to a poor prognosis. Later, CSCs/TICs were isolated as ALDH1$^+$ cells in colon and prostate cancers [32,33].

Functional properties of CSCs/TICs

The CSCc/TICs are defined as the small population of cancer cells that have the properties of tumor initiation abiliy, self-renewal and differentiation as previously described. Based on these properties, CSCs/TICs are thought to make hierarchical model like normal stem cells (Figure 2). However, there are still several questions regarding this model, including: which kind of normal cells do CSCs/TICs originate from (normal stem cells)? Do differentiated non-CSCs/TICs never de-differentiate into CSCs/TICs? Do CSCs/TICs need a niche for their maintenance like normal stem cells? What is the niche for CSCs/TICs? Above all, what are the molecular properties of CSCs/TICs?

As described by several reports, CSCs/TICs have been shown to have higher tumorigenicity than non-CSCs/TICs when xenotransplanted into immune-deficient animals. However, one report had a major question regarding their 'tumorigenicity'. Since even immunodeficient mice, such as nude mice or NOD/SCID mice, still have a very low level immune activity, this may affect the evaluation of the tumorigenicity. Quintana *et al.* reported that they evaluated the melanoma-initiating ability of primary isolated melanoma cells in NOD/SCID IL-2 receptor γ-chain null (*Il2rg*$^{-/-}$; NOG) mice [34,35] using Matrigel. They found that only one in 837,000 human melanoma cells could form into a tumor within 8 weeks of transplantation into NOD/SCID mice, which was almost the same as in other reports. On the other hand, when the tumorigenicity was evaluated with an improved assay in NOG mice using Matrigel, one in four melanoma cells could form tumors, remarkably higher efficiency than in NOD/SCID mice. This suggests that the sensitivity of xenotransplantation might be affected by the host immune status as well as other experimental conditions [36]. At this moment, xenotransplantation using Matrigel is the most sensitive method for evaluation of tumorigenitiy, but a more convenient, time-saving assay is needed for standardized assessment.

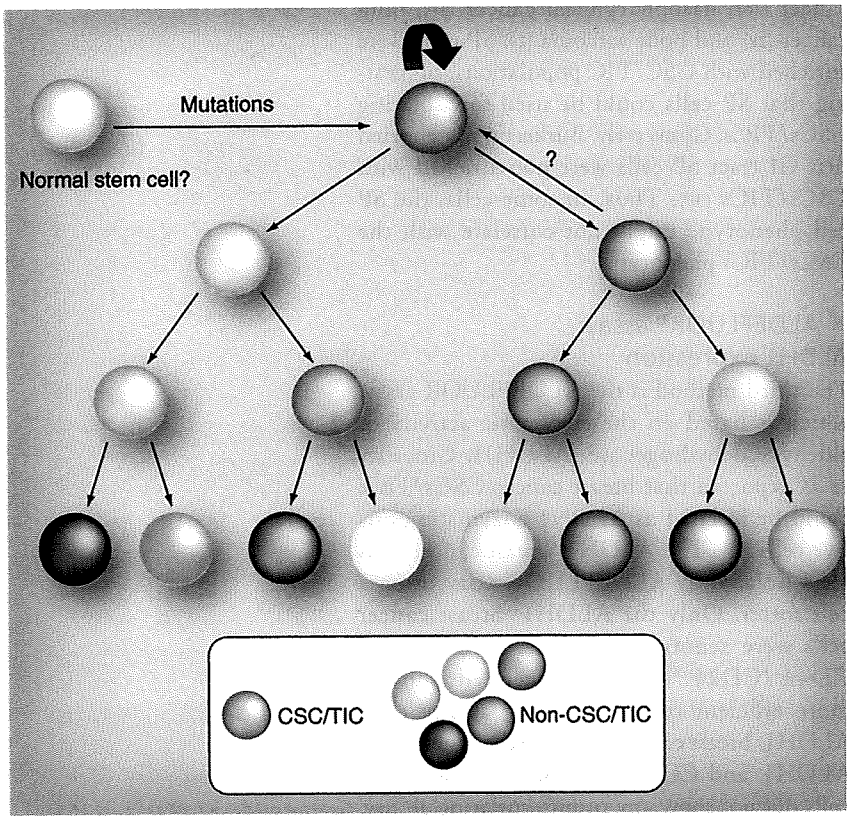

Figure 2. The cancer stem-like cell/tumor-initiating cell model. CSCs/TICs are located on the top of this hierarchical differentiation model. CSCs/TICs self-renew and differentiate to non-CSCs/TICs.
CSC/TIC: Cancer stem-like cell/tumor-initiating cell.

