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T cell anergy, exhaustion, senescence and stemness in the tumor microenvironment

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Abstract

Human tumors progress despite the presence of tumor associated antigen (TAA)-specific T cells. Many different molecular and cellular mechanisms contribute to the failure of T cells to eradicate the tumor. These include immune suppressive networks that impair ongoing T cell function and enable tumor escape. Recent studies have started to reveal the nature of effector T cells in the tumor microenvironment. In this article we discuss T cell anergy, exhaustion, senescence and stemness, and review the phenotype of dysfunctional T cell subsets and the underlying molecular mechanisms in the tumor microenvironments. We suggest that targeting T cell dysfunctional mechanisms and introducing/promoting T cell stemness are important approaches to treat patients with cancer.

Keywords

T cell anergy; exhaustion; senescence; stemness; cancer; PD-1; Tim-3; B7-H1; HIF-1

Introduction

Throughout life, we encounter a multitude of antigens and pathogens that threaten our health and survival. To fend off this antigenic insult, the immune system has evolved and armed itself with an innate immune system for immediate immune attack against the inciting antigen and with an adaptive immune system for long-term protection. The major players in adaptive immunity are effector T cells. Despite tremendous advances in our characterization of effector T cells in infectious disease models in the last decade, the nature of effector T cells is not well understood in patients with cancer. As our current knowledge of effector T cells arises almost exclusively from studies of infectious disease models (particularly mouse models), the induction of memory and effector T cells in cancer is often inadvertently

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thought of as being analogous to that observed in chronic infections. Although we have identified many T cell effector mechanisms in mouse infectious disease model, it is evident that the phenotype and functional profile of effector T cells in cancer are dramatically impacted by the tumor microenvironment in patients with cancer. Over the past few years, immunologists have achieved important insights into cancer immunopathogenesis in patients [1–3], and therefore have started to dissect the nature of T cells in the tumor environment. In this review, we discuss the phenotype, functionality, and the underlying mechanisms of anergic T cells, exhausted T cells, senescent T cells and stem-like T cells in the tumor microenvironment.

T cell anergy

T cell anergy is generally described as the induced hyporesponsive state with low IL-2 production or incomplete activation, to which naïve T cells fall upon low co-stimulatory and/or high co-inhibitory stimulation (Fig. 1). It has been proposed that T cell anergy serves to induce tolerance in the periphery and protect the host from developing autoimmune disease [4–6]. Although this phenomenon has been the focus of many studies of human disease including cancer, it is unclear what causes and/or enforces their anergic state and phenotypic distinctions. Here, we review important observations on T cell anergy in the context of the tumor microenvironment.

The first question is whether T cell anergy is an operative mechanism causing T cell dysfunction in the tumor microenvironment. The evidence for T cell anergy in the tumor context has been indirect. A core problem has been a lack of positive markers to characterize a loss of function state of T cells. Nonetheless, the following observations support that T cell anergy can be an important phenomenon in cancer: (a) There is an active imbalance between stimulatory and inhibitory B7 family members in the tumor microenvironment [1,3,7,8]. Human tumors and tumor associated antigen presenting cells (APCs) often express high levels of B7-H1 (CD274 or PD-L1), B7-H2 (CD275 or ICOS-L), B7-H3 (CD276), B7-H4 (B7S1 or B7x), and B7-DC (CD273 or PD-L2) with low-to-absent expression of B7-1 (CD80) and B7-2 (CD86) [7–10] (Fig. 2). This indicates a poor co-stimulatory, high co-inhibitory and therefore anergy-promoting environment. (b) Animal model studies have shown that introduction of B7-1 into tumors by transfection or blockade of inhibitory B7 family members can reduce tumor growth or result in spontaneous tumor rejection *in vivo* [7–13]. (c) Homeostatic proliferation of anti-tumor T cells in a lymphopenic host both reverses anergy and promotes tumor rejection *in vivo* [14]. (d) There is evidence indicating antigen specific, T cell-intrinsic dysfunction in the tumor microenvironment [15,16]. Therefore, T cell anergy may be a functional mechanism in patients with cancer. However, the relative impact of T cell anergy on tumor immunity remains to be defined.

