ABSTRACT

The tumor immune microenvironment (TIME) is the cellular environment in which tumors exist. This includes: surrounding blood vessels, immune cells, fibroblasts, bone marrow–derived inflammatory cells, lymphocytes, signaling molecules, immune checkpoint proteins and the extracellular matrix (ECM). The TIME plays a critical role in cancer progression and regulation. Tumors can influence the microenvironment by releasing extracellular signals, promoting tumor angiogenesis and inducing peripheral immune tolerance, while the immune cells in the microenvironment can affect the growth and evolution of cancerous cells. The molecules and cells in the TIME influence disease outcome by altering the balance of suppressive versus cytotoxic responses in the vicinity of the tumor. Having a better understanding of the tumor immune microenvironment will pave the way for identifying new targets for immunotherapies that promote cancer elimination.

Keywords: tumor immune microenvironment, immune checkpoints, tumor metastasis, cancer immunotherapy

INTRODUCTION

An established tumor is a complex tissue, comprising not only of tumor cells but also stromal cells, vasculature, extracellular matrices (ECM) and immune cells [1], which can either promote or hinder tumor growth, progression and metastasis. The cross-talk between immune cells and tumor cells has hampered progress in understanding the mechanisms of immunosuppression by which tumor cells circumvent immune responses. Immunoescape is one of the cancer hallmarks that have been proposed in the last decade, which provide a solid foundation for understanding the biology of cancer. Inflammation in the development of cancers has been implicated since the seminal observation made by Virchow in as early as 1863, in which chronic inflammation was shown to create a microenvironment conducive to tumor genesis [3]. This microenvironment is named the tumor immune microenvironment (TIME), and has recently raised much interest. The communication between tumor cells and the TIME is a dynamic process. When the anti-tumor immune response is strong, the cancer stays under control. However, multiple elements of suppression play important roles in the TIME, including immune cells such as T regulatory cells, myeloid suppressive cells, tumor–associated macrophages, and mast cells, and molecules such as checkpoints that control T–cell differentiation (for example, CTLA–4) and effector functions (such as PD–1) [4]. A pharmacological blockade of these inhibitory elements can tip the balance toward anti-cancer effector T cells [5]. Thus, a better understanding of TIME, including classes of TIME, immune cells and immune checkpoints, relationships between tumor genotypes and TIME, as well as the function of TIME in tumor metastasis may benefit the efficiency of cancer immunotherapy.
CLASSIFICATION OF THE TIME

Checkpoint blockade therapy has been proven to be active in many cancer types, but emerging evidence shows it limits the therapeutic benefit to a subset of patients in each cancer entity[6]. It is necessary for us to classify the TIME to make a better understanding about why some patients show no response to checkpoint blockade therapy. The TIME can be characterized into three classes.

Infiltrated–excluded (I–E) TIME is the TIME populated with immune cells, but relatively void of cytotoxic lymphocytes (CTLs) in the tumor core[7]. I–E TIME is associated with various epithelial cancers, in which tumor-associated macrophages (TAMs) along the tumor margin are able to prevent CTLs from entering the tumor core[8]. I–E TIME contains CTLs with low expression of the activation-marker GZMB (GRZB) and IFNG and poor infiltration of CTLs into the tumor core[7]. The lack of activation-marker expression and exclusion from the tumor core shows immunological ignorance, in which state adaptive immunity cannot recognize or respond to a pathogen or malignancy[7]. I–E TIME lacks not only T cells but also the up-regulation of immune inhibitory mechanisms[9]. Therefore, it is not surprising that checkpoint blockade is not effective against this subgroup of patients[10].

Infiltrated–inflamed (I–I) TIME is characterized by a high infiltration of CTLs expressing PD–1 and leukocytes and tumor cells expressing the immune–dampening PD–1 ligand (PD–L1)[8]. Immune escape mechanisms that allow the co–existence of an anti-tumor immune response and the tumor itself in I–I TIME have been reported in several papers[9-11]. CTLs in I–I TIME are related to the up–regulation of immune inhibitory mechanisms which can eliminate immunogenic tumor cells and leave only non–immunogenic tumor cells. This process displays a potent form of immune escape. Immune suppression in I–I TIME is being harnessed to activate the endogenous T–cell response to the blocking of immune inhibitory mechanisms[12].

