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Chapter 3

Immune Checkpoint Blockade and Adaptive Immune Resistance in Cancer

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Abstract

The clinical success of immune checkpoint blockers is a pivotal advancement for treating an increasing number of cancer types. However, immune checkpoint blockers still rarely induce complete remission and show little to no therapeutic efficacy in a significant percentage of cancer patients. Efforts are now underway to identify biomarkers that accurately predict which patients benefit from immune checkpoint blockers. Moreover, adaptive immune resistance can develop in tumors during treatment with immune checkpoint blockers. These adaptive resistance mechanisms in tumors might be disrupted by combining adjunctive immunotherapies, which could potentially improve the therapeutic efficacy of immune checkpoint blockers. This chapter discusses the mechanism of action of cytotoxic T lymphocyte antigen-4 (CTLA-4) and programmed death-1/programmed death-ligand 1 (PD-1/PD-L1) immune checkpoint blockers and biomarkers that might predict clinical responses to these drugs. Lastly, ongoing research on mechanisms of tumor adaptive resistance could facilitate rationale design of adjunctive immunotherapies that can be synergistically combined with immune checkpoint blockers to more effectively treat cancer.

Keywords: immunotherapy, T lymphocytes, immune checkpoints, CTLA-4, PD-1, PD-L1

1. Introduction

Immune checkpoints are inhibitory pathways that are critical for maintaining self-tolerance. Immune checkpoints also control the magnitude and duration of physiological immune responses in peripheral tissues in order to minimize collateral damage. Immune checkpoint receptors and their cognate ligands are naturally expressed on a variety of cell types, including antigen-presenting cells, T cells, B cells, tumor cells, tumor stroma, and also normal tissue.

A number of immune checkpoint pathways have been identified, including cytotoxic T lymphocyte antigen-4 (CTLA-4), programmed death-1 (PD-1), programmed death ligand-1 (PD-L1), T cell immunoglobulin and mucin domain 1 (TIM-1), T cell immunoglobulin and mucin domain 3 (TIM-3), lymphocyte-activation gene 3 (LAG-3), T cell immunoreceptor with Ig and ITIM domains (TIGIT), V-domain Ig suppressor of T cell activation (VISTA), carcinoembryonic antigen-related cell adhesion molecule 1 (CEACAM1), leukocyte-associated immunoglobulin-like receptor 1 (LAIR-1), herpesvirus entry mediator (HVEM), B- and T-lymphocyte attenuator (BTLA), CD160, CD200, CD200 receptor, and adenosine 2A receptor (A2Ar). For brevity, this chapter will focus on CTLA-4 and PD-1/PD-L1, as clinical drugs targeting these pathways have been successfully developed to treat an increasing variety of human cancer types.

2. Main body

2.1. CTLA-4

CTLA-4 is the first immune checkpoint receptor to be clinically targeted. CTLA-4 is expressed mainly on the surface of activated T cells. While certain subsets of T regulatory cells constitutively express CTLA-4, it is virtually undetectable on naïve, inactivated T cells. Upon activation, both $CD4^+$ and $CD8^+$ T cells upregulate CTLA-4 on the surface, reaching maximum level within 2–3 days. $CD4^+$ T cells are reported to express more CTLA-4 mRNA and protein compared to $CD8^+$ T cells, suggesting that CTLA-4 has a more significant regulatory effect on $CD4^+$ T cells [1].

CTLA-4 downregulates T cell activation by sequestering CD80 and CD86 costimulatory molecules on antigen-presenting cells. This prevents CD80 and CD86 from delivering costimulatory activation signals to T cells through the CD28 receptor. CTLA-4 binds to CD80 and CD86 with ~10 times higher affinity than CD28 [2]. CTLA-4 expressed on T cells can also remove CD80 and CD86 molecules from neighboring antigen-presenting cells through a process called trans-endocytosis [3]. CTLA-4 also prevents CD28 recruitment to the immunological synapse, further impairing T cell activation [4].

