Resistance to Targeted Anti-Cancer Therapeutics 7
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Benjamin Bonavida
Salem Chouaib *Editors*

Resistance of Cancer Cells to CTL-Mediated Immunotherapy

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Volume 7

Series Editor
Benjamin Bonavida
Department of Microbiology, Immunology, and Molecular Genetics
University of California at Los Angeles,
Los Angeles, CA, USA

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For several decades, treatment of cancer consisted of chemotherapeutic drugs, radiation, and hormonal therapies. Those were not tumor specific and exhibited severe toxicities in many cases. But during the last several years, targeted cancer therapies have been developed. Targeted cancer therapies are drugs or other agents (e.g. antibodies) that block the growth and spread of cancer by interfering with specific gene products that regulate tumor cell growth and progression. Targeted cancer therapies are also sometimes called "molecularly targeted drugs." We have witnessed in the last decade a significant explosion in the development of targeted cancer therapies developed against various specific cancers. These include drugs/antibodies that interfere with cell growth signaling or tumor blood vessel development, promote the cell death of cancer cells, stimulate the immune system to destroy specific cancer cells and to deliver toxic drugs to cancer cells. One of the major problems that arise following treatment with both conventional therapies and targeted cancer therapies is the development of resistance, preexisting in a subset of cancer cells or cancer stem cells and/or induced by the treatments. Tumor cell resistance to therapies remains a major problem and several strategies are being considered to reverse the resistance by various manipulations.

Resistance to Targeted Anti-Cancer Therapeutics will focus on the basic and translational research behind the molecular mechanisms of resistance found in many kinds of anti-cancer therapeutics.

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Editors

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Editors
Benjamin Bonavida
Department of Microbiology,
Immunology, and Molecular Genetics
University of California at Los Angeles
Los Angeles, CA, USA

Salem Chouaib
Institut Gustave Roussy
Villejuif, France

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Preface

In 1909, Paul Ehrlich proposed that the immune system can recognize and destroy nascent tumor cells. A century later, this principle has been applied successfully for the treatment of patients with various cancers by the use of monoclonal antibodies, adoptively transferred T-cells, genetic amplification of T-cells bearing high affinity TCR, ADCs, CAR T-cells and check point blocking antibodies (see below). Immunotherapy against cancer has recently experienced significant translational clinical applications in the treatment of many cancer types. We witnessed a few decades ago the initial clinical application of T-cell-mediated immunotherapy, initially by the ex vivo culture and activation of cancer patients BPMCs with IL-2, to generate LAK cells and, subsequently, the culture and propagation of TILs from cancer tissues and their adoptive transfer into the patients. Subsequently, various modalities have been examined and applied in cancer, such as ex vivo DCs pulsed with tumor lysates or tumor peptides and administered into the patients with growth factors. In addition, several cancer vaccines have been developed. Further, targeting T-reg cells and MDCS resulted in enhancing the anti-tumor T-cell response. A number of successfully current immunotherapies in cancer patients, including check point targeted antibodies (e.g., anti-CTLA-4, PD-1, and PDL-1) and adoptive T-cell therapies (e.g., genetically transduced T-cell receptors and CARs), are reported to be clinically effective in the treatment of advanced cancers, many of which are resistant to conventional chemotherapy and radiation. The likelihood of responsiveness to these immunotherapies differs strongly depending on tumor type. Targeting check points resulted in significant responses in melanoma, renal cell carcinoma, and non-small cell lung cancer. For CARs, significant clinical responses have been achieved in lymphomas. All of the aforementioned is a testimony to the important role of immunotherapy mediated by T-cells and antibodies that have resulted in the new generation of targeted therapies and reduced toxicity encountered by conventional chemotherapy and radiotherapy. Several of the aforementioned immunotherapy strategies were effective in the treatment of drug-resistant tumor cells. However, there is still a subset of nonresponsive patients who have a cancer with either a naturally acquired resistance or an induced intrinsic resistance to such therapies.

The successful requirements for an adoptive and optimal T-cell response consist of three key elements: the ability to induce a T-cell response; the ability to infiltrate into the tumor microenvironment; and the ability to kill the tumor cells. Most anti-cancer cellular and humoral therapies have given little attention to the generally encountered tumor cell resistance to cytotoxic activities mediated by such therapies. In fact, tumor cells develop several mechanisms to escape tumor cell death. Tumor cell resistance may be responsible, in large part, for the fact that many cancer patients fail to respond to cytotoxic immunotherapy in the presence of anti-tumor cytotoxic T-effector cells and antibodies.

