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REVIEW

Aiming to immune elimination of ovarian cancer stem cells

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Abstract

Ovarian cancer accounts for only 3% of all cancers in women, but it causes more deaths than any other gynecologic cancer. Treatment with chemotherapy and cytoreductive surgery shows a good response to the therapy. However, in a large proportion of the patients the tumor grows back within a few years. Cancer stem cells, that are less responsive to these treatments, are blamed for this recurrence of disease. Immune therapy either cellular or humoral is a novel concept to treat cancer. It is based on the notice that immune cells invade the tumor. However, the tumor invest heavily to escape from immune elimination by recruiting several immune suppressive mechanisms. These processes are normally in place to limit excessive immune activation and prevent autoimmune phenomena. Here, we discuss current knowledge about the immune (suppressive) status in ovarian cancer. Moreover, we discuss the immunological targets of ovarian cancer stem cells.

Key words: Ovarian cancer; Cancer stem cell; Immune therapy; Immune suppression; Tumor microenvironment

Core tip: Ovarian cancer harbors, at a low frequency, cancer stem cells. Those cancer stem cells express stem cell specific antigens. Natural immunity against those antigens exists but is hampered by the suppressive microenvironment that the tumor creates. Erasing this suppressive microenvironment will make immunological elimination of those cancer stem cells is an attractive treatment option.

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EPITHELIAL OVARIAN CANCER

Ovarian cancer is the fourth leading cause of death from cancer in women and the leading cause of death from gynecological cancer. The lifetime risk to get this disease is 1 in 60 women in industrial countries but is less common in Asian and African women. Due to vague symptoms and adequate screening methods at the early stages, more than 60% of the patients are diagnosed at advanced stage. Most patients respond well to primary treatment, either cytoreductive surgery followed by chemotherapy or chemotherapy followed by surgical removal of remaining tumor foci. However, 80% of the patients diagnosed at late stage will eventually develop recurrent diseases, the survival is generally poor. The 5-year survival rates at stage Ⅲ and Ⅳ are 29% and 13%, respectively. The relapse of tumor arises the question about the identity of the cells that give rise to the tumor and somehow escape from the first line treatment, reside in the body undetected, and finally initiate malignant tumor growth in a suitable microenvironment.

Despite intense efforts to improve chemotherapy, *e.g.*, the introduction of paclitaxel, and to improve surgical techniques, over the past 20 years no significant progress has been made (Figure 1).

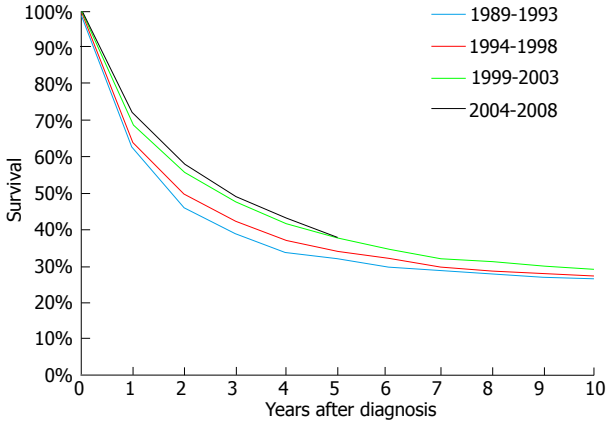

Figure 1 Survival of patients diagnosed with ovarian carcinoma. The percentage of survival after diagnosis is not significantly increased in the past 20 yr (Source from Integraal Kanker Centrum, The Netherlands) .

Novel therapeutic approaches are urgently needed. Since ovarian cancer is immunogenic, immunotherapy should be further pursued and optimized. Stimulating the immune system to attack ovarian tumor is not a new concept, during the last 20 years numerous immunological modalities were involved in clinical trials in ovarian cancer treatment[1]. Targeting a specific tumor antigen plays a decisive role in the success of immunotherapy.

CANCER STEM CELLS

Tumors are composed of phenotypically and functionally heterogeneous cells. There are two theories explaining how this heterogeneity arises[2,3]. According to the stochastic model, tumor cells are biologically equivalent; virtually every tumor cell is able to generate new tumor cells. In contrast, the hierarchy model postulates the existence of tumorigenic as well as non-tumorigenic cells. Only a subset of cells can initiate tumor growth, and these cells are considered as tumor-initiating cells (TICs) or cancer stem cells (CSCs). CSC is a relatively rare cancer cell that has the ability of self-renewal giving rise to another malignant stem cell as well as a cell that undergoes massive proliferation and differentiation to give rise to the phenotypically and functionally more mature cancer cells[4,5]. The similarities of CSCs and normal stem cells (NSCs) point to the origin of CSCs. There are two hypotheses[6]. One states that CSCs can be derived from NSCs, so that they can make use of the already active self-renewal machinery. Another assumes that the CSCs can be derived from progenitor cells by regaining the self-renewal capability. NSCs possess several unique properties. Their self-renewal enables livelong maintenance of all organs of the body. In most cases NSC divide slowly. For hematopoietic cells a doubling time of 30 d was reported[7]. However, for intestinal cells a doubling time of less than 24 h was reported[8]. Those fast regenerating organs have stem cells that are continuously dividing. One of properties of NSC is the expression of pumps of the ATP binding cassette (ABC) superfamily[9-11]. Those pumps can remove toxic components from the cell. Likewise CSC also expresses members of the ABC family[10-19]. For melanoma ABC-B1 and ABC-B5 were reported while other tumors express other members[12,13]. This endows CSC with a nasty property. The pump is able to remove cytotoxic drugs that are given to patients to kill the tumor. Indeed, a common property of CSC is their resistance against cytotoxic drugs, explaining the relapse that is seen in several patients. Traditional therapies that kill primarily non-tumorigenic cancer cells can shrink tumors, but will not cure the patient because the CSCs that survive the treatment will regenerate the tumor. By prospectively identifying and characterizing CSCs, it might be possible to identify more effective therapies[20-24]. CSCs can be eliminated by direct killing, or force them to differentiated cells or by destroying their niche[25]. Accordingly, targeting the CSCs has been put forward as such a new treatment modality for cancer immunotherapy[26,27]. Several studies described in the literature provide several clues for optimizing the immunotherapy against ovarian cancer.

