

## Immune ligands for cytotoxic T Lymphocytes (CTLs) in cancer stem cells (CSCs)

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### 1. ABSTRACT

The immune system has come to the forefront of cancer therapeutics in recent years with the success of immune blockade inhibitors in a variety of cancers whose list is increasing with a quick pace. Despite the efficacy of these drugs across a significant part of the cancer spectrum, responses are still seen only in a minority of patients, that implies that most patients are refractory or promptly develop resistance to these agents. Mechanisms of this resistance are important to decipher as this knowledge may lead to the introduction of additional therapies or manipulations to modulate resistance. The cancer stem cell theory stipulates that a minority of cancer cells in a given tumor are responsible for self-renewal and bulk tumor propagation. These cells, in most instances, are rare and less proliferative but give rise to highly proliferative progeny. In addition, they are, in general, resistant to therapies and endowed with metastatic potential through a process called EMT (Epithelial to Mesenchymal Transition). Cancer stem cells resistance to treatments may relate to inherent insensitivity to external apoptotic stimuli and, thus, may extend to immune therapies by inhibiting the actions of Cytotoxic T Lymphocytes (CTLs) in the tumor micro-environment. This paper examines available data on expression and regulation of immune co-modulatory (co-stimulatory and co-inhibitory) ligands on cancer stem cells in order to devise strategies to circumvent resistance.

### 2. INTRODUCTION

Heterogeneity is a characteristic of cancers with various cell clones arising along the natural history of the disease but also due to selection pressure from treatments as well as the immune system attack (a phenomenon termed immunoediting) (1). The Cancer Stem Cell (CSC) theory hypothesizes that CSCs (also called tumor-initiating cells) are the cells where transformation takes place and are able to give rise to the bulk of the tumor that contains more differentiated progeny in a manner similar to normal tissue physiology where normal adult resident stem cells are able to replace normally lost differentiated tissue cells (2). An alternative hypothesis postulates that transformation happens randomly in any tissue cell that obtains the genetic lesions for developing the required cancer capabilities (3). The two hypotheses are not mutually exclusive and in fact even if transformation may occur in any cell, it requires for this cell to regress towards the stem cell phenotype as part of this transformation, in order to acquire the plasticity enabling it to differentiate towards the various bulk cells of the tumor as well as being able to reproduce itself. CSCs have been confirmed in most types of cancers and are generally a small minority of tumor cells (4). Both anti-cancer treatments and the pressure from the immune system surveillance have been described as able to enrich for CSCs by depleting preferentially non-stem cancer cells (5, 6). This may in fact imply that CSCs are inherently immune resistant in addition to resistant to

chemotherapy and that the CSCs transcription factor network activity promotes concomitantly immune evasion (7).

Immune checkpoint inhibitors are novel antineoplastic therapeutics that enhance the immune response and, thus, they are at the crossroads of both these possible CSC-enriching culprits. In addition, the recent introduction of these inhibitors in the clinical arena with positive results in difficult to treat cancers such as metastatic melanoma and lung cancers has rekindled interest in immune system manipulations as a means to combat cancer. The activity of immune system effectors cytotoxic T lymphocytes (CTLs) towards the CSC compartment could be critical for a successful treatment with immune checkpoint inhibitors or other methods that harness the immune system abilities against an established cancer. Thus, this paper will discuss expression and regulation of immune ligands in CSCs as these surface molecules are in the center-stage of the interaction of immune cells with CSCs.

### **3. BASIC BIOLOGY OF IMMUNE RECOGNITION BY CTLs**

Several surface molecules are involved in the interaction of non-immune cells with immune cells that survey tissues for foreign microbial cells and infected or transformed cells. Central role for the engagement of CTLs is played by Major Histocompatibility Complex type I (MHC I) that presents an oligopeptide antigen in complex with an invariant  $\beta$ 2-microglobulin chain ( $\beta$ 2m) and is the ligand for the T cell Receptor (TCR). This interaction provides the specificity of the engagement and is preceded by several steps known as the tumor-immune cycle that starts with pick-up of an antigen from dying tumor cells by antigen presenting cells (APCs) which then move to lymph nodes and present the antigen to antigen-specific CTLs which in their turn are attracted back to the tumor microenvironment (8). The cycle culminates in recognition of the antigen presented in the MHC I complex by the TCR. In order for an immune attack and lysis of the tumor cell to proceed, TCR engagement must be accompanied by engagement of co-activator ligand-receptor pairs that include CD80 or CD86 and CD28, OX40L and OX40, CD40L and CD40, 4-1BBL and 4-1BB, in target cells and effector cells, respectively (For a more complete list of co-receptor/ ligands discussed in this paper see Tables 1 to 3). Alternatively, co-inhibitory ligand-receptor pairs engagement results in anergy and CTLs death. If, for example, CD80 or CD86 is ligated by cytotoxic T lymphocyte-associated antigen 4 (CTLA-4; alternatively called CD152) instead of CD28, an inhibitory signal is generated (9). The avidity of the interaction of CD80 and CD86 ligands with CTLA-4 is two orders of magnitude higher than the avidity of CD80 or CD86 for CD28 and, thus, the inhibitory signal

predominates when both CTLA-4 and CD28 receptors are present in the surface of immune cells. In addition to the membrane-bound CTLA-4, there exists a soluble form of the ligand produced and secreted from activated lymphocytes. Its role is to contain the immune response beyond the initial stimulation and diffuse the inhibitory signal to neighboring cells (10). Other ligand-receptor couples generating inhibitory signals are, for example, programmed death-ligand 1 (PD-L1; also known as CD274 or B7-H1)/ PD-L2 (also called CD272 or B7-DC)- programmed cell death-1 (PD-1; also known as PDCD1 or CD279) and galectin-9/ CEACAM-1/ HMGB1- TIM-3 (11). Thus, a balance of activating and inhibitory signals determines the final result of CTL immune attack which may result in the death of the target cell or in the neutralization of the attacker if the inhibiting signals are predominant.

Overall immune ligands may be divided into those that interact with both stimulatory and inhibitory receptors and their net effect on T cell activation depends on the availability and affinity of the two types of receptors and those that have only one type of receptor (stimulatory or inhibitory) and thus their expression has an activation or inhibition effect that depends only on the expression of the ligand by the interacting cells. B7-1 and B7-2 and their receptors CD28 and CTLA-4 represent an example of the first, dual activity ligands. Another example is represented by nectin family ligands PVRL1 and PVRL2 and their receptors DNAM-1 and TIGIT, stimulatory and inhibitory, respectively. Both examples of dual type ligands illustrate the principle that the inhibitory receptors, CTLA-4 and TIGIT have higher affinity for the ligands than the respective stimulatory receptors, CD28 and DNAM-1. In contrast, other ligands/ receptors pairs such as PD-L1/ PD-1 and 4-1BBL/ 4-1BB, for example, have only inhibitory and stimulatory interactions respectively. The latter pair belongs to the TNF/ TNFR families of co-stimulators which present a different paradigm of regulation depending on intra-cellular co-regulators to define their ultimate outcome instead of presence of stimulatory-inhibitory receptors on the cell surface.

