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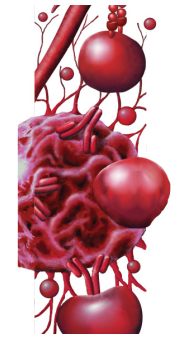

Immunotherapy for breast cancer: is it feasible?

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Immunotherapy invited leading experts in the field to share their thoughts on two key immunotherapeutic strategies in the field of breast cancer research, vaccines and checkpoint inhibitors.

Interviewed by Ellen Clarke (Commissioning Editor, Future Science Group).

Historically breast cancer has been considered immunologically silent. Patients have had limited access to the types of immunotherapy available to melanoma and lung cancer patients, but this could all be set to change as recent preclinical and clinical studies have highlighted the potential of immunotherapy for breast cancer. Breast cancer is now one of the major cancer types for which new immune-based treatments are being developed.

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Q Currently what are the treatment options available to breast cancer patients?

Invasive breast cancer is managed in a multidisciplinary fashion using a combination of surgery, radiation therapy and systemic therapy. Systemic therapies include endocrine therapy, chemotherapy, monoclonal antibody therapy (trastuzumab, perutuzmab, trastuzumab-emtansine (TD-M1) and small molecularly targeted therapies (lapatinib, everolimus and palbociclib). For early stage invasive breast cancer, the goal of therapy is cure. Surgery and radiation therapy are used for local control, where surgical excision removes the bulk of the tumor and radiation is given to treat local microscopic disease that could be left behind. For early stage invasive breast cancer, systemic therapy is used to eradicate disseminated micro-metastatic disease that could ultimately result in relapse and incurable disease. The intensity and type(s) of systemic therapy used with curative intent is matched to the risk of relapse associated with the primary tumor, and to its histologic profile (whether the tumor expresses the estrogen receptor (ER), progesterone receptor (PR) and/or the HER-2). The intensity of systemic therapy is increased in relative proportion to the estimated risk of relapse, with more aggressive chemotherapies generally used for higher risk primary tumors. Chemotherapy is integrated with other systemic therapies based on the histologic profile of the breast tumor. In addition to chemotherapies, endocrine therapy (tamoxifen and/or aromatase inhibitors) is used for early breast cancers that express the ER and/or PR, and HER-2-directed monoclonal antibody therapy (trastuzumab and pertuzumab) for early breast tumors that overexpress HER-2.

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Once distant relapse has occurred, breast cancer is incurable. The primary treatment for unresectable, locally recurrent and frankly metastatic breast cancer is systemic therapy. Because the disease is incurable, the intent of therapy in the metastatic setting is palliative. In this case, therapy is chosen with the goal of both maximizing disease control and minimizing the side effects of therapy so as to maximize quality of life. As with treatment for early stage breast cancer, the choice of systemic therapy is dictated by the biologic profile of the tumor. It is standard to biopsy at first relapse to be sure the biologic profile has not changed, and to tailor therapy to the biologic profile of the relapsed tumor if it has changed. Metastatic breast cancers that express ER and/or PR are managed with endocrine therapies (tamoxifen, aromatase inhibitors, fulvestrant), and metastatic breast cancers that overexpress HER-2 are managed with HER-2-directed therapies (trastuzumab, perutuzmab, trastuzumab-emtansine (TD-M1) and lapatinib). Newer molecularly targeted therapies such as everolimus and palbociclib have shown benefit for metastatic breast tumors that express the ER and/or PR too. Triple negative breast cancer (TNBC) fails to express the ER, PR and HER-2, and there are currently no targeted therapy options available for patients with early or metastatic TNBC in the USA. Chemotherapy remains the only treatment option available for these patients. Surgery and radiation therapy may be used for the palliation of local symptoms.

Q What are the potential advantages of immunotherapy over conventional breast cancer treatments?

Immunotherapy has distinct advantages relative to conventional therapies for breast cancer. One major advantage of immunotherapy is a favorable side effect profile. By contrast, one disadvantage of most conventional breast cancer therapies is lack of specificity, with limiting side effects that result from collateral damage to nonmalignant host tissues. These include nausea and vomiting, hair loss and low blood counts. These side effects are classically associated with most chemotherapy regimens, and neuropathy related to the use of particular cytotoxic drugs poses an added burden for some patients. Additional undesirable side effects of conventional therapy include cardiac dysfunction associated with HER-2-directed therapy, and menopausal symptoms associated with endocrine therapy. By contrast, immunotherapies, particularly vaccines, are generally quite well tolerated. The side effect profile of immune-based cancer therapies, particularly immune checkpoint antagonists, includes autoimmune side effects, which can be serious if not detected and treated early. Importantly, immune-based therapies typically do not cause the types of chemotherapy-related side effects that patients dread (noted above).

A second major advantage of immunotherapy is its ability to circumvent primary or treatment-emergent drug resistance by its unique mechanism of action. Standard cancer therapies frequently fail either due to intrinsic resistance to therapy, or to the emergence of drug-resistant tumor cell clones that result in disease relapse and/or progression. By contrast, cancer immunotherapies are exquisitely specific for and target multiple distinct molecular markers of the tumor, decreasing the likelihood of therapeutic escape and complementing the activities of standard breast cancer therapies. Finally, the greatest advantage of cancer immunotherapies relative to conventional therapies is durability even in the absence of ongoing treatment. A cardinal feature of the immune system is the memory response, where immunity is primed to become activated at the first sign of disease activity. Immunologic memory underlies the durable immune responses observed with immune checkpoint blockade, and the overall survival benefit observed with some vaccine strategies. An established immunologic memory response may obviate the need for continuous therapy as required for conventional systemic therapies. Most importantly, immunologic memory sets the stage for highly effective cancer prevention strategies.

Q Please can you highlight the most promising vaccine candidates for breast cancer, and describe their mechanisms of action?

The most promising vaccine platforms for breast cancer therapy engage the power of dendritic cells to cross prime a coordinated immune response specific for a variety of tumor antigens, and establish a pool of memory T cells for lasting protection from tumor growth. Two strategies come immediately to mind. First, dendritic cells can be isolated from the patient, activated, loaded with tumor-specific antigens and then re-infused into the patient to prime and expand tumor-specific T cells. Second, dendritic cells may be recruited and activated by a vaccine *in situ*. We have used breast tumor cells genetically engineered to secrete the cytokine GM-CSF to cross prime both $CD4^+$ and $CD8^+$ T cells specific for tumor antigens delivered by the vaccinating tumor cells. A combination of dendritic cell-based vaccine or DNA vaccines with GMCSF adjuvant strategies that more effectively engage the innate immune system to optimize immune activation by tumor antigen-specific dendritic cell vaccines are under investigation, with early hints of success in preclinical models and in the clinic.

Q What are the advantages/disadvantages of vaccines over other immunotherapeutic strategies for breast cancer?

Breast tumor vaccines are designed to induce new breast cancer specific T cells, or amplify a pre-existing T-cell response to breast cancer. Other immunotherapeutic strategies-immune checkpoint blockade targeting PD-1, PD-L1, CTLA-4 or TIM-3 among others, low-dose cyclophosphamide or anti-CD25 monoclonal antibodies targeting regulatory T cells, and inhibition of indoleamine 2,3-dioxygenase are designed to alleviate various pathways of immune suppression that keep T-cell responses shut off. Distinct immunotherapeutic strategies – OX-40 and CD137 agonists, for example – help push the T-cell response forward. Still others – VEGF blockade for example – may facilitate T-cell trafficking into the tumor site. These latter immunotherapy strategies require T cells for their therapeutic effect. Tumor vaccines may induce T cells where they did not previously exist, but the efficacy of vaccine-induced T cells may be constrained by active pathways of immune suppression globally or within the tumor microenvironment. An attractive immunotherapy strategy creates and/or amplifies the pool of tumor-specific T cells by effective tumor vaccination or passive transfer (accelerating the immune response), and provides additional signals that promote optimal T-cell activity (co-stimulatory immune modulators or agents that promote T-cell trafficking into the tumor microenvironment) at the tumor site. Regardless, the ultimate goal of cancer immunotherapy is to establish a pool of memory T cells that can control tumor growth and progression in the setting of existing disease, and prevent disease relapse or development in those at high risk of recurrence or first diagnosis of breast cancer. Vaccines have been enormously successful for the prevention of infectious disease. In light of this, their most obvious and powerful role in cancer management may be the prevention of disease in patients at high risk for cancer.

Q Can you highlight any promising results from checkpoint inhibitor trials in breast cancer patients?

Immune checkpoint blockade has been tested in small numbers of breast cancer patients to date. The first report tested tremelimumab, a monoclonal antibody specific for CTLA-4, combined with the aromatase inhibitor exemestane, in patients with advanced ER^+ and/or PR^+ breast cancer. Clinically, the drug combination was relatively well tolerated, and 42% of patients had stable disease for 3 months or more. Treatment was associated with an increase in peripheral $ICOS^+$ T cells, and a significant increase in the $ICOS^+$ effector T cell/$FoxP3^+$ regulatory T-cell ratio. More recently, both the PD-1-specific monoclonal antibody pembrolizumab (MK3475) and the PD-L1-specific monoclonal antibody atezolizumab (MPDL3280A) were reported to have activity in patients with metastatic TNBC. These antibodies have acceptable side effect profiles, and overall response rates of 18–20%. Importantly, the duration of response with PD-1 pathway blockade in TNBC is longer than with standard chemotherapy, with progression-free survival rates at 6 months of 23 and 27% for pembrolizumab and atezolizumab, respectively; some responses were ongoing at the time of report. Both antibodies are now being evaluated in global Phase II and III trials. Notably, a Phase III randomized, double blind trial is testing atezolizumab or placebo in combination with abraxane as first-line therapy for metastatic TNBC. Additionally, a Phase III randomized clinical trial is testing single-agent pembrolizumab versus chemotherapy of physician's choice for metastatic TNBC. Additionally, a Phase II clinical trial is testing single-agent pembrolizumab in distinct populations of metastatic TNBC.

Q What are the limitations of checkpoint inhibitor drugs?