IDENTIFICATION AND CHARACTERIZATION

The first experimental evidence suggests the existence of CSC came from leukemia. Bonnet and co-workers demonstrated that human leukemias are driven by a small population of leukemic stem cells capable of transferring the disease to NOD/SCID mice[28]. This concept was extended to solid epithelial tumors by Al-Hajj and co-workers, who demonstrated that a small population of cells within breast cancer with stem cell properties, bearing the surface marker $CD24^{low}CD44^{high}$[4]. Subsequently, CSCs are identified and prospectively isolated from a variety of epithelial cancers, including pancreas, colon and prostate cancers[29-40].

Ovarian CSC is responsible for ovarian tumor formation

The CSC hypothesis has recently also been explored in ovarian cancer. In 2008, Zhang *et al*[39] claimed that epithelial ovarian cancers derive from a subpopulation of $CD44^{+}CD117^{+}$ cells. Ferrandina and Curley independently found that CD133 expression defines a tumor initiating subpopulation of cells in human ovarian cancer[41,42]. Gao and co-workers reported that CD24 could be utilized as a surface marker to enrich for ovarian CSCs[32]. Ovarian CSCs were also detected in the so-called side population, which are tumorigenic and chemoresistant[38,43,44]. Moreover, Stewart *et al*[45] established a quantitative assay that enables characterization of TICs from serous ovarian cancer, and they also found that the tumor initiating cell phenotype is heterogeneous across patients. And recently, a gene involved in maintaining stem cell pluripotency, Nanog, was proved to be expressed by ovarian tumor cells, and positive Nanog expression indicates poor progression of patients with ovarian serous carcinoma[46].

As described above, increasing experimental evidence suggests that TICs may play a decisive role in the initia-

tion and progression of tumors[4,29-31,35-39,46]. However, TICs with distinct tumorigenic abilities were identified[31,47,48], as well as large variation in their frequency[49,50]. TICs appear not to be a stable entity but show quite some plasticity[2,51-54]. Recently, it was described that the TIC compartment can be subdivided into long-term TICs, tumor transient amplifying cells as well as delayed contributing TICs[48]. Only the long-term TICs are capable of maintaining tumor formation in serial xenografts, and these cells are considered as cancer stem cells.

Phenotypic heterogeneity of ovarian CSCs

CSCs are operationally defined as tumor initiating cells because the CSC assays rely heavily on xenotransplantation[55]. Although it was proven that frequency and tumorigenic ability of melanoma CSCs that can be detected after xenotransplantation were highly dependent on experimental design[50,56], current studies on CSCs all use immunodeficient mice models to check whether putative CSCs can generate secondary tumors *in vivo*. And using this method, phenotypically diverse ovarian CSC populations have been characterized and isolated from both patient material and immortalized tumor cell lines with variable stem cell markers[32,36,38,41,42,46,57,58]. However, due to the fact that a large number of cells was needed to establish a secondary tumor in immunodeficient mice, it is assumed that ovarian CSCs were just enriched in those cell populations[59]. Also, it was questionable whether tumor cell lines can represent the status of primary tumor cells. Moreover, due to the heterogeneity among individuals, it is important to test CSC markers in significant numbers of patients.

The expression of well-known CSC markers, including, CD44, CD117, CD133, CD24, ABCG2 and aldehyde dehydrogenase (ALDH), on tumor and ascites derived cells from patients diagnosed with ovarian cancer is very diverse and is patient-dependent, and no correlation was found between marker expression and tumor histological subtype[60]. In line with these data, another study investigated epithelial and mesenchymal markers expressed by primary ovarian tumors, and they also showed different phenotypic features and expression levels of those markers in different cellular subsets within tumors[59]. Additionally, it has been reported that the CSC marker ALDH show distinct expression pattern in human epithelial cancers, and it can only be used to isolate CSCs for tumors whose corresponding normal tissues express low levels of ALDH[61]. Also CD133 as a marker to identify ovarian CSCs has been questioned, since tumor initiating activities have been detected in both $CD133^+$ and CD133- fractions from primary ovarian masses, and $CD133^+$ cell frequency varies between patients[45]. Similar doubts of CD133 as a putative CSC marker has been reported in colon cancer and melanoma[56]. Moreover, phenotypic heterogeneity of breast CSCs was also reported[34,40,62]. Taken together, these data suggest that CSC phenotypes are heterogeneous, and experimental variables as well as xenograft recipients can dramatically influence CSC frequency[45]. So far a clear set of marker proteins remain to be identified to target ovarian CSCs.

