Review

Immune evasion by cancer stem cells

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Tumor immunity represents a new avenue for cancer therapy. Immune checkpoint inhibitors have successfully improved outcomes in several tumor types. In addition, currently, immune cell-based therapy is also attracting significant attention. However, the clinical efficacy of these treatments requires further improvement. The mechanisms through which cancer cells escape the immune response must be identified and clarified. Cancer stem cells (CSCs) play a central role in multiple aspects of malignant tumors. CSCs can initiate tumors in partially immunocompromised mice, whereas non-CSCs fail to form tumors, suggesting that tumor initiation is a definitive function of CSCs. However, the fact that non-CSCs also initiate tumors in more highly immunocompromised mice suggests that the immune evasion property may be a more fundamental feature of CSCs rather than a tumor-initiating property. In this review, we summarize studies that have elucidated how CSCs evade tumor immunity and create an immunosuppressive milieu with a focus on CSC-specific characteristics and functions. These profound mechanisms provide important clues for the development of novel tumor immunotherapies.

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Abbreviations: ADCC, antibody-dependent cell mediated cytotoxicity; ALDH, alcohol dehydrogenase; AML, acute myeloid leukemia; ARID3B, AT-rich interaction domain-containing protein 3B; CCR7, C–C motif chemokine receptor 7; CMV, cytomegalovirus; CIK, cytokine-induced killer cell; CSC, cancer stem cell; CTL, cytotoxic T lymphocytes; CTLA-4, cytotoxic T-cell-associated antigen-4; DC, dendritic cell; DNMT, DNA methyltransferase; EMT, epithelial–mesenchymal transition; EV, extracellular vesicle; ETO, fat mass and obesity associated protein; HNSCC, head and neck squamous cell carcinoma; KDM4, lysine-demethylase 4C; KIR, killer immunoglobulin-like receptor; LAG3, lymphocyte activation gene 3; IL12, interleukin 12; LOX, lysyl oxidase; MDMC, myeloid-derived suppressor cell; MHC, major histocompatibility complex; NCI, National Cancer Institute; NOD, nonobese diabetic; NSG, NOD/SCID IL-2 receptor gamma chain null; OCT4, octamer-binding transcription factor; PD-1, programmed death receptor-1; PI9, protease inhibitor 9; PSME3, proteasome activator subunit 3; SCID, severe combined immunodeficient; SOX2, sex determining region Y-box 2; TCR, T cell receptor; Treg, regulatory T cell; uPAR, urokinase-type plasminogen activator receptor.

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1. Introduction

Tumor tissues consist of various types of noncancerous cells, including vascular and immune cells. Drugs targeting the VEGF/VEGFR signaling pathway, such as bevacizumab, ramucirumab, aflibercept, and regorafenib, suppress the proliferation of endothelial cells in tumor tissues, thereby inhibiting neoangiogenesis in the tumor microenvironment [1]. The infiltration of immune cells, including CD4+ and CD8+ T cells, dendritic cells (DCs), and natural killer (NK) cells, into tumors is associated with the prognosis of patients with cancer. These immune cells are frequently suppressed by immune checkpoint molecules, such as programmed death receptor-1 (PD-1), its ligands (PD-L1/PD-L2), and cytotoxic T-cell–associated antigen-4 (CTLA-4), which may be restored by checkpoint inhibitors [2]. Within tumors, noncancerous cells with tumor-specific functions, including cancer-associated fibroblasts (CAFs) [3], tumor-associated macrophages (TAMs) [4], tissue-associated neutrophils [5], myeloid-derived suppressor cells (MDSCs) [6], and pericytes [7], also exist. These cells can promote tumor growth, recurrence, and metastasis through direct and indirect interactions with cancer cells [8]. Recently, it has been revealed that bacteria cells may reside within cancer and immune cells. These intratumoral bacteria affect tumor malignancy and response to immunotherapy [9]. Because of their important role in tumor growth and progression, these noncancerous cells have attracted significant interest as therapeutic targets for the development of novel cancer medicines.

Besides noncancerous cells, cancer cells exhibit genetically and nongenetically heterogeneous populations within tumor tissue. The genetic heterogeneity of cancer cells may occur as a consequence of genetic and chromosomal instabilities [10,11] and may cause significant difficulties to the overall effectiveness of precision medicine. In contrast, the nongenetic heterogeneous populations, in particular, cancer stem cells (CSCs) and non-CSCs, were found in tumor tissue. CSCs have distinct properties compared with non-CSCs, including self-renewal, differentiation into non-CSCs, and tumor initiation ability. Interestingly, non-CSCs can revert to CSCs without alteration of genomic sequences [12,13], indicating that the self-renewal property (Fig. 1). The differences observed between NOD/SCID and NSG mice indicate that the tumor initiation property is a more frequent and ubiquitous feature and that the rarity of cancer cells exhibiting tumor-initiating properties may not be so important for CSC definition. Rather, tumor immune evasion may be a more important characteristic of CSCs [21,22]. Because
immune evasion is also observed in normal stem cells, it may represent a fundamental stemness property endowed on CSCs \[14,23,24\].

As described above, immune selection, which is a phenomenon that immune cells preferentially kill non-CSCs, and consequently promote the propagation of CSCs, may play a role in CSC maintenance and induction (Fig. 1). In other words, this phenomenon indicates that CSCs have an immune evasion property, and are capable to grow even under the surveillance of the immune system. A human hepatoma cell line, HepG2 cells, exposed to mouse macrophages or B cells exhibited increased CSC properties, concomitant with the upregulation of CSC markers \[25\]. In mice, cytotoxic CD8$^+$ T lymphocytes (CTL) enriched CD24$^{-}$/C0$^+$/low/CD44$^+$ breast CSCs by inducing the epithelial–mesenchymal transition (EMT) \[26\]. Trastuzumab, a specific antibody against HER2, kills HER2-positive breast cancer cells by NK-cell-mediated antibody-dependent cytotoxicity (ADCC). After ADCC, surviving breast cancer cells formed spheroids exhibiting a CD44$^{\text{high}}$/CD24$^{\text{low}}$ CSC-like phenotype and lower HER2 expression compared with parental cells \[27\]. The immune selection of malignant rhabdoid tumor cells by passaging in immunocompetent mice enriched cells with increased CSC properties and decreased expression of major histocompatibility complex (MHC) molecules and costimulatory molecules \[28\]. Consistently, tumor exposed to an immune challenge \textit{in vivo} exhibited a CSC-like gene expression profile with increased CSC properties, including tumor initiation ability \[29\]. These results suggest that tumor immunity preferentially kills non-CSCs, leading to an increase in CSCs, although it is also worth investigating whether tumor immunity induces CSC properties in non-CSCs.

