Chapter 20
Novel Targets of Immune Inhibitory and Stimulatory Co-signals

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Abstract Great success of anti-CTLA-4 and anti-PD-1 monoclonal antibodies (mAbs) has changed a landscape of cancer immunotherapy. Currently, there is no doubt about an importance of immune checkpoint molecules as one of the most promising targets in anticancer drugs. Thus, identification and characterization of novel checkpoint molecules other than CTLA-4 and PD-1 is a highly anticipated research subject. In addition, agonists of stimulatory co-signal molecules have a capability of enhancing antitumor immunity, rendering them attractive in anticancer drug development. From this perspective, this chapter introduces LAG-3, TIM-3, BTLA, 4-1BB, OX-40, and GITR, as representatives of potential targets which have been explored in cancer immunotherapy. Functions of these molecules in T cell immunity and antitumor effects in preclinical animal models as well as clinical trials, if available, are described here.

Keywords Immune checkpoints • T cell exhaustion • Stimulatory co-signal molecules

20.1 Introduction

In recent years, immune checkpoint blockade has demonstrated substantial advances and a striking success as a novel strategy in cancer immunotherapy. Anti-CTLA4 antibody (Ab) and anti-PD-1 Ab represent approaches of immune checkpoint blockade, which have been approved by FDA as drugs for advanced melanoma in 2011 and 2014, respectively. In future, application of these antibodies (Abs) is anticipated to expand through combinations with other methods of immunotherapies, e.g., tumor vaccine and adoptive T cell transfer, as well as non-immunotherapies including chemotherapeutic drugs, kinase inhibitors, and irradiation. At the same time, further efforts have been made to identify novel checkpoint molecules besides CTLA-4 and PD-1, so as to regulate the functions of

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novel molecules for therapeutic purposes. In addition, agonistic Abs which deliver stimulatory co-signals to activate antitumor T cell responses have been also sought and tested by clinical trials as novel approaches in cancer immunotherapy. In this chapter, preclinical and clinical development of novel checkpoint molecules and stimulatory co-signal molecules is reviewed.

20.2 Novel Targets of Immune Checkpoint Molecules

As definition of immune checkpoint molecules, they deliver inhibitory co-signals into T cells and negatively regulate T cell responses. While they are expressed on T cells either constitutively on naïve status or inducibly in response to activation, the highest expression are often detected on nonfunctional status including exhausted T cells. Typically, checkpoint molecules possess unique intracellular motifs to deliver inhibitory signals, such as ITIM (immunoreceptor tyrosine-based inhibitory motif), which are crucial to render T cells that undergo functionally unresponsive status. Attenuation of immune checkpoint is capable of preventing and restoring the T cell unresponsiveness, providing the rationale for applying checkpoint-blocking Abs to cancer immunotherapy. According to the cells expressing ligands of checkpoint molecules, its blockade mediates the effect at two potential phases of T cell response, i.e., priming phase and effector phase. For instance, as CD80/CD86, ligands of CTLA-4, are expressed on professional APC including DC, blockade of CTLA-4 enhances T cell activation at the priming phase. On the other hand, PD-L1 (B7-H1), a ligand of PD-1, is expressed in the tumor microenvironment, e.g., tumor cells and tumor stromal cells, indicating that PD-1 blockade potentiates T cell functions at the effector phase. Collectively, immune checkpoint molecules for therapeutic targets should meet, at least in part, the following criteria: (1) capacity of delivering inhibitory signal to cause T cell unresponsiveness, (2) blockade of its functions to activate T cells by abrogating unresponsiveness, and (3) its expression on nonfunctional (e.g., exhausted) T cells and its ligand expression on APC or in the tumor microenvironment. LAG-3, TIM-3, and BTLA are among the promising and novel checkpoint molecules which meet these criteria.

20.2.1 LAG-3

LAG-3 (lymphocyte activation gene-3, CD223), a molecule belonging to immunoglobulin superfamily, has structural homology to CD4 [1]. LAG-3 binds MHC class II via its D1 domain at 60 nM K_d, several orders higher affinity than that of CD4 for MHC class II [2]. Intracellular domain of LAG-3 contains a unique motif (KIEELE), which is essential for LAG-3 functions in T cell regulation [3, 4]. Expression of LAG-3 is detected on activated T cells, starting from 24 h after stimulation, peaking at 48 h and then gradually decreasing by day 8.