For better recognition of CSCs, better experimental methods need to be established. One way to identify CSC is to focus on genes involved in stem cell pluripotency, because those genes may be involved in establishment of tumors and may be inherited by their malignant counterparts. Four genes are required for induction of pluripotent stem cells from mouse embryonic or adult fibroblasts *in vitro*, including Oct4, c-Myc, Sox2 and Klf4[63]. A rare cell population, in ovarian tumor tissue as well as ascites, expressing Oct4, Nanog and c-Myc was found. Oct4 expression is crucial for the self-renewing and maintenance of pluripotent properties of embryonic stem (ES) cells [64,65]. The expression of Oct4A indicates that the cells are undifferentiated[66]. Recently, abnormal Oct4 expression level was correlated to several cancers[67-69]. The two isoforms of Oct4, Oct4A and Oct4B, differ in their ability to confer self-renewal, only Oct4A can sustain stem cell properties[70,71]. Several studies have shown that the different isoforms and Oct4 may lead to false positive signals during RT-PCR analysis[72,73]. In order to rule out this, a primer set was described to distinguish the Oct4A from Oct4B and Oct4 pseudogenes[73]. Oct4A mRNA expression was detected by us in ascites-derived tumor cells from all patients tested, regardless of histological subtypes. The c-Myc protein is normally expressed in the nucleus and is virtually undetectable in quiescent cells. It contributes to the long-term maintenance of the ES cell phenotype and is upregulated in many types of malignant human cancers[74]. Moreover, Nanog also sustains ES cell pluripotency[75]. Oct4 and Nanog were described to be higher expressed in side population cells obtained from ovarian cancer cell lines than the bulk of the cells[76], confirming the expression of stem cell markers as described here. To sum up, expression of these genes suggests that those cells are the primitive CSC for ovarian cancer, because all genes needed for reprogramming to induce pluripotent stem are present in the same cell.

According to the hierarchy tumor model, the most "primitive" CSCs are able to self-renew, and develop into more differentiated cells like so-called progenitor cells or CSC-derived transit-amplifying cells, which are not able to self-renew but can generate new tumor cells to support tumor growth[34,48]. In order to adapt to different host microenvironments, CSC-derived progenitors may differ in their phenotypes and functions and in turn differentiate into phenotypically and functionally heterogeneous tumor cells[77]. And a different differentiation status might be generated also to adapt the complicated tumor growth environment[78]. These indicate that CSCs and their progenies may differ between different patient tumors and may be able to change during tumor progression[55]. Collectively, these data may explain why the expression of putative CSC phenotypes are heterogeneous among patients with ovarian cancer and why accumulating evidence shows that solid tumors are initiated by heterogeneous populations of CSCs, and each CSC subset responsible for distinct

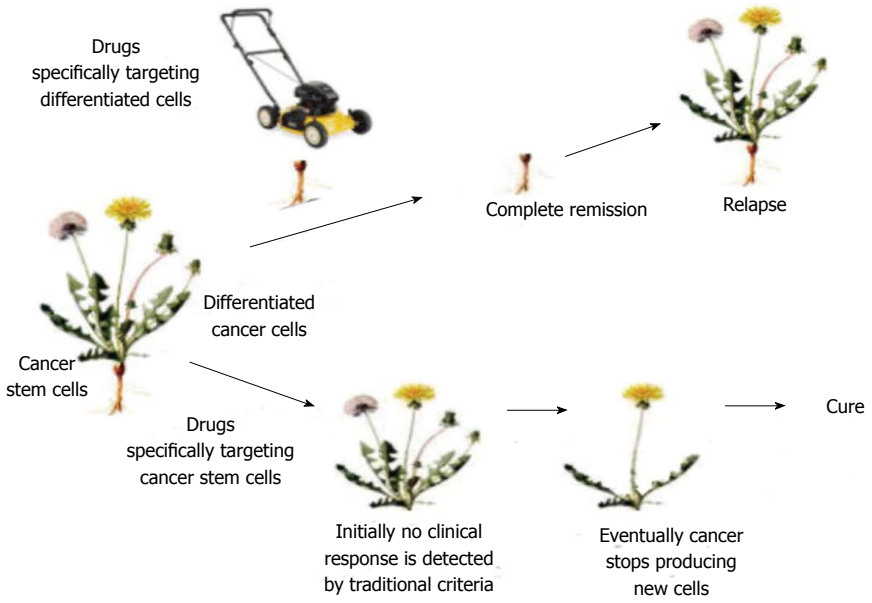

Figure 2 Killing the mature cancer cells leaves the root intact leading to regrowth of the tumor. Killing the root will exhaust the stem cell pool leading to eradication of the tumor. Reprinted from Jones *et al*[154].

functions in tumor progression[33,34,40,45,47,48,50,79-83][Engh, 2011 #756].

Although CSC phenotypes are heterogeneous, current studies suggest ovarian tumor conforms to the CSC hypothesis[45,59], and in this scenario, if the most primitive Oct4-expressing CSC population is eliminated specifically, the tumor will lose its feeding and eventually fade away (Figure 2).

Phenotypic plasticity of ovarian tumor cells

CSC may not be a stable entity. Plasticity describes the dedifferentiation potential of more differentiated cancer cells to acquire stem cell phenotype and characteristics, which further contribute to CSC heterogeneity, and which is an important determinant of the prognosis of tumors[55,84,85]. Thus plasticity in CSCs and their progenies make the situation more complex[51,59]. Two c-Myc expressing populations were found; one is only highly positive for c-Myc, the other also express Oct4. The irelationship between these two subpopulations remains to be investigated. We argue that those intermediate c-Myc$^+$ cells are more differentiated cells than c-Myc$^+$ Oct4$^+$ cells, since in some cases they were not able to survive in serum-free medium. Also, it is possible that the c-Myc$^+$ cells somehow regain Oct4A expression and become a primitive CSC. In fact, phenotypic plasticity of ovarian tumor cells was detected under certain circumstances, *e.g.,* stress created by starvation or co-culture with either epithelial or mesenchymal cells *in vitro*[59].