The immune evasion property, the tumor initiation ability in the presence of immune surveillance, is an additional characteristic of CSCs \[30\]. Thus, tumor-initiating cells investigated in highly immunocompromised animals would underestimate the true frequency of CSCs present in a tumor tissue \[31\]. As such, studies examining the \textit{in vivo} function of CSCs should be performed with partially immunocompromised, syngeneic, or congenic immunocompetent animals \[32\]. For example, transplantation of hematopoietic stem cells transduced with the ovalbumin and MLL/AF9 fusion genes, in which the latter causes acute myeloid leukemia (AML), into wild-type mice resulted in the development of leukemia although ovalbumin-specific CTLs had expanded in the transplanted mice \[33\]. Interestingly, this syngeneic transplantation model revealed that the cancer cells exhibited high expression of immune checkpoint molecules, such as PD-1 and lymphocyte activation gene 3 (LAG3) (Table 1) \[33\]. These results indicate that an immunocompetent mouse represents a good model for the investigation of the immunological features of CSCs.

### 3. Immunosuppressive properties endowed on CSCs

It has been shown that the expression of CSC makers in human tumor tissues is correlated with the number of tumor-infiltrating immune cells \[34,35\], suggesting that CSCs exhibit a close relationship with the tumor immune environment. Accumulating evidence suggests that the immune response to CSCs is frequently compromised because of their immune evasion properties. For example, CSCs may secrete immunosuppressive factors and recruit immunosuppressive noncancerous cells. Subsequently, the immune suppressive cells, in turn, induce and maintain CSCs. However, the dynamics of the tumor microenvironment are complicated, and mechanisms that create the immunosuppressive milieu can vary depending on tumor development. In this section, we summarize the immunosuppressive mechanisms harnessed by CSCs.

#### 3.1. Impaired antigen presentation of CSCs

The interaction between the T-cell receptor (TCR) on CTLs with MHC-I antigens is a prerequisite for a proper T-cell response against tumor cells. Antigens must be sufficiently degraded by
proteosomes, transported to the endoplasmic reticulum by transmembrane proteins, and presented by MHC-I molecules. Otherwise, T cells fail to recognize transformed cells. It has been suggested that these antigen processing and presenting steps are impaired in CSCs.

Cancer cells cultured in an ultralow attachment dish form spheroids enriched with CSCs because CSCs exhibit an anchorage-independent growth [36]. Melanoma spheroids also showed increased CSC properties compared with adherent cells [37]. MHC molecules are shown to be downregulated in melanoma spheroid cells, leading to the inhibition of the allogeneic immune response of T cells [37]. Likewise, defects in MHC-I-mediated antigen presentation are often observed in CSCs and render the cells resistant to the cytotoxic effects of CTL [38]. A similar observation has also been reported in normal hematopoietic cells and Lgr5+-normal epithelial stem cells [39,40], suggesting that impaired antigen presentation may be a common feature of stem cells.

Neurospheres produced from primary glioblastoma were also enriched in glioblastoma CSCs, which exhibited low MHC-I expression levels and lacked MHC-II expression. Moreover, neurosphere cells also showed downregulation of antigen-processing molecules, including low-molecular-weight protein (LMP), transporter associated with antigen processing (TAP), and b2-microglobulin [41], suggesting that not only antigen presentation but also antigen processing is impaired in glioblastoma CSCs. Consistently, T-cell activation and proliferation was more significantly impaired by these CSCs compared with non-CSCs [41,42]. It was shown that IFNs, which are potent immune modulatory cytokines, induced the expression of MHC-I molecules in glioblastoma CSCs. However, the expression level of MHC-I molecules in CSCs treated with IFNs was still lower compared with that in non-CSCs [41]. This suggests that an IFN response suppression mechanism plays a role in the immune evasion property of glioblastoma CSCs.

It was demonstrated that CSCs derived from head and neck squamous cell carcinoma (HNSCC) also exhibit downregulation of MHC molecules [43,44]. CD44+ HNSCC cells exhibited CSC properties and downregulated HLA-A2, HLA class II, and TAP2, suggesting the impairment of antigen presentation and processing such as glioblastoma CSCs [43]. Moreover, in addition to the suppression of the Th1 response, CD44+ HNSCC cells also induced regulatory T (Treg) cells and MDSCs concomitant with high expression of immune modulatory cytokines, including IL-8, granulocyte colony-stimulating factor, and TGFβ [43]. HNSCC spheroid cells, as observed in CD44+ HNSCC cells, also exhibited low expression of MHC-I and resistance to CTLs [44]. However, IFNγ efficiently induced MHC-I expression in spheroid cells and sensitized the cells to CTL-mediated cytolysis [44]. Interestingly, ALDH1A1+ HNSCC spheroid cells were more susceptible to CTLs in the presence of IFNγ than ALDH1A1− spheroid cells [44].

In contrast, mouse breast ALDH1+ CSCs efficiently formed tumors in immunocompetent mice, whereas the growth of bulk cells containing CSCs and non-CSCs was limited [45]. The ALDH1+ CSCs downregulated the expression of TAP genes and a costimulatory molecule, CD80 (B7-1), by DNA methylation (Table 1) [45]. However, CD44+/CD24− CSCs in murine breast cancer did not show decreased TAP and CD80 expression and upregulated C-X-C motif chemokine receptor 4, which is involved in the induction of the CSC and EMT phenotypes in breast cancer [45,46]. It is not surprising that the mechanisms underlying the immune modulatory functions may vary among CSCs expressing different CSC markers, because some of these markers exhibit distinct functions in regulating CSCs [47].

