response is determined by expression of antigen presentation molecules, such as MHC-I and MHC-II, as well as co-stimulatory (e.g., CD80, CD86) and co-inhibitory molecules [e.g., cytotoxic T-lymphocyte antigen 4 (CTLA4), B7-H2, B7-H3, programmed death receptor 1 (PD-1)/-1L] (where co-stimulatory molecule expression is negative for these cells and expression of co-inhibitory molecules is up-regulated) (40). CSCs/TICs subsequently show down-regulation of MHC-I and lack MHC-II molecule expression, resulting in downregulation of low molecular weight protein (LMP) antigen processing systems, a transporter associated with antigen processing (TAP), and beta macroglobulin which elicits escape from immune system attack (41).

CSCs/TICs have been shown to secrete cytokines such as transforming growth factor beta (TGF- β), IL-10 and

IL-13 *in vitro* (42). In glioblastoma, CSC/TIC survival has been found to be dependent on secretion of associated angiogenic factors such as vascular endothelial growth factor (VEGF), macrophage-chemoattractant protein-1 (MCP-1), macrophage inhibitory factor (MIF), growth related oncogene alpha (GRO α) and ecotaxin (43). Also, TGF β , IL-6 and IL-8 expression are downregulated in CSCs/TICs (43). In addition, stromal fibroblasts of the tumor microenvironment may be involved in regulating CSC/TIC generation by release of CCL-2 (44). Breast cancer and glioblastoma CSCs/TICs secrete more TGF β than normal cancer cells (45). Colon CSCs/TICs secrete IL-4, which promotes drug resistance and inhibits anti-tumor immune responses (46). CD200 is also expressed in CSCs/TICs and plays an important role in immune escape (47).

Anti-apoptotic molecules like bcl-2, bcl-xL and survivin protect cells against chemotherapy as well as conferring increased resistance to apoptosis-inducing immune effectors like T or NK cells (48). In a similar manner, the PI3K/Akt pathway mediates chemoresistance and tumor immune escape (49). HER2 interferes with antigen processing and presentation and is key to maintenance of CSCs in luminal breast cancer (50). In summary, CSCs/TICs express soluble and membrane-bound molecules that modulate immune responses and protect cells from immune system attack.

The STAT3 pathway plays an essential role in tumor-mediated immunosuppression by inhibiting macrophage activation (51). STAT3 pathway also reduces the cellular cytotoxicity of NK cells and neutrophils as well as expression of MHC II, CD80, CD86 and IL-12 in dendritic cells (DCs), rendering them unable to activate T cells and initiate antitumor immunity (52). In addition, STAT3 regulates transcription of immunosuppressive factors such as IL-10, VEGF, PGE₂ and TGF- β (53). It has been shown that STAT3 signaling is up-regulated in glioma CSC/TICs, and growth and self-renewal of this subpopulation is dependent on this pathway. CSCs/TICs also secrete some factors that induce STAT3 phosphorylation in immune cells (54).

Tumor-associated antigens (TAAs) expressed by CSCs/TICs

CSCs/TICs express TAAs, which characterize their condition of "stemness" and can be recognized by T cells. TAAs are classed as different subgroups of molecules (41,55) as follows:

- (I) Differentiation antigens from which the tumor derives and which could also be expressed by normal cells, i.e., carcino-embryonic antigen (CEA)

- in colon cancer, mucin-1 (MUC-1) in breast cancer, and gp100 and tyrosinase in melanoma (56);
- (II) hTERT and surviving antigens, and other apoptosis-inhibitory proteins expressed by non-stem cancer cells in addition to subsets of normal cells (57);
 - (III) Cancer-testis (CT) antigens such as Melanoma-associated-antigen-A3 (MAGE-A3) and A4 and NY-ESO1 expressed in normal cells, tumor cells and CSCs/TICs (57);
 - (IV) Mutated antigens deriving from somatic point mutations in tumor cells that can result in entirely new epitopes recognizable by the immune system (58).