A subclass of I–I TIME, termed TLS–TIME, contains tertiary lymphoid structures (TLSs), including and activated conventional T cells, regulatory T cells, B cells and dendritic cells (DCs)[13]. TLSs also act as sites of lymphoid recruitment and immune activation in the settings of enhanced inflammation[13]. TLSs allow T cells to differentiate into effector cells upon contacting with mature DCs and B cells[14]. Thus, TLS represents sites for anti–tumor responses, which confer long–term protection against metastasis and correlate with a good prognosis for the patients. In conclusion, the classifications of the TIME can improve the understanding of how the different kinds of tumor immune microenvironment affect the anti–tumor responses or even the immune escape responses.

IMMUNE CELLS OF THE TIME

Tumors are organized tissues with numerous reciprocal local and systemic connections with immune cells including T lymphocytes, B lymphocytes, as well as myeloid cells including macrophages, myeloid–derived suppressor cells, DCs, and mast cells[15]. These comprise the tumor immune microenvironment (Fig. 1).

Many tumors express antigens that can be recognized by T lymphocytes, suggesting that T cell–mediated immunity may be harnessed for the immunotherapy of cancers[16]. Within the TIME, many T cells have been observed, especially at the invasive tumor margin and in draining lymphoid organs. Among these, CD8+ memory T cells and CD4+ T helper 1(Th1) cells function as the major anti–tumor immune effector cells, which secret many cytokines that can prevent and suppress the development of cancers[17]. CD8+ effector T cells can recognize endogenous peptides displayed on the surface
of tumor cells within the groove of HLA class 1 molecules. After CD8⁺ T cells recognize tumor specific peptides, they induce the killing of target tumor cells, or promote tumor destruction via the secretion of effector cytokines such as interferon-γ (IFN-γ) and TNFα. After activation, naïve CD4⁺ T cells will differentiate into various subsets of T helper cells, including Th1, Th2, and Th17 cells. Th1 cells produce IFN-γ and function as immunity against intracellular pathogens. Th2 cells are able to assist B cells for the production of antibodies. Th17 cell can either suppress tumor cells by promoting the activation of tumor-specific CD8⁺ T cells and recruiting of DCs into TIME or exhibit pro-tumor functions of certain cytokines. Regulatory T cells (Tregs) inhibit the expression of co-stimulatory molecules on the surface of DCs, thus inactivate T cells. Tregs also suppress Th1, CD8⁺ T cells and B cells activity in different ways. B cells in the tumor immune microenvironment can promote disease progression by secreting cytokines.

Studies suggest that among myeloid lineage cells, myeloid derived suppressive cells (MDSCs), mast cells, and most TAMs promote tumor development in the TIME. Human MDSCs can be divided into three subsets, M–MDSCs, PMN–MDSCs and Lin–HLA–DR–CD33⁺ MDSCs. PMN–MDSCs suppress immune responses by producing TGF–β or inducing nitration of T–cell receptors (TCR), which makes T cells unresponsive to antigen stimulation. M–MDSCs can differentiate into TAMs, which promote T cell apoptosis and lead to immune suppression. MDSCs produce large numbers of MMPs, which has a profound impact on tumor progression and metastasis through modulation of tumor angiogenesis and tumor cell invasion.

Macrophages differentiate into two major subsets, including the pro-inflammatory M1-type and anti-inflammatory M2-type. M1-type macrophages are activated by IFN-γ and lipopolysaccharide and are characterized by their high expression of IL–12 and low expression of IL–10. This cytokine profile promotes the development of a type 1 T cell response which facilitates anti-tumor immunity. The TIME can polarize macrophages towards an M2-type, giving rise to TAMs. M2-type macrophages are activated by IL–4, IL–13, IL–10 and glucocorticoid, which can produce high levels of IL–10 and low levels of IL–12 and promote tumor progression.