### **4. IMMUNE LIGANDS EXPRESSION IN NORMAL STEM CELLS (SCS), EMBRYONIC STEM CELLS (ESCS) AND CSCS**

#### **4.1. Main immune CTL signal: The MHC I complex**

The primary signal for CTLs' binding and interaction with tumor cells is provided by MHC I (human HLA-A, HLA-B and HLA-C) harboring the presented antigen and expressed in the cell surface in conjunction with  $\beta$ 2m. This complex represents the ligand for the antigen specific TCR. Each component of the TCR ligand complex is important for the antigen presentation and may be down-regulated in CSCs. Cancer neo-antigens derived from mutated proteins

**Table 1.** Immune ligands and expression in cells of the tumor micro-environment.

| Ligand                    | Alternative name(s) | Type | Chromosome  | Expression/ comments   | Receptor(s)                                     |
|---------------------------|---------------------|------|-------------|--|---|
| <b>B7 family</b>          |                     |      |             |  | <b>CD28 family</b>                              |
| B7-1                      | CD80                | B    | 3q13.3.3    | Down-regulated in various CSCs   | CD28, CTLA-4                                    |
| B7-2                      | CD86                | B    | 3q13.3.3    | Down-regulated in various CSCs   | CD28, CTLA-4                                    |
| B7-H1                     | PD-L1, CD274        | I    | 9p24.1.     | Ubiquitous expression, expressed in sub-sets of gastric, colorectal, breast and lung CSCs. Decreased expression in AML progenitors | PD-1  |
| B7-DC                     | PD-L2, CD273        | I    | 9p24.1.     |  | PD-1  |
| ICOSL                     | B7-H2, CD275        | B    | 21q22.3.    | Colorectal cancer. Unknown whether expressed in CSCs   | ICOS , CD28, CTLA-4                             |
| B7-H3                     | CD276               | B    | 15q24.1.    | Co-expressed with CD133 and EMT markers in colorectal cancer   | TLT-2(debated)<br>Unknown inhibitory receptor   |
| B7-H4                     | B7X, B7S1           | I    | 1p13.1.-p12 | Expressed in various cancers, expressed in GBM CSCs  | Unknown   |
| B7-H5                     | HHLA2, B7H7         | B    | 3q13.1.3    | Expressed in various cancers. Higher expression in triple negative breast cancers than other sub-types                             | TMIGD2<br>Unknown inhibitory receptor           |
| BTLA                      | CD272               | I    | 3q13.2.     | Expressed in CD8+ tumor specific T cells of melanoma patients  | HVEM  |
| CD160                     |                     | I    | 1q21.1.     |  | HVEM  |
| <b>TNF family</b>         |                     |      |             | Ligation of TNF family members is protective against AICD  |   |
| OX40L                     | CD134L TNFSF4       | S    | 1q25.1.     |  | OX40  |
| CD40L                     | CD154, TNFSF5       | S    | Xq26.3.     |  | CD40  |
| 4-1BBL                    | CD137L, TNFSF9      | S    | 19p13.3.    |  | 4-1BB   |
| CD70                      | TNFSF7              | S    | 19p13.3.    |  | CD27  |
| GITRL                     | AITRL, TNFSF18      | S    | 1q25.1.     |  | GITR  |
| LIGHT                     | TNFSF14, CD258      | S    | 19p13.3.    |  | HVEM  |
| <b>Nectin-like ligand</b> |                     |      |             |  |   |
| PVR                       | CD155               | B    | 19q13.3.1   | Expressed in CD34+ HSCs. Expressed in various tumor cells and dendritic cells in the tumor micro-environment                       | DNAM-1, TIGIT                                   |
| PVRL2                     | CD112               | B    | 19q13.3.2   | Expressed in normal murine spermatogonial SCs  | DNAM-1, TIGIT, CD112R                           |
| <b>Other</b>              |                     |      |             |  |   |
| LAG-3                     | CD223               | I    | 12p13.3.1   | Up-regulated in stimulated CD8+ cells  | MHC II  |
| TIM-3                     | CD366               | I    | 5q33.3.     | Exhausted CTLs, melanoma, NSCLC, cervical cancer cells. Increased expression in LSCs compared to normal HSCs                       | Galectin 9. HMGB1, CEACAM1, Phosphatidyl-serine |

B in the type column denotes that the ligand interacts with both stimulatory and inhibitory receptors, S denotes that the ligand has only stimulatory interactions and I denotes that it has only inhibitory interactions. If no specific data for CSCs are available other potentially relevant expressions are mentioned in the Expression column. AICD: Activation Induced cell death. For alternative names of receptors see Tables 2 and 3

are produced in variable abundance in different cancers (12) and their production involves multiple steps including production in the proteasome, and endoplasmic reticulum lumen transfer and MHC I up-loading. Each of these steps may play a role in decreased antigen presentation. For example the proteasome shows decreased activity in CSCs (13). MHC I production and surface presentation is decreased in both the bulk tumor cells of various tumors and in normal human multipotent germ cells and embryonic stem cells (14–16). MHC I is down-regulated in some colorectal cancer cell lines and this down-regulation is

associated with deficiency in specialized proteasome sub-units of the immunoproteasome (17). Glioblastoma and astrocytoma cell lines express MHC I molecules in low percentages and in the CD133 positive fraction where the stem cells reside in even lower level but up-regulation was observed after addition of interferon  $\gamma$  in the culture (18).

$\beta$ 2m loss of expression due to loss of heterozygosity (LOH) and frameshift mutations of the remaining allele have been described and predispose cancers to immune escape (19). Epigenetic silencing

**Table 2.** Immune stimulatory co-receptors

| Receptor                  | Alternative name(s) | Chromosome | Expression  | Ligand (s)         |
|---------------------------|---------------------|------------|---|--------------------|
| <b>CD28 family</b>        |                     |            |   | <b>B7 family</b>   |
| CD28                      |                     | 2q33.2.    | Resting and activated T cells                                     | B7–1, B7–2, ICOS-L |
| ICOS                      | CD278               | 2q33.2.    | CD4+ TILs   | ICOS-L             |
| TLT-2                     | TREML2              | 6p21.1.    | Myeloid and lymphoid immune cells                                 | B7-H3 (debated)    |
| TMIGD2                    | CD28H               | 19p13.3.   | Activated T cells   | B7-H5              |
| <b>TNFR family</b>        |                     |            |   | <b>TNF family</b>  |
| OX40                      | CD134, TNFRSF4      | 1p36.3.3   |   | OX40L              |
| CD40                      | TNFRSF5             | 20q13.1.2  | Expressed in tumor cells from various cancers                     | CD40L              |
| 4–1BB                     | CD137, TNFRSF9      | 1p36.2.3   | Positive TILs have higher antitumor activity                      | 4–1BBL             |
| CD27                      | TNFRSF7             | 12p13.3.1  | T and B cells   | CD70               |
| GITR                      | AITR, TNFRSF18      | 1p36.3.3   | Activated T cells and T <sub>reg</sub>                            | GITRL              |
| HVEM                      | TNFRSF14, CD270     | 1p36.3.2   | Increased expression in colorectal, ovarian cancers and lymphomas | LIGHT              |
| <b>Nectin-like family</b> |                     |            |   |                    |
| DNAM-1                    | CD226               | 18q22.2.   | Expressed in cervical cancer and leukemia cells                   | PVR, PVRL2         |