In melanoma, the CSC/TIC subpopulation that express ATP-binding cassette sub-family B member 5 (ABCB5) elicits tumor cell dissemination through mediation of chemotherapy resistance, has low levels of lineage-related and CT antigens (59). However, the CD133⁺ melanoma cell subpopulation has high expression of NY-ESO1 cancer testis antigen as well as susceptibility to specific T cells (60). The TAA DDX3X has been found in CD133⁺ CSCs/TICs in melanoma and many cancers, conferring immunogenicity on these cells and their ability to induce T-cell dependent protection against murine cancer growth *in vivo* (61). In contrast, the CD271⁺ CSC/TIC melanoma subpopulation is deficient in the expression of both lineage-related and CT antigens, making their removal by immune T cells difficult. This has been correlated with progression and metastasis of these cells. As such, melanoma cells offer a good example of multiple CSC/TIC subpopulations with different antigen expression patterns (62).

None of these potential TAAs seem to be a specific marker of CSCs/TICs since they may also be expressed in both tumoral and normal cells. However, T cell responses against TAAs are expressed by CSCs/TICs, such as IL-13R α 2, SOX2 and CD133 in gliomas (63), CEP55 and COA-1 in colorectal cancer (CRC) (64) and epithelial cell adhesion molecule (EpCAM) in retinoblastoma (65). A possible exception is TAAs resulting from somatic point mutations of tumor cells and their CSCs (66).

Immune targeting of lung CSCs/TICs

Introduction

There is a strong relationship between resistance to conventional therapies and intrinsic mechanisms of CSC/TIC resistance to chemo or radiotherapy. Direct targeting

of CSCs/TICs or specific signaling pathways responsible for resistance can improve treatment benefit (67). Until recently, in contrast to tumors like melanoma, lung cancer was not thought to be immunogenic. Several immunotherapies, such as IL-2, interferon and bacille Calmette-Guerin, have been tried but have not proved successful to control the immune system in NSCLC patients. Therefore, immunotherapy for NSCLC was considered unsuccessful (68). However, immunotherapeutic approaches involving both stimulation of immune responses and inhibition of immune checkpoints have now been tested and could be combined with chemotherapy or targeted therapies with demonstrated efficiency in lung cancer (Figure 1). A body of evidence now suggests lung cancer is immunogenic. Lung cancer cells release growth factors, interleukins, cytokines and prostaglandins that inhibit T-cell response to the microenvironment, and also has been described that increased tumor-infiltrating lymphocytes (TILs), NK cells, DCs, cytotoxic T lymphocytes (CTLs) and T helper cells are associated with improved survival in NSCLC (69). Also, a high ratio of effector T-cells to regulatory T cells (T-reg) is associated with improved long-term survival (70). In addition, increased immunosuppressive T-regs as a proportion of total TILs are associated with poorer survival in lung cancer (69). MHC class I expression is reduced in NSCLC and these tumors can therefore escape routine antigen processing (71).

Immunotherapy tends to produce durable responses in small subpopulations of patients. The challenge currently facing investigators is to identify biomarkers predictive of response. Good examples so far are CTLA4 and PD-1 and its ligand (72). Immune targeting of stem cells carries some risks, one obvious one being that pathways are shared with normal adult stem cells, and autoimmunity could carry toxicity to these normal cells. Therefore, it is crucial to identify markers exclusive to CSCs (73). Other obstacles could also limit immune responses, such as a variety of defense mechanisms like soluble mediators TGF- β and COX-2 which make prostaglandin E, IL-10 and arginase. Also defensive molecules such as Fas ligand, B7-H1, nonconventional HLA molecules, lack of MHC class I and recruitment of suppressor type cells (74). Very low levels of expression of these molecules limit detection and elimination of CSCs/TICs.

Cancer cells express many antigens that can be recognized and presented to T cells, leading to T cell activation and elimination of these tumoral cells. This T cell immune response is modulated by negative regulatory