It has been hypothesized that CSCs/TICs possess several characteristics that make them resistant to conventional chemotherapy and radiotherapy, including high expression of drug transporters, relative cell cycle quiescence, high levels of DNA repair machinery and resistance to apoptosis [37]. There are several reports describing the mechanisms by which CSCs/TICs obtain the resistance to treatments. Costello *et al.* found that CD34$^+$CD38$^-$ cells in both AML patients and normal patients exhibited decreased sensitivity to daunorubicin compared with CD34$^+$CD38$^+$ cells, and that this difference correlated with higher levels of mRNA expression of the drug resistance-related genes, lung resistance-related protein (LRP) and MRP. The decrease in the influx of daunorubicin in CD34$^+$CD38$^-$ cells was associated with increased proliferation and survival [38]. Quiescence is also thought to confer resistance to therapies that target highly proliferating cells. Human AML LSCs have been shown to reside mostly within the G0 phase of the cell cycle. Although CSCs/TICs may be nonproliferative compared with non-CSCs/TICs, quiescence may not be sufficient to mediate drug resistance. Recently, another mechanism of treatment resistance has also been revealed. Diehn *et al.* reported that the low level of reactive oxygen species (ROS) in CSCs/TICs is related to radioresistance as in

normal neural or hematopoietic stem cells [39]. They showed that CSCs/TICs, which contain lower levels of ROS, developed less DNA damage and were preferentially spared after irradiation compared with non-CSCs/TICs, and lower ROS levels in CSCs/TICs were associated with increased expression of free radical scavenging systems.

CSCs/TICs & immunosystems

Cancer stem-like cells/tumor-initiating cells are resistant to several cancer treatments including chemo- and radio-therapies through various mechanisms. However, can immunosystems recognize and kill CSCs/TICs? There are few but convincing reports regarding the immune reactions to CSCs/TICs. Innate immunity plays the front-line of host defence and is composed of pathogen receptors such as Toll-like receptors, and effector cells such as neutrophils, NK cells, part of γδT cells and part of NK T cells. Adaptive immunity plays a prolonged and antigen-specific immunity composed of T cells and antibodies, which must be primed and boosted by antigens. Both innate and adaptive immunity are related to anti-CSCs/TICs immunity. To date, NK cells, γδT cells, CTLs and antibodies are reported to be able to eliminate CSCs/TICs efficiently (TABLE 1).

NK cells

NK cells are one of the key players of innate immunity. Castriconi *et al.* first described the relationship between NK cells and CSCs/TICs [40]. The authors cultured surgically resected glioblastoma cells in stem cell medium and obtained Nestin$^+$ and SOX2$^+$ glioblastoma stem cells. The glioblastoma stem cells expressed both classical (HLA-A, -B and -C) and nonclassical (HLA-E) HLA molecules. The autologous IL-2-(or IL-15)-activated NK cells efficiently killed glioma stem cells, probably through cytotoxity receptor NKp46 and DNAM-1. Although HLA-class I molecules act as inhibitory receptor ligands for NK cells, the blocking of HLA-class I molecules with a monoclonal antibody did not enhance the susceptibility to NK cells, suggesting that the expression level of HLA class I molecules on glioblastoma stem cells was not sufficient for suppressing of NK cell activation. Glioblastoma stem cells and also primary glioblastoma tissues expressed PVR and Nectin-2, which are ligands for DNAM-1. These data suggest that NK cell-mediated immunity might be effective for elimination of CSCs/TICs of glioma cells. However, lymphokine (IL-2 or IL-15) activation is essential for gaining cytotoxic ability, and resting NK cells cannot recognize CSCs/TICs.

