Cancer stem-like cells (CSC)/cancer-initiating cells (CIC) are defined as minor subpopulations of cancer cells that are endowed with properties of higher tumor-initiating ability, self-renewal ability and differentiation ability. Accumulating results of recent studies have revealed that CSC/CIC are resistant to standard cancer therapies, including chemotherapy, radiotherapy and molecular targeting therapy, and eradication of CSC/CIC is, thus, critical to cure cancer. Cancer immunotherapy is expected to become the “fourth” cancer therapy. Cytotoxic T lymphocytes (CTL) play an essential role in immune responses to cancers, and CTL can recognize CSC/CIC in an antigen-specific manner. CSC/CIC express several tumor-associated antigens (TAA), and cancer testis (CT) antigens are reasonable sources for CSC/CIC-targeting immunotherapy. In this review article, we discuss CSC/CIC recognition by CTL, regulation of immune systems by CSC/CIC, TAA expression in CSC/CIC, and the advantages of CSC/CIC-targeting immunotherapy.
Immunological Phenotypes of Cancer Stem-like Cells

Because CSC/CIC are resistant to standard therapies, susceptibility to effectors of immunity is a challenging issue. The immune system is composed of innate immunity and adaptive immunity. Phagocytes, natural killer (NK) cells and γδ T cells are effectors of innate immunity, and these effectors recognize target cells in an antigen non-specific manner. CTL and antibodies are effectors of cellular and humoral immunity, respectively, and they recognize target cells in an antigen specific manner. NK cells, γδ T cells, CTL and antibodies have been reported to be able to recognize CSC/CIC, and only effectors of adaptive immunity can recognize CSC/CIC specifically.\(^{(21)}\)

**Antigen presentation of Cancer stem-like cells/cancer-initiating cells and recognition by cytotoxic T lymphocytes.** Expression of antigenic peptides presented by major histocompatibility complex (MHC) (human leukocyte antigen [HLA] in human) class I is essential for recognition by CTL. MHC class I expression has long been thought to be related to disease progression and survival of cancer patients, and immune surveillance is believed to play a pivotal role in cancer progression.\(^{(22-25)}\) Antigenic peptides are produced from fully or unfolded mature proteins or unfolded immature amino acid fragments known as defective ribosomal products (DRiP). Oligopeptides are produced from proteins by proteasome in the cytosol and are transported into the endoplasmic reticulum (ER) through transporters associated with antigen processing-1 and -2 (TAP1 and TAP2). Oligopeptides are cleaved by endoplasmic reticulum aminopeptidase associated with antigen processing (ERAAP) in the ER to an adequate length (antigenic peptide) and loaded onto MHC class I and -2 (TAP1 and TAP2). Oligo-peptides are produced from proteins by proteasome in the cytosol. Oligopeptides enter the endoplasmic reticulum (ER) through transporters associated with antigen processing-1 and -2 (TAP1 and TAP2). Oligopeptides are cleaved by endoplasmic reticulum aminopeptidase associated with antigen processing (ERAAP), and then loaded onto major histocompatibility complex (MHC) class I with the help of chaperone proteins, calreticulin, tapasin and Erp57. The figure summarizes the expression of antigen presentation machinery proteins in CSC/CIC. N.D., not determined.

In our previous study, colon CSC/CIC derived from colon cancer cells as side population (SP) cells showed MHC class I expression, as did non-CSC/CIC at similar levels.\(^{(13)}\) In contrast, another study showed that colon CSC/CIC isolated as sphere-forming cells from colon cancer tissues had lower expression levels of MHC class I than non-CSC/CIC and were recognized by NK cells.\(^{(26)}\) Similar controversial results have been obtained in gliomas. Wei et al.\(^{(28)}\) report that glioblastoma multiform (GBM) stem cells isolated as sphere-forming cells from GBM tissues showed significant expression of HLA class I. In contrast, Di Tomaso et al.\(^{(29)}\) report that GBM stem cells isolated as sphere-forming cells from GBM tissues showed lower levels of HLA class I expression than the levels in GBM non-stem cells. Wu et al.\(^{(30)}\) report that expression of HLA class I was not detected in CD133+/putative GBM stem cells. Melanoma stem cells isolated as ABCB5+ cells from melanoma tissues showed lower expression levels of HLA class I than those found in ABCB5− melanoma non-stem cells.\(^{(31)}\) Sphere cultured cells derived from several cell lines showed lower levels of HLA class I expression than those found in serum cultured cells. Interestingly, sphere cultured cells did not respond to HLA class I upregulation by IFNγ treatment, suggesting impaired HLA class I induction machinery.\(^{(32)}\) The difference in results might be due to differences in CSC/CIC isolation methods, culture conditions and origins.