Clearly, one of the important, and not completely exploited, area in cancer immunotherapy is the underlying mechanisms of tumor cell resistance to CTL and antibody-mediated cytotoxicities. Several reported studies explored the underlying molecular bases of tumor cell resistance to CTL and shed new light on the improvement of current immunotherapy of cancer and that could significantly improve the clinical response. Resistance of Cancer Cells to CTL-Mediated Immunotherapy reviews, in large part, several of the mechanisms underlying the tumor cell resistance to CTL-Mediated cytotoxicity, and suggests several means to overcome the resistance by the use of combination treatments with agents targeting resistance in combination with CTL-Mediated immunotherapy. This volume comprises the contributions of leaders in the field, and provides numerous examples of molecular bases of CTL resistance. (While this volume does not cover the field in its entirety, due to the vast scope of the subject, subsequent volumes under consideration will cover other areas of CTL resistance in cancer and their clinical implications.)

This volume is divided into four parts. Part I, Factors Regulating Resistance to CTL Cytotoxicity, consists of five review chapters. Doctors Maccalli and colleagues reviewed "Resistance of Cancer Stem Cells to Cell-Mediated Immune Responses." It is clear that, in the majority of cancers, cancer stem cells (CSCs) are believed to be responsible, in large part, for tumor initiation, progression, metastasis, and resistance to cytotoxic therapies. CSCs have been reported to escape immune surveillance, though they exhibit antigenic molecules that can be targeted for immunotherapy, rescuing both the new growth and resistance to CTL-Mediated therapy. Doctors Dolstra and colleagues reviewed "Role of Co-inhibitory Molecules in Tumor Escape from CTL Attack." Tumor cells may express co-inhibitory molecules (CIMs) that can severely inhibit CD-8 T-cell cytotoxicity. These inhibitory molecules on the cancer cell surface, such as PDL-1, will inhibit CTL cytotoxic activity via interaction and cell signaling of PD-1 on the surface of CTL. In addition, CIMs such as CTLA-4, LAG-3, BTLA, Tim-3, and CD200R have been implicated in the inhibition of CTL functions. The authors have discussed the role each of the above CIMs and, as well, suggest various approaches to inhibit their activities and restore cytotoxic activity. Doctors Seliger and Bergner reviewed "Role of the Non-classical HLA Class I Antigens for Immune Escape." One of the mechanisms of tumor escape from immune surveillance is the overexpression of the non-classical class I HLA-G+ antigen that is often overexpressed in solid and hematopoietic tumors. This overexpression leads to its interaction with inhibitory receptors ILT2,

ILT4, and KIR2DL4. HLA-G+ tumors are associated with poor clinical outcomes. The inhibition of HLA-G can increase the sensitivity to tumor cells to CTL and NK cytotoxicities. The authors describe the role of HLA-G+ as a therapeutic target. Doctors Mami-Chouaib and colleagues reviewed "Integrins: Friends or Foes of Antitumor Cytotoxic T Lymphocyte Response." The authors describe the role of integrins and their ligands in the regulation of T-cell effector functions that result in CTL activation and triggering of their cytotoxic machinery. Of particular interest is the authors' description of the integrins CD103 and LFA-1 and their respective ligands, E-cadherin and ICAM-1, in the regulation of T-cell effector functions. Also discussed is the importance of integrin-antagonists in cancer immunotherapy. Doctors Noonan and Murphy reviewed "Cytotoxic T Lymphocytes and their Granzymes: An Overview." In addition to several immunotherapeutic strategies, including antibodies and adoptive transfer of CTLs, novel strategies are aimed at the cell death pathways including granzymes and death ligands (Fas-L TNFalpha TRAIL). In this review, examples of granzymes-mediated cell death using the prototype of granzyme-bound immunotoxin therapy are discussed. In addition, in this review, the authors discuss the initiation and the activation of the effector functions of CTL and how they can be used in cancer immunotherapy.

Part II, "Influence of the Tumor Microenvironment on the Resistance to CTL Cytotoxicity," consists of three review chapters. Doctors Chouaib and colleagues reviewed "Hypoxia: A Formidable Saboteur of the Anti-tumor Response." The tumor microenvironment (TME), in addition to modulating the anti-tumor response, fosters resistance of tumor cells to CTL cytotoxicity. This review emphasizes the influence of hypoxic stress that impacts angiogenesis, tumor progression, and immune tolerance. It includes a discussion on how hypoxia in TME protects tumor cells by modulation of various molecular signaling pathways in the tumor cells and rendering them viable, proliferative, and resistant to CTL. The authors suggest that hypoxia is a target for tumor reactivity to CTL. Doctors Mutis and colleagues reviewed "Mechanisms and Modulation of Tumor Microenvironment-Induced Immune Resistance." The authors discuss the mechanism by which the TME regulates the resistance of tumor cells to CTL cytotoxicity. The authors discuss the modulation of intrinsic, extrinsic, and granzyme/perforin-mediated pathways of apoptosis by the TME and have used multiple myeloma as a cancer model. As well, they discuss strategies to override the resistance of tumor cells to CTL-Mediated immunotherapies. Doctors Sandra Hodge and Greg Hodge reviewed "Evasion of Cytotoxic Lymphocyte and Pulmonary Macrophage Mediated Immune Responses in Lung Cancer." The authors discuss the regulation of tumor cell resistance to CTL cytotoxic therapy, and describe the resistance of lung cancer cells to granzyme B-mediated attack through the expression of a specific inhibitor (such as the intracellular serine protease inhibitor PI-9). PI-9 is expressed in CTLs and protects the tumor cells to killing by granzyme B. The authors cite studies that report that PI-9 expression positively correlated with cancer stage among patients with solid and hematologic malignancies, suggesting that targeting PI-9 may be a strategy to improve immunotherapy in lung cancers.