TILs: Tumor Infiltrating Lymphocytes, T<sub>reg</sub>: CD4+/ CD25+ regulatory T cells. For alternative names of ligands see Table 1

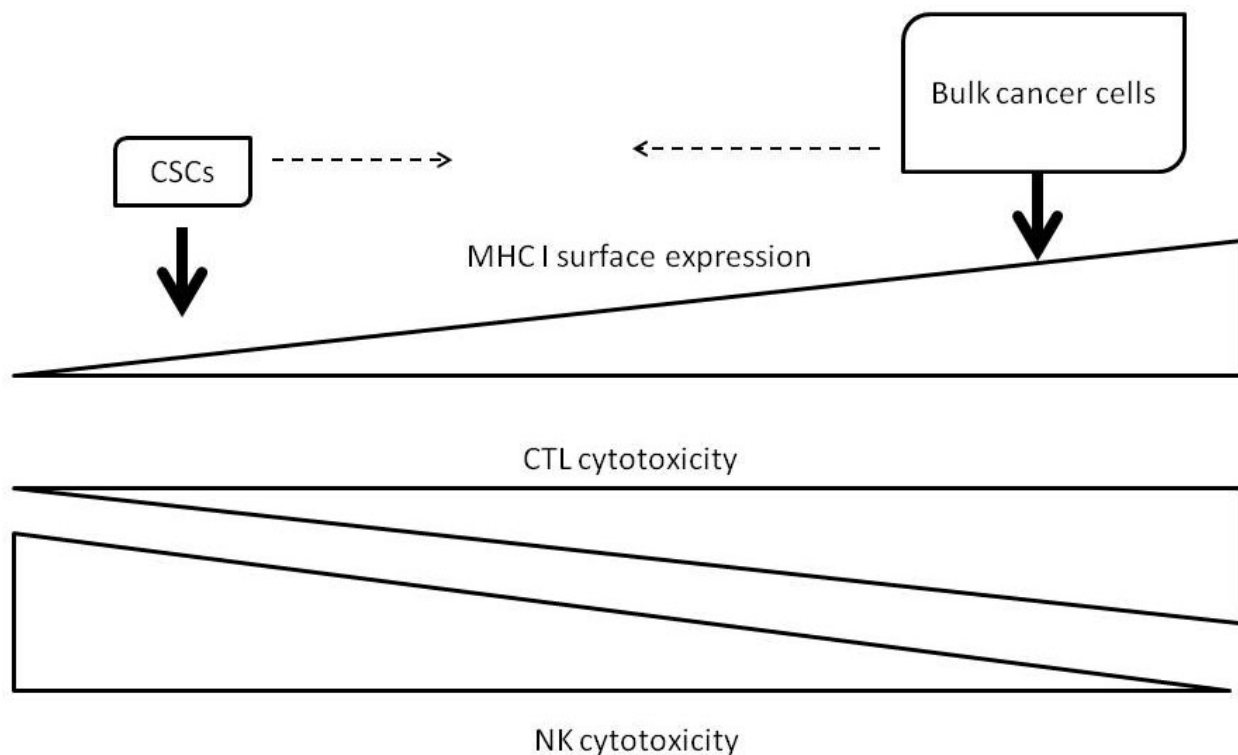
**Table 3.** Immune inhibitory co-receptors

| Inhibitory receptor       | Alternative name(s) | Chromosome | Expression   | Ligand(s)          |
|---------------------------|---------------------|------------|--|--------------------|
| <b>CD28 family</b>        |                     |            |  |                    |
| CTLA-4                    | CD152               | 2q33.2.    | Activated T cells  | B7–1, B7–2, ICOS-L |
| PD-1                      | CD279               | 2q37.3.    | Activated T cells  | PD-L1, PD-L2       |
| VISTA                     | PD-1H, VSIR         | 10q22.1.   | Various hematopoietic cells, activated and resting T cells. Down-regulated in gastric cancer | Unknown            |
| <b>TNFR family</b>        |                     |            |  |                    |
| HVEM                      | TNFRSF14, CD270     | 1p36.3.2   | The only family member with non-TNF ligands  | BTLA, CD160        |
| <b>Nectin-like family</b> |                     |            |  |                    |
| TIGIT                     | WUCAM, VSIG9        | 3q13.3.1   | Expressed by T cells and NK cells in the tumor micro-environment                             | PVR, PVRL2         |
| CD112R                    |                     | 7q22.1.    |  | PVRL2              |
| <b>Other</b>              |                     |            |  |                    |
| MHC II                    | HLA-DR, -DQ, -DP    | 6p21.3.2   | Lower expression in AML progenitor cells than non-stem cells                                 | LAG-3              |
| Galectin-9                |                     | 17q11.2.   | Tumor-associated MSCs, LSCs, HD tissues, cholangiocarcinomas, hepatomas                      | TIM-3              |
| HMGB1                     | HMG1                | 13q12.3.   | Promotes autophagy and inflammation in peritoneal carcinomatosis                             | TIM-3              |
| CEACAM1                   | CD66a, BG-1         | 19q13.2.   | High expression in melanoma, NSCLC, SCLC and in some colon, breast and prostate cancers      | TIM-3              |

LSCs: Leukemia Stem Cells, HD: Hodgkin's Disease For alternative names of ligands see Table 1

of  $\beta 2$ -microglobulin gene expression is also reported to play a role in decreased expression which leads to a parallel MHC I expression down-regulation in human embryonic and induced pluripotent stem cells (20). During *in vitro* differentiation the  $\beta 2$ -microglobulin

gene expression was up-regulated 42 folds and the activating epigenetic trimethylation at lysine 4 of histone 3 (H3K4me3) was observed. Nevertheless, some degree of expression of  $\beta 2m$  in CSCs may be beneficial for tumor progression, given that the protein



**Figure 1.** Schematic representation of MHC I surface expression in the CSCs and bulk tumor cells compartments and expected CTLs and NK cells cytotoxicity. CTLs cytotoxicity increases with increased MHC I dependent antigen presentation while NK cells are inhibited when targets express robustly MHC I through KIRs engagement. CSCs may be under pressure to express at least some amount of MHC I in order to avoid NK cell lysis without engaging a robust CTLs toxicity (dashed arrows).

and its interacting receptor, HFE (Hemochromatosis protein) have been linked to the process of EMT (Epithelial to Mesenchymal Transition) which favors metastasis (21). In addition, EMT is associated with pluripotency (22) and, thus, stemness and CSCs maintenance may be favored by the presence of  $\beta 2m$  expression.

A study that examined the prognostic significance of MHC I complex expression in colorectal cancer disclosed that cancers with low expression of MHC I heavy and light ( $\beta 2m$ ) chains had worse prognosis than both cancers with high MHC I and those with complete absence of MHC I in their surface (23). Authors attributed these results to the fact that low MHC I expression may allow cancer cells to escape both adaptive and innate immunity. Although not specifically addressed in this study, colorectal cancers with intermediate MHC I expression may harbor a higher percentage of stem cells capable of undergoing EMT and with the optimal MHC I level for immune escape (Figure 1).