CTLA-4 knockout mice die within 2–3 weeks of age due to massive lymphoproliferation, resulting in destruction of vital organs [5]. This lethal phenotype is associated primarily with hyperactivated $CD4^+$ T cells, which are skewed toward a T helper type-2 phenotype and have increased resistance to apoptosis. These hyperactivated $CD4^+$ T cells abnormally infiltrate into peripheral tissues, resulting in organ failure. These observations led cancer immunology researchers to hypothesize that blockade of CTLA-4 signaling could potentially induce effective T cell-mediated immune responses against tumor tissue.

A pivotal laboratory study reported in 1996 by James Allison's group showed that treatment of tumor-bearing mice with a CTLA-4-blocking antibody could effectively induce tumor regression [6]. Despite much subsequent investigation, the in vivo mechanism of action of CTLA-4 blockade immunotherapy has remained elusive. The prevailing hypothesis is that CTLA-4 blockade not only enhances T cell infiltration into tumors but also reduces the

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relative presence of immunosuppressive T regulatory cells in tumor tissue [7]. This alteration in the ratio of effector T cells versus T regulatory cells in tumors tilts the immunological balance in favor of T cell-mediated destruction of tumor cells.

These studies led to pharmaceutical development of the first immune checkpoint blocker, ipilimumab (Yervoy®). Ipilimumab is a fully human monoclonal antibody that blocks the CTLA-4 receptor, thereby preventing its ability to sequester CD80 and CD86 costimulatory molecules. It was initially tested in melanoma, and demonstrated extended overall survival in patients versus a comparator melanoma peptide-based immunotherapy vaccine called gp100. In a randomized phase III clinical trial, melanoma patients receiving ipilimumab had a median overall survival of 10.4 months versus 6.4 months in those receiving only the gp100 peptide vaccine (Hodi 2010). Objective response rates (measureable tumor regression) were 10.9% in the ipilimumab group versus 1.5% in the gp100 vaccine group. The responses to ipilimumab were durable, with the 1-year and 2-year survival rate being 46 and 24%, respectively. By comparison, the 1-year and 2-year survival rate in patients receiving only the gp100 peptide vaccine was only 25 and 14%, respectively [8]. These trial results led to US FDA approval of ipilimumab for melanoma in 2011.

2.2. PD-1

PD-1 is another major immune checkpoint receptor that regulates T cell activity against tumor tissue. PD-1 is a cell surface receptor originally identified in a murine T cell hybridoma undergoing programmed cell death [9]. PD-1 is absent on naïve inactivated immune cells but is significantly upregulated on activated T cells, B cells, natural killer cells and myeloid-derived cells [10]. In T cells, PD-1 expression is induced by T cell receptor signaling [11] and also by certain pro-inflammatory cytokines including interleukin-2, interleukin-7, interleukin-15, and interleukin-21 [12].

PD-1 signaling downregulates T cell activity primarily via interaction with its two natural ligands: Programmed Death Ligand-1 (PD-L1) and Programmed Death Ligand-2 (PD-L2). PD-L1 is expressed on a wide variety of cell types including hematopoietic cells, T cells, B cells, myeloid cells, and dendritic cells [10]. It is also expressed on a wide variety of peripheral tissues such as skeletal muscle, lung, heart, and placenta [10]. Notably, PD-L1 is also expressed on a wide variety of cancer cells and generally is associated with poorer patient prognosis [13]. PD-L2 expression is generally more restricted, being found primarily on dendritic cells, macrophages, and occasionally cancer cells [14]. PD-L2 binds to PD-1 with two- to sixfold higher relative affinity than PD-L1 [15]. However, PD-L2 is generally expressed at lower relative levels [16]. Thus, it is believed that PD-L1 is the predominant ligand for PD-1.

Signaling through the PD-1 receptor on T cells results in downstream inhibition of PI3K/AKT activation [17]. The net effect is downregulation of a number of effector functions including cytokine secretion and cytolytic activity. PD-1 knockout mice have various autoimmune pathologies, including autoantibody-induced cardiomyopathy [18], arthritis and lupus-like disease [19], and diabetes [20]. In peripheral tissues, the immunosuppressive activity of PD-1 is mediated primarily by interaction with PD-L1 [21]. PD-L1 expressed in tumor tissue also impairs host antitumor immune responses [22]. PD-L1 and/or PD-L2 in tumor tissue facilitates

evasion from host immune responses via multiple mechanisms including induction of T cell anergy and exhaustion [23], promoting T cell apoptosis [24], and also by enhancing the expansion and activity of immunosuppressive T regulatory cells [25]. Moreover, PD-1 can transmit an antiapoptotic signal to PD-L1-expressing tumor cells, which renders them resistant to lysis by cytotoxic T lymphocytes [26].