The major functions of DCs are to process and present antigen for the CD4⁺ and CD8⁺ T cells. DCs in the TIME act as effective antigen-presenting cells (APCs) inducing specific anti-tumor immune responses. However, DCs often have impaired functions even if they recognize tumor antigen, because DCs may remain immature or may gain the expression of various immunosuppressive molecules. Multiple conditions and factors within the TIME can cause DC abnormalities. The number of DCs often decreases due to tumor-induced immunosuppression. In recent years, DCs presenting tumor-specific antigens are being developed as vaccines to induce immune responses to regress tumors and prevent relapse.

 Mast cells (MCs) infiltrating in the TIME have been found related to tumor development, meaning that MC has become a novel prognostic marker. Mast cells can induce the release of pro-angiogenic factors such as Ang–1 and VEGF–A. Mast cells are also APCs, interact with both T cells and B cells, and modulate immune responses. Mast cells can recruit eosinophils, neutrophils, and activate anti-tumor adaptive T and B cell responses. However, the recruitment of Tregs into the Tumor microenvironment is mediated by mast cell–derived adenosine. Thus, mast cells play a role in either promoting angiogenesis, or regulating the anti-tumor immunity. Investigating the role of immune cells in the TIME may provide a gateway to the development of innovative and effective strategies for cancer immunotherapy.

**IMMUNE CHECKPOINTS OF THE TIME**

Immune checkpoints are important immune regulators in maintaining immune homeostasis and preventing autoimmunity. Under normal circumstances, immune checkpoints allow the immune system to respond to infection and malignancy while protecting tissues from any harm that may derive from the condition. However, some of these immune–checkpoint proteins expressed on malignant cells may deregulate antitumor immunity and favor tumor growth. So far, many immune checkpoint molecules have been studied, such as PD–1, PD–L1/2, cytotoxic T lymphocyte antigen–4 (CTLA–4), lymphocyte–activation gene 3 (LAG–3), T–cell immunoglobulin and mucin–domain–containing molecule–3 (TIM–3), B and T lymphocyte attenuator (BTLA), and so on.

Present immune checkpoint molecules research is mainly focusing on CTLA–4, PD–1 and its ligands PD–L1 (B7H1) and PD–L2 (B7–DC). CTLA–4 is thought to play a role in regulating the magnitude of early T cell activation, while PD–1 is expressed upon T cell activation and regulates effector T cell activity, and restricts CTLA–4 expression only to T cells; while PD–1 is found on T cells, B cells and NK cells. CTLA–4 is a cell–surface receptor expressed by acti–
vated T cells with homology to the T cell costimulatory molecule CD28. Although CD28 and CTLA-4 are both ligands for B7-1 (CD80) and B7-2 (CD86), they serve opposing roles in regulating T cell activation. CD28 provides costimulatory signals required for T cell activation while CTLA-4 modulates T cell responses by raising the activation threshold for T cell priming.

In response to immune attack, cancer cells over-express PD-L1. They bind to the PD-1 receptor on T cells, inhibiting the activation of T-cells, thus suppressing T-cell attack and inducing tumor immune escape. Tumor cells form a suitable tumor immune microenvironment and continue to proliferate. The PD-1/PD-L1 pathway regulates immune suppression by multiple mechanisms, specifically the performance of: ① CD28, which sustains T cell survival by a driving expression of the anti-apoptotic gene Bcl-xL. PD-1 prevents Bcl-xL expression by inhibiting PI3K activation (which is essential for upregulation of Bcl-xL). PD-1 induces apoptosis of activated T cells. ② PD-L1 is able to promote the generation of induced Tregs by down-regulating the mTOR, AKT, S6 and the phosphorylation of ERK2 and increasing PTEN, thus restraining the activity of the effector T-cell. ③ PD-1 inhibits the proliferation of T cells by preventing the phosphorylation of PKC-theta, which is essential for IL-2 production, and arresting T cells in the G1 phase. ④ The exhaustion of tumor infiltrating lymphocytes (TILs) in the TIME is closely related to the PD-L1 expression of tumor cells or myeloid cells derived from tumor. ⑤ PD-1/PD-L1 blocks the downstream signaling events triggered by Ag/MHC engagement of the TCR and co-stimulation through CD28, resulting in impaired T cell activation and IL-2 production.

There are also some newly emerging immune check-points, such as LAG-3, BTLA, and TIM-3.