#### 4.2. Co-stimulatory immune signals

Several members of the B7/ CD28 families of the Immunoglobulin superfamily (IgSF) and of the

TNF/ TNFR super-families (TNFSF/ TNFRSF) are important co-stimulatory immune ligands/ receptors for CTLs (Table 1 and 2). In the case of B7 and CD28 the distinction of ligand and receptor is blurred as both families interact mainly on cell surface and can produce intra-cellular signaling after engagement. Members of both families may also be expressed in CTLs and target cells. For this discussion and in tables, B7 family members are considered ligands and CD28 members the receptors.

CD80 (B7-1) and CD86 (B7-2) are prototypic co-activator molecules for CTL signaling and are ligands for CD28 but they may transduce inhibitory signals if they ligate CTLA-4. Stem cells from various cancer types have been shown to down-regulate their B7-2 and B7-2 expression in order to avoid immune attack, as discussed in the next section. ICOSL (B7-H2) may provide immune co-stimulatory signals by binding ICOS but also, similarly to B-1 and B7-2, by binding CD28 (24). In addition, also similarly to those other B7 family members, it can act as a co-inhibitory molecule by binding CTLA-4. In colorectal cancer, ICOSL is expressed in tumor cells and macrophages in the tumor micro-environment while the ICOS receptor is expressed in CD4<sup>+</sup> cells (25). Higher expression of ICOS in these cells was associated with a better overall

survival in colorectal cancer patients (25). Expression and engagement of ICOS act synergistically with CTLA-4 blockade to produce anti-tumor activity in mice bearing melanoma and prostate cancers (26). There are no specific data in the literature regarding ICOSL expression in CSCs but these cells as well as their progeny might be under pressure to down-regulate it in order to avoid immune attack, especially in cases that immune infiltrating cells express ICOS instead of CTLA-4.

Two additional B7 family members, B7-H3 and HHLA2 (B7-H5) may provide co-stimulatory or co-inhibitory signals for CTLs. The putative ligand for B7-H3 is the TREM (Triggering Receptor Expressed on Myeloid cells) family member TLT-2, although this remains somewhat controversial (27). B7-H3 expression has been observed in colorectal cancer patients expressing the stem cell marker CD133 using immunohistochemistry (IHC) techniques (28). In contrast, cases not expressing the CD133 marker by IHC (probably having a smaller number of CSCs) were more often B7-H3 negative. The CD133/ B7-H3 co-expressing cases had a worse prognosis than patients expressing only one of the two or none of the markers (28). These data argue for an inhibitory signal provided by B7-H3 that protects CSCs from the immune attack possibly through expression of an unidentified B7-H3 inhibitory ligand by CTLs in the colorectal cancer micro-environment rather than expression of TLT-2 or another unidentified stimulatory ligand. In addition, B7-H3 expression in colorectal cancer cell lines was associated with up-regulation of mesenchymal markers and down-regulation of epithelial markers, as well as increased migration potential of those cells (29). Epithelial-Mesenchymal Transition (EMT) is a state promoting metastatic potential and is correlated, as previously mentioned, with the stemness potential of cancer cells, arguing for an inbuilt interrelationship of B7-H3 with CSCs and EMT.

The ligand of HHLA2 (B7-H5) for immune co-stimulation is a molecule homologous to CD28, TMIGD2 (Transmembrane and Immunoglobulin Domain-containing 2, also called CD28H). TMIGD2 is expressed in T cells, contributing to their activation, but repetitive stimulation leads to its down-regulation (30). HHLA2 is expressed in various cancers with the higher percentage of expression in breast, lung, thyroid, melanomas and pancreatic cancers (31). In triple negative breast cancer a high expression of HHLA2 was observed in 56% of cases and the gene was amplified in a minority of cases (30%) of the basal sub-type of breast carcinomas compared to 18% in non-selected cases. This may imply an association of HHLA2 expression with CSCs, given that the CSC phenotype is associated with ER-negativity (32). HHLA2-positive triple-negative breast cancers presented more commonly with lymph node positivity,

suggesting that HHLA2 may promote tumors by suppressing the immune system through binding to a currently unknown inhibitory ligand on immune cells. Alternatively, HHLA2 expression may be associated with the presence of different proteins or pathways activation that may promote progression of malignancy.

In contrast to members of the B7 family that have alternative co-stimulatory or co-repressive receptors and, as a result, may act as both immune co-activators and co-repressors, TNFSF ligands are pure co-activators. They include 4-1BBL (CD137L, TNFSF9), CD70 (TNFSF7), CD154 (TNFSF5), OX40L (CD252, TNFSF4), GITRL (AITRL, TNFSF18) and LIGHT (TNFSF14) (Table 1). The respective receptors are: 4-1BB (CD137, TNFRSF9), CD27 (TNFRSF7), CD40 (TNFRSF5), OX40 (CD134, TNFRSF4), GITR (AITR, TNFRSF18) and HVEM (TNFRSF14, CD270) (Table 2). The main expression of these TNF/ TNFR pairs is in immune cells and they play a role in both CTLs survival and proliferation in later stages of immune stimulation, particularly in escape from Activation-Induced Cell Death (AICD) (33). Data on expression of each of these receptors or ligands beyond the immune system and specifically either in neoplastic cells or CSCs are less abundant. Nevertheless, their importance in immune stimulation has been investigated and confirmed for both anti-tumor and anti-infection immunity (34). Activating antibodies, for example, of 4-1BB are able to promote CD8<sup>+</sup> CTLs antitumor cytotoxic activity in several models of cancer xenografts in mice (35). Tumor-infiltrating CTLs expressing 4-1BB in the ovarian cancer micro-environment are reactive to tumor cells, in contrast to 4-1BB-negative CTLs (36). CD40 is expressed in cancer cells from a variety of tumors such as melanoma, breast, lung and colorectal carcinomas as well as gliomas (37, 38). Tumor cells would be under pressure to down-regulate these molecules in order to avoid immune attack. This pressure could theoretically be greater than the pressure to down-regulate B7 family members, because, in contrast to these latter, TNFRSF members have no respective suppressive members that, if expressed, would offset the effect of co-stimulatory members. The only notable exemption is HVEM which, in addition to a stimulatory ligand, LIGHT, has two co-inhibitory ligands BTLA and CD160 that, also exceptionally, are not TNFR members (Table 1).

It is worth mentioning, at this point, that other members of the TNFRSF, TNFRSF1 (p55), Fas (TNFRSF6), DR4 (TNFRSF10A) and DR5 (TNFRSF10B) are the main effectors of the immune cytotoxicity (together with the granzyme/ perforin system) after recognition of tumor cells by CTLs (39). These members, that are effectors of the immune execution, are regulated in the intra-cellular level and under certain conditions may promote cell survival through NF- $\kappa$ B activation and cell motility instead of cell death. Similar intra-cellular regulations may



exist for the co-stimulatory members which under certain conditions may promote apoptosis of immune cells. This is the case, for example, for OX40, that, when activated by an agonist antibody following chemotherapy in a mouse model of melanoma, leads to tumor regression by promoting apoptosis of regulatory T cells in the tumor micro-environment (40). CD40 activation on tumor cells independently of interactions with immune cells also induces apoptosis (41, 42). CD40 also facilitates immune attack by up-regulating MHC I expression as well as by promoting lymphocyte interactions with endothelial cells in tumor vasculature (43). Evidently, parallel signal inputs shape the signal transductions from co-stimulatory receptors of TNFSF and determine the effect of their stimulation similarly to the main effector family members.