In line with this, plasticity has been described in other tumor stem cell studies, showing that non-tumorigenic cells can convert to a tumorigenic cell[50,86,87]. For instance, knocking down of JARID1B in slow cycling melanoma cells exhausted the tumor, however, expression of JARID1B is dynamic since negative cells can become JARID1B positive[47]. This indicates that the cancer cells might reversibly transit between tumorigenic and non-tumorigenic status, generate reversible heterogeneity[85,88].

In addition to tumor cells, plasticity was also described in normal development procedures. Endothelial cells could simply be converted into multipotent stem-like cells by Transforming growth factor β2 or Bone morphogenetic protein 4[89]. Also in spermatogonial development more differentiated cells can go back to the stem cell state when the stem cell niche is emptied and the number of stem cells is decreased. In this way the normal number of stem cells is recovered by differentiated cells that regain stem cell properties[90]. Plasticity would have major implications for the CSC model and for future therapeutic approaches, as discussed in[52].

INTERPLAY BETWEEN TUMOR AND THE IMMUNE SYSTEM

The immune system affects cancer development and progression. Before the tumor cells cause clinically detectable disease, they have already resided in the body for a while. The immune system can recognize and interact with the transformed cells before and after the formation of tumormass; this process is termed "cancer immunoediting". Cancer immunoediting consists of three distinct phases: elimination, equilibrium and escape[91,92]. During the elimination phase, tumor specific immune cells and molecules are recruited to the tumor site and destroy the developing tumor cells. The equilibrium phase is a dynamic state; the interaction between tumor growth and immune prevention represents a type of tumor dormancy, in which tumor outgrowth is also limited by the immune system[93]. Meanwhile, due to the immune selection, some malignant cell can acquire the ability to circumvent immune recognition, or no longer sensitive to immune effector mechanisms, and escape. And then their growth is no longer blocked by the host immunity anymore. In addition, the malignant tumor cells can even manipulate the immune system to promote their own growth[91,92].

Immune elimination of tumors

The effectors mechanisms of both cell-mediated immunity and humoral immunity have been shown to kill tumors *in vitro*. In several cases also *in vivo* killing of tumor cells was observed. During the elimination phase of cancer immunoediting, different types of immune cells are recruited to the tumor site, including T cells, antibody-secreting B cells, different subsets of dendritic cells (DCs), tumor-associated macrophages (TAMs), myeloid-derived suppression cells (MDSCs), Th17 cells, natural killer (NK) cells, NK T cells and γδT cells[94,95]. And those intratumoral T cells were functionally active since interleukin-2 (IL-2) and interferon-γ (IFN-γ) was produced, which may enhance T cell proliferation and anti-tumor immunity[96,97].

An effective antitumor immune response is direct killing of tumor cells by $CD8^+$ cytotoxic T lymphocytes (CTLs), which recognize tumor antigens presented by MHC I molecules. $CD8^+$ T cell responses specific for tumor antigens may require cross-presentation of the tumor antigens by professional antigen presenting cells (APCs), such as DCs. Most tumor cells do not express the co-stimulatory molecules needed to initiate T cell responses or the class Ⅱ MHC molecules needed to stimulate helper T cells that promote the differentiation of $CD8^+$ T cells. It is possible that tumor cells or their antigens are ingested by host DCs, the tumor antigens are then processed inside the DCs, and peptides derived from these antigens are displayed bound to class I MHC molecules for recognition by $CD8^+$ T cells. The APCs expressing co-stimulatory molecules that provide the signals needed for differentiation of naïve $CD8^+$ T cells into anti-tumor effector CTLs, and the APCs express class Ⅱ MHC molecules that may present internalized tumor antigens and activate $CD4^+$ helper T cells as well. Once effector CTLs are generated, they are able to recognize and kill the tumor cells without a requirement for co-stimulation. CTLs mediate lysis of target cells by two major mechanisms, the predominant mechanism appears to be perforin-granzyme-dependent, and the other is FasL dependent[98,99]. The ability of CTLs to provide effective anti-tumor immunity *in vivo* is most clearly seen in animal experiments. However, tumor-specific CTLs can be isolated from animals and humans with established tumors, such as melanomas[100].

The importance of $CD4^+$ helper T cells in tumor immunity is less clear. $CD4^+$ cells may play a role in anti-tumor immune responses by providing cytokines for effective CTL development. In addition, $CD4^+$ T cells specific for tumor antigens may secrete cytokines, such as tumor necrosis factor (TNF) and IFN-γ, that can increase tumor cell class I MHC expression and sensitivity to lysis by CTLs. IFN- γ may also activate macrophages to kill tumor cells. In addition to T cells, tumor-bearing hosts may produce antibodies against various tumor antigens[101-104]. Whereas it has also been documented that CD4 T cells can be more effective than CD8 T cells in tumor killing in tumor bearing mice[105]. Moreover, NK cells may kill many types of tumors, especially "missing" cells that have reduced class I MHC expression and can escape killing by CTLs[106,107]. $CD4^+$ T cells cooperate with NK cells to accomplish the maximum tumor killing[105]. Macrophages can kill many tumor cells more efficiently than they can kill normal cells[108]. Several studies showed the existence of tumor infiltrating T cells in ovarian cancer associated with favorable clinical outcome[109,110]. Distribution of tumor infiltrating lymphocytes (TILs) were studied in patients with late stage ovarian cancer, $CD3^+$ T cells were detected in more than 50% of the patients and $CD4^+$ and $CD8^+$ T cells were either both present or absent. The presence of TILs correlates with a better 5 year survival as well as progression-free survival[39]. It has also been documented that patients with higher TIL counts showed improved overall survival than patients with lower TIL counts[111]. Moreover, Sato and co-workers demonstrated intraepithelial $CD8^+$ TILs and the high $CD8^+$ TIL/Treg ratio indicates better survival of ovarian cancer patients[112].