Immune checkpoint blockade is an exciting class of agents for cancer patients, with activity across a broad range of tumor types and durable clinical benefit for most patients who respond. While these immunotherapies transform the cancer experience for those patients who do respond, the fact remains that most patients do not respond. Moreover, the inflammatory side effects associated with these agents can sometimes be life threatening, and must be recognized and managed early in their course. Developing therapeutic strategies that increase the number of patients who can respond to immune checkpoint blockade is critical, and this will be undoubtedly be achieved through combination therapies. Current data suggest that tumors that do not harbor a significant number of T cells or a significant mutational load may be less likely to respond to immune checkpoint blockade, and devising combination strategies to circumvent these limitations to response is critical for increasing the number of patients who can benefit. Furthermore, strategies for dissociating the serious immune-related adverse events associated with immune checkpoint blockade and its antitumor activity will be critical for maximizing the impact of these drugs in the clinic. Finally, at a societal level, the cost of immunotherapy must be considered, and cost–benefit analyses incorporated into late-stage clinical evaluation are essential for determining the proper place of immune checkpoint agents in the management of cancer. It is likely that the unique mecha-

nism of action cancer immunotherapy will render it far more cost-effective than standard cancer therapy.

Q Does immunotherapy have the potential to become a first-line treatment for breast cancer?

Immunotherapy is the next great frontier for breast cancer therapy. It has already shown promise in advanced disease. It is clear that there is place for immunotherapy in the first-line treatment of both metastatic and high-risk early stage breast cancer. One active clinical trial is already evaluating immune checkpoint blockade – atezolizumab, or MPDL3280A – as one component of treatment for metastatic TNBC at first relapse. A major unmet need is for those patients with residual breast cancer after standard neoadjuvant therapy, and immunotherapy is likely to play a major role in the management of these patients.

Q Where do you see the field of breast cancer immunotherapy heading in the next 5 years?

Given emerging data demonstrating the clinical activity of immune checkpoint blockade in metastatic TNBC, immunotherapy has arrived as a potentially viable treatment strategy for breast cancer. It is likely that immune checkpoint blockade will have an important role in the management of locally advanced breast cancer that is treated with systemic neoadjuvant therapy. A variety of other immunotherapies that circumvent immune resistance mechanisms in advanced disease are likely to play a role in the locally advanced and metastatic disease settings. Perhaps the greatest potential for breast cancer immunotherapy lies in decreasing the likelihood of relapse in early stage disease, and in preventing disease in high-risk patients. These advances in immunotherapy truly represent a revolution in cancer therapy, and they will transform the management of breast cancer beyond anything we have seen before. The greatest strength of immunotherapy lies in its ability to prevent disease, and breast cancer prevention is the next great frontier for breast cancer immunotherapy. The next 5 years should see significant efforts applying immune-based strategies for breast cancer prevention.

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Q What are the potential advantages of immunotherapy over conventional treatments?

Surgical intervention has an immediate effect on reducing tumor load. Likewise, chemotherapy and radiation therapy typically induce rapid measurable tumor shrinkage. These interventions may result in enduring beneficial effects in extending overall survival, but all too often the effects of these therapies have limited durability beyond the treatment period. Hormone therapy does not typically induce rapid measureable effects on tumor growth, and the long-term beneficial effects of hormone therapy require extended administration of drugs that block the tumor growth effects of estrogen and progesterone. In all cases, these conventional therapies are accompanied by deleterious side effects that are often debilitating and frequently preclude patient compliance with additional or extended treatments. Immunotherapy has the advantage of inducing long-term immune memory which once established will persist indefinitely to provide a long-lasting immune attack against the tumor. Immunotherapy may require periodic booster vaccinations or intermittent treatments with checkpoint inhibitors for optimized outcomes and may occasionally induce autoimmune sequelae. However, such additional booster interventions and potential autoimmune complications compare favorably to the harsh toxicities and extended side effects of conventional current standards of care and to the morbidity often associated with breast cancer, particularly with the more aggressive forms of breast cancer. The unique elegance of immunotherapy is that once the immune system is effectively induced to attack and destroy the breast tumor, it keeps doing it relentlessly day and night without requiring any frequent, long-term additional interventions. Moreover, this feature of established long-term memory is not restricted to cancer vaccines and appears to occur even following short-term treatment with checkpoint inhibitors that seem capable of inducing effective *de novo* priming to tumor antigens and durable long-lasting immunity with long-term survival.

Q Please can you highlight the most promising vaccine candidates for breast cancer, and describe their mechanisms of action?

There are several proposed vaccines that target the HER2 receptor for EGF known to be overexpressed in a subset of breast tumors. The ongoing Phase III clinical trial headed by Dr Elizabeth Mittendorf at the MD Anderson Cancer Center (TX, USA) involves 11 immunizations with Neuvax™, a DNA vaccine that incorporates an immunogenic HER2 peptide as well as an immune activating cytokine. This trial is designed to prevent recurrence of breast tumors with low-to-intermediate levels of HER2 expression. Another vaccine developed by Dr Brian Czerniecki at the University of Pennsylvania (PA, USA) involves activation of the patient's own autologous dendritic cells (DCs) against peptides derived from HER2. This approach has shown promise in preventing invasion of ductal carcinoma *in situ* (DCIS), a transformed precancerous breast lesion widely believed to be the precursor of many cases of invasive ductal carcinoma. Dr Czerniecki's DC vaccine appears to be selective against estrogen receptor negative/HER2-positive breast tumors. Dr Mary Disis at the University of Washington (WA, USA) has clinical trials in DNA vaccines derived not only from HER2 but also from other self-proteins overexpressed in DCIS and invasive breast cancer including IGFBP2, IGF1R, and a series of breast cancer stem cell antigens. Dr Olivera Finn at the University of Pittsburgh School of Medicine (PA, USA) has long proposed that immunity targeted against MUC1 may provide safe and effective protection and therapy against breast cancer because the heavy native glycosylation of MUC1 presumably protects normal tissues from immune attack whereas the deficient glycosylation of MUC1 in tumors precludes this protection. Dr Joseph Baar at Case Western Reserve School of Medicine in collaboration with Dr Walter Storkus at the University of Pittsburgh School of Medicine have recently initiated vaccination of patients with metastatic TNBC with a DC vaccine that targets peptides derived from tumor-associated blood vessel proteins including among several others, the notch antagonist DLK1. This unique approach is designed to induce a T-cell-mediated response in the tumor microenvironment that compromises the blood supply to the tumor rather than targeting any direct immunity against the tumor itself. Drs Edith Perez and Keith Knutson at the Mayo Clinic (MN, USA) have recently proposed that vaccination against FOLR1 may be effective in preventing recurrence of TNBC, and investigators at the Roswell Park Cancer Center have proposed that vaccination against the cancer testis antigens MAGE-A and NY-ESO-1 may also be effective in treating TNBC. Dr William Gillanders at Washington University has proposed that DNA vaccination against mammaglobin-A (SCGB2A2; secretoglobin, family 2A, member 2) may be effective in preventing breast cancer recurrence due to its overexpression in many human breast tumors, and Dr Songdong Meng at the Chinese Academy of Sciences (Beijing, China) has proposed that vaccination against the heat shock protein 90 kDa beta, member 1 (gp96 or HSP90B1) extracted from human placentas may be effective against breast cancer by inducing immunity against numerous developmentally related peptides expressed in both placenta and breast tumors. Dr Stephen Johnston at Arizona State University (AZ, USA) has proposed that a finite number of nonself neoantigens resulting from frameshift mutations common to many breast tumors can be targeted in a multivalent prophylactic breast cancer vaccine. Finally, we have proposed that safe and effective prevention and therapy against TNBC may be induced by vaccination against α-lactalbumin (LALBA), a protein overexpressed in the majority of human TNBC tumors but 'retired' from expression with age in normal tissues. Clinical trials for our vaccine are planned to begin by the end of 2015 or early 2016.

Q Which stages of the disease are vaccines most effective for?

In our experience with animal models of breast cancer, we have found that the best clinical outcomes occur when the tumor immunity is established early. Indeed, when the tumor has a substantial head start in growing, the induction of immunity has minimal chance to produce any substantive growth inhibition. One can compare the delayed establishment of tumor immunity to giving a world record holder like Usain Bolt a big head start in a sprint race and expecting to catch him and win the race. It is simply not realistic. The induction of an effective immune response involves clonal expansion of high affinity T cells to frequency levels that can produce a clinically relevant immune response. It also involves production of high titers of high affinity antitumor antibodies that may participate effectively in the ultimate demise of the tumor. The completion of this immune process often requires multiple booster vaccinations and typically takes several months to develop and reach maturity. Thus, there seems to be little sense in waiting until the tumor has taken root and has a complete array of dozens of mutations capable of maintaining the adaptive plasticity and immortality of the tumor even in the presence of a powerful vaccine-induced immunity. Considering these issues, it remains perplexing that we still focus predominantly on using tumor vaccines as therapy when we know that vaccines provide

their best impact when used early and pre-emptively to prevent disease. The current paradigm for controlling breast cancer involves waiting for the tumor to manifest and then initiating an offense in the form of surgery, chemotherapy, radiation therapy, hormone therapy, etc. to prevent progression or recurrence of the tumor. Even though preventing recurrence of the breast tumor is often discussed and referred to in terms of disease prevention, it is clearly a treatment and not designed to provide primary pre-emptive immunity against the emergence and growth of newly forming breast tumors. What is urgently needed for optimized control of breast cancer is primary prevention in the form of a prophylactic vaccine that induces immunity in cancer-free and otherwise healthy women, particularly those at high risk for developing breast cancer including previvors with mutations in their BRCA genes. We have proposed that 'retired' self-proteins no longer expressed in normal tissues with age but expressed in emerging tumors may substitute for unavailable viral targets for vaccination and primary immunoprevention of many adult-onset cancers including breast cancer. Our results from extensive preclinical studies provide a rational basis for inducing safe and effective pre-emptive immunity against the emergence and growth of TNBC, the most lethal form of breast cancer and by far the most common form of this disease occurring in women at high genetic risk with mutations in their BRCA genes.

Q How effective are checkpoint inhibitors for hard to treat TNBC cancer tumors?

Many tumors produce PD-L1 that can bind to PD-1 on tumor infiltrating immune cells and transduce a signal that kills the invading immune cells thereby thwarting an effective antitumor immunity. Results from a recent Phase I trial sponsored by Genentech (CA, USA) and led by Dr Leisha Emens at the Johns Hopkins Kimmel Cancer Center (MD, USA) showed that 19% of patients with metastatic TNBC responded to treatment with MPDL3280A, a humanized monoclonal antibody that binds and blocks PD-L1 and thereby allows the tumor-invading immune cells to respond to the tumor. In this way, the drug acts to prevent the TNBC tumors from inhibiting the immune response against itself thereby allowing the patient's own immune system to mount an effective uninhibited antitumor response that inhibits tumor growth. Remarkably similar results were obtained from another recent Phase I trial led by Dr Rita Nanda at the University of Chicago (IL, USA). In this study, the objective response rate was 18.5% in patients treated with pembrolizumab, a checkpoint inhibitor that blocks PD-1. The results of these two Phase I trials are exciting and show great promise for using checkpoint inhibitors against TNBC. However, it is important to recognize that in both studies, less than 20% of evaluable patients responded to treatment with these different checkpoint inhibitors. This significant but modest response rate clearly indicates that there is much room for improvement. Such improvement may occur in ongoing Phase II trials or in future trials involving complementary combination therapies.