Cellular and molecular mechanisms controlling T cell anergy are insufficiently understood. It is generally accepted that T cells that are presented antigen along with suboptimal CD28 co-stimulation [4,5] and/or high co-inhibition [17] result in anergic phenotypes, as characterized by their low IL-2 production and cell cycle arrest at the G_1/S phase. Early growth response gene 2 (Egr2) may be a central transcription factor that regulates T cell anergic state [*18]. It has been suggested that the anergy program is initiated by improper mTOR and Ras/MAPK signaling in the cell, a pathway which lies directly downstream of TCR/CD28 engagement. Specifically, sole binding of TCR by MHC promotes Ca^{2+} imbalance on T cells and retention of active-RAP-1 in the cytosol, an imbalance that would normally be corrected by co-stimulation through CD28 (Ras/MAPK) [**19,20]. The effects of this imbalance on the genetic reprogramming of these cells have been hypothesized to be mediated by NFAT homodimer formation and transcription of anergy-inducing genes [21,22]. The E3 ubiquiting ligase family can affect PI3K, mTOR, and Ras/MAPK signaling

pathways and help to actively maintain anergy [21,23,24]. Epigenetic factors such as IKAROS (through acetylation) [25] and Sirt1 are involved in histone modifications that promote T cell anergy [26,27]. Thus, anergy is the combined result of factors that negatively regulate proximal TCR-coupled signal transduction, together with a program of active transcriptional silencing that is reinforced through epigenetic mechanisms [6].

In summary, tumor induced T cell anergy may be one of the immune evasion mechanisms in patients with cancer. Egr2 may be the potential transcriptional factor controlling T cell anergy. However, the downstream molecular mechanisms involved in the anergic state have been incompletely understood. The lack of surface marker(s) to define anergic T cells makes T cell anergy research a difficult challenge for immunologists.

T cell exhaustion

Exhausted T cells are described as effector T cells with decreased cytokine expression and effector function, and being resistant to reactivation [28](Fig. 1). T cell exhaustion occurs when T cells are chronically activated at sites of chronic inflammation, such as cancer, autoimmunity, and chronic infection. Dissecting the mechanism by which an exhaustive phenotype is ensured has been the focus of much research with the molecular enforcers just being revealed.

Initial mouse studies have proposed that B7-H1/PD-1 signaling pathway mediates $CD8^+$ T cell functional exhaustion in the context of chronic infection, and PD-1 was proposed to be a marker for exhausted T cells [*29]. Interestingly, far before these mouse studies in chronic infectious disease models [29], it was demonstrated that human tumor cells and/or tumor associated APCs expressed B7-H1, and B7-H1/PD-1 pathway mediated immune suppression [**9], and blockade of B7-H1/PD-1 pathway was investigated as therapeutic targets in solid human tumors [9,30] (Fig. 2). Exhausted $CD8^+$ T cells were found in patients with melanoma [*31], ovarian cancer [9] and hepatocellular carcinoma (HCC) [30]. Recent clinical trials have validated that blockade of B7-H1/PD-1 signaling is a meaningful immune therapeutic regimen [**32,**33]. Although the detailed molecular mechanism of T cell exhaustion is incompletely defined, it is suggested that recruitment of SH2-domain containing protein tyrosine phosphatases (SHP-1 and/or SHP-2) to the immunoreceptor tyrosine-based switch motif (ITSM) within the PD-1 cytoplasmic tail inhibits signaling events, particularly PI3K/AKT activation, downstream signals of the T-cell receptor [34], and in turn results in T cell dysfunction. Notably, activated T cells and effector T cells in the early stage may express PD-1 and remain functional [35,*36]. Given that members of the inhibitory B7 family are widely expressed by malignant cells and APCs within the human tumor microenvironment [7], the development of novel therapeutic strategies targeting the inhibitory B7 family members in malignancies is under active clinical investigations and show exciting clinical promise [32,33].