γδT cells

The susceptibility to γδT cells was evaluated using colorectal CSCs generated by culture in the presence of EGF and bFGF [41]. The authors isolated colon CSCs from primary colon cancer tissues under serum-free culture conditions and evaluated their susceptibility to Vγ9Vδ2T cells. The CSCs/TICs from colon cancer tissues showed susceptibility to Vγ9Vδ2 T cells, but of nine colon cancer CSC/TIC patients, two were resistant. Interestingly, colon cancer CSCs/TICs from primary tissues were relatively resistant to Vγ9Vδ2T cells. Zoledronate, a small nonpeptidic phosphorylated compound, sensitized colon cancer CSCs/TICs to Vγ9Vδ2 T cells. These data suggest that naturally expressed ligands for Vγ9Vδ2 T cells might not be sufficient to activate Vγ9Vδ2 T cells completely and in some colon cancer cases CSCs/TICs might lack the expression of ligands for Vγ9Vδ2 T cells. The killing activity of Vγ9Vδ2 T cells involved TCR and NKG2D. Vγ9Vδ2 T cells have been detected in the majority of colon cancer tumor-infiltrating lymphocyte populations, and Vγ9Vδ2 T cells can recognize colon cancer cells. These observations suggest that the natural immune response mediated by these lymphocytes might contribute to the immunosurveillance of these tumors. Immunotherapy targeting colon cancer CSCs/TICs using Vγ9Vδ2 T cells (active immunization or adoptive cell transfer) might be effective as an alternative therapy.

T cells: CTLs

CD8-positive effector cells (CTLs) play an important role for acquired immunity. CTL-based cancer immunotherapy, such as cancer vaccine therapy and adoptive cell transfer, is one representative approach to cancer immunotherapy. CTL-based CSC/TIC-targeting therapy can be performed using CSC/TIC-specific TAAs. Ishiyama *et al.* reported that Numb-1- and Notch-derived antigenic peptides, both related to the Notch signal, could be recognized by CD8$^+$ T cells from ovarian cancer patient ascites [42]. Furthermore, the same group demonstrated that treatment of the MCF-7 breast cancer cell line and SK-OV-3 ovarian cancer cell line with 5-fuorouracil and paclitaxel caused increases of CD133$^+$ cells and also CD44$^+$CD24$^-$ cells, both putative CSC/TIC markers. Incubation of these CSC/TIC-enriched cells with Numb-1 or Notch

Table 1. Immune effectors and cancer stem-like cells/tumor-initiating cells.

Target antigens	Effectors	Types of cancer	CSC/TIC phenotype	Antigenic peptide	Presenting molecule	Locus	Functions of antigens	Ref.
CTL targets								
Numb-1	CTL	Breast cancer	$CD44^+CD24^-$	VLWVSADGL	HLA-A2	87–95	Notch signal	[43]
Notch	CTL	Breast cancer	$CD44^+CD24^-$	RLLDEYNLV	HLA-A2	2112–2120	Notch signal	[43]
ALDH1A1	CTL	HNSCC	Aldeflour	LLYKLADLI	HLA-A2	88–96	Enzyme	[47]
P2X5	CTL	Leukemia	$CD34^+$	TPNQRQNVC	HLA-B7	110–118 (frameshift)	Minor antigen	[48]
Potential CTL targets								
SOX2	CTL	Glioblastoma	–	TLMKKDKYTL	HLA-A2	118–127	Stem cell marker, self-renewal	[49]
EZH2	CTL	Hepatocellular carcinoma	–	YMSCSFLFNL	HLA-A2	666–674	DNA methylation	[50]
Survivin	CTL	Several	–	Several	Several	–	Anti-apoptosis	[51,54]
Livin	CTL	Several	–	Several	Several	–	Anti-apoptosis	[55]
Aurora-A	CTL	Several	–	YLILEYAPL	HLA-A2	207–215	Cell division	[56]
Ep-CAM	CTL	Several	–	RYQLDPKFI	HLA-A24	173–181	Cell adhesion	[57]
Antibody targets								
IGF-IR	Antibody	Colon cancer	$CD44^+$ or $CD133^+$	–	–	–	Growth factor receptor	[58]
DLL4	Antibody	Colon cancer	$ESA^+CD44^+CD166^+$	–	–	–	Notch signal	[59]
CD47	Antibody	AML	$CD34^+$	–	–	–	Inhibition of phagocytosis	[60]
CD47	Antibody	Bladder cancer	$CD44^+$	–	–	–	Inhibition of phagocytosis	[12]
Innate immunity								
Nonspecific	NK cell	Glioblastoma	$Nestin^+SOX2^+$	–	–	–	–	[40]
Nonspecific	$V\gamma9V\delta2$ T cell	Colon cancer	Sphare formation	–	–	–	–	[41]