Q Do combinations of immunotherapies warrant investigation?

Checkpoint inhibitors that target the CTLA-4 immune inhibitory pathway (e.g., ipilimumab) appear to have their predominant impact on the priming phase of T-cell activation whereas those acting on the PD-1 inhibitory pathway (e.g., pembrolizumab, nivolumab, pidlizumab, MK–3475) or PD-L1 inhibitory pathway (e.g., BMS–936559, MPDL3280A) appear to have their predominant impact on the activity of T cells that are already primed. Thus, it seems reasonable to consider that inhibition of a single inhibitory T-cell pathway may not be sufficient to establish optimized tumor immunity, and that treatment involving both forms of checkpoint inhibitors may provide both enhanced immune priming against the tumor as well as enhancement of any established tumor immunity already in place. The potential synergy that may occur when both pathways are inhibited simultaneously may allow for effective treatment regimens involving lower doses, shorter time courses and diminished toxicities. Combination therapies may also involve co-treatment with a targeted breast cancer vaccine plus ipilimumab during the priming phase of vaccination. Indeed, such combination therapy has recently shown promise in improving overall survival in prostate cancer patients receiving escalating dose of ipilimumab after vaccination with PROSTVAC, a poxvirus-based vaccine against prostate-specific antigen (PSA). Taken one step further, one could consider combining targeted breast cancer vaccination in combination with ipilimumab during the priming phase followed by treatment with an anti-PD-1 or anti-PD-L1 checkpoint inhibitor during the postpriming effector stage of the immune response. In this way, one could sequentially orchestrate an enhanced response to the vaccine in the first phase of combination therapy with one checkpoint inhibitor followed by the induction of an enhanced response to the tumor with another checkpoint inhibitor during a subsequent second phase of treatment. This aggressive combination therapy may be

particularly useful and effective against tumors like TNBC that appear to be only modestly immunogenic and are known to be aggressive and notoriously resistant to currently available treatments.

Q Where do you see the field of breast cancer immunotherapy heading in the next 5 years?

In the next 5 years, we may likely see approval of the first therapeutic breast cancer vaccine and may also see the results of several clinical trials showing efficacy of checkpoint inhibitors in breast cancer treatment, particularly when used in rational combination therapies with each other and with immunogenic breast cancer vaccines. We are already seeing an increase in the number of clinical trials designed to introduce immunotherapies much earlier in the adjuvant setting as soon as possible after surgical intervention so that the activated immune response has the greatest chance to eliminate any residual tumor cells. We may see dramatic advances in the identification of immunogenic breast tumor specific neoantigens that may be highly specific for each tumor and lead to the development of personalized immunotherapies involving tumor-specific customized vaccines. There may also be a breakthrough in the identification of a group of neoantigens common to many breast tumors that could form the basis for developing a multivalent vaccine for treatment and perhaps prophylaxis against defined subtypes of breast cancer. Over the next 5 years, customized immunotherapies will likely become more prominent and successful including active and DC personalized vaccines targeted against individual breast tumor neoantigens or overexpressed self-proteins as well as passively transferred tumor-specific immunity in the form of genetically modified cloned T cells. Finally, clinical trials designed to test safety and efficacy of primary immunoprevention of breast cancer will likely be initiated with the ultimate goal of providing safe and effective immune protection in otherwise cancer-free women, particularly those women at high genetic or familial risk for developing breast cancer.

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Q What are the limitations associated with standard breast cancer treatment?

There is significant heterogeneity in breast cancer even within the subtypes therefore patients develop resistance to current therapies or to progress through multiple lines of established therapies. Currently, there are no validated biomarkers to accurately predict patients that will not respond to specific therapies. Hormone receptor positive tumours do not respond as well to neoadjuvant chemotherapy as triple negative and HER2-positive tumors and the mechanism of this is incompletely understood. Furthermore, adjuvant endocrine therapy provides prolonged disease-free survival; however there are significant differences in recurrence rate and response to therapy between luminal A and luminal B disease. There remain patients that have hormone-positive recurrence even during adjuvant hormone therapy or develop recurrent disease years after initial diagnosis. In metastatic hormone receptor positive disease, patients have to return to chemotherapy after progressing through endocrine options because the tumor develops endocrine resistance. Resistance is also a major limitation in HER2-positive breast cancer, although disease-free survival is much improved after trastuzumab. Furthermore, the hormone receptor positive and hormone receptor negative HER2-positive disease are different disease entities, may have different levels of immune infiltrate and seem to respond to therapy differently therefore these need to be examined separately. Finally, with the addition of adjuvant HER2 targeted therapies, patients are presenting with increased recurrent disease in the brain because it is a privileged site where the targeted therapy cannot infiltrate, therefore studies to determine how to prevent brain recurrence is needed. The highest need for therapy remains in TNBC where there is no targeted therapy. TNBC has also emerged as having five subtypes all of which have different prognoses and response to therapy. More needs to be understood about how different subtypes of TNBC should be treated. Finally, in metastatic triple negative disease the only therapeutic option currently is cytotoxic chemotherapy. TNBC remains the worst prognosis of all the breast cancer subtypes. In pre-invasive ductal carcinoma *in situ* (DCIS), there is a wide range of disease despite all being currently treated as similar. Low-grade DCIS may be able to be followed by active observation whereas high-grade DCIS has a much higher likeliness to progress to invasive breast cancer but currently there are no biomarkers to identify which of these tumors can be safely observed versus need therapy.

Q What are the challenges of developing a personalized breast cancer vaccine?

Breast cancer is typically less genetically unstable than melanoma and lung cancer which may explain why most breast cancers are not effectively recognized and infiltrated by the immune system. Therefore breast cancer also typically has fewer neoantigens to target for personalized tumor-specific neoantigen vaccines. However, many of the overexpressed proteins in breast cancer are conserved between the different breast cancer subtypes. This suggests that conserved tumor-associated antigens may be developed into vaccines that could broadly treat many breast cancer patients and target all of the breast cancer subtypes.

Q Have there been any significant adverse events reported in response to breast cancer vaccines?

No there have not been any significant grade 3 or 4 adverse events currently reported in breast cancer vaccine clinical trials. The most common side effects have been pain and inflammation at the injection sites, transient flu-like symptoms and self-limiting inflammation symptoms. There have also been transient not clinically significant increases in autoimmune serum markers and cytopenias. Most importantly, the significant nonspecific autoimmune side effects such as nephritis, pulmonitis and endocrinopathies seen in other immune therapies have not been seen with breast cancer vaccine therapies.

Q Are there any potential issues that could prevent patients responding to checkpoint inhibitors?

The two issues with breast cancer patients responding to checkpoint inhibitors is that breast cancer overall does not have high levels of immune infiltrate and the immune infiltrates present are typically immunosuppressive. The most common breast cancer subtype is hormone receptor positive breast cancer and this subtype has the lowest lymphocyte infiltrate with only 7% of tumors with greater than 50% lymphocytic infiltrate. Both overexpression of estrogen and treatment with hormone therapy (tamoxifen and raloxifene) have been shown to stimulate a Th2 immune suppressive immune environment. However, even though hormone-positive tumors have not been shown to have prognostic benefit from immune infiltrate, increased FOXP3 immunosuppressive infiltrate predicts worse survival suggesting that the tumor immune environment is important in hormone-positive disease. For hormone receptor positive disease, immune therapy such as vaccines to improve the tumor immune infiltrate and ensure that the immune infiltrate is immune activating (Th1) may then make checkpoint therapy more effective. Unlike hormone receptor positive disease, HER2-positive disease has more immune infiltrate with 11% with lymphocyte predominant (>50% infiltrate). The role of immune infiltrate in HER2-positive breast cancer remains unclear. There is some evidence that increased immune infiltrate has better prognosis in HER2-positive breast cancer but other studies which show no effect. One possible explanation for this discrepancy is that there is some evidence that hormone receptor negative HER2-positive tumors and hormone receptor positive HER2-positive tumors may have different responses to increased immune infiltrate, with the hormone receptor negative, HER2-positive tumors having better prognosis with increased $CD8^+$ T-cell infiltrate. Finally, for the TNBC which does not have high immune infiltrate, vaccination, adoptive T-cell therapy or other immune therapies which increase immune infiltrate to the tumor may allow for better responses to checkpoint inhibitors.

Q Does immunotherapy have the potential to become a first-line treatment for breast cancer?

Yes, immunotherapy has the potential to be first line in both treatment and prevention for breast cancer. However before this can occur, careful evaluation of the immune environment in specific subtypes need to be performed to best understand how to best use immune therapy. Much as has been found in cytotoxic and targeted therapies, no one immune-based therapy will treat all breast cancer subtypes equally. One exception may be in prevention, where the ideal vaccines would target all of the subtypes to activate the immune system and destroy any developing malignancy. The side effects of checkpoint inhibitors may limit their use in prevention, although whether these can be improved with different dosing and treatment schedule remains to be examined. With established tumors, the existing immune environment and stage of disease may determine which immune therapy is most appropriate. For example, in the population of TNBC which has high immune infiltrate, a checkpoint inhibitor may be the only first-line therapy that may be needed. However, for a hormone-receptor positive breast cancer with no immune infiltrate in the tumor a vaccine may be needed to increase immune infiltrate before checkpoint therapy. Finally, there may be a subset of breast cancers which do not respond to immune therapy and require further targeted or cytotoxic chemotherapies, and biomarkers for this subset should be identified so that immunotherapy is not used frontline.

Q Do combinations of immunotherapies warrant investigation?