Spheroid cells derived from TC-1 cells, a lung cancer cell line transduced with viral oncopgene E6/E7, showed lower MHC-I expression compared with adherent cells [48]. IFNγ sensitized spheroid cells to E6/E7-specific immune attack by inducing MHC-I expression [48]. In contrast, the spheroid cells derived from 12 solid tumor cell lines (colon, pancreas, melanoma, and breast cancer) exhibited higher expression of antigen processing and presentation molecules, including LMP2/LMP7, MECL1, and TAP1/TAP2, whereas the expression levels of HL-A-I and HL-A-II were lower compared with those in adherent cells [49], suggesting that T-cell activation by the spheroid cells may be impaired. However, IFNγ failed to induce the expression of HLA molecules in spheroid cells [49]. Therefore, the sensitivity of CSCs to IFNγ differs among cell lines. In addition, mechanisms responsible for the regulation of antigen presentation and antigen-processing molecules in CSCs may also differ. The expression of MHC-I molecules was found to be transcriptionally downregulated in glioblastoma [50], whereas, in peripheral blood mononuclear cells, a serine protease, cathepsin G, degraded MHC-I molecules in the endosome and lysosome [51]. Although active cathepsin G is not present in glioblastoma cells.

### Table 1

| Tumor cells | Effector T cells | Effector function | Function in CSCs |
|-------------|-----------------|------------------|------------------|
| MHC class I & II | TCR | activation | [37–41,43,44,48–50,132,133] |
| CD80/B7-1 | CD28/CD44 | activation | [45] |
| CD68/B7-2 | CD152/CTLA-4 | inhibition | |
| CD274/PD-L1/B7-H1 | CD279/PD-1 | inhibition | [130,141,143,151–163,166,167,169] |
| CD273/PD-L2/B7-DC | | | |
| CD275/ICOS-L/B7-H2 | CD278/ICOS/AILIM | activation | ? |
| CD276/B7-H3 | ? | ? | ? |
| VTC1/B7-51/B7-H4 | ? | ? | ? |
| VISTA/CD24/B7-H5 | CD28H/IGP1/1/TM1G2 | inhibition? | ? |
| HHLA2/B7-H7 | | | |
| NCR3LG1/B7-H6 | CD37/NKp30/NCR3 | activation | [78–80] |
| MHC class II | CD223/LAG3 | inhibition | [33,186,187] |
| FGL1, galec-tin-3, LSECtin | CD220 | inhibition | ? |
| CEACAM1, galecin-9, HMGB1 | TIM3 | ? | [179–182] |
| CD200 | CD200R | inhibition | |

ALILM, activation-inducible lymphocyte immunomodulatory molecule; CEACAM1, carcinoembryonic antigen-related cell adhesion molecule 1; CTLA-4, cytotoxic T-cell-associated antigen-4; FGL1, fibrinogen-like protein 1; HHLA2, human endogenous retrovirus-H long terminal repeat-associating protein 2; HMGB1, high-mobility group box-1; ICOS, inducible T-cell co-stimulator; ICOS-L, ICOS ligand; IGRP1, immunoglobulin and proline-rich receptor-1; LAG3, lymphocyte activation gene 3; LSECtin, liver and lymph node sinusoidal endothelial cell C-type lectin; MHC, major histocompatibility complex; NCR3, natural cytotoxicity triggering receptor 3; NCR3LG1, NCR3 ligand 1; PD-1, programmed cell death-1; PD-L1/2, PD-1 ligand 1/2; TCR, T-cell receptor; TIM3, T-cell immunoglobulin and mucin domain 3; TM1G2, transmembrane and immunoglobulin domain containing 2; VISTA, V-domain immunoglobulin suppressor of T-cell activation; VTCN1, V-set domain-containing-T-cell activation inhibitor 1.  

*Expressed on NK cells.*
CSCs may utilize multiple pathways to downregulate MHC-I expression at the transcriptional and posttranscriptional levels.

### 3.2. Downregulation of tumor-associated antigens in CSCs

As observed in NOD/SCID and NSG mice, the melanoma initiation property depends on host immunity, and only CSCs can evade immune surveillance to form tumors in NOD/SCID mice [17,52]. In addition to the downregulation of MHC-I and MHC-II molecules, melanoma CSCs evade tumor immune surveillance through several mechanisms, one of which involves the downregulation of melanoma-associated antigens [53,54]. Consequently, T cells failed to efficiently recognize melanoma CSCs; however, CSCs highly express the marker proteins specific for CSCs, which are necessary for CSC induction and maintenance [47]. Therefore, CSC-specific proteins are desirable targets as tumor-associated antigens for cancer immune therapy. Consistently, it was demonstrated that activating the immune response to octamer-binding transcription factor 4 (OCT4), which is a well-known stemness transcription factor, suppressed the growth of teratoma cells in mice [55]. Intriguingly, it was reported that naturally occurring T cells specific for OCT4 were found in the ascites and peripheral blood of patients with ovarian cancer, although they lost the ability to eliminate OCT4+ CSCs [56,57]. However, these OCT4-specific T cells were activated by DCs primed with an OCT4-derived peptide [57].

Antibodies against another stemness transcription factor, sex-determining region Y-box 2 (SOX2), were also found in the serum of patients with lung cancer, prostate cancer, and glioblastoma [58,59]. The presence of SOX2-reactive antibodies predicted a good prognosis in patients with lung cancer [58]. SOX2-reactive T cells were also found in patients with monoclonal gammopathy, a benign disease, that sporadically progresses to multiple myeloma [60]. However, the SOX2-reactive T cells disappeared in patients with myeloma [60], suggesting that the collapse of anti-SOX2 immunity may be a mechanism of progression to multiple myeloma.

It has been clinically and experimentally demonstrated that immunotherapy targeting CSC markers, such as CD133, CD44, CD24, CD44+/low, and EPCAM [61,62], can suppress tumor growth. However, these CSC markers are also expressed in normal cells. Therefore, embryonic stemness markers, such as OCT4 and SOX2, may be preferential targets for CSC-specific immunotherapy [68].

### 3.3. Modulation of NK-cell activity by CSCs

In general, it is known that NK cells attack “missing self” cells exhibiting a lack or low expression of MHC-I. This antigen-presenting molecule suppresses NK activity by interacting with NK-cell inhibitory receptors on the surface of NK cells (Fig. 2) [69]. The preferential targeting of NK cells suggests that, although CSCs could escape from MHC-restricted T-cell recognition, they would be eventually eliminated by NK cells. Nonetheless, the sensitivity of CSCs to NK-cell-mediated cell lysis remains under debate because CSCs, not non-CSCs, can form tumors in NOD/SCID mice [16]. This indicates that the tumor microenvironment containing immuno-suppressive noncancerous cells should also be considered to increase our understanding of the relationship between CSCs and NK cells [70]. However, because of space limitations, only the intrinsic properties of CSCs are discussed in the following sections.