ALHD1A1: Aldehyde dehydrogenase family 1, subfamily A1; AML: Acute myeloid leukemia; CSC/TIC: Cancer stem-like cell/tumor-initiating cell; CTL: Cytotoxic T lymphocyte; DLL4: Δ-like 4; Ep-CAM: Epithelial cellular adhesion molecule; EZH2: Enhancer of zeste, drosophila, homolog 2; HNSCC: Head and neck squamous cell carcinoma; IGF-IR: IGF I receptor; SOX2: SRY (sex-determining region Y)-box 2.

peptide-activated peripheral blood mononuclear cells (PBMCs) caused a CSC/TIC population-specific decrease [43]. This report suggests that Numb-1 or Notch peptide-specific CTLs might recognize and kill CSC/TIC populations; however, the authors did not show any direct cytotoxic activity of CTLs *in vitro* or *in vivo* anti-tumor effects. This was the first report demonstrating that CTL could recognize CSCs/TICs. Other groups reported that model antigen (cytomegalovirus [CMV]) transduced $CD133^+$ glioma stem cells or HER2-expressing $CD133^+$ glioma stem cells are also susceptible for CTLs [44,45].

We also recently observed that, CSCs/TICs were recognized by CTLs both *in vitro* and *in vivo* [Inoda S *et al.*, Unpublished Data]. We isolated CSC/TIC populations by SP analysis from lung cancer, breast cancer and colon cancer cell lines. These SP cells induced tumors in immune-deficient mice with ten- to 100-fold efficiency, suggesting that CSC/TIC populations are enriched in these SP cells. Next, we examined the susceptibility to CTL clones specific for Cep55/c10orf3 [46]. The SP cells could be recognized by the CTL clone at the same level as non-SP cells, which represent non-CSC/TIC population. This suggests that CSCs/TICs are also as sensitive to CTLs as non-CSCs/TICs both *in vitro* and *in vivo*. Thus, CTL-based tumor immunotherapy, for example peptide vaccine therapy or adoptive cell transfer, is potentially effective for the elimination of the CSC/TIC population. Since CTLs need TAA expression for recognition, CSC/TIC-specific TAAs need for further investigation. ALDH1A1- [47] and minor histocompatibility antigen- [48] specific CTLs are able to recognize head and neck squamous cell carcinoma (HNSCC) and leukemia CSCs/TICs, respectively. Furthermore, several other CTL target TAAs are expressed in CSC/TIC populations. Such CTL target TAAs are candidates for CSC/TIC-targeting immunotherapy (potential CTL targets). SOX2 [49], EZH2 [50], survivin [51–54], livin [55], Aurora-A [56] and Ep-CAM [57], all targets of CTLs, are expressed in CSCs/TICs. These TAAs might be useful for CSC/TIC-targeting cancer immunotherapy.

Antibodies

Following the success of monoclonal antibody (mAb) therapy with rituximab (anti-CD20) for B-cell malignancies, several mAbs have been developed and evaluated for other malignancies, including solid tumors. mAb therapy is based on functional activity (both inhibitory and antirational functions) and NK cell-mediated antibody-dependent cell-mediated cytotoxicity (ADCC). Very recently, some groups have reported that mAbs for IGF-I receptor (IGF-IR), δ-like 4 ligand (DLL4) and CD47 efficiently eliminate colon cancer and leukemia CSCs/TICs.