Several molecules are involved in antigenic peptide processing, transportation into the ER and loading onto MHC class I, and studies on antigen presentation machinery in CSC/CIC are limited. Busse et al.\(^{(32)}\) characterized sphere cultured cells derived from several cell lines and found that sphere-forming cells show equal or higher expression levels of antigen-presenting machinery molecules, including LMP2, LMP7, LMP10 (MECL1), TAP1 and TAP2. However, sphere cultured cells derived from primary GBM samples and colon cancer samples showed lower expression levels or no expression of antigen-presenting machinery molecules, including LMP2, LMP7, LMP10, TAP1, TAP2, calnexin, calreticulin, Erp57 and tapasin.\(^{(29,33)}\) Chikamatsu et al.\(^{(34)}\) report that CD44+ putative head and neck CSC/CIC showed expression levels of LMP2, LMP7, LMP10 (MECL1), TAP1 and tapasin that were equal to those in CD44− cells, whereas CD44+ cells showed a lower expression level of TAP2 than that in CD44+ cells (Fig. 1).

In our previous study, SP cells derived from colon cancer cells showed susceptibility to CEP55, a colon cancer-related antigen-specific CTL clone #41, that was equal to that in non-SP cells.\(^{(13,35)}\) Furthermore, CTL clone #41 showed a significant anti-tumor effect in immune-deficient mice transplanted with SP cells. Similar results were obtained using a novel cancer testis (CT) antigen, DNAJB8. DNAJB8 is preferentially expressed in CSC/CIC and has an essential role in the maintenance of CSC/CIC derived from renal cell carcinoma cells and colon cancer cells.\(^{(15,36)}\) These observations indicate that CSC/CIC have the ability to produce antigenic peptides and to be recognized by CTL both in vitro and in vivo. Ahmed et al.\(^{(37)}\) report that CD133+ GBM stem cells could be...
recognized by HER2-specific CTL, as could CD133⁺ GBM cells. Sphere-forming GBM stem cells showed higher susceptibility to CTL than did NK cells. HER2-specific peptide vaccination decreased aldehyde dehydrogenase (ALDH)-positive breast cancer stem cells in MMTV-PyMT transgenic breast cancer model mice. All of these observations in humans and mice suggest that treatment-resistant CSC/CIC are sensitive to differentiated CTL both in vitro and in vivo.

**Immune regulation by cancer stem-like cells/cancer-initiating cells.** For induction of CTL from CD8⁺ naïve T cells, naïve T cells must encounter an antigenic peptide presented by antigen-presenting cells, and then they are activated and clonally expand. Not only signaling through the T cell receptor (TCR) but also second signaling through co-stimulatory molecules is necessary for full activation (Fig. 2).

Sphere-forming GBM stem cells express low levels of ligands for second signals (B7.1 [CD80] and B7.2 [CD86]); however, they express high levels of the inhibitory co-stimulatory molecule ligand PD-L1 (CD274 or B7-H1). Furthermore, GBM stem cells release immune suppressive cytokines, including IL-10, IL-13 and TGF-β, and induce regulatory T cells (Treg cells) in vitro culture with peripheral blood CD4⁺ T cells. Similar observations were reported by another group. The inhibitory co-stimulatory molecule PD-L1 (B7-H4) is expressed in non-dividing glioma stem cells. Volonté et al. report that sphere-forming colon CSC/CIC suppress the proliferation of peripheral blood T cells by membrane-bound IL-4 on the CSC/CIC. These immune suppressive profiles of co-stimulatory molecules and cytokines suggest that CSC/CIC might contribute to making an immune-suppressive microenvironment and escaping from CTL. Therefore, CSC/CIC might inhibit differentiation of CD8⁺ naïve T cells into CTL; however, CSC/CIC cannot inhibit the cytotoxicity of differentiated and activated CTL (Fig. 2).

**Antigens Expressed in Cancer Stem-like Cells**

Antigen expression is essential for recognition by CTL, and antigen expression profiles in CSC/CIC have been reported in several malignancies. TAA can be classified into several groups, including (i) CT antigens; (ii) overexpressed antigens; (iii) mutated antigens (neoantigens); and (iv) differentiation antigens. Overexpressed antigens and differentiation antigens are expressed not only in cancer cells but also in normal somatic cells. CT antigens are expressed in cancer cells and germ cells. Germ cells do not express MHC class I molecules and CTL cannot access antigens expressed in germ cells. Thus, CT antigens and mutated antigens show strict expression in cancer cells and are thought to be more immunogenic (probably more effective in cancer immunotherapy) (Fig. 3).