Immune-regulatory functions of LAG-3 were first revealed by experiments using anti-LAG-3 mAb, in which human CD4+ T cell clone exhibited persistent proliferation in vitro when LAG-3 was blocked [5]. Later, inhibitory function of LAG-3 was further consolidated by the studies that T cells in LAG-3 knockout (KO) animals augment proliferation, accumulation, and effector functions in response to mitogenic stimuli or cognate antigens [6, 7]. Regarding T cell inhibitory mechanism of LAG-3, transfection of LAG-3 gene lacking intracellular signaling domain lost its effects, indicating an intrinsic inhibitory mechanism [4]. On the other hand, LAG-3 expression on regulatory T cells (Treg) and its role in T cell suppression was also reported [8]. Anti-LAG-3 mAb abrogated suppressive effects of Treg, and Treg from LAG-3 KO mice reduced the suppressive activity. Ectopic expression of LAG-3 on CD4+ T cells confers them suppressive potential toward other T cells. These findings suggested extrinsic mechanisms of LAG-3 in T cell inhibition. As a cell surface marker, LAG-3 expression is associated with T cell exhaustion caused by chronic infection [9]. Recent studies further indicated that functionally impaired T cells in cancer also express LAG-3 simultaneously with PD-1 [10, 11].

Therapeutic application regulating LAG-3 functions for cancer immunotherapy has been attempted by means of LAG-3-Ig fusion proteins and anti-LAG-3 mAb. Administration of LAG-3-Ig induced growth retardation and regression of various types of tumor in mouse models [12]. It has been reported that mechanisms of antitumor effects by LAG-3-Ig are dependent on its binding to MHC class II and consequent maturation and activation of APC, including upregulated expression of co-stimulatory ligands and IL-12 production [13]. A potential role of LAG-3-Ig to block LAG-3 inhibitory signal in T cell activation remains largely unexplored. In clinical studies, LAG-3-Ig has been developed as IMP321 by Immutep and tested in renal cell carcinoma by a single agent and in breast cancer in combination with chemotherapy [14, 15]. Besides LAG-3-Ig, antagonistic anti-LAG-3 mAb has been shown to restore T cell exhaustion in mouse tumor model [11]. Accordingly, phase I clinical trial of anti-LAG-3 mAb (BMS-986016 developed by Bristol-Myers Squibb) with or without anti-PD-1 mAb in solid tumor as well as hematological malignancies has been initiated. Results of the clinical studies of LAG-3-Ig and anti-LAG-3 mAb are currently awaited with great expectations.

20.2.2 TIM-3

TIM-3 belongs to TIM (T cell immunoglobulin) family molecules, type I membrane protein, which structurally consists of N-terminal IgV domain followed by a mucin domain, a transmembrane domain, and an intracellular domain [16]. In T cells, TIM-3 is uniquely expressed on those differentiated into IFN-γ-producing cells, such as Th1-type CD4+ and Tc1-type CD8+ T cells. Galectin-9, a soluble molecule that is upregulated by IFN-γ, was identified as a ligand of TIM-3 [17]. Binding of galectin-9 with TIM-3 triggers T cell death by dissociating Bat3

(HLA-B-associated transcript 3) from intracellular domain of TIM-3 [18]. Thus, TIM-3 plays an essential role in termination of IFN-γ-mediated inflammatory T cell responses. Consistent with this notion, blockade of TIM-3 by anti-TIM-3 or TIM-3-Ig fusion protein augments T cell responses, leading to exacerbation of autoimmune diseases and abrogation of T cell tolerance in animal models [19, 20]. Mice deficient of TIM-3 gene also demonstrated similar phenotypes [20].