Dallas *et al.* reported that treatment of a colon cancer cell line with 5-fluorouracil and oxaliplatin induced a drug-resistant subline [58]. This subline expressed higher levels of $CD133^+$ and $CD44^+$, both putative colon cancer CSC/TIC markers, than the wild-type colon cancer cell line, and also showed higher colony-formation efficiency, suggesting that the drug-resistant subline had an enriched CSC/TIC population. The authors demonstrated that the CSC/TIC-enriched population expressed a higher level of IGF-IR than the wild-type cell line, and treatment with an anti-IGF-IR mAb (AVE-1642) inhibited CSC/TIC growth more efficiently than in the wild-type both *in vitro* and *in vivo*. Drug resistance depends on IGF-IR signaling and inhibition of this signal by AVE-1642 is a reasonable treatment. This mAb will have a synergistic effect with chemotherapy. Very recently, another group also reported a mAb for colon cancer CSCs/TICs. Hoey *et al.* reported that blockade of DLL4 with mAb reduced the colon cancer CSC/TIC population and also tumor growth *in vivo* [59]. DLL4 acts as a ligand for the Notch signal pathway and contributes to stem cell self-renewal and vascular development, and inhibition of DLL4 caused tumor growth suppression. This was partially due to inhibition of angiogenesis, but Hoey explained the tumor inhibitory mechanism also included anti-CSC/TIC direct effect. Treatment of tumor-bearing mice with the DLL4-specific mAb suppressed tumor growth and, importantly, reduced the CSC/TIC population. This suggests that suppression of Notch signaling by the DLL4 mAb abrogated self-renewal of CSCs/TICs *in vivo*. Thus, this mAb appears to have both anti-CSC/TIC and antiangiogenesis effects.

Majeti *et al.* and Jaiswal *et al.* published two serial papers regarding CD47, which is expressed on leukemia stem cells [60,61]. CD47 is expressed on $CD34^+CD38^-$ AML LSCs, and protects LSCs from phagocytosis by macrophages. The expression of CD47 is related to a poor prognosis, suggesting that surveillance by macrophages plays an important role in inhibition of the disease. An anti-CD47 mAb did not have any lytic activity on LSCs, but the mAb did cancel the phagocytic-inhibitory activity and caused phagocytosis by macrophages. Anti-CD47 mAb treatment also inhibited tumor cell growth

Table 2. Candidates of cancer stem-like cell/tumor-initiating cell antigens.

Molecule	Types of malignancies	Localization	Functions	Application
SOX2	Lung cancer, colon cancer and breast cancer	Nucleus	Self-renewal	Antigenic peptide
SMCP	Lung cancer, colon cancer and breast cancer	Mitochondria	Unknown	Antigenic peptide
OR7C1	Lung cancer and colon cancer	Cell surface	Unknown	Antigenic peptide and antibody
pCDH19	Lung cancer and colon cancer	Cell surface	Unknown	Antigenic peptide and antibody

OR7C1: Olfactory receptor, family 7, subfamily C, member 1; pCDH19: Protocadherin 19; SMCP: Sperm mitochondria-associated cysteine-rich protein; SOX2: SRY (sex determining region Y)-box 2.

in vivo, but did not show any inhibitory effect on normal hematopoietic stem cells, suggesting that the phagocytosis-inducing effect was specific for LSCs. The same group also reported that CD47 is expressed on the bladder cancer CSCs/TICs, and treatment with anti-CD47 antibody enhanced the phagocytosis by macrophages [12]. Thus, CD47 can be the general CSCs/TICs target not only for hematopoietic malignancies, but also solid malignancies.

NKT cells

NKT cells play an important role in cancer immunity involving both innate immunity and adoptive immunity [62]. NKT cells express T-cell receptor (TCR) like other T cells, but show very restricted diversity. NKT cells recognize α-galactosylceramide presented by the MHC-like class Ib molecule CD1d, and secrete robust IFN-γ. IFN-γ activates NK cells and enhances innate immunity. IFN-γ also induces IL-12 secretion by dendritic cells (DCs) and upregulates CD40/40L on NKT cells and DCs. NKT cells and DCs crosstalk through CD40/CD40L and cause maturation of DCs, which induce adaptive immunity including CTLs. Since both effector cells (NK cells and CTLs) can recognize CSCs/TICs, activation of NKT cells can potentially regulate innate and adaptive immunity positively and cause elimination of CSCs/TICs.