This fundamental understanding of the PD-1/PD-L1 axis in suppressing host antitumor immune responses led to development of the first clinical PD-1 blockers, nivolumab (Opdivo®) and pembrolizumab (Keytruda®). Both nivolumab and pembrolizumab are fully human monoclonal antibodies that block the PD-1 receptor, thereby preventing its ability to bind its natural ligands PD-L1 and PD-L2. In large phase I clinical trials, nivolumab and pembrolizumab each demonstrated durable clinical response rates with acceptable safety profiles in patients with advanced melanoma, non-small cell lung cancer, renal cell carcinoma or Hodgkin's lymphoma [27–30]. Nivolumab and pembrolizumab are now both FDA approved for treating melanoma and non-small cell lung cancer. Nivolumab is additionally approved for treating renal cell carcinoma, Hodgkin's lymphoma, and also for use in combination with the CTLA-4 blocker, ipilimumab, for treating melanoma. Remarkably, in two separate melanoma clinical trials, the combination of nivolumab and ipilimumab induced objective responses in ~60% of patients, with complete responses seen in ~11.5–22% of patients [31–32].

Pembrolizumab and nivolumab (and a third investigational PD-1 blocker, pidilizumab) are now collectively continuing in 500+ clinical trials. Virtually all cancer types are now being targeted with PD-1/PD-L1 blockers in some capacity. Notably, there is a significant effort to test nivolumab or pembrolizumab with other adjunctive therapies to determine synergistic combinatorial regimens. Conventional treatments like chemotherapy and radiation have shown in animal tumor models to potentially synergize with PD-1/PD-L1 blockers [33–35]. In addition, PD-1 blockers are now also being tested in combination with small molecule drugs (investigational and Food and Drug Administration (FDA) approved) and also experimental immunotherapies such as vaccines and chimeric antigen receptor T cells.

All clinical PD-1 blockers have the same mechanism of action. Slight variances in the protein structure among different PD-1 blockers could potentially confer differences in binding affinity for the PD-1 receptor and also differences in half-life (i.e. persistence in the body). The physiological significance and clinical effectiveness of such variances remain undetermined.

2.3. PD-L1

Expression of PD-L1 is found on diverse cell types, including normal and malignant tissue, antigen presenting cells, myeloid cells, B cells, and T cells. PD-L1 downregulates T cells via multiple mechanisms. PD-L1 expressed on various cells primarily interacts with PD-1 expressed on T cells, delivering an inhibitory signal that downregulates T cell activity. PD-L1 also binds to CD80 expressed on both antigen-presenting cells and activated T cells [36]. Interaction of PD-L1 with CD80 on antigen-presenting cells prevents CD80 from delivering costimulatory activating signals to T cells. When PD-L1 binds to CD80 expressed on activated T cells, an inhibitory signal is delivered to T cells. Currently, it is unknown exactly what intracellular

signaling pathways are altered when PD-L1 binds to CD80 on T cells. Nonetheless, it is now generally understood that blocking PD-L1 results in enhanced T cell activation.

Atezolizumab (Tecentriq®) was the first PD-L1 blocker to enter clinical trials. Atezolizumab is a fully human monoclonal antibody that prevents PD-L1 from binding to PD-1 and CD80. It was initially tested in patients with PD-L1-positive metastatic bladder cancer [37]. Bladder cancer patients with PD-L1-negative tumors were subsequently included for treatment. Clinical response rates were ~15% of PD-L1-negative patients and ~25% of PD-L1-positive patients [37]. Because of the higher clinical activity of atezolizumab in PD-L1-positive bladder cancer, a companion diagnostic called the Ventana PD-L1 (SP142) assay is offered to provide tumor PD-L1 expression status of patients considering atezolizumab treatment. In 2016, atezolizumab was FDA approved for urothelial carcinoma, the most common form of bladder cancer. Like nivolumab and pembrolizumab PD-1 blockers, atezolizumab is now continuing in clinical trials for a wide variety cancer types and also being tested in combination with conventional cancer treatments, small molecule drugs and other investigational immunotherapies. Alternative PD-L1 blockers, such as avelumab and durvalumab, are also now in clinical trials.