Yes, a combination of immunotherapies will be important particularly to treat established breast tumors. Furthermore, the role of the immune system with breast cancer therapy is not limited to immune therapies. For example, trastuzumab and other monoclonal antibodies have been shown to be immune therapies, activating antigen-dependent cellular cytotoxicity through natural killer cell recognition of the Fc receptor of the monoclonal antibody. Trastuzumab also can trigger the adaptive immune response, stimulating an adaptive HER2-specific immune response in a subset of HER2 tumors. Cytotoxic chemotherapy can also increase the inflammatory immune environment of the tumor with the tumor cell destruction increasing immune recognition of the tumor and increasing tumor infiltrating lymphocytes. Doxorubicin has been shown to increase cytotoxic $CD8^+$ T cells in breast tumors and paclitaxel has been shown to decrease $CD4^+$ regulatory T cells. Even zolendronic acid has been shown to change the bone immune environment preventing growth and establishment of breast cancer metastases in the bone. Combination immunotherapy can include vaccines with adoptive T-cell therapy and using vaccines or adoptive T-cell therapy with checkpoint inhibitors. However, combining immune therapy with cytotoxic or targeted therapies may further expand the possibilities of immune-mediated options to improve breast cancer therapies.

Q Where do you see the field of breast cancer immunotherapy heading in the next 5 years?

The field of breast cancer immunotherapy will be expanding into both the prevention and the therapeutic setting in the next 5 years. Since clinically the immune system has emerged as important in breast cancer prognosis and development, now the field has to carefully and systematically evaluate the tumor immune environment of the individual breast cancer subtypes to be able to develop rationally designed clinical trials of combination immune therapies to best treat the individual subtypes. Careful evaluation of the tumor immune infiltrate in the breast tumors with the good response to checkpoint inhibitors as well as the breast tumors that do not respond to checkpoint therapies may demonstrate what components of the immune system are necessary for these therapies to function. The role of checkpoint inhibitors in breast cancer and particularly how it differs depending on breast cancer subtypes also has to be evaluated including evaluation of the patients who have failed checkpoint therapy to determine whether they have low immune infiltrate or increased immunosuppressive immune infiltrate preventing response. For prevention studies, it is important to identify clinically relevant antigen targets, particularly as currently early breast cancer biomarkers remain unknown. Also for prevention it is essential to develop a very safe and immunogenic therapy which will allow for the development of memory and a durable immune response, and ideally which will prevent development of all subtypes of breast cancer. The current advances in breast cancer immunotherapy demonstrate that immunology will have a widespread impact on the future of breast cancer therapy.

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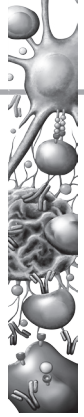

Immunotherapy targeting colon cancer stem cells

In the last 10 years, cancer stem cells have interested the scientific community because this small tumorigenic population is also associated with tumor progression in human patients and specific targeting of cancer stem cells could be a strategy to eradicate cancers currently resistant to conventional therapy. Clinical studies have recently demonstrated that adding immune therapy to chemotherapy has survival benefits in comparison with chemotherapy alone that can sensitize tumors to immune cell-mediated killing (e.g., increasing sensitivity of tumor cells to subsequent cytotoxicity by T cells via upregulation of death receptors DR5 and Fas). However, loss of MHC molecules is often observed in cancer cells, rendering tumor cells resistant to CD8 T-cell-mediated cytotoxicity. For this reason, we review the role of other T-cell subsets, such as γδ T and NK cells that are able to efficiently recognize and kill tumor cells and that could be used in passive or active immunotherapy in cancer stem cell eradication.

KEYWORDS: γδ T cells ■ cancer stem cells ■ chemoresistance ■ immunotherapy ■ NK cell

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Stem cells are unspecialized cells that are able to go through asymmetric replication to give rise to a daughter cell that is identical to the original stem cell (self-renewal ability) and another cell that is termed the progenitor cell, which divides to generate differentiated progeny.

According to differentiation ability, stem cells are named totipotent, pluripotent or multipotent. Totipotent stem cells can differentiate into embryonic and extraembryonic cell types, so they can give rise to a complete organism. These cells are produced from a fertilized egg until the first few divisions (morula state). Pluripotent stem cells are able to differentiate into all cellular types of the body because they originate as inner-mass cells within a blastocyst and can provide any of the three germ layers (ectoderm, mesoderm and endoderm). Multipotent stem cells or adult stem cells (ASCs) maintain the self-renewal ability but are committed to a specific organ lineage. Useful sources of adult stem cells are actually detectable in all organs of the body and take part in tissue homeostasis and tissue repair [1,2].

According to the number of cell division, there are two types of stem cells. The first functions as a cellular reserve and includes the cells with a limitless replicative potential, normally quiescent (G0 phase of the cell cycle) and rarely enter mitosis. The second group plays an important role during development or tissue repair, when ASCs go through symmetric divisions and replace damaged tissue. Cycling cells might accumulate DNA errors that could cause carcinogenesis: mutations in stem cells are extremely dangerous because they are transmitted to all generations of derived daughter cells. Growing experimental evidence has revealed that an accumulation of genetic abnormalities in tissue ASCs [3–5] or their more committed progenies together with changes in their niches, result in their malignant transformation into leukemic or tumorigenic stem cells [6].

Cancer stem cells (CSCs) might derive from normal stem cells that acquire genetic or epigenetic hits necessary for cancer lesions or from progenitors that acquire stem cell features [7,8]. In the case of stem cells, such mutations would be passed on to progenitors, allowing evolution towards malignancy over time and ultimately resulting in a pool of stem cells that feeds neoplastic formation.

The discovery of CSCs (also termed 'tumor-initiating cells') in a variety of tumors has changed the view of carcinogenesis and therapeutic strategies in recent years. Tumors have been widely described to evade death signals induced by therapeutic drugs through multiple mechanisms, and the molecular basis concerning the failure of chemotherapy has not been defined. The CSCs are characterized by high resistance to drugs and in general to toxins, which target rapidly proliferating cells and spare the slow-dividing cells, due to an upregulation of several ATP-binding cassette (ABC) transporters, active DNA-repair capacity and overexpression

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of antiapoptotic molecules that cause changes in the signaling pathways controlling proliferation, differentiation and apoptosis.

Although these cells comprise only a small amount within the tumor mass, they are necessary and sufficient to drive and sustain tumor growth and promote the metastatic spread of cancer. Resistance to induction of cell death of cancer cells represents one of the major obstacles to successful cancer treatment. Many mechanisms may contribute to the development of therapeutic resistance, including the stochastic selection of resistant genetic subclones, microenvironmental factors (e.g., hypoxia and acidosis) and cell extrinsic factors. The development of the CSC hypothesis has led to the expectation that tumor-initiating cells may display resistance to cytotoxic cancer therapies, permitting the repopulation of tumors treated with conventional therapies.

Cancer stem cell source

In the last 10 years, CSC isolation has interested the scientific community and remains a topic of considerable controversy owing to the lack of morphological criteria or specific markers able to recognize the heterogeneous cell populations inside the tumor mass [9].

Some references used the side population (SP) analysis for isolation of CSCs based on the ability of stem cells to efflux the DNA-binding dye Hoechst, due to expression of the ABC-dependent transporter ABCG2 [10]. However, the extension of the SP analysis to thyroid cancer cell lines showed that both SP and non-SP cells can form tumors when subcutaneously implanted into nude mice. Accordingly, these findings suggest that CSCs are not exclusive or identical to SP cells. According to the slow cycling, bromodeoxyuridine labeling was applied to SC detection, but this method is not precise and does not permit us to isolate viable cells. Cell surface molecules can be detected easily and specifically by flow cytometry.

The existence of cancer cells with stem cell-like features was first demonstrated in acute myeloid leukemia (AML), whereas recently, this principle has also been extended to other tumors, such as breast, brain, prostate, colon, ovarian and melanoma. The $CD34^+CD38^-$ phenotype was associated with AML and $CD44^+CD24^-$ with breast CSCs.

Recent evidence has demonstrated the expression of CD133 transmembrane glycoprotein in cells with self-renewal and tumor-initiating properties in colon, retinoblastoma, brain, kidney and prostate. A small fraction of cells within a colon carcinoma expresses CD133 and it is capable of initiating tumor outgrowth [4,5,11]. Although experimental evidence supports the role of CD133 in CSCs, its physiological function is not yet well determined. In hematopoietic progenitors, CD133 polarized localization should regulate proliferation because it is concentrated in the spindle pole region during metaphase. Cell biological studies have shown that CD133 localizes in particular membrane microdomains in a cholesterol-dependent manner and this could elucidate new aspects of CSC biology [12]. Within the intestine, CD133 would mark SCs prone to neoplastic transformation due to disruption of normal tissue homeostasis [13] and consequent neoplastic lesion. One contribution to understanding tumor initiation and growth comes from developmental biology of the stem cell system. Of particular interest is the recent observation that transient Hh, Notch and Wnt pathway activities promote stem cell self-renewal in normal tissues, whereas continuous activation is associated with the initiation and growth of many types of human cancer. These pathways thus provide a potential link between the normal self-renewal of stem cells and the aberrantly regulated proliferation of CSCs because the sustained stimulation of these growth factor pathways may result in an upregulation of diverse gene products in cancer stem/progenitor cells [14].

The adult intestinal epithelium has a well-defined structure ordered into crypts and villi, with a hierarchical organization that consists of cells displaying stem cell features; rapidly dividing cells, also called 'transit-amplifying cells', with little or no stem cell attributes; and differentiated cells, which constitute all the intestinal lineages. The intestinal epithelium possesses a high turnover rate and thus epithelial cells with a brief lifespan. The long-lived stem cells or transit-amplifying cells, which undergo a large number of cell divisions, should be the source of cells with mutations and epigenetic changes (Figure 1).