Part III, "Resistance to Death Ligands-Mediated Apoptosis and Sensitization" consists of four review chapters. Doctor Bonavida reviewed "Sensitization of Immune-Resistant Tumor Cells to CTL-Mediated Apoptosis via Interference at the Dysregulated NF-/Snail/YY1/PI3K/RKIP/PTEN Resistant Loop." He discusses the mechanisms by which tumor cells develop resistance to CTL-Mediated apoptosis via a dysregulated loop consisting of the NF-kB/Snail/YY1/RKIP/PTEN. This dysregulated loop further regulates cell growth, proliferation, MET, metastasis, and the resistance to both CTL and chemotherapeutic drugs. The role of each of the gene products in the loop and its direct involvement in the regulation of the above functions and, in particular, to CTL-Mediated apoptosis via death ligands (Fas-L, TNFa, DR4, and DR5) is discussed. Also discussed is the manner in which each of the gene products in the loop has potential for reversal of resistance as well as inhibition of tumor cell growth and metastasis. Several examples are provided with different agents that target different gene products of the loop and resulted in the reversal of resistance to CTL cytotoxicity. Doctors Zhang and colleagues reviewed "Overcoming Cancer Cell Resistance to Death Receptor Targeted Therapies." Targeting death receptors for cancer treatment has been explored in both the laboratory and in human clinical trials. Among the death ligands that are not toxic to normal tissues, TRAIL, is being investigated in clinical trials through the use of either recombinant TRAIL or agonist antibodies to TRAIL receptors DR4 and DR5. While these studies are ongoing clinically, both alone and in combination with conventional chemotherapy, it must be noted that many patient cancer cells are resistant to such therapies and require sensitizing agents that can be used in combination to reverse resistance. The authors discuss various approaches to reverse resistance. Doctors Chen and colleagues reviewed "Pancreatic Cancer Resistance to TRAIL Therapy: Regulators of the Death Inducing Signaling Complex." The authors discuss the resistance of pancreatic cancer to TRAIL-induced apoptosis. They have identified several factors in the death receptor activated DISC (which include FLIP, calmodulin, Src, and PARP-1) that contribute to the resistance of cancer cells to death receptor-mediated apoptosis. Also discussed are mechanisms that regulate the DISC that result in the resistance of TRAIL apoptosis. In addition, they suggest, for pancreatic cancer, various therapeutic targets for immunotherapy. Doctors Thiery and colleagues reviewed "Resistance of Carcinoma Cells to CTL-Mediated Immunotherapy." The authors discuss the role of EMT and cancer stemness in the resistance to both chemo and CTL-Mediated therapeutics. As well, they explored the immunological synapse and how it is affected by EMT and discuss the manner in which the inhibition of EMT can restore cytotoxic immune function.

Part IV, "Future Directions" consists of two chapter reviews. Doctors Kawakami and colleagues reviewed "Cancer Induced Immunosuppression and Its Modulation by Signal Inhibitors." The authors describe various signal-mediated pathways that regulate the immune response, and how signal inhibitors may enhance anti-tumor responses. The authors suggest personalized treatments (as the oncogenic signal activities are different for each cancer patient). They also consider and recommend personalized treatment for immunotherapy. Doctors Mehrotra and colleagues

reviewed “Quality of CTL Therapies: A Changing Landscape.” The authors discuss the various mechanisms by which tumor cells escape immune surveillance and discuss several strategies that they recommend be investigated in order to restore the immune functions and, in particular, the response of tumor cells to T-cellmediated therapy. This chapter also provides various challenges for consideration in the future.

Benjamin Bonavida
Salem Chouaib

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Contents

Part I
Factors Regulating Resistance to CTL Cytotoxicity

Chapter 1
Resistance of Cancer Stem Cells to Cell-Mediated Immune Responses

Veronica Catalano*, Cecilia Eleuteri*, Gaia Campoccia, Gianluca Giacobini, Mariangela Zane, Giorgio Stassi, Giorgio Parmiani, and Cristina Maccalli

Abstract In the past decades, the hierarchical organization of tumors, governed by Cancer Stem Cells (CSCs), have been reported with regard to several tumor types. Advances in sequencing technologies have demonstrated that diverse genetic CSCs subclones, derived from the branching evolution, compete with each other within the tumor mass, thereby contributing to the functional heterogeneity. It is becoming increasingly clear that epigenetic modifications and microenvironmental influences are important determinants of tumor fitness resulting in disease progression, recurrence and reduced patient survival. Therefore, more effective therapies will require gaining insights into the role of genetic and non-genetic influences in coordinating tumor maintenance.