### 3.4. NK cell-sensitive phenotype of CSCs

It has been demonstrated that NK-cell-mediated cell lysis activity is enhanced by coculture with CSCs [71,72]. Consistently, NK cells preferentially killed CD24+/CD44−, CD133+, and ALDH1+ CSC lines and primary tumors in vitro [73]. In addition to downregulating MHC-I, NK cells may preferentially kill CSCs because of the upregulation of NK-cell-activating receptor ligands in CSCs (Fig. 2). Furthermore, it was revealed that CSCs exhibited high expression of MHC-I polypeptide-related sequence A/B (MICA/MICB; ligands for a NK-cell-activating receptor, NKG2D) and were killed by NK cells in a manner depending on NKG2D [73].

In addition, NK cells utilize not only the secretory granule-mediated cell lysis pathway involving perforin and granzymes but also the FAS/FASL and DR4/DR5/TRAIL death receptor pathways to kill tumor cells [74]. CSCs from a colon cancer cell line showed c-MYC-mediated expression of DR4 and were susceptible to TRAIL-induced apoptosis [75]. Breast CD44+/CD24− low CSCs isolated from a spontaneous breast carcinogenesis model (B6 PyMT-MMTV transgenic mouse) expressed FAS and DR5 and were more susceptible to FASL- and TRAIL-induced apoptosis compared with non-CSCs [76]. Although these CSCs exhibited lower expression of MHC-I and higher expression of Rae-1 (NKG2D ligand) and CD155/PVR (ligand for a NK-cell-activating receptor, DNAM-1) compared with non-CSCs, the sensitivity to NK-cell-mediated cell lysis was not significantly different between the CSCs and non-CSCs [76]. Therefore, these results suggest that the sensitivity of CSCs to NK cells may not be determined by these cell surface molecules. It is now known that cancer cells need to be resistant against the death receptor pathways by modulating the expression of proapoptotic and antiapoptotic regulators [77].

Nonetheless, it has been suggested that various ligands for NK-cell-activating receptors are involved in the sensitization of CSCs to NK cells. Colon CSCs displayed reduced MHC-I expression and upregulation of CSC markers (CD133, CD44, Lgr5, and CD166) and ligands for the NK-cell-activating receptors, Nkp30 and Nkp44. As a result, they were more sensitive to allologic NK-cell-mediated cell lysis compared with non-CSCs [78]. Ovarian CD24+ CSCs also exhibited downregulation of MHC-I molecules and upregulation of NKG2D ligands compared with non-CSCs [79]. Similarly, ovarian CSCs were more susceptible to NK-cell-mediated lysis compared with non-CSCs [71]. In patient-derived melanoma cell lines, it was found that a subpopulation of C–C motif chemokine receptor 7 (CCR7)-positive cells exhibited certain CSC characteristics, including sphere formation, tumorigenesis, metastatic potential, and CSC marker expression [79]. These CCR7-positive melanoma CSCs also exhibited reduced MHC-I molecule and CD155/PVR expression, but upregulation of ligands for Nkp30 and Nkp46 [79]. Consistently, allologic NK cells more efficiently lysed CCR7-positive CSCs compared with non-CSCs, although CCR7-positive CSCs also displayed higher expression of the two immune checkpoint ligands PD-L1 and galectin-9 [79]. As a result of Nkp30 ligand expression (Nkp46, DNAM-1, and NKG2D), medulloblastoma cells were sensitive to NK-cell-mediated cell lysis, regardless of their CD133 expression status [80]. Further investigation on the expression of NK-cell-activating receptor ligands in CSCs may benefit the development of NK-cell-based immunotherapy.

It was demonstrated that glioblastoma CSCs were more sensitive to NK-cell-mediated cytotoxicity compared with non-CSCs because of the higher expression of NKG2D ligands including UL16 binding protein 1 (ULBP1) and ULBP3 [81]. In contrast, another report [82] showed that glioblastoma CSCs were resistant to NK cells. However, when NK cells were activated by lectin and IL-2, these CSCs were more efficiently killed by the NK cells compared with non-CSCs [82]. Primary cancer cells isolated from nine patients with glioblastoma expressed neuronal stem cell markers and exhibited a multi-lineage differentiation potential when cultured in a stem cell medium [83]. Glioblastoma CSCs derived from all patients expressed HLA-I molecules (A, B, C) and HLA-E, which suppressed the activation and function of NK cells through killer immunoglobulin-like receptors (KIRs) and the NKG2A receptor,
respectively [83,84]. However, these CSCs concomitantly expressed DNAM-1 ligands, CD155/PVR and CD112/Nectin-2, and showed low, but detectable, expression of one of the NKG2D ligands (MICa/MICb and ULBPs) [83]. Consistently, allogenic and autologous NK cells activated by IL-2 or IL-15 efficiently lysed these glioma CSCs [83].

As described in this section, NK-cell-based immunotherapy is expected to efficiently eliminate CSCs [85]. These NK cells need to be activated by cytokines to exhibit complete NK activity. Inevitably, cytokine-induced killer cells (CIKs) attract attention for immune cell therapy targeting CSCs. CIKs are induced from peripheral blood mononuclear cells following treatment with specific cytokines, such as IL-2 and IFNγ [86]. Accordingly, CIKs are a mixture of CD3⁺/CD56⁺ NK cells, CD3⁺/CD56⁻ T cells, and CD3⁻/CD56⁻ bona fide CIKs [86]. CD3⁺/CD56⁺ CIKs express the NK-activating receptor, NKG2D, and exhibit MHC-unrestricted cytotoxicity against a wide variety of malignant cells, possibly through NKG2D, but not toward normal cells [86]. CIKs were shown to kill osteosarcoma CSCs without enriching CSCs [87]. CIKs were also shown to directly lyse nasopharyngeal CSCs in an NKG2D-dependent manner and prolong survival in patients with nasopharyngeal carcinoma treated with gemcitabine and cisplatin [88,89]. Moreover, CIKs combined with DCs also demonstrated significant therapeutic effects on patients with metastatic breast cancer, advanced colorectal cancer, and metastatic renal cell carcinoma [90–92]. CIK-mediated cytotoxicity against CSCs was also experimentally demonstrated in lymphoma [93], melanoma [94,95], and hepatocellular carcinoma [96].