Colon CSCs can be expanded *in vitro* as tumor spheres that express CD133 and are able to outgrow the xenograft. Besides CD133, tumor spheres are characterized by the expression of CSC markers such as CD166, CD44, CD29, CD24 and nuclear β-catenin [15]. Recent data have identified that cytosolic aldehyde dehydrogenase (ALDH)1 is an isoenzyme responsible for the oxidation of aldehydes as stem cell markers. Strong experimental evidence supports the

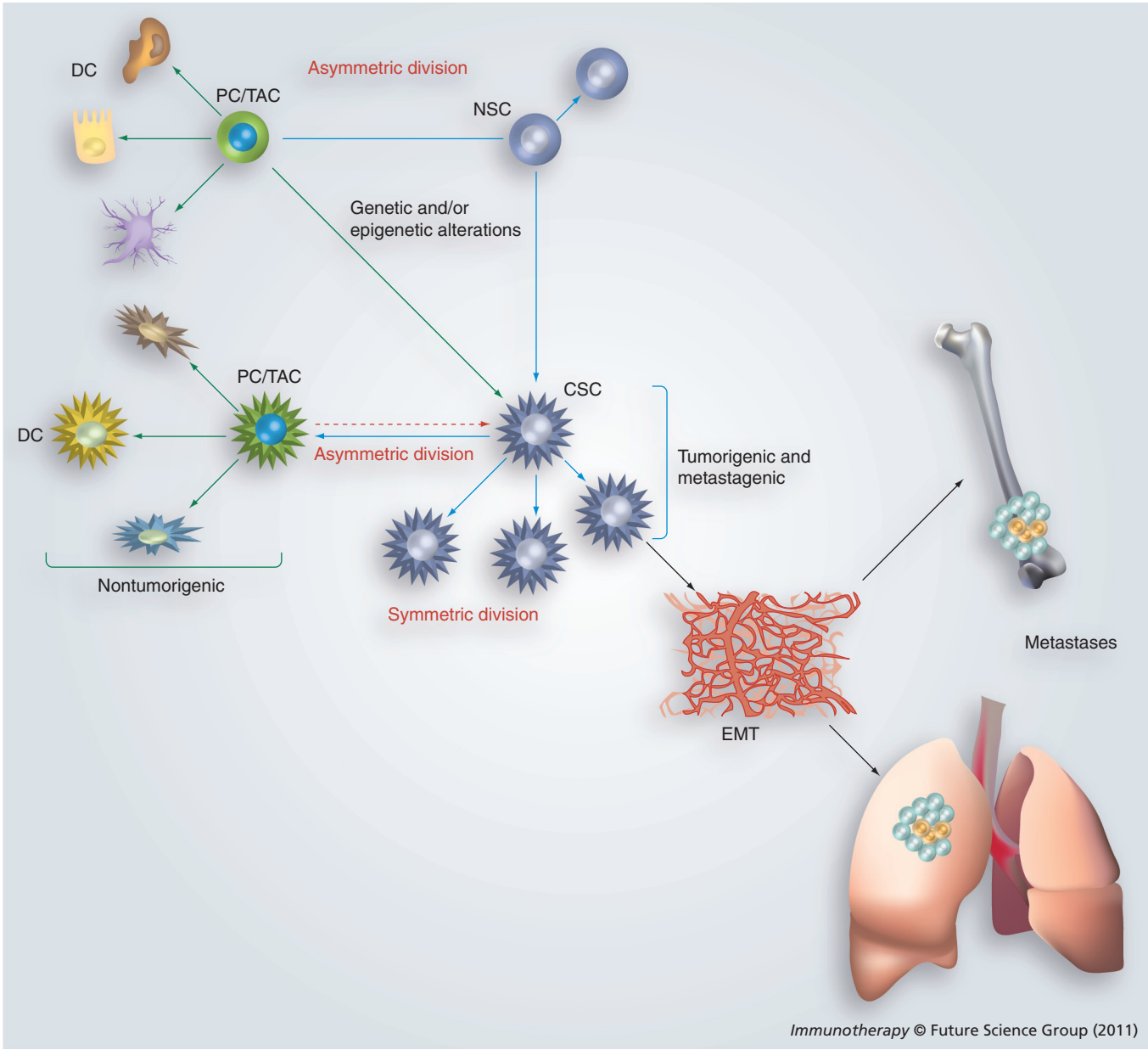

Figure 1. Stem cell division. NSCs undergo asymmetric division that provides a daughter cell identical to the original one and a progeny of more differentiated cells, called PCs or TACs. PCs sustain the generation of the pool of DCs. CSCs could arise from genetic and epigenetic alterations of NSCs or PCs. CSCs divide both symmetrically and asymmetrically, generating a progeny of nontumorigenic PC/TAC. The differentiated state is not irreversible but could revert to a stem cell-like state (red arrow). The acquisition of a migratory phenotype by CSCs during the EMT causes their invasion from primary neoplasms, dissemination through the peripheral circulation and tumor formation in different sites.
CSC: Cancer stem cell; DC: Differentiated cell; EMT: Epithelial–mesenchymal transition; NSC: Normal stem cell; PC: Progenitor cell; TAC: Transit-amplifying cell.

role of ALDH in early stem cell differentiation regulating the conversion of retinol to retinoic acids. Increased ALDH activity has been described in cells with stem/progenitor features of murine and human brain, breast and hematopoietic compartments. High ALDH activity has also been found in cancers of multiple tissues, including AML, multiple myeloma, pancreas, lung and breast carcinomas. Cells with high ALDH activity possess the ability to self-renew, reinitiate serial transplantable tumors and recapitulate the phenotypic characteristics and metastatic potential of the parental tumor [16,17]. Circulating tumor cells expressing ALDH1 and EMT-associated molecules (Twist, PI3Ka and Akt2) have been detected in blood samples from metastatic breast cancer patients [18]. Moreover, $CD44^{+}CD24^{low}$ breast CSCs were detected in all bone marrow specimens from early breast cancer patients: 72% of disseminated tumor

cells express CSC markers compared with primary tumors where this phenotype is reported in fewer than 10% of cells [19]. These data are in agreement with the role of CSCs in invasion and metastatic spread at definite distant sites.

Cancer stem cells & metastasis

The EMT is a biological process typically activated in metastatic cancer, which allows a polarized epithelial cell to go through biomolecular programs that permit it to assume a mesenchymal phenotype [20]. This complex mechanism is characterized by disruption of cell–cell junctions, loss of contact inhibition and extensive reorganization of the actin cytoskeleton and remodeling of the extracellular matrix (ECM) components that, consequently, lead to degradation of the basement membrane and the acquisition of migratory capacity, invasiveness and homing in distant sites from the epithelial layer in which the cell originated. There is currently considerable debate on whether EMT must necessarily result in a complete, irreversible transition to a mesenchymal state, or whether a partial or transient EMT may be more common [21].

However, disseminated cancer cell characteristics overlap with properties of CSCs such as self-renewal capability that permits the spread of metastases. The acquisition of a migratory phenotype by CSCs during the EMT process concomitant with the changes in the activated stroma may lead to their invasion from primary neoplasms, dissemination through the peripheral circulation and tumor formation in different sites (Figure 1) [22–24]. Consistent with this hypothesis, the so-called metastasis-initiating cells have been detected at invasion sites in primary tumors, as well as isolated from peripheral blood and secondary tumor samples of cancer patients and metastatic cancer cell lines [25,26]. Developmental genetics research has revealed a number of transcription factors that play critical roles in embryogenesis by regulating EMTs [27] and conferring malignant traits [28,29]; decreased expression of E-cadherin is concomitant with an upregulation of different signaling elements such as N-cadherin, vimentin, tenascin C, NF-κB, Snail, Slug, Twist, β-catenin, CXCR4 and antiapoptotic factors [30–32].

A relationship between EMT and the CSC phenotype has been demonstrated in immortalized human mammary epithelial cells (HMECs) that can acquire mesenchymal traits, the expression of stem-cell markers and an increased ability to form mammospheres, soft-agar colonies and tumors after EMT induction by ectopic expression of Twist or Snail transcription factors [33]. In addiction, $CD44^{high}$ $CD24^{low}$ breast cancer fractions express low levels of E-cadherin and high levels of mRNA encoding mesenchimal markers (e.g., N-cadherin, vimentin, fibronectin and Snail2).

Weinberg *et al*. also demonstrated that EMT could promote a dedifferentiation program of differentiated cells. Accordingly, HME-flopc (a floating subpopulation of basal-like normal HMECs) and hTERT, SV40 T/t and H-RasV12 cancer cell lines highlighted the ability of spontaneous dedifferentiation of differentiated cells into new stem cells/CSCs as judged by their changing CD44/CD24 profile [34]. This supports the hypothesis that the differentiated state is not irreversible but could revert to a stem cell-like state.

γδ T cells & cancer stem cells

New strategies for complete tumor eradication are necessary; they comprise inhibitors of survival pathways, differentiation-inducing agents and immunotherapy [35,36].

Recent evidence shows that $CD133^+$ melanoma cells show increased expression of NY-ESO-1 cancer/testis (CT) antigens and can be targeted by specific cytolytic T lymphocytes (CTLs) [37]. Since CD133 marks melanoma stem cells and these cells express CT antigens, it is likely that the heterogeneity of CT antigen expression, often seen in melanoma, may be due to differentiation of melanoma stem cells into differentiated cells with limited proliferative potential and loss of CT antigen expression. Even if the specifities of these T lymphocytes have not yet been elucidated, *in vivo* stimulation of colon rectal cancer-specific T lymphocytes could be used as adjuvant therapy to tumor resection. $CD8^+$ T lymphocytes are recognized by CT antigens (also known as cancer/germline or shared tumor-specific antigens), which are particularly interesting targets because they are predicted to be expressed specifically by the tumor cells and not by the adjacent normal epithelial cells.

Cancer/testis antigens are expressed in melanoma cell lines grown in embryonic stem cell media [38]; treatment of ovarian cancer cell lines with 5-aza-2-deoxycytidine upregulates CT antigen expression, perhaps by the selection of chemoresistant cells [39].

Targeting these antigens could be a novel therapeutic approach even if loss of MHC molecules often renders tumor cells resistant to CD8 T-cell-mediated cytotoxicity. For this reason, we review the role of other T-cell subsets, such as

γδ T and NK cells, which are able to efficiently recognize and kill tumor cells and that could be used in passive or active immunotherapy in CSC eradication.

It has been demonstrated that within the colon tumor [40], the CD133 subpopulation (CSCs) is more resistant than differentiated primary cells to the conventional chemotherapeutic drugs and to TNF-α-related apoptosis-inducing ligand (TRAIL) therapy [41], which is able to kill many tumor cell lines but not most nontransformed cells, and the selective efficacy of histone deacetylase inhibitors versus AML cells involves TRAIL induction *in vivo* [42,43].

γδ T cells exhibit potent MHC-unrestricted lytic activity against different tumor cells *in vitro*, suggesting their potential utility as an anticancer therapy. γδ T cells have been consistently identified and isolated from tumor-infiltrating lymphocytes in various types of cancer, including prostate carcinoma [44,45].

Moreover, γδ T cells are a natural component of resistance to cutaneous carcinogenesis in mice [46] and in humans display potent MHC-unrestricted cytotoxic activity *in vitro* against various tumors including prostate cancer cell lines [47]. Indeed, human Vγ9Vδ2 T cells expanded *ex vivo* and then adoptively transferred to SCID mice xenografted with tumor cells demonstrated efficacy against B-cell lymphoma, melanoma and renal, pancreatic and nasopharyngeal carcinoma [48].