CSCs are believed to be responsible for tumor initiation, progression and resistance to therapeutic agents. Therefore, CSC-targeted therapeutic interventions are desirable to achieve complete tumor eradication. Immunotherapy can represent a valuable treatment thanks to its antigen-specificity. The molecular and immunological characterizations, though still not definitive, of CSCs revealed their low efficiency in eliciting adaptive immune responses and the presence of features correlating with escape from immunosurveillance. Nevertheless, CSC-specific molecules may represent novel targets for immunotherapy and immunomodulatory agents may able to rescue their immunogenicity. This information might be exploited to design novel CSC-targeting therapies, possibly in association with inhibitors of

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V. Catalano • C. Eleuteri • M. Zane • G. Stassi
Department of Surgical and Oncological Sciences, University of Palermo, Palermo, Italy

G. Campoccia • G. Giacobini
Medical Oncology and Immunotherapy, University Hospital of Siena,
V.le Bracci, 16, Siena 53100, Italy

G. Parmiani • C. Maccalli (✉)
Medical Oncology and Immunotherapy, University Hospital of Siena,
V.le Bracci, 16, Siena 53100, Italy

NIBIT-Italian Network for Bio-therapy of Tumors, Siena, Italy
e-mail: crimaccalli@gmail.com

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survival pathways and/or with differentiation agents and cytotoxic drugs. These therapeutic strategies are desirable in order to target the entire cancer and can represent a promising strategy to achieve complete tumor regression.

Keywords Cancer stem cells • Cancer stem cell markers • Signaling pathways of cancer stem cells • T cell responses • NK Cells • Immunomodulatory molecules • CSC-targeted therapies • Immune escape

Abbreviations

ABC	ATP-binding cassette
ADAM	A disintegrin and metalloprotease
APC	Adenomatosis polyposis coli
APCs	Antigen presenting cells
APM	Antigen processing machinery
B7-H1, 3, 4	B7 homolog family members
BMP	Bone morphogenetic protein
CEA	Carcino embryonic antigen
CK1	Casein kinase 1
COA-1	Colon antigen-1
CRC	Colorectal cancer
CSC	Cancer stem cells
CSL	CBF1/Su(H)/Lag-1
CXCR-4	C-X-C chemokine receptor type 4
EMT	Epithelial-to-mesenchymal transition
Ep-CAM	Epithelial cell adhesion molecule
EphB	Ephrin B
ESC	Embryonic stem cells
FZ	Frizzled
GBM	Glioblastoma multiforme
GDF-15	Growth differentiation factor 15
Gp100	Glycoprotein 100
GS	γ-Secretase
GSK3	Glycogen synthase kinase 3
Hh	Hedgehog
HLA	Human leukocyte antigen
IDO	Indoleamine 2,3-dioxigenase
IFN	Interferon
IL-10	Interleukin-10
IL-13α2	α2 chain of IL-13 receptor
IL-4	Interleukin-4
LRP	Low-density-lipoprotein-related protein5/6
M	Mastermind-like protein 1

MAA	Melanoma associated antigen
MAGE	Melanoma-associated antigen gene
MART-1	Melanoma antigen recognized by T cells
MDSC	Myeloid derived suppressor cell
Melan-A	Protein melan-A, *see also* MART-1
MHC	Major histocompatibility complex
MSC	Mesenchymal stem cell
MUC-1	Mucin 1
NKG2D	Natural killer group 2, member D
Notch-IC	Receptor intracellular domain
NY-ESO-1	New York esophagus 1 antigen
PD-1	Programmed cell death protein 1
PD-L1	Programmed death ligand 1
PGE2	Prostaglandin E2
PSA	Prostate specific antigen
PTCH	Patched
R	Recombining binding protein suppressor of hairless
Runx2	Runt-related transcription factor 2
SMO	Smoothened
STAT3	Signal transducer and activator of transcription 3
SVV-1	Survivin 1
TAA	Tumor associated antigen
TCF	T-cell factor-1
TGF-β1	Tumor growth factor beta 1
Treg	T regulatory cell

1.1 Introduction

According to the classical model of tumorigenesis, every cell of the body is equally susceptible to acquire an unlimited and uncontrolled proliferative potential, following genetic and epigenetic mutations. Clonal evolution of different subclones, dictated by environmental influences and continuing mutagenesis, explains the phenotypic differences observed in a tumor population [1]. Accumulating evidences suggest that tumor growth and progression are driven by a subset of cells with "stemness" properties, called cancer stem cells (CSCs). Located at the top of tumor hierarchy, these cells possess the long-life capacity to self-renew and generate the heterogeneous population of differentiated descendants, which constitute the tumor bulk [2]. The practical translation of this definition is their ability to generate a serially transplantable phenocopy of the original malignancy when injected into immuno-compromised mice [3].