Immune reactivity towards CSCs

When the immune system is directed to eliminate the CSC, it will also destroy CSC reverting from more differentiated progeny. We consider Oct4 as a suitable antigen for immunological targeting ovarian CSCs, since it is neither expressed in normal adult stem cells nor somatic cells. Once the progenitors re-express Oct4 and become CSCs, they can be recognized and eliminated by Oct4-reactive T cells. Removing of the CSCs from the pool will diminish the feeding of more mature tumor cells. Further understanding of the relationship between CSCs and their differentiated progenies can help us to develop better immunotherapeutic strategies that can prevent the emergence of tumor cell variants that are capable of generate a new tumor and metastases[55,113].

OCT4-REACTIVE T CELLS ARE DETECTABLE

Naturally occurring T cells directed against tumor-associated antigens (TAAs) can be frequently detected in cancer patients (reviewed in[114]). Amazingly, Oct4 reactive $CD4^+$ as well as $CD8^+$ T cells were detected in both healthy people and patients with ovarian cancer[115]. This finding suggests that the host immune system has the ability to target the primitive ovarian CSCs. The frequency of Oct4 specific T cell was low in peripheral blood, while it was higher in the ascites of patients. This means those cells are either recruited to the tumor or proliferate upon exposure to Oct4. Moreover, lymphocytes isolated from ascites from patients with ovarian tumor contained Oct4 specific T-cells. It was shown that Oct4-reactive $CD8^+$ T cells produce IFN-γ-inducible protein 10 (IP-10) and IFN-γ, and were capable of proliferation upon Oct4 peptide loaded or Oct4 mRNA pulsed dendritic cell stimulation. The $CD8^+$ cytotoxic T cells were able to release lysosomal components as indicated by CD107a expression. Moreover, Oct4-reactive $CD4^+$ T cells were also detected,

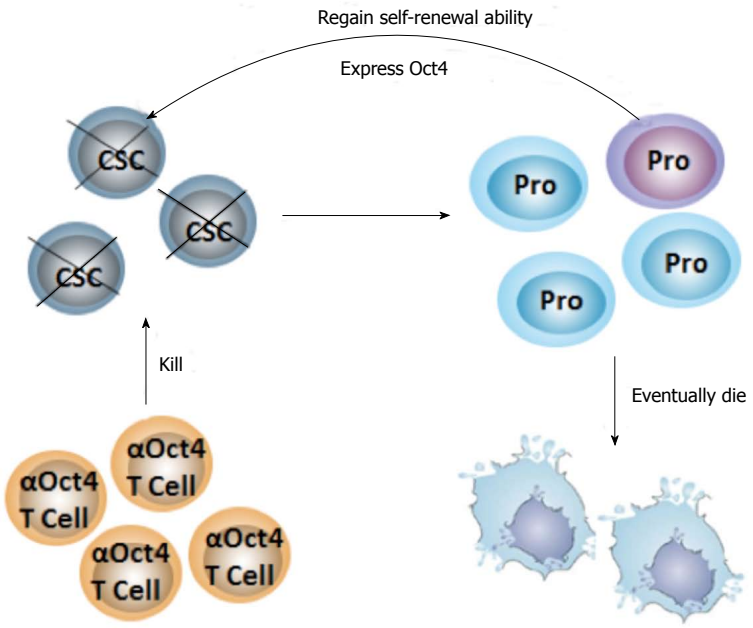

Figure 3 Hypothesis of specific targeting of primitive cancer stem cells. In a non-immunosuppressive tumor microenvironment, Oct4-specific T cells (αOct4 T cell) can recognize the primitive cancer stem cells (CSCs). and destroy them. Progenitor cells (Pro) differentiate to more mature tumor cells and will eventually undergo apoptosis or necrosis. Once some progenitors regain the self-renewal machinery and re-express Oct4 to become a CSC, T cells will also eliminate it. In this way, the tumor loses its ability to generate new tumor cells.

and also capable of proliferating upon stimulation. These results proved the existence of anti-CSC specific T cells in patients with ovarian cancer.

Natural immunity against genes involved in pluripotency has been shown. Dhodapkar *et al*[115] claim the Oct4 responsive T cells were detected in PBMCs from 83% of healthy donors, although they showed the Oct4-specific cells were $CD4^+$ T cells. They also found 38% of patients with germ-cell tumors had measurable Oct4-specific T cell immunity at baseline, and after chemotherapy, 83% of the patients developed Oct4-reactive T cells. Also, it has been documented that $CD8^+$ Sox2-specific T cells were frequently detected in patients with monoclonal gammopathy of undetermined significance (MGUS). MGUS is a precursor lesion to myeloma, whereas Sox2-specific T cell immunity was not detectable in patients with myeloma[116].

Taken together, these data indicate that the ovarian CSCs are prone to immunological attack because CSC specific T cells are present in the T cell repertoire (Figure 3). Meanwhile, this raises the question about why CSCs and their progenies escape from immune elimination, and why the already activated Oct4-reactive memory T cells do not kill those cells.