3.5. NK-cell-resistant phenotype of CSCs

As described above, many CSCs exhibit downregulation of MHC-I molecules and upregulation of NK-cell-activating receptor ligands. They are susceptible to NK-cell-mediated cell lysis and, in some cases, more compared with non-CSCs. However, the fact that CSCs, not non-CSCs, can initiate tumor formation in NOD/SCID mice in which NK activity is low but detectable [16] indicates that tumor-initiating cells have an immune evasion ability, in particular, from NK cells. Hereafter, we present studies demonstrating the ability of CSCs to evade NK-cell surveillance.

It has been shown that adult T-cell leukemia downregulates NKG2D ligands, concomitant with increased expression of CD44 and the EMT marker, vimentin, and acquires an apoptosis-resistant phenotype [97]. Glioblastoma CSCs also downregulate NK-cell-activating receptor ligands, including MICa/MICb and ULBPs, and are resistant to NK-cell-mediated cytotoxicity [41]. Downregulation of NK-cell-activating receptor ligands was also observed in colon cancer cells with activated STAT3, a transcription factor that regulates colon CSCs [98,99]. STAT3 suppression in colon cancer cells induced MICA expression, which enhanced NK-cell activation [99], suggesting that STAT3 confers not only CSC properties but also NK-cell resistance.

CSCs may upregulate MHC-I molecules to acquire NK-cell resistance. It was shown that EPCAM⁺/CD45⁺ ascitic cells in patients with epithelial ovarian carcinoma possessed CSC properties, including drug resistance, high metastatic potential, and high CSC marker expression [100]. These cells also upregulated MHC-I molecules and exhibited resistance to NK-cell-mediated cell lysis by suppressing the activation of CD3⁺/CD56⁺ NK cells, whereas EPCAM single-positive cells were efficiently lysed by NK cells [100]. Another study reported that spheroid cells derived from a renal carcinoma cell line exhibited higher tumorigenicity and increased expression of stemness factors, including OCT4, NANOG, and BMI, compared with monolayer cells [101]. In these spheroid cells, MHC-I molecules were also significantly expressed, whereas NK-cell-activating receptor ligands were downregulated compared with

Fig. 2. Pathways for the recognition and killing of cancer cells by NK cells. Details are discussed in the main text.
monolayer cells. This suggests that these renal CSCs are less sensitive to NK-cell-mediated cell lysis [101].

HLA-G and HLA-E are nonclassical MHC-I molecules that may be involved in the underlying immune evasion mechanism of cancer cells from NK-cell surveillance [84,102,103]. KIR2DL4 and NKGA2 on the surface of NK cells represent HLA-G- and HLA-E-specific receptors, respectively (Fig. 2), and provide an inhibitory signal to NK cells upon ligand binding [104]. In colorectal carcinoma tissues, the expression of HLA-G, HLA-E, and CSC markers was significantly higher compared with noncancerous colorectal tissues [105]. This suggests that HLA-G and HLA-E are involved in immune evasion of colorectal CSCs from NK-cell surveillance. Another study demonstrated that HLA-E was expressed in CSCs from patient-derived glioblastoma cell lines, although the expression levels were different among individual cell lines [106]. In contrast, under differentiation culture conditions, HLA-E expression was downregulated in some glioblastoma cell lines [106]. Consistently, the differentiated glioblastoma cells exhibited increased sensitivity to NK-cell-mediated cell lysis. Knockdown of HLA-E sensitized the glioblastoma cells to NK cells regardless of CD133 status [106], suggesting that HLA-E contributes to immune evasion in glioblastoma. Notably, stimulation with IFNγ induced HLA-E expression in all glioblastoma CSCs [106]. However, it has also been suggested that IFNγ enhances the immunogenicity of CSCs by upregulating MHC-I molecules [48]. Therefore, these results suggest that HLA-E is an important factor for glioblastoma CSCs to survive immune selection. Interestingly, HLA-G regulates the cell surface localization of HLA-E and thereby indirectly inhibits NK-cell activation [107]. HLA-G also has additional effects against Dcs. CD105-positive renal CSCs exhibited increased expression of HLA-G, which was delivered to Dcs by extracellular vesicles (EVs) [108]. Consistently, the EVs secreted by these CSCs inhibited the maturation of Dcs more significantly compared with those secreted by CD105-negative non-CSCs [108]. In contrast to human HLA-G, the murine homolog, Qa-2, has been reported to be an immunogenic molecule and contribute to tumor rejection [109]. In murine CD44+/CD24− breast CSCs, Qa-2 expression was abrogated, possibly by the Src signaling pathway, whereas Src inhibitor treatment suppressed the CSC properties of breast cancer cells and restored Qa-2 expression [110]. Moreover, Qa-2 overexpression suppressed breast cancer growth and metastasis [110]. Therefore, caution is needed when interpreting the Qa-2 and HLA-G data obtained from mouse studies.

3.6. Inhibition of cytolytic granules by CSCs

Upon recognition of target cells, CTL and NK cells release cytolytic granules that contain perforin and granzymes (Fig. 2). Perforin oligomerizes and forms pores in the membranes of target cells. At high concentrations, perforin induces necrosis because of a loss of membrane integrity, whereas perforin at low concentrations induces apoptosis in a granzyme-dependent manner, which are delivered to the cytosol through perforin-formed pores or by perforin-triggered endocytosis [111]. Granzymes are serine proteases that activate pro-caspases and cleave various nuclear and mitochondrial proteins [112]. CSCs deploy several strategies to inhibit these cytolytic molecules. In spheroids of an estrogen receptor-α-positive breast cancer cell line, protease inhibitor 9 (PI9), a potent granzyme B inhibitor, was shown to be upregulated [113]. Estrogen enhanced spheroid formation and increased PI9 expression in the cells [113]. These results suggest that breast CSCs acquire resistance to CTL- and NK-cell-mediated cell lysis by PI9.

Serglycin, a proteoglycan with high affinity for granzyme B, contributes to the stabilization of granzyme B in lytic granules and delivery to target cells. Internalization of granzyme B into target cells requires an electrostatic exchange from serglycin to cell surface proteoglycans [114]. Mast cells infiltrated into glioma tissues induced serglycin expression, as well as CD44 and ZEB1, CSC and EMT markers, respectively [115]. Moreover, serglycin expression in glioma tissues was significantly associated with poor prognosis [115]. These results suggest that mast cell-induced serglycin contributes to the immune evasion of glioma CSCs from CTL and NK cells. However, multiple functions of serglycin [116] suggest that other mechanisms may also be involved in the malignant function of serglycin.

