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Jie Xu *Editor*

Regulation of Cancer Immune Checkpoints

Molecular and Cellular Mechanisms and Therapy

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Chapter 1
Introduction

Jie Liu and Jie Xu

Abstract Cancer immunotherapy, especially immune checkpoint blockade therapy, represents a hotspot in cancer research. However, the low response rate, adaptive/acquired resistance, and adverse effects still keep most cancer patients from obtaining sustained clinical benefits. To overcome these limitations, it is essential to improve our understanding on the regulation of immune checkpoints under physiological and pathological contexts. Recent researches have gained insights into the molecular control of immune checkpoint receptors and ligands, which extended our knowledge on the immune system and provided alternative strategies for developing checkpoint inhibitors.

Keywords Immune checkpoint · Cancer immunotherapy · Gene regulation · Biomarkers · Antibody

Immune checkpoints represent negative regulators of the immune system that mediate self-tolerance, preventing autoimmunity and protecting tissues from immune attack (Webster 2014). During the past decade, immune checkpoint blockade (ICB) therapy has changed the palette of cancer biotherapy (Allison 2015). Unlike many cancer studies that only demonstrated therapeutic effects in cellular or animal models, ICB research has achieved unprecedented success in clinical applications (Tang et al. 2018). While compared to other proposed immunotherapies such as interferon and cancer vaccines, ICB targeting CTLA-4 and PD-1/PD-L1 checkpoints showed stronger and more durable therapeutic effects. The FDA has approved the clinical use of antibody-based ICB drugs such as Opdivo (nivolumab, Bristol-Myers

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Squibb), Keytruda (pembrolizumab, Merck), and Tecentriq (atezolizumab, Genentech Inc./Roche Holdings AG) that function by blocking PD-L1 on the cell surface. Recently, ICB therapy has been proposed to treat infectious diseases (Wykes and Lewin 2018).

However, there are still outstanding challenges for ICB therapy, and one major concern is the low response rate. Taking the humanized anti-PD-L1 inhibitor Tecentriq as an example (approved by the FDA for treating urothelial carcinoma in 2016), the IMvigor 210 study revealed an objective response rate of only 27% in patients with metastatic urothelial carcinoma expressing medium and high levels of PD-L1. This means as many as 73% of patients did not show objective response although PD-L1 is expressed in these cancers. To improve the response rate of ICB, efforts have been made to identify more accurate biomarkers and more effective therapeutic approaches, which rely on further mechanistic insights into the dark box of checkpoint regulation (Villanueva 2017; Nishino et al. 2017).

For the patients who respond to ICB, the therapeutic effects may be eventually lost after long-term treatment (acquired resistance). Unfortunately, the molecular and cellular events driving the acquired resistance are yet to be illustrated, rendering the sustainability of ICB treatment a puzzling question. Due to the lack of comprehension on the physiological roles of checkpoints, the adverse effects of PD-1/PD-L1 blockade were often discovered rather unexpectedly (Samaan et al. 2018). Till now, a diverse panel of adverse effects have been found in patients receiving ICB (Byun et al. 2017; Sury et al. 2018), with the underlying mechanisms largely unclear.

To improve the response rate, durability, and safety of ICB therapy, it is essential to gain insights into the regulation of immune checkpoints at physiological and pathological conditions. From at least two aspects, it is reasonable to expect a huge space for growth of researches on checkpoint regulation in the future:

(1) The number of questions remaining to be unanswered. As a core biomarker for ICB therapy, PD-L1 expression may vary considerably among tumor types, stages, cases, and tissue sections. However, our current knowledge on the regulation of this molecule can adequately explain few of these observations (Wang et al. 2018). Actually, some of the most fundamental questions still remain unclear, such as the identification of key immune checkpoints in different physiological and pathological conditions.

(2) The unmatched advances in basic and translational studies. When compared to some relatively well-studied pathways such as p53, the mechanistic researches on immune checkpoints still have vast space for growth. The clinical trials related to PD-1 (n = 721) are fivefold more than those related to p53 (141), according to ClinicalTrials.gov (until March 2019). In contrast, the papers for PD-1 (n = 10,132) indexed by PubMed only account for 1/9 of those for p53 (n = 9463). Although both related to the concept of "checkpoint", the studies on PD-1 (immune checkpoint) and p53 (cell cycle checkpoint) have displayed drastically distinct characteristics in translational and basic researches (Fig. 1.1).

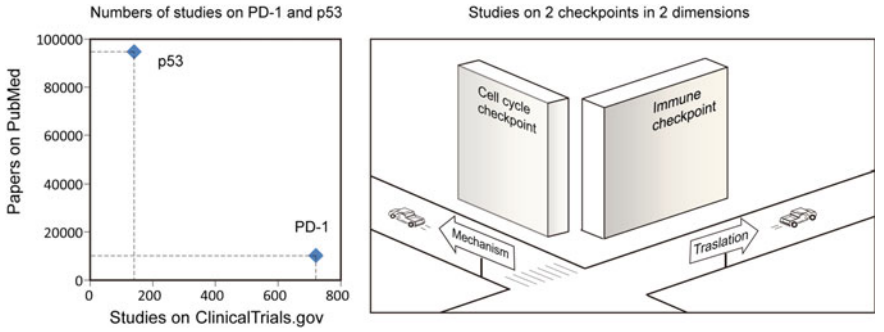

Fig. 1.1 Researches on immune checkpoints have made considerable progresses in therapeutic development, with the basic mechanistic studies lagging behind. The left panel plots the numbers of studies related to PD-1 and p53 on PubMed and ClinicalTrials websites. The right panel is a metaphor for the unmatched study progresses for the two "checkpoint" pathways in two dimensions (mechanistic and translational researches)

In addition to the two aspects mentioned above, the higher complexity and variability of immune checkpoint signaling also deserve more efforts in mechanistic studies. Sometimes being called "the shield of tumour cells" (Brahmer 2013), immune checkpoints are engaged in the "intercellular Star War" by modulating cell–cell interactions. This complex feature differs from housekeeping pathways involved in essential cellular activities such as energy metabolism and cell cycle control. As an example, the p53 tumor suppressor is more conserved in different species, and p53-like proteins are also found in squid, oyster, worm, and other lower organisms. In various physiological and pathological conditions, the wild-type p53 is expected to trigger cell cycle arrest and/or apoptosis but not to promote cell proliferation (Hafner et al. 2019; Minton 2014; Liang et al. 2018). In contrast, the PD-1/PD-L1 checkpoint signaling has been found with higher variability in different contexts. In immune cells, the activation of PD-1 transduces an inhibitory signal and induces T-cell exhaustion, but the stimulation of cancer-intrinsic PD-1 instead promotes cell proliferation through the mTOR pathway (Yao et al. 2018; Li et al. 2017; Kleffel et al. 2015). A potential explanation for the higher variability in immune checkpoint signaling may be due to its evolvement together with the complexity of the organisms. According to the UniProt.org database, the genes encoding PD-L1 (CD274) are only found in higher organisms such as vertebrates (Fig. 1.2).

The regulation of immune checkpoints may be more complicated in cancerous conditions, due to the variations in cancer origins, mutational backgrounds, subtypes, stages, and treatment contexts. Thus, enough caution should be paid while trying to generalize the regulatory mechanisms found in one cancer type/condition to others. Also, the characterizations on checkpoint regulation should be preconditioned by the clarification on the genetic status and transcriptional isoform(s) of reported genes, because these factors may contribute to the complexity of checkpoint signaling in cancers (Hassounah et al. 2019).

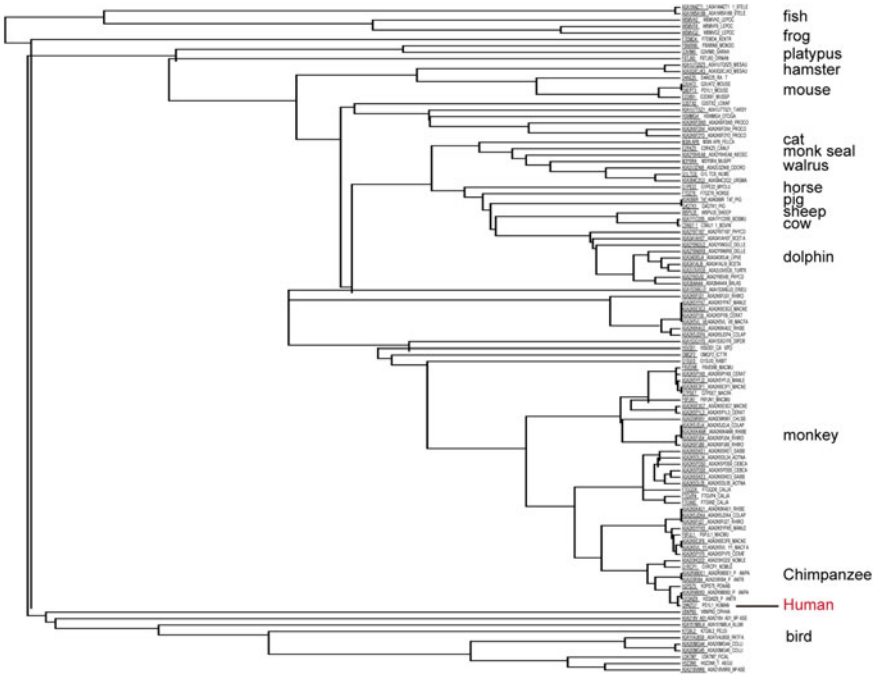

Fig. 1.2 Homology of PD-L1 in different organisms. The hierarchical tree presents the similarity between the sequences of PD-L1 in different organisms. The human PD-L1 gene is marked in red

Even in a well-defined context, the expression and functions of immune checkpoints may be collaboratively determined by many factors. Taking the PD-1/PD-L1 pathway as an example, previous studies have revealed sophisticated regulatory mechanisms at genetic, epigenetic, transcriptional, translational, posttranslational, and trafficking levels to control the expression and functions of PD-L1 (Wang et al. 2018). In addition to the direct regulations on the checkpoint ligands and receptors, various molecular or cellular events may affect immune checkpoint signaling by perturbing the microenvironment and inflammatory signaling networks. Although understanding these regulatory mechanisms requires tremendous research work, such efforts are certainly worth well. The new insight into the regulation of immune checkpoint may bring new opportunities for discovering more accurate biomarkers and developing more effective, durable, and safer ICB therapy.

In this book, we systematically describe current understandings on the regulation of immune checkpoints. In the following two chapters, we first introduce the CTLA-4 and PD-1 pathways that have been studied earlier with translational success, and in another chapter, the emerging immune checkpoints have been summarized. As the driving force for mechanistic studies, several major challenges in ICB therapy are discussed, including the primary and acquired resistance, adverse effects, as well as the quest for more accurate biomarkers. The drastic variation in the expression

of immune checkpoint molecules is discussed in an independent chapter, given the outstanding feature of this pathway. Then we focus on the functions of checkpoint molecules beyond immune evasion, in order to give a comprehensive view on the biology of immune checkpoints. In several chapters to follow, the regulatory mechanisms on immune checkpoint molecules are elaborated at genetic, transcriptional, translational, posttranslational, and trafficking levels. In the final chapters related to translational researches, the targeting strategies using macromolecules and antibodies, peptides, and small molecular compounds are, respectively, described. Before the concluding remarks, we provide an overview of therapeutic development of immune checkpoint blockers. To improve the readability of our book, we try to present key mechanisms and concepts by schematic drawings.

Together with all chapter authors, we made efforts to present a comprehensive and in-depth perspective model for the regulation of immune checkpoints, which may promote the basic and translational studies on cancer immunology and immunotherapy.

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Chapter 2
Molecular and Cellular Functions of CTLA-4

Samya Van Coillie, Bartosz Wiernicki and Jie Xu

Abstract Cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) is an inhibitory receptor belonging to the CD28 immunoglobulin subfamily, expressed primarily by T-cells. Its ligands, CD80 and CD86, are typically found on the surface of antigen-presenting cells and can either bind CD28 or CTLA-4, resulting in a costimulatory or a co-inhibitory response, respectively. Because of its dampening effect, CTLA-4 is a crucial regulator of T-cell homeostasis and self-tolerance. The mechanisms by which CTLA-4 exerts its inhibitory function can be categorized as either cell-intrinsic (affects the CTLA-4 expressing T-cell) or cell-extrinsic (affects secondary cells). Research from the last decade has shown that CTLA-4 mainly acts in a cell-extrinsic manner via its competition with CD28, CTLA-4-mediated trans-endocytosis of CD80 and CD86, and its direct tolerogenic effects on the interacting cell. Nonetheless, intrinsic CTLA-4 signaling has been implicated in T-cell motility and the regulation of CTLA-4 its subcellular localization amongst others. CTLA-4 is well recognized as a key immune checkpoint and has gained significant momentum as a therapeutic target in the field of autoimmunity and cancer. In this chapter, we describe the role of costimulation in immune response induction as well as the main mechanisms by which CTLA-4 can inhibit this process.

Keywords CTLA-4 · CD28 · CD80 · CD86 · Immune tolerance

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2.1 T-Cell Costimulation and Costimulatory Molecules

Cancerous cells develop as a result of genetic mutations of oncogenic (promoting growth) or suppressor (inhibiting mitosis) genes. Their unchallenged proliferation leads to tumor formation, metastasis, and ultimately death. Abnormally growing cells, however, are detected and fought off by the immune system with both its innate and adaptive branch. Because of the frequency of DNA mutations in cancer cells, they very often carry their own set of tumor-associated antigens (TAA). This makes them prone to recognition by T-cells as non-self tissue, which in turn can provoke immunogenic response against the tumor.

T-cells are blood cells of lymphoid lineage that develop in the bone marrow and are transported to and mature in the thymus. Their marker is simply called "T-cell receptor" (TCR), a molecule that is responsible for the most important function of the T-cells—antigen recognition. Each T-cell carries a TCR specific only for one epitope, which is presented by an "antigen-presenting cell (APC)"—usually a dendritic cell (DC). DCs reside in different peripheral tissues and are first responders to potential threats. When they encounter infected or cancerous cells, they phagocytize them and present their specific antigens via major histocompatibility complex (MHC) molecules. These DCs further migrate to the lymphoid tissue where they are able to present the newly loaded epitopes to specific, naive T-cells. Epitopes loaded on MHC-I will interact with a cytotoxic subpopulation of the T-cells (CTL), while those presented on MHC-II will induce activation of so-called helper T-cells (Th). It is worth noting, however, that MHC-TCR interaction alone is not enough for proper functional activation of TAA T-cells. This primary signal has to be supported by so-called costimulatory molecules in order to develop an effective immunogenic response. T-cell subtypes with their function and markers are listed in Table 2.1.

2.1.1 Two-Signal Model of CD4+ T-Cell Activation

The interaction between the TCR and MHC molecules presenting specific epitopes is detrimental to ensure the specificity unique to the adaptive immune response. However, proper activation of T-cells requires an additional signal ensured by costimulatory molecules. Only upon receiving both the antigen receptor—antigen signal and the secondary signal, the lymphocyte's survival and proliferation is promoted (Murphy and Weaver 2017) (Fig. 2.1). Because of the necessity of these two signals, the process was coined the "two-signal model of activation" (Lafferty and Cunningham 1975; Lafferty and Woolnough 1977; Jenkins et al. 1987). The basis for this model was laid in 1970 by Cohn and Bretscher, who proposed that in order to become activated, a lymphocyte not only had to interact with an APC but also with a different lymphocyte specific to that antigen (Bretscher and Cohn 1970). Their model became generally accepted for CD8+ T-cells since the activation of most CD8+ T-cells indeed

Table 2.1 T-cell subtypes, CTLA-4 levels, and function

	Type of T-cell	Markers	CTLA-4 levels	Function	Main transcription factor regulating differentiation
Conventional T-cells (responding to the threat)	CTLs	CD3, CD8	Upregulated in activated cells. Higher in exhausted (functionally incapable after prolonged activation) CD8 CTLs (Wherry et al. 2007)	Possess cytotoxic activity against infected or cancerous cells	T-bet
	Th1	CD3, CD4	Lower than in Th2 (Wherry et al. 2007)	Stimulate cell-mediated response. Important for the proper activation of CTLs	T-bet
	Th2	CD3, CD4	Higher than in Th1 (Wherry et al. 2007)	Stimulate antibody-based response. Important for B-cells, eosinophils	GATA3
	Th17	CD3, CD4	High in memory Th17 compartment (Krummey et al. 2014)	Neutrophil and macrophage recruitment	Stat3, JunB, ROR-ɣ
Regulatory T-cells (regulate function of T-cells and other immune system cells)	iTreg	CD4, CD25, CD39	Constitutively expressed (Takahashi et al. 2000)	Regulation of immune system response	Foxp3

requires costimulation from CD4+ Th-cells. However, for CD4+ T-cells the mechanism of activation is somewhat different, with the secondary signal originating from costimulatory signals between the T-cell and an APC, rather than another lymphocyte (Lafferty et al. 1978). In the context of this book, we will focus on the two-signal model of activation as described for CD4+ T-cells, not going into any further details

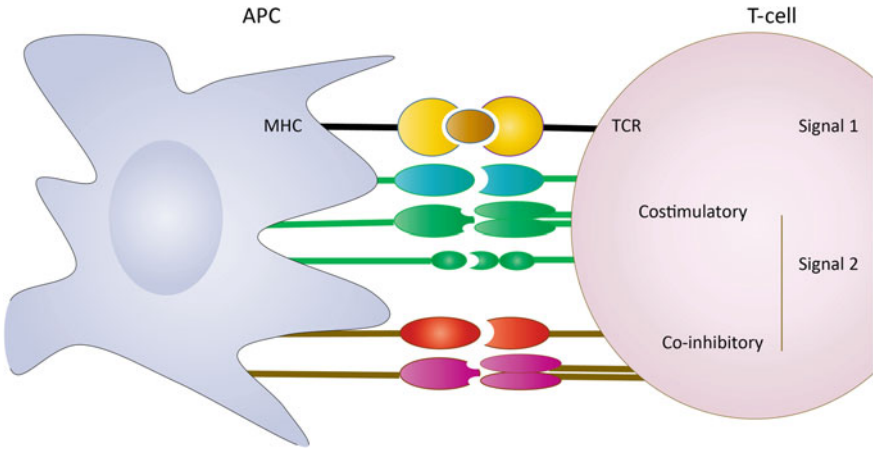

Fig. 2.1 Two-signal model of T-cell activation. Signal 1 in this model comprises the binding of the TCR complex to an antigen presented by an APC. The binding of one or more costimulatory receptors expressed by the naïve T-cell to their respective ligands on the APC constitutes signal 2. Only if these positive signals predominate the negative signals mediated by co-inhibitory molecules will the T-cell become fully activated. TCR: T-cell receptor; APC: antigen-presenting cell

on CD8+ T-cell activation, as it falls outside the scope of the book. For simplicity, CD4+ T-cells will be referred to as just T-cells in the rest of this chapter, unless stated otherwise.

The two-signal model of activation for T-cells consists of signal 1—the already mentioned binding of the T-cell receptor complex to an antigen presented by an APC—and signal 2: the binding of costimulatory molecules to costimulatory receptors on the APC and the naïve T-cell, respectively. These costimulatory receptors on the surface of the T-cell contain, next to their extracellular domain, also a transmembrane part and an intracellular tail inducing a signaling cascade that modulates the T-cell response (Schwartz 1990; Mir 2015; Murphy and Weaver 2017). In the absence of costimulatory signal 2, the T-cell can become anergic toward stimulation or even undergo apoptosis (Jenkins et al. 1987; Mueller 1989; Schwartz 1990; Kroczek et al. 2004).

The different costimulatory molecules known to date are either members of the immunoglobulin superfamily or the tumor necrosis factor (TNF) receptor superfamily. Within the immunoglobulin superfamily, various subfamilies can be distinguished, of which the CD28 family is by far the most important—and well characterized—in the context of costimulation (Sharpe and Freeman 2002). Prior to ligation, the costimulatory receptors are often somewhat spread out over the surface of the T-cell. Only upon peptide: MHC recognition by the TCR will these costimulatory molecules migrate toward the immunological synapse where the interaction between T-cell and APC takes place. This colocalization of the costimulatory receptors and the TCR was proven to be crucial to ensure optimal activation of naïve T-cells (Saito et al. 2010; Chen and Flies 2013).

Once a costimulatory receptor binds its costimulatory ligand on the APC a signaling cascade is induced in the T-cell, mediated via the transfer of signal through the receptor's cytoplasmic tail. Depending on the type of receptor, different signaling motifs are expressed thereby creating a variety of signaling pathways within the cell. Commonly activated pathways include the PI3K/AKT, RAS/ERK, NFAT, AP1/2, C-MAF, and NF-κB pathway (Dower et al. 2000; Isakov and Altman 2002; Rao et al. 2002; Oh-hora and Rao 2009; Chen and Flies 2013; Haining and Weiss 2018). Activation of these pathways stimulates T-cell survival, growth, and functioning. However, co-inhibitory signaling mediated by co-inhibitory receptors on the surface of the cell may interfere with these positive signals via a number of different mechanisms, one of which is by dephosphorylation of major signaling nodes essential to the proper activation of T-cells (Sinclair 1990; Sinclair and Anderson 1996). The various signaling pathways used by most co-inhibitory and costimulatory molecules were found to overlap greatly, resulting in complex interaction and crosstalk patterns. Which pathways will eventually predominate is highly context specific as even the precise function of both costimulatory and co-inhibitory molecules is prone to change (Chen and Flies 2013; Kumar et al. 2018). Additionally, costimulation has proven to be important not only in naïve T-cell activation but also in effector, memory, and regulatory T-cell functioning (Chambers and Allison 1997; Collins et al. 2002; Appleman and Boussiotis 2003).

2.1.2 Costimulatory and Co-inhibitory Molecules

2.1.2.1 The CD28 Subfamily

The most well-studied costimulatory receptor on the surface of a T-cell is a protein called CD28. Its presence is essential for proper T-cell activation, and hence, it is expressed on the surface of all murine naïve T-cells and on approximately 80% of CD4+ T-cells and 50% of CD8+ T-cells in humans (Esensten et al. 2016). CD28 binds to either CD80 or CD86, also known as B7-1 and B7-2, which are costimulatory molecules or ligands from the same family, found mainly on the surface of specialized APCs. Consequently, the antigen receptor signaling of the T-cell is stimulated resulting in the promotion of proliferation, cytokine secretion and cell survival (Murphy and Weaver 2017). However, CD28 is not merely an amplifier of the TCR signal; it also transfers signals controlling complex biochemical events involved in posttranslational modifications such as phosphorylation or signals altering the epigenetic code, thereby changing the gene expression profile of the T-cell (June et al. 1987; Shapiro et al. 1997; Acuto and Michel 2003; Boomer and Green 2010; Esensten et al. 2016).

CD28 is one of many proteins defining a subfamily of costimulatory receptors and ligands. The receptors within this family all harbor a variable extracellular immunoglobulin-like domain, a single transmembrane domain, and a cytoplasmic tail containing critical signaling motifs (Carreno and Collins 2002; Chen and Flies

2013; Esensten et al. 2016). A second important member of this family is the CTLA-4 receptor. In humans, the genes encoding CD28 and CTLA-4 are located next to one another on chromosome 2q33 and their amino acid sequences share roughly 30% identity (Linsley et al. 1991; Rudd et al. 2009; Mir 2015). Despite these similarities, CD28 and CTLA-4 have opposing functions as CTLA-4 inhibits T-cell activation rather than enhancing it (Tivol et al. 1995a, b; Krummel and Allison 1995). CTLA-4 also binds CD80 and CD86 ligand on APCs but with higher affinity than the CD28 molecule, thereby preventing its functional interaction with the ligands. This is believed to be one of the mechanisms CTLA-4 uses to counteract T-cell stimulation (Thompson Emma et al. 2000; Engelhardt et al. 2006).

The binding of both CD28 and CTLA-4 with CD80 or CD86 is mediated by a MYPPPY motif within the extracellular V-set immunoglobulin domain of the receptors. CD28 its cytoplasmic tail contains several signaling motifs such as the YXN motif and the YMNM motif which undergo phosphorylation on tyrosine residues during activation. Upon phosphorylation, these signaling motifs bind SH2 domain-containing proteins, which together with SH3 binding of the proline-rich motive PYAP will initiate the downstream signaling cascade (Esensten et al. 2016; Murphy and Weaver 2017). The N-terminal YXXM motif is not only characteristic of CD28, but is also found back in the cytoplasmic tail of CTLA-4 and ICOS (Esensten et al. 2016; Yang et al. 2017). Inducible T-cell costimulator or ICOS, as suggested by its name, is an inducible costimulatory receptor with homology to both CD28 and CTLA-4 (Hutloff et al. 1999; Mages et al. 2000; Rudd and Schneider 2003). Although ICOS and CD28 probably arose from gene duplication (Coyle et al. 2000; Mages et al. 2000) and ICOS resembles CD28 its structure and functionality as stimulator of T-cell proliferation, the two molecules promote the secretion of different types of cytokines and cannot be interchanged (Hutloff et al. 1999; Yoshinaga et al. 1999; Esensten et al. 2016; Murphy and Weaver 2017). Moreover, ICOS lacks the MYPPPY motif, which is necessary for the binding to CD80 and CD86 ligands. Unlike CD28 and CTLA-4, it has a slightly altered FDPPPD motif via which it binds a specific ligand named ICOS ligand or ICOSL (Yoshinaga et al. 1999; Mir 2015).

Programmed Death receptor 1 (PD-1) and its ligands PD-L1 and PD-L2 together with the more recently discovered CD28 homolog member B and T lymphocyte attenuator (BTLA) and two B7 homologs—B7-H3 and B7-H4—belong to the CD28 subfamily as well. In contrast to CD28, CTLA-4 and ICOS which genes are clustered and which amino acid sequences share a "PPP" ligand-binding domain and the SH2 binding YXXM motif, PD-1 and BTLA are found elsewhere in the genome (2q37.3 and 3q13.2, respectively) and show more resemblance to one another than they resemble the other family members (Shinohara et al. 1994; Ravetch and Lanier 2000). PD-1, just as CTLA-4, is an important co-inhibitory receptor. Yet, the mechanism by which these two molecules function differs significantly (Parry et al. 2005; Buchbinder and Desai 2015), as will become clear in the next chapter of this book. BTLA seems to provide mainly inhibitory signals as well, although in contrast to CTLA-4 and PD-1, it interacts with a TNF receptor family member called herpesvirus entry molecule or HVEM to initiate its signaling (Sedy et al. 2005; Murphy et al. 2006). Nonetheless, more recent research also showed a role for BTLA in the costimulation of T-cells,

highlighting once more the complexity of the binding characteristics found within the CD28 family (Gavrieli and Murphy 2006).

2.1.2.2 The TNF/TNFR Subfamily

Next to the CD28 family, certain molecules belonging to the TNF/TNFR family were found to contribute to T-cell costimulation, albeit mostly after the initial activation has been established (Watts 2004; Mir 2015; Murphy and Weaver 2017). Costimulatory TNFR molecules are type I transmembrane proteins, which means they have an extracellular N-terminal and in intracellular C-terminal domain, and their N-terminal extracellular domain is characterized by cysteine-rich motifs. The best studied costimulatory receptor of the TNF subfamily is CD40, which plays a critical role in B-cell activation (Bretscher 1999; Elgueta et al. 2009). Much less is known about the costimulatory molecules in the context of T-cell activation such as OX40, CD27, CD30, 4-1BB, HVEM, and GITR. In general, TNFR-mediated costimulation results in the non-canonical activation of NFκB via the recruitment of TNFR-associated factor (TRAF) proteins harboring an E3 ubiquitin ligase potential (Watts 2004; Murphy and Weaver 2017). Except for CD27, which is constitutively expressed on naïve T-cells, all TNFR costimulatory receptors known to date are induced only after T-cell activation (Mir 2015). Hence, this type of costimulation is thought to contribute to a secondary stimulus toward cell proliferation, maintained effector response, and acquired memory (Croft 2003; Mir 2015; Sturgill and Redmond 2017).

Other co-signaling molecules of the immunoglobulin or TNF/TNFR family include PD-1 homolog (PD1H), Tim-3, LAG-3, CD160, LAIR1, DR3, and others (Chen and Flies 2013; Flies et al. 2014; Simons et al. 2019). However, these more recently uncovered molecules are discussed in chapter four.

2.2 Inhibitory Function of CTLA-4

CTLA-4 is a type 1 transmembrane glycoprotein of the immunoglobulin superfamily, expressed in the form of a covalent homodimer on the surface of T-cells (Brunet et al. 1987; Linsley et al. 1995). Yet, unlike regulatory T-cells (T_{reg} cells) which constitutively express CTLA-4 (Read et al. 2000; Takahashi et al. 2000a), conventional naïve CD4+ and CD8+ cells only bring CTLA-4 to the surface following activation (Lindsten et al. 1993), as all cells harboring the capacity to bring CTLA-4 to expression sequester the molecule intracellularly within clathrin-coated vesicles, allowing its rapid translocation to the cell membrane (Chambers et al. 2001). The cytoplasmic domain of CTLA-4 has no intrinsic enzymatic activity but comprises several tyrosine- and proline-rich motives, comparable to the cytoplasmic portion of CD28 (Baroja et al. 2002). Nonetheless, there is only limited conservation between the cytoplasmic regions of these proteins (Ward 1996). Intriguingly, the extracellular domains of both CTLA-4 and CD28 interact with the same ligands, CD80 and CD86,

which are primarily found back on the surface of APCs (Linsley et al. 1990; Freeman 2004).

CTLA-4 was identified as an inhibitory molecule counteracting CD28 in its T-cell activation in 1995 by Krummel and Allison (Krummel and Allison 1995). It has since proven to be a crucial regulator of the immune system with a critical impact on self-tolerance and T-cell homeostasis as evidenced by the work of both the lab of Sharpe and Mak who independently generated CTLA-4 deficient mice showing fatality around three to four weeks of age (Tivol et al. 1995a, b; Waterhouse et al. 1995). Nowadays CTLA-4 is well recognized as a key immune checkpoint and has gained significant momentum as a therapeutic target in the field of autoimmunity and cancer. The exact mechanisms underlying its suppressive function, however, remain to be fully elucidated.

Shortly after the discovery of CTLA-4 and its inhibitory effect on costimulation, various hypotheses concerning the mechanism of inhibition were postulated. Several of these suggested CTLA-4 to interfere with molecules downstream of CD28, which as the main costimulatory molecule of T-cells exerts its functions via a multitude of intrinsic signaling pathways. Similarly, CTLA-4 was believed to inhibit effects downstream of TCR signaling (Teft et al. 2006). As such, phosphorylation of the YVKM motif in CTLA-4 cytoplasmic tail was reported to lead to binding of the SH2-containing phosphatase-2 (SHP2) (Marengère et al. 1996) in the same manner that the tyrosines in the signaling motifs of CD28 can be phosphorylated to trigger binding of the motive to SH2-containing proteins (Boomer and Green 2010). SHP2 recruitment would then hinder early TCR signaling through decrease of the level of tyrosine phosphorylation on multiple molecules of the TCR complex (Schneider et al. 2008b). However, these and other findings related to TCR signaling, amongst which a role for CTLA-4 in alteration of the phosphorylation of CD3z chains (Lee et al. 1998), could not be confirmed (Calvo et al. 1997; Schneider and Rudd 2000; Schneider et al. 2001; Yokosuka et al. 2010). Likewise, the most crucial nodes suggested to be shared between CD28 and CTLA-4 are those from the PI3K/Akt pathway (Hu et al. 2001; Schneider et al. 2008a). Yet, conflicting results on the ability of CTLA-4 to interact with these molecules made it impossible to deduce any solid mechanism (Walker and Sansom 2015).

In recent years, however, great research efforts have led to substantial evolution of our knowledge on CTLA-4, with a completely altered paradigm as a result. In fact, only the early hypothesis of competition between CTLA-4 and CD28 for binding to their shared ligands CD80 and CD86 as discussed below was acknowledged to hold true value. Accordingly, the mechanisms by which CTLA-4 exerts its function are now categorized as either cell-intrinsic (affects the CTLA-4 expressing T-cell) or cell-extrinsic (affects secondary cells). Although some novel findings still support an intrinsic signaling model initiated by CTLA-4 its cytoplasmic tail leading to posttranslational modifications in downstream targets (Arra et al. 2017; Lingel et al. 2017), compelling evidence from multiple groups now indicates that CTLA-4 mainly acts in a cell-extrinsic manner (Walker 2017). The most important mechanisms in our current understanding of CTLA-4 functioning are thus cell-extrinsic and are discussed below. Nonetheless, intrinsic CTLA-4 signaling was found to regulate

CTLA-4 its cellular localization and holds the capacity to increase T-cell motility and reduce T-cell: APC dwell time, which might indirectly support its inhibitory function by preventing adequate T-cell binding and activation (Schneider et al. 2006; Rudd 2008).

2.2.1 Predominant Binding of CTLA-4 to CD80 and CD86

CD28 is critical in the regulation of a whole range of different stimulatory molecules in the T-cell. Through its activation of NFAT, NFκB, mTOR, GLUT1, AP-1, and other transcription factors, it drives T-cell proliferation, survival, differentiation, IL-2 production and immunoglobulin isotype switching (Sharpe and Freeman 2002; Chen and Flies 2013). One of the earliest postulated mechanisms of CTLA-4 inhibition, which is still widely accepted today, is the blockage of all these different pathways at once through competition of CD28 with CTLA-4 for the binding of CD80 and CD86.

Unlike CD28, CTLA-4 is not constitutively present on the membrane of naïve T-cells. It is stored intracellularly until its relocation to the cell membrane is induced upon T-cell activation, after which it is quickly taken up again by endocytosis. When present in the membrane of a cell, both CTLA-4 and CD28 exist as homodimers capable of binding CD80 and CD86 via their extracellular MYPPPY motif (Rudd et al. 2009). CTLA-4 has a substantially higher affinity (Kd 0.2 and 2.6 μm resp. CD80 and CD86) and avidity than CD28 (affinity Kd 4.0 and 20 μm resp. CD80 and CD86) toward these ligands, thereby outcompeting CD28 and simply preventing it from eliciting its stimulatory signals (Ikemizu et al. 2000; Van Der Merwe and Davis 2003; Teft et al. 2006). Additionally, the accumulation of CTLA-4 takes place in the same region of the immunological synapse where CD28 is present, thereby physically excluding it from the active site (Pentcheva-Hoang et al. 2004; Yokosuka et al. 2010).