Immune escape by tumors

Many malignant tumors possess mechanisms that enable them to disturb the balance in the equilibrium phase and shift to escape phase, including down-regulation of MHC Ⅰ expression on tumor cells, loss or hidden of tumor-antigen expression, production of immune suppressive molecules, and inhibition of co-stimulatory or MHC Ⅱ molecules expression on APCs, leading to immunologic tolerance[92,117,118]. Tumors escape not only from the host immune system, but also effectively benefit from infiltrating cells and create a microenvironment that favors its progression by modifying TIL functions[119]. Ovarian tumor can effectively create its suppressive microenvironment. Curiel *et al*[120] showed the first evidence that tumor associated $CD4^+CD25^+$ regulatory T cells (Treg) were correlated with a poor clinical prognosis of ovarian cancer. They showed the presence of Treg in both tumor tissue and malignant ascites, and also proved that tumor cells and microenvironmental macrophages produced the chemokine CCL22, which attracted Tregs to the tumor site. Tumor infiltrating Tregs suppress tumor-specific T cell immunity by blocking T cell proliferation as well as IFN-γ and IL-2 production. Similarly, Woo *et al*[121] found that $CD4^+CD25^+$ Tregs contribute to $CD8^+$ T cell dysfunction by secreting the immunosuppressive cytokine transforming growth factor-β (TGF-β). Later on, forkhead box protein-3 (FoxP3) expressing Tregs were also detected and emerged as an independent prognostic factor for both poor progression-free and overall survival[122]. Conrad et al. demonstrated that majority of these $FoxP3^+$ Tregs accumulated nearby the tumor and also express inducible co-stimulator (ICOS)[123]. The expansion and immunosuppressive function of these $FoxP3^+ICOS^+$ Treg cells are dependent on their interaction with plasmacytoid DCs (pDCs) which provide ICOS-ligand (ICOS-L) stimulation. The presence of immature pDCs was also found in the vicinity of ovarian tumor and associated with poor clinical outcome of patients with ovarian tumor[124]. pDCs are recruited by CXCL12 produced by tumor cells and produce type Ⅰ IFN in response to toll-like receptor (TLR) ligand triggering[125,126]. In addition to $CD4^+$ Tregs, $CD8^+$ Tregs also exist in ascites produced by malignant ovarian tumor. Wei et al. showed that tumor pDCs induce suppressive $CD8^+$ Tregs in ascites. These $CD8^+$ Tregs inhibit T cell proliferation and IFN-γ production, while they induce IL-10 production[126]. Moreover, ovarian tumor infiltrating DCs express programmed death 1 (PD-1), which interacts with B7-H1 on tumor-associated macrophages. This reaction can lead to suppressed NFκB

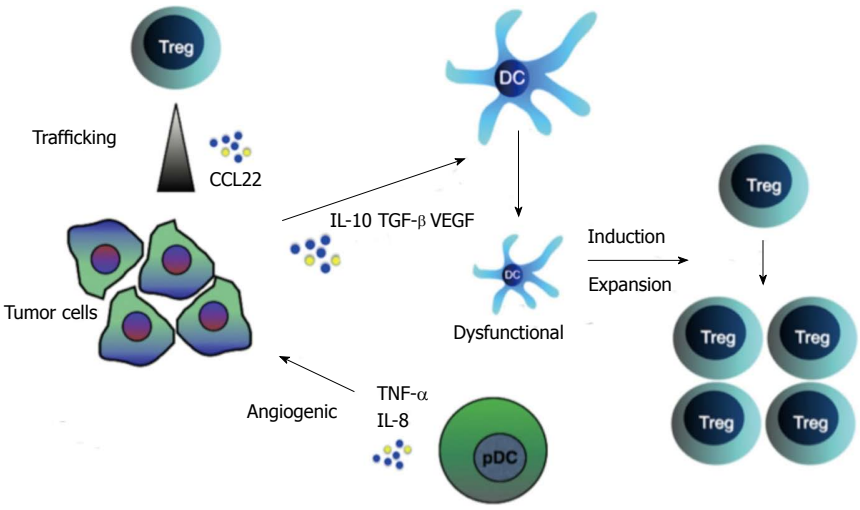

Figure 4 Immune-suppressive pathways in ovarian cancer. Tregs are attracted to the tumor environment by CCL22, secreted by the tumor. The tumor microenvironment expresses molecules that can convert functional antigen presenting cells (APCs) into dysfunctional ones. These dysfunctional APCs in turn stimulate Treg differentiation and expansion. pDCs are also present in the tumor environment and stimulate tumor growth by releasing tumor necrosis factor-α and interleukin-8. (modified from[132]). pDCs also facilitate immunosuppressive function of FoxP3$^+$ICOS$^+$Treg[123].

activation and downregulated co-stimulatory molecule expression on DCs[127] (Figure 4).

Ovarian tumor infiltrating T cells are anergic

A remarkable characteristic of ovarian cancer is the typical metastasis behavior. Metastases are found but hardly in other organs. As the tumor spreads in a diffuse intra-abdominal fashion and even after recurrence, it is in most cases confined to the peritoneal cavity. There are several papers that report the presence of metalloproteases in ascites[128-130]. Those enzymes are found in metastasizing tumors by chopping tissues to make room for the metastasis. Moreover, ovarian tumors orchestrate suppressive mechanisms that enable them to evade or resist host immune responses[131-135]. The fact that CTLs against human tumors can be easily generated *in vitro* using peripheral blood lymphocytes indicates that the tumor microenvironment has immunosuppressive capacities[131]. Tumor infiltrating immune cells together with fibroblasts and extracellular matrix form a scaffold supporting tumor cell expansion, contribute to establish an inflammatory milieu that nourishes the tumor and promotes its growth[131,136]. And apparently, the weak anti-CSC immunity generated by Oct4-reactive T cells is counterbalanced (Figure 5). Collectively, this metastasis behavior suggest that as soon as tumor cells escape from the immune suppressive microenvironment in the peritoneal cavity and enter sites where full immune responses are possible in the periphery, they cannot survive[132,134,137]. This opens enormous possibilities to treat patients by boosting the immune response.