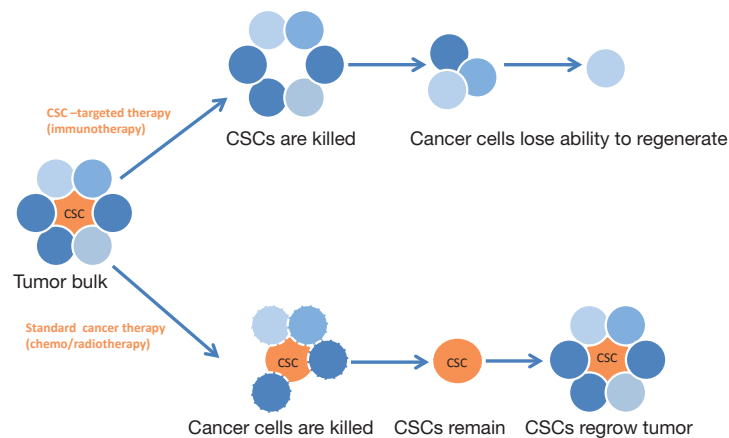


Figure 1 Heterogeneous subpopulation of cancer cells could be treated with standard therapy, as chemo- or radio-therapy. This treatment eliminates cancer cells but not CSCs and tumor grows back. If the cancer cells are treated with CSC-targeted therapy, as monoclonal antibodies or vaccines, the immune system could be stimulated or immune checkpoints inhibited, and then CSCs are killed and tumor loses its ability to generate new cancer cells. CSC, cancer stem cell.

Table 1 Immune checkpoint blockade

Compound	Target
Ipilimumab	CTLA4
Tremelimumab	CTLA4
Nivolumab	PD-1
Pembrolizumab	PD-1
BMS-936559	PD-L1
MPDL3280A	PD-L1
MEDI4736	PD-L1
Bec2	GD3
Bevacizumab	VEGF
Urelumab	CD137
TRX518	GITR
Anti-OX40	OX40
Anti-CD40	CD40
Solitomab	EpCAM
Anti-CD133	CD133
Lirilumab	KIR
BMS-9896016	LAG-3
Racotumomab	N-glycolil-GM3 ganglioside

CTLA4, cytotoxic T-lymphocyte antigen 4; PD-1, programmed death receptor 1; PD-L1, programmed death ligand 1; VEGF, vascular endothelial growth factor; GITR, glucocorticoid-induced tumor necrosis factor receptor; EpCAM, epithelial cell adhesion molecule; KIR, killer cell immunoglobulin-like receptor; LAG-3, lymphocyte-activation gene 3.

molecules such as the immune checkpoint molecules CTLA-4, PD-1, killer cell immunoglobulin-like receptor (KIR) and lymphocyte-activation gene 3 (LAG3); these molecules prevent overstimulation of immune responses. The T cell immune response could be also modulated by co-stimulatory molecules such as glucocorticoid-induced tumor necrosis factor receptor (GITR), OX-40, CD28 and CD137 (75). Deregulation of these molecules in the tumor leads to tolerance of the tumor by the immune system and cancer cell escape from surveillance. A description of the different compounds tested to target these regulatory molecules follows (Table 1).

Immune checkpoint blockade

Immune checkpoints are inhibitory pathways crucial for maintaining self-tolerance and to escape to immune system control by the tumor (76). It has observed that inhibitory ligands and receptors are usually overexpressed in cancer cells or their microenvironment (77). Inhibition of these immune checkpoints releases the brakes on the immune system, resulting in antigen-specific T-cell responses. Such inhibition of immune checkpoints relies on the presence of TILs. Stimulation of TILs and/or modulation of the tumor microenvironment could weaken immune responses (78). In lung cancer, targeting the immune checkpoint molecules, CTLA4, PD-1 and its ligand PD-L1 has achieved promising and durable responses but it

remains unclear why some patients have only transient or no response (79). One strategy is to target CSCs/TICs with monoclonal antibodies targeting antigens that are differentially overexpressed in these cells. These could be used alone as unmodified antibodies to allow antibody-dependent cytotoxicity (ADCC) to occur, or used with radioisotopes, chemotherapy, cytokines or enzymes to target cancer. A problem of this treatment is that stem cells could escape the cytotoxic effect of specific antibodies by decreasing expression of surface antigen, developing chemotherapy resistance or acquiring multiple mutations. Therefore, antibody treatment is used in combination with conventional cancer therapies (80).