CD86, which is a monomer, has weaker binding properties than CD80 for both its interaction with CD28 and CTLA-4. As a result, the CD28:CD86 interaction is the weakest of the four possible combinations. CD80 is mostly present in its dimeric form on the surface of APCs, which is a crucial element causing the CTLA-4:CD80 complex to be the strongest (Collins et al. 2002). Structurally, CD28 and CTLA-4 are quite alike, as evidenced by both their ability to form a complex with CD80 or CD86. Yet, the complex between a CD80 homodimer and CTLA-4 is more robust because the ligand is capable of binding CTLA-4 in a bivalent manner while in the CD28 complex there is steric interference between the most membrane-proximal domains of the dimer, making a strong binding impossible (Fig. 2.2) (Collins et al. 2002; Esensten et al. 2016). It has been suggested that instead of forming a monovalent CD28:CD80 complex, reorientation of the cytoplasmic tail of CD28 upon T-cell receptor signaling could beget bivalent binding of CD28 on the two sites of the CD80 homodimer (Sanchez-Lockhart et al. 2014). These findings might indicate

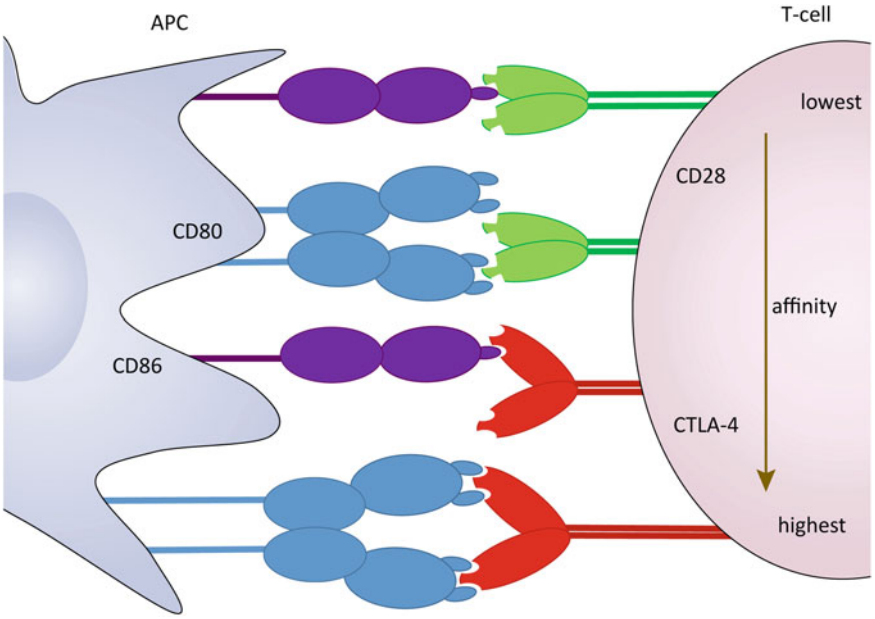

Fig. 2.2 Relative CD28 and CTLA-4 ligand-binding affinities. Both ligand CD80 and CD86 expressed on APCs are shared between the CD28 and CTLA-4 receptor on T-cells. CD80 is present on the membrane mostly in its dimeric form, while CD86 is a monomeric ligand. CTLA-4 binds both ligands with a higher affinity and avidity than CD28 and CD80 has a stronger binding capacity than CD86. The relative affinities are presented from low (top) to high (bottom). CTLA-4: cytotoxic T-lymphocyte antigen 4; APC: antigen-presenting cell; and CD28/80/86: cluster of differentiation 28/80/86

that the current view of CTLA-4 inhibition by competition for ligand needs to be revisited.

2.2.2 CTLA-4-Mediated Trans-endocytosis of CD80 and CD86

Recently, a new multiscale model of the T-cell: APC interaction predicted that sole outcompetition of ligand binding of CD28 by CTLA-4 is insufficient to fully eliminate the positive costimulatory activation of the T-cell (Sugár et al. 2017). It was found that CTLA-4—next to having a higher binding affinity toward CD80 and CD86—is also capable of removing these ligands from the APC's cell surface which is followed by their degradation in CTLA-4 expressing cells—a process called trans-endocytosis. Hereby the APC would thus be deprived from its activating potential (Qureshi et al. 2011).

CTLA-4, unlike most immunomodulatory proteins, is characterized by a peculiar and highly dynamic spatiotemporal migration pattern within the cell. In fact, in steady state most CTLA-4 molecules are gathered in intracellular vesicles which are transported to the surface of the cell upon activation. While the extracellular domain of CTLA-4 binds its target ligands CD80 and CD86, the cytoplasmic tail is essential to the tight control of CTLA-4 its cellular localization (Teft et al. 2006). More specifically, endocytosis is regulated via binding of the YVKM domain to the μ2 subunit of the clathrin-associated adapter protein AP-2 (Shiratori et al. 1997; Zhang and Allison 1997). Subsequent CTLA-4 degradation or recycling has been associated to the adaptor protein AP-1 and the cytoplasmic YVKM motif, respectively (Schneider et al. 1999; Kaur et al. 2013). Lastly, several different motifs have been suggested to play a role in the recruitment of CTLA-4 to membrane lipid rafts and in the regulation of its expression on the cell surface in general (Walker and Sansom 2015). This sophisticated trafficking of CTLA-4 is of great importance in the process of trans-endocytosis as along with CTLA-4, the captured ligands are taken up and degraded inside the CTLA-4 expressing cell (Qureshi et al. 2011). Consistently, in silico predictions suggest that efficient and persistent ligand uptake requires a short (maximally 2.3 h) T-cell: APC contact duration and rapid CTLA-4-mediated ligand depletion (Khailaie et al. 2018).

Novel findings, however, indicate that conventional T-cells have a rather limited ligand capturing capacity. The inhibitory mechanism of trans-endocytosis would be of particular importance in T_{reg} which, in contrast to conventional T-cells, constitutively express CTLA-4 on their cell membrane (Ovcinnikovs et al. 2019). These observations together with the finding that CTLA-4 is absolutely crucial to T_{reg} functioning as evidenced by the spontaneous death of mice lacking CTLA-4 in the T_{reg} population (Wing et al. 2008; Yamaguchi et al. 2013), really shifted the focus within the field of CTLA-4 biology from its relatively minor function in conventional T-cells to its major function in T_{regs} (Walker 2017). Further in the chapter, the function of CTLA-4 on T_{reg} cells in relation to the regulation of immune homeostasis is discussed in more detail.

2.2.3 Direct Tolerogenic Effects of CTLA-4 on the Interacting Cell

Next to the more drastic removal of CD80 and CD86 via trans-endocytosis, interaction of CTLA-4 with these ligands has also been shown to impact the intrinsic signaling of the APC presenting CD80 and CD86 (Bourque and Hawiger 2018). Using this mechanism, T_{reg} cells are capable of stimulating the PI3K/Akt pathway within dendritic cells (DCs), leading to activation of mTOR and consequent inhibition of autophagy (Alissafi et al. 2017). The DC's antigen-presenting capacity will therefore be compromised, since it depends largely on autophagy-regulated degradation of pathogenic proteins (Mak et al. 2014). Moreover, T_{reg} cells were found

to enhance the expression of active dendritic indoleamine 2,3 dioxygenase (IDO), which leads to the production of proapoptotic tryptophan metabolites in the DC (Fallarino et al. 2003; Munn et al. 2004). Interestingly, in vitro results show that CTLA-4 is expressed not only on T-cells but also on the DCs themselves, where it seems to affect cytokine production, antigen presentation, DC maturation, and DC-mediated T-cell proliferation (Laurent et al. 2010; Wang et al. 2011). Next to the membrane bound isoform of CTLA-4, the molecule exists in a soluble monomeric form as well (Metzler et al. 1997; Magistrelli et al. 1999). Secretion of this CTLA-4 by DCs appears to result in decreased levels of CD80 and CD86 on surrounding dendritic cells (Halpert et al. 2016). In line with these findings, in vitro results indicate that binding of CTLA-4 to CD80 expressed by DCs triggers the phosphorylation of STAT3, resulting in NF-κB-mediated downregulation of CD80 and CD86 gene transcription in these DCs (Alissafi et al. 2017). Lastly, CTLA-4 on T_{reg} cells would also stabilize the interaction between regulatory and conventional T-cells, allowing for T_{reg}-mediated suppression of the conventional T-cell (Matheu et al. 2015). An overview of the different inhibitory mechanisms described for CTLA-4 is depicted in Fig. 2.3.

2.3 Role of CTLA-4 in Immune Homeostasis and Disease

The primary function of the immune system is to eliminate pathogens, remove early malignant cancer cells, and prevent tumor progression (Dunn et al. 2004). Equally important, however, is the strict control of this system, thereby preventing immune reactions mounted against self-antigens. Consequently, the immune system comprises a multitude of distinct cell types, all under tight regulation in order to ensure the fine balance of effective protection while preventing deleterious autoimmune responses (Crimeen-Irwin et al. 2005). Although various different subsets of T-cells exist, in general, they can be divided in two main groups: The conventional effector T-cells (T_{eff}) and the regulatory T-cells (T_{reg}). These two groups have opposing functions, with the T_{eff} cells fighting off immunogenic antigens (viruses, bacteria, tumor cells, etc.) and T_{reg} cells dampening the immune reaction (Sakaguchi et al. 1995).

CTLA-4 and its suppressive function have been found to be involved in the physiological functioning of both T_{eff} and T_{reg} cells. In the former group, cytoplasmic CTLA-4 vesicles are trafficked to the membrane shortly after CD28-mediated activation (Chambers et al. 2001). Its inhibitory action thereby prevents continuous activation of the T-cell leading to an excessive immune response and subsequent tissue damage (Hirahara and Nakayama 2016). CTLA-4 on the T_{reg} is critical in the prevention of autoimmunity and acts via the regulation of other immune cells such as APCs or naïve T-cells (Rowshanravan et al. 2018). Specific deletion of CTLA-4 in T_{reg} cells in an in vivo mouse model provided the first insight into its crucial role in maintaining self-tolerance. The mice lacking T_{reg} specific CTLA-4 developed severe splenomegaly and lymphadenopathy and showed high titers of autoantibodies with subsequent fatality at the age of 7 weeks (Wing et al. 2008). The phenotype of these

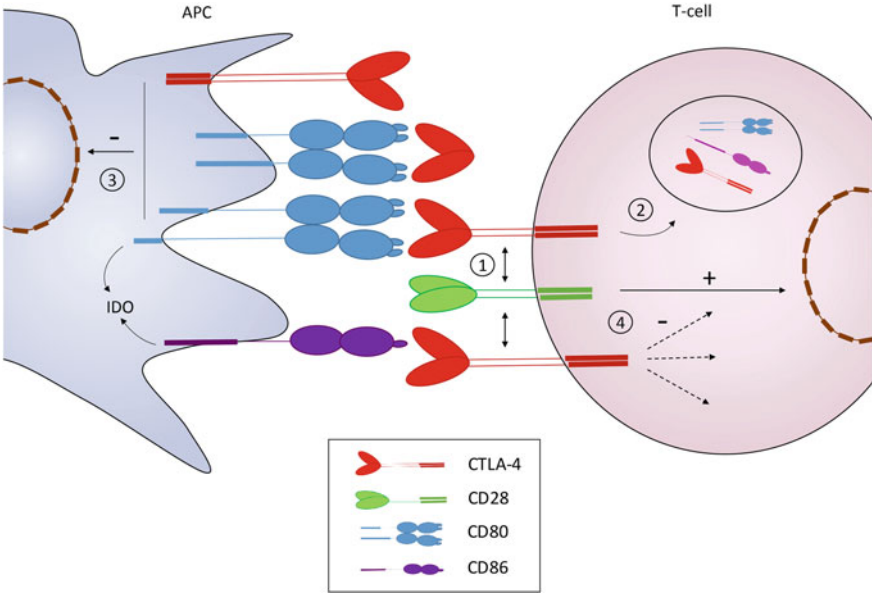

Fig. 2.3 Mechanistic models of CTLA-4 inhibition. First, CTLA-4 has a greater binding affinity toward CD80 and CD86 by which it outcompetes CD28 (1). Additionally, binding of CTLA-4 to CD80 and CD86 promotes the uptake and breakdown of these ligands via the process of trans-endocytosis (2). Both membrane bound and soluble CTLA-4 stimulate negative signaling within the APC via various pathways including the PI3K/Akt/mTOR pathway, the STAT3/NFκB axis and the kynurenine degradation pathway in which IDO is the rate-limiting enzyme. Likewise, dendritic cells can themselves also express CTLA-4 which inhibits their proper functioning (3). Lastly, CTLA-4 might function via cell-intrinsic signaling pathways in the T-cell, affecting positive CD28 signaling, TCR-mediated signaling, T-cell motility, and other pathways (4). CTLA-4: cytotoxic T-lymphocyte antigen 4; APC: antigen-presenting cell; CD28/80/86: cluster of differentiation 28/80/86; PI3K: phosphatidylinositol 3-kinase; Akt: protein kinase B; mTOR: mammalian target of rapamycin; STAT3: signal transducer and activator of transcription 3; NF-κB: nuclear factor kappa-light-chain-enhancer of activated B-cells; and IDO: indoleamine 2,3 dioxygenase

mice was comparable to the phenotype of CTLA-4 full knock-out mice, although the additional lack of CTLA-4 on T_{eff} cells exacerbated the outcome as evidenced by mortality of the mice at 20 days of age already (Tivol et al. 1995a, b; Waterhouse et al. 1995). Soon after, the dominant role of CTLA-4 on T_{regs} controlling inappropriate T-cell activation was confirmed and CTLA-4 on T_{eff} cells in the context of tolerance was suggested to contribute to *in cis* suppression of improperly activated T_{eff} cells, thereby preventing organ tissue destruction (Jain et al. 2010).

Given the importance of CTLA-4 its inhibitory property in remaining self-tolerance, it is not surprising that defects in CTLA-4 expression or function cause auto-immunogenic diseases to develop (Verma et al. 2017). In this context, the use of synthetically developed CTLA-4 fusion proteins has proven to be a valuable treatment option (Linsley et al. 1992). Contrarily, cancer cells typically evade immune surveillance by various mechanisms including upregulation of CTLA-4 and increased

presence of T_{reg} cells in the tumor microenvironment (Zou and Chen 2008; Pardoll 2012). Hence, CTLA-4 targeting therapy was developed in order to shift the balance toward immune activation, thereby stimulating the immune system to fight malignant cells (Leach et al. 1996).

2.3.1 CTLA-4 Enrichment in Autoimmune Diseases

Several different mutations in the human CTLA-4 gene cause patients to present with severe clinical symptoms of both immune deficiency and loss of self-tolerance resulting in autoimmunity (Schubert et al. 2014; Sun et al. 2014). Accordingly, various autoimmune diseases have been associated with genetic defects in or altered posttranslational modifications of the CTLA-4 gene or its promoter (Zhang and Vignali 2016). These include both systemic diseases such as rheumatoid arthritis (RA) and systemic lupus erythematosus (SLE) and organ-specific diseases such as type 1 diabetes (T1D), Graves' disease, and multiple sclerosis (MS) (Saverino et al. 2010; Zhang and Vignali 2016). Successful in vivo experiments with a CTLA-4 extracellular domain—IgG2a Fc fusion protein (CTLA-4Ig) lead to the development of a similar human fusion protein by the name of abatacept (Abrams et al. 1999; Moreland et al. 2002). Abatacept was subjected to various clinical trials and found to be effective against RA, juvenile idiopathic arthritis, and psoriatic arthritis, but so far it had only minor or no positive impact on most other tested autoimmune diseases including T1D, MS, SLE, asthma and ulcerative colitis (Kuemmerle-Deschner and Benseler 2008; Ruperto et al. 2008; Adams et al. 2016; Mease et al. 2017). However, multiple new clinical trials are ongoing for these and previously excluded autoimmune diseases (NIH US National Library of Medicine 2019). Meanwhile, a second-generation CTLA-4Ig (belatacept) has been developed and approved for patients undergoing kidney transplantation, illustrating the distinct applications CTLA-4 fusion proteins might have in clinic (Vincenti et al. 2010).

2.3.2 CTLA-4 Blockage as Immunotherapy

Initially, therapeutic interference based on CTLA-4 was focused mainly on exploiting its suppressive function in autoimmune diseases. The Allison lab, however, approached CTLA-4 from a different angle early on and discovered the potential of CTLA-4 targeting therapy in the context of cancer (Leach et al. 1996). By blocking CTLA-4, the immune system is no longer repressed and a stronger response toward the tumor cells can be mounted (Korman et al. 2006). This prompted the concept of immune checkpoint blockade resulting in the development of ipilimumab, a monoclonal antibody targeting CTLA-4 (Hodi et al. 2010). As yet however, the drug could only be approved as therapy against melanoma worldwide and renal cell carcinoma in the United States (Motzer et al. 2018; Barquín-García et al. 2019). A second

CTLA-4 directed antibody called tremelimumab has been subjected to clinical trials investigating various cancers as well. Although multiple trials are still ongoing, no positive outcome has been reported thus far (Borrie and Maleki Vareki 2018).

Recently, it has been suggested that resistance toward therapeutic immune checkpoint blockade is dependent on the same mechanisms used by tumors to escape detection by the immune system. This so-called immunoediting would prevent treatments such as ipilimumab and tremelimumab from functioning to their full potential (O'Donnell et al. 2019). Growing evidence indicates that combination of different anticancer treatments, both immunotherapeutic and conventional (e.g., chemotherapy, radiation), could help overcome this resistance (Mellman et al. 2011; Topalian et al. 2011; Stephen and Hajjar 2018). Fortunately, CTLA-4 is not the only immune checkpoint against which therapy has been developed. PD-1 and its ligand PD-L1 are a second set of co-inhibitory molecules belonging to the CD28 family, which are of major importance as targets in immunotherapy (Ghahremanloo et al. 2019). PD-1 and PD-L1 are discussed in great detail in the following chapter and the role of CTLA-4 in the context of cancer immunotherapy is further elucidated in the last five chapters of this book.

2.3.2.1 Intermezzo: Cell Death and Its Relevance in Immunotherapy

In recent decades the field of cell death has developed immensely. The early, dichotomic view of cell death as either apoptotic—programmed, regulated, immunologically silent—and necrotic—accidental and pro-inflammatory, has been gradually rejected as several other, previously unknown cell death modes were discovered. Today, more than 10 specific regulated cell death (RCD) types have been defined (Galluzzi et al. 2018) and each and every one of them is differently regulated and executed.

Different types of RCD do not only differ in the way that cell death is executed, but also in their ability to impact surrounding tissue, particularly immune system cells—macrophages and DCs, responsible for cleaning the residue of the dead cells. Interestingly, however, the way in which cell death is executed will impact the response of body cleaners. Certain types of cell death—termed immunogenic—will engage APCs and cause their maturation, which will end up in T-cell proliferation, while other form of cell death (non-immunogenic) will not be able to do so and will suppress the activity of APCs. Inducing immunogenic cell death (ICD) is, therefore, a very attractive tool in cancer therapy, as different cell death modes can engage the adaptive immune system and sustain its activation which may help overcome the usually immunosuppressive tumor microenvironment.

As more treatments and cell death inducers were described as immunogenic, it became clear that induced cell death has to fulfill several conditions. Functionally, dying cells should be able to engage DCs and cause their maturation, which is the first step for a successful immunogenic response. This process has been linked to the release of specific molecules—damage-associated molecular patterns (DAMPs) from dying cells (Kepp et al. 2014). Several DAMPs have been described as absolutely

crucial in the induction of immunogenicity. They involve endoplasmic reticulum (ER) chaperone protein calreticulin (CRT) exposure, ATP secretion, and high mobility group box 1 protein (HMGB-1) release (Kepp et al. 2014). CRT serves as a so-called eat me signal—presence of CRT on the surface of the dying cell facilitates engulfment by DCs. Moreover, blocking CRT (genetically or by a specific antibody) prevents the induction of an immunogenic response (Obeid et al. 2007). ATP, on the other hand, serves as a "find me signal" for APCs and stimulates chemoattraction of DCs to dying cells. Last, but not least, HMGB-1 is mostly responsible for inducing maturation and activation of APCs. Its targets involve well known pro-inflammatory receptors like toll-like receptor (TLR-) 2 and 4 as well as RAGE receptor. It is probably TLR-4 receptor that is of the greatest importance as ICD cannot be induced in Tlr4-/- mice (Apetoh et al. 2007). In vitro, HMGB-1 is able to activate DCs and increase the levels of costimulatory molecules (CD83 and CD86) on their surface (Gao et al. 2019). Of note, this set of DAMPs is the most crucial for inducing immunogenic apoptosis and not necessarily other forms of cell death (see below).

Several types of cell death have been implicated as immunogenic with apoptosis induced by chemotherapeutics—anthracyclines—pronounced immunogenic as first. While this cell death requires caspases and involves the formation of apoptotic bodies, there were several things making it different than classical apoptosis, mainly the presence of ER stress as well as release of aforementioned DAMPs. Other types of immunogenic cell death involve necroptosis—a caspase-independent programmed necrotic cell death type. While necroptosis has been described as an immunogenic type of cell death by several groups, the exact mechanism of this type of immunogenicity is not fully understood. It seems, however, that necroptotic cells were able to successfully induce maturation of DCs without ER stress or calreticulin exposure on the surface of the dying cells (Aaes et al. 2016; Ren et al. 2017). Perhaps it is the necrotic morphology of the dying cells that involves massive release of HMGB-1 and ATP that stimulated DCs, although this notion has not been properly tested.

Apart from specific types of cell death, cell death induced by certain stimuli can be either immunogenic or not. Oncolytic viruses have been proposed as an attractive tool to induce ICD. In this form of cell death (which is a mix of several morphologically distinct forms of cell death modes), tumor cells are infected with special strains of viruses, whose replication leads to lysis of the tumor cells. The evidence showed that dying cells underwent ER stress and managed to engage a strong APC response. Similarly, irradiated cells have been proclaimed as undergoing ICD and present release of ICD-related DAMPs (Golden and Apetoh 2015). Interestingly, however, the immunogenic effect is dependent on the dose of radiation as well as on the frequency of the treatment (Poleszczuk and Enderling 2018).

Apart from already described cell death modes that can be proclaimed as immunogenic or non-immunogenic, there are still several cell death modes that need to be studied in this context. One of the most important ones being ferroptosis. This iron-dependent type of cell death was first described in 2012 (Dixon et al. 2012) and since then gained much attention in the context of cancer treatment. Ferroptosis is dependent on the activity of glutathione peroxidase 4 (GPX4) enzyme that is able

to detoxify oxidized phospholipids. When the activity of that enzyme is compromised—either by drugs that bind directly to GPX4 or those that deplete the enzyme's cofactor, glutathione, levels in the cell—lipid peroxidation can occur in an unchallenged way, which ultimately leads to cell membrane permeabilization and cell death. There are several reasons which make ferroptosis a very attractive tool for cancer therapy. First, cancer cells have a different metabolism compared to healthy cells and they often have higher levels of iron making them more prone to ferroptosis (Jung et al. 2019). Second, ferroptosis seems to be a more effective type of cell death when it comes to eradication of tumor cells compared to drugs inducing apoptosis (Hassannia et al. 2018). Last, but not least, ferroptosis inducers seem to be targeting cancer cells preferably compared to healthy tissue (Hangauer et al. 2017). As of now, however, the relationship between dying ferroptotic cells and immune system cells, particularly DCs is not established. On the one hand, necrotic cell death is usually pro-inflammatory as it causes massive release of intracellular DAMPs. On the other hand, however, ferroptotic cells produce a lot of oxidized phospholipids (OxPL) and these have been described as anti-inflammatory (Oskolkova et al. 2010). Moreover, OxPLs are able to block dendritic cell maturation and their antigen processing capacity (Cao et al. 2014) and can cause T-cell functional impairment in terms of their cytotoxic potential and proliferation (Bochkov et al. 2010).

2.4 Conclusions

CTLA-4 is an inhibitory receptor belonging to the CD28 family which is expressed mostly on T-cells and consists of a cytoplasmic tail, a transmembrane part, and an extracellular domain. By counteracting the activating signals provided by costimulatory molecules such as CD28, it contributes to the safe-keeping of immune homeostasis. Specifically, CTLA-4 is critically involved in T-cell priming and contributes substantially to regulation of T_{eff} cells by T_{reg} cells. The different expression patterns of CTLA-4 on conventional T_{eff} cells and T_{regs} are indicative for its distinct functions on both cell types. CTLA-4 on a T_{eff} cell mainly acts to balance the T-cell's own activation during priming and is only transiently present on the membrane. T_{reg} cells, on the other hand, constitutively express CTLA-4 which exerts its inhibitory effect on the T_{reg} partner cell, thereby ensuring self-tolerance. This is necessary since despite the removal of most self-reactive T-cells during development, a small proportion of mature T-cells targets host-specific antigens. CTLA-4-dependent downregulation of T_{eff} cell activity prevents these T-cells from provoking a harmful autoimmune response.

Owing to its critical role in the maintenance of immune homeostasis, CTLA-4 is recognized as one of the most important immune checkpoints. As such, its function has been exploited for therapeutic purposes from two opposing angles. First, synthetic CTLA-4 is administered in the form of an immunoglobulin fusion protein to counter the pathological immune response in the context of autoimmunity.

Second, CTLA-4 targeting antibodies are used as anticancer therapy. Blockage of endogenous CTLA-4 dampens its suppressive effect on the immune system, thereby eliciting a stronger immune response against malignant cells. This type of therapy is known as immunotherapy. Despite its great success for other immune checkpoints, resistance of most cancer types toward CTLA-4-targeted therapy has led the drug to be applicable in clinic only against melanoma and renal cell carcinoma.

Various models have been proposed to attribute to CTLA-4 its mechanism of action: both cell-intrinsic and cell-extrinsic. Currently, competition between CTLA-4 and CD28 for their ligand binding, trans-endocytosis of the ligands, and direct APC signaling effects are believed to be the most credible models involved in CTLA-4 inhibition. Still, there is little consensus in the field concerning intrinsic signaling pathways downstream of CTLA-4 and the relative contribution of each model. In order to fine-tune CTLA-4-targeting immunotherapy, it is of great importance to gain better understanding of these precise biological mechanisms. This book aims to provide an overview of the current understanding of all immune checkpoints crucial to cancer immunotherapy, describing their mechanisms and how these are—and could be—used for therapy.

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Chapter 3
Roles of PD-1/PD-L1 Pathway: Signaling, Cancer, and Beyond

Luoyan Ai, Antao Xu and Jie Xu

Abstract Immunotherapies that target PD-1/PD-L1 axis have shown unprecedented success in a wide variety of human cancers. PD-1 is one of the key coinhibitory receptors expressed on T cells upon T cell activation. After engagement with its ligands, mainly PD-L1, PD-1 is activated and recruits the phosphatase SHP-2 in proximity to T cell receptor (TCR) and CD28 signaling. This event results in dephosphorylation and attenuation of key molecules in TCR and CD28 pathway, leading to inhibition of T cell proliferation, activation, cytokine production, altered metabolism and cytotoxic T lymphocytes (CTLs) killer functions, and eventual death of activated T cells. Bodies evolve coinhibitory pathways controlling T cell response magnitude and duration to limit tissue damage and maintain self-tolerance. However, tumor cells hijack these inhibitory pathways to escape host immune surveillance by overexpression of PD-L1. This provides the scientific rationale for clinical application of immune checkpoint inhibitors in oncology. The aberrantly high expression of PD-L1 in tumor microenvironment (TME) can be attributable to the "primary" activation of multiple oncogenic signaling and the "secondary" induction by inflammatory factors such as IFN-γ. Clinically, antibodies targeting PD-1/PD-L1 reinvigorate the "exhausted" T cells in TME and show remarkable objective response and durable remission with acceptable toxicity profile in large numbers of tumors such as melanoma, lymphoma, and mismatch-repair deficient tumors. Nevertheless, most patients are still refractory

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to anti-PD-1/PD-L1 therapy. Identifying the predictive biomarkers and design rational PD-1-based combination therapy become the priorities in cancer immunotherapy. PD-L1 expression, cytotoxic T lymphocytes infiltration, and tumor mutation burden (TMB) are generally considered as the most important factors affecting the effectiveness of PD-1/PD-L1 blockade. The revolution in cancer immunotherapy achieved by PD-1/PD-L1 blockade offers the paradigm for scientific translation from bench to bedside. The next decades will without doubt witness the renaissance of immunotherapy.

Keywords PD-1 · PD-L1 · T cell inhibition · Self-tolerance · Cancer immune evasion

3.1 Introduction

Modern efforts aimed to use the potency of immune system to fight against cancer can date back to the work of William Coley, a surgeon in the nineteenth century. He first associated the occurrence of postoperative infection with improved clinical outcomes in cancer patients. After a series of fits and starts throughout the ensuing century, cancer immunotherapy swagged and progressed slowly until very recently, we turn our focus from systemic immunity to tumor microenvironment (TME) (Zou et al. 2016) and shift the paradigm of immunotherapy from enhancement to normalization (Sanmamed and Chen 2018).

T cell-based host immune system can recognize and eradicate tumor cells that expressed tumor-specific antigens, with TME being the primary interacting location. A two-signal model was proposed for detection of cancer cells (Bretscher 1999): binding of TCR on T cells to peptide-major histocompatibility complex (p-MHC) on target (tumor) cells ("signal 1"), and an additional signal ("signal 2", the costimulatory or coinhibitory signal) controlling the magnitude and duration of the response. Receptors delivering coinhibitory signals act as immune checkpoints and have a decisive role in the maintenance of peripheral tolerance and the prevention of autoimmunity (Boussiotis 2016). The pathways involving cytotoxic T-lymphocyte-associated antigen-4 (CTLA-4) and programmed cell death 1 (PD-1; also called CD279) and its ligands, PD-L1 (B7-H1 or CD274) and PD-L2 (B7-DC or CD273), are the best-characterized coinhibitory pathways. Tumors and pathogens can exploit these pathways to paralyze the immune system, escaping from immune surveillance and leading to cancer progression and immune tolerance. Our previous efforts were aimed to enhance the antitumor immune response. Hence, several immunotherapeutics such as bacillus Calmette–Guerin, interferon-α, and interleukin-2 (IL-2) were approved for use in cancer (Ribas and Wolchok 2018), with limited benefit. It was the discovery of CTLA-4 functioning as a potent negative regulator of immune responses that led to a radical shift in cancer immunotherapy from enhancement to normalization. The preferred approach would not be the augmentation of the immune system

to attack cancer cells but rather the removal of the coinhibitory signals that block antitumor T cell responses.

Indeed, Ipilimumab (a CTLA-4 blocker), the first immune checkpoint inhibitor (ICI) approved by the Food and Drug Administration (FDA) in 2010, allowed for durable clinical responses in up to 20% of patients with metastatic melanoma, who previously lacked any effective treatment options. Subsequently, based on the same concept, the PD-1/PD-L1 coinhibitory pathway was exploited therapeutically and stood out because of its extraordinary outcomes: 20–50% response rate with favorable toxicity profile for various types of malignant cancer in multiple clinical trials (Boussiotis 2016; Topalian et al. 2012). These drugs revolutionized cancer immunotherapy in two ways. First, they do not target tumor cells. Instead, they target the soldiers of immune system, T cells. They unleash a patient's own T cells to kill tumors. Second, perhaps in a more radical shift, the goal of the therapy is to normalize the compromised immune system by removing coinhibitory signals rather than to enhance the immune attack (Sharma and Allison 2015).

So far, antibodies targeting PD-1-PD-L1 axis are being evaluated in more than 1000 clinical trials (Sun et al. 2018), across a spectrum of different tumor types spanning both solid tumors and hematologic malignancies including melanoma, non-small-cell lung cancer (NSCLC), Hodgkin's lymphoma, head and neck squamous cell carcinoma (HNSCC), hepatocellular carcinoma, renal cell carcinoma (RCC), urothelial cancer, microsatellite-instability-high (MSI^{high}), or mismatch-repair deficient (dMMR) solid tumors (Huang et al. 2019). It is critical to note that the apparent rapid clinical progress reported in the past few years was the result of decades of investment in basic science in numerous fields. Without basic mechanistic knowledge in molecular biology, virology, immunology, cell biology, and structural biology, clinical advances in cancer immunotherapy would never have been realized.

Despite the considerable improvement in patient outcome that has been achieved with PD-1 pathway blockade, durable responses to these therapies are observed in only a minority of patients and intrinsic therapy resistance is common. Even in melanoma, the majority of patients show limited or only temporary benefit of checkpoint blockade (Patel and Minn 2018; Pitt et al. 2016). And large groups of patients with such common cancers as prostate, ovarian, breast, and non-MSI colorectal cancer have been completely refractory to checkpoint blockade therapy (Topalian et al. 2012; Patel and Minn 2018; Llosa et al. 2015; Kroemer et al. 2015), despite strong evidence that immune surveillance holds key to control the rate of recurrence and progression of these cancers (Galon et al. 2006; Sato et al. 2005). Furthermore, toxicity and immune-related adverse events (IRAEs) have been observed, with particularly high rates occurring when PD-1-targeted therapy is used in combination with CTLA-4-targeted therapy. These clinical findings underscore the need for a better mechanistic understanding of why PD-1 pathway modulation leads to significant clinical benefit in some patients but not in others. In addition, a better understanding of the causes of IRAEs is sorely needed to guide safer use of PD-1 pathway blockers.

3.2 PD-1/PD-L1 Signaling Overview

Initially, PD-1 was identified as being preferentially expressed in apoptotic cells (Ishida et al. 1992), but it was later understood that it is actually a critical immune checkpoint that regulates the threshold of antigen responses of T cells and B cells. Both PD-1 and PD-L1 are type I transmembrane proteins that belong to the immunoglobulin (Ig) superfamily. PD-1 contains one Ig-V like extracellular domain, a transmembrane domain, and a cytoplasmic domain with two tyrosine signaling motifs: immunoreceptor tyrosine inhibitory motif (ITIM) and immunoreceptor tyrosine-based switch motif (ITSM) (Ishida et al. 1992; Zhang et al. 2004). PD-L1 contains two extracellular domains (Ig-V- and Ig-C-like), a transmembrane domain, and a short cytoplasmic tail which lacks known signaling motifs (Dong et al. 1999; Lin et al. 2008). Unlike CTLA-4 completely blocks costimulation by CD28 through its stronger affinity for B7 molecules (Stamper et al. 2001), PD-1 exerts its inhibitory role in a more indirect manner.

During T cell activation, PD-1 changes its conformation after engagement with PD-L1 (Freeman et al. 2000) or PD-L2 (Latchman et al. 2001), translocating to dynamic TCR microclusters and accumulating at the signaling central supramolecular activation cluster (c-SMAC). The cytoplasmic tail of PD-1 becomes phosphorylated by Src family kinases (Gauen et al. 1994; Zak et al. 2015). These phosphorylated tyrosine motifs, perhaps ITSM motif, serve as a docking site of the tyrosine phosphatase SHP-2 (Okazaki et al. 2001; Sheppard et al. 2004; Chemnitz et al. 2004; Yokosuka et al. 2012) and SHP-1. Although both SHP-1 and SHP-2 were found to bind to PD-1, live-cell imaging technique revealed that only SHP-2 interacts with PD-1 during T cell activation in real time in live cells (Yokosuka et al. 2012). The recruitment of SHP-2 in proximity to TCR attenuates key TCR proximal signaling events such as Lck-mediated phosphorylation of ZAP70 (Sheppard et al. 2004) and affects downstream signaling pathways including those involving phosphoinositide 3-kinase (PI3K)–AKT, RAS, extracellular-signal-regulated kinase (ERK), VAV, and phospholipase Cγ (PLCγ) (Riley 2009; Parry et al. 2005; Patsoukis et al. 2012; Hui et al. 2017).