Human Vγ9Vδ2 T cells can be activated by a variety of nonpeptide phosphoantigens or by agents that cause their accumulation within cells; among the latter are aminobisphosphonates [49,50]. Aminobisphosphonates, in addition to their effect of inhibiting osteoclastic bone resorption [51], exhibit direct antitumor activity by both inhibiting proliferation and inducing apoptosis in tumor cells [52]. Their unique ability to render tumor cells susceptible to Vγ9Vδ2 T-cell attack makes these drugs particularly interesting candidates for use in T-cell therapy [53–57].

In addition to the binding of the antigenic molecules to γδ T-cell receptors (TCRs), γδ T cells express NK cell-activating receptors such as NKG2D, which recognizes target cells expressing stress-inducible NKG2D ligands, such as MICA, MICB and UL-16-binding proteins (ULBPs) [58]. After recognizing target cells via γδ TCR, NKG2D, CD6 and so on, γδ T cells use the Fas/FasL killing signal [59] as well as the perforin–granzyme pathway [60] for cytotoxicity against target cells such as tumor cells. In addition, upon activation with antigenic molecules, γδ T cells secrete Th1 cytokines such as IFN-γ and TNF-α, which have cytotoxic activity against tumor cells directly and indirectly via stimulating adaptive immune-competent cells such as αβ T cells [61] and dendritic cells [62].

Phosphoantigens or aminobisphosphonates together with IL-2 can trigger the selective outgrowth of Vγ9Vδ2 T cells *in vitro* and *in vivo* in both preclinical (nonhuman primate) models and in cancer patients [63]. A pilot study of adoptive immunotherapy using *in vitro*-activated γδ T cells was performed against advanced renal cell carcinoma, in which the synthetic 2-methyl-3-butenyl-1-pyrophosphate was used for activation and expansion of γδ T cells [64]. Results from this pilot study indicate that adaptive immunotherapy using *in vitro*-activated autologous γδ T cells is well tolerated and induces antitumor effects.

Regarding active immunotherapy, both preclinical studies and Phase I/II trials performed in myeloma, lymphoma, metastatic renal carcinoma and prostate cancer patients have demonstrated efficient but transient *in vivo* Vγ9Vδ2 T-cell systemic expansions after treatment with γδ agonists and IL-2. These treatments are generally well tolerated with limited side effects and may lead to disease stabilization or partial tumor regression in some treated patients.

Vγ9Vδ2 T cells have been detected in the majority of colon cancer tumor-infiltrating lymphocyte populations, and the response of this T-cell subpopulation to colon cancer cells suggests that a natural immune response mediated by these lymphocytes contributes to the immunosurveillance of these tumors.

Vγ9Vδ2 T-cell activation by zoledronate may represent a novel strategy for colon cancer immunotherapy as suggested by Todaro *et al.* in 2009 [65]; in fact, the authors demonstrated that the treatment of CSCs with zoledronate induces the activitation of Vγ9Vδ2 T cells in terms of proliferation, cytokine production as IFN-γ and TNF-α and cytotoxic molecules release as TRAIL and BLT esterase, while untreated CSCs fail to activate γδ T cells. Conversely, zoledronate-treated CSCs were more susceptible to the killing of γδ T cells by perforin release, mainly after TCR-mediated recognition. The encouraging prospect that the activation of peripheral blood Vγ9Vδ2 T cells can be efficacious against CSCs requires further follow-up investigation, in order to assess whether activated lymphocytes are indeed infiltrating the tumors and/or are helping other cells to do so.

NK cells & immunotherapy

NK cells are important players of the innate immune response characterized by strong cytolytic activity against susceptible target cells, including tumor cells, and by the ability to release several cytokines. NK cell activation and function are regulated by triggering and inhibitory surface receptors [66–68]. Inhibitory receptors include HLA class I-specific killer immunoglobulin-like receptors and CD94/NKG2A. These receptors allow NK cells to discriminate between normal MHC class I-positive cells and cells that have lost expression of surface MHC class I molecules, as frequently occurs in tumor cells.

The loss or downregulation of MHC class I antigens is one of the best analyzed mechanisms of cancer evasion from T-lymphocyte-mediated immune recognition [69,70]. There are different molecular mechanisms that lead to altered MHC class I expression: loss of heterozygosity in human chromosomes 6 and/or 15 [71,72]; mutations of genes coding class I heavy chain or β2-microglobulin [73]; coordinated downregulation of HLA A, B or C loci [74]; and downregulation of the antigen-processing machinery, such as *TAP* and *LMP* genes [75]. MHC alterations can be reversible since it can be recovered by immunomodulators (i.e., interferons) or pharmacological agents. Instead, a structurally irreversible defect is responsible for the loss of class I antigens on the tumor cell surface when normal HLA-I expression cannot be recovered by cytokines.

In the absence of inhibitory signals (as in MHC class I-negative tumor cells), activating NK receptors (including NKp46, NKp30, NKp44, NKG2D and DNAX accessory molecule-1 [DNAM-1]) mediates NK cell triggering and target cell lysis, upon interaction with specific ligands. The best characterized ligands are represented by the NKG2D ligands (i.e., the stress-inducible molecules MHC class I-related chain A/B [MICA/B] and ULBPs) [76,77] and the DNAM-1 ligands (Nectin-2 and the poliovirus receptor [PVR]) [78]. In most cases, these molecules are not expressed by normal resting cells, while they may become highly expressed in tumor cells belonging to different histotypes.

The ability of NK cells to kill tumor cells and the mechanism involving inhibitory and activating NK receptors provided a rational basis for their exploitation in novel immunotherapy approaches, primarily in the treatment of AML. The susceptibility of AML to NK-mediated lysis in haploidentical bone marrow transplantation was shown to correlate with the existence of a mismatch between killer immunoglobulin-like receptors expressed by donor NK cells and HLA class I alleles expressed by the patient [79].

On the basis of this knowledge, NK cell-based immunotherapy might be considered a possible approach for cancer immunotherapy, but the susceptibility of CSCs to NK-mediated lysis has been poorly explored so far.

In 2009, Pietra *et al.* evaluated the susceptibility to NK cell-mediated lysis of $CD133^+$ or $CD133^-$ melanoma cells [80]. The authors demonstrated that NK cells can kill melanoma cell fractions enriched in CSCs according to both phenotypic and functional criteria. Since long-term survival of patients with cancer requires effective removal of CSCs, these data may encourage promising NK cell-based immunotherapeutic strategies.

Although the results are exciting, an open question remains: which human cancers may be targeted by NK cells? With respect to tumor cell types, it is already evident from studies performed *in vitro* and even in some clinical trials that certain tumor types may be better suited than others for NK cell-based immunotherapy. The presence on human tumors of ligands for activating receptors provides an important prerequisite for NK cell activation, and thus for the potential of achieving good clinical results [81]. An illustration of this is the inefficient NK cell killing of lymphoid compared with myeloid leukemias that may be caused, at least in part, by the absence of LFA-1 ligand expression [82].

In 2007, Wu *et al.* investigated the immunogenicity of $CD133^+$ cells in two human astrocytoma and two glioblastoma multiforme samples [83]. Flow cytometry analyses revealed that the majority of $CD133^+$ cells do not express detectable MHC-I or NK cell-activating ligands, which may render them resistant to adaptive and innate immune surveillance. Incubating $CD133^+$ cells with IFN-γ significantly increased the percentage of $CD133^+$ cells that expressed MHC-I and NK cell ligands. Furthermore, pretreatment of $CD133^+$ cells with IFN-γ rendered $CD133^+$ cells sensitive to NK cell-mediated lysis *in vitro*. This indicates that $CD133^+$ and $CD133^-$ glioma cells may be similarly resistant to immune surveillance, but that IFN-γ may partially restore their immunogenicity and potentiate their lysis by NK cells.

Very recently, we studied the susceptibility of colon CSCs to NK cell-mediated lysis. $CD133^+$ colon CSCs express high levels of many different NK cell ligands (NKp30, NKp44 and NKp46) compared with differentiated colon cancer cells

that virtually lack expression of these ligands. The expression of MHC class I molecules shows an inverse pattern, with undetectable levels of expression on colon CSCs and high expression levels on differentiated colon cancer cell lines. Moreover, further analysis, although preliminary, shows an effective NK cell-mediated lysis of colon CSCs, supporting their role in antitumor response and their application in immunotherapy.

Conclusion & future perspective

Tumor-initiating cells capable of self-renewal and differentiation, which are responsible for tumor growth, have been identified in human hematological malignancies [1,2] and solid cancers [3–5]. Cancers are believed to arise from a series of sequential mutations that occur as a result of genetic instability and/or environmental factors. A better understanding of the consequences of these mutations on the underlying biology of the neoplastic cells may lead to new therapeutic strategies. While targeted immunotherapies offer new possibilities to harness the immune response to treat cancer patients, effective manipulation of the immune system may require overcoming barriers, while avoiding potential hazardous complications. Furthermore, it must be recognized that therapies designed to establish adaptive immune responses toward a tumor mass may not effectively target the minor subpopulation of CSCs that support tumor development and recurrence, since those progenitor cells may express distinct antigens.

Chemotherapeutic agents can sensitize tumors to immune cell-mediated killing (e.g., by increasing the sensitivity of tumor cells to subsequent cytotoxicity by T cells via upregulation of death receptors DR5 and Fas, ligands of TRAIL and CD95L [FasL], respectively) [84]. Most current immunotherapeutic approaches are aimed at inducing an antitumor response to stimulate the adaptive immune system, which is dependent on MHC-restricted αβ CD4 and, particularly, CD8 cytotoxic T cells. However, loss of MHC molecules is often observed in cancer cells, rendering tumor cells resistant to CD8 T-cell-mediated cytotoxicity [85,86]. T-cell subsets, such as γδ T and NK cells, are able to efficiently recognize and kill tumor cells, and this could be used in passive or active immunotherapy.

Executive summary

Cancer stem cell model

- Tumor cells are heterogeneous.
- Cancer stem cell (CSC) subpopulation are capable of dividing in an asymmetric way.
- CSCs might derive from normal stem cells that acquire genetic or epigenetic hits necessary for cancer lesions or from progenitor cells that acquire stem cell features.
- These cells are necessary and sufficient to drive and sustain tumor growth and promote the metastatic spread of cancer.

Cancer stem cell isolation

- CSC isolation remains a topic of considerable controversy owing to the lack of morphological criteria.
- Side population analysis is based on the stem cells' ability to efflux Hoechst dye due to transporter ATP-binding cassette G2 expression.
- The $CD34^+CD38^-$ phenotype was associated with acute myeloid leukemia and $CD44^+CD24^-$ with breast CSCs.
- A $CD133^+$ fraction of cells within a colon carcinoma is capable of initiating tumor outgrowth.
- Aldehyde dehydrogenase 1 has a role in early stem cell differentiation, regulating the conversion of retinol to retinoic acids.