From a clinical point of view, CSCs have been defined by multiple resistant mechanisms against anti-cancer therapies contributing to tumor recurrence and metastatic dissemination [4]. Similar to normal stem cells, CSCs were reported to

shuttle between quiescence, slow-cycling and active states [5, 6]. Despite the loss of the CDK4/6 pathway regulation, the retention in a non-proliferating or G0 state, depends on the activation of p21 and p27 cell-cycle inhibitors, which block the transition from G1 to S-phase [7]. Interestingly, CSCs are stimulated to enter in a proliferative state in response to signals produced by the tumor microenvironment, such as the TGF-β family members which abrogate the p21 and p27 activation [8]. Although conventional cancer therapies are targeting the cell cycle and/or rapidly dividing cells, most patients relapse because of the quiescent regrowth of CSCs [9]. Complementary mechanisms responsible for chemoresistance are represented by high levels of anti-apoptotic factors (FLIP, BCL-2, Bcl-xl, IAP family members) [10], active DNA repair capacity [11], up-regulation of cell pumps such as the multidrug resistance transporter (MDR1) [4] and increased metabolic activity through ALDH1 [12]. By stabilizing the cysteine transporter subunit xCT and, thereby, regulating the intracellular levels of reduced glutathione (GSH), a primary intracellular antioxidant, CSCs are also able to protect themselves from ROS-inducing anticancer drugs [13]. Lastly, CSCs can be difficult to reach because they reside in a permissive environment that protects them from diverse genotoxic insults [14, 15].

In addition, to sustain CSCs functional traits [16, 17], the tumor microenvironment is also involved in the CSC generation through induction of "stemness" features into differentiated tumor cells [18, 19]. Along this line, HGF-producing myofibroblasts are able to provide a CSC phenotype to non-CSC, by reactivating the Wnt signaling pathway. These dedifferentiated cancer cells acquire the expression of stem cell-associated genes but also gain tumorigenic potential [20]. The unexpected plasticity of CSCs enables these cells to change their phenotype and to assume different functions and properties, including a stem cell state. Epithelial cells undergoing the epithelial-to-mesenchymal transition (EMT) lose polarity and cell-to-cell adhesion properties. However, they acquire a mesenchymal-like phenotype associated with increased motility, invasiveness and resistance to apoptosis [21]. CSCs can be also generated by inducting the EMT program, which stimulates the expression of CSCs markers and increases tumorigenic potential [22]. By either down-regulating "stemness"-repressed microRNAs [23, 24] or by inducing expression of Bmi-1 [25], EMT-inducing factors, like cytokines and hypoxia, stimulate the expression of transcription factors associated to self-renewal program.

Recent data show that cytokines secreted by the tumor microenvironment, including HGF, osteopontin and stromal-derived factor 1α, reprogram colorectal $CD44v6^-$ progenitors in metastatic stem cells by increasing the CD44v6 expression via the Wnt pathway activation. Survival analysis, conducted by using Kaplan-Meier curves, revealed that in patient cohorts, low levels of CD44v6 predict increased probability of disease-free survival. Importantly, the inhibition of phosphatidylinositol 3-kinase (PI3K) that selectively killed CD44v6 expressing CSCs has been shown to be effective in reducing the metastatic process initiated by CSCs, through the expression of CD44v6 [26].

These evidences underline the importance of studying the complex interplay between CSCs and the tumor microenvironment, which may lead to the identification of novel drug candidates.

It has been extensively demonstrated that the immune system plays a relevant role in the control of tumor growth; in fact, lost of immunity is associated with cancer risk, and on the other hand efficient systemic immune responses can lead to tumor killing [27, 28]. The interplay between tumor development and the immune system has been re-defined by a step-wise process that includes 3 different phases (3E), early elimination, equilibrium and escape [29]. The concept that the immune infiltrate at tumor site can have prognostic significance has been initially proposed by Mihm et al. [30] for melanoma; then it was extended to other neoplastic tissues and, more recently, it was quantitatively and molecularly defined leading to the development of the immunoscore as an efficient prognostic tool for solid tumors [31].

Despite the fact that in the last two decades a variety of molecular and regulatory features of tumor immunology have been extensively dissected, effective therapeutic vaccines for solid tumors have not yet been convincingly obtained; an overall 10–20 % of clinical responses have been observed. A possible explanation for these disappointing clinical results may lie in the failure to elicit effective and persistent immune responses by tumor vaccine in cancer patients. On the other hand, many factors can work in concert to inhibit anti-tumor immunity, including the release by tumor cells of suppressive cytokines/factors, the induction of regulatory T lymphocytes (Tregs) and/or myeloid derived suppressor cells (MDSCs) [32–34].