LAG-3 is a single transmembrane protein with the three-α extracellular domains, which expresses on activated T cells, regulatory T cells, NK cells, DCs, and B cells. LAG-3 acts to inhibit the immune response by inhibiting effector T cell killing, and through Treg-mediated immune suppression.

BTLA is an immunoglobulin domain-containing glycoprotein expressed on T cells, resting B cells, macrophages, and DCs. BTLA acts as an inhibitory receptor on T cells, and anti-BTLA treatment can cause T cell proliferation. TIM-3 is upregulated in various types of cancer, which can inhibit Th1 cell responses.

**TUMOR GENOTYPES AND THE TIME**

To have a better understanding of the tumor developing mechanisms, we have to figure out how tumor onco-genes and mutation landscapes determine the composition of the TIME. There are several examples showing relationships between tumor genotypes and the TIME.

Deficient DNA mismatch repair (MMR) results in a strong mutate phenotype known as microsatellite instability (MSI), which is characterized by length alterations within simple repeated sequences. In MSI cancers, MMR deficiency generates many aberrant proteins truncated by frameshift mutations. Inactivating mutations in the HLA and β2-microglobulin genes are often observed in MSI cancers, by which cancer cells may escape immune recognition by CTLs. Analyses of tumor genotypes have revealed that most tumors within the Th1/CTL immune microenvironment have defects in mismatch repair, as evidenced by MSI. To counterbalance this active Th1/CTL immune microenvironment, MSI cancers selectively showed highly upregulated expression of multiple immune checkpoint proteins, such as PD-1, PD-L1, CTLA-4, and LAG-3. These findings link tumor genotype with the immune microenvironment and explain why MSI tumors are not naturally eradicated, despite the active Th1/CTL microenvironment.

**BRAF** is a mutated and oncogenic form of the MAPK family member BRAF in melanoma. In BRAF mutant melanoma patients, MAPK pathway inhibitors increase the number of TAMs and TNF-α expression. BRAF has also been shown to drive expression of IL-6, IL-10 and VEGF, cytokines that promote a tolerogenic monocyte–derived DC phenotype *in vitro*, a process that affects anti-tumor T cell
function in vivo[51].

The tumor suppressor p53 is the most mutated gene in human cancer[52]. Compelling evidence suggests that p53 mutation leads to the gain-of-function or activation of non-autonomous pathways, which either directly or indirectly promote tumorigenesis[53]. P53 dys-function in various cellular compartments of the tumor immune microenvironment leads to immune suppression and immune evasion[54].

Multiple reports have showed that the oncogene KRAS[52] can drive pancreatic ductal adenocarcinoma cells (PDAC) to secrete high levels of the growth factor GM-CSF, which is associated with an increase in tumor-associated CD11b+ myeloid cells with reported immunosuppressive function[54]. These data show that oncogenes promote the establishment of an immunosuppressive TIME, which is likely to support malignant development. Therefore the systematic analysis of the correlation between cancer genome and the TIME will generate new perspectives on the comprehension of the carcinogenesis and cancer treatment.

CHARACTERS OF THE TIME DURING TUMOR METASTASIS

Tumor metastasis is responsible for more than 90% of cancer mortality, yet still remains the least understood stage of tumor development. It has been proven that primary tumors can secrete cytokines, growth factors and proteases that mediate the establishment of the specific TIME in distant organs that are sites of future metastasis, in terms of the formation of the "pre-metastatic niche."[55]

Epithelial-to-mesenchymal transition (EMT) of tumor cells is associated with distant metastasis. During EMT, the tumor cells undergo morphological changes that confer enhanced motility and reduced intercellular adhesion. It is reported that tumor recruitment of MDSC promotes EMT[56]. Mast cells in the breast tumor microenvironment secrete tryptase, which results in enhanced rates of metastasis to the lymph nodes and high-grade breast tumors[57]. In the poly-some middle T antigen (PyMT) model of breast cancer, studies showed that macrophages exposed to IL-4 are able to upregulate the expression of cysteine protease cathepsin B and lead to increased lung metastases[58]. In the colorectal cancer model, immature CCR1+ myeloid cells recruited from bone marrow migrate toward the CCR1 ligand (CCL9) expressed in the tumor epithelium and also promote tumor invasion[59]. Immunosuppressive CD4+ T cells recruited to the TIME can secrete IL-4 and IL-13 to convert tumor suppressive M1 macrophages to a tumor-promoting M2 phenotype, which have been shown to support breast cancer metastasis to the lung[60]. In particular, breast cancer cells are able to recruit tumor-associated macrophages through the production of CSF1 and CXCL12. Lewis lung carcinoma (LLC) cells can activate macrophages by the secretion of TNF-α and IL-6, which induce invasion and metastasis by signaling to toll-like receptor 2 (TLR2) and toll-like receptor 6 (TLR6)[61]. These data emphasize that the immunosuppressive mediators in the TIME may encourage the tumor metastasis.