CTLA4

CTLA4 is an immunomodulatory molecule expressed in T cells which plays a role in regulating T-cell activity at early stages of activation; its expression on T cells increases after exposure to an antigen. Binding of the CTLA-4 receptor to CD80/86 expressed on APCs has a co-inhibitory effect on T cells. By competing with the CD28 molecule for the same ligands, albeit with a higher binding affinity than CD28, CTLA-4 inhibits T-cell activation (68). Negative signals are delivered to T cells upon binding to APC CD80/CD86 molecules via CTLA4, T cell function is inhibited and T cells can then be eliminated via apoptosis. Lung cancer could stimulate abnormal expression of CTLA-4 in T cells and these T cells exhibit an anergic phenotype (81). There are currently several clinical trials in lung cancer with human monoclonal antibodies against CTLA4 like ipilimumab or tremelimumab. To date, the response rate is low but these responses are more durable than with cytotoxic therapies (82).

Ipilimumab is a human monoclonal antibody that blocks binding of CTLA-4 to its ligand. As a single agent it has virtually no effect (83) but does seem to provide modest benefit in NSCLC and small cell lung cancer (SCLC) patients in combination with chemotherapy. A phase II study of chemotherapy, paclitaxel and carboplatin with and without ipilimumab in stage IV NSCLC showed a significant improvement in progression-free survival (PFS) when ipilimumab was given after chemotherapy (5.7 *vs.* 4.6 months) (84). Patients with squamous histology showed better response than non-squamous histology. Now are several clinical trials ongoing. A phase III trial is currently comparing ipilimumab to placebo in SCLC patients receiving platinum and etoposide, and another phase II is comparing ipilimumab to pemetrexed in non-squamous NSCLC. Ipilimumab is also being evaluated with the

anti-KIR antibody BMS-986015 that recognizes KIR in NSCLC, castration-resistant prostate cancer (CRPC) and melanoma (85). These antibodies must be used carefully as they can cause autoimmunity and other severe side effects that limit their use (86). Tremelimumab, which also targets CTLA4, has been tested as maintenance therapy compared with observation in patients with stable or responding disease after first line chemotherapy, however, no improvement in PFS was seen (87).

PD-1

PD-1, like CTLA-4, is a member of the CD28 family. PD-1 is expressed in T cells and inhibits their survival, proliferation and immune function through interaction with its ligands PD-L1 and L2. PD-1 is also expressed in B cells and in some myeloid cells (88). Interactions between PD-1 and its ligands attenuate immune responses (89) and serve to protect tumor cells from cytotoxic T cells since T cells become triggered for apoptosis upon signal transduction with PD-1 family proteins (90). Clinical trials with humanized monoclonal antibodies against PD-1 have shown good antitumor activity in subsets of patients with metastasis disease with a good safety profile (80). Several PD-1 antibody trials are ongoing and one study has found a strong correlation between pretreatment tumor expression and responses (72).

Nivolumab, a human monoclonal antibody that binds to PD-1, has been tested in several clinical studies in NSCLC and in two trials specifically for primary squamous cell carcinoma (SQCC), either as a single agent or in combination with chemotherapy or ipilimumab (68). In other studies it was combined with anti-KIR antibody (91). In a phase I clinical study it was administered to 306 patients with different tumor types, including 129 NSCLCs. Overall response rate (ORR) of this study was 17% and the median duration of response was 47 weeks. Another 10% of patients showed stable disease for 6 months with median survival of 9.6 months. Thirty seven patients who received nivolumab at doses of 3 mg/kg showed 24% response rate and 14.9 months median survival (68). Ongoing phase III studies are comparing nivolumab *vs.* docetaxel in the second-line setting and a phase III first line trial of nivolumab *vs.* standard chemotherapy in PD-L1 positive metastatic NSCLC is currently recruiting (92). An ongoing phase I clinical trial is combining nivolumab plus ipilimumab with an ORR of 22% at time of interim analysis (93).

Another anti-PD-1 antibody similar to nivolumab, pembrolizumab (also known as MK-3475 or lambrolizumab) is a humanized IgG4 antibody that contains a mutation

at C228P designed to prevent Fc-mediated ADCC. In a phase I study, 38 NSCLC patients were treated with pembrolizumab, achieving 24% of lasting responses in previously treated patients. Pembrolizumab is now being examined in the relapsed/refractory setting (NCT01905657) and in combination with first-line chemotherapy (NCT01840579) (85,86).