Moreover, the modulation by the complex interactions of co-stimulatory or negative regulatory molecules, defined as immune checkpoint molecules, on antigen presenting (APC)/tumor cells and on effector immune cells has been shown to play a key role in the regulation of anti-cancer immune responses [35]. The clinical development of immune-checkpoint blockade agents showed durable clinical responses and increase of survival for patients with solid tumors with different histological origins [36]. These evidences indicate that immunotherapy represents a promising treatment for cancer patients as it can induce efficient anti-tumor immune responses in these patients. Notably, the effectiveness of immunotherapy could be increased by targeting CSCs that represent the component of the tumor responsible of resistance to standard therapy, such as chemotherapy and radiotherapy, and to immunotherapy as well [11, 37–39].

The characterization of the immunological profile of CSCs and of the relationship between CSCs and anti-tumor immunity, thus, represent a relevant issue to design novel and more effective immunotherapy interventions for cancer patients.

1.2 CSCs Markers

CSCs are hypothesized to derive from normal stem cells through an aberrant step of differentiation or after a reprogrammed leading to a less differentiated status [3]. In light of this, it is possible to identify CSCs by using stemness characteristic markers, such as transcription factors acting during early embryogenesis, or genes involved in pluripotency maintenance. Similarly, cancer stem/progenitor cells can

be recognized by following specific proteins that intervene in early organogenesis, from the three different germ layers.

In association with Oct4, Sox2 forms a trimeric complex involved in embryonic development. These markers are transcription factors that perform their function by binding to DNA and activating some important genes, such as *YES1*, *FGF4*, *UTF*, and *ZFP206*. Nanog is a transcription factor induced by Oct4 involved in stem cell self-renewal and pluripotency and hence, preventing differentiation. CSC identification can be obtained by following genes belonging to stem cell pathways, such as *Wnt*, *Hedgehog*, and *Notch* (classified also by EMT-inducing signaling pathways) (www.uniprot.org).

In proceeding with differentiation, embryonic stem cells undergo a phenotype change in their tissue destination. To analyze the differentiation towards each lineage, it is possible to use ectodermal (i.e., Notch, Nestin, p63), mesodermal (i.e., BMP4, Nodal, CD34, Cryptic), and endodermal (i.e., a-fetoprotein, beta-catenin, CXCR4, SOX17) markers. In relation to tissue differentiation and development, these marker classes belong to all cells with the same tissue derivation. For this reason, they are commonly used and constitute a simple screen panel for CSC characterization. Being that most markers are intracellular, they cannot be used for FACS sorting or beads separation. Hence, the challenge of many research groups is the identification of membrane markers that can be stable and specific to a definite pathology.

The cells with the capacity to efflux Hoechst 33342 vital dye, that were first identified in mouse bone marrow, are referred to as "side population" (SP) because they are composed of unstained cells in the left lower quadrant of a FACS profile [40]. SP has been used to isolate malignant cells since their ability to efflux dyes correlates to multidrug resistance mediated by the ABC transporters over-expression [41]. Moreover, these cell subsets are highly enriched for the capacity to initiate tumor formation upon serial transplantation and express stem-like genes [42].

The use of Hoechst dye to isolate stem-like cells has met with criticisms. In fact, this is a dynamic process, based on dye efflux, in which variables in staining times, dye and cellular concentrations can affect the SP phenotype. Since the DNA binding induced by Hoechst staining promotes a toxic effect in living cells, the SP cells, isolated through this method, may be a population able to resist the lethal effects rather than stem-like cells. Furthermore, flow cytometry gating strategies, used to define SP cells, cannot be associated with gating strategies involved in staining using other markers [43].

A similar method of characterization of CSCs is the analysis of the cell subset expressing an high Aldehyde Dehydrogenase (ALDH) activity, which is involved in early cellular differentiation, detoxification, and drug resistance, through the oxidation of intracellular aldehydes [44]. ALDH belongs to the oxidoreductase enzyme family and is highly expressed in stem and progenitor cells, thus it is used as a functional marker for CSC isolation from solid tumors (i.e., breast, lung, ovarian, prostate, head-neck, and thyroid cancer), as well as in multiple myelomas and acute leukemia [45]. Using the ALDEFLUOR™ assay, it is possible to isolate cancer stem and progenitor cells through cell sorting with a positive selection, without compromising their vitality.

In solid tumors, several cell surface markers are used to isolate cell subsets enriched with CSCs, such as CD44 [46–49], CD24 [57, 50], EpCAM [46, 51], THY1 (also called CD90) [52], and CD133 [51, 53–57].