**Cancer testis antigens.** Cancer testis antigens are as highly tumor-specific antigens that are expressed in cancer cells and germ cells but not in normal cells. In transcriptome analysis of CSC/CIC, we found that some CT antigens are preferentially expressed in CSC/CIC. Therefore, we compared the expression of known CT antigens in SP cells and non-SP cells derived from colon cancer cells, lung cancer cells and breast cancer cells and found that 18 CT antigens (MAGEA2, MAGEA3, MAGEA4, MAGEA6, MAGEA12, MAGEB2, GAGE1, GAGE8, SPANX1, SPANXB1, SPANXC, XAGE2, SPA17, BORIS, PLU-1, SGY-1, TEX15 and CT45A1) are preferentially expressed in CSC/CIC. DNAJB8 (DnaJ [Hsp40] homolog, subfamily B, member 8) belongs to the heat shock protein (HSP) 40 family and is preferentially expressed in SP cells derived from kidney cancer cells. DNAJB8 has an essential role in the maintenance of kidney CSC/CIC, and a DNA vaccination model revealed the immunogenicity of DNAJB8. In the present study, we found that DNAJB8 is preferentially expressed in SP cells derived from colon cancer cells and that it has a role in the maintenance of colon CSC/CIC. HLA-A24-bound antigenic peptide was identified from the DNAJB8 amino acid sequence, and CTL specific for DNAJB8 peptide recognized SP cells of colon cancer cells more efficiently than did non-SP cells of colon cancer cells. These observations indicate that

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**Fig. 2.** Immune regulation by cancer stem-like cells (CSC)/cancer-initiating cells (CIC). The first signal through T cell receptors and the second signal through co-stimulatory molecules are essential for naïve CD8⁺ T cells to differentiate into cytotoxic T lymphocytes (CTL). DC, dendritic cells; IL-10, interleukin-10; TGF-β, transforming growth factor-β.
DNAJB8-derived antigenic peptides are promising candidates for colon and kidney CSC/CIC-targeting immunotherapy. In contrast, Boiko et al.\(^\text{(45)}\) report that CD271\(^+\) melanoma stem cells show lower expression levels of MAGE-C1/MAGE-C2, CT antigens than the levels in CD271\(^+\) differentiated melanoma cells. However, they do not describe expression profiles of other CT antigens.

**Overexpressed antigens.** Cancer cells express TAA that are expressed in normal cells at low levels, and these TAA are named overexpressed antigens. CSC/CIC express several overexpressed antigens. Interestingly, CSC/CIC express antigens that are also expressed in normal stem cells, such as SOX2, OCT3/4 and ALDH1A1.\(^\text{[12,46-48]}\) Expression of these antigens is shared by normal stem cells, and these antigens might, therefore, be sub-categorized as stem cell-related antigens. In generating CSC/CIC-targeting immunotherapy, the severe side effect risks of targeting normal stem cells should be considered with stem cell-related antigens.

Other types of overexpressed antigens have been reported to be expressed in CSC/CIC. Antigens of this category are expressed in ubiquitous normal organs at very low levels; they are expressed in CSC/CIC and often have pivotal roles in carcinogenesis. The apoptosis inhibitor proteins Survivin, Livin and Bcl-2 are expressed in several CSC/CIC.\(^\text{(15,49-50)}\) hTERT is expressed in CD44\(^+\) breast cancer stem cells.\(^\text{(51)}\) HER2, a proto-oncogene, is expressed in glioma stem cells.\(^\text{(37)}\) CEP55 and COA-1 are expressed in colon CSC/CIC.\(^\text{(13,33)}\) SP cells derived from breast cancer cells showed expression of MUC1.\(^\text{(52)}\) These overexpressed antigens are thought to be good candidates for CSC/CIC-targeting immunotherapy.