PD-L1 (B7-H1)

Another therapeutic strategy is inhibition of PD-L1. PD-L1 is overexpressed in around 50% of NSCLC patients and is associated with poor prognosis. Its overexpression induces T-cell anergy and circumvents recognition and processing of tumor antigens by APCs (90). A potential advantage of this approach is lack of interference with T-cell PD-1 receptor interaction with APCs via other ligands, such as B7-H2 (94). A human anti-PD-L1 antibody, BMS-936559, has been tested in a phase I trial and showed promising clinical activity and good safety profile in NSCLC with partial response in 5 of 49 patients (68,95). Other antibodies in clinical development are MPDL3280A (RG7446), a human IgG1-kappa anti PD-L1 monoclonal antibody with a single amino acid substitution in its Fc region that docks with Fc receptors in circulating immune cells, thus preventing ADCC and inadvertent killing of bystander immune cells that also express PD-L1, such as activated T cells. In a phase I trial, 85 NSCLC patients received MPDL3280A as a single agent, with 23% best overall response and 24-week PFS of 46% (96,97). Another IgG1-kappa PD-L1 inhibitor is the antibody MEDI4736, engineered with a triple mutation in the Fc domain that also avoids ADCC as does MPDL3280A. MEDI4736 is currently being tested in a phase I clinical trial. In this study, of 11 NSCLC patients evaluated for efficacy, three achieved partial response, two showed stable disease and one had disease progression (68).

In conclusion, a few patients have good responses to anti-PD-L1 antibodies like nivolumab, MPDL3280A or MEDI4736, despite the absence of PD-L1 expression by immunohistochemistry. However, robust predefined cut-points or independent external validation methodology are not available in the literature. In addition, use of fresh or paraffin-embedded tumor samples could affect results in fresh samples due to the influence of cytokines, such as IFN- α , that upregulate PD-L1 expression (86,98).

GD3

GD3 is a cell surface ganglioside highly expressed in SCLC but not in NSCLC. Bec2/bacille Calmette-Guerin is an

anti-idiotypic antibody that binds to the idiotype of the antibody against GD3. Therefore, Bec2/bacille Calmette-Guerin is thought to mimic GD3. In a phase III clinical trial in 515 limited stage patients, use of Bec2/bacille Calmette-Guerin showed no improvement in survival, PFS, or quality of life in the vaccination arm compared with control arm (median survival 16.4 *vs.* 14.3 months, respectively) (99,100). 1E10 is an anti-idiotypic antibody against Neu-glycosylated sialic acid ganglioside (NeuGc-GM3). It was used in clinical trials in SCLC and NSCLC, and a survival benefit of about 6 months was noted in those patients that developed immunity to NeuGc-GM3 (101).

Vascular endothelial growth factor (VEGF)

Bevacizumab is an anti-VEGF antibody that plays a role in tumor angiogenesis and inhibition of immune response by switching off the action of DCs. A phase III clinical trial in metastatic NSCLC demonstrated improved PFS and overall survival (12.5 *vs.* 10.2 months) (102).

Other immunotherapy compounds

CD137, GITR and OX40 are positive regulatory molecules of T cell immune responses. Now we describe some compounds that target these molecules.

Urelumab (BMS-663513) is a human IgG4 monoclonal antibody that targets CD137 receptor of the tumor growth factor alpha (TNF α) family and acts as co-stimulatory molecule of T cell activation. Urelumab activates a component of the TNF receptor expressed on the cell membrane of activated white blood cells, subsequently activating CD137-expressing immune cells and stimulating a cytotoxic T cell response against tumor cells. Clinical development in NSCLC has been stopped but is continuing in other cancers (NCT014712109) (85,86).

GITR is a member of the TNF receptor family. GITR co-stimulates CD4⁺ and CD8⁺ naïve T cells, leading to T cell proliferation and effector function (85,103). TRX518 is an anti-GITR antibody currently being tested in a phase I trial in melanoma (NCT01239134).