CD133, also known as Prominin-1, is a pentaspan transmembrane glycoprotein originally identified as a marker for human $CD34^+$ hematopoietic stem and progenitor cells by Miraglia et al. [58]. It was recognized as an important marker in the identification and isolation of cell subsets with "stemness" properties in many tumor tissues, such as brain [55], kidney [59], prostate [56], hepatic [60], and colon [53, 57]. Nonetheless, the usage of CD133 as an identification and isolation marker in colon CSCs is controversial because its expression pattern is not completely elucidated. In line with this, $CD133^+$ and $CD133^-$ cell fractions have been reported to display similar "stemness" and differentiation potential, including the ability to generate tumors similar to the parental ones [61]. Kamper and colleagues explained the contradictions found in the literature by studying possible regulation mechanisms of epitope expression. CD133 is expressed in both CSCs and differentiated tumor cells. Whereas the CD133 mRNA and protein expression remained unchanged, differentiation led to down-regulation of the AC133 epitope, correlating with differential glycosylation and reduced antibody detection [62].

The CD133 polarized localization suggests its role in regulating proliferation but its functions remains still unclear. Recent studies highlight that CD133 could be involved in tumor angiogenesis since $CD133^+$ glioma cells have shown to produce vascular endothelial growth factors [63]. In the intestine, CD133 has been proposed as a stem cell marker susceptible to neoplastic transformation, being prone to activate Wnt signaling [64]. Therefore, it is important to note that CSC identification and isolation requires the use of more than one specific marker.

1.3 Survival Pathways in CSCs

The signaling pathways, which regulate normal stem cell self-renewal, lead to tumorigenesis when dysregulated; a comprehensive understanding of the pathways involved in development, "stemness" and apoptosis, is considered to be a very important goal in cancer therapy. The most important signaling pathways that regulate normal and cancer stem cell functions are: Notch, Wnt, BMP and Sonic-Hedgehog.

The Notch signaling pathway is evolutionarily conserved and has profound, context-dependent phenotypic consequences because it is involved in the maintenance of stem cells and in differentiation regulation. In both humans and rodents, the Notch genes encode four distinct members (from Notch1 to Notch4) of a transmembrane heterodimeric receptor family. In physiologic conditions, Notch ligands (Delta and Jagged) binding induces the Notch receptor intracellular domain (Notch-IC) release via a cascade of proteolytic cleavages catalyzed by a disintegrin and metalloprotease (ADAM) and γ-secretase (GS) proteases. Notch-IC translocates into the nucleus and modulates the gene expression by binding the transcription factor,

CBF1/Su(H)/Lag-1 (CSL), and recruiting co-activators, such as recombining binding protein suppressor of hairless (R) and mastermind-like protein 1 (M) [65] (Fig. 1.1a). The aberrant activation of this pathway contributes to tumorigenesis [66–70]. With the notable exception of epidermal keratinocytes where Notch-1