T cell exhaustion may be a layered or progressive process to which T cells fall upon repeated activation. T cells acquiring multiple inhibitory surface molecules in persistent disease settings such as chronic infection [37,38] and malignancies [30,**39,40], which effectively prevent T cell activation. In the course of defining exhausted $PD\text{-}1^+$ T cells, T cell immunoglobulin and mucin-domain-containing molecule-3 (Tim-3) [39,41], lymphocyte-activation gene (LAG)-3 [38], and the B and T-cell lymphocyte attenuator (BTLA, CD272) [42,43] were found to be co-expressed with PD-1, and the co-expression has been strongly correlated with immune dysfunction in patients with cancer. In these studies, T cells co-expressing these surface molecules show a significant decrease in IL-2, IFNγ, and TNFα expression as well as cell cycle arrest, which defines T cell exhaustion. In line with the concept that Tim-3 and PD-1 may define and maintain T cell exhaustion,

blockade of these surface molecules allows rescue of their effector functions as shown by cell cycle progression and acquired effector cytokine expression and cytotoxicity [40,43,44]. Notably, exhausted T cells may highly express multiple “inhibitory” receptors, including PD-1, 2B4 (CD244), BTLA, CTLA-4, CD160, LAG-3 and Tim-3 [38,42,43,45]. However, exhausted T cells may not necessarily co-express these molecules. Furthermore, it is controversial if the co-expression of inhibitory molecules is functionally important to determine T cell functional state. For example, in patients with HCC, the expression of PD-1 and Tim-3 is minimally overlapping in tumor infiltrating T cells. HCC-associated Tim-3^{+}T cells expressed reduced CD28, suggesting that these cells may be early senescence stage [40]. The question remains unanswered if these “inhibitory molecule” expressing T cells share similar molecular and genetic signature in patients with chronic infection and cancer.

Nonetheless, it is assumed that the tumor microenvironment provides the necessary conditions for effector T cells to become functionally exhausted as well as being able to maintain this state during disease progression. The detailed molecular signals remain undefined. A promising aspect is that clinical blockade of B7-H1/PD-1, the key T cell exhaustion pathway, may rescue T cell effector functions *in vivo,* and results in significant objective clinical responses [32,33].

T cell senescence

Senescent T cells are characterized by telomere shortenings, phenotypic change (loss of CD28 expression), and cell cycle arrest [46,47] (Fig. 1). Telomere shortening is an inherent byproduct of cellular division, which affects cellular function and leads to cell senescence [48]. Cell cycle controlling proteins p16, p21, and p53, normally inhibit cell cycle progression and have been shown to be accumulated in senescent cells [49–51]. In addition to phenotypic alteration, senescent T cells manifest defective killing abilities and the development of negative regulatory functions [52,53].

It is naturally thought that senescence is associated with physiological ageing process. Indeed, the cell has its natural life-span and proliferation exhaustion results in cell senescence. However, high levels of senescent T cells were found in younger patients with autoimmune disease and chronic viral infection [54]. This suggests that cells in younger patients may become senescent, and chronic activation and proliferation may still cause T cell senescence [55]. In line with this notion, tumor cells can induce T cell senescence in *in vitro* co-cultures [56]. Phenotypically, senescent $CD28^{-/dim}CD8^{+}$T cells are observed in patients with lung cancers [57], head and neck cancer [58]. DNA damage can cause mouse thymic precursor lymphocytes to withdraw from the cell cycle and undergo senescence [59]. This is sufficient to inhibit oncogenic chromosomal abnormality and suppress tumorigenesis. However, there is no direct evidence that similar mechanisms are applied for mature T cell senescence in the tumor microenvironment.

In addition to low expression of CD28, high expression of Tim-3, CD57, killer cell lectin-like receptor subfamily G, member 1 (KLRG-1) are thought to be associated with T cell senescence [40,60–64](Fig. 2). For example, human HCC- associated T cells highly express Tim-3 and cyclin dependent kinase inhibitors, and interact with galectin-9^{+}myeloid APCs. These cells fail to enter the cell cycle [40]. Similar findings were obtained in patients with melanoma [*63] and lymphoma [64]. It suggests that cancer associated Tim-3^{+} T cells may contain senescent cells and experience cell cycle arrest in G_1/S phase.