2.4. Predictive biomarkers for CTLA-4 and PD-1/PD-L1 blockers

CTLA-4 and PD-1/PD-L1 immune checkpoint blockers have proven to be pivotal advancements in cancer treatment. However, a significant proportion of cancer patients still experience little to no clinical benefit from treatment. Even among responding patients, only a small minority achieve complete remission. Studies using clinical tumor specimens from patients treated with immune checkpoint blockers have revealed some potentially important differences between responders versus nonresponders.

During early clinical development of PD-1 blockers, it was hypothesized that differential expression levels of PD-L1 in tumor tissue would correlate with clinical responses. It was anticipated that PD-L1 expression in tumor tissue could therefore be a predictive biomarker to accurately identify patients likely to respond to PD-1 or PD-L1 blockers. However, a definitive correlation has thus far not been established. Both PD-L1-positive and PD-L1-negative tumors can respond to PD-1 or PD-L1 blockers. Further confounding factors include variability of PD-L1 expression in different anatomical areas of tumor tissue. In addition, PD-L1 expression in tumor tissue may be transient—appearing and disappearing due to treatments or other poorly understood influences. Lastly, assays measuring PD-L1 in tumors have yet to establish a clear threshold of expression that defines what is considered "PD-L1-positive." For instance, the FDA-approved Ventana PD-L1 assay defines ≥5% PD-L1-positive cells in bladder cancer tissue to be associated with higher clinical response rates to atezolizumab [38]. However, alternative PD-L1 assays used in various other clinical trials of nivolumab or pembrolizumab have wide variability in PD-L1 expression analysis methodologies. Overall, it is generally agreed upon that low or absent PD-L1 expression in tumors is not sufficient to preclude a patient from treatment with PD-1/PD-L1 blockers [39].

Alternative predictive biomarkers for clinical response to PD-1/PD-L1 blockers are currently being explored. $CD8^+$ T cell infiltration into tumors might be predictive of clinical response to

PD-1 blockers. Specifically, the density of pretreatment $CD8^+$ T cells at both the tumor invasive margin and tumor center may be correlated with clinical response to pembrolizumab. In serially biopsied tumors from melanoma patients undergoing pembrolizumab treatment, it was shown that responding patients generally had higher densities of $CD8^+/PD\text{-}1^+$ cells in close proximity to PD-L1-expressing tumor cells [40]. Furthermore, serial analysis of tumor biopsies showed that intratumoral $CD8^+/PD\text{-}1^+$ T cells actively proliferate during pembrolizumab treatment [40]. These data offer insights on a potential mechanism of PD-1 blockade efficacy, whereby presence of pretreatment $CD8^+$ T cells in tumors is a prerequisite for clinical response. However, like tumor PD-L1 expression assays, establishing a standard cut-off threshold value for $CD8^+$ T cell levels in tumors that accurately predicts clinical response to PD-1/PD-L1 blockade will be challenging. Tumors of various tissue origins often contain infiltrating T cells that can vary greatly in absolute number, density, and also anatomical location within the intratumoral space. Nonetheless, establishing a "scoring system" based on pretreatment $CD8^+$ T cell infiltration warrants further investigation as a potential predictive biomarker.

Another intriguing biomarker with predictive potential may be intratumoral expression of indoleamine-2,3-dioxygenase (IDO). IDO is a tryptophan catabolizing enzyme that is occasionally expressed in various tumor types. Depletion of tryptophan within tumors by IDO may be a rate-limiting step for effective antitumor T cell activity. Studies in melanoma patients treated with ipilimumab suggest a correlation between pretreatment IDO expression and clinical response. In one study, intratumoral IDO was detected in 37.5% of responding melanoma patients and only 11.1% in nonresponders [41]. It remains to be seen if similar patterns are seen in other cancer types and also patients treated with PD-1/PD-L1 blockers.