PTEN-PI3K-Akt and RAS-MEK-ERK signaling are two major pathways targeted by PD-1 ligation (Parry et al. 2005; Patsoukis et al. 2012). PD-1 blocks activation of PI3K by recruiting SHP-2, but the targeting of PTEN is mediated by CK2. PTEN is a serine–threonine phosphatase that opposes the activation of PI3K and suppresses the signals delivered by the PI3K–Akt pathway. During T cell activation, CK2 phosphorylates and stabilizes PTEN, but it suppresses PTEN phosphatase activity. PD-1 inhibits the stabilizing phosphorylation of PTEN, thereby resulting in diminished PTEN abundance but increased PTEN phosphatase activity (Patsoukis et al. 2013). For MEK–ERK-MAP kinase pathway, the attenuation of which by PD-1 is mainly mediated by inhibiting activation of PLC-γ1 and Ras (Patsoukis et al. 2012). By altering this way, PD-1 is likely to influence a plethora of downstream biochemical events. In addition, PD-1 can inhibit T cell functions by increasing the expression of transcription factors such as basic leucine zipper transcriptional factor ATF-like

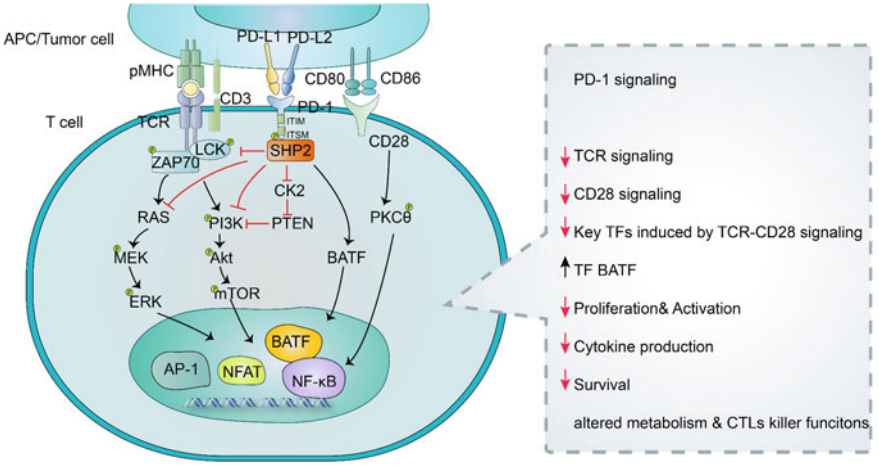

Fig. 3.1 Mechanisms of PD-1-mediated inhibition in T cells. The ligation of PD-L1/PD-L2 to PD-1 recruits the phosphatase SHP-2, which attenuates LCK-induced ZAP70 phosphorylation and reduces RAS-MEK-ERK/PI3K-Akt-mTOR pathway. In addition, PD-1 activation induces expression of BATF, which represses effector genes. Collectively, PD-1 signaling leads to inhibition of T cell proliferation, activation, effector function, and survival

(BATF), which can further repress expression of effector genes. The functional outcome of these effects is multiple including the inhibition of T cell proliferation, activation, survival, cytokine production, altered metabolism and cytotoxic T lymphocytes (CTLs) killer functions, and eventual death of activated T cells (Dong et al. 1999; Freeman et al. 2000; Butte et al. 2007; Chang et al. 1999; Curiel et al. 2003; Keir et al. 2006; Latchman et al. 2004; Honda et al. 2014). The mechanism of PD-1-mediated inhibition is illustrated in Fig. 3.1.

It should be noted that T cell functions are differentially susceptible to PD-1. Wei et al. (2013) observed that high levels of PD-1 expression were required to inhibit macrophage inflammatory protein 1 β production, lower levels were required to block cytotoxicity and IFN-γ production, and very low levels could inhibit TNF-α and IL-2 production as well as T cell expansion. In general, PD-1 is believed to function mainly at the effector, but not in the activation, phase of T cell responses. One explanation why PD-1 function is restricted at the activation stage may be that CD80 interacts with PD-L1 in cis on primary activated DCs, hence interferes with the ability of PD-L1 to engage PD-1 on T cells (Sugiura et al. 2019). While traditional PD-1/PD-L1 binding was thought to reduce the strength of TCR signal itself (Sheppard et al. 2004; Chemnitz et al. 2004), recent work suggests that the costimulatory receptor CD28, rather than the TCR, may be the primary target for the SHP-2 (Hui et al. 2017; Kamphorst et al. 2017). Hence, PD-1 may target both TCR and CD28 to take action.

The signaling capacity of PD-L1 and PD-L2 is another area of active investigation. Although the cytoplasmic tail of PD-L1 and PD-L2 does not contain canonical known signaling motifs, there is evidence of "reverse signaling" through PD-1 ligands

into PD-1 ligand-expressing cells, enhancing their resistance to proapoptotic effects of Fas, interferons, and CTLs (Azuma et al. 2008; Gato-Canas et al. 2017). Still, which intracellular factors participate in such proposed signal transduction remain obscure. In addition to interacting with PD-1, PD-L1 and PD-L2 have other binding partners. PD-L1 can interact with CD80, interfering the binding between PD-1 and PD-L1 (Sugiura et al. 2019). Antibody-based PD-L1 targeting can reduce mTOR activity and their glycolytic pathway of metabolism in cancer cells in the absence of T cells (Chang et al. 2015). PD-L2 was reported to interact with RGM domain family member B (RGMB), which may be involved in the maintenance of respiratory tolerance (Xiao et al. 2014). These alternative binding partners may also partially account for the differences seen in the efficacy of anti-PD therapy in different biological settings. Further characterization of when these interactions are biologically active, their pathway and functional effects in different types of cells may provide insights into how to optimally modulate the PD-1 pathway in cancer immunotherapy while minimizing adverse events. Nevertheless, much of our understanding of PD-1 signaling comes from studies of acutely activated T cells (Riley 2009). The mechanisms by which PD-1 modulates the functions of other types of T cells (including regulatory, exhausted, memory, tolerant, and anergic T cells) and other cell types remains less clear.

3.3 Expression and Regulation of PD-1

Although the PD-1 pathway has received considerable attention for its roles in T cell exhaustion and tumor immunosuppression, PD-1 is not an exhaustion-specific molecule. Instead, it is a marker of effector T cells (Sharpe and Pauken 2018), as PD-1 is typically absent in naïve and resting T cells but induced in all T cells during activation (Agata et al. 1996; Barber et al. 2006; Day et al. 2006). Several transcription factors regulate PD-1 expression in antigen-activated T cells, including nuclear factor of activated T cells, cytoplasmic 1 (NFATC1), fork-head box protein O1 (FOXO1), T-bet (also known as TBX21), and B lymphocyte-induced maturation protein 1 (BLIMP1) (Keir et al. 2008; Schildberg et al. 2016), as well as the serine–threonine kinase glycogen synthase kinase 3 (GSK3) (Taylor et al. 2016). If the activating antigen is acutely cleared, PD-1 levels decrease on responding T cells. However, if the antigen is not cleared, as seen in chronic infections and cancers, PD-1 expression would be high and sustained (Barber et al. 2006; Crawford et al. 2014). The mechanism by which sustains high PD-1 expression may be attributable to substantial and irreversible demethylation (opening) of CpG dinucleotides in the promoter region of *Pdcd1* (Youngblood et al. 2011). During naïve to effector $CD8^+$ T cell differentiation, a transient loss of DNA methylation was accompanied that was directly coupled to the duration and intensity of TCR signaling. Further differentiation into functional memory cells coincided with *Pdcd1* remethylation, inversely correlated with PD-1 expression. In contrast, in exhausted $CD8^+$ T cells, the Pdcd1 locus remained unmethylated even when virus titers decreased. Besides, that region

contains two transcription factor–binding sites (NFAT and ISRE), which are activated by TCR- and interferon-dependent pathways, respectively. It is likely that continuous stimulation through antigen receptors, acting together with inflammatory cytokines causes the demethylation of the locus, which results in high expression of PD-1.

Other factors such as TGF-β (Park et al. 2016) and IL-10 (Sun et al. 2015) and "bystander T cells" can also induce PD-1 expression. The interplay between cell metabolism and PD-1 signaling also emerges as a hotspot (Chang et al. 2013; Scharping et al. 2016). Switching from oxidative phosphorylation to aerobic glycolysis during T cell activation enables effector T cells to satisfy their energy requirements for proliferation and differentiation (O'Sullivan and Pearce 2015). PD-1 signaling antagonizes TCR and CD28 signaling-induced upregulation of glucose and glutamine metabolism (Parry et al. 2005; Patsoukis et al. 2015). Giving the metabolic competition in the TME can drive tumor progression by inducing a T cell hyporesponsive state through glucose deprivation (Chang et al. 2015; Scharping et al. 2016), understanding how PD-1 interfered with metabolism and vice versa becomes increasingly important. Posttranslational modification such as ubiquitination also regulates PD-1 expression. A recent study found that FBXO38, an E3 ligase of PD-1, mediates Lys48-linked poly-ubiquitination and subsequent proteasome degradation of PD-1 (Meng et al. 2018).

In addition to activated T cells, PD-1 is expressed by subsets of tolerant T cells, regulatory T (T_{reg}) cells, T follicular helper (T_{FH}) cells, T follicular regulatory (T_{FR}) cells and memory T cells, and several other cell types, including B cells, natural killer (NK) cells (Benson et al. 2010; Terme et al. 2011), macrophages (Gordon et al. 2017), and cancer cells. Yet, the mechanisms regulating PD-1 expression are best described for T cells.

3.4 Expression and Regulation of PD-L1 and PD-L2

PD-L1 and PD-L2 have distinct expression patterns. PD-L1 is constitutively expressed at low levels on APCs, including T cells, B cells, dendritic cells, mast cells, and macrophages, as well as on a wide variety of nonhematopoietic cells, including pancreatic islet cells, astrocytes, vascular and stromal endothelial cells, and cells in sites of immune privilege, such as the testis, placenta, and eye (Boussiotis 2016). By contrast, PD-L2 has more restricted expression predominantly in professional APCs such as DCs, macrophages, and B cells (Baumeister et al. 2016; Yamazaki et al. 2002), and its expression is generally low at steady state as well. But similar to PD-L1, PD-L2 is induced by inflammatory stimuli. Both PD-L1 and PD-L2 can be expressed by tumor cells and tumor stroma, with PD-L1 is more commonly found on these cells. Engagement of PD-L2 at such tumor sites may potentially contribute to PD-1-mediated T cell inhibition (Yearley et al. 2017). But there is no compelling evidence indicating that antibodies against PD-1, which block binding to both PD-L1 and PD-L2, show higher clinical activity than antibodies against PD-L1. These data are consistent with the model in which PD-L1 is the dominant inhibitory ligand of

PD-1 on T cells in the human TME. Here, we mainly focus on the regulation of PD-L1 expression.

The regulation of PD-L1 expression has undergone extensive investigation. A complex regulatory network exists to control PD-L1 expression, which can largely be divided into two parts: primary (nonimmune-driven) mechanisms and secondary (immune-driven) mechanism (Fig. 3.2). Primary elevation of PD-L1 mainly consists of (1) genomic aberrations, (2) microRNA-based control, (3) oncogenic transcription factors and pathways, and (4) posttranslational modulation and trafficking. Secondary mechanism mainly means inflammatory signaling activation driven by soluble factors that are produced by immune cells in TME (Sun et al. 2018).

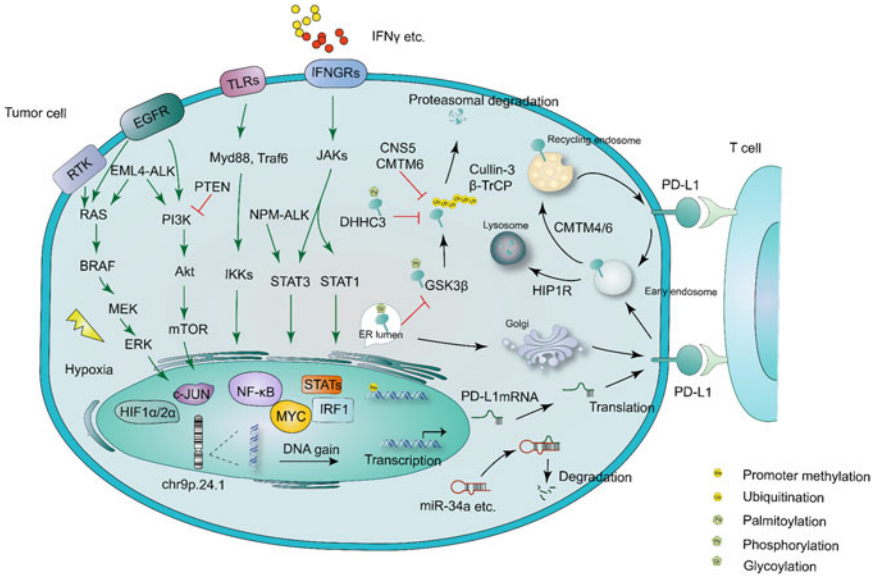

Fig. 3.2 Overview of the regulatory mechanisms of PD-L1 expression. Primary elevation of PD-L1 mainly consists of (1) genomic aberrations, (2) microRNA-based control, (3) oncogenic transcription factors and pathways, and (4) posttranslational modulation and trafficking. RAS/MEK/ERK pathway, PI3K/Akt/mTOR pathway, JAK/STATs signaling and TLRs/IKKs are major pathways regulating PD-L1 expression. IRF1, STATs, MYC, NF-κB, c-JUN, and HIF1α/2α are main downstream transcription factors. Posttranslational modifications of PD-L1 include phosphorylation, ubiquitination, glycosylation, and palmitoylation. The induction of PD-L1 by cytokines such as IFN-γ is regarded as the secondary mechanism

3.4.1 Primary Regulation of PD-L1

3.4.1.1 Genomic Aberrations in PD-L1/PD-L2

PD-L1 and PD-L2 are both located in close proximity to each other on chromosome 9p24.1. Over the past decades, both amplifications and translocations have been implicated in "primary" elevation of PD-L1 expression in several types of tumors. 9p copy number amplifications were initially detected and found to be positively correlated with increased expression of PD-L1/PD-L2 in cases of Hodgkin's lymphoma and mediastinal large B cell lymphoma (Green et al. 2010). In a subsequent study that analyzed 571 cases of mediastinal large B cell lymphoma, genetic aberrations were also observed frequently, with translocations being identified in 20% of the cases and amplification in 29% of the cases (Twa et al. 2014). Further, in-depth analysis of a larger cohort of Hodgkin's lymphomas showed alterations of the PD-L1/PD-L2 locus in 97% of the cases tested (Roemer et al. 2016). In both of these studies, samples harboring genomic aberrations show increased PD-L1 and PD-L2 expression. Evidence for a functional role of locus amplification in pathogenesis is given by the fact that Hodgkin's lymphomas respond particularly well to PD-1 blockade (Ansell et al. 2015; Armand et al. 2016; Younes et al. 2016). PD-L1/PD-L2 amplification has likewise been observed in cases of small cell lung cancer (George et al. 2017), squamous cell carcinoma of the oral cavity (Straub et al. 2016), and in Epstein–Barr virus (EBV)-positive gastric cancer (Cancer Genome Atlas Research Network 2014).

The PD-L1 3′ untranslated region (3′ UTR) played a negative regulatory role in PD-L1 expression. Loss of this gene segment due to different structural variations has been described to correlate with increased PD-L1 expression in a fraction of human tumors of diverse histology. Further deletion of the 3′ UTR of the PD-L1 gene using CRISPR Cas9 technology leads to enhanced PD-L1 mRNA stability in human and murine cells, thereby increasing their resistance to T cell attack (Kataoka et al. 2016). Similarly, when Mezzadra et al. (2017) tried to find factors that modulate PD-L1 expression in a genetic screen, gene trap vector integrations that result in loss of the 3′ UTR of the PD-L1 gene were enriched in cells with high PD-L1 levels.

3.4.1.2 MicroRNA-Based Control of PD-L1 Expression

In agreement with the regulatory role of PD-L1 3′ UTR, a considerable number of microRNAs (miRNAs) that bind to the 3′ UTR of the PD-L1 mRNA were revealed to regulate PD-L1 protein levels. miR-513 was the first miRNA that identified as a PD-L1 negative regulator by direct binding to the 3′ UTR of PD-L1 mRNA. IFN-γ suppresses miR-513 expression and overexpression of miR-513 is able to block IFN-γ-induced PD-L1 expression in cholangiocytes (Gong et al. 2009, 2010). miR-155, which can be induced by TNFα and IFN-γ, suppresses PD-L1 expression at the protein level by binding PD-L1 3′ UTR in human primary cells (Yee et al. 2017). Moreover, miR-34a in AML (Wang et al. 2015) and NSCLC (Cortez et al. 2016),

miR-142-5p (Jia et al. 2017), miR-93, and miR-106b (Cioffi et al. 2017) in pancreatic cancer, miR-200 in NSCLC (Chen et al. 2014) and gastric cancer (Xie et al. 2017), miR-17-5p in melanoma, miR-152 (Xie et al. 2017) and miR-570 (Wang et al. 2013) in gastric cancer, and miR-15a, miR-193a, miR-16 in malignant pleural mesothelioma (Kao et al. 2017) have all been identified as suppressors of PD-L1 expression.

3.4.1.3 PD-L1 Upregulation by Oncogenic Transcription Factors and Pathways

Activation of multiple oncogenic pathways and transcription factors is a major cause of the primary PD-L1 overexpression in tumor cells. A number of oncogenic transcription factors such as MYC, STAT3, HIF1α, HIF2α, c-JUN, and RELA (p65) have been identified that directly regulate PD-L1 transcription. The *MYC* gene is a canonical oncogene that regulates the expression of a multitude of gene products involved in cell proliferation, growth, differentiation, and apoptosis. Approximately 70% of human cancers showed elevated expression of MYC. In NSCLC, MYC expression was reported to be positively correlated with PD-L1 expression (Kim et al. 2017). Genetic or pharmacological inactivation of MYC leads to reduced PD-L1 expression in multiple tumor cell models including melanoma, leukemia, lymphoma, NSCLC, and HCC (Kim et al. 2017; Atsaves et al. 2017; Wang et al. 2017). Mechanically, MYC directly binds to the PD-L1 promoter (Casey et al. 2016), indicating that MYC may be able to regulate PD-L1 expression at the transcriptional level. Similarly, active STAT3 in human HNSCC and lymphoma cells (Atsaves et al. 2017; Marzec et al. 2008; Bu et al. 2017) and RELA (p65; a subunit of NF-κB) in NSCLC cells, monocytes, and breast cancer cells (Bouillez et al. 2017; Huang et al. 2013; Xue et al. 2017) and STAT1 (Cerezo et al. 2018) can also directly act on the promoter of PD-L1 to increase its expression.

Hypoxia represents a key character of tumor microenvironment, as expanding tumor mass always outgrows the oxygen supply. To promote angiogenesis, hypoxic TME induces a series of hypoxia-inducible factors such as HIF-1α activation (Brown and Wilson 2004), but also leading to local PD-L1 expression. Both HIF-1α and HIF-2α have been shown to physically interact with the hypoxia-responsive element in the PD-L1 promoter (Barsoum et al. 2014; Messai et al. 2016). And evidence has gathered that PD-L1 expression is regulated by HIF-1α in mouse melanoma, human breast cancer, prostate cancer, NSCLC cells, myeloid-derived suppressor cells (MDSCs) (Barsoum et al. 2014; Koh et al. 2016; Noman et al. 2014), and by HIF-2α in renal cell carcinoma cells (Messai et al. 2016).

The PI3K-Akt signaling pathway impacts cancer cell survival, proliferation, metabolism, and mobility. The positive correlation between PI3K-Akt signaling and PD-L1 expression is well documented in NSCLC, CRC, glioma, breast cancer, and melanoma cells (Atefi et al. 2014; Lastwika et al. 2016; Parsa et al. 2007; Xu et al. 2014; Song et al. 2013). In human gliomas, activation of PI3K pathway and loss of PTEN enhanced PD-L1 expression (Parsa et al. 2007). In renal cell carcinoma,

melanoma (Atefi et al. 2014) and NSCLC (Lastwika et al. 2016), inhibition of PI3K decreased PD-L1 expression. In a mouse lung SCC model that resulted from biallelic inactivation of Lkb1 and PTEN, increased PD-L1 levels was observed (Xu et al. 2014). In addition, type I and type II interferons can activate the PI3K-Akt-mTOR cascade, and PI3K-Akt-mTOR controls interferon-dependent mRNA translation, implying a certain level of cooperation between Akt-mTOR pathway and interferon receptor signaling pathway. In line with this, pharmacological inhibition of PI3K-Akt signaling suppressed IFN-γ-induced PD-L1 expression (Zhang et al. 2017).

The MEK-ERK pathway, often resulting from upstream receptors activating mutations such as KRAS and EGFR, is another commonly activated pathway in human cancers and plays a vital role in upregulating PD-L1 level. Evidence has gathered that hyperactivation of MEK-ERK signaling can directly promote PD-L1 gene expression in multiple cancers including lung cancer, breast cancer, multiple myeloma, bladder cancer, and lymphomas (Liu et al. 2007; Loi et al. 2016; Sumimoto et al. 2016; Qian et al. 2008; Karakhanova et al. 2010; Yamamoto et al. 2009). Suppression of MEK reduced PD-L1 expression through inactivation of JUN and STAT3 (Sumimoto et al. 2016; Jiang et al. 2013). In some KRAS mutant NSCLC cells, silencing of KRAS decreased ERK activation which then suppressed PD-L1 expression. Consistently, ectopic expression of mutant KRAS results in increased PD-L1 expression in bronchial epithelial cells (Chen et al. 2017). Activating mutations in EGFR induced PD-L1 expression in bronchial epithelial cells, NSCLC, head and neck cancer (HNC), and breast cancer cells, which can be blocked by pharmacological inhibition of EGFR (Akbay et al. 2013; Concha-Benavente et al. 2016), rapamycin (Lastwika et al. 2016) and by ERK inhibitors (Chen et al. 2015).

Oncogenic anaplastic lymphoma kinase (ALK) signaling that often results from gene translation or amplification also induced PD-L1 expression. In lymphomas harboring nucleophosmin-anaplastic lymphoma kinase (NPM-ALK) fusion gene, NPM-ALK oncoprotein activates STAT3 through JAK3 activation, binds physically to the PD-L1 gene promoter, and induces its expression in vitro and in vivo (Marzec et al. 2008). In NSCLC cells that harbors EML4-ALK fusion gene, the upregulation of PD-L1 by the constitutively active ALK kinase was dependent on MEK/ERK and PI3K/Akt signaling pathways (Ota et al. 2015). In addition, STAT3 and HIF1a were also reported to be downstream mediators for EML4-ALK-induced PD-L1 upregulation (Koh et al. 2016). Vice versa, PD-L1 overexpression can also reflect activation of KRAS, EGFR, and ALK, as observed in NSCLC, head and neck cancer, breast cancer, lymphomas, and other malignancies (Akbay et al. 2013; Concha-Benavente et al. 2016; Coelho et al. 2017). These observations explain frequent observations of the elevated levels of PD-L1 and PD-L2, even in the absence of active immune cells infiltrate and indicate the common role of these molecules in the primary escape of cancer cells from immune attack.

3.4.1.4 Posttranslational Regulation and Trafficking of PD-L1

Posttranslational regulation including ubiquitination, deubiquitylation, glycosylation, phosphorylation, and palmitoylation is the final "primary" mechanism by which PD-L1 protein expression is modulated. Poly-ubiquitination of PD-L1 by the E3 ligases cullin-3 promotes degradation of PD-L1 (Zhang et al. 2018), while deubiquitylation of PD-L1 by COP9 signalosome 5 (CSN5) (Lim et al. 2016) and the CKLF-like MARVEL transmembrane domain-containing protein CMTM6 (Mezzadra et al. 2017) prevents PD-L1 degradation. Glycogen synthase kinase 3β (GSK3β) interacts with PD-L1 and induces phosphorylation and subsequent ubiquitination-dependent proteasome degradation of PD-L1 by β-TrCP. Glycosylation blocks GSK3β binding, and thus stabilizes PD-L1 (Li et al. 2016). Consistently, a strategy targeting glycosylated PD-L1 exhibited promising therapeutic effects (Li et al. 2018). More strikingly, PD-L1 is also found in tumor-derived exosomes and suppresses T cell activation in the draining lymph node, adding the complexity of PD-L1 regulation (Chen et al. 2018; Poggio et al. 2019).

The controlled trafficking of PD-L1 represents another hotspot in PD-L1 posttranslational research. At the glycosylation stage, targeted blockade of PD-L1 transportation from the endoplasmic reticulum to the Golgi apparatus was found to trigger endoplasmic reticulum-associated degradation (ERAD) of PD-L1 (Cha et al. 2018). The mechanisms by which CMTM6 and CMTM4 stabilize PD-L1 involve not only ubiquitination-dependent degradation but also lysosome-dependent proteolysis (Burr et al. 2017). We also recently demonstrated that huntingtin interacting protein 1 related (HIP1R) binds to PD-L1 and delivers it to lysosomal degradation, thus altering T cell-mediated cytotoxicity (Wang et al. 2018). Furthermore, for the first time, we (Yao et al. 2019) and another group (Yang et al. 2019) arrived at the same conclusion that PD-L1 is palmitoylated and thus stabilized in tumor cells. We further depicted a detailed mechanism: the palmitoyltransferase DHHC3 palmitoylates PD-L1, suppressing the mono-ubiquitination of PD-L1 and thereby preventing its trafficking to the multivesicular body and blocking lysosomal degradation of PD-L1.

3.4.2 Immune Induction of PD-L1 as a Secondary Mechanism

PD-L1 expression is greatly increased in cancer tissues undergoing immune attack, representing the "secondary" mechanism of tumor-related immune suppression. A number of soluble cytokines can induce PD-L1 expression, with IFN-γ being some of the most potent one. In one of the first reports indicating that PD-L1 could be exploited by tumor cells as a defense mechanism against T cell attack (Dong et al. 2002), regulation of PD-L1 by IFN-γ was described for various tumor types, healthy tissues, and immune cells, and this phenomenon has been extended in further studies (Brown et al. 2003; de Kleijn et al. 2013; Kondo et al. 2010; Mazanet and Hughes

2002; Nakazawa et al. 2004; Schoop et al. 2004; Wintterle et al. 2003). IFN-γ is a pro-inflammatory cytokine that is abundantly produced by T cells and NK cells upon activation. Binding of IFN-γ to its receptor leading to signaling through the classical JAK-STAT pathway, preferentially through STAT1, thereby inducing enhanced expression of a series of transcription factors, called the interferon-responsive factors (IRFs). Of those factors, IRF1 is crucial in the IFN-γ-mediated induction of PD-L1 (Lee et al. 2006). Other signaling pathways such as MAK14, CRKL, and PI3K may also involve in IFN-γ-mediated PD-L1 expression. As IFN-γ is generally considered the most prominent soluble inducer of PD-L1, and expression of PD-L1 may therefore be viewed as a crude measure of local IFN-γ signaling and T cell activity in most settings. Next to IFN-γ, type I interferons, i.e., IFN-α and IFN-β, can also induce PD-L1 expression in cultured melanoma cells, endothelial cells, monocytes, and dendritic cells (Garcia-Diaz et al. 2017). The ability of interferons to regulate PD-L1 expression has contributed to the concept of "adaptive immune resistance" in cancer biology, which proposes that the pro-inflammatory cytokines produced by infiltrating T cells further promote PD-L1 expression and protect cancer cells from immune cells-mediated killing in the TME.

Apart from interferons, other inflammatory stimuli such as LPS (Mezzadra et al. 2017; Qian et al. 2008; Loke and Allison 2003), TNFα (Kondo et al. 2010; Ou et al. 2012; Quandt et al. 2014; Wang et al. 2017), IL-17 (Wang et al. 2017; Zhao et al. 2011), IL-10 (Curiel et al. 2003), IL-4 (Quandt et al. 2014), IL-27 (Karakhanova et al. 2011; Matta et al. 2012), IL-2 (Eppihimer et al. 2002; Xiong et al. 2014) and other NF-κB activators such as Toll-like receptor ligands (Mezzadra et al. 2017; Loke and Allison 2003; Boes and Meyer-Wentrup 2015; Cole et al. 2011) have been shown to induce PD-L1 expression. Finally, TGF-β, a molecule that is in general regarded as an anti-inflammatory cytokine, appears to have a context-dependent effect on PD-L1 expression. Specifically, whereas exposure of tubular epithelial cells (Starke et al. 2007) or monocytes (Ou et al. 2012) to TGF-β suppressed PD-L1 expression, TGF-β upregulated PD-L1 protein expression in dendritic cells (Ni et al. 2012; Song et al. 2014). In line with it, production of TGF-β by $CD8^+$ T cells in an in vivo model of pancreatic islet transplantation was shown to be necessary for sustained PD-L1 expression by the same cells (Baas et al. 2016).

While the data above indicate that a substantial number of inflammatory mediators can modulate PD-L1 expression, in many cases it is unclear whether such induction occurs in an indirect manner, for example, via regulation of IFN production. In addition, further investigations are needed to establish in which cell types these different stimuli alter PD-L1 expression in vivo and whether the altered PD-L1 expression impacts on local T cell function.

3.5 PD-1/PD-L1 Pathway in Cancer

PD-1 is a "rheostat" that regulates the threshold of antigen responses and maintains peripheral tolerance (Okazaki et al. 2013). Perturbing the PD-1 pathway can

profoundly impact host physiology. PD-L1 blockade can impair fetomaternal tolerance, and PD-1-deficient mice have altered thymic T cell education and are prone to develop autoimmune diseases. In contrast to the devastating fatal autoimmune disease of mice deficient in CTLA-4, PD-1-deficient mice showed much milder, chronic, and strain-specific autoimmune phenotypes. Aged PD-1 deficient mice on a C57BL/6 background spontaneously developed arthritis and lupus-like glomerulonephritis (Nishimura et al. 1999). On a BALB/c background, aged PD-1 deficient mice develop dilated cardiomyopathy via the generation of autoantibody against troponin I (Okazaki et al. 2003; Nishimura et al. 2001). These observations were the first experimental evidence for the autoimmune basis of dilated cardiomyopathy and provided the rationale for immune-absorption therapy for this deadly disease. In NOD and MRL mice, PD-1 deficiency specifically accelerated the onset and frequency of type 1 diabetes (Wang et al. 2005) and myocarditis (Wang et al. 2010), respectively. The variations in the disease phenotype depend on the genetic backgrounds, which suggests that immunoregulation by PD-1 is rather antigen-specific and mainly cell intrinsic. Moreover, the target specificity for PD-1 regulation of autoimmunity also holds true for the association between single-nucleotide polymorphisms in Pdcd1 and various kinds of human autoimmune diseases, including systemic lupus erythematosus, rheumatoid arthritis, type I diabetes, Grave's disease, multiple sclerosis, and ankylosing spondylitis (Prokunina et al. 2002; Nielsen et al. 2003; James et al. 2005).

The first evidence for PD-1 in impairing immunosurveillance for tumor cells was provided by the observation that overexpression of PD-L1 on P815 tumor cells markedly inhibits the cytolytic activity of $CD8^+$ T cells through engagement with PD-1 and enhances their tumorigenesis and invasiveness in vivo (Iwai et al. 2002). Subsequent studies discovered aberrant elevation of PD-L1 in clinical tumor samples and their positive correlation with poor prognosis (Thompson et al. 2004; Okazaki and Honjo 2007), supporting the idea of applying PD-1 pathway blockade to cancer treatment. How does PD-1 signaling mediate immune escape? $PD\text{-}L1^+$ cells, particularly PD-L1–expressing tumor cells and APCs, engage $PD\text{-}1^+$ T cells, causing T cells apoptosis, anergy, exhaustion, and interleukin-10 (IL-10) expression. PD-L1 just acts as a molecular "shield" sending a critical "don't find me" signal to the adaptive immune system to protect $PD\text{-}L1^+$ tumor cells from $CD8^+$ T cell-mediated lysis. Consistently, T cell dysfunction is a hallmark of many cancers (McLane et al. 2019). Additionally, as mentioned above, PD-L1 can transmit signals back into T cells (Dong et al. 2003) and tumor cells (Azuma et al. 2008) to affect their survival. These observations provide scientific rationales to design drugs to block PD-1 pathway. Until now, five drugs targeting PD-1 pathway were approved by FDA to treat a wide spectrum of different tumors: monoclonal antibodies nivolumab (anti-PD-1; Bristol-Myers Squibb, USA), pembrolizumab (anti-PD-1; Merck, USA), atezolizumab (anti-PD-L1; Genentech, USA), avelumab (anti-PD-L1; EMD Serono, USA), and durvalumab (anti-PD-L1; AstraZeneca, UK). Detailed information about anti-PD-1/PD-L1 drugs are discussed in the following chapters.

The objective response rate for PD-1 pathway blockade varies greatly among different cancer types. Lymphomas (Ansell et al. 2015), melanoma (Topalian et al.

2012, 2014; Brahmer et al. 2012; Herbst et al. 2014; Hamid et al. 2013; Wolchok et al. 2013), bladder cancer (Powles et al. 2014), and mismatch-repair-deficient cancers (Le et al. 2015, 2017) may be among the most responsive cancer types based on current clinical data. Given that only a percentage of patients are responsive to immunotherapy, identifying the biology of treatment response and resistance are a priority to optimize drug selection and improve patient outcomes. There are numerous ongoing efforts to identify predictive biomarkers of PD-1 pathway blockade. PD-L1 expression, cytotoxic T lymphocytes infiltration, and tumor mutation burden (TMB) are generally considered as the most important factors affecting the effectiveness of immune checkpoint blockade therapy in different tumor types and individual patients (Topalian et al. 2012; Llosa et al. 2015; Herbst et al. 2014; Tumeh et al. 2014; Junttila and de Sauvage 2013; Fridman et al. 2012; Galluzzi et al. 2015; Weichselbaum et al. 2017).

Cancer cells exploit the expression of PD-L1 to subvert T cell-mediated immunosurveillance. It is reasonable to assume that PD-L1 in TME may predict or be associated with the clinical response of anti-PD therapy. Most lymphoma patients have an amplification of PD-L1/PD-L2. Consistently, lymphoma is among the most responsive cancer type to anti-PD therapy. An 87% objective response is observed in patients with relapsed or refractory Hodgkin's lymphoma treated with nivolumab (Ansell et al. 2015). The positive correlation between tumor tissue PD-L1 expression and the likelihood of responsiveness to PD pathway blockade has also been observed in patients with melanoma (Topalian et al. 2012; Brahmer et al. 2012), NSCLC, and RCC (Topalian et al. 2012). In contrast, most progressing patients show a lack of PD-L1 upregulation by either tumor cells or tumor-infiltrating immune cells (Herbst et al. 2014). However, it should be noted that the expression of PD-L1 in tumor tissues should not be used as a biomarker for selection or exclusion of patients for anti-PD-1/PD-L1 therapy. As patients whose tumors exhibited negative PD-L1 staining can have objective responses.

The scientific rationale for anti-PD-1/PD-L1 therapy is to reinvigorate the "exhausted" T cells. Hence, the preexistence of antitumor T cells whose function is compromised by specific immune checkpoints is the main premise for inducing an immune response. Indeed, intratumoral T cell infiltration, T_H1-type gene expression, and a clonal TCR repertoire predict and shape the clinical response to anti-PD-1/PD-L1 therapy (Tumeh et al. 2014; Cristescu et al. 2018). PD-L1 expression in the tumor is most compelling when it is paralleled with an active T cell response. The ongoing T cell response itself, not PD-L1 expression, may be the key factor to elicit clinical response. However, a central question is why some tumors are "inflamed" with effector T cell infiltration, whereas others are not. One supposition is that tumor intrinsic β-catenin signaling activation leads to poor CCL4 expression, limiting DC trafficking to tumor sites and DC-mediated T cell activation (Spranger et al. 2015). Potent epigenetic silencing of tumor T_H1 type chemokines CXCL9 and CXCL10 was also reported to be responsible for poor T cell tumor infiltration (Peng et al. 2015; Nagarsheth et al. 2016). These two chemokines mediated effector T cell and NK cell tumor migration.