A few studies have examined how Tim-3 carries out its function [65]. Galectin-9 is the Tim-3 ligand. Galectin-9 induces intracellular calcium flux, aggregation and death of Th1 cells in Tim-3-dependent manner [65]. Yet, it has been postulated that the human leukocyte antigen (HLA)-B-associated transcript 3 (Bat3) modulates Tim-3 function on T cells by

interacting with Tim-3 cytoplasmic tail [66]. Bat3 localization at this protein region allows T cells continued proliferation and IFNγ production, an effect that was lost on Bat3 deficient cells and upon Tim-3 binding to galectin-9. However, how Tim-3 and Bat3 interaction links to T cell senescence remains to be elucidated.

T cell stemness

The self-renewal, expansion and multi-lineage developmental potential define the unique properties attributed to stem cells. Based on these properties, the concept of T cell stemness was proposed and "stem-like memory T cells" may encompass the capability to both self-renew and to generate more differentiated, memory T cells (Fig. 1).

The concept of T cell stemness was initially stemmed from the observations that mouse central memory T cells are arrested at a pre-differentiation stage by transcriptional inhibitors and retain replicative potential and long-term production of effector T cells after a second antigen challenge [67]. The capacity to continuously generate effector memory T cells will replenish the effector memory T cell pool, and help maintain a constant repertoire of memory T cells for a human lifetime, despite the finite lifespan of individual effector cells and reduced thymus function [68]. This notion has recently received certain experimental support. In a mouse model, $CD44^{low}CD62L^{high}$ memory $CD8^+$ T cells express high levels of stem cell antigen-1 (Sca-1), Bcl-2 and common IL-2 and IL-15 receptor β chain (CD122). Because these cells showed robust self-renewal and the multipotent capacity to generate central memory and effector memory T cells, they were designated T memory stem cells [69]. More recent mouse work has shown that by blocking T cell differentiation, Wnt signaling promoted the generation of this memory stem cell population [70]. These studies support the concept that certain subsets of memory T cells endow the capability both to self-renew and to generate more effector T cells. Therefore, by manipulating these "stemness properties" of memory T cells, it is possible to generate and maintain long-lived, self-renewing antigen-experienced memory T cells with stem cell-like properties for treating patients with cancer.

In line with these limited reports in non-tumor system, human tumor associated Th17 cells expressed stem cell markers and exhibited stem cell like features. When measured for their biological activity, mouse and human Th17 cells displayed greater survival potential, persistence as well as the ability of repopulating sub-lethally irradiated mice [**71,**72]. Furthermore, these cells achieved a higher anti-tumor response when compared to effector and central memory T cells. Interestingly, Th17 cells retain a stem cell-like phenotype through the coordinated effects of HIF1α/Notch/Bcl-2 and are also potent anti-tumor effectors [71], suggesting that stemness might correlate with better immune responses. Human Th17 cells were shown to give rise to distinct Th lineages, as measured through the expression of IFNγ, and $Foxp3^+$ cells, heightened self-renewal, and survival capabilities [71,72].

Human Th17 cells have certain "stem cell properties" at the genetic, molecular and functional levels, and are long-lived cells. This property may be critically important for controlling Th17 cell biology. Manipulation of Th17 stemness may be therapeutically interesting for treating patients with Th17-associated chronic diseases.

Conclusions

Compelling evidence demonstrates the co-existence of T cell anergy, exhaustion, senescence and stemness in the tumor microenvironment. When we interpret the current literature, the following points may need to be taken into consideration: (a) T cell subset markers. Are there specific markers to phenotypically define anergic, exhausted, senescent and stem-like