The assumption that without this suppressive microenvironment the immune system is able to eradicate tumor cells needs further prove. Furthermore, as argued for immunotherapy, only boosting the antitumor immune response is not enough. It is of great importance to "repair" the already existing tumor specific T cells *in vivo*. It was found that ovarian tumor infiltrating lymphocytes fail to proliferate in response to CD3/CD28 stimulation and adding IL-2 cannot reverse this unresponsiveness. The inhibited T cell proliferation was due to reduced cyclin E expression (unpublished data). So even though the host immune system can recognize the tumor, they lack the ability to eliminate it. The observed effects were reversible after culture of the cells *ex-vivo* for 10 d. This demonstrates that the impaired functions are reversible and can be repaired. The results are in line with recent findings from other groups proved that TIL isolated from melanoma, oral carcinoma, colorectal carcinomas were also functionally impaired, as manifested by decreased proliferative responses and decreased ability to mediate cytotoxicity[138]. Abnormalities in signal transduction molecules associated with reduced expression of T-cell receptor (TCR) ζ chain[139] and/or hampered Fas/FasL signaling pathway[140]. Moreover, it has been shown that T cells isolated from ascites of patients with ovarian tumor were deficient in expression of ζ chain, lower basal levels of protein tyrosine phosphorylation, altered patterns of protein phosphorylation when stimulated via surface CD3 or CD16, and declined expression and kinase activity of p56^{lck}. These deficiencies in expression and function of signaling molecules were associated with reduced proliferation and an altered profile of cytokine secretion by the NK or T cells isolated from ascites and stimulated with IL-2 or by cross-linking of surface CD3[141]. In addition, tumor-associated CD8$^+$ T cells might be dysfunctional due to upregulation of programmed death 1 (PD-1) and T cell immunoglobulin and mucin-domain-containing molecule 3 (Tim-3)[142,143]. We could not detect PD-1 expression in ascites-derived lymphocytes, however, both ascitic CD4$^+$ and CD8$^+$ cells showed upregulation of Tim-3 (Figure 6). These findings indicate that infiltrated immune cells are not only suppressed, but also impaired in their signaling pathways resulting from the yet unknown factors present in tumor associated ascites.

Furthermore, except for harming of immune cells, ovarian cancer cells also secrete immunosuppressive and pro-inflammatory cytokines into the tumor microenvironment to support tumor growth[144,145]. Previous studies demonstrated that IL-6 is significantly increased in cyst fluid, serum as well as ascites of patient with advanced

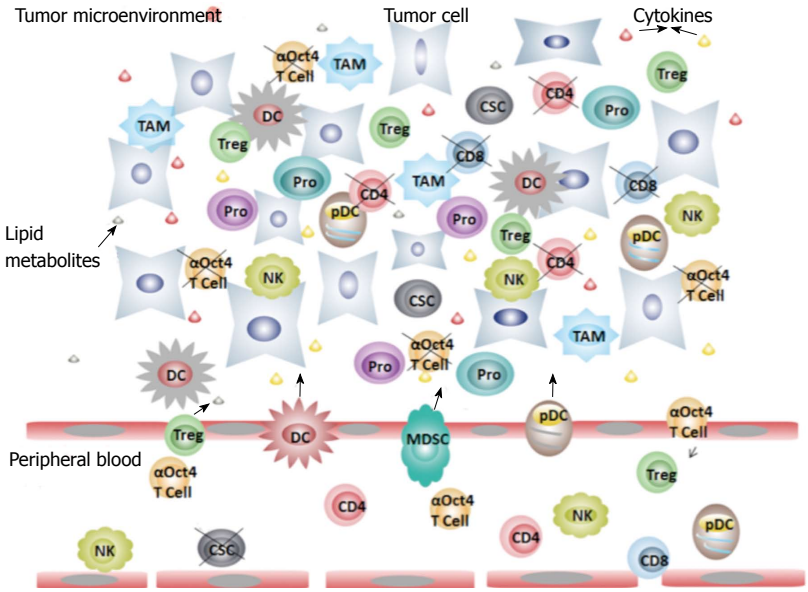

Figure 5 Dysfunctional immune system in peritoneal cavity of patients with ovarian cancer. Many types of immune cells are recruited to the ovarian tumor site, including regulatory T cells (Treg), dendritic cells (DC), tissue associated macrophages (TAM) myeloid derived suppressor cells (MDSC)[155], plasmacytoid DCs (pDC), natural killer cells and T cells (CD4, CD8). Once being recruited, most cells function abnormally and become immune suppressive. T cells specific for Oct4 (αOct4 T cell), $CD4^+$ T cells, $CD8^+$ T cells are damaged, due to dysfunctional DCs, pDCs and suppressive Tregs. Also the secretion of immune suppressive cytokines and lipid metabolites contribute to establish such an immunosuppressive tumor microenvironment, and may also be required for cancer stem cells (CSCs) maintenance. So even if the CSC is recognized, T cells lack the ability to eliminate it. Whereas such suppression mechanisms are not operative in the peripheral blood of the patients, once the CSC migrates to the peripheral, it is killed.