Somatic mutations have the potential to encode "non-self" immunogenic antigens to elicit host T cell response. High mutational burden, particularly the accumulation of insertion–deletion (indel) mutational load (Mandal et al. 2019), renders tumors immunogenic and thus sensitive to PD-1/PD-L1 pathway blockade. Several lines of evidence have been obtained to support a link between TMB and responsiveness to anti-PD-1/PD-L1 therapy (Cristescu et al. 2018). Lung cancer and melanoma, cancers with high numbers of mutations due to exposure to cigarette smoke and ultraviolet radiation, respectively, are among the best responses to anti-PD therapy. In NSCLC patients treated with pembrolizumab, high nonsynonymous mutation burden is associated with objective response, durable clinical benefit, and progression-free survival. And neoantigen-specific $CD8^+$ T cell responses parallel tumor regression, indicating neoantigen-specific T cell reactivity is enhanced by anti-PD-1 therapy (Rizvi et al. 2015). Specifically, a recent study using a clinicogenomic database analyzed 1290 NSCLC patients received anti-PD-1/PD-L1 therapies found TMB of 20 or more was significantly associated with improved overall survival from therapy initiation (16.8 months vs. 8.5 months) and increased clinical benefit rate (80.7% vs. 56.7%) compared with TMB less than 20 (Singal et al. 2019). In CRC patients, mismatch-repair deficiency status predicts clinical benefit of pembrolizumab (Le et al. 2015). In 2017, FDA approved pembrolizumab and nivolumab for the treatment of microsatellite-unstable cancers of any origin. This is the first class of reagents to be granted FDA approval based on a genetic characteristic as opposed to the site of origin of the cancer (Ribas and Wolchok 2018; Le et al. 2017). Although the antigen specificity is unknown, the number of predicted MHC class I-associated neoantigens was identified to be correlated with local immune cytolytic activity by large-scale genomic data sets of solid tumor tissues (Rooney et al. 2015).

3.6 The Future of Immune Checkpoint Therapy

PD-1/PD-L1 offers one of the best examples of scientific translation from bench to bedside and a powerful demonstration to all scientists, funding agencies, and pharmaceutical companies—of the extreme significance of basic research for progress in medicine. However, we have to bear it in mind that most patients still do not respond to anti-PD therapy. Several questions need to be addressed in future.

First, what mechanisms constitute the primary immune checkpoint inhibitor therapy resistance? Accumulating evidence converges on the antigen presentation and interferon-γ signaling pathways. A deeper understanding of basic mechanisms underlying clinical successes versus failures sheds lights on how human immune system responds to and is shaped by different TME. More importantly, it helps guide rational PD-1-based combinational therapy, which may overcome resistance mechanisms to immune checkpoint blockade. Despite extensive efforts on combinational therapy are ongoing, some of these combinational therapies are irrational, or even contradictory in mechanisms which may lead to worse outcomes than PD-1/PD-L1 blockade alone.

Rational PD-1-based combinational therapy clinical trials, for instance, mechanism-based ones, are needed to determine which combinations will work best for which patients. In parallel, precise indications, effectiveness, and side effects of given combinations in treating a specific type of cancer are also needed to be conclusively demonstrated in future clinical trials.

Second, what is the best time for PD-1/PD-L1 modulation? PD-1 is not required for the induction but required for maintaining T cell exhaustion (Odorizzi et al. 2015), as PD-1 blockade "reinvigorates" exhausted T cells (Barber et al. 2006). Is earlier better for checkpoint blockade (Robert 2018)? A recent neoadjuvant approach relying on the administration immune checkpoint inhibitors before surgery is evaluated in macroscopic stage III melanoma patients (Blank et al. 2018) and in glioblastoma patients (Cloughesy et al. 2019; Schalper et al. 2019), respectively. Encouraging clinical, pathological and immunological responses to neoadjuvant therapy were observed, highlighting that this concept warrants further exploration. However, an unfavorable toxicity profile was also observed in these trials, which must be addressed in future studies.

Third, future strategies of precision medicine will likely rely on novel diagnostic tools with which to identify and correct defects in current therapy. For example, further development of molecular PET imaging for assessment of PD-L1 status (Bensch et al. 2018) may represent a potential better detection method.

Fourth, beyond conventional $CD4^+$ and $CD8^+$ T cells, what are the functions of PD-1 on T_{reg} cells, B cells, myeloid cells, and NK cells? As systemic modulation of the PD-1/PD-L1 pathway will broadly impact all immune cell subsets, additional work is needed to understand how PD-1 functions on these cells.

Fifth, what are the unique and overlapping functions of PD-1/PD-L1 compared with other inhibitory coreceptors (including CTLA-4, LAG3, TIM3, and TIGIT)? It is not clear if there is a hierarchy by which inhibitory coreceptors operate, such that if one receptor is lost, other receptors will compensate. Also unclear is how to optimize synergies between different inhibitory receptors. We have a very rudimentary understanding of these signaling pathways, let alone how blockade of one receptor impacts another. Without doubt, the next decade will witness a boost in the clinical application of immunotherapy, and these issues deserve to be well addressed.

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Chapter 4
Discovery of New Immune Checkpoints: Family Grows Up

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Abstract The first generation of immune checkpoint inhibitors (ICIs) including anti-CTLA-4 and anti-PD-1/anti-PD-L1 has achieved profound and great success. Till 2019 Q1, there are nine ICIs landing the oncology market: Ipilimumab (anti-CTLA-4, Bristol-Myers Squibb), Nivolumab (anti-PD-1, Bristol-Myers Squibb), Pembrolizumab (anti-PD-1, Merck), Atezolizumab (anti-PD-L1, Roche/Genentech), Durvalumab (anti-PD-L1, Astra Zeneca), Tremelimumab (anti-CTLA-4, Astra Zeneca), Cemiplimab (anti-PD-1, Sanofi/Regeneron), Toripalimab (anti-PD-1, Junshi), and Sintilimab (anti-PD-1, Innovent), which have covered the majority of hematologic and solid malignancies' indication. Beyond the considerable benefits for the patients, frustrated boundary still exists: limited response rate in monotherapy in late-stage population, poor effectiveness in neoplasms with immune desert and immune excluded types, and immune-related toxicities, some are life-threatened and with higher incidence in I-O combination regiment. Moreover, clinicians observed some cases switching to progression after achieving partial or complete response, indicating treatment failure or drug resistance. So people begin looking for the next generation of immune checkpoint members.

Keywords Immune checkpoint inhibitors · Receptor identification · Ligand screening · Cell-based assay · T cell inhibition

4.1 Introduction

Generally speaking, there are two types of novel immune checkpoints, one takes part in co-stimulatory interaction with T cells (e.g., 4-1BB and its ligands) and another functions as suppressive factors (e.g., LAG-3). Besides, some investigators found that tumor glycol code also plays a critical role in cancer immunity and could

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be considered as novel immune checkpoint. In this chapter, we will introduce the mechanism of these new members and brief the preclinical and early clinical data in I-O treatment.

4.2 Co-stimulatory Targets

4.2.1 4-1BB (CD137) and 4-1BBL (CD137L)

4.2.1.1 Mechanism of Action

4-1BB, also called as CD137 or TNFRSF9, is a surface glycoprotein which belongs to tumor necrosis factor receptor superfamily (TNFRSF). It was discovered in 1989 during screening for novel receptors on murine T cell lines. There were 255 aa containing two potential N-linked glycosylation sites in human 4-1BB protein, of which aa 1–17 was a putative signal peptide, followed by an extracellular domain of 169 aa and then a transmembrane region of 27 aa between positions 187 and 213, and finally a short intracellular domain of 42 aa revealed by hydrophobicity analysis. In the cytoplasmic domain, five regions of amino acids sequences were conserved between mice and human, indicating that these residues might be important for 4-1BB function. The molecular weight of 4-1BB was calculated to be 27 kDa (Cheuk et al. 2004).

4-1BB functions as an inducible co-stimulatory molecule expressed on diverse immune cell population. On T cells, natural killer (NK) cells, regulatory T cells (Treg), and NK T cells (NKT), 4-1BB expression is activation dependent. When FcRγIII Fc receptor (CD16) is ligated by the Fc portion of mAb, the expression of 4-1BB would be elevated on NK cells. In addition to activated immune effectors, 4-1BB is also expressed on innate immune cell populations, including neutrophils, granulocytes, monocytes, mast cells, eosinophils, and dendritic cells (DC) (Cheuk et al. 2004; Croft 2009; Chester et al. 2018).

4-1BB can be activated by binding to its ligand 4-1BBL (CD137L; TNFSF9). The human 4-1BBL was first isolated in 1994 using direct expression cloning from an activated $CD4^+$ T lymphocyte population, and it consisted of 254 aa and shared a 36% identity with murine 4-1BBL. 4-1BBL is predominantly expressed by activated antigen-presenting cells (APCs) including dendritic cells (DC), B cells, and macrophages. It could also express on $CD4^+$/$CD8^+$ T cells, mast cells, and NK cell when induced by T cell receptor/FcεRI activated. 4-1BBL was also present at high levels in the sera of some patients with hematological diseases as well as on some carcinoma cell lines. It is of highly affinity with 4-1BB; the latter could associate with the TNF-associated factors 1 and 2 (TRAF1 and TRAF2) and activate the master immune-regulatory transcription factor NF-κB and activating protein-1 (AP-1) once ligating and cross-linking with its ligand (Croft 2009; Chester et al. 2018).

In primary immune response, the activation of 4-1BB:4-1BBL pathway leads to $CD4^+$ and $CD8^+$ T cells' activation and proliferation via co-stimulation signals by

NF-κB, JNK, and p38 MAPK downstream pathways, also producing several pro-inflammatory cytokines like interferon-gamma (IFN-γ) and IL-2. Furthermore, 4-1BB stimulation increases signaling through the T cell receptor (TCR) and amplifies the cytotoxicity of $CD8^+$ T cells. 4-1BB also plays a critical role in the differentiation of effector memory $CD8^+$ T cells. Besides, 4-1BB regulates the activation of NK cell via antibody-dependent cell-mediated cytotoxicity (ADCC) manner. When NK cells were activated via CD16 pathway, 4-1BB will be upregulated and mediated cytotoxic function in response to 4-1BB agonism; however, this phenomenon would be in a negative impact when 4-1BB agonism on resting NK cells. In the cancer setting, tumor-targeted mAb is recognized by NK cell FcγRIII, triggering release of perforin and granzyme. It also reported that 4-1BB engaged fine-tune synergistic IL-15- and IL-21-driven NK cell proliferation (Vidard et al. 2019). After interacting with some inflammatory factors such as TNF-α, liposaccharide, and IL-1β, endothelial cells could also upregulate 4-1BB; in that case, 4-1BB expression on vascular walls might lead to tumor microangiogenesis and migration. Some studies found that on tumor-infiltrating lymphocytes (TILs) 4-1BB was highly expressed by hypoxia-induced factor-1α (HIF-1α) mediating. Blocking 4-1BB via specific antagonistic antibody may cause severe depletion of CD4, CD8, B cells, and NK cells. Given that all together, 4-1BB and 4-1BBL pathway could be considered as a potential therapeutic target for autoimmune disease and malignancy disease (Croft 2009; Chester et al. 2016, 2018).

4.2.1.2 Preclinical and Early Clinical Data

Based on its mechanisms of action, agonistic antibody of 4-1BB could lead to T cells' remodeling and activation, enhancing the tumor suppression immunity. In the classic mouse model of OVA antigen-induced T cell energy, OVA could result in $CD8^+$ T cells exhausted. While treating with anti-4-1BB, antibody could restore the cytotoxicity $CD8^+$ function and induce IFN-γ secreting. In the B16.SIY model of melanoma, treatment with anti-4-1BB mAb restored the function of $CD8^+$ TILs that had lost the capacity to secrete IL-2. Anti-4-1BB mAb induced liver tumor regression in mice model via activating CD8 cells, so did limit the infiltration of cells suppressing antitumor immunity such as MDSC and regulatory T lymphocytes. It was also reported that agonistic 4-1BB mAb has synergy effect when combing with other antitumor activity agents, e.g., anti-CD20 mAb and anti-PD(L)-1mAb. However, some general autoimmune disease exacerbations have been found in mice model, for example, systemic lupus erythematosus and autoimmune encephalomyelitis, mediated by autoreactive $CD4^+$ T cells that seem to experience activation-induced cell death upon in vivo co-stimulation with the antibodies but not with the natural ligand. In another study, a mild degree of $CD8^+$ T cell-mediated hepatitis was induced in anti-4-1BB mAb treated mice. These antibodies have been expanded to clinical studies after demonstrating potent anticancer efficacy in murine models. Currently, two ongoing clinical trials have been being set for two anti-4-1BB mAbs, namely, Urelumab (BMS-663513) and Utomilumab (PF-05082566).

Urelumab, a fully human IgG4 mono antibody, was the first anti-4-1BB therapeutic to enter clinical trials. Urelumab showed a promising cancer treatment potential in a preclinical study. It showed activity signal in its dose-escalation phase 1 study and did not reach the MTD; nevertheless in phase 2 study enrolling advanced malignancies patients, it was suspended due to severe drug-related liver toxicity when occurred in one-third of the subjects. This toxicity was mainly due to S100A4 protein secreted by tumor and stromal cells. To ensuring the safety and tolerance for subjects, the doses were reduced to 0.1 and 0.3 mg/kg, and unfortunately it was confirmed the loss of potent under this dosage, and only few of responders with non-Hodgkin's lymphoma. Ensuing phase 1b single-arm combination of such low-dose Urelumab combining with the Nivolumab resulted in good tolerability, but the efficacy was disappointing. In the monotherapy cohorts, none of the solid tumor patients had an objective response, including 17 CRC, 15 SCCHN, and 31 other solid malignancies. Very few of objective response was observed when subjects were given Urelumab at 8 mg every 4 weeks plus nivolumab at 240 mg every 2 weeks. However, among 46 metastatic melanoma patients receiving such combo therapy who were naïve to treatment with ICIs, the ORR was satisfied, including 1 CR, 17 PR, and another 5 unconfirmed PR (Segal et al. 2016). These responses could be attributed to the activity of Nivolumab, but the responses in PD-L1-negative cases suggest that Urelumab may be contributing to the observed efficacy (NCT01471210 and NCT02253992). Data from other clinical studies, e.g., Urelumab combining with Rituximab, Cetuximab, and Elotuzumab, have not come out yet.

Utomilumab is a humanized IgG2 monoclonal antibody that activates 4-1BB while blocking binding to endogenous 4-1BBL. Utomilumab is safe in patients with tolerability up to 10 mg/kg, even though it has very modest therapeutic activity only seen against Merkel cell carcinoma. In the early phase 1 study, among 27 treated patients, the best clinical response was disease stabilization, occurring in 22% (6/27) of patients. Importantly, Utomilumab did not induce any dose-limiting toxicities (DLTs). Combination regimens were also found to be safe in phase 1 clinical trials with Pembrolizumab 2 mg/kg Q3W and Utomilumab dose escalation ranging from 0.45 to 5.0 mg/kg. In total, 23 patients with advanced solid malignancies (including NSCLC, RCC, and SCCHN) were treated. The safety profile was promising that Utomilumab showed no additional toxicity when combining with Pembrolizumab, and no higher grade TRAE or DLT occurred. The combination demonstrated clinical benefit with two CR and four PR confirmed and one unconfirmed PR (Tolcher et al. 2017). Utomilumab is tested in combination with Avelumab (anti-PD-L1 from Pfizer) in DLBCL (NCT02951156) and in one of the first triple-agent combination immunotherapy regimens with Avelumab and an anti-OX40 (NCT02554812) most recently. Ongoing clinical studies test Utomilumab in combination with Rituximab and Mogamulizumab (anti-CCR4).

4.2.2 GITR and GITRL

4.2.2.1 Mechanism of Action

Glucocorticoid-induced tumor necrosis factor receptor-related protein (GITR), also referred as TNFRSF18/activation-induced TNFR (AITR)/CD357, was first identified by differential display following treatment of a T cell hybridoma with dexamethasone in 1997. GITR was firstly caught attention due to its highly expression on $Foxp3^+$ regulatory Tregs. Human (h)GITR is a 241-residue type I transmembrane protein that shares 55–60% sequence identity with murine (m)GITR. The murine and human Tnfrsf18 loci are found on chromosomes 4 and 1, respectively, clustered with TNFRs OX40 and 4-1BB. GITRL (also referred to as TNFSF18, AITRL), a type II transmembrane protein, is the specific activate ligand of GITR. hGITRL is a 177-residue protein with 51% sequence identity with mGITRL; it locates on chromosome 1 and clusters with TNFSF members FasL and OX40L (Clouthier and Watts 2014; Knee et al. 2016; Gurney et al. 1999).

As previously mentioned, GITR is constitutively enriched on Tregs, and it could be expressed promptly when Treg is stimulated. Besides, GITR is original expressed at low levels on resting effector $CD4^+$ and $CD8^+$ T cells but rapidly upregulating when they are activated. It was also reported that human GITR would express on DC, macrophages, and NK cells. GITRL is widely expressed on APCs, including DCs, macrophage, and activated B cells. Foxp3 is a key factor of regulating GITR expression in Tregs (Beek et al. 2019; Shimizu et al. 2002), but in effector $CD4^+$ and $CD8^+$ T cells, GITR is regulated by NF-κB and nuclear factor of activated T cells (NFAT), with NF-κB inducing and NFAT repressing GITR expression downstream of TCR signals. GITR is also upregulated by CD28 signaling in both conventional and regulatory T cells (Zhan et al. 2008). Expression of GITR increases upon activation immediately within hours, and then reduction followed 2–3 days till below pre-stimulation level. It is very interesting that GITR and GITRL expressions are not limited to hematopoietic cells. It has been confirmed that GITR could be expressed at intermediate levels on epidermal keratinocytes and osteoclast precursors, whereas on type 1 IFN-stimulated endothelial cells GITRL would be highly expressed. Based on all the evidence, GITR/GITRL axis may participate in multiple cytological functions other than immune modulation, like mediating immune cell adhesion and migration (Clouthier and Watts 2014).

Like other members of the TNFR superfamily (e.g., 4-1BB and OX40), GITR represents a class of targets referred to as co-stimulatory receptors. In the thymus, GITR is expressed during T cell development and plays a crucial role in thymic Treg differentiation and expansion. In the periphery under the scenarios of GITR activation including some agonist antibodies (e.g., DTA-1), recombinant GITRL or GITRL transfectants leads to suboptimal TCR stimulation, so that T cells would be activated as consequence. MAPK and NF-κB pathways play important role in mediating GITR signaling. TRAF2/5-dependent NF-κB induction following GITR engagement is associated with upregulation of Bcl-xL expression on activated $CD8^+$

cells, suggesting a potential role for GITR in enhancing cell survival. Highly expression of GITR has been demonstrated as another maker and modulator for Tregs with the function of inducing Treg expansion, inhibiting Treg suppressive function, and promoting Teff overcome to Treg restraint.

4.2.2.2 Preclinical and Early Clinical Data

In multiple mouse cancer models including concomitant immunity to B16 melanoma, Meth-A sarcomas, and CT26 colon carcinoma, anti-GITR has demonstrated its active signals in cancer suppression. With similar results from GITRL-expressing tumors, using GITRL-Fc showed a potent antitumor immunity in vivo and in vitro. GITR agonist therapy could lead to activation of conventional T cells with increasing IFN-γ production and elevating the level of multifunctional $IFNg^{+}TNF^{+}CD107a^{+}$ cells in preclinical. DTA-1 could enhance the production of Granzyme B via agonizing GITR. In another study, it reported that more IL-12^{+} DC in tumor metastatic LN and sustained IFN-γ^{+} effector CD4 T cells were observed when given CD4 T cells from mouse that is previously tumor resistance and DTA-1 together. Besides, CD8-depletion could not completely ablate the effects of GITRL-Fc in some models, indicating that there are potential non-CD8-dependent effects on tumor clearance. For example, some have found that tumor rejection was dependent on CD4, CD8, and $NK1.1^{+}$ cells and required IFN-γ and FasL, but was perforin-independent in B16 model. In mice, DTA-1 abrogates Treg-mediated suppression either by eliminating GITR-expressing tumor-infiltrating Tregs or by causing them to become unstable, thereby attenuating their suppressive activity.

TRX518 is a first-in-human, fully humanized Fc-dysfunctional aglycosylated IgG1κ monoclonal antibody that triggers hGITR signaling (Rosenzweig et al. 2010). The two phase 1 trials are as follows: NCT01239134: dose-escalation study in melanoma and other solid tumors; and NCT02628574: repeated administrations of TRX518 in combination with PD-1 pathway blockade in patients with advanced refractory solid tumors. In the first dose-escalation study, TRX518 was well tolerated (from 0.0001 up to 8 mg/kg) and no DLT was reported. There was also no related SAE or TRAE. Efficacy data was available in the limited small sample size (28/40 patients); the BOR was SD in four patients. TRX518 preferentially induces loss of activated proliferating Treg cells, thus contributing to the decrease in peripheral and intratumoral Treg cells, in particular, activated potentially pathogenic tumor-induced Treg cells, and in TRX518-treated patients. Despite Treg reductions and increased Teff: Treg ratios, substantial clinical responses were not seen. From this study, it was concluded that TRX518 treatment was safe and well tolerated and further investigation is warranted. When combining with anti-PD-1 antibody, three of the first patients enrolled in this study have demonstrated clinical responses (1 CR, 1PR, and 1 SD), including one patient that had progressed on previous anti-PD-1 therapy (Knee et al. 2016).

MK-4166 is another humanized IgG1 agonist anti-GITR mAb, which shows high-affinity interaction with GITR that enhances TCR-driven in vitro proliferation of

human and cynomolgus monkey naïve $CD4^+$ T cells, similar to the effect of DTA-1 on mouse T cells. Besides, MK-4166 enhanced the proliferation of human tumor-infiltrating lymphocytes (TILs). MK-4166 decreased proliferation and suppressive functions of Tregs in vitro (Sukumar et al. 2017). MK-4166 induced phosphorylation of NF-κB and increased expression of dual-specificity phosphatase 6 (DUSP6), indicating that MK-4166 activated downstream NF-κB and ERK/MAPK signaling pathways in human TIL cultures. Furthermore, it was found that FOXP3 mRNA in human tumor-infiltrating Tregs could be downregulated by MK-4166, indicating that MK-4166 may attenuate the Treg-mediated suppressive tumor microenvironment in order to enhancing the activation of TILs. MK-4166 and MK-4126 (Merck & Co., Kenilworth, USA) are other agonistic monoclonal antibodies; they are now developed in several phase 1 studies (combination of MK-4126 and/or Pembrolizumab, NCT02132754 and NCT02553499) (Knee et al. 2016).

BMS-986156 is another anti-GITR antibody. It is now investigated in a phase 1 study with mono or combo regimen (with nivolumab) in patients with advanced solid malignancies (NCT02598960). Preliminary results showed no DLT, and no active signal was observed (Siu et al. 2017). AMG 228, an agonistic IgG1 monoclonal antibody of GITR, was also recently studied in a first-in-human clinical trial in 30 patients with refractory CRC, SCCHN, urothelial carcinoma, and melanoma. No responder was observed, and no DLT was identified. Most of the subjects (27/30) experienced mild AEs, including electrolyte imbalances, anemia, and fever. Besides antibodies (INCAGN1876, GWN323, and MK-1248), agents in development include synthetic GITR ligands fused to an Fc: OMP-336B11, a GITRL trimer, and MEDI1873, a GITRL hexamer. No clinical results are available (Tiguea et al. 2017).

4.2.3 OX40 and OX40L

4.2.3.1 Mechanism of Action

OX40 (CD134; TNFRSF4) is a member of the TNFR superfamily. It is a 50KDa type I transmembrane glycoprotein which contains four cysteine-rich domains (CRDs). OX40 has only one known ligand called OX40L (CD252), which is classically expressed on activated APCs (Willoughby et al. 2017; Aspeslagh et al. 2016). OX40 and OX40L are encoded by TNFRSF4 and TNFSF4 on chromosome 1, where they are in close proximity to other TNF family molecules. Upon activation, three OX40 molecules bind to the OX40L trimer, which is typical formation in the TNFRSF; in the case of OX40, CRDs 1, 2, and 3 all interact with OX40L (Croft 2009; Willoughby et al. 2017).

Once TCR is stimulated, OX40 is transiently expressed on both $CD4^+$ and $CD8^+$ T cells, and its expression level is higher on $CD4^+$ compared with $CD8^+$ T cells in vitro and at tumor sites. Thus, both $CD4^+$ and $CD8^+$ T cells could be recognized as potential targets of OX40-directed immunotherapy in cancer. OX40 expression is induced following TCR/CD3 cross-linking, and by the presence of inflammatory cytokines,

including IL-1, IL-2, and TNF-α (Lane 2000; Bansal-Pakala et al. 2004; Takeda et al. 2004). Upon OX40 co-stimulation of T cells, intra-cytoplasmic pathways associated to T cell signaling are activated such as NF-κB and NFAT, which can enhance the expression of molecules such as survivin, cyclin A, cyclin-dependent kinases, Bcl-2 anti-apoptotic molecules, cytokines, and cytokine receptors. Furthermore, lower level OX40 expression is reported on NKT cells, NK cells, and neutrophils, OX40 is not expressed on naïve T cells. TCR ligation alone is sufficient to induce OX40 expression on $CD4^+$ T cells, and additional augment signals including CD80/CD86-CD28 and CD40-CD40L could enhance the expression of OX40. Other inflammatory factors like TNF-α, IL-1, and IL-2 may induce OX40 on both CD4 and CD8 T cells. As with OX40, OX40L expression is upregulated in response to antigen presentation on multiple APCs, including B cells, macrophages, and DCs. Other cells that can be induced to express OX40L including mast cells, endothelial cells, and malignancy cells. There is constitutive expression on lymphoid tissue inducer cells (Takeda et al. 2004; Zaini et al. 2007).

The most recognized function of OX40 is to enhance proliferation and survival of CD4 and CD8 T cells. OX40 could function as immune modulator via affecting on various cytokine productions; on one hand, it could lead to differentiation of CD4 T cells into either Th1 or Th2 subsets; on the other hand, it could also regulate IL-17 production so that to mediate Th17-mediated diseases. On naïve CD4 T cells (after its TCR-mediated upregulation), the OX40 interacts with OX40L that could preferentially lead to the differentiation of Th2 cells as a result of autocrine IL-4 production. OX40 engaging with its ligand leads to the migration of OX40 and TRAF2 into lipid rafts, which is a key step for subsequent activation of NF-κB. Other than TRAF2, OX40 also binds to TRAFs 1, 3, and 5; these proteins share a highly overlapping sequence GGSFRTPIQEE on their binding site with OX40. However, it is not known whether these TRAFs could also co-localize with OX40 in lipid rafts to activate downstream pathway, nor what the mechanisms lie within any given TRAF interacting with OX40 (Willoughby et al. 2017; Aspeslagh et al. 2016). OX40 plays a critical role in both maintenance and effective reactivations in memory T cell; it is reported that numbers of memory cells are increased after administration of OX40 agonists in a TRAF2-dependent manner in an antigen-specific mouse model. It is also important for adhesion of activated T cells to endothelium and their subsequent transmigration by OX40-OX40L interaction; this function could be inhibited in cultured vascular endothelial cells via blockade of OX40L. OX40-L interactions upregulate a number of molecules implicated in migration: CXCR5, CXCR4, and RANTES/CCL5. In animals, there is evidence that OX40-deficient T cells may be impaired from reaching sites of inflammation in addition to their reduced effector function (Curti et al. 2013).

4.2.3.2 Preclinical and Early Clinical Data

As previously mentioned, OX40 could be recognized as a therapeutic target and it has been investigated in several preclinical tumor models using either anti-OX40 mAbs

or OX40L-Fc fusion proteins (Sugamura et al. 2004). OX40 therapy has demonstrated its potency in suppressing tumor growth in immunogenic models. Till now, there are lots of OX40 modulators in development, including monoclonal antibodies (e.g., MEDI6469, GSK3174998, INCAGN01949, MEDI0562, BMS-986178, and KY-B602) and bispecific antibodies co-targeting PD-L1 (MEDI1109) or CTLA-4 (ATOR-1015). Besides those, OX40L-Fc fusion proteins and oncolytic viruses armed with OX40L (DNX-2440) are also under exploration. In a phase 1 study using OX40 agonistic antibody (9B12, a murine IgG antibody) monotherapy in patients with advanced solid malignancies (NCT01644968), 40% subjects (12/30) observed tumor regression of at least one metastatic lesion with only one cycle of treatment, and no severe drug-related AE were reported. Currently, other agonistic OX40 antibodies (e.g., MOXR0916, PF-04518600, and MEDI6383) are under evaluating in several phase 1/2 clinical trials either as monotherapy or in combination with other IO agents (e.g., anti-PD-1/PD-L1, anti-CTLA-4, and anti-4-1BB) (Linch et al. 2015; Turner et al. 2001; Infante et al. 2016; Hamid et al. 2016).

Moreover, it is clearly demonstrated additional benefits in combo regimens of OX40 agonists and IO agents to cancer patients. In terms of this, lots of trials incorporating multiple complementary interventions are under development (Linch and Redmond 2014). Novel clinical settings and combinations are also being explored: MEDI0562 in the neoadjuvant setting of SCCHN and melanoma (NCT03336606); MEDI6469 in combination with radiotherapy in breast cancer (NCT01862900) and with radiotherapy plus cyclophosphamide in prostate cancer (NCT01303705).

4.3 Inhibitory Targets

4.3.1 VISTA (B7-H5)

4.3.1.1 Mechanism of Action

VISTA (V-domain Ig-containing Suppressor of T cell Activation), gene Vsir, RIKEN cDNA 4632428N05, also known as c10orf54, PD-1H, DD1α, Gi24, Dies1, and SISP1, is a member of the B7 family of negative checkpoint regulators and represents a new target for immunotherapy. There are 930 base pairs in the murine Vsir gene transcript, and it could be translated into a type I transmembrane protein with 309 aa. There is a 136 aa containing single IgV extracellular domain in murine VISTA protein; it is linked to a 23-aa stalk region, a 21-residue transmembrane segment, and a 97-aa cytoplasmic domain that does not contain ITAM, ITIM, or ITSM motifs (Wang et al. 2011). Phylogenetic analysis of the full VISTA molecule shares similarities with PD-1, CD28, and CTLA-4, with the highest identity with PD-1. Besides these similarities, there are some different characters of VISTA IgV domain from the B7 family ligands and receptors, including the most significant differences of the three additional cysteines (Cys44, Cys83, and Cys144) within the

IgV domain, one cysteine (Cys177) in the stalk region, and the insertion of a loop (IRNFTLQHLQHHGSHLKAN) between the C″ and D strands. These features are highly conservative among VISTA orthologues but absent in all other B7 superfamily members. Moreover, unlike VISTA has a conserved Src homology 2 (SH2)-binding (YxxQ, potentially capable of binding STAT proteins) motif in the middle of the cytoplasmic tail and three C-terminal SH3-binding domains (PxxP, two in CD28 and one in CTLA-4) without a classic ITIM/ITAM motif, distinguish from other B7 molecular family members. Whether these motifs within the VISTA tail actually recruit SH2/SH3 domain adapter proteins remains to be confirmed (Xu et al. 2018; Nowak et al. 2017). Taken together, these data suggest that VISTA may act as both a ligand and receptor in regulating immune responses.

VISTA is found the highest expression in myeloid cells, including expression on macrophages, conventional DCs, monocytes, and circulating neutrophils. Among conventional T cells, VISTA expression is highest in naïve cells and FoxP3$^+$ Tregs, and Memory CD4$^+$ T cells also express VISTA in some extent. Meanwhile, the expression of VISTA is relatively low in CD8$^+$ T cells and NK cells, while B cells do not express VISTA at all. This unique surface expression pattern suggests that VISTA may function to restrict T cell immunity at different stages compared to PD-1/PD-L1 and CTLA-4 axes. Similar to the murine homologue, the human VISTA gene is predominantly expressed in hematopoietic cell lineages and in tissues rich in infiltrating leukocytes. In human PBMC, VISTA is expressed on CD4$^+$ and CD8$^+$ T cells, myeloid CD11c$^+$ DCs, neutrophils, and CD14$^+$ monocytes. VISTA is not expressed on CD56^{Hi} NK cells (Xu et al. 2018; Mercier et al. 2014; Lines et al. 2014a, b).

Based on its expression, VISTA has been demonstrated to exert both ligand and receptor functions. First, VISTA can function as a ligand to negatively regulate T cell activation. In vitro, VISTA-Ig suppressed the production of IL-2 and IFN-γ in both CD4$^+$ naïve and memory T cells as well as in CD8$^+$ T cells. VISTA-Ig fusion protein also promotes in vitro conversion of naïve CD4$^+$ T cells to Tregs in both mouse and human. Second, it has been demonstrated that VISTA function as a negative receptor on T cells. Comparing with WT CD4$^+$ T cells, VISTA-/- CD4$^+$ T cells respond more vigorously to both polyclonal and antigen-specific stimulations, which shows increased proliferation and production of IFN-γ, TNF-α, and IL-17A. Transfection of monocytes from healthy donors to overexpress VISTA led to the spontaneous secretion of inflammatory cytokines IL-8, IL-1β, IL-6, TNF-α, and IL-10 (Xu et al. 2018; Mercier et al. 2014; Lines et al. 2014a, b).

4.3.1.2 Preclinical and Early Clinical Data

VISTA expression is upregulated in TME and dominant in shaping antitumor immunity in several in vivo models. VISTA expression is specifically upregulated on myeloid DCs and MDSCs, and enriched on tumor-infiltrating Tregs compared to periphery, indicating that tumors with infiltrating immune cells may harbor abundant

levels of VISTA available for targeting therapy. It has been reported that tumor regression has been observed when given anti-VISTA monotherapy in several preclinical melanoma models. Anti-VISTA could not only enhance T cell response within TME, but also lead to increased accumulation, proliferation, and IFN-γ and TNF-α production. Besides, natural Treg-mediated suppression of T cells was reduced, and tumor-induced differentiation of Tregs was also diminished when blocked VISTA expression. Finally, anti-VISTA suppressed tumor-infiltrating MDSCs in the B16OVA and PTEN/BRAF melanoma models (Mercier et al. 2014; Liu et al. 2015).

Two molecules are being tested on early-phase clinical trials: JNJ-61610588 and CA-170. JNJ-61610588 is a fully human IgG1 kappa anti-VISTA monoclonal antibody, also the first-in-human developed in clinical studies. NCT02671955 is a phase 1 PK/PD study in advanced malignancy patients; safety and activity signals also were observed in this trial currently. Other clinical studies combinations with IO agents are also under planning (Xu et al. 2018). CA-170 is a first-in-class oral, small-molecule antagonist that selectively targets PD-L1 (EC50: 17 nM) and VISTA (EC50:37 nM). In preclinical toxicology studies, CA-170 appeared to be safe when administered at multiple dose levels using a once-daily oral dosing schedule. Now CA-170 is investigated in a phase 1 study in patients with advanced solid tumors and lymphomas (NCT02812875) to demonstrate its PK/PD profile and safety (Lee et al. 2017).