THE TIME AS A TARGET FOR IMMUNOTHERAPY

Immunotherapy represents the third important historical wave in the systemic treatment of cancer after chemotherapy[62], its first clinical application was in the late 19th century when William B. Coley observed tumor shrinkage (and in some cases disappearance) following the injection of bacterial products in and around tumors.

Immune checkpoint therapies that use blocking antibodies for CTLA-4, and PD-1 have shown impressive efficacy in clinical trials. Cancer Immunotherapy was voted as 2013’s Breakthrough of the Year by the journal Science[63]. In 2011, the US Foods and Drug Administration (FDA) approved anti–CTLA-4 antibodies (ipilimumab) for use of treating melanoma, which marked the beginning of a new era in cancer immunotherapy[64]. The FDA also approved sipuleucel-T (trade name Provenge®) for advanced prostate cancer[65]. Leukocyte migration are regulated by chemokines, which raises the idea that manipulating the TIME chemokine profile may recruit sufficient numbers of effector cells into tumor tissues for tumor destruction[66]. CXCL10 is a kind of chemokine that recruits T cells and dipeptidyl peptidase 4 (DPP4) (an enzyme that inactivates CXCL10 in tumor tissues)[67]. DPP4 inhibitors can increase the number of tumor-infiltrating T cells and can also be used in combination with other immunotherapies[68]. In contrast to chemokines, cytokines are a broad group of proteins that can modulate immune responses. The fusion protein generated by linking IFN-α to an anti–CD20 antibody can induce tumor regression, since IFN-α plays important roles in anti–tumor responses[69]. In another study, the anti–EGFR–IFN-α fusion protein also showed potent anti–tumor activities, such as targeting DCs to increase antigen cross-presentation, leading to increased T-cell priming in the TIME[70]. The approach that holds the most clinical promise today is the generation of chimeric antigen receptors (CARs). CARs are fusion proteins which incorporate antigen-recognition domains and T-cell activation domains[71].
T cells can be changed to express CARs and transferred to patients. Two CART19 products, CTL019 (Kymriah®, Novartis) and KTE–19 (Yescarta®, Kite Pharma/Gilead) have already been approved by the US FDA to treat refractory or relapsed paediatric and young–adult B–ALL or refractory or relapsed adult diffuse large–B cell lymphoma, respectively.[22] The development of immunotherapy underscores the importance of understanding basic tumor immunology and the roles of the immunosuppressive TIME, and thus is shaping the direction of current cancer research and having a large influence on cancer therapy.

PERSPECTIVES

With the rapid development of science and technology, large progress has been made in the tumor immune microenvironment. Increasing evidence suggests that the disruption of TIME may serve as a novel and critical therapeutic paradigm to fight cancer.[23] Clinical trials of combination therapy that target both the tumor and TIME are being conducted in several types of advanced cancer.[74,75].

To date, there are still challenges in this field: since the different subsets of TIME may lead to different clinical outcomes, further studies will need to be conducted in order to find more functions and mechanisms in the subsets of TIME which can instruct the clinical therapy. Proper in vitro and in vivo models need to be constructed for analyzing the interactions between cancer cells and the immune microenvironment. It is still largely unknown how different immune cells cooperate with each other within tumor tissues.[76]. Thus, the molecular mechanisms that regulate cell–to–cell interaction need to be uncovered. The composition of the immune cells in the TIME in primary tumors and metastases is highly varied,[77] therefore immune therapies that target different kinds of TIME are much required for better efficacy.

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