Chemoresistance of cancer stem cells

- CSCs are characterized by high resistance to drugs and to toxins in general.
- CSCs are slow-dividing cells and so are unable to escape death signals induced by therapeutic drugs that target rapidly proliferating cells.
- CSCs present an upregulation of several ATP-binding cassette transporters responsible for efflux of chemotherapeutics.
- CSCs overexpress antiapoptotic molecules that cause changes in the signaling pathways controlling proliferation, differentiation and apoptosis.
- The failure of chemotherapy against CSCs could pave the way for new strategies such as immunotherapy.

γδ T-cell-mediated immunotherapy

- γδ T cells have potent MHC-unrestricted lytic activity against different tumor cells *in vitro*.
- Vγ9Vδ2 T cells can be activated by a variety of nonpeptide phosphoantigens or by aminobisphosphonates.
- The ability of NK cells to kill tumor cells and the mechanism involving inhibitory and activating NK receptors have provided a rational basis for their exploitation in novel immunotherapy approaches.
- The majority of $CD133^+$ cells do not express detectable MHC-I or NK cell-activating ligands; incubation with IFN-γ significantly increased MHC-I and NK cell ligand-expressing cells.

Conclusion

- γδ T and NK cells are able to efficiently recognize and kill tumor cells and could be used in passive or active immunotherapy.
- Clinical benefit could be improved by a combination with strategies aimed at activating T cells.
- Targeted immunotherapy offers new possibilities to treat CSCs, avoiding potential hazardous complications.

In conclusion, future targeted immunotherapies might be necessary, which specifically direct immune reactivity toward these CSCs.

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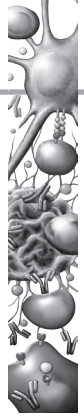

Immune response against tumor antigens expressed on human cancer stem-like cells/tumor-initiating cells

Cancer stem-like cells (CSCs)/tumor-initiating cells (TICs) are a small population of cancer cells that have the properties of tumor-initiating ability, self-renewal and differentiation. These properties suggest that CSCs/TICs are essential for tumor maintenance, recurrence and distant metastasis. Thus, elimination of CSCs/TICs is essential to cure malignant diseases. However, there are several studies reporting that CSCs/TICs are more resistant to standard cancer therapies, including chemotherapy and radiotherapy, than non-CSC/TIC populations. How then, can we eliminate CSCs/TICs? Immunotherapy might be the possible answer. In recent analysis, innate immunity (natural killer cells and γδT cells) and also adaptive immunity (cytotoxic T lymphocyte-based cellular immunity and antibody-based humoral immunity) can recognize CSCs/TICs *in vitro* efficiently. Furthermore, CSC/TIC-specific monoclonal antibody therapies are also efficient *in vivo*. In this article, we describe the potency, possibilities and problems of CSC/TIC-targeting immunotherapy.

KEYWORDS: antigenic peptide ■ cancer stem cell ■ CTL ■ immunotherapy ■ monoclonal antibody ■ tumor antigen

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Antigen-specific immune responses based on $CD8^+$ cytotoxic T lymphocytes (CTLs) and antibodies are essential for tumor rejection in the immune reaction against cancer cells. At the end of the 20th Century, cancer immunity research moved into a new stage when the first human tumor-associated antigen (TAA) recognized by CTLs was identified by van der Bruggen *et al.* [1]. With the identification of TAAs, cancer immunotherapy moved into a new era of specific cancer immunity, and cancer immunotherapy based on TAAs is becoming a reality [2]. CTLs recognize 9- to 14-mer antigenic peptides that are derived from endogenously expressed proteins digested by several proteases, including proteasomes and endoplasmic reticulum-associated aminopeptidase associated with antigen processing (ERAAP). Antibodies are other major players in specific immunity. Antibody-based immunotherapy can target only the cell surface proteins or secreted proteins such as $p185^{HER2/neu}$ for breast carcinoma, CD20 for B-cell lymphoma and VEGF for colorectal carcinoma. These CTL-based and antibody-based cancer immunotherapies are being developed in several ways and have already been launched all over the world. Of course, for cancer immunotherapy, various problems remain to be overcome. These problems include several immune escapse mechanisms such as antigen loss, HLA loss, immune suppressive cytokines, immunosuppressive cells such as regulatory T cells, regulatory dendritic cells and myeloid-derived suppressor cells, and immunosuppressive molecules such as indoleamine 2,3-dioxygenase (IDO).

Research into cancer stem-like cells (CSCs)/tumor-initiating cells (TICs) have made huge progress recently. CSCs/TICs are defined as the small population of cancer cells, which have the abilities of tumor initiation, self-renewal and differentiation. When we focus on clinical aspects of CSCs/TICs they have huge impact, since CSCs/TICs are resistant to standard therapeutic modalities, including chemotherapy and radiotherapy in various mechanisms. Thus, CSCs/TICs are thought to cause disease recurrence post-therapy, making the disease untreatable. Therefore, efficient CSC/TIC targeting therapy is needed to cure cancer. In this article, we focus on cancer immunotherapy as one of the representative way to treat CSCs/TICs.

Cancer stem cell hypothesis & isolation of cancer stem cell/tumor initiating cell population

It is well known that tumors are composed of morphologicaly and functionaly heterogeneous cells, and it has long been suspected that cancers may contain a small stem cell-like population (cancer stem cell hypothesis). Lapidot *et al.* had first given an explanation to this hypothesis [3]. They showed that human acute myeloid leukemia (AML) contained a small percentage of

cells (0.1–1%) capable of transferring human AML into immunodeficient mice. The resulting leukemia recomposed morphological and immunophenotypic heterogeneity resembling the original disease. The leukemia-initiating cells/leukemia stem cells (LSCs) could be isolated by cell surface markers ($CD34^+CD38^-$). More importantly, the nonleukemia stem cell phenotype ($CD34^+CD38^+$) did not engraft mice. This study clearly showed that LSCs were located at the top of the hierarchical organization of AML and harbor the ability to differentiate into matured differentiated leukemic cells, and only LSCs can initiate the disease. In subsequent studies, CSCs/TICs were isolated from several solid tumors by various methods. The use of cell surface markers (e.g., $CD34^+CD38^-$, $CD44^+CD24^-$ and CD133), side population (SP) and ALDEFLUOR® (StemCell Technologies Inc., USA) assay are representative methods for isolating CSCs/TICs today, as described in the following sections (Figure 1).

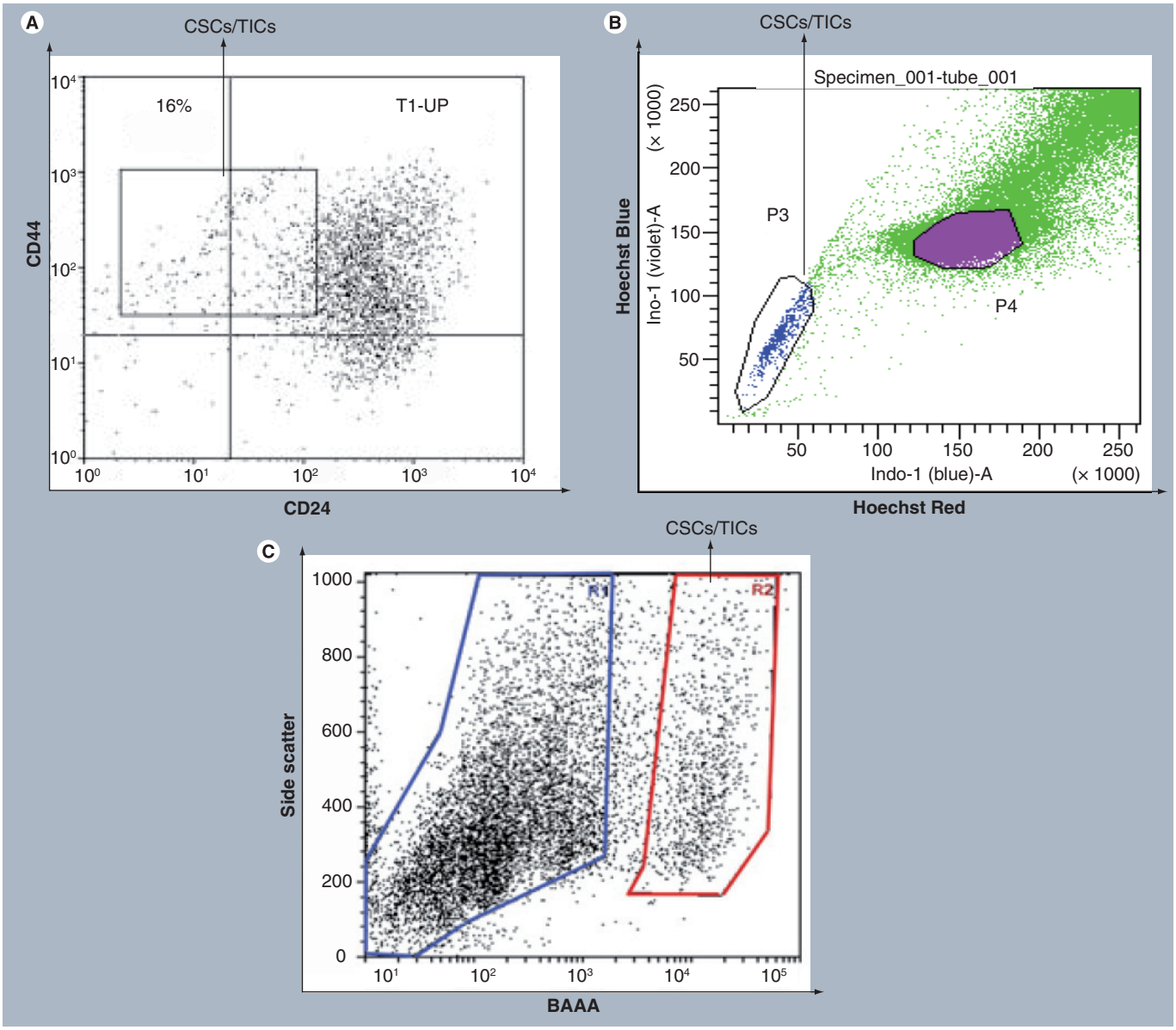

Figure 1. Isolation of cancer stem-like cells/tumor-initiating cells population. (A) Isolation of CSCs/TICs by cell surface markers. Breast cancer CSCs/TICs can be isolated as $CD44^+CD24^-$/low population. **(B)** Isolation of CSCs/TICs as side population (SP) cells. CSCs/TICs express ABCG2 protein, which export Hoechst33342 dye, thus, CSCs/TICs can be isolated as low-stained populations. **(C)** Isolation of CSCs/TICs as ALDEFLUOR®-positive cells. CSCs/TICs express ALDH1 enzyme, thus, CSCs/TICs can be isolated as BAAA fluorescence.
CSC/TIC: Cancer stem-like cell/tumor-initiating cell.
(A) is reproduced with permission from [5]. **(C)** is reproduced from [31].