4.3.2 LAG-3

4.3.2.1 Mechanism of Action

LAG-3 (also called lymphocyte-activation gene 3, CD 223) is a surface molecule located closely to CD4 but sharing less than 20% homology at the amino acid level, which belongs to an immunoglobulin superfamily member composed of four extracellular Ig-like domains and a type I transmembrane domain and hence structurally resembles the CD4 co-receptor. Similar to CD4, LAG-3 binds to MHC-II on APCs with a much stronger affinity, directly hindering TCR signaling in immune response (Workman et al. 2002; Huard et al. 1995). It is expressed on activated $CD4^+$ and $CD8^+$ effector T cells, $CD4^+Foxp3^+$ Treg, Tr1 cells, B cells, pDCs, and a subset of NK cells. Cross-linking of LAG-3 and CD3 can impair T cell proliferation and cytokine secretion by inhibiting calcium ion fluxes. Besides MHC-II, LAG-3 also binds to additional two proteins, LSECtin and galectin-3. LSECtin belongs to DC-sign family; it is dominantly expressed in the liver and on tumor cells; galectin-3 is a soluble lectin; unlike LSECtin it is expressed in a wide spectrum of cell types including tumor cells; it could interact with LAG-3 serves to broaden LAG-3's immune-regulatory impacts on tumor-infiltrating $CD8^+$ T cells within TME. The cytoplasmic tail of LAG-3 is quite different from other immune checkpoints, of which has three conserved domains: the first domain plays as a potential serine phosphorylation site; the second is KIEELE motif that is important in modulating $CD4^+$ T cell function; and

the last one is glutamic acid–proline (EP) repeat, binding to LAG-3-associated protein (LAP); hence, it could localize LAG-3. The cytoplasmic KIEELE motif mediates LAG-3 intrinsic signaling transition; via this method it prevents T cells entering the S-phase and results in suppressing T cell expansion consequently. However, which protein/domain plays the role of the intracellular binding partners of KIEELE motif is still unknown.

As an MHC-II ligand, LAG-3/MHC-II interaction negatively modulates $CD4^+$ T cells expansion and suppresses cytokine response like the way of CTLA-4 (Huard et al. 1995). LAG-3 has strong affinity with MHC-II so that it substantially upregulates in inflammatory conditions. IL-2, IL-7, and IL-12 could stimulate the expression level of LAG-3 on human-activated $CD4^+$ T cells, whereas IL-4, IL-6, TNF-α, and TNF-β do not have such functions. In particular, IL-12 is recognized as the most robust IFN-γ inducer; once given IL-12 stimulation, it will result in LAG-3 significant expression and elevate the numbers and frequency of LAG-3 positive T cells and NK cells. Moreover, LAG-3 expression is also mediated by the zinc-dependent a disintegrin and metalloproteinase (ADAM) through TCR signaling-dependent mechanisms (Workman et al. 2002; Huard et al. 1995; Xu et al. 2014; Goldberg and Drake 2011; Andrews et al. 2017). Besides, in tumor microenvironment it has reported that LAG-3 and PD-1 are commonly co-expressed and upregulated on TILs, which leads to immune exhaustion and tumor growth consequently. Thus, LAG-3 blockade not only improves antitumor immune responses but also potentiates other forms of immunotherapy given its different mechanisms of action mainly mediated by impeding cell cycle progression (Woo et al. 2012). Inactivated $CD8^+$ T cells express very low level of LAG-3, while LAG-3 expression remarkably elevates in response to antigenic stimulus. On $CD8^+$ T cells, LAG-3 expression is induced by T cell activation and, like in $CD4^+$ T cells, blockade of LAG-3 improves CTL proliferation and effector function. Importantly, LAG-3 is also highly expressed on exhausted $CD8^+$ T cells in both chronic viral infections and cancer, such as ovarian cancer, HCC, RCC, and other solid tumors. In most recently, it is reported that there may be another two additional LAG-3 ligands in the tumor microenvironment to function as supplement of regulating $CD8^+$ T cells—galectin-3 and LSECtin, which could effectively block the ADCC of $CD8^+$ T cells via LAG-3 for tumor immunity privilege. Furthermore, LAG-3 has been identified that it could concurrently express with multiple co-inhibitory immune checkpoints especially PD-1 by $CD8^+$ T cells, which could be recognized as biomarker of dysfunction of $CD8^+$ TILs. In another way, it has been reported that LAG3 is highly expressed in regulatory IL-10 producing Tr1 cells and $Foxp3^+$ Tregs, and LAG-3 has been confirmed to identify IL-10 producing Tr1 cells in both mice and humans together with CD49b. In Tregs, loss of LAG-3 reduced the suppressive function of Tregs, while forced expression of LAG-3 conferred effector T cells with suppressive capacity. Furthermore, LAG-3 cross-linking of MHC-II on DCs was shown to inhibit DCs differentiating and thus suppress the priming of effector T cell responses. As aberrant LAG-3 expression has been found in a broad spectrum of human tumors such as melanoma, NSCLC, CRC, breast cancer, HCC, HNSCC, etc., which is significantly associated with aggressive tumor progression and clinicopathological characteristics (Long et al. 2018).

4.3.2.2 Preclinical and Early Clinical Data

More and more evidence shows that LAG-3 has the remarkable synergistic effect with PD-1/PD-L1, which leads to immune suppression and conjointly abrogate autoimmune disease and enhance tumor-induced immune escape. This striking synergy phenomenon has been reported in melanoma, fibrosarcoma, and CRC animal models, and dual blockade of LAG-3 and PD-1 could effectively lead to remission of most established tumors, which are commonly resistant to single-agent treatment. Genetic knockout of LAG-3 and PD-1 can suppress tumor growth and prolong the lives of xenograft mice (Sharma and Allison 2015). Interestingly, it also reported that LAG-3 and PD-1 are synergized to attenuate $CD8^+$ T cell effector function in a murine ovarian cancer model. A recent study in human NSCLC revealed that both LAG-3 and PD-L1 higher expressions could predict the poorer prognosis of patients, and overexpression of LAG-3 on TILs significant correlates with PD-1/PDL1 expression. Overall, based on these preclinical data, demonstrating a synergistic effect between LAG-3 and PD-1 provides the backbone for combination regimen strategy. What is more, in light of the interaction between LAG-3 and other immune checkpoints, targeting LAG-3 along with other IO agents such as PD-1/PD-L1 and CTLA-4 has potent possibility in cancer immunotherapy (Nguyen and Ohashi 2015). Currently, two inhibitory approaches have been developed: a LAG-3-Ig fusion protein (IMP321, Immuntep®) and mAbs targeting LAG-3.

IMP321 is a soluble form of LAG-3; it does not function as immune suppressive way, of which could upregulate co-stimulatory molecules and increase IL-12 production to enhance tumor immune responses. Till now, there are two phase 1 studies ongoing using IMP321 monotherapy, one in advanced RCC and another in advanced pancreatic adenocarcinoma. In both trials, it showed an increasing in tumor-reactive T cells, but no clinical meaningful response was observed. In another phase 1 clinical trial using IMP321 in combination with paclitaxel in metastatic breast cancer patients, preliminary data showed an objective response rate of 50%. This promising result has prompted a phase 2b clinical trial that is currently recruiting patients with metastatic BC (NCT02614833) (Brignone et al. 2009; Duhoux et al. 2017).

Antagonistic mAbs interferes with the LAG-3 interaction between MCH-II molecules expressed by tumor and/or immune cells. Relatlimab (BMS-986016), an anti-LAG-3 conducting in a phase 1 study in melanoma patients to determine the PK/PD profile and safety, with and without nivolumab in various ranges of dosages (NCT01968109). Interim analysis results show promising efficacy with an ORR of 16% and DCR of 45% among patients who had progressed despite previous therapy with IO (Ascierto et al. 2017). And more impressively is that add-on Relatlimab didn't show additional toxicity, the safety profile is similar to nivolumab alone. LAG525 is another anti-LAG-3 mAb being studied on a phase 1/2 clinical trial with metastatic solid malignancies (NCT02460224), and currently no data are available.

4.3.3 TIM-3

4.3.3.1 Mechanism of Action

T cell immunoglobulin mucin-3 (TIM-3), also known as HAVCR2, belongs to the TIM gene family. It was first discovered in 2001, which plays a critical role in immune regulation. The Tim gene family comprises eight members (TIM-1 to 8) on mouse chromosome 11B1.1 and three members include TIM-1, TIM-3, and TIM-4 and is located on human chromosome 5q33.2 (McIntire et al. 2001; Freeman et al. 2010). TIM-3 protein consists of 281 amino acids. The human TIM family is conserved with type-1 membrane proteins, which share a similar structure, including a variable immunoglobulin domain (IgV), a glycosylated mucin domain of variable length in the extracellular region, and a single transmembrane domain. Except for TIM-4, all the other TIM molecules contain a C-terminal cytoplasmic tail with a conserved tyrosine-based signal motif. Most TIM IgV regions contain four cysteines, which form two disulfide bridges that contribute to the formation of a unique binding surface; however, there are six cysteines within the IgV domain of TIM-3; the CC' loop is reoriented closer to the FG loop and a unique binding pocket (FG-CC0 cleft) is created, which is required for interactions with its ligands (Anderson et al. 2007).

TIM-3 is originally identified as a specific marker for Th1 and Tc1 cells, and its expression is regulated by the Th1 transcription factor T-bet together with another transcription factor NFIL3. It is further expressed on many types of immune cells, including T cells, DCs, macrophages, NK cells, cancer stem cells, and so on. TIM-3 is also expressed on Th1, Th17, and $CD8^+$ T cells—cells of myeloid lineages in mice. Suppression of Th1 and Th17 responses by TIM-3 and its ligands interaction could induce peripheral immune tolerance, indicating an inhibitory role of TIM-3 in T cell-mediated immune responses. In chronic infection, TIM-3 expression is also one of the characteristics for exhausted T cells (Das et al. 2017). So far, there are four relevant ligands shown to interact with the IgV domain of TIM-3, including galectin-9 (Gal-9) (Zhu et al. 2015), carcinoembryonic antigen cell adhesion molecule 1 (Ceacam-1) (Huang et al. 2015), phosphatidylserine (PtdSer), and high mobility group protein B1 (HMGB1) (Chiba et al. 2012). Gal-9 binds to the N-linked sugar moieties in the TIM-3 IgV domain, and this interaction triggers cell death in Th1 and Tc1 cells; it is important to note that glycosylation of the IgV is required for Gal-9 binding; Ceacam-1 is co-expressed with TIM-3 on T cells, and Ceacam-1-TIM-3 interaction is mandatory for the inhibitory function of TIM-3. In that way, this engagement could suppress T cell function like proliferation and cytokine secretion. Although Ceacam-1 and Gal-9 bind to different regions on the IgV domain of TIM-3, the same two tyrosine residues were phosphorylated by Gal-9 and Ceacam-1 ligands and were mandatory for functional activity of TIM-3; PtdSer is a molecule exposed on the surfaces of apoptotic cells, which was shown to bind to a pocket within the IgV domain of TIM-1, TIM-3, and TIM-4 (Cheng and Ruan 2015). This interaction of PtdSer and TIM-3 facilitates the clearance of apoptotic bodies and also promotes the cross-presentation of antigens by DCs; TIM-3 binding to HMGB1 could inhibit the

transport of nucleic acids to endosomes, so that it interferes with nucleic acid-sensing and danger signaling pathways in DCs consequently; in this way, this interaction promotes the negative function of conventional T cells. Whether interactions of TIM-3 and PtdSer or HMGB1 take place in T cells and whether such contacts have functional consequences is still unknown (Chiba et al. 2012).

TIM-3 was originally identified as a receptor expressed on Th1 and Tc1 cells, where it acts as a negative regulator of type 1 immunity (McIntire et al. 2001; Han et al. 2013). TIM-3 blocking antibodies were shown to exacerbate experimental autoimmune encephalomyelitis (EAE); in contrast, activation of TIM-3 by administration of Gal-9 dampened Th1 responses through induction of cell death in TIM-3$^+$ Th1 cells and ameliorated EAE. TIM-3 also plays an important role in the induction of T cell tolerance, and loss of TIM-3 abrogates the induction of antigen-specific tolerance. TIM-3 inhibited antitumor immunity by mediating T cell exhaustion. Stat5 and p38 signaling pathway would be attuned by TIM-3$^+$ CD8$^+$ T cells. Blocking the TIM-3 pathway could enhance tumor immunity and increased the production of IFN-γ in T cells (Das et al. 2017; Han et al. 2013). It has been reported that the expression of CD8$^+$ TIM-3$^+$ T cells was correlated with PD-1 expression both in vivo and in vitro (Ngiow et al. 2011). So far as we knew, TIM-3 was constitutively expressed on innate immune cells and was confirmed to suppress innate antitumor immunity. TIM-3 inhibited the convention T cells proliferation and effector of cytokine production, such as IL-2. PD-1 and TIM-3 positive CD8$^+$ T cells produced less IFN-γ than TIM-3 negative CD8$^+$ T cells. Under steady-state conditions, TIM-3 is barely expressed on Foxp3$^+$ Tregs. However, it is important to note that a substantial proportion of CD4$^+$TIM-3$^+$ TIL is Foxp3$^+$, suggesting a role for TIM-3 in Treg within TME. Furthermore, multiple suppression function-related molecules such as CTLA-4, PD-1, and LAG-3 are up-expression in TIM-3 expressing Tregs; what is more, the level of suppressive cytokines such as IL-10 and TGF-β was also higher in this subset of Treg as a result. More importantly, tumor-resident TIM-3$^+$ Tregs may play a role in impairing effector T cell function observed in TILs, as their depletion restores functionality to effector T cells. TIM-3 is also found to be updated on CD4$^+$ T cells in patients with chronic infection and cancer. It is possible that TIM-3 is an exhaustion marker for Th1 cells. It is important to note that a substantial proportion of CD4$^+$TIM-3$^+$ TIL is Foxp3$^+$, suggesting a role for TIM-3 in Treg within TME (Sakuishi et al. 2013). Lastly, TIM-3 is highly expressed on mature human NK cells and is variably expressed on immature NK cells. TIM-3 marks NK cells with greater effector function, including cytokine production (e.g., IFN-γ) and cytotoxicity. However, cross-linking of TIM-3 inhibits NK cell-mediated cytotoxicity, suggesting that interaction of TIM-3 with one or more of its ligands negatively regulates NK cell activity.

4.3.3.2 Preclinical and Early Clinical Data

In preclinical research, TIM-3 inhibitors share the similar biological function with PD-1 inhibitors. It was reported that PD-1 antibodies might lead to an increase in

TIM-3 expression in in vivo models of lung cancer, indicating that TIM-3 might be a negative feedback loop of PD-1 blocking antibody to induce drug resistance. In HCC tissues, TIM-3, PD-1, and LAG-3 were also found upregulated on tumor-associated antigen-specific T cells. PD-1, TIM-3, or LAG-3 inhibitors had a synergistic function that enhances T cells' response to tumor antigens. The combination of TIM-3 inhibitor with PD-1 inhibitor could be more effective than single agent of TIM-3 or PD-1 alone (Das et al. 2017; Ngiow et al. 2011).

In preclinical models, given TIM-3 mAbs has produced variable antitumor effects. TIM-3 has been identified upregulated in TIL in mouse models such as CT26 colon adenocarcinoma, 4T1 mammary adenocarcinoma, and B16F10 melanoma. Another study showed that TIM-3 mAbs could suppress tumor growth and delay the progression in various mouse tumor models, including MC38 colon carcinoma, WT3 sarcoma, CT26 colon adenocarcinoma, and TRAMP-C1 prostate tumor. Furthermore, combo regimens of anti-TIM-3 with either anti-CTLA-4 or anti-PD-1 could enhance the antitumor effects to a great extent. These studies have established that anti-TIM-3 could be a new approach for cancer immunotherapy. In addition, combo regimens of anti-TIM-3 with anti-CTLA-4 and/or anti-PD-1 have great potential in improvement of the current immunotherapeutic approaches to cancer (Das et al. 2017).

Till now, three TIM-3 antagonistic monoclonal antibodies are under exploration in early-phase clinical development (MBG453, TSR-022, and LY3321367). TSG-022 (Tesaro, Waltham, USA) is currently evaluated as monotherapy in a phase 1 trial in patients with advanced solid malignancies (NCT02817633). Furthermore, the part 2 of the study will look into the safety and clinical activity of TSR-022 alone or combining with anti-PD-1 in patients with select tumor types. MGB453 (Novartis, Basel, Switzerland) is evaluated in a phase 1-1b/2 open-label multicenter study as monotherapy (PK/PD, safety and efficacy) and in combination with PDR001 (novel anti-PD-1 antibody) in patients with advanced malignancies (NCT02608268). LY3321367 (Eli Lilly, Indianapolis, USA) started a phase 1 study of LY3321367 alone or combined with anti-PD-L1 in advanced solid tumors who was current no available therapies (NCT03099109). All trials are currently recruiting patients and no data released (Lee et al. 2017; Das et al. 2017).

4.3.4 TIGIT

4.3.4.1 Mechanism of Action

T cell immunoglobulin and ITIM domain (TIGIT), also known as VSig9, Vstm3, or WUCAM, was first identified by bioinformatic algorithm at 2009 as a novel member of the CD28 family. TIGIT has the similar structure with the larger poliovirus receptor (PVR)/nectin family of molecules, which is highly conserved between mouse and human including an extracellular IgV domain, a type 1 transmembrane region, and a cytoplasmic tail containing an ITIM and an immunoglobulin tail tyrosine (ITT)-like

motif (Yu et al. 2009). The PVR/nectin family comprises PVR (CD155, NECL-5, TAGE-4), PVRL2 (CD112, Nectin-2), CD112R (PVR-related immunoglobulin domain containing, PVRIG), DNAX accessory molecule-1 (DNAM-1, CD226), CD96 (Tactile), and PVRL3 (Nectin-3, PPR3, CD113), whereas PVR binds with high affinity to TIGIT, PVRL2, and PVRL3 have been described as low-affinity binding partners in an artificial cell line model (Stanietsky et al. 2009; Stamm et al. 2018; Pauken and Wherry 2014; Lozano et al. 2012; Liu et al. 2012).

Similar with LAG-3 and TIM-3, TIGIT plays as a co-inhibitory receptor, and it is widely expressed on NK cells and T cells specifically those of a subset of regulatory T cells like activated, memory, and follicular T helper cells (Joller et al. 2011). TIGIT is part of a complex ligand/receptor network in which it binds with high affinity to PVR and weaker interacts with PVRL2. Both of these ligands are expressed on APCs and a variety of non-hematopoietic cell types including tumor cells and are shared with DNAM-1. DNAM-1 is expressed on monocytes, T cells, and NK cells (Stanietsky et al. 2009; Stamm et al. 2018). Unlike TIGIT's inhibitory effects on immune cells, DNAM-1 could enhance cytotoxicity of $CD8^+$ and NK cells; it could bind to PVR and likely provide positive co-stimulation to induce IFN-γ production. DNAM-1's interactions with PVR and PVRL2 were found to enhance NK-mediated lysis of tumor cells. Conversely, TIGIT binding of PVR suppresses IFN-γ production leading to downregulation of NK cells. The phosphorylation of ITT-like domain of the cytoplasmic tail of TIGIT accounts for downregulation of NK cell activity. The phosphorylation site of this ITT-like domain is at Tyr225 and binds Grb2, recruiting SHP-1 to terminate PI3K, MAPK, and NF-κB signaling in the NK cell. NK cell maturation is dependent on the presence of the TIGIT receptor and essential for development of self-tolerance. Besides, CD96 also acts as a supplementary role to this ligand/receptor network, which belongs to the Ig gene superfamily and plays an important role as receptor allowing adhesive interactions of NK and T cells in immune response. It has been confirmed that CD96 has the similar immunosuppressant effects as TIGIT, binding to PVR with an affinity higher than DNAM-1 but weaker than TIGIT. It was proposed that TIGIT/DNAM-1/PVR/CD96 form a dynamic axis of inhibitory signals from TIGIT and CD96 opposing stimulatory signals from DNAM-1 (Blake et al. 2016).

As previously mentioned, TIGIT is highly expressed in regulatory cells, which could facilitate their suppressive function. TIGIT expression is correlating with IL-10 level in Tr1 cells ($CD4^+Foxp3^-IL\text{-}10^+$). TIGIT is also a direct target of Foxp3 and is expressed in a subset of predominantly natural $CD4^+Foxp3^+$ Tregs. TIGIT is also commonly co-expressed with other immunosuppressive gene signature including CTLA-4 and PD-1 in Tregs. Given the agonistic anti-TIGIT antibodies could induce the expression of fibrinogen-like protein 2 (FGL2) in activation of $TIGIT^+$ Treg cells. Neutralization of FGL2 could attenuate the suppressive function of $TIGIT^+$ Treg cell to the similar levels as $TIGIT^-$ Treg cells. In inflammatory tissues, FGL2 could induce the pro-inflammatory Th1 and Th17 cells to Th2 cells via modulating $TIGIT^+$ Treg cells function. $TIGIT^+$ Treg cell could express a spectrum of gene to inactivate $CD8^+$ T cells. It was proposed that upregulation of IL-10 production

by TIGIT$^+$ Tregs and CD8$^+$ T cells leads to the dysfunctional phenotype of CD8$^+$ cytotoxic TILs (Joller et al. 2014; Kurtulus et al. 2015).

Nevertheless, TIGIT engagement downregulates transcription of central components of the TCR signaling pathway as well as the TCR complex itself (e.g., TCRα, CD3ε), thereby inhibiting productive T cell activation. Except for its inhibitory effects on the TCR signaling pathway, TIGIT engagement promotes T cell survival via two mechanisms: the induction of anti-apoptotic molecules (e.g., Bcl-xL) and function as receptors for pro-survival cytokines such as IL-2, IL-7, and IL-15. TIGIT thus not only inhibits T cell activation but also promotes T cell survival and maintenance (Joller et al. 2011).

In preliminary, in vitro studies and animal models indicate a synergistic effect in immune cell proliferation, cytokine release, and reversal of T cell exhaustion with subsequent tumor rejection and induction of protective memory responses via both inhibit of TIGIT and PD-1 or TIM-3. More importantly, TIGIT appears to be enriched in TME than periphery, which indicates that anti-TIGIT would offer the advantage of a more targeted-directed therapy with less immune-related toxicities theoretically. Ultimately, TIGIT appears to function as limiting cytokine competency and CD8 T cell function which results as complementary effects when used with other forms of ICIs.

4.3.4.2 Preclinical and Early Clinical Data

Similar with previously mentioned, TIGIT expression is rarely found in the peripheral lymphoid organs of tumor-bearing mice but highly expressed in tumor tissue. It has been confirmed that TIGIT and PD-1 were upregulated in a 15-gene signature of multiple tumor-associated T cells, especially in colon, endometrial, breast, and renal clear cell carcinoma. Besides, in advanced melanoma patients, upregulation of TIGIT and downregulation of DNAM-1 was observed in CD8$^+$ TILs and most of these cells co-expressed PD-1. Moreover, it was found that DNAM-1 expression was decreased on NK cells and CD8$^+$ cells in the peripheral blood of these patients, which indicates potential inhibitory manners of DNAM-1 and TIGIT in tumor suppression (Chauvin et al. 2015). PVR and PVRL2 were also found to be strongly expressed on tumor cells in patients with cutaneous T cell lymphoma along with TIGIT. Interestingly, it seems that TIGIT's role in TME might correlate with the microbiome to some extent. *Fusobacterium nucleatum*, a bacterium indigenous to the oral cavity which was considered participating in tumorigenesis, was found in humans to produce Fap2, a protein that directly interacts with TIGIT causing inhibition of NK cell cytotoxicity (Gur et al. 2015). We already knew that TIGIT has the synergistic effect with both PD-1 and TIM-3 in impairing protective antitumor responses. Therefore, dual blockade of either TIGIT with PD-1 or with TIM-3 might enhance antitumor potency and induce tumor regression.

In preclinical trials, anti-TIGIT candidate drug OMP-313M32 demonstrated a statistically significant reduction of tumor volume in human melanoma PDX in humanized NSG mice. It has reported that plus the PVRIG inhibitor COM701 together with

dual blockade of PD-1 and TIGIT could result in increasing effector $CD8^+$ T cell activation in an in vivo model, which led to tumor growth suppression and prolonged survival. OMP-313R12, another novel TIGIT antibody, was found to induce tumor growth suppression in a murine CRC model (CT26 WT). It was also confirmed that combination of OMP-313R12 and anti-PD-L1 could significantly improve overall survival in mice models as compared to controls (Solomon and Garrido-Laguna 2018).

BMS-986207 (Bristol-Myers Squibb), an anti-TIGIT mAb initiated a phase 1/2 clinical studies combining with Nivolumab in about 170 malignancy patients (NCT02913313); MTIG7192A (Genentech) is another anti-TIGIT mAb that combines with Atezolizumab in phase 1 trial enrolling 300 patients (NCT02794571). No early clinical data are available currently (Solomon and Garrido-Laguna 2018).

4.4 Conclusion

Significant advances have been made in cancer immunotherapy in the last decade. Taken altogether, we found that most of these novel immune checkpoints have such new characteristics other than "first generation" ICIs, e.g., PD-1, PD-L1, CTLA-4: (1) not only negative impact on immune system, but also function as stimulators, even dual modulator; (2) participate in full perspective of innate immunity and humoral immunity; (3) involve in tumor microenvironment and have complementary function with current I-O therapy via mechanisms on immune cells. Expanding clinical benefit to the majority of patients and preventing drug resistance still require a deeper understanding of the mechanisms. The discovery of new immune inhibitory, stimulatory pathways, and rational combination strategies would shed the lights to the future of I-O therapy.

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Chapter 5
Mechanisms of Resistance to Checkpoint Blockade Therapy

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Abstract Immune checkpoint blockades (ICBs), as a major breakthrough in cancer immunotherapy, target CTLA-4 and the PD-1/PD-L1 axis and reinvigorate antitumor activities by disrupting co-inhibitory T-cell signaling. With unprecedented performance in clinical trials, ICBs have been approved by FDA for the treatment of malignancies such as melanoma, non-small-cell lung cancer, colorectal cancer, and hepatocellular carcinoma. However, while ICBs are revolutionizing therapeutic algorithms for cancers, the frequently observed innate, adaptive or acquired drug resistance remains an inevitable obstacle to a durable antitumor activity, thus leading to non-response or tumor relapse. Researches have shown that resistance could occur at each stage of the tumor's immune responses. From the current understanding, the molecular mechanisms for the resistance of ICB can be categorized into the following aspects: 1. Tumor-derived mechanism, 2. T cell-based mechanism, and 3. Tumor microenvironment-determined resistance. In order to overcome resistance, potential therapeutic strategies include enhancing antigen procession and presentation, reinforcing the activity and infiltration of T cells, and destroying immunosuppression microenvironment. In future, determining the driving factors behind ICB resistance by tools of precision medicine may maximize clinical benefits from ICBs. Moreover, efforts in individualized dosing, intermittent administration and/or combinatory regimens have opened new directions for overcoming ICB resistance.

Keywords PD-1 · PD-L1 · CTLA-4 · Resistance · Precision medicine

5.1 Introduction

In recent years, medical oncology has undergone a dramatic transformation with the advent of immunotherapy. Immune checkpoint blockades (ICBs), as a major breakthrough in cancer immunotherapy, target CTLA-4 and the PD-1/PD-L1 axis and

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reinvigorate anti-tumor activities by disrupting co-inhibitory T-cell signaling (Brahmer et al. 2012). Since the first US Food and Drug Administration (FDA) approval of ICB (ipilimumab for the treatment of melanoma) in 2011, monotherapy and combinatory regimens of ICBs have been proved as effective and powerful modalities across types of cancers. While ICB has exerted a response rate as high as 60% in selected subsets of patients (Larkin et al. 2015; Robert et al. 2015), more patients remain non-responsive (innate/intrinsic resistance) (Sharma et al. 2017). In responders, adaptive resistance may occur shortly after an initial clinical response, which result from the phenotype changes in cancer cells and/or in the tumor microenvironment (TME) from sensitive to resistant (Sharma et al. 2017; Ribas 2015). Moreover, late relapses were also observed in some responders after a period of response as a result of acquired resistance.

As is shown, drug resistance is one of the major barriers that prevent a larger-scale of patients from benefiting. From the current understanding, the molecular mechanisms for the resistance of ICB include tumor-derived mechanism, T cell-based mechanism and TME-determined mechanism. In this chapter, we summarized the current understanding of molecular mechanisms for the resistance of ICB, hoping to outline potential strategies to overcome the resistance, to improve treatment efficiency and response rates, and to guide the optimization of ICB regimens in cancer patients for better outcomes.

5.2 Current Status of Immune Checkpoint Blockade Therapy in Clinical Practice

5.2.1 Clinical Application of FDA Approved Checkpoint Blockades

CTLA-4, a co-inhibitory receptor upregulated early in the process of T cell activation, was the first described negative regulator of T cell activation in1987 (Brunet et al. 1987; Walunas et al. 1994). Another checkpoint receptor PD-1 expressed by activated T cells was cloned in 1992 (Ishida et al. 1992), and its cell surface ligand PD-L1 was subsequently characterized (Freeman et al. 2000; Dong et al. 1999). The interaction between PD-1 and PD-L1 negatively regulates the effector phase of T-cell responses (Blank et al. 2004), leading to the down-regulation of immune system and enhanced self-tolerance. Immune checkpoint inhibitors that block the PD-1/PD-L1 pathway significantly intensify the function of T-cells (Brahmer et al. 2012), and therefor exert antitumor activity. In 2011, ipilimumab, a monoclonal antibody that target CTLA-4 to activate the immune responses (Lipson and Drake 2011), became the first immune checkpoint inhibitor approved by U.S. Food and Drug Administration (FDA) after decades of bench-side researches. Later in 2014, PD-1 inhibitors nivolumab and pembrolizumab were approved for the treatment of melanoma and the indications have been expanded in a broader spectrum of tumors (Fares et al. 2019).

To date, a total of seven ICB agents have received FDA approval, including one CTLA-4 blockade (ipilimumab), three PD-1 blockades (nivolumab, pembrolizumab, and cemiplimab), and three PD-L1 blockades (atezolizumab, avelumab, and durvalumab) (Fares et al. 2019). These agents have proved their efficacies across various types of tumors including melanoma (Robert et al. 2015; Weber et al. 2015; Hamid et al. 2013), non-small-cell lung cancer (NSCLC) (Nishio et al. 2017), and Hodgkin's lymphoma (Maruyama et al. 2017).

i. *CTLA-4 blockade*

Ipilimumab (marketed as Yervoy®) is the first and only CTLA-4 blockade approved by FDA by now (Hargadon et al. 2018; Hodi et al. 2010; Robert et al. 2011). In 2010, unexpected results came from a phase 3 trial of the GP100 peptide vaccine with ipilimumab, that the patients with unresectable stage III/IV melanoma treated with ipilimumab exhibited prolonged survival over those treated with the peptide vaccine alone or with the combination of vaccine and ipilimumab (Hodi et al. 2010). Following the outcomes, ipilimumab were initially approved for the treatment of unresectable or metastatic melanoma. Additionally, pooled data from phase 2 and 3 clinical trials showed that ipilimumab treatment in advanced-melanoma patients resulted in a 22% 3-year survival rate and durable clinical responses that lasted beyond 10 years (Schadendorf et al. 2015). In 2015, the FDA approved expanded indications for ipilimumab, allowing it to be used as adjuvant therapy for patients with stage III melanoma, to lower the risk of disease relapse following surgery, and for the treatment of patients with BRAF V600 wild-type unresectable or metastatic melanoma as a combinatory regimen with nivolumab. In addition to melanoma, ipilimumab has also been investigated as monotherapy or combination therapy in other cancer types, including renal cell carcinoma (Motzer et al. 2018; Cella et al. 2019), colorectal cancer (Overman et al. 2018), NSCLC (Hellmann et al. 2017; Govindan et al. 2017), prostate cancer (Beer et al. 2017), and others (Topalian et al. 2015). To date, the approved indications of ipilimumab include advanced melanoma, post-surgical cutaneous melanoma with positive regional lymph nodes as monotherapy; and intermediate or poor-risk, previously untreated advanced renal cell carcinoma and previously treated microsatellite instability-high/deficient mismatch repair (MSI-H/dMMR) metastatic colorectal cancer in combination with nivolumab (Squibb and Sons 2018).

Tremelimumab is the other ICB that blocks CTLA-4 checkpoint pathways. It is a fully human IgG2 anti-CTLA-4 monoclonal antibody marketed by AstraZeneca (Sadreddini et al. 2019; Ribas et al. 2013). Tremelimumab has not improved patient survival as monotherapy in any trials by now and it is yet approved for clinical utility. Still, it is being investigated with durvalumab or other agents as part of combinatorial regimens, and further results of ongoing trials are anticipated.

ii. *PD-1 blockade*

Nivolumab (marketed as Opdivo®) is a high-affinity human monoclonal immunoglobulin G4 antibody inhibitor of PD-1 (Hargadon et al. 2018; Hardy et al. 1997). It was the CheckMate-037 trial (NCT01721746) that laid the foundation for

the clinical application of nivolumab. This open-label, randomized phase 3 clinical trial reported improved objective response rates (ORRs) to nivolumab over investigator's choice chemotherapy in patients with advanced melanoma progressed after treatment of ipilimumab with/without a BRAF inhibitor (Weber et al. 2015). Base on the outcome of CheckMate-037 trial, nivolumab was approved by the FDA as the first PD-1 inhibitor for cancer in 2014. Subsequently, following a phase 3 trial (Checkmate-066, NCT01721772) that revealed improved ORR (40% versus 14%), progression-free survival (PFS) (5.1 months versus 2.2 months), and overall survival (OS) at 1 year (72.9% versus 42.1%) in patients receiving nivolumab compared to dacarbazine (Robert et al. 2015), nivolumab received approval as first-line therapy for previously untreated melanoma without a BRAF mutation.

Apart from melanoma, nivolumab has also exhibited therapeutic benefits against traditional therapies in a wide range of cancers (Sharma et al. 2017; El-Khoueiry et al. 2017; Overman et al. 2017). Notably, two independent phase 1/2 trials revealed a combined ORR of 65% of nivolumab in patients with classical Hodgkin lymphoma (Ansell et al. 2015; Younes et al. 2016), which led to its approval as the first ICB agent in the treatment of hematological malignancy. By now, the indication spectrum of nivolumab include first- and second-line therapies for metastatic melanoma, metastatic NSCLC, advanced renal cell carcinoma with prior anti-angiogenic therapy, advanced renal cell carcinoma, relapsed or progressed classical Hodgkin lymphoma, recurrent or metastatic head and neck squamous cell carcinomas (HNSCC), locally advanced or metastatic urothelial carcinoma, previously treated MSI-H/dMMR metastatic colorectal cancer as well as hepatocellular carcinoma, either as a single agent or in combination with ipilimumab (Brahmer et al. 2015; Borghaei et al. 2015; Motzer et al. 2015; Ferris et al. 2016; Squibb 2015). Furthermore, nivolumab has also been assessed in other cancer types. In a two-cohort phase 2 trial (UMIN000005714) of 20 patients with platinum-resistant ovarian cancer, nivolumab at a dose of 3 mg/kg revealed an ORR of 20%, and complete response was reached in two cases (Hamanishi et al. 2015).