Genetic signatures of tumors are yet another parameter with potential for yielding predictive biomarkers for clinical response to immune checkpoint blockers. Certain tumors, such as colorectal cancer, are highly refractory to treatment with PD-1 blockers. In early clinical trials of nivolumab, it was found that only 1 in 33 colorectal cancer patients responded to treatment [27–28]. Subsequently, it was hypothesized that the single responding colorectal cancer patient harbored a defect in DNA mismatch repair in tumor tissue, resulting in a significantly high load of somatic mutations [42]. Defects in tumor tissue mismatch repair can result in thousands of somatic mutations, providing a larger pool of neo-antigens for immune recognition. Immune checkpoint blockade therapy could therefore amplify the natural adaptive immune response to mutated neo-antigens. Hence, mutational load in pretreatment tumor tissue might be predictive of clinical response to immune checkpoint blockers. To test this hypothesis, a small clinical trial focusing primarily on colorectal cancer showed that patients with defects in tumor tissue mismatch repair harbored significantly higher loads of somatic mutations versus those with mismatch repair-proficient tumors. Upon treatment with pembrolizumab, higher response rates and longer survival times were seen in patients with mismatch repair defects versus those with proficient mismatch repair [42]. This pivotal study has catalyzed further investigation of tumor mutational profiles to determine if a correlation with clinical responses can be established in large studies of diverse cancer types.

2.5. Adaptive immune resistance

Mechanisms of inherent and acquired resistance to immune checkpoint blockade are poorly understood. Clinical responses to CTLA-4 and PD-1/PD-L1 blockers are often durable, sometimes lasting years. However, complete regressions are still relatively rare and eventual disease relapse among responding patients is frequent. Recent studies have offered insights that immunological parameters of tumor tissue adapt in response to T cell-mediated attack induced by immune checkpoint blockers. Enhanced T cell activity within tumors involves local production of inflammatory mediators, such as interferon (IFN)-γ, which is known to upregulate PD-L1 on peripheral tissues [43]. Upregulation of PD-L1 on various cell types within tumor tissue might result in heightened CD80-mediated inhibition of proximal effector T cells.

Furthermore, augmentation of effector T cell activity in tumor tissue via PD-1 blockade may subsequently induce compensatory upregulation of alternative immune checkpoint receptors, TIM-3. TIM-3 is a receptor expressed primarily on IFN-γ-secreting $CD4^+$ and $CD8^+$ T cells [44]. TIM-3 is bound by multiple ligands, including galectin-9, CEACAM-1, and high-mobility group box 1 (HMGB-1). Signaling through TIM-3 in activated T cells triggers the release of human leukocyte antigen B-associated transcript 3 (BAT3) from the TIM-3 cytoplasmic domain. This results in defective production of IL-2, IFN-γ, and likely other pro-inflammatory cytokines [44]. Although the TIM-3 signaling pathway has yet to be fully elucidated, it seems clear that TIM-3 affects T cell receptor downstream signaling via a mechanism distinct from PD-1 and CTLA-4.

TIM-3 appears to be co-expressed with PD-1 in tumor-infiltrating lymphocytes of cancer patients and is upregulated on T cells upon therapeutic PD-1 blockade [45]. This may provide a mechanism of immunological escape and a possible reason for incomplete clinical responses upon PD-1 blockade immunotherapy. It might also be a contributing factor toward acquired resistance to PD-1 blockade clinically, whereby patients initially respond to treatment but eventually relapse despite continuous therapy. Preclinical studies in animal tumor models show that PD-1 blockade immunotherapy results in upregulation of TIM-3 on T cells. Co-blockade of both TIM3 and PD-1 can prevent resistance to PD-1 blockade immunotherapy [45]. As such, TIM-3 blocking antibodies are now in early phase clinical trials to evaluate their safety, tolerability, and dosing ranges. **Figure 1** illustrates how PD-1/PD-L1 blockade may result in compensatory upregulation of TIM-3 and/or PD-L1 on T cells and tumor cells.

Downregulation of major histocompatibility (MHC) receptor expression in tumors might also contribute to acquired resistance to PD-1 blockers. Loss-of-function mutations in the MHC beta-2 microglobulin antigen-presenting protein have been noted in selected melanoma patients who initially responded to pembrolizumab therapy but subsequently relapsed [46]. Further studies in larger patient populations are necessary to confirm the association of MHC-related mutations and acquired resistance to PD-1 blockers.