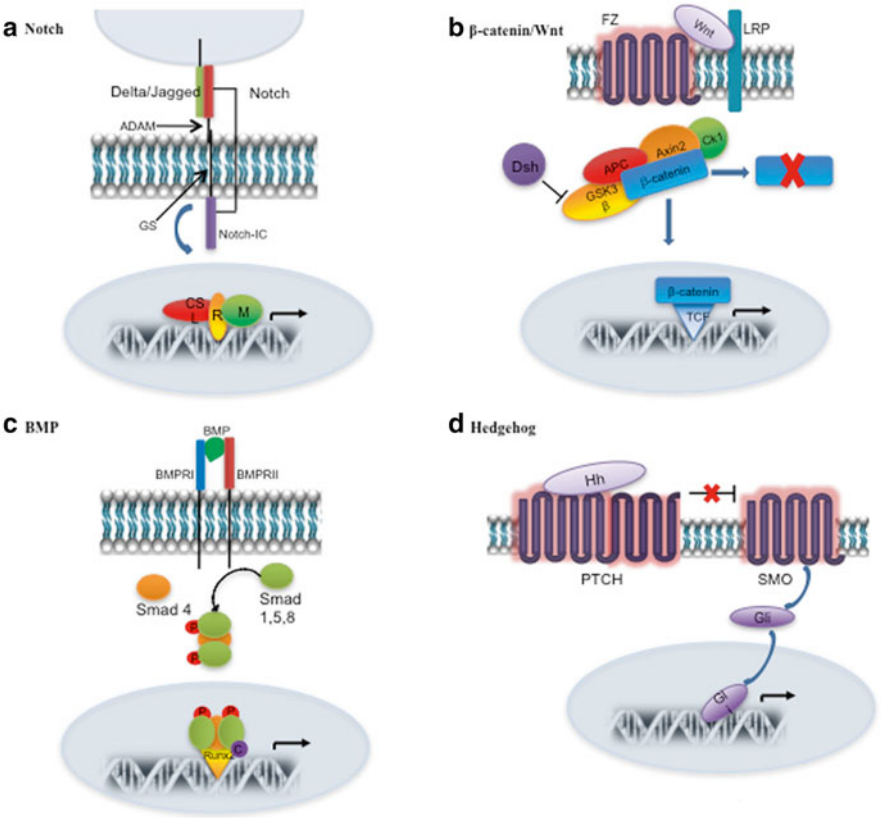

Fig. 1.1 Pathways involved in CSC survival and differentiation. (**a**) Notch signaling. Notch signaling relies on the activation of Notch receptors by Delta and Jagged ligands expressed in a neighbor cell. The release of Notch-IC, subsequent to the two proteolytic events catalyzed by ADAM and GS proteases, leads to the transcription of target genes by binding the transcription factor CSL and recruiting the co-activators R and M. (**b**) Canonical Wnt/β-catenin signaling. Wnt binds to FZ, which recruits LRP5/6 as co-factor and interacts with Dsh. β-catenin cytoplasmatic localization is regulated by a destruction complex formed by APC, Axin2, GSK3βCK1, which directs its degradation by ubiquitination. In presence of Wnt ligands, Dsh inhibits GSK3 and the destruction complex disassembles allowing β-catenin to shift to the nucleus. (**c**) BMP signaling. The heterodimerization of BMPR receptors induced by BMP proteins promotes the phosphorylation of SMAD 1,5,8 and their association with SMAD 4. The complex formed enters into the nucleus and stimulates the target genes' transcription aided by Runx2 and a cofactor (C). (**d**) Hedgehog signaling. Signaling by Hh depends on the interaction between the membrane proteins SMO and PTCH. When bound to Hh, PTCH does not repress SMO, which in turn activates GLI transcription factors

functions as a tumor suppressor [71], the inappropriate activation of the Notch pathway results in the stimulation of proliferation, restriction of differentiation and prevention of apoptosis in T-cell acute lymphoblastic leukemia [69], breast cancer [72, 73], melanoma [74], lung adenocarcinoma [75] and others. Therefore, a possible anticancer therapy goal may be the Notch signaling inhibition that is achieved at many different levels. It is possible to interfere with receptor activation by blocking ligand-induced conformational changes [76] and releasing the Notch-IC receptor by blocking the ADAM [77] or GS proteases cleavage [53, 65, 78]. In addition, Notch signaling could be inhibited by disrupting protein–protein interactions involved in nuclear events, including the assembly of co-activators [79, 80]. The γ-secretase inhibitors (GSIs) and monoclonal antibodies (mAbs), which block Notch receptors, are currently in the beginning stages of clinical trials [81, 82]. Moreover, mAbs that target Notch ligand Delta-like 4 have been shown to inhibit Notch signaling in endothelial cells by inducing disorganized angiogenesis [83]. In the platinum-resistant ovarian cancer, the inhibition of Notch signaling by a GSI and conventional Paclitaxel chemotherapy, synergistically reduced xenograft growth [84]. In intestinal crypts, where the staminal cell niche is located, Notch directs proliferation when Wnt signaling activity is high and promotes enterocyte differentiation when Wnt activity levels are reduced [85].

Wnt proteins constitute a family of signaling molecules that regulate cell-to-cell interactions during development. They are secreted glycoproteins that bind to the extra-cellular domain of the Frizzled (FZ) receptor, a seven-transmembrane protein that requires different co-receptors to mediate three different Wnt pathways:

(1) the canonical Wnt/β-catenin cascade; (2) the non canonical planar cell polarity (PCP) pathway; and (3) the Wnt/Ca^{2+} pathway.

In the canonical Wnt pathway, the co-factor low-density-lipoprotein-related protein5/6 (LRP5/6) interacts with the cytoplasmatic phosphoprotein Dishevelled (Dsh) [86]. This interaction causes an accumulation of β-catenin in the cytoplasm and its translocation into the nucleus, where it attracts, as co-activator, some transcription factors belonging to the T-cell factor-1 and lymphoid enhancing factor-1 TCF-1/LEF-1 family as well as regulating gene transduction. In the absence of Wnt ligands, a destruction complex formed by Axin2, adenomatosis polyposis coli (APC), glycogen synthase kinase 3 (GSK3) and casein kinase 1 (CK1), degrades β-catenin by targeting for ubiquitination. The canonical Wnt pathway activation produces the translocation of the negative Wnt regulator, Axin2, to the plasma membrane where it binds to the cytoplasmatic tail of LRP-5/6. Thus, Axin2 becomes de-phosphorylated and its stability is decreased. Moreover, Dsh inhibits the GSK3 activity of the destruction complex allowing the β-catenin accumulation in the nucleus (Fig. 1.1b) [85].

The Wnt canonical signaling is important in many developmental processes and in the regulation of self-renewal in normal and CSCs. In particular, the Wnt target gene *leucine-rich repeat-containing G protein-coupled receptor 5* (*Lgr5*) marks stem cells in multiple adult tissues and cancers [87]. A germline APC mutation is the genetic cause of a hereditary colorectal cancer syndrome called Familiar