PVR (Poliovirus Receptor, CD155) is a nectin-like ligand and can provide co-stimulatory signals to CTLs when ligating DNAM-1 receptor (also called CD226). CD34<sup>+</sup> Hematopoietic Stem Cells (HSCs) co-express PVR (44) that could promote their lysis by CTLs if these cells express DNAM-1 instead of the co-inhibitory receptor TIGIT. DNAM-1 may also have direct inhibitory effects in leukemia and cervical cancer cells through ligation of PVR and the alternative receptor PVRL2 (PVR-like 2, CD112), in addition to immune activation (45). PVRL2 is also able to bind the same couple of stimulatory and inhibitory ligands and binds, additionally, another inhibitory receptor, CD112R. A sub-set of normal murine spermatogonial stem cells express the PVR homolog (46). These expressions would make these stem cells or CSCs vulnerable to CTLs attack but this would mostly depend on the nature of CTLs and whether they express the stimulatory or inhibitory ligand rather than the stem cells per se (see also next section). This is a feature of all molecules that have both stimulatory and inhibitory interactors.

### 4.3. Co-repressive immune signals

Co-repressive immune signals are produced mainly from interactions of the B7 family ligands with repressive receptors of the CD28 family. Several ligands such as B7-1 and B7-2 have a dual role, while others such as PD-L1 are dedicated repressive signals generators (Tables 1 and 3). Stem cells as well as non-stem fractions of head and neck cancer cell lines are devoid of both B7-1 and B7-2 co-activators (47). AML progenitors with the CD34<sup>+</sup>/CD38<sup>-</sup> phenotype have a lower expression of B7-1 and B7-2 than CD34<sup>+</sup>/CD38<sup>+</sup> counterparts (48). These progenitors displayed also a decreased expression of the TNF/ TNFR family members FasL/ Fas and of MHC II molecules. Renal carcinoma cells with stem cell properties, growing in spheres, had lower expression of B7-1 and B7-2 than monolayer-growing counterparts of the same cell line (49). Cells with stem cell properties were more resistant to radiation and were expressing higher levels of the

transcription factors of the stemness circuitry, Oct4 and Nanog. Moreover, they displayed lower levels of PD-L1 and lower intensity of PVR staining (49). CSCs may have modulatory effects on the tumor micro-environment by suppressing B7 molecules expression in other cells. This is the case in ovarian CSCs that decrease B7-2 expression on tumor macrophages and promote M2 polarization that favors tumor growth (50). B7-1 and B7-2 down-regulation in CSCs may counter-intuitively provide a window of opportunity for treatment if it is a result of immune pressure to the tumor by the presence of CD28<sup>+</sup> TILs. In this case, treatment with anti-CTLA4 monoclonal antibodies may have the ability to tip the balance of engagement of the low level of B7-1 and B7-2 expressing CSCs towards CD28 in order to activate TILs.

PD-L1 is expressed in a subset of gastric CSCs and, in addition to preventing CTLs from attacking these cells, it may transmit signals of proliferation and survival to the expressing cells when stimulated (51). Colorectal CSCs expressing CD133 and a higher level of Oct4 and Sox2 mRNA co-express PD-L1 (52). These cells display, additionally, up-regulation of EMT markers Snail, Twist and vimentin and grow more aggressively as xenografts in mice than CD133<sup>-</sup> xenografts. PD-L1 expression may be an inherent property of CSCs together with EMT. This is evident in claudin-low type of triple negative breast cancer where PD-L1 was expressed in a manner dependent on PI3K/ Akt signalling (53). In contrast, when PD-L1 expression was knocked-down with shRNA in breast cancer cells, CD44<sup>+</sup> CSCs lost CD44 expression and up-regulated CD24. Expression of PD-L1 was also documented in CSCs from human squamous cell carcinomas of the lung (LSCC) and a mouse model of LSCC derived from targeted inactivation of kinase Ikb1 and phosphatase pten (54). In addition, Tumor Infiltrating Lymphocytes (TILs) in the mouse tumors displayed higher expression of pd-1. Human glioblastoma CD133<sup>+</sup>/ Sox2<sup>+</sup> stem cells also express PD-L1 but so do their progeny cells (55). Expression of PD-L1 was higher in high grade versus low grade gliomas and tumors with higher expression of PD-L1 had a lower number of CD8<sup>+</sup> TILs. A multiple myeloma cell sub-set express the hematopoietic stem cell marker CD34 and co-express PD-L1 (56). Another study has shown that PD-L1 is broadly expressed in myeloma plasma cells and in plasma cells from patients with smoldering myeloma and monoclonal gammopathy of undetermined significance (MGUS) (57). In all three cases PD-L1 expression was higher than in normal plasma cells. In contrast to the above data, in cholangiocarcinoma cell lines, it was shown that the cancer initiating capacity was associated with a subset expressing low levels of PD-L1, which also express high ALDH1 levels (58). These PD-L1<sup>low</sup> cholangiocarcinoma cells were able to generate tumors in nude mice when infused

in lower numbers than PD-L1<sup>high</sup> cells from the same cell lines. These data support expression of PD-L1 in CSCs but also in progeny cells from several types of cancer. Nevertheless, the cholangiocarcinoma data is a reminder that heterogeneity between various cancer types exist.

VISTA (V domain Immunoglobulin-containing Suppressor of T cell Activation) is a new inhibitory member of the immunoglobulin superfamily, although it is not clear whether it belongs to the CD28 or the B7 family. Its ligand is not known (59). One of the groups that identified VISTA named it PD-1H (PD-1 Homolog), implying that it belongs to the CD28 family (60) and this is supported by the fact that it has a single IgV domain similarly to the other CD28 members (CD28, CTLA-4, PD-1, ICOS and TIMGD2). In addition, a study that included VISTA in a phylogenetic analysis of the B7 family showed that it is clearly an outlier (61). VISTA expression is predominantly observed in immune cells both of the myeloid and lymphoid lineages. In contrast to PD-1 and CTLA-4 that are expressed only in activated T cells, VISTA expression is also observed in the surface of resting T cells (62). VISTA is down-regulated in gastric cancer tissues compared to normal tissue mucosa and in several cancer cell lines compared to non-cancer cell lines (63). In addition, in an immortalized breast cancer cell line, VISTA was down-regulated after exposure of the cells to TGF- $\beta$  which produced an EMT and re-expressed in even higher levels after the end of the exposure which allowed cells to return to an epithelial state. Re-expression was associated with VISTA promoter demethylation (63). It would be interesting to determine if VISTA's currently unknown ligand would prove to be expressed in tumor cells, similarly to other co-inhibitory ligands of the B7 family.