OX-40 (CD134) is also a member of the TNF receptor family. Like CD137 and GITR (101), OX-40 is a co-stimulatory molecule in activated T cells at sites of inflammation and regulates antigen-specific T-cell expansion, survival and cytokine production (IL-2, IL-4, IL-5, IFN-gamma) (104). In a phase I trial, 30 patients with solid tumors were treated with an anti-OX-40 antibody with tumor reduction in 12 patients and enhanced humoral and cellular immunity (75,105).

Table 2 Vaccines

Compound	Target
Stimuvax	MUC-1
Anti-NY-ESO1	NY-ESO 1
GSK1572932	MAGE-A3
CimaVax	EGF
Anti-WT-1	WT-1
Anti cyclophilin B	Cyclophilin B
Lucanix	TGF- β 2
IDM-2101	CEA, p53, HER2, MAGE 2 and 3
Dendritic vaccine	p53
TG4010	MUC-1
Anti-IDO	IDO
GV1001	Telomerase

MUC-1, mucin-1; MAGE-A3, melanoma associated antigen A3; EGF, epidermal growth factor; WT-1, Wilms tumor antigen-1; TGF- β 2, transforming growth factor beta 2; CEA, carcino embryonic antigen; IDO, indoleamine-2,3-dioxigenase.

CD40, a member of the TNF receptor family, is expressed in APCs and its ligand is expressed in T cells. Binding of both enhances APC ability to present antigens and activate T cells. Preclinical studies have demonstrated that anti-CD40 antibodies have the potential to suppress tumor growth and metastasis (106).

Racotumomab (1E10) is an anti-idiotypic murine monoclonal antibody against the human monoclonal antibody for N-glycolil-GM3 ganglioside. N-glycolil-GM2 is a glycolipid present within gangliosides, sulfatides, and other antigens expressed in some solid tumors which seems to correlate with survival and suppression of immune activity in NSCLC. A phase III clinical trial (NCT01460472) is currently ongoing with a planned accrual of 1,018 participants (85).

EpCAM is a transmembrane glycoprotein overexpressed in most human carcinomas (107). Solitomab (MT110) is a single-chain bispecific T-cell engager (BiTE) antibody targeting EpCAM (108) which has been tested in dose escalation phase I clinical trials in patients with locally advanced, recurrent or metastatic lung cancer (109). CD133 is reported in CSC/TICs in lung cancer (110). A bispecific antibody against CD3 and CD133 has been designed to eradicate CD133⁺ cancer cells (111).

KIR and LAG3 are negative regulatory molecules of T cell immune responses, like PD1 and CTLA-4. Several

monoclonal antibodies are designed to target these molecules.

KIR is a receptor on NK cells that downregulates NK cytotoxicity activity (86). Lirilumab (IPH2102), an anti-KIR human monoclonal antibody, was used in combination with nivolumab and demonstrated efficacy in preclinical models. A clinical trial in 32 NSCLC patients is ongoing (NCT01714739) as is another combining lirilumab plus ipilimumab in 20 NSCLC patients (NCT01750580) (86).

LAG3 (CD223) is a receptor expressed with PD-1 on tolerant T cells and T-regs which suppresses APC activation by binding with MHC II (112) and becoming an inhibitory molecule of T cell activation in the same manner as KIR. A clinical trial is ongoing with BMS-9896016, an anti-LAG3 monoclonal antibody, alone and in combination with nivolumab (NCT01968109) (86).

Vaccines

TAAAs contain more than 70 proteins, including CT antigens such as MAGE-A3, and antigens like MUC-1 that are overexpressed in tumor cells. Using protein or peptide vaccines such as Stimuvax (tecemotide or L-BPLP25) and GSK1572932, TAAAs can be targeted for subsequent killing of tumor cells (113). There are many TAAAs expressed by tumors not identified, and to recognize them whole tumor vaccines were designed. Vaccines such as Lucanix can be harvested from the patient's own tumor (autologous) or from established cancer cell lines (allogeneic) and express many TAAAs found in patient tumors, theoretically generating an immune response to the tumor (113).

Adaptative T-cell therapy is a passive strategy that involves the transfusion of T-lymphocytes to attack cancer cells in the patient. NY-ESO-1 is one such vaccines (113).

Vaccines currently in clinical trials in lung cancer (*Table 2*).