2.6. Strategies to counteract adaptive resistance to immune checkpoint blockade

The mechanism of inherent and acquired/adaptive resistance to CTLA-4 and PD-1/PD-L1 immune checkpoint blockers is not fully understood and could possibly vary between

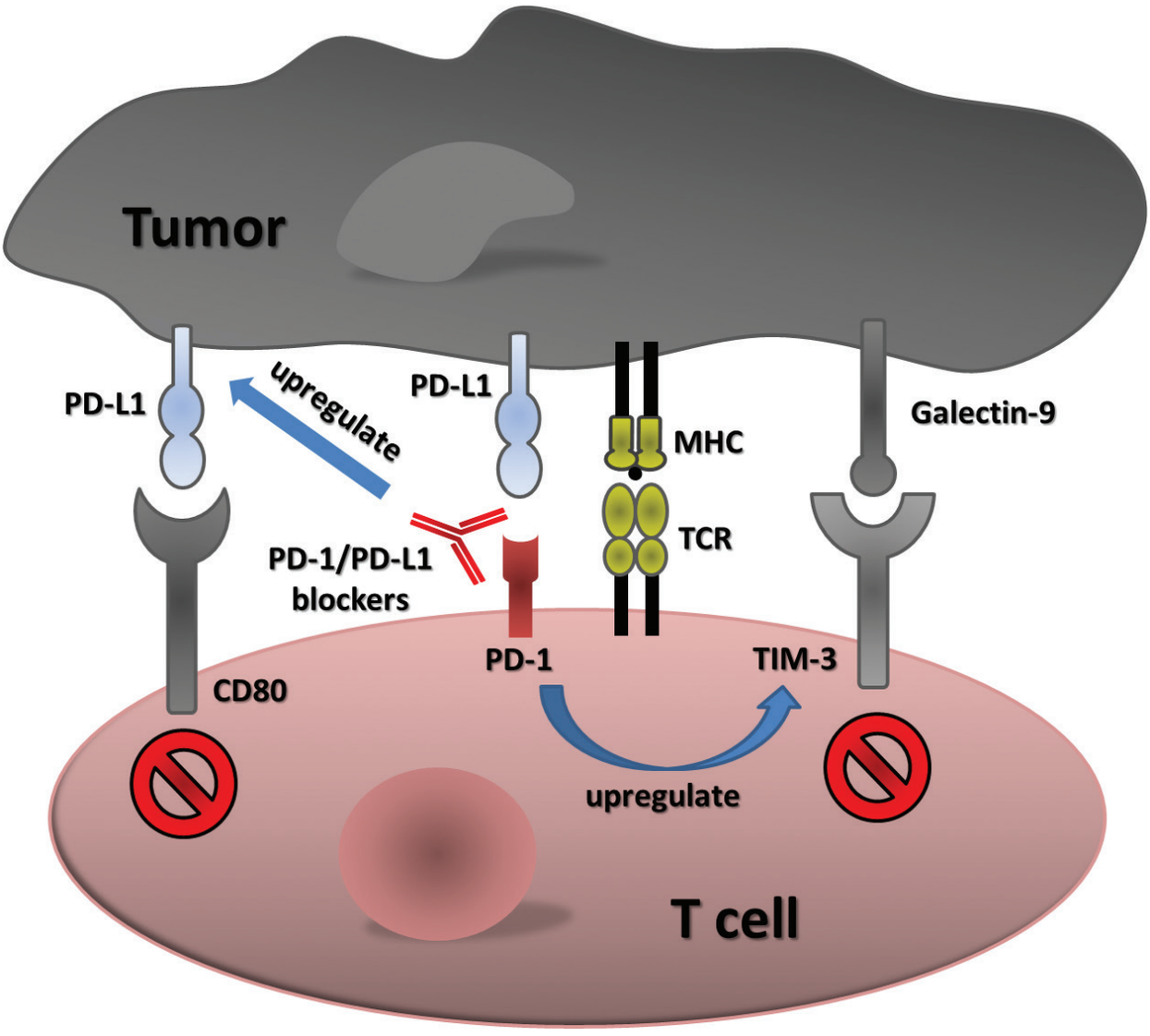

Figure 1. PD-1/PD-L1 blockade promotes T cell-mediated inflammation in tumors. In turn, this can trigger upregulation of PD-L1 on various cells within tumor tissue. This can also trigger compensatory upregulation of TIM-3 on effector T cells. Upregulation of PD-L1 and TIM-3, even during continuous treatment with PD-1 blockers, can impair T cell activity and result in clinical resistance.