In cancer immunotherapy, TIM-3 is a potential target as an immune checkpoint molecule to interfere with. TIM-3 expression is detected on tumor-infiltrating lymphocytes (TIL) in various types of cancer and associated with T cell exhaustion [21]. It should be noted that T cells expressing both PD-1 and TIM-3 represent the most deeply exhausted phenotype, in terms of proliferation and cytokine production of IL-2, TNF-α, and IFN-γ. Based on this finding, combined blockade of TIM-3 and PD-1 was tested and revealed a striking effect in tumor growth inhibition, more potent than a single blockade of either molecule [21, 22]. Restoration of T cell effector functions by dual blockade of TIM-3 and PD-1 has been reported in animal tumor models as well as T cells from melanoma patients [21, 23]. Besides direct effects on antitumor T cells, TIM-3 has been reported to promote granulocytic MDSC (myeloid-derived suppressor cells) via cognate interaction with galectin-9, which is expressed on $CD11b^+$ $Ly6G^+$ cells [24]. As MDSC expand in tumor-bearing hosts and facilitate immune suppression at tumor microenvironment, TIM-3 blockade could indirectly stimulate antitumor immunity by attenuating MDSC functions. In addition, recent intriguing studies discovered an increased expression of TIM-3 on leukemic cancer stem cells in patients with acute myeloid leukemia, suggesting a potential use of TIM-3 as a target for tumor killing [25, 26]. Collectively, TIM-3 could serve as a multifunctional molecule in tumor growth and antitumor immunity. Clinical trials to examine TIM-3-targeting reagents such as anti-TIM-3 mAb have yet to be initiated in cancer patients, and such studies are eagerly awaited.

20.2.3 BTLA

BTLA (B and T lymphocyte attenuator, CD272) was cloned from activated T cells as a molecule structurally homologous to immunoglobulin superfamily [27]. Similar to PD-1 and CTLA-4, BTLA has one IgV domain in extracellular domain, followed by transmembrane domain and intracellular domain, where two ITIM motifs exist. The ligand of BTLA is HVEM (herpesvirus entry mediator, CD270), which belongs to TNF (tumor necrosis factor) receptor superfamily [28]. By interacting with HVEM, BTLA delivers inhibitory signal into activated T cells by recruiting SHP-1/2 via its intracellular ITIM motifs [28, 29]. Consistent with these findings, mice deficient of BTLA gene exhibited exacerbated autoimmune and inflammatory diseases [27, 30] and enhanced memory T cell responses [31]. While an increased expression of BTLA on anergic T cells was reported [32], another study indicated

no correlation between BTLA expression level and a severity of T cell exhaustion [33].

Based on the findings described above, a role of HVEM-BTLA interaction in tumor immunity and its potential as a therapeutic target have been explored. In animal model, blockade of BTLA signal facilitated the effects of antitumor vaccine and inhibited tumor growth in vivo [34]. In melanoma patients, HVEM expression was detected on tumor cells, and tumor Ag-specific T cells persistently express high levels of BTLA [35]. $CD8^+$ T cells expressing BTLA were partially dysfunctional, and blockade of BTLA restored T cell proliferation and cytokine production in response to tumor Ag in vitro [33]. Thus, anti-BTLA mAb can be a novel approach of immune checkpoint blockade, although no clinical trial has initiated yet. It should be noted that HVEM-BTLA interaction can deliver bidirectional signal to both sides, where HVEM transmits stimulatory co-signal to T cells [36]. Thus, HVEM-BTLA pathway should be carefully manipulated for cancer immunotherapy, as simple blockade could diminish HVEM-mediated positive effects as well as BTLA negative signal.

20.3 Novel Targets of Immune Stimulatory Co-signal Molecules

Quality and quantity of T cell responses are determined by a fine balance between stimulatory and inhibitory co-signals. When stimulatory co-signals surmount inhibitory co-signals, T cells activate and generate productive responses. On the other hand, when inhibitory co-signals are dominant, T cells undergo dysfunctional state, such as anergy and exhaustion, leading to a termination of immune responses. Thus, in order to accelerate antitumor immunity, triggering stimulatory co-signals, in addition to blockade of inhibitory co-signals (=immune checkpoints), would be an important strategy. Accordingly, agonistic Abs against stimulatory co-signal molecules have been developed, and some of them are currently under clinical investigation. Abs against 4-1BB, OX-40, and GITR are among the most promising and advanced reagents in this strategy.