MUC1 is a highly glycosylated transmembrane protein overexpressed and abnormally glycosylated in many cancers including NSCLC (114). High levels of MUC1 could enhance immunosuppression and predict poor prognosis in patients with adenocarcinoma (115). Stimuvax is a 25-aminoacid MUC-1 peptide formulated into liposomes targeting MUC1 (116). Several clinical trials have already been performed, including a phase IIb study in stage IIIB and IV NSCLC (117). Median survival time in patients receiving Stimuvax was 17.2 *vs.* 13.0 months for those receiving best supportive care. Three year survival was 31% with Stimuvax *vs.* 17% for supportive care (118). Following this study, a phase III clinical trial in NSCLC

was carried out (START trial) in 1,513 patients with median overall survival of 25.6 months for patients treated with Stimuvax and 22.3 with placebo. Therefore, the trial did not achieve its primary endpoint of improvement in overall survival. However, analysis of treatment with Stimuvax plus chemotherapy and radiotherapy did show an improvement in median overall survival of 30.8 months compared to 20.6 months for placebo. Inspired by these results, a new phase III clinical study is currently ongoing (START 2 trial) with a primary end-point of overall survival in patients receiving Stimuvax plus chemotherapy and radiotherapy (113). Similar to the START trial, a phase III clinical trial in Asian NSCLC patients is ongoing (INSPIRE) comparing Stimuvax with placebo. In another phase III–IV trial, NSCLC patients were treated with Stimuvax plus bevacizumab following chemotherapy. In a stage III–IV trial, 16 of 65 patients showed a T-cell immune response and had median survival of 30.6 months compared to 13.3 months for best supportive care (85,119).

NY-ESO-1 is a fusion protein vaccine currently being tested in NSCLC (120). In a clinical trial with other tumors a measurable response rate of 66% (four of six patients) was reported in synovial cell sarcomas and 45% in melanoma (five of eleven patients) (120).

MAGE-A3 is an antigen present in about 35% to 55% of NSCLC patients. GSK1572932 is a recombinant DNA vaccine composed of MAGE-A3 and immunoadjuvant AS15. In a phase II clinical trial, 182 stage I and II patients were enrolled with a 27% improvement in time to progression and disease-free survival in patients receiving the vaccine. A phase III clinical trial is ongoing studying the combination of the vaccine with adjuvant chemotherapy in 2,270 NSCLC patients (121).

Mutations in the *epidermal growth factor receptor (EGFR)* gene are associated with cell proliferation, apoptosis, angiogenesis and metastasis. The epidermal growth factor (EGF) ligand is often overexpressed in lung cancer and its receptors frequently mutated (122). The CimaVax vaccine is a humanized recombinant EGF fusion protein that targets the EGF ligand circulating to prevent EGFR activation. Circulating anti-EGF antibody titers increased as a result of vaccination. These findings were then correlated with decreased levels of serum EGF and patient survival. A phase II trial included 80 patients with NSCLC (stage IIIB or IV) after first-line chemotherapy and demonstrated a decrease in EGF concentration in patient serum. A strong correlation was found between antibody titer and reduction in EGF concentration. Reduction of EGF concentration to

below 168 pg/mL is associated with prolongation of overall survival (13 months with 168 pg/mL or less *vs.* 5.6 months above 168 pg/mL). High initial concentration is a predictive factor of vaccine response and an adverse prognostic factor for non-vaccinated patients. A phase III clinical trial is ongoing (85,123).

A phase I trial in stage III–IV NSCLC is investigating vaccines targeting indoleamine-2,3-dioxygenase (IDO), an immune regulatory protein that suppresses activity of CD8⁺ cytotoxic T cells. To date, long-lasting clinical benefits have been demonstrated in almost half of the patients (124).

GV1001 is a telomerase-based vaccine used in clinical trials in NSCLC patients previously treated with chemotherapy and radiotherapy (85). In a phase II trial (CTN-2006), 23 stage III patients received radiotherapy and docetaxel followed by GV1001 vaccination. Long-term immunomonitoring showed durable responses in 13 patients. Immune responders achieved a median of 371 days survival, compared with 182 days for non-responders. In another clinical trial (CTN-2000), 26 patients were vaccinated with two telomerase peptides (GV1001 and I540). Thirteen developed a GV1001 response and achieved increased survival compared with non-responders (median survival 19 *vs.* 3.5 months, respectively) (125).