However, sometimes CSC/TIC-like populations isolated by those methods do not show the cancer stem cell properties, so it is essential to qualify the CSC/TIC-like populations as enriched cancer stem cell populations by methods such as xenotransplantation.

Cell surface markers

The first major method of isolating CSCs/TICs is the use of cell surface markers, such as LSCs, as previously described. Cell-surface markers for solid tumors have also been identified. Previously, we isolated CD44 as one of the cell-surface antigens that is related to the transformation of rat fibloblast cells with H-Ras oncogene [4]. Conversely, CD44 is not expressed in the normal fibroblast counterpart. This suggests that CD44 might be one of the highly tumorigenic cell (tumor-initiating cell) markers. We also found that CD44 can be the target of NK cells, raising the possibility that tumor-initiating cells may be eliminated by NK cells. In a later study, CD44 was identified as a marker for solid tumor CSCs/TICs by Al-Hajj *et al* [5]. They demonstrated that $CD44^+CD24^{-/low}$ breast cancer cells have high tumor-initiating ability in non-obese diabetic (NOD/SCID) mice. This population, representing 11–35% of cells in primary breast tumors, gave rise to tumors that recapitulated the morphologic and immunophenotypic features of the original tumor. In addition, these same cells could be sorted from the primary grafts and serially transplanted, demonstrating their self-renewal capability. The same group demonstrated that $CD44^+CD24^{-/low}$ expressed 186 genes (termed as invasive gene signature) compared with normal breast epithelia. Invasive gene signature positive group showed high risk of both metastasis-free survival and overall survival. This supports the idea that CSC/TIC populations have great impact *in vivo* [6]. In the following works, several other solid tumor CSCs/TICs have been identified in colon [7], pancreatic [8], prostate [9], head and neck squamous cell carcinomas [10], gastric carcinoma [11] and bladder carcinoma [12] using CD44 as a marker of CSCs/TICs. CD90 could be used for isolating CSCs/TICs of hepatocellular carcinoma [13]. The neural stem cell marker CD133 has been used for isolating CSCs/TICs of glioblastoma, medulloblastoma [14,15], colon cancer [16,17], pancreas cancer [18] and lung cancer [19]. However, Shmelkov *et al.* reported controversial data concerning CD133. They found that both $CD133^+$ and $CD133^-$ colon cancer cells could be xenotransplanted serially, and $CD133^-$ colon cancer cells made more aggressive tumors than $CD133^+$ colon cancer cells [20]. One ATP-binding cassette (ABC)-transporter gene product, ABCB5, was isolated as a melanoma-initiating cell marker [21].

Side population

The second method is using SP cells, that can be isolated by the effulux of $Hoechst_{33342}$ dye [22]. CSCs/TICs express various types of ABC transporters, including those encoded by the multidrug-resistant (*MDR*) gene 1, the multidrug-resistance-like protein (MRP) and ABCG2, which contribute to drug resistance in many cancers by pumping the drugs out of the cells. Importantly, some of these transporters are also expressed by small populations that have stem cell phenotypes. ABCG2 pumps out the fluorescent dye $Hoechst_{33342}$, which enables identification of the unlabeled SP cells as CSCs/TICs. Kondo *et al.* reported that mouse C6 glioma SP cells have multidifferentiation ability and high tumorigenicity [23]. Several reports demonstrated that GI tract cancer [24], hepatocellular carcinoma [25], thyroid cancer [26], nasopharyngeal cancer [27], lung cancer [28] and bone sarcoma [29] SP cells were enriched with CSC/TIC populations, suggesting that SP cells could be used for isolating CSCs/TICs. Conversely, Burkert *et al.* reported that GI tract SP cells were not enriched with CSCs/TICs [30]. Thus, in some cells, the SP cell phenotype might not correlate with the CSCs/TICs phenotype.

ALDEFLUOR® assay: ALDH1 expression

The third method is the ALDEFLUOR assay, which is based on the enzymatic activity of aldehyde dehydrogenase (ALDH)1. Ginestier *et al.* reported that breast cancer CSCs/TICs could be isolated as ALDH1 enzymatic positive cells [31]. The $ALDH1^+$ breast cancer cells were isolated by ALDEFLUOR assay using a cell sorter. Only the $ALDH1^+$ breast cancer cells were xenotransplantable. $ALDH1^+$ and $CD44^+/CD24^{-/lin-}$ breast cancer cells showed more efficient tumor-initiating ability than $ALDH1^+$ breast cancer cells. On the other hand, $ALDH1^-$ and $CD44^+/CD24^{-/lin-}$ breast cancer cells did not show any tumor-initiating ability. These data suggest that ALDH1 is more essential than $CD44^+/CD24^{-/lin-}$ in tumor-initiating ability. More importantly, $ALDH1^+$ breast cancer cases show the basaloid phenotypes related

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 tumorigenitiicy, but a more convenient, time-saving assay is needed for standardized assessment.

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

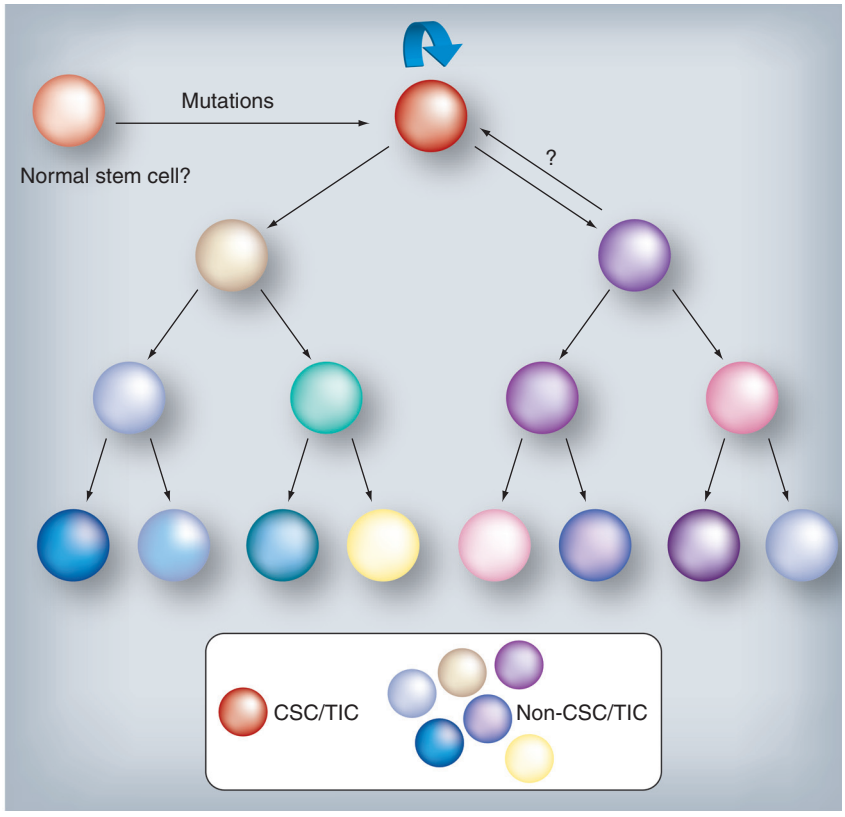

Figure 2. The cancer stem-like cell/tumor-initiating cell model. CSCs/TICs are located on the top of this hierarchical differentiation model. CSCs/TICs self-renew and differentiate to non-CSCs/TICs.
CSC/TIC: Cancer stem-like cell/tumor-initiating cell.

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	Vγ9Vδ2 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].

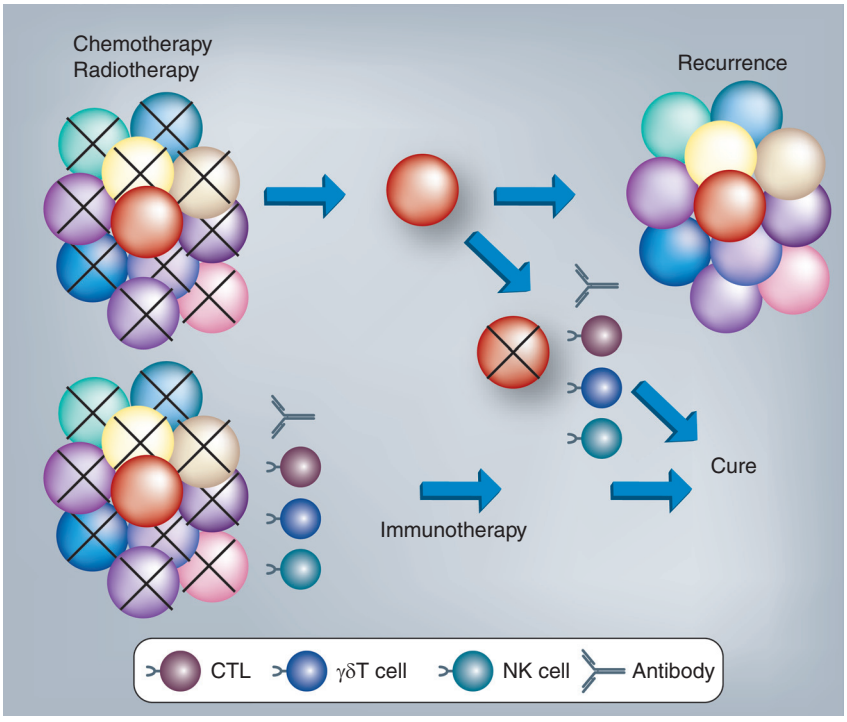

Figure 3. Immunotherapy targeting cancer stem-like cells/tumor-initiating cells. CSCs/TICs are thought to be resistant to chemo- or radio-therapies. However, immunosystems can recognize and kill CSCs/TICs. CSC/TIC-targeting immunotherapy, or chemo- or radio-therapy following immunotherapy can possibly '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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