B7-H4 (alternatively called B7x or B7S1) is another B7 family member with CTL inhibitory functions. It is expressed in several cancers and its ligand in T cells is currently still unknown (64). Although B7-H4 is expressed at the mRNA level in normal human tissues, protein expression is restricted (65). In contrast, various types of carcinomas such as ovarian, breast, lung, pancreatic and melanomas express B7-H4 at the protein level, suggesting a post-translational dysregulation (64). Prostate, renal cell and gastric carcinoma patients with higher B7-H4 had worse survival than patients with lower expressions of the proteins (66–68). Curiously, in breast cancer, although B7-H4 is expressed in most cases (69), a higher expression was observed to be associated with a better outcome (70). The expression of B7-H4 in breast cancer patients was positively correlated with MHC I expression and thus, in this cancer, the higher expression of B7-H4 that inhibits CTLs may be counter-balanced by a higher MHC I expression promoting immunologic recognition of the tumors.

Alternatively, it is conceivable that there may exist also a co-stimulatory ligand for this B7 family receptor that mediates CTLs activation. A sub-set of human glioblastoma cells with stem cell properties growing as xenografts in nude mice have been shown to express B7-H4 (71). In addition, glioblastoma cell line U251 cells growing in serum-free conditions favoring stem cell maintenance express a higher level of B7-H4 than cells of the same cell line growing in serum-containing medium promoting differentiation (72). Medium from the stem-like cells was able to induce higher levels of B7-H4 when added in cultures of human monocytes than medium from the differentiated U251 cells, implying that these stem-like cells secrete or shed a soluble factor that promotes B7-H4 up-regulation.

As discussed in the previous section, most other members of the B7 family (ICOSL, B7-H3, HHLA2) have co-inhibitory functions if bound by inhibitory instead of stimulatory ligands on CTLs. ICOSL may bind CTLA-4, an interaction that transmits inhibitory signals interfering with the stimulation produced by the interaction of ICOSL with stimulatory receptors ICOS and CD28 (24). This redundancy may increase the effectiveness of anti-CTLA-4 antibodies currently used in the clinical treatment of cancers, as, in this case, anti-CTLA-4 blockade will favor the B7-1 or B7-2 interaction with CD28 but also the interactions of ICOSL with ICOS and CD28. As mentioned previously no specific data exist for the expression of ICOSL in CSCs.

Interactions not through B7 family members that produce additional co-repressive immune signals involve TIM-3, LAG-3, HVEM and nectin-like family molecules. TIM-3 (T cell Immunoglobulin and Mucin-containing protein 3) is expressed in populations of exhausted CD4<sup>+</sup> Helper T cells and CD8<sup>+</sup> CTLs during chronic viral infections, often in combination with PD-1, and is ligated by three different molecules, CEACAM-1, HMGB1 and galectin-9 (73). In addition, similarly to other TIM family members, TIM-3 binds phosphatidylserine on the surface of apoptotic cells, facilitating their uptake by phagocytes in an immunologically silent manner (74). CTLs with co-expression of PD-1 and TIM-3 in the tumor micro-environment have a severely exhausted phenotype and fail to proliferate and produce cytokines (75). Their exhaustion can be reversed by combined targeting of the two molecules. Leukemic stem cells (LSCs) as well as leukemia bulk cells of all AML sub-types express higher levels of TIM-3 compared to normal hematopoietic stem cells (76). LSCs have been found to produce and secrete the ligand galectin -9 which then bind TIM-3 in an autocrine manner activating  $\beta$ -catenin and NF- $\kappa$ B pathways that promote stemness (77). Expression of TIM-3 has been documented in various other cancers such as melanoma, NSCLC and cervical cancer, but no information specifically for expression in CSCs is available (78–80). On the other



hand, expression of the ligands of TIM-3 by CSCs may protect them from CTLs attack. One of these ligands, HMGB1 has an intracellular role in promoting autophagy and as an extracellular secreted protein is able to bind several other proteins, in addition to TIM-3 after been released from cells as a danger signal (81). HMGB1 released from colorectal cancer cells attract neutrophils and sustain inflammation in the tumor microenvironment in a peritoneal carcinomatosis model (82). When ligating the Toll-like Receptor 2 (TLR2) in human and murine breast CSCs, HMGB1 promotes self-renewal and tumor metastasis (83). In a mouse model of colon carcinogenesis, APC gene loss produced Wnt signalling activation, HMGB1 induction and crypt CSCs expansion, a phenotype that was partially reversed by HMGB1 neutralizing antibodies (84). In gastric cancer HMGB1 is involved in the activation of transcription factor NF- $\kappa$ B, which results in increased cell growth and invasion (85). Galectin-9, another TIM-3 ligand and member of a carbohydrate-binding gene family, may be produced by mesenchymal stromal cells (MSCs) in the tumor micro-environment in response to interferon  $\gamma$  and suppresses T cell proliferation (86).

The third TIM-3 ligand CEACAM1 (also called Biliary glycoprotein-1 or CD66a) was also shown to require MSCs for optimal signaling and gland formation of breast cancer cells *in vitro* and *in vivo* (87). A study of breast cancer tissues has found down-regulation of CEACAM1 compared to adjacent benign tissue (88). Cancer cell lines were expressing CEACAM1, albeit in a lower level than the benign breast line MCF10A. Expression of CEACAM1 in breast cancer has a proliferation-suppressing effect and, in addition, it inhibits Epithelial–Mesenchymal Transition (EMT) by interacting with  $\beta$ -catenin in the plasma membrane preventing nuclear signalling of this transcription factor (89). In melanoma, cancer cells may shed or secrete CEACAM1 and its elevated levels in serum of melanoma patients is associated with tumor progression (90). This may be due to both the loss of EMT-inhibiting effects and T cell inhibition through ligation of circulating CEACAM1 to TIM-3, an effect corroborated by the fact that elevated serum CEACAM1 leads to resistance to adoptive cell transfer (90). A monoclonal antibody against CEACAM1 enhanced the effects of melanoma reactive lymphocytes in a mouse model of human melanoma xenograft (91). Precancerous lesions of the uterine cervix that harbor HPV genetic elements co-express CEACAM1, allowing for viral replication (92). Thus all TIM-3 ligands have multiple functions, besides inhibiting CTLs that affect various aspects of carcinogenesis. Data linking TIM-3 ligands with EMT programs imply that CSCs, also linked to EMT, may use these ligands to suppress immune system attack.

LAG-3 (Lymphocyte-Activated Gene-3, CD223) is another immunoglobulin superfamily

member and an inhibitory co-receptor for MHC II. LAG-3 is homologous to the stimulatory co-receptor CD4 and has much higher affinity for MHC II than CD4 (93). It is expressed in a variety of immune cells and its expression may render CTLs unresponsive to tumor cells expressing MHC II. Expression is low in resting CD8<sup>+</sup> cells but is up-regulated after stimulation which may play a role in avoiding an excessive or persistent immune response. In other instances, some CSCs such as CD34<sup>+</sup>-CD38<sup>-</sup> leukemic blasts negate the co-stimulatory influence of MHC II by down-regulating expression of these molecules in their surface, avoiding attack from CTLs not expressing LAG-3 (48).