20.3.1 4-1BB

4-1BB (CD137), a molecule of TNF receptor superfamily, is inducibly expressed on T cells along with their activation. Interaction with its ligand, 4-1BBL, triggers 4-1BB stimulatory co-signal, which activates NF-κB and MAPK via recruitment of TRAF [37]. 4-1BB signal enhances T cell activation and cytokine production and promotes their survival by inducing antiapoptotic molecules such as Bcl-X_L, especially in CD8+ T cells [38]. While mice deficient of 4-1BB gene exhibited a

reduced number of memory CD8+ T cells in bone marrow, there is an accumulation of effector memory T cells in 4-1BB-overexpressing transgenic mice [39, 40]. Expression of 4-1BB is also detected on NK cells and DC, and stimulatory effects of 4-1BB on these cells have been also reported [41, 42].

In mouse tumor models, triggering 4-1BB stimulatory co-signal by agonistic Ab or gene transfection induced prominent effects of tumor regression [43, 44]. Mechanistically, these effects are dependent on activation of CD8+ T cells and NK cells and associated with an increased accumulation of TIL by IFN-γ secretion [45]. Based on these studies, fully human anti-4-1BB mAbs with agonistic capacity have been developed by at least two pharmaceutical companies. Although early results from clinical trials indicated a substantial liver toxicity, more detailed examinations of anti-4-1BB mAbs as monotherapy or in combination with other mAbs are currently performed in patients with solid tumors and hematological malignancies [46].

20.3.2 OX-40

OX-40 (CD134) is a member of TNF receptor superfamily and originally identified as an activation marker on rat CD4+ T cells [47]. Subsequent studies revealed that OX-40 is expressed on both CD4+ and CD8+ T cells upon activation, as well as NK cells, and OX-40 signal promotes proliferation, cytokine production, migration, and effector functions of these cells [48]. Mice deficient of OX-40 or OX-40L, a ligand of OX-40, exhibited impaired T cell responses in vivo, indicating a role of this pathway in providing a stimulatory co-signal to T cells [49, 50]. In animal experiments, administration of OX-40 agonists, including anti-OX-40 mAb and OX-40L-Ig fusion protein, prolonged the mouse survival in various tumor models [51]. In addition to direct effects in stimulating T cell activation, there is also evidence that OX-40 agonists dampen suppressive function of Treg, thus indirectly facilitate antitumor immunity [52, 53]. In cancer patients, the existence of OX-40-positive T cells in TIL and tumor-draining lymph nodes has been reported [54]. Phase I clinical trial using anti-OX-40 mAb demonstrated that the drug was tolerated, promoted T cell proliferation, and induced tumor shrinkage in some patients [55]. Further studies of OX-40 agonists are currently underway in clinical trials to evaluate its antitumor effects as monotherapy or in combination with other drugs.

20.3.3 GITR

GITR (glucocorticoid-induced TNF receptor, CD357) is expressed on various immune cells including activated T cells. GITR signal delivers stimulatory co-signal into T cells and enhances their proliferation, cytokine production, and

survival [56]. Compared to other stimulatory co-signal molecules, GITR has a unique feature that is constitutively expressed on Treg at high levels, and triggering GITR signal in Treg abrogates their suppressive function [57, 58]. As expected from these findings, treatment with anti-GITR agonistic mAb caused regression of tumor in animal models [59, 60]. Further studies suggested that GITR agonists decrease the number and suppressive function of Treg at the tumor microenvironment by causing Treg instability and depletion [61, 62]. A phase I clinical trial using humanized anti-GITR agonistic mAb in advanced melanoma and other solid tumors is currently ongoing.

20.4 Summary

Recent development of anti-CTLA-4 and anti-PD-1 mAbs represents magnificent success in cancer immunotherapy. Accordingly, approaches to manipulate inhibitory or stimulatory co-signal functions are considered to be a rising star in the field, and identification of novel targets with a potent therapeutic potential is eagerly anticipated. While this review focuses on several novel molecules which are among the most promising and progressive in clinical translation, there are many other intriguing targets which are not introduced here. Development of novel reagents to regulate these molecules as monotherapy or combined immunotherapy with current medical interventions including kinase inhibitors, chemotherapy, and radiotherapy will establish next generation of cancer treatment.

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