Recently, Bellone M *et al.* reported the relation of NKT cells and tumor initiation. Transgenic adenocarcinoma of the mouse prostate (TRAMP) mice develop spontaneous prostate cancer, and, TCR Ja18$^{-/-}$ mice lack NKT cells. TRAMP Ja18$^{-/-}$ mice lacking NKT cells develop more precocious and aggressive prostate cancer than TRAMP mice, suggesting that NKT cells control the tumor initiation of prostate cancer cells, probably by inhibiting the CSCs/TICs directly or indirectly [63].

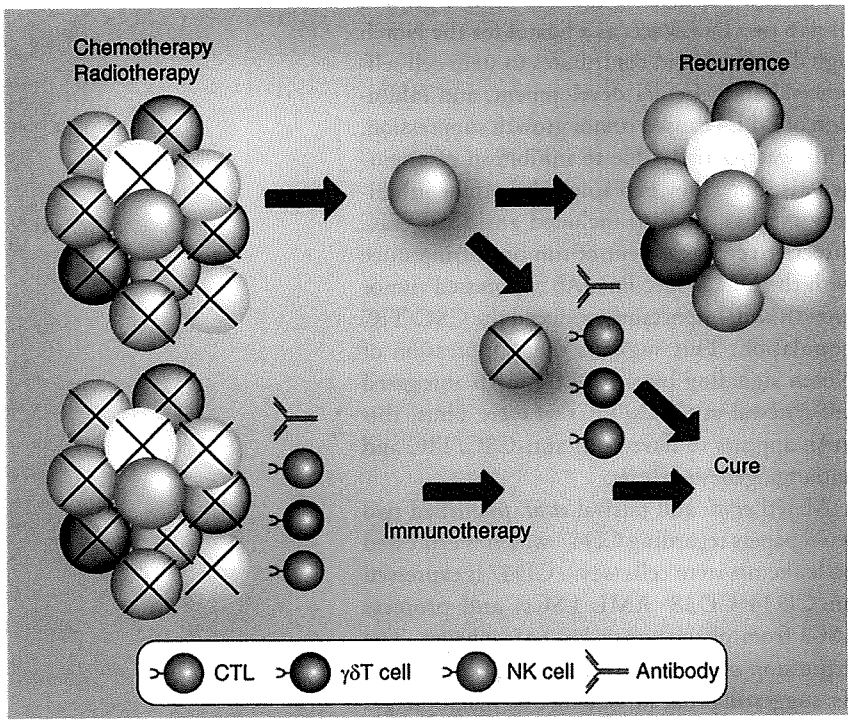

Figure 3. Immunotherapy targeting cancer stem-like cells/tumor-initiating cells. CSCs/TICs are thought to be resistant to chemo- or radio-therapies. However, immunosystems can recognize and kill CSCs/TICs. CSC/TIC-targeting immunotherapy, or chemo- or radio-therapy following immunotherapy can possibly 'cure' cancer.
CSC/TIC: Cancer stem-like cell/tumor-initiating cell; CTL: Cytotoxic T lymphocyte.

Feasibility of immunotherapy targeting cancer stem-like cells

As previously described, CSCs/TICs express TAAs that can be recognized by CTLs or antibodies. Furthermore, several effector cells can recognize CSCs/TICs. So, it is possible that CSCs/TICs can also be killed *in vivo*. However, it is not clear whether CSC/TIC-targeting immunotherapy is feasible, as CSCs/TICs exist and self-renew under interactive conditions with connective tissues in a 'niche' that may protect CSCs/TICs from immunological reactions.