Watanabe et al. found that calreticulin was upregulated on the cell surface of pancreatic CSCs and that the expression of calreticulin was significantly associated with poor prognosis in patients with pancreatic cancer [117]. Calreticulin is a chaperone protein present in cytolytic granules of CTL and NK cells to prevent the unnecessary activation of perforin [118,119]. Accordingly, pancreatic CSCs may escape perforin-mediated cytosis by expressing calreticulin. However, calreticulin also appears on the cell surface of apoptotic cells by binding to phosphatidylserine and promotes phagocytosis as an “eat-me signal” [120,121]. Therefore, further studies are necessary to clarify the precise role of calcineurin in CSC.

3.7. Humoral factors modulating immune response to CSCs

A number of humoral factors are involved in the regulation of immune response, which is also the case with immune evasion by CSCs. Such immunomodulatory factors are secreted not only by CSCs but also by tumor-associated noncancerous cells. In this section, we nonetheless summarized the factors that are secreted by CSCs or directly influence the immune evasion property of CSCs.

TGFβ is a multifunctional cytokine that induces CSCs and immune suppression, as well as EMT [122,123]. CD44+/CD24− breast CSCs were found to secrete a higher level of TGFβ compared with non-CSCs. In these CSCs, activation of the TGFβ signaling pathway was observed [124,125]. Consequently, TGFβ induced an EMT phenotype and enhanced stemness in not only breast cancer cells but also in normal mammary epithelial cells [126–128]. In contrast, metformin suppressed the TGFβ-induced EMT phenotype, self-renewal, and proliferation of trastuzumab-resistant breast CSCs [127,128]. In breast CSCs, the Sca-1 stem cell marker was suggested to be involved in TGFβ signaling [129]. It was shown that the human Sca-1 homologs Ly6K and Ly6E play an important role in TGFβ-induced SMAD2/SMAD3 activation, induced immune evasion, and eventually were associated with poor prognosis in patients with breast cancer [130]. In glioma CSCs, another CSC marker, CD133, was suggested to be involved in TGFβ production since CD133+ glioma CSCs exhibited higher expression of TGFβ compared with CD133− glioma CSCs [129]. Cytomegalovirus (CMV), with which glioma cells are frequently infected, is also suggested to be involved in TGFβ production in glioma. Interestingly, CMV-infected glioma CSCs secreted CMV IL-10 encoded by the CMV genome, induced immunosuppressive M2 macrophages, and enhanced the production of TGFβ by monocytes [131]. The CSC- or monocyte-induced TGFβ, in turn, downregulated NKGD and MHC-II molecules in glioblastoma cells [132,133]. In addition, TGFβ secreted from glioblastoma CSCs induced the differentiation of Tregs from naïve T cells [42], enabling the escape of glioblastoma CSCs from immune recognition. Meanwhile, retinoic acid-inducible gene 1 (RIG-I) exhibits suppressive effects on virus infection and CSC induction. Consistently, RIG-I knockout increased CSC marker expression in hepatocellular carcinoma [134]. These hepatic CSCs secreted TGFβ to enhance hepatic CSC properties through the SMAD2/3/AKT pathway in an autocrine manner [134]. TGFβ further inhibited the differentiation and maturation of Dcs and recruited immature Dcs with decreased expression of costimulatory molecules to
hepatocellular carcinoma tissues [134]. Another mechanism involving urokinase-type plasminogen activator receptor (uPAR) was proposed for TGFβ production in CSCs. In breast cancer, uPAR increased the CD44+/CD24- CSC population with upregulation of a breast CSC marker, integrin α6β4, and enhanced tumor initiation [135]. The expression of uPAR was found in breast cancer, pancreatic cancer, and glioblastoma and was associated with the secretion of TGFβ and IL-4 [136]. These immunosuppressive cytokines induced the differentiation of macrophages into an arginase 1-positive M2 phenotype and thereby created an immunosuppressive tumor microenvironment [136]. These results suggest that TGFβ is not only a direct target of cancer therapy but also enhances tumor immunity. Other TGFβ family members, such as activin A and nodal, secreted by CSCs in pancreatic adenocarcinoma promoted cathelicidin-18 secretion from TAMs [137]. Cathelicidin-18, in turn, enhanced CSC function by binding to the formyl peptide receptor 2 and P2X purinoreceptor 7 purinergic receptor [137]. The fact that TGFβ and nodal/activins share SMAD2/SMAD3 as downstream effectors renders these signaling molecules attractive targets for cancer therapy. IL-6 is a pleiotropic cytokine that also plays an important role in the induction of CSCs. In lung cancer, IL-6 upregulated the expression of DNA methyltransferase (DNMT) 1 through JAK2/STAT3 signaling. This subsequently silenced transcription of cell cycle regulatory factors and thereby induced lung CSCs [138]. IL-6 secreted by TAMs was shown to induce and maintain CSC properties through STAT3 in breast cancer [139]. CEBPα also mediated IL-6- and hypoxia-induced breast CSCs by activating the NOTCH1 pathway [140]. In prostate cancer, IL-6 induced CD44+ CSCs in an autocrine manner [141]. IL-6 and CD44 expression was significantly associated with tumor malignancy and poor prognosis [141]. IL-6 also upregulated PD-L1 expression in CD44+ CSCs and recruited MDSCs to tumors and suppressed tumor infiltration of T cells [141]. Inhibition of the IL-6/STAT3 pathway resulted in the downregulation of PD-L1 and CD44 expression, decreased CSC properties, and suppressed tumor growth [141]. STAT3 is a transcription factor required for the maintenance of pluripotency in stem cells and plays an important role in the maintenance and induction of CSCs in both IL-6-dependent and IL-6-independent manners. In bladder CSCs, histone 3 K9 trimethylation by KMT1A upregulated STAT3 expression in an IL-6-independent manner by suppressing GATA3, which suppressed STAT3 transcription [142]. Consistently, deletion of the KMT1A gene decreased CSC properties in bladder cancer [142]. CD44+ CSCs of HNSCC exhibited constitutive activation of STAT3, which upregulated PD-L1 expression [143]. These results suggest that IL-6- or STAT3-targeting therapy will benefit patients with malignant tumors.