HVEM (Herpes Virus Entry Mediator) is a unique member of the TNFRSF that provides repressive signals, besides co-stimulatory ones. The relevant inhibitory ligands are Immunoglobulin superfamily (IgSF) members BTLA (B and T Lymphocyte Attenuator) and CD160 (94). This is also unique among TNFRSF members which usually are ligated by TNFSF members. Ligation of HVEM on TILs by BTLA leads to inhibition of proliferation and cytokine production but concomitantly protects TILs from apoptosis through activation of Akt kinase (95). In the presence of both its stimulatory (LIGHT and LT $\alpha$ ) and inhibitory (BTLA and CD160) ligands which may bind concomitantly through different domains of the extracellular part, HVEM delivers inhibitory signals (94, 96). HVEM is shown to be increasingly expressed in colorectal neoplasia as tumors progress from normal epithelium to adenomas and carcinomas (97). Moreover, carcinomas with a higher HVEM expression display lower TILs infiltration and worse prognosis. Similarly, in ovarian cancer, HVEM mRNA was up-regulated compared with non-malignant tissues (98). Silencing HVEM on ovarian cancer cells by shRNA knock-down increased T cell-induced apoptosis of cancer cells *in vitro*. CD160 is expressed in neoplastic B cells of CLL and hairy cell leukemia as well as in a minority of mantle cell lymphomas and other B cell lymphomas (99). In contrast, normal B cells in all developmental stages including precursor stem cells are not expressing the receptor (99).

Nectin-like molecules PVR and PVRL2 may provide repressive signals when ligated by TIGIT (and CD112R in the case of PVRL2), instead of stimulatory DNAM-1. TIGIT and DNAM-1 inhibitory/ stimulatory pair has several analogies with the CTLA-4 and CD28 pair including a higher affinity of the inhibitory member of the pair for the respective receptors and expression on a wide range of immune cells (100). Thus, expression of PVR and PVRL2 by tumor cells would favor CTLs inhibition and protection from immune attack if both DNAM-1 and TIGIT are expressed. Expression and activation of family member ligands and receptors may regulate one another as suggested from a PVR knockout mouse model of methylcholanthrene-induced

carcinogenesis where PVR knockout results in higher levels of PVRL2 in tumors and higher levels of DNAM-1 and lower levels of TIGIT in peripheral blood CD8<sup>+</sup> T cells (101). PVRL2 expression was decreased in hepatocellular carcinoma (HCC) tissues and patients with the lower expression of PVRL2 had worse prognosis (102).

Different co-inhibitory receptors have non-redundant functions modulating various aspects of the cancer-immunity cycle and various immune cell subsets (103). VISTA inhibition for example has a more pronounced effect for CD8<sup>+</sup> CTLs and a lesser effect in Tregs (104). In human ovarian serous carcinomas, CD8<sup>+</sup> TILs expressing PD-1 were not expressing TIM-3, CTLA-4 or LAG-3 (105). Thus, opportunities arise for combinations of therapeutics blocking various inhibitory molecules as already witnessed by the success of combinations of anti-PD-1 and anti-CTLA4 antibodies in NSCLC and melanoma (106). Nevertheless, as glimpsed from the limited data available, CSCs from different cancers may express different subsets of co-inhibitory molecules and effective combinations against each case may possibly prove to be cancer type specific.

## **5. PATHWAYS AND TRANSCRIPTION FACTORS REGULATING EXPRESSIONS OF IMMUNE CO-REGULATORS**

Control of co-regulatory immune molecules by cancer-related pathways has been documented in several instances and some examples are discussed in this section. Components of the antigenic peptides production machinery that create and up-load these peptides on MHC I molecules are down-regulated in human glioblastoma multiforme cells through increased IGF-1 signaling (107). Components down-regulated include the immunoproteasome sub-units LMP-2 (also called  $\beta 1i$  or PSMB9) and LMP-7 (also called  $\beta 5i$  or PSMB8), which participate in the immunoproteasome-mediated production of antigenic peptides from digestion of cellular proteins, and transporter proteins TAP-1 and TAP-2, which transport produced peptides into the endoplasmic reticulum for MHC I up-loading. In contrast, these components are up-regulated and MHC I expression is increased in cell surface after IGF-1 signaling down-regulation through an antisense RNA or through monoclonal antibody blockade of its receptor (107).

PD-L1 regulation has become a subject of particular interest given that together with its main receptor PD-1 is a target of monoclonal antibody-based cancer therapy. Carcinogenesis-related pathways emanating from membrane associated growth factors and proceeding through the Ras/ Raf/ Erk and the PI3K/ Akt cascades culminating in activation of STAT3 and NF- $\kappa$ B are able to activate transcription of PD-L1

(108). These transcription factors possess binding sites in the promoter of PD-L1 gene. The same is true for transcription factor HIF-1 which is involved in hypoxia-induced PD-L1 up-regulation (109, 110). All three pathways are involved in stem cell biology. In contrast, PD-L1 mRNA may be down-regulated by several miRNAs, among which the miR-200 family features prominently (111). miR-200 suppresses, additionally, ZEB family members and EMT, which is interwoven with stemness plasticity in CSCs. ZEB-1 transcription factor was recently found to up-regulate PD-L1 in breast cancer cell lines undergoing EMT (112). Knock-down of ZEB-1 with shRNA or over-expression of miR-200 miR family was able to reverse PD-L1 up-regulation. Thus, PD-L1 expression may be an additional element of the CSCs- EMT network's multiple connections with immunity. The stemness transcription factor network, for example, is up-regulated by hypoxic conditions and then promotes autophagy, associated with tumor immune escape (113) (Figure 2).

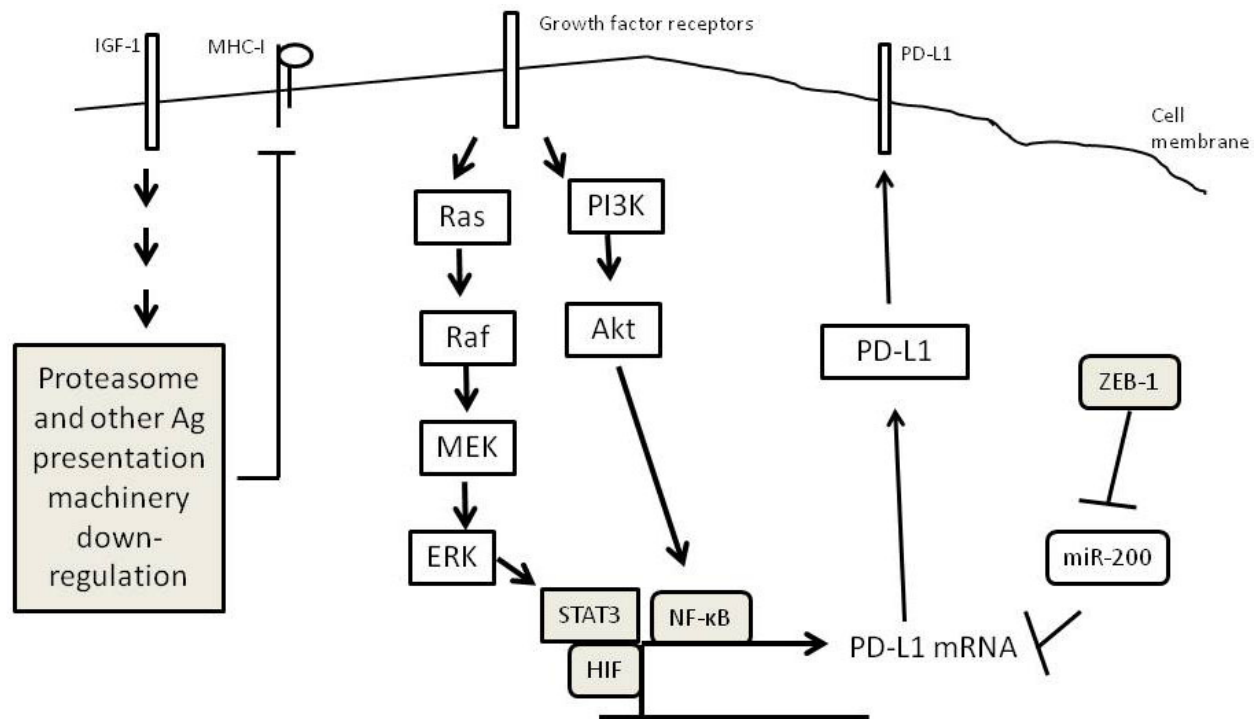
TIM-3 expression is observed in leukemic HSCs as well as bulk leukemic cells but not normal HSCs (114). This expression is seen in several subtypes of leukemia but is particularly associated with translocations or mutations involving the core binding factor CEBPA. Thus, CEBPA lesions may either directly or indirectly up-regulate TIM-3 in leukemias.