Pembrolizumab (marketed as Keytruda®), previously known as lambrolizumab, is a highly selective, IgG4-kappa humanized isotype monoclonal antibody against PD-1 (Hamid et al. 2013). In 2014, pembrolizumab obtained accelerated approval as an alternative to nivolumab for the treatment of patients with unresectable or metastatic melanoma after prior ipilimumab with/without a BRAF inhibitor. Later in 2015, approved indication of pembrolizumab expanded to the first-line therapy for unresectable or metastatic melanoma based on outcomes from further trials (Ribas et al. 2015; Robert et al. 2015). The subsequent multicenter, randomized, open-label phase 3 trial (KEYNOTE-006) confirmed the durable survival benefits of pembrolizumab in advanced melanoma, with 2-year overall survival rates of 55% compared to that of 43% in ipilimumab group (Schachter et al. 2017). Moreover, the outcomes of KEYNOTE trial series resulted in accelerated or full approval of pembrolizumab in multiple cancer types such as classical Hodgkin lymphoma, HNSCC, urothelial carcinoma, and colorectal cancer. Currently, indications of pembrolizumab include melanoma, NSCLC, small cell lung cancer (SCLC), HNSCC, classical Hodgkin

lymphoma, primary mediastinal large B-cell lymphoma (PMBCL), urothelial carcinoma, gastric cancer, esophageal cancer, cervical cancer, hepatocellular carcinoma, Merkel cell carcinoma, renal cell carcinoma as well as endometrial carcinoma (Chen et al. 2017; Chow et al. 2016; Bellmunt et al. 2017; Fuchs et al. 2018; Administration UFaD 2016). Significantly, the pan-approval of pembrolizumab for the treatment of MSI-H/dMMR solid tumors marked it as the first anti-cancer agent that received tissue/site-agnostic approval based on biomarker statuses (Prasad et al. 2018).

iii. *PD-L1 blockade*

Atezolizumab (marked as Tecentriq®) is a human IgG1 monoclonal anti-PD-L1 antibody that contains an engineered Fc-domain to target PD-L1 (Festino et al. 2016). During the application of atezolizumab, the immune homeostasis is theoretically maintained because it does not blockade the interaction of PD-1 and its second ligand PD-L2 (Chen et al. 2012). A phase 1 trial (NCT01375842) of patients with locally advanced or metastatic solid tumors including melanoma, NSCLC, renal cell carcinoma, colorectal cancer and gastric cancer demonstrated durable responses and an acceptable safety profile, with the ORR and 24-week PFS in non-selected solid tumors as 21% and 44%, respectively (Herbst et al. 2013). In 2016, atezolizumab became the first approved PD-L1 blockade for cancer treatment, with accelerated approval for selected indications of urothelial carcinoma (Rosenberg et al. 2016) and full approval for similar indications of NSCLC (Hargadon et al. 2018). With better OS compared to that with docetaxel, atezolizumab has been proved superior to conventional chemotherapy in patients with previously treated NSCLC (Rittmeyer et al. 2017). Atezolizumab has also been evaluated in other tumor types, including metastatic melanoma (NCT01375842) (Hamid et al. 2013) and renal cell carcinoma (NCT01375842) (McDermott et al. 2016). By now, the approved indications of atezolizumab include urothelial carcinoma, NSCLC, SCLC, and triple-negative breast cancer (Administration UFaD 2017).

Avelumab (marked as Bavencio®), is a fully human IgG1 recombinant monoclonal antibody directed against PD-L1. Subsequent to durable ORR in phase 1/2 studies (Patel et al. 2018; Kaufman et al. 2016), avelumab received accelerated approved for the treatment of metastatic urothelial carcinoma after prior chemotherapy. In addition, avelumab has also been investigated in other cancer types such as recurrent/refractory ovarian cancer (NCT01772004) (Disis et al. 2016) and advanced NSCLC (NCT01772004) (Gulley et al. 2015). Currently, avelumab is indicated for the treatment of adult and pediatric patients 12 years and older with metastatic Merkel cell carcinoma, selected patients with locally advanced or metastatic urothelial carcinoma and as first-line treatment for advanced renal cell carcinoma in combination with axitinib (Administration UFaD 2017).

Likewise, durvalumab (marked as Imfinzi®) is a human IgG1- kappa monoclonal antibodies of PD-L1 with an engineered Fc domain. In 2017, durvalumab received accelerated approval as second-line treatment for progressive metastatic urothelial carcinoma. In 2018, according to the outcome of the phase 3 PACIFIC trial (Antonia et al. 2017), it further obtained the full approval for the treatment in the patients with

unresectable, stage III NSCLC whose disease has not progressed following concurrent platinum-based chemotherapy and radiation therapy (Administration UFaD 2018).

5.2.2 *Ongoing Trials and Clinical Responses of Checkpoint Blockades*

Currently, there are over ten ICBs in various stages of clinical testing in many different tumor types. Apart from the above, the efficacy and safety profiles of novel agents such as camrelizumab (Huang et al. 2019), pidilizumab (Fried et al. 2018), sintilimab (Ishizuka et al. 2019), BMS-936559 (MDX-1105) (Tykodi et al. 2012), and toripalimab (JS001) (Tang et al. 2019) are undergoing clinical trials. Camrelizumab (SHR-1210) is a selective, humanized, high-affinity immunoglobulin G4-kappa monoclonal antibody against PD-1 (Huang et al. 2019). The results of a large phase 1 clinical trial (NCT02742935) in nasopharyngeal carcinoma (Fang et al. 2018), esophageal carcinoma (Huang et al. 2018), and gastric cancer (Huang et al. 2019) revealed promising anti-tumor efficacy. Unlike a varied spectrum of adverse events with other ICBs, reactive capillary hemangiomas (RCHs) were dominantly observed in patients treated with camrelizumab, most of which could be well managed with supportive care. Another PD-1 inhibitor, pidilizumab (CT-011) has been tested in patients with metastatic melanoma (Atkins et al. 2014) and has entered a phase 1 ascending-dose trial for 17 patients with advanced hematologic malignancies (Berger et al. 2008).

To improve therapeutic efficacy, efforts have been made in seeking and determining novel immune targets, optimized dosage regimens and combinatory strategies of ICBs with chemotherapy, targeted therapy, radiation therapy, and other immunotherapeutic modalities. So far, while the most favorable prognosis has been seen with combination of CTLA-4 blockade and PD-1 blockade (Park et al. 2018), the pool of patients benefiting from ICBs remains relatively small. Possible reasons include tumor-intrinsic resistance, which occurs when cancer cells alter bioactivities that are related to immune recognition, cell signaling, gene expression, and DNA damage (Fares et al. 2019) and/or -extrinsic resistance, which is external to tumor cells throughout the T-cell activation. In the clinical application of ICBs, the patients who never responded to ICBs and those who relapsed after duration of responses may suffer from various adverse events while gaining little benefit in survival. Therefore, understanding and overcoming drug resistance is one of the biggest challenges and the urgent need in the field of ICBs.

5.3 Resistance Mechanisms to Immune Checkpoint Blockades in Cancer

Immune checkpoint therapy is more and more widely used in clinical application and curative effect has been obtained in various human cancers. However, drug resistance to agents targeting immune checkpoints is a prominent restriction for patients treated with immunotherapy. Many studies are ongoing to elucidate the functional mechanisms underlying resistance to ICBs. Meanwhile, researches have shown that resistance could occur at each stage of the tumor's immune responses. Herein, we summarized and discussed the resistance mechanisms in the aspect of tumor-derived resistance, T cell-based resistance and TME-determined resistance (Fig. 5.1).

5.3.1 Tumor-Derived Resistance

The genetic and epigenetic alternations of tumor cells are the innate and tractive force that drives the resistance to ICBs. More specifically, they prevent tumor cells from being recognized and killed by immune cells, and promote immune evasion, excessive growth, recurrence and metastasis of tumor cells when they are under the stress of ICBs.

i. *Absence of antigenic proteins on tumor cell surface*

Absence of antigenic proteins, such as cancer-testis antigens (CTAs), viral antigens (VAs), tumor specific antigens (TSAs) and tumor associated antigens (TAAs), is the

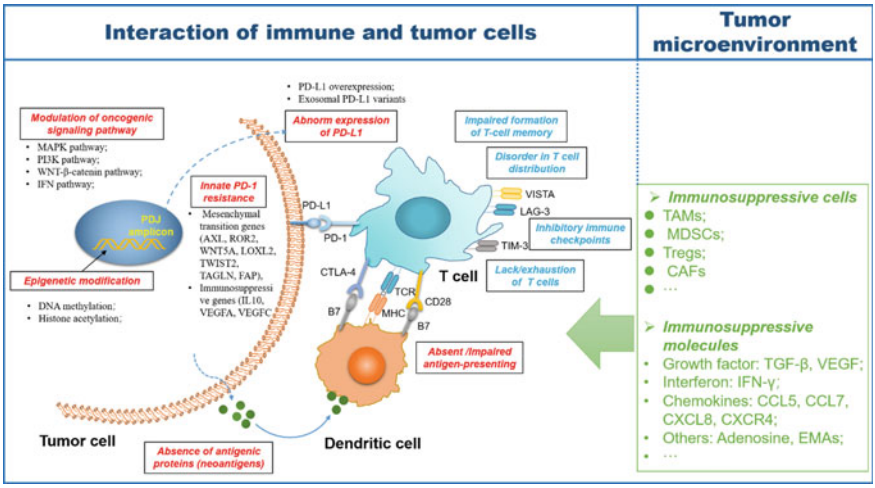

Fig. 5.1 Schematic diagram of immune checkpoint blockade resistance in cancer therapy

most direct factor in determining the recognition of restricted T cells, which alleviate tumor's responses to immune checkpoint therapy (Gubin et al. 2014). Schumacher and van Rooij et al. found that tumor-targeted T cells reactivated by immune checkpoint inhibitors tend to recognize mutational tumoral neoantigens under the treatment of ICBs. Therefore, any tumor cell-related factor that could lead to the deficiency of cell surface antigens, such as gene mutation, genetic deletion, and epigenetic modification, is likely to induce primary or acquired resistance to immune checkpoint therapy (Schumacher and Schreiber 2015; van Rooij et al. 2013). Moreover, low mutational burden and overlapping surface proteins were also reported to contribute to intrinsic resistance (Locarnini and Yuen 2010; Hodges et al. 2017; Hellmann et al. 2019).

ii. *Mutations and modulations in oncogenic signaling pathway*

Tumor cell-based resistance is inevitably related to cellular oncogenic signaling pathway. For example, proteins like IL-8 and VEGF, secreted subsequent to the activation of MAPK pathway, may inhibit the recruitment and effectiveness of T cells (Liu et al. 2013). Additionally, IL-6 assembly induced by STK11/LKN1 deletion in the mutated oncogenic KRAS pathway could decrease T cell infiltration and increase the expression of T cell exhaustion receptors, such as T-cell immunoglobulin and mucin domain-containing 3 (TIM-3), CLTA-4, and PD-1 (Koyama et al. 2016). Similarly, downregulation of the dendritic cell-recruiting cytokine CCL4 by Wnt/β-catenin pathway was also capable to prevent T cell infiltration (Spranger et al. 2014). The oncogene MYC and STAT3 could upregulate the expression of CD47 and PD-L1 by directly binding to their promoters to disturb antitumor immunity (Atsaves et al. 2017; Casey et al. 2016). As for the tumor suppressor gene PTEN, either its inactivation or loss would give rise to immunosuppression and interfere with therapeutic effect of anti-PD-1 therapy, thus leading to resistance to ICBs (Peng et al. 2016; George et al. 2017). Besides, it has been described that Wnt signaling transduction upregulated by β-catenin stabilization could result in T cell exclusion from tumor cells. Consistent with this conclusion, the effectiveness of tumor's response to ICBs in murine model was positively related to the expression level of β-catenin, as β-catenin-positive tumors responded poorly to ICBs (Spranger et al. 2015). IFN-γ signaling pathway plays inconstant but important roles in many intracellular and intercellular physiological processes. As for the tumor response and resistance to targeted therapy, researchers had elucidated that downregulated or mutated molecules in IFN-γ pathway (IFN-γ/JAK/STAT3) could help tumor cells escape from its killing effect, or directly suppress IFN-γ's killing effect, which contribute to tumor cell's insensibility to T cells (Darnell et al. 1994; Kaplan et al. 1998; Dunn et al. 2005).

iii. *PD-L1 expression*

The immunosuppressive cell surface ligand PD-L1, which is constitutively expressed by tumor cells, is an indispensable character in the therapeutic effect or resistance to ICBs. The expression of PD-L1 on cell surface makes tumor cell possible to "silence" the activated T cells that recognize tumoral neoantigens. By now, many signaling pathways and molecules have been found related to PD-L1 expression,

including EGFR mutation (Akbay et al. 2013), MYC overexpression (Casey et al. 2016), PARP inhibition (Jiao et al. 2017), aberrant IFN-γ pathway (Abiko et al. 2015), CDK5 disorder (Dorand et al. 2016), PDJ amplication (Ansell et al. 2015; Rooney et al. 2015), PTEN loss, and PI3K/AKT mutations (Lastwika et al. 2016; Parsa et al. 2007). These alternations are driving factors that influence anti-tumor T cell responses. Likewise, it was revealed that truncated PD-L1 transcripts could induce PD-L1 expression (Kataoka et al. 2016). Malignant tumors expressing PD-L1 tend to exert better response to anti-PD-1 therapy. In particular, recent data has shown that some PD-L1 variants secreted by tumor cells could work as "decoys" of PD-L1 targeted antibody and induce resistance to PD-L1 blockade in NSCLC (Gong et al. 2019).

iv. *Innate PD-1 resistance (IPRES)*

The deeper mechanisms of signal network underlying tumor cell's resistance to anti-PD-1 therapy remains to be explored. Hugo et al. uncovered that a cluster of genes related to mesenchymal transition (AXL, ROR2, WNT5A, LOXL2, TWIST2, TAGLN, and FAP), immunosuppression (IL10, VEGFA, and VEGFC), and monocyte and macrophage chemotaxis (CCL2, CCL7, CCL8, and CCL13) were enriched in non-responding tumors. And it was reasonably inferred that these genes participate in PD-1 resistance through their functions on immunosuppression, mesenchymal transformation, and stemness maintaining (Hugo et al. 2016).

v. *Epigenetic modification*

Changes in gene expression are observed in the resistance to various oncotherapy treatments, and abnormal epigenetic modification is a crucial trigger of the disorder of gene expression. Histone deacetylase inhibitors were proved to induce the expression of major histocompatibility complex (MHC) and tumor associated antigens, thus to improve the anti-tumor effect of immune therapy (Vo et al. 2009). We could speculate that deacetylation of histone might play important roles in the development and maintaining of ICB resistance. Likely, as methylation of tumoral DNA was repressed by hypomethylating agents, CD80 expression was upregulated in tumor cells, which could lead to enhanced tumor infiltration of effector T cells (Wang et al. 2013).

vi. *Absence of antigen presentation*

B2-microglobulin (B2M) and human leukocyte antigen (HLA) are components of the MHC-I molecules that are required for antigen presentation. Downregulation of HLA class I molecules and loss of B2M have been described. More specifically, loss of B2M expression results in impaired cell surface expression of MHC class I, which in turn impairs antigen presentation to cytotoxic T cells. As reported in an analysis of 4512 tumors across 11 tumor types, deletions and deleterious alterations in B2M and HLA alleles have been demonstrated to be associated with a gene expression signature of cytotoxic immune cells, which is consistent with the previous findings that downregulation of antigen presentation by the tumor evades a cytotoxic T-cell antigen-specific immune response. An acquired deleterious mutation in B2M was found in a late-progressing lesion from a melanoma patient with initial response

to PD-1 blockade. In a larger longitudinal cohort of 17 melanoma patients treated with ICB with subsequent progression, loss of B2M or deleterious mutations in it were found in progressive lesions in three patients with initial response to therapy and two patients with intrinsic resistance. In a study of ICB-resistant lung cancer patients, B2M loss and concomitant loss of MHC-I expression was found in a non-responsive patient, and downregulation of B2M in patient derived xenografts was found in two other patients. Subsequent functional validation demonstrated that B2M knockout conferred resistance to PD-1 blockade in vivo in an immunocompetent mouse model of lung cancer. In a murine model, tumors with elevated β-catenin lacked a subset of dendritic cells (DCs) known as $CD103^+$ DCs, due to decreased expression of CCL4, a chemokine that attracts $CD103^+$ DCs. In addition, murine tumors lacking β-catenin responded effectively to ICBs whereas β-catenin-positive tumors did not. Importantly, alterations in genes encoding components of the antigen processing and/or presentation apparatus (e.g., class I MHC, B2M) can also lead to ICB resistance.

5.3.2 T Cell-Based Resistance

T cell is a kind of lymphocytes that plays core roles in cancer's immune response, as T cell can recognize fragments of specific antigens on tumor cells, which are presented by DCs with its MHC. The function of T cells is mainly controlled by the activation of T-cell receptors and downstream signaling pathways, so that cancer cells can be recognized and killed to prevent the formation of solid tumor. During the action of ICBs, the killing effect of T cell is usually derepressed by ICBs to restore its recognition and cytolytic effect on cancer cells. Once the functional phenotype of these reactivated anti-tumor T cells changes, the therapeutic effect of ICB declines. In the perspective of T cell alteration, ICB resistance is mainly determined by the number, distribution, effect, and activation status of T cells.

i. *Absence of T cells*

T cell is the executor at the forefront of innate defense of cancer, and its killing effect is indispensable in the ICB therapy. Absence of tumor-specific T cells, or loss of T cell function, results in the immunotherapy inefficiency, namely, nonresponsiveness/resistance to ICBs. It is worth mentioning that failed tumor infiltration and abnormal distribution of functional T cells can be regarded as lack of T cells in the regional TME. For example, cancer-associated fibroblasts (CAFs) were found to have an impact on T-cell distribution in solid tumors (Feig et al. 2013). In human melanoma, β-catenin signaling pathway is more intensely activated in tumors and T cells and $CD103^+$ DCs are rarer in TME. Similar results suggested that β-catenin signaling pathway also suppresses $CD8^+$ T-cell infiltration in colorectal cancer (Xue et al. 2019), and eventually lead to T cell exclusion in TME.

ii. *Inhibitory immune checkpoints*

In addition to the most commonly targeted immune checkpoints CTLA-4 and PD-1, alternative checkpoints like TIM3, LAG3, TIGIT, B7-H3, CD38, CD73, and A2A receptor are being explored in the ongoing researches (Kalbasi 2019). Because inhibitory immune checkpoints, such as LAG-3, TIGIT, and VISTA, which are expressed on the surface of T cells act as compensatory inhibitors of T cell function, the reactivation of cytotoxic T lymphocytes (CTLs) is erased as a result (Topalian et al. 2015). Clinical researches in lung adenocarcinoma concluded that the expression level of TIM-3 was upregulated after anti-PD-1 therapy (Koyama et al. 2016), and TIM-3 might contribute to ICB resistance. In accordance with this inference, it is reported that combinatory ICB therapies that target LAG3 and PD-1, or, TIM-3 and PD-1, have gained enhanced therapeutic effects in preclinical studies (Woo et al. 2012; Sakuishi et al. 2010). Similarly, Leach et al. found that in the TCR activated and CD28 co-stimulated T cells, CTLA-4 itself could also be increased eventually (Leach et al. 1996).

iii. *Impaired formation of T-cell memory*

In order to obtain long-term immune memory, a subtype of effector T cells differentiate into effector memory T cells with the assistance of helper $CD4^+$ T cells and DCs. Therefore, impaired formation of T cell memory could cause the failure of ICB therapy (Jenkins et al. 2018). Pauken et al. revealed that epigenetics modifications were capable to limit the durability of immune memory by interfering with T cell memory formation to alleviate the killing effect on tumor cells (Pauken et al. 2016). It was also uncovered that in patients with higher tumor burden, there is limited reacquisition of memory T-cell response when tumor antigen persists for a long time. As a result, the damage of memory effector T cells could result in the attenuation of clinical outcome, acquired ICB resistance or tumor recurrence after drug withdrawal (Huang et al. 2017).

5.3.3 Tumor Microenvironment-Determined Resistance

The broad spectrum of other functional components within the TME indicated a separate pool of paramount modulators of immune activities against cancers, apart from tumor cells and T-cells. These modulators mainly involve immunosuppressive cells, molecules, cytokines, and chemokines.

i. *Immunosuppressive cells*

In TME, myeloid derived suppressor cells (MDSCs), Tregs, tumor-associated macrophages (TAMs), and CAFs are major non-tumor cellular elements of the tumor-extrinsic mechanisms that contribute to primary and/or adaptive resistance to ICBs.

MDSCs are a heterogeneous population of immature myeloid cells that are recruited by tumors. Human MDSCs are positive for CD11b and CD33, and are

usually negative for lineage-specific antigens and HLA-DR. Monocytic MDSCs are $CD14^+$ and granulocytic MDSCs are $CD15^+$. Instinctively, mature monocytes are positive for HLA-DR (Wesolowski et al. 2013). MDSCs haven been implicated in pro-tumor bioactivities such as angiogenesis, invasion and metastasis of cancer cells (Yang et al. 2004, 2008). Importantly, MDSCs are known to play pivotal roles in the regulation of immune responses, in that they impair T cell responses through local nutrient depletion, reactive oxygen production, and nitrosylation of local chemokines (Gabrilovich et al. 2012). The clinical findings suggested that the presence of MDSCs in TME was associated with reduced survival in patients with colorectal cancer and breast cancer (Solito et al. 2011). Besides, a low frequency of MDSCs in the TME may promote the responses to ICBs (Meyer et al. 2014), and that depletion of intratumor MDSCs restores the effectiveness of PD-1 blockade (Highfill et al. 2014; Steinberg et al. 2017). Therefore, the regulation and manipulation of MDSCs might be a potential strategy to address ICB resistance.

Another major immunosuppressive cell type within the TME is the Tregs. Tregs can be identified by the expression of the FoxP3 transcription factor. They are a subtype of $CD4^+$ T cells that suppress the proliferation and function of local effector CD8 T cells (Teffs), either directly through cell contact or indirectly by secreting inhibitory cytokines, such as transforming growth factor beta (TGF-β), IL-10, and IL-35 (Oida et al. 2003; Sakaguchi et al. 2008; Sundstedt et al. 2003). Studies in murine models indicated that the depletion of Tregs in TME restores anti-tumor immunity (Linehan and Goedegebuure 2005; Viehl et al. 2006) and improves the effectiveness of PD-1/PD-L1 blockades (Vargas et al. 2017; Taylor et al. 2017). Furthermore, an increased Teff/Treg ratio has been related with response to CTLA-4 blockade (Quezada et al. 2006). Therefore, tumors with high Teff/Treg ratio are more likely to be resistant to ICB treatment. Previous studies suggested that many human tumors are infiltrated by Tregs (Chaudhary and Elkord 2016; Ormandy et al. 2005; Woo et al. 2002), and the presence of Tregs in TME results in a poor immunologic anti-tumor response (Bettelli et al. 2006; Elpek et al. 2007). Of note, tumor-infiltrating Tregs may also co-exist with other immune cells, demonstrating a potentially immune-responsive tumor. As reported in a retrospective study, cancer patients with a high baseline expression of $FoxP3^+$ Tregs exhibited better outcomes of CTLA-4 blockade therapy (Hamid et al. 2011). According to the current evidence, future studies on the functions of Tregs and MDSCs in TME may further elucidate the mechanisms of resistance to ICBs.

Macrophages are immune cells derived from bone marrow hematopoietic cells and are widely distributed in the human body (Davies and Taylor 2015). They are an important component of effector cells in the innate immune system and are also involved in specific immunity. Macrophages function in phagocytosis, antigen presentation and secretion of various cytokines, and also play an important role in inflammation, immune regulation, repair, metabolism and tumor behavior. Among the macrophages, TAMs are a population of immune cells involved in the response to immunotherapy. TAMs are highly plastic and may present as classically activated macrophages (M1 macrophage) or alternatively activated macrophage (M2 macrophage) within different microenvironments. The surface molecules, secreted

cytokines and physiological functions of the two phenotypes vary greatly, even antagonistic (Atri et al. 2018; Biswas and Mantovani 2010; Hu et al. 2016). More specifically, M1 macrophages contribute to an anti-tumor immunity, while M2 macrophage tend to promote pro-tumorigenic activities (Chanmee et al. 2014). In the mice model of lung adenocarcinoma, the depletion of TAMs downregulated the recruitment of M2 and/or TAMs, thereby suppressing tumor growth. The possible mechanisms might involve the inactivation of CCL2/CCR2 signaling pathway (Fritz et al. 2014). Besides, similar results have been observed in murine models of multiple tumor types such as breast cancer (Luo et al. 2006), cutaneous T cell lymphoma (Wu et al. 2014), and melanoma (Tham et al. 2015). Furthermore, the results of clinical studies suggested that high frequency of TAMs was associated with a poor prognosis in malignancies (Hu et al. 2016).

Investigators have explored the function of TAMs in the resistance to cancer immunotherapy. And the results indicated that TAMs may restrain T cell responses through B7-H4 in ovarian carcinoma (Kryczek et al. 2006) and PD-L1 in hepatocellular carcinoma (Kuang et al. 2009). In a murine model of pancreatic cancer, the blockade of colony-stimulating factor 1 receptor (CSF1R), the receptor for macrophage-colony stimulating growth factor (M-CSF) lead to a decreased frequency of TAMs, increased IFNs and suppressed tumor progression (Zhu et al. 2014). While PD-1 inhibitor or CTLA-4 inhibitor alone was unable to significantly reduce tumor growth in the murine model (Zhu et al. 2014; Le et al. 2013), the combination of CSF1R blockade and ICB in addition to gemcitabine may improve tumor regression (Zhu et al. 2014). Several initial trials are ongoing to elaborate the efficacy of CSF1R blockade in the management of cancers and ICB resistance.

T cells can interact with cancer cells only when they reach the vicinity of the tumor. CAFs, especially those positive for fibroblast activation protein-a (FAP), contribute to ICB resistance by regulating the spatial distribution of T cells in tumor (Feig et al. 2013). CAFs produce extracellular matrix that physically separate T cells from the tumor (Salmon et al. 2012). Moreover, FAP^+CAFs secrete CXCL12 and recruit MDSCs into TME, thereby blocking T cells from the tumors (Feig et al. 2013; Yang et al. 2016). As indicated in the model of pancreatic cancers, FAP^+CAF-dominated CXCL12/CXCR4 signally pathway might be a potential target to reverse ICB resistance (Feig et al. 2013).

ii. *Immunosuppressive molecules*

In TME, tumor and macrophages may release immunosuppressive cytokines to assist the local suppression of immune responses (Sharma et al. 2017). TGF-β is a powerful negative regulator of effector T cells (Park et al. 2018), and it functions in the process of immunosuppression and angiogenesis through the stimulation of Tregs (Lebrun 2012). As indicated in several types of cancers, the increase in the level of TGF-β was associated with a poor prognosis (Lin and Zhao 2015; Massague 2008). Another study of patients with metastatic bladder cancer, who were primarily resistant to PD-L1 blockade (atezolizumab) revealed that TGF-β can be upregulated by CAFs and collagen-rich extracellular matrices, that prohibit the recruitment of $CD8^+$ T-cells into TME (Mariathasan et al. 2018). As evidenced by a preclinical study, TGF-β

receptor kinase inhibitor may work synergistically with CTLA-4 blockade (Hanks et al. 2014).

Another typical immunosuppressive molecule that might be promoted by IFNγ is indolaimine-2, 3-deoxygenase (IDO), a tryptophan-metabolizing enzyme that participate in peripheral tolerance and suppress the functions of effector T cells (Gajewski et al. 2013). More specifically, IDO can be expressed by tumor cells and myeloid cells to atalyze tryptophan into kynurenine, and dysfunctions T cells by the impairment of essential amino acid (Platten et al. 2012). When combined with ICBs, IDO inhibitors exhibited anti-tumor effects, and the results in clinical trials are awaiting (Holmgaard et al. 2013; Spranger et al. 2014). Moreover, other immunosuppressive molecules with potential competent in ICB resistance include carcinoembryonic antigen cell adhesion molecule-1 (CEACAM1) (Takahashi et al. 1993; Gray-Owen and Blumberg 2006), adenosine (Zhang et al. 2004), CD73 (Stagg et al. 2010), cyclin-dependent kinases (CDKs) (Choi et al. 2012) and TIM-3 (Koyama et al. 2016).

Particular chemokines and their receptors may recruit MDSCs and Tregs toward the tumors. For instance, CCR4 in richly expressed by Tregs in TME (Sugiyama et al. 2013), and CCR4 inhibitors reduce the recruitment of Tregs. Besides, it also promotes antibody-dependent cell-mediated cytotoxicity (ADCC), thereby further decreasing Tregs (Chang et al. 2012). Other examples include CXCL12 and its receptor CXCR4 (Gil et al. 2014), as well as CCL5, CCL7, and CXCL8 with their receptors CCR1 or CXCR2 (Highfill et al. 2014). The blockade of these chemokine receptors may sabotage immune evasion and promote anti-tumor responses of T cells, thus potentially addressing the resistance of ICBs.

iii. *Aberrant regulation of signaling pathways*

The PI3K/AKT/mTOR signaling pathway regulates a variety of cellular bioactivities including proliferation, survival and motility (Polivka and Janku 2014). Aberrant activation of PI3K/AKT/mTOR pathway is associated with the innate resistance to PD-1/PD-L1 blockade (Bai et al. 2017). Moreover, it has been reported that the loss of PTEN in melanoma patients may lead to the overexpression of immunosuppressive cytokines, thus influencing resistance to ICBs (Peng et al. 2016). Besides, the inhibition of the PI3Kβ isoform could strengthen the effectiveness of PD-1/PD-L1 blockades. Another signaling pathway of note is Wnt/β-catenin pathway, in that the activation of Wnt/β-catenin axis may trigger T cell exclusion from TME and induce primary resistance to PD-1/PD-L1 blockades (Spranger et al. 2015). Still, other cellular signaling pathways involved in ICB resistance include JAK/STAT/IFN-γ (Marzec et al. 2008; Shin et al. 2017) and ERK/Erk MAPK pathways (Tumeh et al. 2014; Hugo et al. 2015).

5.4 Novel Strategies to Overcome Resistance to Immune Checkpoint Inhibitor

Since the first FDA approved of ICB agent in 2011, ICBs have become a breakthrough in the treatment of multiple malignancies and have achieved significant clinical benefits (Hodi et al. 2010). Unfortunately, drug resistance is an inevitable obstacle to a durable antitumor activity, thus leading to tumor relapse. To date, there have been various mechanisms of resistance to ICBs proposed, some of which have been clinically validated. On the basis of above mechanisms, great efforts need to be made to overcome ICB resistance by exploring promising therapeutic strategies.

According to the anti-tumor immune responses, therapeutic strategies to overcome ICB resistance can be categorized into the following aspects, enhancing antigen procession and presentation, reinforcing the activity and infiltration of T cells, and destroying immunosuppression microenvironment. Several combinational therapies are currently being evaluated in preclinical models and clinical trials.

5.4.1 Enhancing Antigen Procession and Presentation

In view of the above, the presentation of tumor associated antigens plays a pivotal role in tumor immune response and it is thought to be the first step of antitumoral response. Therefore, the deficiency of antigens will contribute to the escape of immune surveillance, leading to the development of ICB resistance. This phenomenon can be reversed by several strategies, such as personalized vaccines, oncolytic viruses, radiotherapy, chemotherapy, and targeted therapy.

i. *Cancer vaccine*

When it comes to immunogenicity strengthening, cancer vaccines must be the first consideration. Cancer vaccine can directly enhance immunogenicity, stimulate effector immune cells and induce broad immune responses. It eradicates tumor cells through educating host immune system to recognize cancer cells as foreign pathogens. To date, various tumor vaccines have been developed, such as DNA/RNA vaccines, synthetic peptide vaccines, viral component vaccines, and conjugate vaccines. Recently, next-generation sequencing and bioinformatics analysis have made neo-antigens encoded by somatic mutation in individual cancer attractive vaccine targets based on the loss of expression in healthy tissues (Sahin and Tureci 2018). A variety of preclinical and clinical trials have been investigating the antitumor activities of neo-antigen vaccines in several tumors including melanoma, NSCLC, breast cancer and others. The increase of tumor immunogenicity can convert “cold” tumors into “hot” tumors and induce PD-L1 expression in TME, which sensitize tumors to PD-1/PD-L1 blockades. Moreover, tumor response to checkpoint blockade therapy can be influenced by HLA class I genotype that maximal heterozygosity at HLA-I loci with improved overall survival, which can be drawn on to design

rational combination of neo-antigen vaccines and ICB (Chowell et al. 2018). In a more recent trial, Patrick A. Ott and their colleagues demonstrated that neo-antigen vaccines induced powerful muli-functional effector T cell responses and four of six patients with melanoma were recurrence-free at 25 months after neo-antigen vaccine treatment (Ott et al. 2017). Meanwhile, extra anti-PD-1 therapy prolonged and broadened $CD8^+$ and $CD4^+$ T cell responses, as evidenced in two patients who experienced complete tumor eradication after post-vaccination relapse. Clinical trials such as NCT04072900, NCT03532217, and NCT03289962, accessing combinatory regimens of ICBs and neo-antigen vaccines are currently recruiting patients.

ii. *Oncolytic virus*

Oncolytic virus immunotherapy is an effective therapeutic regimen to treat tumors that makes use of native or genetically modified viruses which can selectively replicate in tumor cells and kill them, resulting in innate and adaptive antitumoral immunity (Prestwich et al. 2008; Twumasi-Boateng et al. 2018). The antitumor activity of oncolytic virus is mediated through multiple mechanisms. However, the most important one is that oncolytic viruses promote the maturation and function of antigen-presenting cells (APCs) by releasing tumor-associated antigens, pathogen-associated molecular patterns (PAMPs) as well as danger-associated molecular patterns (DAMPs). In addition to strengthened antigen procession and presentation, the improvement of cancer cell recognition by immune system also involves upregulation of MHC class I and MHC class II expressed on APCs and tumor cells. Furthermore, promoting T cell recruitment and tumor infiltration by forming a pro-inflammatory environment is another important mechanism of oncolytic virus to activate immune system (Bommareddy et al. 2018). Based on these mechanisms, combination therapeutic regimens of viruses and ICBs are attractive. A preclinical study demonstrated that localized tumor therapy of B16 with oncolytic Newcastle Disease Virus (NDV) increased local and distant tumor-specific $CD4^+$ and $CD8^+$ T cell infiltration and increased therapeutic efficacy of CTLA-4 blockade (Zamarin et al. 2014). A recent research also suggested that oncolytic virus therapy increased sensitivity of triple-negative breast cancer to ICBs and prevented tumor relapse, which made the combination of oncolytic virus and ICBs a novel neoadjuvant regimen (Bourgeois-Daigneault et al. 2018). Increasing evidence showed that oncolytic virotherapy induced expression of PD-L1 and CTLA-4 on immune and tumor cells (Puzanov et al. 2016; Saha et al. 2017). In a phase 1b trial (NCT01740297), combination of talimogene laherparepvec and ipilimumab significantly improved PFS and OS without extra adverse effects compared to ipilimumab alone in the treatment of unresectable stage III–IV melanoma (Puzanov et al. 2016). A randomized phase 2 study (NCT01740297) is ongoing to assess whether talimogene laherparepvec plus ipilimumab is superior to ipilimumab alone for advanced melanoma. Furthermore, several clinical trials are underway to evaluate the efficacy of combinatory regimens with oncolytic viruses and ICBs (NCT02263508, NCT03206073, and NCT02965716).