One interesting paper by Xu *et al.* has shown how glioma CSC/TIC-targeting immunotherapy might be more effective and have prognostic benefits in a rat glioma model [64]. The authors demonstrated that several TAAs, including EGFR, HER2, TRP2, MRP3, AIM2, SOX2

and IL13Rα2, were also expressed in glioma stem cells. Furthermore, glioma CSC/TIC-pulsed DCs stimulated CTLs from a healthy volunteer and showed TRP2, HER2, CD133, IL13Rα2 and SOX2 peptide-specific IFN-γ-secreting activity in ELISA. Interestingly, these CTLs also recognized a glioma CSC/TIC line, but not glioma non-CSCs/TICs, suggesting that they recognized glioma CSC/TIC antigens specifically. The authors also presented a rat glioma treatment model. Rat immunized with apoptotic glioma neurosphere (enriched with glioma stem cells)-pulsed DCs had a better prognosis than those immunized with adherent glioma (differentiated glioma cells)-pulsed DCs. This suggests that glioma stem cells express several TAAs, and that immunization with such TAAs is a more efficient strategy than using nonstem cell antigens. This strategy uses a glioma-derived CSC/TIC population for immunization. Since it is difficult to maintain glioma CSCs/TICs, it might be difficult to standardize. Identification of novel CSC/TIC-specific TAAs should make possible their further application to clinical studies.

We screened CSC/TIC-specific TAA candidates with a gene expression microarray using colon cancer SP cells. We isolated several genes that were specifically expressed in CSCs/TICs rather than non-CSCs/TICs (summarized in Table 2). As aforementioned, SOX2 can be a good candidate for CSC/TIC-targeting immunotherapy. The immunogenicities of SMCP, OR7C1 and pCDH19 are still unclear, but their gene products are reasonable candidates for CSC/TIC-targeting immunotherapy.

Conclusion

Cancer stem cell theory was unclear for a long time, but now the molecular mechanisms are emerging, and the CSC/TIC research is moving to focus on how to eliminate the small population. As previously described, immune systems can recognize CSCs/TICs and can be reasonable candidates for CSC/TIC-targeting therapy. Thus, solo immunotherapy or immunotherapy following chemo- and radio-therapies, can possibly eliminate CSCs/TICs and cure the disease (Figure 3). As described by Koebel *et al.*, immunity controls carcinogen-induced or spontaneous tumors [65]. The mice models suggest that a considerable proportion of occurring tumors are suppressed by immune surveillance, probably by inhibiting CSCs/TICs. Thus, immunosystems potentially recognize CSCs/TICs. Conversely, clinical cancer must survive (probably several years) immunosurveillance. This phenomenon is termed 'immunoediting', and clinical cancers including CSC/TIC populations must have some sort of immunosuppressing mechanisms. In the recent study, the epithelial–mesenchymal transition, which is related to development, but also plays a significant role in cancer progression, is related to cancer stem cell properties [66]. In addition, cancer cells suppress immunity during epithelial–mesenchymal transition [67]. Thus, these reports strongly suggest that CSCs/TICs have the higher potential to inhibit immunosystems than non-CSCs/TICs. However, as aforementioned, each immunocyte (NK cells, γδT cells and CTLs) has the potential to kill CSCs/TICs if they are accurately activated. Thus, we may break immunosuppressing conditions by cancer cells (CSCs/TICs) by accurate activation of immunosystems (adaptive immunotherapy, adoptive immunotherapy and inhibitors of immunosuppressive cytokines).

Future perspective

The CSCs/TICs research has made a breakthrough with the establishment of CSCs/TICs isolating-methods. At present, the molecular mechanisms and properties of CSCs/TICs are emerging gradually, and several basic researches on CSCs/TICs-targeting therapies, including immunotherapy, have being established. As CSCs/TICs focus on the functional and phenotypical aspects of small population of cancer cells (not cell line level), the *in vivo* effect and analysis of CSCs/TICs-targeting therapy will have significant meanings. CSCs/TICs markers are useful for detecting CSCs/TICs *in vivo* and are also required to evaluate the CSC/TIC-targeting therapies. In the near future, the focuses of CSC research will move into analysis of mice treatment models or human preclinical and clinical trials.

Executive summary

Cancer stem-like cells (CSCs)/tumor-initiating cells (TICs) can be isolated by cell surface markers, side population and ALDEFLUOR® assay.
CSCs/TICs are resistant to several therapies, including chemo- and radio-therapies by several molecular mechanisms.
Cytotoxic T lymphocytes, antibodies, NK cells and γδT cells can recognize CSCs/TICs.
CSCs/TICs express tumor-associated antigens that can be CTL targets of antibodies.
Immunotherapy is a possible and promising approach to eliminating CSCs/TICs and by curing cancer.

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