T cell subsets? It is arguable but experimentally operative that PD-1 may be a marker for exhausted cells, Tim-3 and KLRG-1 may be markers for senescent cells, and mouse stem-like T cells may express Sca-1 [70]. However, these markers are not mutually exclusive and inclusive in a given T cell subset. Our opinion is that these T cell subsets are functionally generated and defined. Therefore, genetic and functional pattern, but not specific surface phenotypes will define their nature and fate. For example, despite their phenotypic markers of terminal differentiation, Th17 cells have stem cell feature with powerful functionalities [71–73]. (b) Functional and phenotypic overlap. Although these cell subsets are conceptually distinct, they may be functionally and phenotypically overlapped. PD-1^+ cells may express Tim-3 and LAG-3. Regardless of these different immunological concepts, it is evident that B7-H1/PD-1 and Tim-3/galectin-9 signaling pathways may synergistically and/or additively mediate T cell dysfunction, and simultaneous blockade of these pathways may result in improved T cell immunity. Preclinical and clinical studies suggest that T cell dysfunction may be functionally reversible. This paves the way for targeting cancer therapy. (c) Mechanistically intertwined. Although the underlying mechanisms causing T cell anergy, exhaustion and senescence are not well defined, compelling evidence indicate that dysfunctional T cells express in different degrees the “inhibitory” molecules including PD-1, Tim-3, LAG-3, 2B4, CD160, and KLGR-1. It suggests that different categories of T cell abnormalities may be mechanistically intertwined [28,74–76].

In conclusion, peripheral T cell tolerance mechanisms including regulatory T cells, T cell anergy, exhaustion, and senescence impair ongoing T cell immunity and enable tumor immune escape. Further clarification of their pathogenic mechanisms and roles will have significant implications for the development of novel therapeutic strategies targeting these mechanisms in malignancies. We suggest that targeting T cell dysfunctional mechanisms and introducing/promoting T cell stemness are important approaches to treat patients with cancer.

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Highlights

- We provide an updated view on effector T cell compartment in the tumor microenvironment.
- We describe dysfunctional T cell subsets in the tumor microenvironment.
- We discuss co-inhibitory and co-stimulatory molecular networks in the tumor microenvironment.
- We review stem like-memory T cell subsets (e.g. Th17) in the tumor microenvironment.
- We suggest anti-cancer modality by targeting T cell dysfunction and stemness.

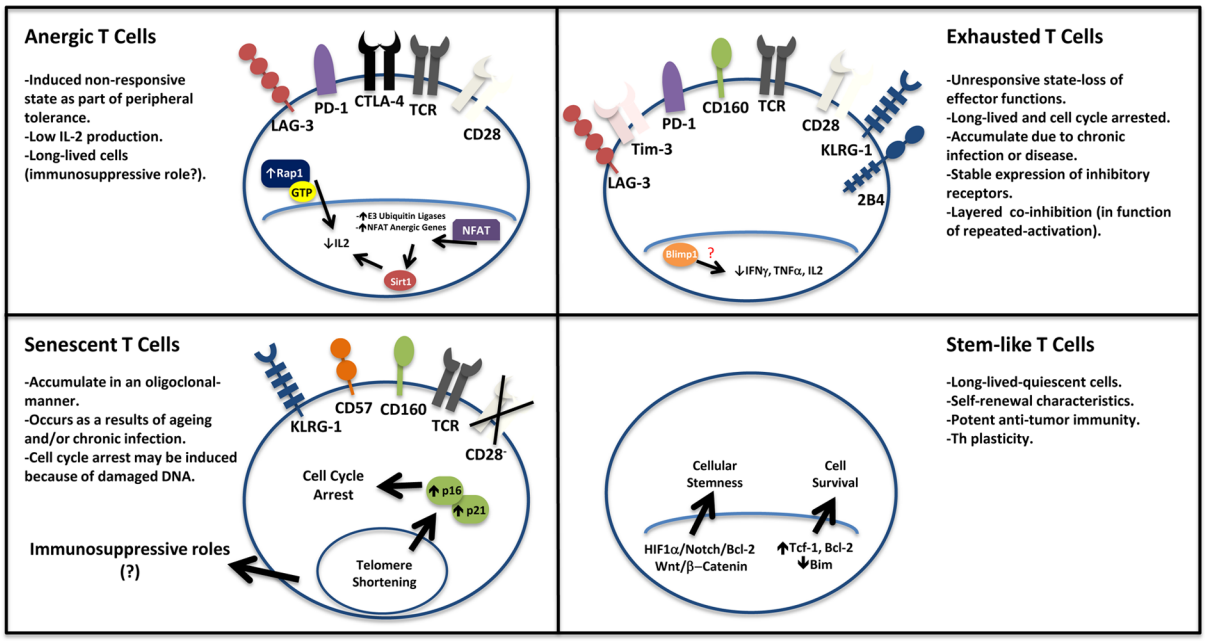

Figure 1. General characteristics for anergic, exhausted, senescent and stem-like T cells