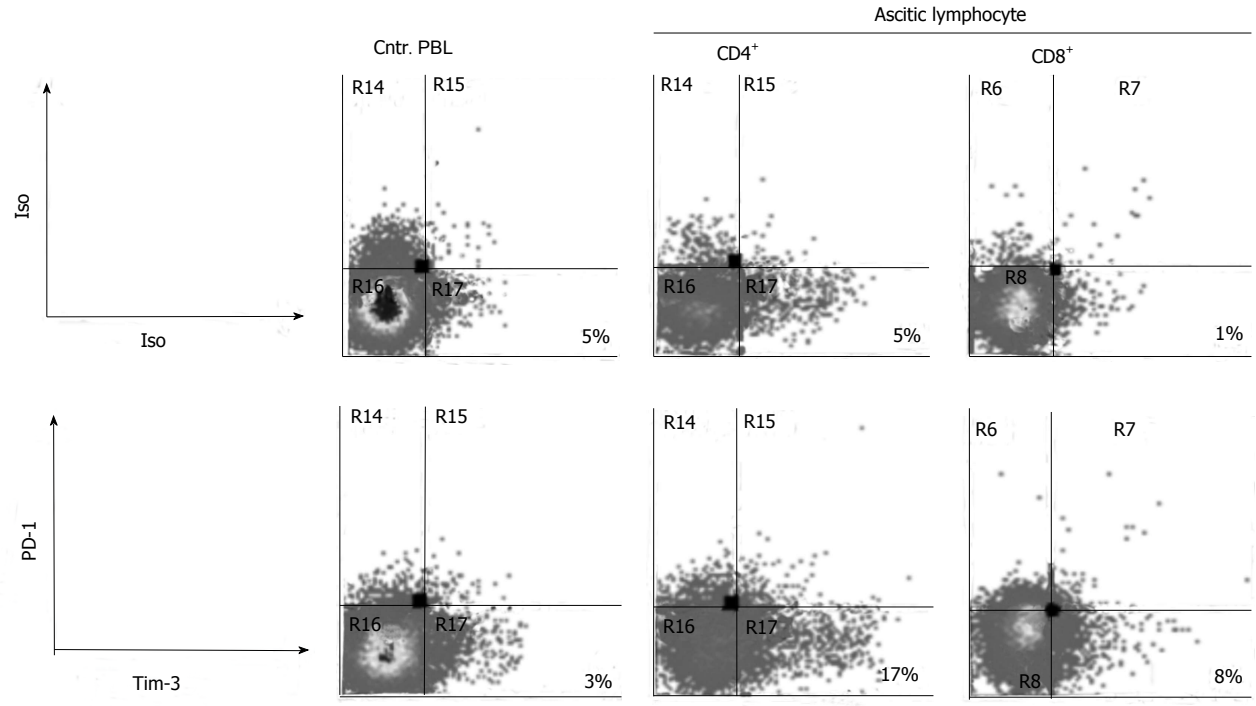

Figure 6 PD-1 and Tim-3 expression by ascitic lymphocytes from patients with ovarian cancer. PD-1 expression was undetectable in ascitic lymphocytes. Compare to isotype control, peripheral blood lymphocyte (PBL) from healthy express 2% Tim-3, ascitic $CD4^+$ and $CD8^+$ cells express four times more Tim-3 than control PBL.

ovarian cancer, and associated with poor prognosis[144,146]. IL-6 is a pro-inflammatory cytokine. It has multiple effects on T cell function, and it has already been reported to be an important factor in promoting the progression of epithelial of ovarian cancer[147]. IL-6 also plays a role in enhancing tumor growth by inducing abnormal c-Myc expression *in vitro*. It has been shown that IL-6 can induce c-Myc translation in multiple myeloma cells and meanwhile c-Myc is shuttled to cytoplasm by the RNA-binding protein, hnRNP A1[148]. Our research demonstrated that c-Myc was expressed in both nucleus and cytoplasm in ovarian tumor tissue as well as ascitic cells, while c-Myc is only expressed in the nucleus of normal stem cells. Similarly, except for being expressed in the nucleus, c-Myc was also detected in the cytoplasm of leukemia patients[149]. Regulation of stem cell genes or even tumor development by cytokine indicates a strong correlation between the tumor and its microenvironment. Taken together, these results indicate that in addition to its suppressive property, the tumor successfully creates a favorable microenvironment to support tumor growth.

In conclusion, ovarian cancer is an extremely complicated disease, because the tumor growth might be driven by heterogeneous CSCs and multiple immunosuppressive

mechanisms are functional in the abdomen. To enable an immunological attack on CSC either the response has to strengthened or the immunosuppressive milieu has to be reversed or both.

FUTURE PERSPECTIVES

For future studies, it is of great importance to investigate how somatic cells are reprogrammed *in vivo* to become malignant pluripotent cells, and how the self-renewal pathways are orchestrated in such transformed cells. Furthermore, it remains unclear why the pluripotent genes were upregulated in a small subset of tumor cells. We sequenced both Oct4 and c-Myc isolated from ovarian patient ascitic cells, however, no mutation was found (unpublished data). It is important to elucidate what went wrong in the self-renewal pathways in the patients and why. Understanding this might help to stop tumor growth before it happens.

Another challenge is how to boost the favorable host immune response in the suppressive tumor microenvironment and train the immune system to fight against ovarian cancer. To overcome this, it is of great importance to determine the mechanisms that contribute to protective immune responses against tumors and to enhance these effector mechanisms in a tumor specific way. And apparently, only boost the immune system is not enough to eliminate tumors, due to functional crippling of TILs.

Moreover, the role of ascites in tumor progression remains to be elucidated. Ascitic fluid is produced by ovarian tumor. The cellular fraction of ascites consists of tumor cells, lymphocytes and mesothelial cells; and the acellular fraction harbors cytokines, growth factors, bioactive lipids, angiogenic factors, and extracellular matrix constituents[150-152]. Although the role of ascites as tumor cell microenvironment remains poorly understood, recent research suggests that it may affect cell growth, invasion and induction of resistance of ovarian cancer cells and thus may play a decisive role in ovarian tumor progression[153].

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