The Wilms tumor antigen-1 (WT-1) is found in most NSCLC and SCLC patients (126) and a clinical trial tested a 9-mer of WT-1 in several tumor types. Three of 10 lung cancer patients showed an immunological response and one patient continues to survive following repeated vaccinations over more than 2 years (127). WT2725 is a peptide vaccine derived from Wilms tumor protein; a clinical trial in SCLC is also ongoing (85).

Cyclophilin B is found in lung cancer patients and can be a target of CTLs (128). A cyclophilin-based vaccine is being tested in a phase I trial, though no significant increases in cellular response have been observed.

TGF- β 2 is released by tumor cells in their microenvironment to protect themselves from immune system. Expression of TGF- β 2 has been correlated with poor prognosis in NSCLC (129). Lucanix (Belagenpumatucel-L) is a vaccine consisting of allogeneic NSCLC cell lines transfected with an antisense plasmid to TGF- α 2, designed to block TGF- β secretion. A phase II clinical trial in 75 NSCLC patients (stages II–IV) has been completed. The estimated probability of surviving 1 or 2 years was 39% and 20% for patients receiving a low dose of the vaccine and 68% *vs.* 52% for the higher doses. Estimated median survival time for patients on the low dose was 252 *vs.*

581 days for the high dose (129). This vaccine is now in a phase III study (STOP) with 532 patients enrolled. This trial did not meet its primary endpoint, with median overall survival of 20.3 months in vaccine-treated patients treated *vs.* 17.8 months in the group control, but a marked improvement in survival has been detected in specific subgroups of patients (85,113).

The IDM-2101 peptide vaccine is based upon ten different HLA-A2 restricted epitopes against five different antigens (CEA, p53, HER2, MAGE-2 and MAGE-3 antigens along with a pan-DR epitope). A phase II study has been completed and demonstrated immune response (130).

DC vaccines: most smoking-related cancers have p53 mutations and DC vaccines are based on infecting DCs with p53 adenoviruses (131). In *in vitro* experiments, when these transfected DCs are activated they can generate CTLs against p53 (132). In SCLC patients, a significant immune response is induced and patients are sensitized to chemotherapy (133). Cyclophosphamide followed by vaccinations with tumor-antigen-loaded, DC-derived exosomes inhibits Treg functions, restoring T and NK cell effector functions and activating cell immunity. This is currently being studied in phase I trials (85).

Conclusions

The study of two different scientific fields such as stem cell research and cancer immunology and the links between the two could be crucial to develop new therapeutic approaches to prevent metastasis and development of therapy resistance. CSCs/TICs are characterized by low immunogenicity and immunosuppressive activity. They defend themselves from the immune system and adapt to modifications in the tumor microenvironment caused by chemotherapy or radiotherapy. After chemotherapy and radiotherapy, some resistant cells remain that could be detected and partially killed by the immune system. Equilibrium subsequently occurs between immunological elimination and growth of cancer cells and during this period cells may suffer some changes, giving rise to a poorly immunogenic stem cell subpopulation that is not recognized by the immune system. The molecular identification of immunomodulating agents that can reverse or inhibit CSC/TIC escape from immunosurveillance should allow design of new immunotherapy protocols targeting CSCs/TICs. Immune checkpoint blockade has shown promising results in clinical trials in lung cancer. Responses tend to be durable, but there are problems with inter-patient heterogeneity of responses and appropriate

patient subpopulations need to be identified. The tumor microenvironment could play a major role in modulating immune response. The success of immunotherapeutic approaches will depend on a better understanding of the basic biology of immune responses and, in particular, the role that tumor microenvironment plays in shaping immune responses. Vaccines targeting stem cells genes, however, are not without potential risks and adverse effects. The most obvious risks relate to pathways shared with normal stem cells. Research into combination of CSC/TIC-targeting antibodies and/or vaccines with conventional cancer therapies at the optimum moment during the course of the disease, and the identification of suitable biomarkers could improve cancer treatment, is therefore crucial.

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Footnote

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