CCL20 is also implicated in the immune evasion of CSCs. Breast cancer cells induced CSC properties by secreting CCL20, which activated NFκB via PKCζ and p38MAPK [144]. NFκB, in turn, induced CCL20 transcription by forming a positive feedback loop [144]. CCL20 and its receptor, the CCR6 axis, may recruit Tregs to tumors to suppress tumor immunity (Table 1) [146]. PD-L1 expression has been frequently observed in tumor cells. Upon binding to PD-1 on immune cells, PD-L1 suppresses their effector functions, and induces their exhaustion [150].

PD-L1 was found to be expressed in patient-derived Lgr5+ gastric CSCs, and its expression in gastric CSCs was associated with tumor growth [151]. In breast cancer and malignant mesothelioma, PD-L1 expression was positively correlated with CSC marker expression [152,153]. Epithelial CSCs derived from the ascites of patients with bladder cancer also exhibited PD-L1 expression as well as E-cadherin, CD24, and VEGFR2, and had more potent tumorigenicity compared with mesenchymal CSCs that exhibited constitutively active TGFβ signaling [154]. An ALDH isozyme, ALDH3A1, was shown to increase CSC properties, EMT, and the expression of pro-inflammatory factors in non-small cell lung cancer and melanoma and was significantly correlated with PD-L1 expression [155]. Moreover, ALDH3A1 overexpression also upregulated PD-L1 expression, which inhibited the proliferation of peripheral immune cells [155]. It was demonstrated that the population of CD44+/CD133+ CSCs was higher in PD-L1high pancreatic tumors compared with PD-L1low tumors [156]. However, higher infiltration of CD8+ T cells was associated with a favorable prognosis for patients even with PD-L1high pancreatic tumors [156]. These observations suggest that CSCs clinically correlate with PD-L1 expression and rationalize the activation of T cells by immunomodulatory checkpoint inhibitors to overcome the PD-L1-mediated immune checkpoint pathway.

It has been suggested that several factors are involved in PD-L1 expression in CSCs, although further studies are necessary. In breast cancer cells, it was determined that Ly6K/E were required for not only TGFβ-induced SMAD activation but also IL-4-induced PD-L1 expression [130]. Consistently, PD-L1 expression was significantly correlated with Ly6K/E [130]. Notably, proteasome activator subunit 3 (PSME3) was suggested to be involved in breast CSCs [157]. PSME3 overexpression upregulated PD-L1 expression, as well as EMT and CSC markers, leading to the suppression of chemotaxis of CD8+ T cells and the induction of T-cell apoptosis [157]. Conversely, PSME3 knockdown downregulated PD-L1 expression, promoted CD8+ T-cell activation, and suppressed tumor growth [157]. On the other hand, in ALDH1α−-radiosensitive CSCs of oral squamous cell carcinoma, PD-L1 and DNMT3B expression was positively correlated and associated with increased MDSC infiltration into tumors [158]. Interestingly, pharmacological inhibition of DNMT3B downregulated PD-L1 expression, impaired the radiosensitivity of ALDH1α− cells, and suppressed MDSC infiltration [158]. In CD133+/CD44+ colorectal CSCs and drug-resistant colorectal cancer cells, increased PD-L1 expression was observed [159]. Moreover, PD-L1 increased CSC properties and expanded the CSC population in colorectal cancer cells through the PI3K/AKT and MEK/ERK pathways that were activated by direct interaction of PD-L1 with HMGAI [159]. Another report showed that the AT-rich interaction domain-containing protein 3B (ARID3B) recruited lysine-specific demethylase 4C (KDM4C) to chromatin in colorectal cancer, which subsequently induced the expression of NOTCH-target genes, intestinal stem genes, and PD-L1 [160]. Conversely, a KDM4C inhibitor decreased ARID3B-induced CSC properties and PD-L1 expression [160]. Insulin induced PD-L1 expression through the PI3K/AKT/mTOR pathway in colon CSCs [161]. Interestingly, EGF promoted the transfer of PD-L1 protein to the cell membrane, although it did not affect PD-L1 expression in colon CSCs [161]. On the contrary, in AML, STAT3 was also suggested to play a role in PD-L1 expression since STAT3 knockdown downregulated PD-L1 expression [162]. Consistently, STAT3 knockdown in combination with toll-like receptor 9 activation resulted in the activation of tumor immunity [162]. In thyroid CSCs, acetylcholine secreted by tumor-invading neurons also increased CSC properties and PD-L1 expression through the CD133/PI3K/AKT pathway, whereas an
acetylcholine receptor antagonist, 4-DAMP, suppressed CSC properties and PD-L1 expression [163]. These results suggest that the mechanisms underlying PD-L1 expression in CSCs represent novel targets to improve the therapeutic efficacy of immune checkpoint inhibitors.

3.8.2. IFNs

IFNs are potent antiviral and antineoplastic cytokines. However, in a mouse model of chronic virus infection, the blockade of the type-I IFN pathway downregulated the immunosuppressive factors IL-10 and PD-L1 and resulted in a substantial reduction in viral titers in the infected animals [164,165]. This dual role of IFNs (immunostimulative or immunosuppressive) may, in part, depend on a period of IFN exposure time (acute phase or chronic phase) [164,165]. In tumors, it was shown that PD-L1 expression was induced by IFNγ secreted from activated T and NK cells in a negative feedback manner [166]. Moreover, IFNγ also induced PD-L1 expression in CD44+ CSCs of HNSCC, possibly through STAT1 [143]. In melanoma, IFNγ also upregulated the expression of CD271/NGFR and PD-L1 and thereby increased CSC properties via NGF stimulation [167]. Interestingly, avelinab is a fully humanized anti-PD-L1 IgG1 capable of inducing ADCC in addition to inhibiting the PD-L1/CD80 pathway [168]. The PD-L1 expression in chondoma cells was upregulated by coculture with CDB+ T cells, leading to further enhancement of avelinab-induced ADCC [168]. Therefore, the clarification of the precise mechanisms of IFNγ-induced PD-L1 expression in CSCs may provide further improvement in PD-1/-PD-L1-targeted cancer therapy.