**Mutated antigens (neoantigens).** Mutated antigens are now known as neoantigens and are defined as antigens derived from tumor-specific genomic DNA mutations.\(^\text{(53)}\) Antigens that belong to this category depend on the DNA mutations; therefore, the epitopes are case-specific. The T cell pool specific for neoantigens may be less affected by T cell tolerance compared with nonmutated self-antigens. Recent studies in mice revealed that the neoantigen-specific CTL response has a role in tumor regression in checkpoint blockade cancer immunotherapy,\(^\text{(54)}\) and mass spectrometry and exome sequencing using next-generation sequencer are expected to be powerful tools for identification of case-specific neoantigens.\(^\text{(55)}\)

Neoantigens may be promising targets of CSC/CIC-targeting immunotherapy; however, there are several issues to be overcome. First, neoantigens are mostly derived from passenger gene mutations (not driver gene mutations); therefore, tumor cells carrying neoantigens of passenger gene mutations may lose the expression of neoantigens due to immune selection by CTL because passenger gene mutations do not play a role in tumor cell growth. Second, expression of neoantigens depends on the expression of mutated genes. Thus, limited numbers of neoantigens may be expressed in CSC/CIC, suggesting the difficulty of identification of CSC/CIC-specific neoantigens.

**Differentiation antigens.** Tissue-specific differentiation antigens from several organs are reported to be targets of CTL. CSC/CIC are thought to be in an immature state of cancer cell differentiation, and show lower expression levels of differentiation antigens. Melanoma differentiation antigens MART-1 and tyrosinase are expressed at low levels in CD271\(^+\) melanoma stem cells and ABCB5\(^+\) melanoma stem cells.\(^\text{(31,45)}\) A prostate differentiation antigen, PSA, is expressed at a low level in prostate CSC/CIC.\(^\text{(56)}\) Therefore, differentiation antigens are not suitable for CSC/CIC-targeting immunotherapy.

**Cancer stem-like cell-targeting immunotherapy: From bench to bedside.** Susceptibility to CTL and expression of TAA in CSC/CIC potentially enable the design of CSC/CIC-targeting immunotherapy, and several trials of both pre-clinical and clinical settings have been reported. We previously reported that CTL can be isolated in in vitro culture with SP cells derived from malignant fibrous histiocytoma (MFH).\(^\text{(57)}\) Interestingly, a CTL clone preferentially recognized SP cells rather than non-SP cells, indicating that this CTL clone recognizes MFH stem cell-specific antigens. However, the antigen is not known. Xu et al.\(^\text{(58)}\) report that rats immunized with dendritic cells (DC) pulsed with sphere-forming cells derived from 9L rat glioma cells demonstrated a significantly stronger anti-tumor effect than rats immunized with DC pulsed with differentiated glioma cells. Similar results were obtained in a mouse immunization model using DC pulsed with ALDH\(^+\) cells derived from D5 mouse melanoma cells and SCC7 mouse squamous cell carcinoma cells.\(^\text{(59)}\) These observations indicate that a CSC/CIC-specific CTL response can be induced and that a CSC/CIC-specific CTL response might give rise to a better anti-tumor effect than an all cancer cell-reactive CTL response. To examine the efficacy of CSC/CIC-specific antigens, we used a mouse DNA vaccination model. We immunized mice using DNAJB8-coding DNA and Survivin-coding DNA. DNAJB8 is a novel CT antigen, and the CTL response to DNAJB8 can target only CSC/CIC. In contrast, Survivin is an overexpressed antigen, and the CTL response to Survivin can target all cancer cells. Interestingly, vaccination using DNAJB8 DNA showed a sig-
nificantly stronger anti-tumor effect than that using Survivin DNA, indicating that a CSC/CIC-specific CTL response corresponds to a better anti-tumor effect.15

The results of a clinical trial based on these models of CSC/CIC-targeting immunotherapy are reported. DC transfected with RNA isolated from sphere-cultured GBM stem cells were used for immunotherapy in GBM patients. There were no severe adverse effects, and the patients had prolonged progression-free survival compared with survival in the control group.60

Conclusion and Perspectives
Previous studies have shown that treatment-resistant CSC/CIC are sensitive to cancer immunotherapy using differentiated CTL, and CT antigens are a reasonable and promising candidate for CSC/CIC-targeting immunotherapy. However, several reports suggest that CSC/CIC might have immune suppressive potential. The expressions of immune suppressive cytokines and the inhibitory co-stimulatory molecules in CSC/CIC are potentially major concerns. To overcome this problem, peptide vaccination at sites distant from cancer lesions using appropriate adjuvants, peptide vaccination using immune checkpoint inhibitors (CTLA-4 antibody, PD-1 antibody and PD-L1 antibody), and adoptive transfer of differentiated CTL specific for CSC/CIC are possible options in the future.

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The authors have no financial conflict of interest to declare.

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