individual patients and different tumor types. However, research on predictive biomarkers and mechanisms of adaptive resistance to PD-1 blockers have yielded insight that might be extrapolated to rationally design combination immunotherapies that synergistically enhance the efficacy of immune checkpoint blockers. For instance, it is now generally understood that PD-1 blockers augment T cell-mediated inflammation in tumor tissue. In turn, this can promote upregulation of PD-L1 on various cells in tumors, likely due to IFN-γ signaling [43]. Upregulation of PD-L1 expression in tumor tissue can promote enhanced CD80 signaling in T cells, which impairs T cell activity [36]. PD-1 blockade may also induce compensatory upregulation of alternative immune checkpoint receptors, such as TIM-3, on T cells within tumor tissue [45]. TIM-3 signaling results in downregulation of T cell activity. Next-generation immunotherapeutic regimens might combine PD-1 blockers such as

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nivolumab/pembrolizumab with PD-L1 blockers like atezolizumab, to counteract PD-L1 upregulation induced by T cell-mediated inflammation in tumor tissue. Other rational combinations might include PD-1/PD-L1 blockers combined with investigational TIM-3 blockers, to counteract the effects of TIM-3 upregulation on activated T cells.

Another strategy to enhance the efficacy of immune checkpoint blockers might involve improving T cell trafficking to tumor tissue. The extent of T cell infiltration into tumor tissue may be a predictive biomarker and a prerequisite for efficacy of both CTLA-4 and PD-1/PD-L1 blockers. As such, therapies that promote T cell trafficking to tumors could potentially improve tumor sensitivity to immune checkpoint blockers. Studies of human melanoma tumors have identified a set of chemokines that are associated with enhanced recruitment of T cells toward tumor tissue. These chemokines, including CCL2, CCL3, CCL4, CCL5, CXCL9, and CXCL10, might have utility as clinical therapies to improve T cell trafficking to tumors [47]. However, such chemokines or other T cell recruitment factors must be targeted specifically to tumor tissue in order to effectively recruit T cells. T cell recruitment factors might be coupled to antibodies that bind to tumor cell receptors, thus providing a vehicle for tumor targeting. In animal tumor studies, a T cell recruitment factor called LIGHT (also called tumor necrosis factor superfamily member 14) was fused to an anti-epidermal growth factor receptor (EGFR) antibody. This LIGHT-anti-EGFR fusion molecule was able to promote more extensive T cell infiltration into EGFR-expressing tumors. In turn, this prevented resistance to PD-L1 blockade immunotherapy [48]. Similar strategies that target other T cell recruitment factors toward tumors might be feasible.

Our group at the Pacific Heart, Lung & Blood Institute (Los Angeles, CA) is conducting research on gene-modified human mesenchymal stem cells (MSCs) as a strategy to alter the tumor microenvironment and prevent resistance to immune checkpoint blockers. MSCs can be isolated and expanded from various adult tissues including bone marrow, fat, umbilical cord blood, and term placentas. MSCs are known to preferentially migrate to tumor tissue, making them potentially useful drug delivery vectors to alter the immunological microenvironment of tumors [49]. In animal tumor models, MSCs have been genetically modified in diverse ways to effectively treat tumors. These include modification to produce immunostimulatory cytokines (e.g. IFN-α, IFN-β, IL-12) and T cell trafficking molecules such as LIGHT [50–53].

Both autologous and allogeneic MSCs have been used extensively in clinical trials for treating severe inflammatory disorders and certain degenerative conditions, and generally have an acceptable safety profile [54]. Autologous gene-modified MSCs have recently entered clinical trials for cancer [55]. It remains to be seen if MSCs and other tumor-targeting systems can effectively deliver pro-inflammatory agents to tumor tissue and improve sensitivity to clinical immune checkpoint blockers.

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