Apart from oncolytic virus, cytotoxic radiotherapy, chemotherapy, and targeted therapy can directly kill cancer cells, leading to the release of tumor-associated antigens and causing inflammatory reactions.

iii. *Radiotherapy*

Recently, a large number of studies demonstrated that combination of radiotherapy and ICB is superior to ICB monotherapy in a spectrum of malignancies (Deng et al. 2014; Dovedi et al. 2017; Twyman-Saint Victor et al. 2015). Preclinical studies suggested that the expression of PD-L1 was increased on tumor cells after the treatment of radiotherapy, which made tumors sensitive to anti-PD-1/PD-L1 therapy (Gong et al. 2017; Dovedi et al. 2014). Additionally, radiotherapy and ICB worked synergistically to demolish immune-inhibitory environment through reducing the accumulation of MDSCs (Deng et al. 2014) and increasing the diversity of TCR repertoire of tumor-infiltrating lymphocytes (TILs) (Twyman-Saint Victor et al. 2015). In the clinical settings, optimistic responses to combination of radiotherapy and PD-1/PD-L1 blockade were observed in neuroendocrine cervical carcinoma with high TMB (Sharabi et al. 2017) and melanoma (Haymaker et al. 2017). To date, numerous clinical trials are ongoing to evaluate the safety and efficiency of combination therapy of radiotherapy and ICB in multiple types of cancers (NCT03898895, NCT04017897, and NCT03480334).

iv. *Chemotherapy*

Similar to oncolytic virus, chemotherapeutic agents also promoted antigen presentation through increasing the expression of tumor antigens and MHC class I molecules (Ohtsukasa et al. 2003; Rubinfeld et al. 2006) and creating comfortable immune environment, although they were previously known to produce systemic immunosuppressive effects because of the bone marrow toxicity (Nowak et al. 2003). Jin Peng et al. demonstrated that the expression of PD-L1 was upregulated in a NF-κB-dependent manner with the treatment of chemotherapy and suggested that combination of chemotherapy and anti-PD-1 therapy could be a prospective regimen (Peng et al. 2015). In view of above, a serial of clinical trials were conducted to verify the synergism of chemotherapy and ICB. A phase 1 trial confirmed that combination of pembrolizumab and platinum-based chemotherapy exhibited encouraging antitumor response. In another random phase 2 trial, pembrolizumab combined with chemotherapy significantly improved PFS (8.8 versus 4.9 months) and overall survival at 12 months (69.2% versus 49.4%) compared to chemotherapy alone in patients with advanced NSCLC (Gandhi et al. 2018). Besides, trials on chemotherapy combined with PD-1 blockade (NCT03904537 and NCT02961101), PD-L1 blockade (NCT03456063 and NCT03164993), and CTLA-4 blockade (NCT03215706 and NCT02659059) are underway.

v. *Targeted therapy*

Over the past decade, multiple hyper-activated oncogenic signaling pathways that drive the survival, proliferation, migration, and apoptosis phenotype of tumor cells have been identified (Flaherty 2012). These activated pathways or mutant proteins have been verified to play important roles in the progression of malignant tumor, and the development of reasonably designed inhibitors or antibodies that block alternative pathways and proteins made targeted therapy clinically valuable for the treatment of cancers (Mcconnell and Wadzinski 2009). These agents can significantly

arrest tumor cell growth and shrink regression in specific patients who are screened for molecular classification such as BRAF mutation, EGFR mutation, and ALK gene fusions, which made targeted therapy another breakthrough in the landscape of cancer therapy. Amounting data indicated that targeted therapy could directly affect tumor-immunity cycles by intensifying the recognition and effector function of T cells and downregulating immunosuppressive environment except for the antitumor activity. In melanoma, MAPK inhibition with BRAF or MEK inhibitors can increase the expression of melanocyte differentiation antigens (MDAs) such as MART-1, gp-100, Trp-1, Trp-2, and MHC class I molecules on tumor cells (Boni et al. 2010; Bradley et al. 2015). Possible explanation is that transcriptional expression of melanocyte pivotal transcription factor (MITF) was upregulated with MAPK blockade, thus leading to the upregulation of MDAs, the targets of MITF. Moreover, loss of expression of melanoma antigens along with reduction of MITF conferred resistance to several targeted drugs in melanoma (Muller et al. 2014). A range of preclinical and clinical researches have revealed that targeted therapies that exhibit anticancer effects with tumor-intrinsic and -extrinsic mechanisms can coordinate with immunotherapy, especially ICBs. For example, RAF inhibitors combined with PD-1/PD-L1 blockade or CTLA-4 blockade significantly inhibited tumor growth and prolonged survival via enhancing effector T cell infiltration (Cooper et al. 2014; Callahan et al. 2014). Furthermore, a phase 1 study suggested that combination of MAPK blockade and antibody (MEDI4736) against PD-L1 enhanced durable antitumor responses without excessive toxicity (Ribas et al. 2015). In a recent phase 1 trial (NCT02130466), combination of BRAF inhibitor, MEK inhibitor, and PD-1 blockade showed durable responses with manageable toxicity in majority of patients with BRAF mutant melanoma (Ribas et al. 2019). As previously reported, this triple-combined therapy increased the expression of MHC class I molecules and induced immune cell infiltration. Considering the toxicity of this triple-combined therapy, efforts are made to select the most idea timing and sequencing of the three agents (NCT02625337, NCT03149029, and NCT02858921). Apart from MAPK targeted therapy, other targeted agents against oncogene pathways that showed synergistic effect with ICBs include PI3K-AKT and EGFR signaling pathways (Hughes et al. 2016).

5.4.2 Strengthen the Function and Infiltration of T Cells

Although the generation of antitumor immune responses is complicated with the involvement of diverse immune cells and a variety of steps, T cells, especially TILs is a determinant in this process. Mechanisms of strengthened function and infiltration of T cells to produce durable tumor regression include adoptive T cell transfer, inhibition of negative molecules, and activation of stimulatory molecules.

i. *ACT (TCR, CAR-T)*

Chimeric antigen receptor (CAR) T cells therapy involves adoptive cells from patient with genetically engineered with CAR to eradicate tumors specifically after cell expansion ex vivo and reinfusion back into patients. CAR-T therapy not only improve the function of effector T cell, but also strengthen the ability to recognize antigens expressed by tumor cells with high avidity and specificity, which is independent to MHC restriction (Restifo et al. 2012). Therefore, it may over resistance to immune checkpoint blockade due to the deficiency of the function and infiltration of T cells. Additionally, the blockade of immune checkpoint using mAb may further reinforce the function of CAR-T cells via inhibition immunosuppressive signaling pathways (Liu et al. 2016). Based on the advantages of CAR-T and immune checkpoint blockade therapy, combination of them has become a promising research area. Preclinical studies have confirmed that combination of CAR-T and immune checkpoint blockade are synergistic, leading to durable antitumor responses and improving survival outcome in multiple cancers. John LB and his colleagues demonstrated that specific PD-1 blockade with antibody enhanced and proliferation and function of anti-HER CAR-T cells in vitro and in vivo, leading to better tumor regression without causing autoimmunity in mouse model (John et al. 2013). Similar result had been confirmed by other researches, combination of CAT-T and PD-1/PD-L1 axis blockade through genetic approach were more effective to inhibit tumor growth than monotherapy (Liu et al. 2016; Suarez et al. 2016). Moreover, the efficiency of immune checkpoint blockade combining with CAR-T therapy having been further assessed in clinical trials (NCT03615313, NCT02862028, and NCT03182803). Better understanding the mechanisms of combination therapies will be helpful to develop more rational and favorable regimens.

ii. *Inhibition of other immune checkpoints*

Although PD-1 and CTLA-4 are the most predominant regulators of T cell activation and exhaustion, to some extent, inhibitory molecules of other signaling pathways may also control the activation and exhaustion of T cells. Upregulation of other immunosuppressive molecules including TIM-3, LAG-3, TIGIT, BTLA, and VISTA in immune cells are thought to be another major reason for the failure of PD-1/PD-L1 and CTLA-4 blockades. Accordingly, combination of these molecules with ICBs may be a prospective therapeutic strategy. Inspiringly, these combination therapies have resulted in tumor elimination in multiple preclinical models through reversing the suppression of effector T cells. For example, Tim-3^+ $PD1^+$ and $TIGIT^+$ $PD1^+$ TILs against specific antigens have presented the most severe exhausted phenotype, with weakened functions in eliminating tumors, thus co-targeting TIM-3 or TIGIT and PD-1 pathways exerted remarkable antitumor activity through reversing T cell exhaustion (Sakuishi et al. 2010; Chauvin et al. 2015). Dual LAG-3/PD-1 blockade showed better antitumor responses than monotherapy in mouse model of chronic lymphocytic leukemia (CLL) (Wierz et al. 2018).

It is well known that VISTA can inhibit T cell activation as an ICB agent. A recent phase 2 trial (NCT01194271) demonstrated that ipilimumab could increase the infiltration of T cells in tumors. However, it was insufficient to produce durable antitumor

responses since other immune checkpoint molecules, such as PD-L1 and VISTA, were upregulated in $CD68^+$ TAMs after the treatment (Gao et al. 2017). Therefore, combination of different ICBs may be a prospective strategy. In the mouse model, VISTA and PD-1 synergistically mediated T cell immune responses, and targeting VISTA and PD-1 improved antitumor effects without severe adverse events (Liu et al. 2015). Several clinical trials are currently underway to test the efficacy of antibodies against these inhibitory molecules as monotherapy or as part of combination therapy in cancer treatment (Anderson et al. 2016; Sharma et al. 2015).

iii. *Costimulatory agonist*

Co-stimulatory signaling is indispensable to maintain the function of effector T cells except co-inhibitory activities. The co-stimulatory receptors mainly include two families, B7-CD28 and TNFR (Mayes et al. 2018). Targeting immune-stimulatory receptors with antibodies can reverse immune resistance in several tumors, and a variety of such drugs have been developed and approved for the treatment of cancers, such as antibodies against 4-1BB, CD27, OX40, ICOS, and GITR (Mayes et al. 2018). The profound antitumor activity of these agents combined with PD-1/PD-L1 or CTLA-4 blockade have been confirmed by preclinical data in mouse models. For instance, the substantial synergism of 4-1BB agonist and PD-1 blockade was validated in a poorly immunogenic tumor model (Chen et al. 2015). Whereas, concurrent PD-1 blockade diminished the anti-tumor activity of 4-1BB agonist in the model of spontaneous B-cell lymphoma (McKee et al. 2017). It might be possibly explained that the timing of anti-PD-1 therapy is important to the elimination of tumors. Similar result has been obtained in the mouse model of MMTV-PyMT breast cancer. Anti-OX40 therapy sequenced with PD-1 blockade significantly strengthened tumor elimination and survival in a $CD4^+$ and $CD8^+$ dependent manner. However, simultaneous PD-1 blockade reduced the therapeutic effect of anti-OX40 antibody (Messenheimer et al. 2017). Considering this, we should further explore the mechanisms of antitumor activity of OX40 stimulation to discover more rational and efficient combinatorial regimens. A recent research demonstrated that the combination of anti-GITR therapy and PD-1 blockade have promoted tumor rejection through reinvigorating intratumoral dysfunctional T cells (Wang et al. 2018). Moreover, anti-CD40 mAb can reverse immune resistance to PD-1 blockade by inducing IL12 to regulate PD-1 expression on $CD8^+$ T cells and diminishing immunosuppressive phenotype of Tregs (Ngiow et al. 2016). Such regimen eradicated melanoma even in the brain when combined with PD-1 inhibition (Singh et al. 2017). Encouraged by the results from preclinical studies, numerous clinical trials are ongoing to evaluate the safety and efficiency of agonist antibodies against costimulatory receptors combining with ICBs (Mayes et al. 2018).

5.4.3 *Undermining Immunosuppression Microenvironment*

It is now clear that tumor cells interplay closely with immune cells, stromal cells, and extracellular matrix that together form the TME, rather than working alone (Hanahan and Coussens 2012). Therefore, apart from tumor-intrinsic influences, tumor-extrinsic influences involving TME also contribute to evasion of immune surveillance, leading to immune tolerance and drug resistance to immunotherapy. Accordingly, how to destruct hostile tumor immunosuppressive microenvironment that is hijacked by tumors becomes a research interest. The negative regulatory elements employed by cancer cells include MDSCs, Tregs, TAMs, CAFs, and immunosuppressive cytokines.

i. *MDSC*

As described above, one important obstacle to ICB efficacy might be the recruitment of MDSCs into TME. Therefore, eradicating or re-educating MDSCs could enhance antitumor responses to anti-PD-1/PD-L1 or anti-CTLA-4 therapies. A growing body of evidence demonstrated that several drugs such as selective inhibitor of PI3Kδ/γ, HDAC and DNA methyltransferase inhibitors eliminated tumors through neutralizing MDSCs except for directly acting on tumors cells (Davis et al. 2017; Orillion et al. 2017; Kim et al. 2014), which contributed to the strengthened antitumor effect of PD-1/PD-L1 or CTLA-4 inhibitors. For example, in mouse model of HNSCC, PI3Kδ/γ inhibitor IPI-549 have reversed T cell suppression by MDSCs and enhanced response to antibodies against PD-L1 (Davis et al. 2017). Additionally, Jingying Zhou et al. verified that the accumulation of MDSCs were regulated by hepatoma-intrinsic CCRK signaling pathways and provided a promising strategy to eradicate hepatocellular carcinoma, i.e. combining tumorous CCRK depletion with PD-L1 blockade (Zhou et al. 2018). A recent research of rhabdomyosarcoma showed that reduced infiltration of MDSCs in TME resulting from the interaction of CXCR2 and its ligands could enhance anti-PD-1 efficiency (Highfill et al. 2014). Moreover, the functions of MDSCs were also influenced by long non-coding RNAs (lncRNAs), which predicted another hopeful therapeutic regimen. Encouraged by promising results from preclinical models, a clinical trial (NCT03302247) is ongoing to determinate the function of MDSCs depletion in the ICB treatment.

ii. *Treg*

In addition to MDCSs, Tregs (CD25 and FOXP3 expressing $CD4^+$ T cells) are another immunosuppressive subset of T cells in TME. Tregs play important roles in resistance to ICBs (Saleh and Elkord 2019). Therefore, therapies targeting Tregs rather than checkpoint blockades may overcome resistance and improve clinical outcomes. The depletion of Tregs can be achieved by several methods, including targeting immune checkpoint molecules and targeting kinase signaling in Tregs (Togashi et al. 2019). Delightfully, lots of researches have affirmed the efficiency of these combination therapies. In a mouse model of claudin-low breast cancer, combination of Treg depletion and anti-PD-1 and anti-CTLA-4 therapy strengthened the function of effector T cells and significantly improved survival (Taylor et al. 2017). The finding

that depletion of tumor-infiltration Tregs can synergize with PD-1 inhibitors to eliminate tumor was confirmed by Arce Vargas F et al. with Fc-Optimized Anti-CD25 antibody (Vargas et al. 2017). Recently, Yenkel Grinberg-Bleyer et al. demonstrated that NF-kB c-Rel played a vital role in the differentiation and function of Tregs and c-Rel inhibitor PTXF significantly reduced the tumor growth (Grinberg-Bleyer et al. 2017). Furthermore, they showed that ablation of PTFX potentiated the antitumor effects of PD-1 blockade in melanoma without any extra adverse effects, providing a viable combinatorial approach. Besides, combination of PI(3)K p110δ inhibitor might potentiate effects of ICBs since this inhibitor breaks immune tolerance, which is regulated by Tregs (Ali et al. 2014).

iii. *TAM*

Emerging evidence demonstrated that the infiltration of TAMs in TME is associated with poor prognosis in many tumors. In general, TAMs contribute to immunosuppression through promoting the immunosuppressive activity of Tregs, inhibiting DC maturation, producing metabolic starvation of T cells, and overexpressing immune checkpoint molecules such as PD-L1, PD-L2, CTLA-4, and VISTA (Mantovani et al. 2017). Consequently, targeting TAMs might complement the function of ICBs through eradicating extra inhibitory effects that might contribute to restrained T cell function though checkpoint blockade. One of the effective regimens that target TAMs is the inhibitor of CSF1R which plays pivotal roles in the recruitment, differentiation, and function of TAMs (Mantovani et al. 2017). Actually, in a mouse model, combining checkpoint agents with CSF1R produced appreciable antitumor responses in pancreatic ductal adenocarcinoma (PDAC) (Zhu et al. 2014). A recent study by Megan M. Kaneda et al. indicated that PI3Kγ regulated the polarization of TAMs, and that NF-κB, C/EBP and PI3Kγ inhibitors combined with PD-1 blockade showed significant synergistic effects in most tumor types (Kaneda et al. 2016). Additionally, the immunosuppressive function of TAMs can also be undermined by Class IIa HDAC inhibition (Guerriero et al. 2017). Notably, TMP195 treatment enhanced the efficiency of anti-PD-1 inhibitor and induced durable response in mouse model of breast cancer. Another potential target of TAMs is Fc-gamma receptor (FcγR) which was described by Sean P. Arlauckas et al. (Arlauckas et al. 2017). In their research, FcγRs inhibition prolonged the binding to PD-1 antibodies and tumor-infiltrating effector T cells, thereby enhancing the efficacy of ICB therapy. The above evidence demonstrated that combination of TAM inhibitor and ICBs is another promising strategy.

iv. *CAF*

CAFs, a stromal cell population, promote resistance to ICB mainly through providing immunosuppressive microenvironment to restrain function or deletion of effector T cells. A research reported that the overriding immunosuppression of CAFs was exerted by regulating the interaction CXCL12 with its receptor CXCR4 (Feig et al. 2013). Depletion of CAFs or targeting CXCL12 derived from CAFs could coordinate with PD-L-1 blockade in pancreatic cancer. In another preclinical study, TGF-β signaling contributed to CAF immunosuppression to escape from immunosurveillance

and TGF-β blockade might facilitate efficiency of ICBs (Chakravarthy et al. 2018). Consistently, simultaneous inhibition of TGF-β and PD-L1 can produce remarkable antitumor response in multiple mouse models as previously reported (Lan et al. 2018). Given these facts, the deletion of CAFs may be a promising approach to enhance ICB effects.

v. *Immunosuppressive cytokines*

Immunosuppressive factors such as interleukins (IL-6, IL-10), chemokine CCL2, VEFG, TDO and IDO, are produced by tumor cells or immune suppressive cells to demolish immune responses. IL-6/STATs axis is important for tumorigenesis and tumor progression by providing immunosuppressive microenvironment. Therefore, combination of IL-6 inhibitor and PD-1/PD-L1 blockade has significantly improved the therapeutic outcome in murine model of melanoma and PDAC by abrogating immunosuppressive effects (Tsukamoto et al. 2018; Mace et al. 2018). Similar to IL-6, IL-10 blockade combined with PD-1 inhibitor enhanced the function of tumor antigen-specific $CD8^+$ T cells (Sun et al. 2015). VEGF, a proangiogenic factor, may suppress the differentiation of DCs to maintain tumor cell growth. VEGF blockade could synergize with anti-PD-1 therapy through enhancing T cell infiltration (Meder et al. 2018). Several clinical trials combining VEGF or VEGFR inhibitors and ICB agents are ongoing (NCT02210117, NCT02348008, and NCT01472081). Additionally, CXCL12 inhibitors have strengthen the infiltration of NK and T cells, leading to improved efficacy of PD-1 blockade therapy (Zboralski et al. 2017). In addition to these factors above, modulators, which act in immunometabolism such as IDO/TDO, play a pivotal role in construction of immunosuppressive microenvironment (Prendergast et al. 2017). Delightfully, IDO deficiency or inhibition combined with CTLA-4 blockade significantly promoted tumor regression in melanoma mouse model through increasing T cell infiltration and Teff/Treg ratio (Holmgaard et al. 2013). Consistent with the results from preclinical models, a variety of clinical studies also demonstrate the synergism between IDO inhibitor and ICB. For instance, an open-label phase 1/2 trial showed that IDO inhibitor combined with PD-1 blockade produced encouraging antitumor activity without high-grade adverse effects in multiple tumors (Mitchell et al. 2018). In conclusion, it is promising to inhibit IDO and immune checkpoints simultaneously. Regrettably, a phase 3 trial demonstrated that IDO inhibitor failed to improve the efficiency of anti-PD-1 therapy in advanced melanoma. Therefore, combination of IDO inhibitor and ICBs still needs a long way to go.

5.4.4 Convert a "Cold" Microenvironment to a "Hot" One

Currently, the immediate challenge facing immunotherapy is how to convert "cold" tumor into "hot" tumor. "Cold" tumor means lacking immune cell infiltration, which is also referred to as low immunogenic tumor, while "hot" tumor means tumors with very dense T cell infiltration and are sensitive to immunotherapy. If there are no

specific-$CD8^+$ T cells within a tumor, ICBs is unlikely to work. There are multiple approaches to make immune "cold" tumors "hot", T-EVC and novel drug CMP-001 (TLR9 agonists) have received profound results in advanced melanoma patients, even though some patients did not response to anti-PD-1 antibody or had recurrence after treatment with PD-1 blockade (Jbag 2017). Surprisingly, tumor cell-intrinsic factor CXCL1 played a predominate role in T cells infiltration in TME, which provided an innovative strategy of combination with ablation of CXCL1 to promote tumor rejection.

5.5 Future Prospects of Immune Checkpoint Blockade Therapy

Management strategies for cancer patients have been largely transformed along with the advent of immunotherapy in recent decades. ICBs, as an innovative modality of immunotherapy, have been tested in a broad spectrum of tumor types. With unprecedented performance in clinical trials, ICBs have been approved by FDA for the treatment of malignancies such as melanoma, NSCLC, colorectal cancer, lymphoma and hepatocellular carcinoma. However, while ICBs are revolutionizing therapeutic algorithms for cancers, drug resistance remains a major barrier to a higher rate of and more durable clinical responses. No matter innate, adaptive or acquired, ICB resistance is a result of sophisticated interactions between cancer cells and the immune system. This chapter comprehensively summarized the resistance mechanisms of ICBs in cancer immunotherapy in the aspects of tumor-derived, T cell-based and TME-determined mechanisms, and discussed potential therapeutic strategies to overcome resistance. In order to maximize clinical benefits from ICBs, tools of precision medicine shall be utilized to select patients who are most likely to respond to ICBs, and to exploit tailored therapeutic settings, such as individualized dosing, intermittent administration and/or combinatory regimens of ICBs with other anti-tumor agents. Moreover, inhibitors of novel immune checkpoints shall be explored apart from CTLA-4, PD-1 and PD-L1.

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Chapter 6
Molecular Events Behind Adverse Effects

Shan Sun and Feng Wang

Abstract Immune checkpoint blockade (ICB) therapy has become a promising way of overcoming cancers, whereas the therapy can induce immunopathology due to the disruption of the immune homeostasis. These adverse events caused by ICB are named as immune-related adverse events (irAEs), which can be severe and life-threaten. Understanding the mechanisms and managements of irAEs is critical for improving the efficacy of immune checkpoint therapy. Immune-related adverse events can occur on various organs, and gastrointestinal tract has the highest rate for severe irAEs. Accumulated evidences indicate the ability of the gut microbiota in regulating the response to immune checkpoint therapy, but the function of microbiota in irAEs remains unclear. T cells, including functional subsets: Th17 T cells and regulatory T (Treg) cells, play significant roles in determining the inflammatory microenvironment. The gut immune tolerance toward dietary antigens and commensals, and anti-inflammatory function in intestines are maintained mainly by Treg cells. Furthermore, tissue residency of functional T cells depends on the homing/trafficking to the locations of inflammation. Here, we review the role of microbiota and the interaction between microbiota and intestinal Treg cells in irAEs, and discuss the function of gut-trafficking blockade antibodies in the context of ICB therapy.

Keywords IrAEs · Microbiota · Intestinal T cells · T cell metabolism · T cell homing

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6.1 Adverse Effects Induced by Immune Checkpoint Blockade

Recently, immune cancer therapy has become a promising way of overcoming cancers. In the tumor microenvironment, immunosuppressive molecules are markedly overexpressed. These immune inhibitors are named as immune checkpoints. Tumors can manipulate some of these pathways to evade immune destruction. Relying on the knowledge of immune checkpoints and immune response against tumor, researchers have developed agents targeting immune checkpoints such as cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4), programmed cell death protein (PD-1), and its ligand PD-L1. Targeting on the co-stimulatory signal of T cell activation, CTLA-4 is involved in inhibiting the response of naïve and memory T cells; PD-1 is related to the effector phase of T cell activation. Immune checkpoint antibodies have shown promising efficacy in tumor treatment by enhancing the antitumor immune response. However, blockade of immune self-tolerance molecules can induce immunopathology, which is named as immune-related adverse events (irAEs).

6.1.1 Overview of Immune-Related Adverse Effects (IrAEs) Induced by Anti-CTLA-4 and Anti-PD-1/PD-L1

Reports show that the frequency of irAEs is worthy of attention. Although irAEs occur in both anti-CTLA-4 therapy and anti-PD-1/PD-L1 agent treatment, the proportion of patients with irAEs is higher in CTLA-4 blockade condition. 19.9% of ipilimumab-treated advanced melanoma patients occurred irAEs, and the proportion for pembrolizumab-treated patients was 10.1–13.3% in a phase III trial (Robert et al. 2015). In a phase I study of 296 patients with advanced melanoma, non-small-cell lung cancer, castration-resistant prostate cancer, or renal cell or colorectal cancer after receiving PD-1 blockade antibody BMS-936558, 14% antibody receivers occurred irAEs and 5% of them discontinued treatment owing to treatment-related adverse events (Topalian et al. 2012).

Immune-related adverse events can occur on various organs. Immune-related adverse events most commonly affect gastrointestinal tract, skin, endocrine glands, and liver. The hypophysis, cardiovascular, pulmonary, and hematologic systems are less often involved in irAEs. It is reported that using the Common Terminology Criteria for Adverse Events is helpful to quantify irAEs.

Among irAEs grade 1–2, skin is the most frequent place for immunopathology (Michot et al. 2016). Dermatological disease, like vitiligo, is of high frequency in both CTLA-4 blockade therapy and PD-1/PD-L1 blockade therapy (Spain et al. 2016). It is reported that melanoma tends to have higher possibility to relate to vitiligo, comparing lung cancer or renal cancer after immune checkpoint blockade antibody treatment (Robert et al. 2015; Weber et al. 2015; Larkin et al. 2015). It

is more prevalent in ipilimumab-received patients to have pruritus, with the rate of 35%, while the rate in anti-PD-1 treated patients is 6–20% among several types of malignancies (Larkin et al. 2015; Rizvi et al. 2015). Severe skin autoimmune problems are reported to occur in combination immune therapy (Larkin et al. 2015).

Gastrointestinal tract has the highest rate for irAE grade 3–5, such as diarrhea, colitis, enteritis, and coeliac disease (Michot et al. 2016). It is important to distinguish diarrhea with colitis in clinic. Diarrhea is more common in irAEs. It is reported that 23–33% of ipilimumab receiving patients occur diarrhea and 8–19% of those treated with anti-PD-1 antibodies. The combination of ipilimumab and nivolumab therapy is reported with 44% proportions to occur diarrhea, and grade 3–4 diarrhea is most frequent in combination immune therapy (Robert et al. 2015; Weber et al. 2015; Larkin et al. 2015; Garon et al. 2015). Colitis is used to describe diarrhea related with abdominal pain, per rectal bleeding, or when imaging findings confirm large bowel inflammation, like lymphocytic and neutrophil inflammation with cryptitis. Mesenteric engorgement and bowel wall thickening also reported in immune checkpoint therapy-related colitis (Kim et al. 2013a). Enteritis and coeliac disease are relatively rare, although described in ipilimumab studies (Gentile et al. 2013; Venditti 2015).

Liver dysfunction can occur in some cases of immune checkpoint therapy treatment. Ipilimumab and anti-PD-1 antibodies are related with 1–7% hepatitis in patients, while the combination therapy is reported to induce 30% of patients with hepatitis (Robert et al. 2015; Weber et al. 2015). General pneumonitis is an uncommon side effect of immune checkpoint therapy, with the frequency of 2% in melanoma and around 5% in renal cancer and NSCLC. For anti-PD-1 antibody BMS-936558 therapy, the most common adverse events were fatigue, rash, diarrhea, pruritus, decreased appetite, and nausea, while in this case 3 of 296 antibody-receiving patients ended up with fatal pulmonary toxicity (Topalian et al. 2012). The highest rate of pneumonitis is 5–10%, reported in the combination of ipilimumab and nivolumab (Robert et al. 2015; Weber et al. 2015).

Knowledge about when irAEs onset is helpful. IrAEs typically occurs between 6 and 14 weeks after therapy initiation. Gastrointestinal irAEs tend to onset in the first 6–7 weeks after immune checkpoint antibody treatment. It is reported that diarrhea appears at 7 weeks after ipilimumab or nivolumab administration. Hepatitis is reported to onset between the first 6 and 14 weeks.

6.1.2 Clinical Significance of Ameliorating IrAEs During Immune Checkpoint Blockade

9.4% of ipilimumab treatment-related irAEs can lead to therapy discontinuation (Robert et al. 2015). After receiving ipilimumab at a dose of 10 mg/kg, administration of steroids did not appear to alter clinical benefit, and 1% of patients is reported to death due to therapy-related intestinal dysfunction (Spain et al. 2016; Eggermont et al. 2015; Kwon et al. 2014).

It is of great importance to manage these therapy-induced inflammatory outcomes. As toxicity becomes more severe, management is focused on preventing life-threatening bowel dysfunction. In most cases of immune checkpoint inhibitor-related colitis, the appropriate treatment is immunosuppressive therapy, including corticosteroids, agents targeting tumor-necrosis factor-α (TNF-α), the anti-metabolite mycophenolate mofetil, and calcineurin inhibitors to limit interleukin 2 (IL-2) transcription. Steroids are administered when the severity of an irAE warrants reversal of inflammation. But if the irAEs recur despite treatment, the discontinuation of the immune checkpoint therapy must be considered.

When the first immune checkpoint therapy has been discontinued due to severe irAEs, whether the second treatment can be helpful for tumor suppression in patients remains elusive. Most of patients who had irAEs grade 3 or 4 are not included in further immune checkpoint therapy trails, although the clinic practice and judgment are based on physicians. An interval of at least 4 weeks is necessary when changing different immune checkpoint blockade agents. A pooled analysis indicates that nivolumab treatment after ipilimumab seems to be safe, while ipilimumab treatment after anti-PD-1 treatment appeared to relate with atypical irAEs (Horvat et al. 2015; Danlos et al. 2015). But the reports hardly draw the conclusion currently.

IrAEs is critical for early detection and management for patients receiving immune checkpoint therapy. Researchers have approaches with combining clinic observations to imaging methods to diagnose irAEs accurately. Most irAEs are reversible with steroids, if the management is treated with a sufficient dose level in the initiation of irAEs. Clinical phase 2 studies showed that early administration of corticosteroids is important to the management of irAEs.

Communication with patients timely also contributes to proper management of irAEs and outcome of immune checkpoint therapy. An informed discussion with patients about the toxicities and benefits of immune checkpoint therapy is helpful for patients to prepare for the toxicity. Also, regular communications between patients and physicians may be useful in early identifying of signs and symptoms, promoting intervention and probably reducing the progression of adverse events. Appropriate education on patients is critical to maximize the possibility of patient early signs recognition (Wood et al. 2019).

6.2 The Role of Microbiota in Immune Checkpoint Blockade-Related IrAEs

Mammals have trillions of gut bacteria. Gut bacteria co-exist with the host and are highly related to the digestive system and immune system. Considering the influences of these bacteria on the host, gut bacteria can be divided into two groups: the pathogens that can lead to infection and inflammation and the bacteria that help the host digest food, act as a barrier for pathogen defence, and regulate the development of the immune system (Kato et al. 2014).

Host genetics, diet choices, diseases, and treatments influence the gut environment and alter the bacterial composition (Faith et al. 2013). Alterations in the gut environment might increase pathogens and induce inflammation. Through exposure to various complex bacterial antigens, the gut evolves the accurate innate and adaptive immune systems against non-self-antigens and self-antigens. Accumulating evidences show the function of the gut microbiota in modulating host carcinogenesis and the antitumor immune response.

6.2.1 *Microbiota Changes During Immune Checkpoint Blockade Therapy*

The accumulating evidence shows the ability of the gut microbiota to regulate the response to immune checkpoint therapy. Antibiotic-treated or germ-free tumor-bearing mice do not respond to anti-CTLA-4 antibody therapy. In a study assessing how bacteria regulate anti-CTLA-4 therapeutic efficacy, researchers found that anti-CTLA-4 antibodies lose control of tumor progression in germ-free mice and antibiotic-treated mice. CTLA-4 treatment altered the bacterial composition with significant increases in the relative abundances of *Bacteroides* species, such as *B. thetaiotaomicron* and *B. fragilis*. The colonization by *Bacteroides* species enhanced host antitumor immunity by affecting *Bacteroides*-specific T cells. Fecal microbial transplantation (FMT) from melanoma patients receiving anti-CTLA-4 therapy favored the outgrowth of *B. fragilis* with anticancer properties, revealing the immune-stimulatory role of bacteria in anti-CTLA-4 therapy (Kwon et al. 2014).

Gut microbiome diversity is associated with the immune response and cancer progression (Drewes et al. 2016; Garrett 2015). Low intestinal bacterial diversity is also associated with poor outcomes of cancer therapies, such as allogeneic stem cell transplantation (Taur et al. 2014). Specific gut-resident bacteria are beneficial in immune checkpoint therapy responses. In a recent clinical study, researchers compared microbiome samples from metastatic melanoma patients collected at the start of anti-PD-1 treatment and approximately 6 months after treatment initiation. Higher gut microbiome diversity was found in the treatment responders who achieved an objective response than in the nonresponders who showed progressive disease or stable disease lasting less than 6 months. Compositional differences in the gut bacteria populations were also associated with responses to anti-PD-1 treatment. The responders harbored an enrichment of *Faecalibacterium* species, while the nonresponders showed enrichments of *B. thetaiotaomicron*, *Escherichia coli*, and *Anaerotruncus colihominis*. The authors suggested that a favorable gut microbiome composition is related to enhance systemic and antitumor immunity by presenting data showing that an enhanced $CD8^+$ T cell density in the peripheral blood mononuclear cells (PBMCs) from the anti-PD-1 therapy-treated patients correlated with the increased abundance of *Faecalibacterium*. Transplanting gut bacteria from the responders to germ-free mice also increased the $CD8^+$ T cell density and enhanced the antitumor immune

response. An unfavorable gut microbiome composition was associated with impaired systemic and antitumor responses that shaped the responses of the nonresponding patients to anti-PD-1 immunotherapy (Gopalakrishnan et al. 2018).