Transcription factor p53, besides being an important tumor suppressor and guardian of the genome, is a guardian of the epithelial state and impedes pluripotency (115). Thus, it needs to be down-regulated or disabled in CSCs and during EMT induction. Interestingly, VISTA has been observed to be a target of p53 (116) and this may contribute to its down-regulation associated with cells undergoing EMT (63).

In summary, cancer-associated circuitries are, in many occasions, in control of the expression and regulation of immune co-regulators resulting in dysregulation of these immune molecules in cancer and CSCs. This may be favoring immune editing, as cancer promoting pathways and combinations of signaling that produce up-regulation of immune co-inhibitors or down-regulation of immune co-stimulators would be selected.

## **6. THE CSC NETWORK AND CYTOTOXICITY AGAINST CSCS**

The ability (or lack thereof) of CTLs to kill CSCs has been examined in several studies since the introduction of the CSC theory that advocates for the importance of these cells in tumor propagation and thus proposes that their elimination could be of therapeutic relevance. Exposure of a cervical cancer cell line to CTLs in culture results in the development



**Figure 2.** Pathways leading to MHC I down-regulation and PD-L1 expression through IGF-1 and other growth factor receptor signalling. This signalling leads to antigen presentation machinery down-regulation and activation of PD-L1 transcription. In addition, ZEB-1 inhibits miR-200-dependent PD-L1 mRNA down-regulation. Shaded elements represent down-regulations and transcription factors operating in CSCs. Arrows denote activation and inverted T symbols denote inhibition or down-regulation.

of an increasing percentage of resistant cells through consecutive generations of cultured cells (7). These resistant cells have an up-regulated expression of the stem cell factor Nanog that leads to activation of kinase Akt. The activated Nanog- Akt circuitry is related to CTLs resistance as inhibition of Nanog reverses the resistance. In addition, CTLs-resistant cells had an increased expression of EMT markers Twist and BMI1 and were able to produce more metastases when injected in mice (117). Nanog knock-down resulted also in decreased invasiveness of cervical, colorectal and hepatocellular carcinoma cell lines *in vitro*.

Another member of the stemness transcription factors network, Sox2 has been examined as a determinant of immunotherapy effectiveness in NSCLC (118). Patients with pre-existing CTLs reactive to Sox2 had better therapeutic responses to treatment with a PD-1 inhibitor, suggesting that promoting a pre-existing immunity against CSCs contributes to the effectiveness of immune checkpoint inhibitor immunotherapy. In contrast, pre-existing CTLs against various viral antigens or the NY-ESO-1 cancer-associated antigen were not correlated with treatment responses in this study.

The above study did not examine whether healthy individuals harbor anti-Sox2 reactive CTLs but

this was the subject of a similar study by the same investigators with another stemness transcription factor, Oct4 (119). This report found that 80% of healthy individuals possess anti-Oct4 reactive CTLs in their peripheral blood, while this percentage drops to 35% in patients with germ cell tumors (GCTs). GCTs are known to express Oct4 in the majority of cases (120). After chemotherapy treatment, some patients developed anti-Oct4 CTLs which may contribute to the effectiveness of treatment.

Myc oncogene is an additional member of the pluripotency transcription factor circuitry and is one of the most common cancer associated oncogenes (115). The Myc family member MYCN is commonly amplified in neuroblastoma and has been observed to be co-expressed with Oct4 in tumor cells with plasticity allowing them to produce tumor-associated endothelial cells (121). In addition, they could theoretically serve as tumor-associated antigens to be targeted by the immune system, under the appropriate conditions (122).

## 7. PERSPECTIVES ON TREATMENT

As discussed, data support the presumption that resistance to cancer therapies may be mediated by CSCs, and immune therapies may promote the arising of clones with CSC characteristics (32). As a

further example, cells with the breast CSC phenotype arise after treatment of Her2-positive tumors with trastuzumab and immunotherapy with polyclonal NK cells, mediating antibody-dependent cell-mediated cytotoxicity (ADCC) (123). Clones that are observed after treatment have undergone an epithelial to mesenchymal transition that is associated with expression of pluripotency phenotypes (6). Decreased expression of immune response molecules such as MCH I and II by CSCs may mediate their lower immunogenicity as suggested for CSCs in the case of glioblastoma (124). Immunotherapy with immune blockade inhibitors has already proved efficacious in a sub-set of cases from several types of cancers. Part of this efficacy may be derived from the fact that immune checkpoint proteins are expressed in CSCs and could contribute to their decreased immunogenicity and is reversed by these treatments. Given that multiple immune checkpoint proteins may be having a non-redundant role in CSCs resistance, combination treatments could be the future avenue for successful immunotherapy. This strategy has already been successful in the treatment of melanoma where combination of ipilimumab and nivolumab was more effective than monotherapy with ipilimumab (125). Other combinations with anti PD-L1/PD-1 monoclonal antibodies as a backbone or alternative targets are intensively investigated (126). Alternative approaches using transfer of engineered receptor CTLs to attack tumor neo-antigens in combination with immune checkpoint inhibitors or agonistic antibodies for immune co-activators could be worth of further investigation. Data discussed in this paper argue that, because of the complicated biology of immune co-regulation, and despite success of immune checkpoint blockers across multiple cancers, an one-size-fit-all approach is unlikely to produce good results in resistant cancers. In contrast, treatments will have to be tailored to the specific genetic composition of the individual cancer in order to activate the immune system through the relevant players in each case. Fortunately, given the progress of genomic approaches to characterize both the whole genome but also the antigenome (127), individualized approaches against both bulk tumor cells but also against more cryptic but equally or even more relevant sub-sets such as the CSCs may become feasible in the foreseeable future.

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