a) Anergic T cells are T cells stimulated with low co-stimulatory and/or high co-inhibitory signaling. These cells are unresponsive to subsequent activating conditions with limited IL-2 expression. (b) Exhausted T cells are effector T cells that have lost their effector functions including effector cytokine expression due to repeated stimulation. These cells express multiple regulatory receptors. (c) Senescent T cells are described as unresponsive/terminally differentiated T cells. The hallmark is their cell cycle arrest along with limited CD28 expression and/or high levels of regulatory receptor expression. (d) Stem-like T cells may show a naïve or memory phenotype. Importantly, these cells are capable of self-renewal, have enhanced anti-tumor responses and are long-lived effector cells.

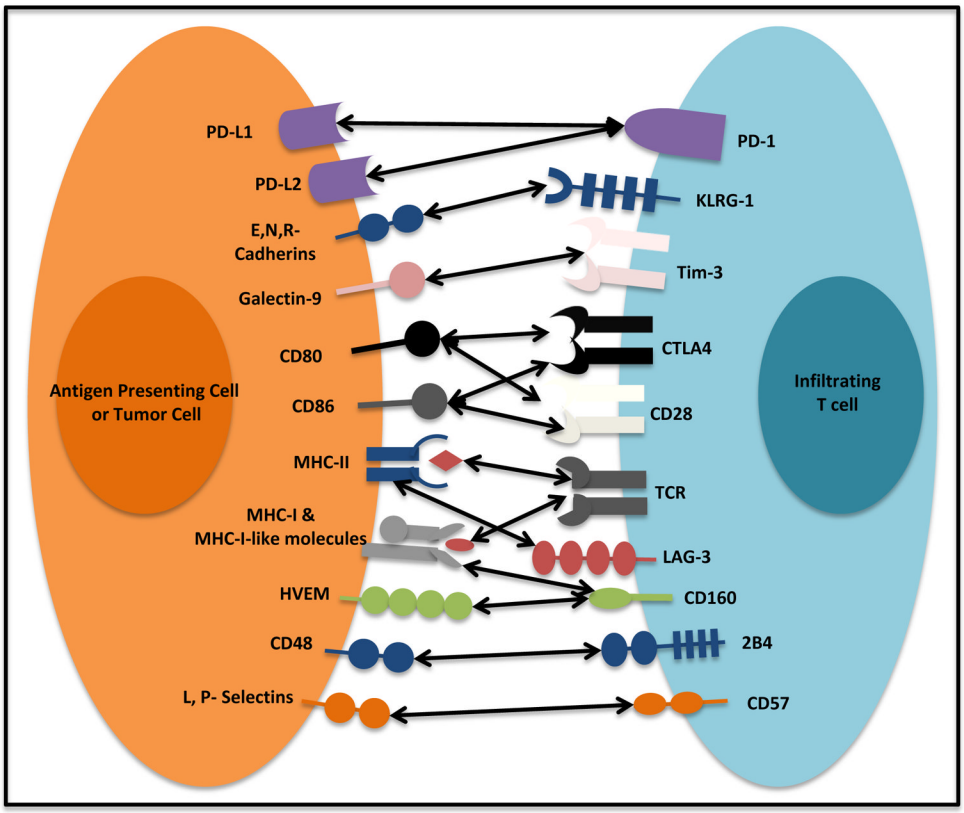

Figure 2. Immunoregulatory receptors and their ligands

T cell activation relies on the T cell receptor (TCR) recognizing its cognate antigen in the context of MHC molecules from an antigen presenting cell (APC) or an APC-like cell (tumor cell). Interaction between co-stimulatory molecules CD80, and CD86 and CD28 is crucial for appropriate T cell activation. Immunoregulatory receptors such as CTLA-4 and PD-1 are to fine tune T cell activation. High levels of multiple immunoregulatory receptors (LAG-3, 2B4, CD160, KLRG1, Tim-3, CTLA-4, and CD57) or their ligands are found in the tumor microenvironment. Potent and lasting immunoregulatory signaling results in reduced T cell function and tolerance.