IFNs are also involved in CSC induction and maintenance [167]. In methylcholanthrene-induced sarcoma in mice, treatment with anti-PD-L1 or anti-CTLA-4 antibodies expanded the Sca-1+/CD90+ CSC population, suggesting that an alternative immune evasion mechanism may be employed by the CSCs [169]. IFNγ is involved in the expansion of the Sca-1+/CD90+ CSC population in methylcholanthrene-induced sarcoma because IFNγ blockade decreased its expansion [169]. On the other hand, IFNα upregulated the expression of CSC markers and induced the EMT phenotype in pancreatic adenocarcinoma [170]. Moreover, IFNβ secreted from pancreatic adenocarcinoma cells induced TAMs to produce IFN-stimulated gene 15, which subsequently promoted the induction of pancreatic adenocarcinoma CSCs [171]. The underlying mechanism of IFNβ-induced CSC properties may be dependent, at least in part, on its concentration. Thus, IFNβ at low concentrations induced CSC properties in non-small cell lung cancer through the ICAM1/P3K/AKT/NOTCH1 pathway, whereas it induced apoptosis through the JAK1/STAT1/caspase pathway at high concentrations [172].

IFNs inhibit the tumor-initiating property and expansion of CSCs [173], and the response to IFNs was suppressed in CSCs to resist the anti-neoplastic effects. The underlying mechanisms remain to be investigated; however, they involve the suppression of autophagy [174], cyclooxygenase 2, and its product, prostaglandin E2 [175], as well as the downregulation of LCOR [176] and IFN signaling molecules [173,177]. Therefore, the two-edged functions of IFNs must be considered in IFN-based cancer therapy.

3.8.3. CD200

CD200 provides a negative signal to CD200R-expressing macrophages and DCs to induce immune tolerance [178]. The CD200/CD200R axis may be involved in immune evasion of CSCs by suppressing T-cell activation and Th1 cytokine production and inducing regulatory DCs and Tregs [179]. For example, CD200 was coexpressed with CSC markers in various types of cancers [180–182]. The CD200 expression in leukemia predicted poor prognosis in multiple myeloma and AML [179,183]. In B cell lymphoma, the inhibition of CD200 by a neutralizing antibody or siRNA increased the CTL response and the expression of inflammatory cytokines (IFNγ and TNFα), resulting in lymphoma cell death [184]. In HNSCC, CD200 overexpression induced the expression of BM11 and sonic hedgehog, both of which are involved in the maintenance of CSCs [182]. Moreover, CD200 promoted chemo- and radioresistance of squamous cell carcinoma in vivo [182]. Therefore, CD200 may also be involved in the induction of CSCs.

3.8.4. Other immunosuppressive molecules

Galectin-3, a β-galactoside-binding protein of the lectin family, impairs tumor immunity through several mechanisms that remain to be defined [185]. Lung CSCs were shown to express galectin-3, which was significantly associated with CD133 and β-catenin in lung cancer tissues [186]. Consistently, the overexpression of galectin-3 induced CSC properties by activating β-catenin, which promoted the transcription of stemness-related genes [186]. Galectin-3 also increased CSC properties of prostate cancer and endowed CSCs with immune evasion and metastatic properties [187].

Inhibitory leukocyte immunoglobulin-like receptors (IILRs) on immune cells receive a negative signal from MHC-I molecules and other unidentified ligands [188]. LILRB2 and LILRB3 were shown to be expressed in AML and lung cancer and promoted their tumor-growth and stemness [189,190]. Fat mass- and obesity-associated protein (FTO) is an RNA N6-methyladenosine demethylase that expanded the CSC population of various cancers through LILRB4 upregulation [191]. Conversely, knockdown of FTO or treatment with the FTO-specific demethylase inhibitors, CS1 and CS2, down-regulated LILRB4 expression in leukemia cells, resulting in the activation of the tumor immune response and the subsequent suppression of tumor growth [191].

As summarized in Table 1, it is anticipated that CSCs employ other molecules to create an immunosuppressive tumor microenvironment. Investigation of these mechanisms will assist in the development of novel immunotherapeutics.

3.9. Immune modulation by CSC-intrinsic functions

Compared with non-CSCs, CSCs retain a more intact DNA repair function that reduces immunogenicity [192]. As such, CSCs employ their intrinsic functions to evade tumor immunity. Antiapoptosis proteins, such as BCL2, BCLXL, and P33K, may protect CSCs from T-cell- and NK-cell-induced apoptosis [193,194]. Apoptosis inhibitor-5, which was upregulated in CD44high CSCs, induced CSC properties and suppressed the antigen-specific T-cell response in a fibroblast growth factor 2-dependent manner [195,196]. STAT3 in CSCs may also be involved in the expression of galectin-3 [42], suppression of phagocytosis, and secretion of IL-10 from TAMs [197]. Likewise, several CSC-regulating molecules, including NANOGL [198,199], SOX2 [200], DCLK1 [201,202], and CD73 [203,204], were shown to be involved in immune evasion by CSCs.

4. Conclusions

Tumor immunity has opened new avenues for cancer therapy; however, further improvements in efficacy are needed. The mechanisms through which cancer cells evade the immune response must be precisely clarified. In this article, we summarized studies that clarified how CSCs suppress tumor immunity and create an immunosuppressive milieu, which are expected to provide important clues for the development of novel tumor immunotherapies. Moreover, in agreement with the fact that cancer cells must evade tumor immunity to form tumors, our literature survey
suggests that the immune evasion property, rather than the tumor-initiating property, is a more fundamental feature of CSCs. In the present article, the underlying mechanisms of immunosuppressive properties of CSCs were summarized as: 1) the impairment of antigen processing and presentation machinery; 2) the downregulation of tumor-associated antigens; 3) the downregulation of NK-cell-activating ligands and upregulation of NK-cell inhibitory ligands; 4) the inhibition of cytolytic granules released from CTL and NK cells; 5) the secretion of immune suppressive humoral factors, such as TGFβ, IL-6, and CCL20; 6) the upregulation of immunosuppressive molecules including immune checkpoint molecules; and, 7) others. In order to suppress tumor immunity, CSCs employ these mechanisms in combination. Therefore, CSCs are expected to be a more effective target to restore tumor immunity than targeting each of the mechanisms individually. However, because of space limitations, we have reluctantly omitted immune modulatory functions of noncancerous cells in the tumor microenvironment, which also endow CSCs with the immune evasion property and vice versa. Moreover, we would also like to emphasize that additional factors, including hypoxia, cancer-specific metabolites, and EMT, remain to be discussed but must be considered for the development of immunotherapies. We expect that an integrated understanding of these factors will lead to significant improvements of current cancer immunotherapies.

Declaration of competing interest

G.S. holds more than 5% of the total shares of KanonCure Inc., and receives compensation as a member of KanonCure Inc. The other author has no competing interests.

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