Similarly, Matson et al. found a great abundance of favorable bacteria (*Bifidobacterium longum*, *Collinsella aerofaciens,* and *Enterococcus faecium*) in the gut of responding melanoma patients (Matson et al. 2018). Routy et al. found that an antibiotic consumption-induced abnormal gut microbiome composition led to anti-PD-1 therapy resistance in mouse tumor models and epithelial tumor patients. For NSCLC and renal cell carcinoma (RCC), nonresponding patients showed a lower abundance of *Akkermansia muciniphila*, which is a species that can restore the response of antibiotic-treated mice to anti-PD-1 antibodies (Routy et al. 2018). The evidence reveals that a favorable gut microbiome helps to enhance the host immune response to immune checkpoint therapy.

Bifidobacterium are gram-positive anaerobic species that are considered beneficial. Previous studies found that *Bifidobacterium* species have efficacy in treating IBD. A recent study compared B16 melanoma progression in two different facilities, Taconic Farms (TAC) and The Jackson Laboratory (JAX) and found differences in tumor progression. The authors then identified *Bifidobacterium* species as candidate drivers of tumor rejection in the JAX mice (Sivan et al. 2015). In study of melanoma patients who responded efficiently to anti-PD-1 therapy, the relative abundance of *Bifidobacteriaceae* operational taxonomic units (OTUs) was abundant, as suggested by the 16S rRNA sequence and shotgun sequence. Responders had enrichments in *Bifidobacterium longum*, *Collinsella aerofaciens*, and *Enterococcus faecium* in the gut. Fecal material from the responders enhanced the anti-PD-L1 response and $CD8^+$ T cell infiltration in a mouse model of melanoma (Matson et al. 2018). The evidence suggests a beneficial role for gut commensals in modulating the outcome of immune checkpoint therapy.

6.2.2 Correlation of Microbiota Status and Severity of IrAEs

The intestinal microbiota affect the development of inflammatory disorders in anti-CTLA-4 therapy. Wang et al. found that antibiotic administration affects the sensitivity toward CTLA-4 blockade-related toxicity in mice (Wang et al. 2018a). Metastatic melanoma patients treated with CTLA-4 blockade therapy that progress to colitis harbor bacterial compositions distinct from those who are colitis free (Dubin et al. 2016). The complex intestinal microbiota must maintain a balance between beneficial microbes and pathogens to maintain host immune tolerance and appropriate inflammatory responses.

The composition of the gut microbiome modulates immune checkpoint therapy-related immunopathology. Chaput et al. found that among metastatic melanoma patients receiving ipilimumab, those who progressed to colitis tended to harbor a gut microbiome most related to *Firmicutes*. The *Faecalibacterium*-driven microbiome community also caused a low proportion of Tregs in patient peripheral blood.

Bacteroidetes species were related to phylotypes without colitis development and extended PFS and OS, which is consistent with previous works (Chaput et al. 2017; Vetizou et al. 2015).

Recent clinical trials have reported the beneficial function of bacteria in immune checkpoint-associated colitis by FMT. Researchers transplanted fecal samples from healthy donors into patients with refractory immune checkpoint therapy-associated colitis. In this trial, one patient received a combination of CTLA-4 and PD-1 blockade (NCT1928394) and developed severe colitis, while the other patient received ipilimumab after chemotherapy and hormonal therapy and showed Crohn's colitis-like presentation with fever and diarrhea. FMT treatment altered the immune infiltrates in the patients' colonic mucosa, decreasing the $CD8^+$ T cell density and increasing the Treg density. The microbiomes in these patients were also altered by FMT treatment, with an expansion of *Bifidobacterium*, which was reported to ameliorate anti-CTLA-4 therapy-related intestinal immunopathology. After FMT, the gut microbiome of one patient showed an immediate enrichment in *Akkermansia*, which are considered beneficial bacteria for an efficient response to anti-PD-1 therapy (Routy et al. 2018; Wang et al. 2018a, b). *Clostridium difficile*-associated colitis and IBD can also be successfully treated by modulating the gut microbiome via FMT, suggesting that FMT can serve as an additional approach to ameliorate irAEs during immune checkpoint therapy (Borody and Khoruts 2011).

6.3 Interaction Between Immune System and Microbiota Under Immune Checkpoint Blockade Condition

The intestine is the largest surface of the body that is constantly exposed to dietary and bacterial antigens. The gastrointestinal tract of mammals harbors a complex bacterial community containing trillions of members, comprising commensals and pathogens. The complexity of microbiota is matched with the complexity of the host immune system. An efficient host immune system is essential for maintaining the delicate balance between immune tolerance toward commensals and effective immune response against pathogens. In addition to the intestinal homeostasis maintenance, the innate immunity and the adaptive immunity have critical role in establishing the complex bacterial communities in gut.

6.3.1 Overview of Effects of Immune System on Microbiota Community

The innate immune system relies on the immune cells that code pattern recognition receptors (PRRs) to sense microorganisms by conserved molecular structures. Several families of PRRs and their signaling pathways are now known, including the toll-like receptors (TLRs), the nucleotide-biding oligomerization (NOD)-like receptors (NLRs), the RIG-1-like receptors, the absent in melanoma 2 (AIM2)-like receptors, and OAS-like receptors (Thaiss et al. 2016a, b). A variety of cells has the ability to express these sensors that contribute to not only a continuous surveillance system for the presence of microbiota but also a symbiotic relationship between host and microbiota. The observation of a diminished microbiota in myeloid differentiation factor 88 (MyD88)-deficient mice that lack the key adaptor protein in TLR signaling marked the importance of innate immune system for microbiota community (Rakoff-Nahoum et al. 2004). The microbiota in TLR-5-deficient mice were also reported significantly different from that in wild-type (WT) mice, although *Firmicutes* and *Bacteroides* were similar relative abundance in the gut of both TLR-5-deficient and WT mice (Kato et al. 2014; Vijay-Kumar et al. 2010). In addition to TLR signaling, NOD containing protein 1 (NOD1)-deficiency led to aberrant bacterial expansion of specific bacteria, like *Clostridiales*, segmented filamentous bacteria (SFB), *Bacteroides* and *Enterobacteriaceae*. NOD2 is reported to promote cytokines secretion and antimicrobial peptides production, which cause the alteration of microbiota community. NOD2 deficiency resulted in decreasing the expression of antimicrobial peptides like α-defensins and RegIIIγ, changing the composition of gut microbiota. The deficiency of NOD2 was found to induce the outgrowth of *Bacteroidetes*, which can also expand in NLRP6-deficient condition (Elinav et al. 2011; Kobayashi et al. 2005; Vaishnava et al. 2008). The innate immune system is critical for eliminating gut pathogens and keeping the ecological balance of gut microbiota community.

The adaptive immune system is critical for gut homeostasis. The adaptive immunity has its unique strategy to sensing antigens and response efficiently, which are the key molecules like B and T cell receptors and co-stimulatory signals, the major histocompatibility complex (MHC) family members, and the transposable element-containing Rag genes. The colitis observed in T cell receptor (TCR) alpha mutant, TCR beta mutant, and class II MHC mutant mice indicated that dysfunction of mucosal T cell response led to intestinal pathogenesis (Mombaerts et al. 1993). Lack of adaptive immune system affects the microbiota significantly. Rag-1-deficient mice that lack the adaptive immunity were reported to harbor significantly lower microbiota diversity with skewed community of an expansion in *Firmicutes* and *Deferribacteres* and a decrease in *Bacteroidetes* compared to WT mice (Kato et al. 2014).

B cells and T cells are the key mediators in mucosal adaptive immune response. T cells are critical for intestinal homeostasis and inflammation. Early studies characterized $CD4^+$ T cells into T-bet^+ Th1 cells, $Gata3^+$ Th2 cells, $Rorgt^+$ Th17 cells, and $Foxp3^+$ regulatory T cells. Among different T cell subtypes, Th17 cells and

Treg cells are higher dominant in intestine, mainly resident in intestine lamina propria (LP). Th17 cells preferentially locate in the intestinal LP of small intestine and colon, where they produce IL-17A. The Th17 cells were absent in antibiotics-treated condition or germ-free housing mice illustrated that gut microbiota was required for Th17 cell differentiation (Ivanov et al. 2008). Accumulating evidence shows that intestinal Th17 cells is highly related with specific microbiota, like segmented filamentous bacteria (SFB). SFB belong to a nonculturable group of *Clostridia*-related Gram-positive bacteria and are commonly colonized in the flora of terminal ileum in mouse colonies. SFB can regulate the development of Th17 cells and coordinated pro-inflammatory T cell response in intestine, while Th17 cells control SFB burden in feedback loop (Gaboriau-Routhiau et al. 2009). Disruption of IL-17 signaling by IL-17R-deficient in the enteric epithelium caused SFB dysbiosis owing to reduced expression of α-defensin, polymeric immunoglobulin receptor (Pigr), and NADPH oxidase 1 (Nox1) (Kumar et al. 2016).

The Forkhead box P3 (Foxp3) expressing regulatory T cells are dominant in intestinal lamina propria (LP) and function as important roles in gut homeostasis and immune tolerance. Mice deficient in a regulatory region of the Foxp3 promoter and lacking the peripherally induced subset of $Foxp3^+$ cells shown an overall decrease in the ratio of *Firmicutes* to *Bacteroidetes* (Josefowicz et al. 2012). Adoptive transfer $Foxp3^+$ cells to CD3-deficient mice led to the increase of gut microbial diversity. $Foxp3^+$ cells are considered to be required for bacterial diversity and *Clostridia* species abundance in gut (Kato et al. 2014).

6.3.2 *Effector Strain-Induced Global Microbiota Change Under Immune Checkpoint Blockade*

The gut bacteria form a complex community consisting with pathogens, bacteria from food intaking, and commensals. Bacteria groups change dynamically during the change of dietary habit, treatment of antibiotics, and onset of diseases. The domain bacteria among the trillions of microbes in host has the most important role in shaping the gut bacteria community. Current studies compared healthy adults in the USA and Europe and found that around 100 species of gut bacteria colonized in those respondents. The colonized gut bacteria were of various combinations and could be responsible for various functions and effects (Faith et al. 2010; Qin et al. 2010). This study suggests the importance to identify some "effector strains" that individually or in concert influence the biological functions, from the vast number of possible dominant species.

Studies with mouse models have found some potential "effector strains" that affect immune system development and pathogenesis. After being characterized as an effector strain to modulate Th17 cell development, SFB was found to alter bacteria composition. Researchers found the bacterial taxa in gut of conventionally raised C57BL/6 mice from Jackson Laboratory facility and C57BL/6 mice from Taconic

Farms are significantly different, with an enrichment (>25 fold) of SFB in Taconic Farms-raised mice (Ivanov et al. 2008, 2009). In anti-inflammatory cytokines transforming growth factor β (TGF-β) and IL-10-deficient mice that are spontaneously mouse models for inflammatory bowel disease (IBD), *Bacteroides thetaiotaomicron* was causally associated to gut inflammatory, but its relative abundance among gut microbiome was not significantly different between noninflammatory and inflammatory states. The spontaneous colitis state in this study was related with bacteria abundance alteration with an enrichment (>100 fold) of commensal Enterobacteriaceae, a bacteria family of Gram-negative facultative anaerobes. Even though Enterobacteriaceae was not sufficient for disease induction, the study suggested its role in shaping gut bacteria community after antibiotics administration (Bloom et al. 2011). Garret and colleagues applied *T-bet*$^{-/-}$ x *Rag2*$^{-/-}$ ulcerative colitis (TRUC) model to investigate the relationship between individual intestinal bacteria and entire microbial community. The presence of *Klebsiella pneumoniae* and *Proteus mirabilis* were related with colitis in TRUC mice in which the fecal microbial communities were characterized. A maternally transmitted endogenous microbial community for maximal intestinal inflammation helped TRUC-derived strains elicit colitis in *Rag2*$^{-/-}$ and WT mice, illustrating the correlation between gut microbial communities and individual species in colitis (Garrett et al. 2010).

Bacteroidetes and *Firmicutes* are identified as the majority of gut commensals in healthy adults, but the relative abundance of individual strains or even of the phyla varies greatly from person to person (Skelly et al. 2019; Lozupone et al. 2012). In the context of immune checkpoint therapy, the response also varies greatly from person to person, which limits the application of this promising immune cancer therapy. Previous studies show that microbiome involves in the response efficacy of immune checkpoint blockade therapy. Vetizou et al. found that the administration of anti-CTLA-4 antibody altered the gut bacteria composition. The colonization of "good" bacteria, like *B. thetaiotaomicron* or *B. fragilis*, to germ-free mice improved the response to anti-CTLA-4 mAb. The results demonstrated that specific commensals have the possibility to promote the response efficacy, which might function through orchestrating the gut bacteria community (Viaud et al. 2013). Additionally, Sivan and colleges found that mice harbored different gut bacteria community from TAC and JAX facilities had different tumor progression states after receiving anti-PD-1 antibodies, which indicate that some benefit bacteria shaped the microbiota community and regulated the treatment response (Sivan et al. 2015). In human samples, the distinct bacteria composition between response patients and nonresponders also suggested that the potential benefit commensals exist in the intestines and promote the response efficacy of immune checkpoint therapy (Fig. 6.1).

Though specific microbiota configurations are related with disease, defining the composition of a healthy microbiota has proved difficult. In the further studies, researchers may take the specific bacteria strains into considerations and identify the function of the bacteria in depth, which would contribute to the application on both researches and clinical treatments.

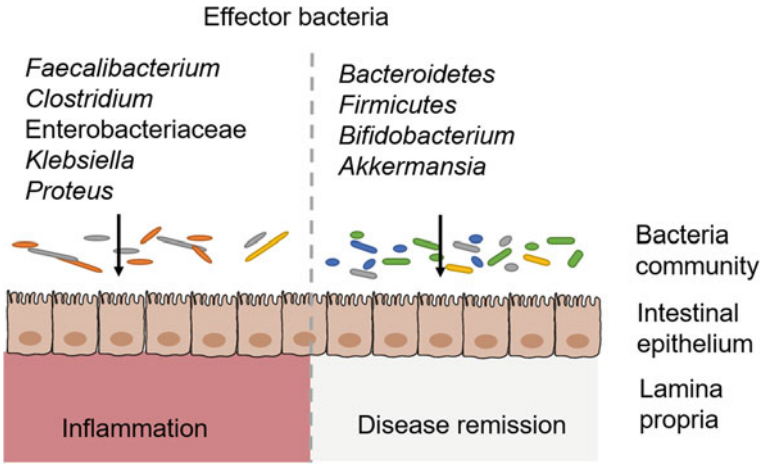

Fig. 6.1 The microbiome community affects the response to immune checkpoint therapy. "Harmful strains" like *Faecalibacterium* and *Clostridium*, were shown to change the bacteria community and result in inflammation. "Beneficial strains" like *Firmicutes* and *Bifidobacterium* were reported to be associated with disease remission or homeostasis

6.4 Regulatory T Cell and Its Metabolism Under Immune Checkpoint Blockade Condition

6.4.1 Overview of Treg Function and Metabolism

Treg cells are a subpopulation of $CD4^+$ T cells. The study that deficiency of $CD25^+$ subpopulation of $CD4^+$ T cells led to the spontaneous multi-organ autoimmunity revealed the immune suppressive function of these cells (Sakaguchi et al. 1995). Following the development of a $CD4^+CD45RB^{hi}$ T cell transfer model of IBD, Fiona Powrie and colleagues then discovered that co-transfer of $CD4^+CD25^+$ T cells could suppress the colitis symptoms (Mottet et al. 2003). Subsequently, researchers identified Foxp3 as a crucial transcription factor for Treg cells. Foxp3-mutate mice were found to have spontaneous multi-organ autoimmunity and Foxp3 was then clarified as the key reason for the above autoimmune disorders (Fontenot et al. 2003). Constitutive presence of Treg cells was required to prevent autoimmune inflammation (Mayer et al. 2014). It is well established that $Foxp3^+$ $CD4^+$ Treg cells are central to maintain the immune homeostasis and tolerance.

Treg cells exist in every organ of the body and are around 10% of the total $CD4^+$ T cell population. In intestinal LP, Treg cells constitute a higher proportion: approximately 30% of $CD4^+$ T cells in the lamina propria of colon and about 20% in the LP of small intestinal (Geuking et al. 2011; Round and Mazmanian 2010; Tanoue et al. 2016). To regulate the gut immune response, intestinal Treg cells apply a variety of functional pathways, including CTLA-4, T cell co-stimulator (ICOS), IL-10, TGFβ,

and IL-35 (Collison et al. 2007; Herman et al. 2004; Li et al. 2007; Rubtsov et al. 2008; Wing et al. 2008). Besides, Treg cells contribute to immune tolerance against dietary components and commensals by inhibiting effector T cells. Treg cells can suppress bystander effector T cells and also mediate the population of T follicular helper cells (Tfh cells) and germinal center (GC) responses to regulate intestinal Immunoglobulin A (IgA) production (Cong et al. 2009; Kawamoto et al. 2014; Zheng et al. 2009). The intestinal Treg cells have important roles in regulating the gut microbiome. The reduction of colonic Treg cells resulted in the susceptibility to pathogen infection in mice (Maloy et al. 2003). The Treg cells are showed to contribute to the diversification of commensals, such as the species belonging to Firmicutes (Kawamoto et al. 2014).

The major population of Treg cells are thymus-derived Tregs (tTreg), a percentage of Treg cells are generated from $Foxp3^-$ T cells in peripheral sites, which has been termed as pTreg (Curotto de Lafaille and Lafaille 2009). The presence of TGFβ can promote the generation of Foxp3-expressing cells from Tconv cells in vitro. The antigen activation in the intestine can enhance the expression of Foxp3 in naïve T cells (Coombes et al. 2007). Studies have identified that the microenvironment of intestine is suitable for pTreg development by identifying the peripheral conversion of naïve T cells into Treg cells in gut-associated lymphoid tissue (GALT) after exposing to antigens (Sun et al. 2007). The bacterial and dietary antigens are the main inducer of pTreg cell development in the intestine (Tanoue et al. 2016). To explore the functions of different types of Treg cells, it is necessary to distinguish tTreg cells and pTreg cells. Researchers have found potential specific markers, Helios and Neuropilin 1 (NRP1). The nuclear protein Helios and cell surface protein NRP1 are expressed by tTreg cells but not by in vitro-induced Treg cells, leading to the specific identifier for tTreg cells ($Helios^+NRP1^+Foxp3^+$) and pTreg cells ($Helios^-NRP1^-Foxp3^+$). The abundance of $Helios^-NRP1^-Foxp3^+$ cells in intestinal LP indicates that pTreg cells may participate in gut homeostasis. Additionally, the absence of gut bacteria reduced the population of $Helios^-NRP1^-Foxp3^+$ cells in the colon of germ-free mice and the small intestine of antigen-free mice (Kim et al. 2016; Weiss et al. 2012). These findings suggest a potential role of pTreg cells in participating immune tolerance toward gut microbiota.

The maintenance of Treg cell function requires proper metabolism state. During T cell development, oxidation provides the ATP for naïve T cells to migrate from the thymus, which involves oxidative phosphorylation (OXPHOS) in the mitochondria and fatty acid oxidation (FAO). T cell activation demands higher energy than T cell development, leading T cells to enhance nutrient uptake and to change the metabolism pathways. Once the activation onsets, $CD4^+$ T cells shift their metabolic pathway from oxidation to an anabolic state by enhancing the glycolysis and mitochondria metabolism. This process helps T cell to transfer glucose to pyruvate and generate metabolic intermediates, promoting T cell growth and proliferation. Different from effector T cells that rely mostly on glycolysis, Treg cells mainly use OXPHOS and FAO for their metabolic phenotype. Additionally, activation signal of T cells trigger downstream pathways like PI3K/Akt/mTORC1, the pentose phosphate pathway (PPP), and AMP-activated kinase (AMPK) pathway.

6.4.2 Treg Function and Metabolism Change in Immune Checkpoint Blockade Condition

T cells, along with other immune cell types like macrophages and natural killer cells (NKs), play significant roles in tumor microenvironment. T cells are the most important immune regulators in attacking specific antigen-expressing tumor cells. This process requires TCR to recognize tumor-derived peptides binding to MHC and the activation of co-stimulatory signaling. The mediation between T cell activation and tolerance is critical for a proper antitumor immune response, though it is complicated.

The immune checkpoint therapies mainly target on the co-stimulatory signaling on T cells. CTLA-4, the homology to co-stimulatory receptor CD28, has been applied in treating various cancer types clinically, including advanced melanoma, prostate cancer, and renal cell carcinomas with significant improved PFS and OS. The low responding rate of anti-CTLA-4 therapy limits its application. It is important to understand the mechanism of how the immune cell functions in the immune checkpoint blockade condition. CTLA-4 is constitutively expressed by the Treg cells and Teff cells after activation. Treg cells use CTLA-4 to inhibit the downstream CD80/CD86 on antigen-presenting cells, and downregulate the activation of Teff cells. The deficiency of CTLA-4 affected the stability of Foxp3 of Treg cells, which leads to the dysfunction of Treg cells. The specific depletion of CTLA-4 in Treg cells resulted in hyperproliferation of Teff cells systematically and multi-organ autoimmune diseases (Wing et al. 2008). The results suggest that CTLA-4 function is relatively specific to Treg cells, and Treg-expressed CTLA-4 is essential for maintaining immune tolerance.

Researchers thought that the treatment of anti-CTLA-4 antibody upregulated the activation and function of tumor-infiltrating T cells at first. Accumulating evidences show the possibility that Treg cells could be dominantly influenced by CTLA-4 blockade to enhance host antitumor immune responses. With Fc-receptor-deficient mouse model, researchers also found that besides regulating the activation of Teff cells, the cellular toxicity of tumor-infiltrating Treg cells contributes to the antitumor efficacy of anti-CTLA-4 antibodies in tumors such as melanoma and colon adenocarcinoma (Bulliard et al. 2013; Simpson et al. 2013; Selby et al. 2013). A preclinical trial in patients with localized bladder cancer revealed that ipilimumab administration markedly increased the expression of ICOS on $CD4^+$ T cells, leading to an increase in the ratio of Teff cells to Treg cells, in peripheral blood and tumor tissues (Liakou et al. 2008).

Another immune checkpoint molecular PD-1 also has the influence on Treg cells. Anti-PD-1 antibodies enhance host antitumor immune response by suppressing the transduction of activate TCR signal and co-stimulatory signals in Tconv cells and resulting in their dysfunctional or exhausted state. Previous studies found that Treg cells in tumor microenvironment were found to express PD-1 comparable to Teff cells, and the activation of Treg cells relies on TCR and co-stimulatory signaling, suggesting that PD-1 might regulate the activation or function of Treg cells. The deficiency of PD-1 could increase the immunosuppressive function of Treg cells,

which success to rescue the autoimmune pancreatitis in mice (Togashi et al. 2019). With the samples from patients who received anti- PD-1 mAb nivolumab as gastric cancer treatment, researchers found that anti-PD-1 treatment decreased the immunosuppressive function of Treg cells (Togashi et al. 2018). The studies suggested the important role of PD-1 blockade in downregulating the activity of Treg cells.

Further studies on how immune checkpoint regulates immune cell activity would contribute to predict the response efficacy of treatment and guide the strategy of clinical therapies.

6.4.3 Treg's Role in Ameliorating Gut Inflammation Under Immune Checkpoint Blockade Condition

CTLA-4 is a crucial regulator in host immune homeostasis. As discussed above, anti-CTLA-4 has the promising efficacy in enhancing antitumor immune response, along with high risk in fatal adverse events, particularly gut inflammation. The gut immune tolerance toward dietary antigens and commensals is performed mainly by intestinal Treg cells.

The function of Treg cells in immune checkpoint blockade antibody-related gut dysfunction is unclear. Wang et al. found that Treg cells participate in the immune checkpoint therapy-related gut inflammation in mice. After pretreating vancomycin to disturb the gut microbiome in mice, researchers found that treatment of a commensal, *Bifidobacterium*, led to the remission of CTLA-4-induced colitis. During this period, Treg cells were indispensable, as Treg cell-deficient mice showed severe colitis after *Bifidobacterium* administration (Wang et al. 2018a). This study demonstrates the essential role for Treg cells in regulating the CTLA-4-related gut inflammation.

The regulation mechanism of Treg cells on the *Bifidobacterium*-induced colitis remission remains elusive. Our unpublished work found that *Bifidobacterium* can alter the gut bacteria community in genera level, which depended on the presence of Treg cells. In the absence of Treg cells, *Bifidobacterium* failed to increase the abundance of major beneficial gut bacteria, like *Lactobacillus*. Meanwhile, the treatment of *Bifidobacterium* enhanced the functional metabolism of colon Treg cells, which contributed to the amelioration of intestinal inflammation and the maintenance of immune homeostasis. This work further clarifies the mechanism of the interaction between gut microbiota and immune system in the context of CTLA-4 blockade condition (Fig. 6.2).

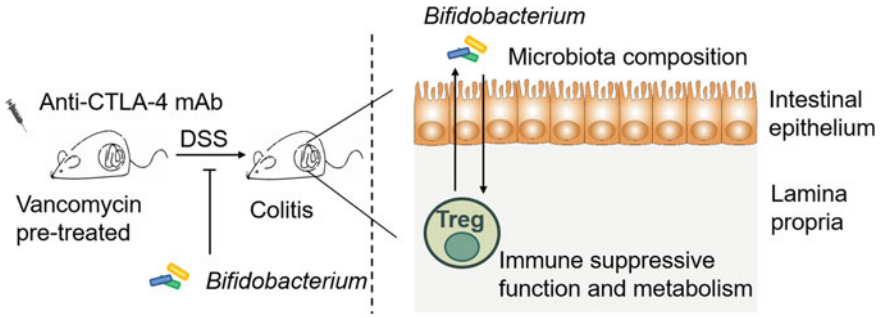

Fig. 6.2 *Bifidobacterium* alters the outcome of anti-CTLA-4 therapy in a Treg-dependent manner. *Bifidobacterium* ameliorates the CTLA-4-related colitis by altering the microbiota composition. Treg cells are required for this modulation process, and the function and metabolism of LP Treg cells are influenced by *Bifidobacterium*-induced microbiota alteration

6.5 T Cell Trafficking and Its Effect Under Immune Checkpoint Blockade Condition

6.5.1 Overview of T Cell Gut Homing

Immune responses depend on the trafficking of immune cells to the locations of diseases. During an inflammatory response, circulating immune cells migrate to the target tissues through a homing process. This process begins with the capture of immune cells in bloodstream and rolling to a specific place. Then the immune cells arrest and adhere to vascular endothelial cells, and finally undergo trans-endothelial migration. This process is mediated by the specific interactions of immune cells and adhesion molecules that are normally expressed by endothelial cells (Ley et al. 2007).

T cell migration across vascular endothelium is essential for T cell response. Through the specific tissue-homing receptors, T cells access peripheral tissues aiming to eliminate invading pathogens or tumor cells. The T cell homing to small and large intestines is important to gut homeostasis, mediate immune response, and regulate inflammation. Researchers have studied the mechanisms of T cell recruitment to intestines and GALT for decades. T cells are normally stimulated by antigens in draining lymph nodes, and upregulate selective homing molecules that allow them to migrate to specific extra-lymphoid tissues. The homing process of T cells is important in both homeostasis and inflammatory conditions. This process is highly regulated as homing molecules help T cells migrating to different tissues and within tissues to specialized microenvironments (Habtezion et al. 2016).

When the priming and activation occurs in the GALT, gut-homing programs of T cells onset by first upregulating the expression of integrin α4β7 and chemokine receptor CCR9. Integrin α4β7 and CCR9 are identified as essential molecules that can direct T cells to the intestine LP (Habtezion et al. 2016). Studies by adoptive transfer and intravital microscopy found that the small intestine lamina propria T cells, but

not spleen T cells, can bind to the small intestine via the binding of integrin α4β7 and CCR9. The ligand of CCR9 is the chemokine CCL25, which is produced by the small intestine epithelium. The ligand of α4β7 is Mucosal Addressin Cell Adhesion Molecule-1 (MADCAM-1), which is expressed by lamina propria. The expression of α4β7 and CCR9 in activated effector and regulatory lymphocytes could be regulated by mucosal dendritic cells in a retinoic acid-dependent manner. The efficient homing of lymphocytes relies on these migration molecules. A study found that the expression of CCL25 increased from the distal to the proximal small intestine in mice, and T cells can migrate to the distal small intestine with the CCR9-independent manner (Stenstad et al. 2007). To the large intestine that expresses little CCL25, few colon T cells express CCR9, and CCR9 is not required for the homing of T cells to the large intestine (Lazarus et al. 2003). Instead, the colon epithelium expresses the chemokine CCL28 to bind to its receptor CCR10, which mediates the trafficking of plasmablasts. Both naïve and effector T cells apply α4β7 integrin to interact with MAdCAM-1, and then to migrate from blood to gut mucosal tissues, like GALT, MLN, and LP (Gorfu et al. 2009).

Treg cells that homing to the small intestine also express CCR9 and integrin α4β7 in the MLN. The homing of Treg cells to the intestine provides the tolerance to mucosal antigens. The upregulation of integrin α4β7 of circulating Treg cells is related to the decreased risk of intestine graft-vs-host disease, but few circulating Treg cells are reported express α4β7. In the naïve T cell transfer-induced colitis model, Treg cells lead to remission of the gut dysfunction. During this process, CCR7 rather than β7 integrins is required for Treg cells (Schneider et al. 2007). In the *Citrobacter rodentium*-induced colitis model, Treg cells contribute to the resolution of inflammation (Kim et al. 2013b). In mice with chronic ileitis, CCR5 was reported to participate in the migration of Treg cells to the inflamed location of the intestine (Kang et al. 2007).

Additionally, for colon T cells, the orphan G-protein-coupled receptor 15 (GPR15) takes the responsibility to direct T cells. The preferential expression of *Gpr15* in colon memory $CD4^+$ T cells initially suggested the role of GPR15 in lymphocyte migrating. GPR15-mediated Teff homing to colon is required for pathogenesis in T cell transfer model, where the homing of Teff cells to colon is critical for disease onset (Nguyen et al. 2015). The knowledge of the mechanisms on T cell migration may guide the development of strategies to induce T cell homing to particular compartments where T cells are necessary to protect host from particular infections or tumors.

6.5.2 Effects of Traffic-Blocking Antibody in Immune Checkpoint Blockade Condition

As discussed above, the treatment of immune checkpoint antibodies has their promising potential to improve the therapy of advanced malignancies but is also related to a substantial risk of immune-related adverse events. The gastrointestinal toxicity

is one of the most frequent immune-related adverse events. Researchers are focusing on ameliorating the gastrointestinal toxicity to improve the outcome of immune checkpoint therapy.

As the gastrointestinal toxicity of immune checkpoint blockade therapy assembles the symptoms of IBD, the treatment of IBD could provide ideas for immune-related adverse gut dysfunction. Besides the conventional treatments that target inflammation-related pathways or biologics like anti-TNF agents used in IBD treatment, the leukocyte trafficking blockade antibodies can inhibit the homing to the inflamed sites. By targeting the actions of integrin, these agents selectively prevent the intestinal recruitment of lymphocytes to the site of inflammation (Danese and Panes 2014). Clinical studies with blockade antibody integrin α4β7 and MAdCAM-1 are proving the value of lymphocyte trafficking mechanisms as therapeutic targets for IBD. These humanized monoclonal IgG antibodies approved by U.S. FDA can decrease the trafficking immune cells to the endothelium and suppress the recruitment of inflammatory cells such as to intestinal lesions. In the GEMINI studies, the α4β7 blocking antibody, Vedolizumab, contribute to the remission in both Crohn's disease (CD) and ulcerative colitis (UC) by specifically targeting the gut-trophic α4β7 heterodimers and inhibiting lymphocyte trafficking selectively in the intestine (Wyant et al. 2015).

The effects of integrin blockade antibody in immune checkpoint blockade condition remain unclear, but the application of trafficking blockade antibodies in IBD suggests the possibility to use them in ameliorating immune checkpoint-related adverse events. A recent clinical trial has adapted Vedolizumab in the treatment of immune checkpoint blockade antibody-induced enterocolitis. Researchers have found the successful resolution on the intestinal inflammation in patients who had received ipilimumab or nivolumab therapy. Though the administration of Vedolizumab was after corticosteroids, a common treatment for irAEs, this study highlighted the possibility that trafficking blockade antibody may play a key role in the gastrointestinal toxicity remission under the immune checkpoint blockade condition (Fig. 6.3). Further studies need to be conducted to clarify the function of various types of gut-trafficking immune cells together with the application of immune checkpoint blockade antibody.

6.6 Conclusions

Immune checkpoints are pathways that naturally restrain the immune system to prevent excessive immune responses. The immune checkpoint blockade has improved the treatment of a wide range of malignancies, including melanoma, renal cell carcinoma, non-small-cell lung cancer, and urothelial carcinoma in some clinical cases. Importantly, these treatments are related with substantial risks of immune-related adverse events. These toxicities can be severe or even life-threatening. The gastrointestinal toxicity is one of the most frequent adverse events of immune checkpoint therapy. Accumulating evidences show the regulation of gut microbiota in immune checkpoint therapy, and beneficial bacteria community is reported to be sufficient

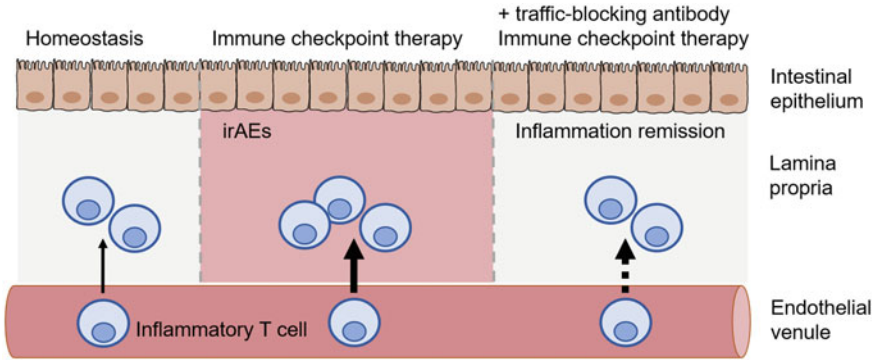

Fig. 6.3 The possible role of traffic-blocking antibodies in inflammation remission. Inflammatory T cells accumulate in intestinal lamina propria in the gut irAEs situation. The application of traffic-blocking antibodies, like anti-α4β7 antibody, could inhibit the migration of pro-inflammatory T cells to gut tissue, leading to the inflammation remission

to improve the efficacy of the immune checkpoint therapy. Treg cells are important for gut immune tolerance against dietary and commensal-derived antigens both in homeostasis and immune checkpoint blockade conditions. Recent study showed that Treg cells were required for gut microbiota to form a beneficial community and ameliorate the intestinal dysfunction in the CTLA-4 blockade condition. The migration of immune cells in the gut also contributes to the inflammations and immune responses, and the trafficking blockade antibodies could inhibit the colitis symptoms in clinic. Further studies are supposed to explore the mechanism about the interregulation between gut bacteria and host immune system, and the potential molecular mechanisms that contribute to the resolution of immune checkpoint blockade-related adverse events. Understanding the mechanism of these immune checkpoint blockade therapy-related adverse events is essential to improve the outcome of cancer immune therapy.

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Chapter 7
Rational Discovery of Response Biomarkers: Candidate Prognostic Factors and Biomarkers for Checkpoint Inhibitor-Based Immunotherapy

Qianqian Hou and Heng Xu

Abstract Immunotherapy with checkpoint inhibitor has been successfully applied in treatment for multiple cancer types, especially for patients at advanced stage. However, response rate of this promising therapy is low, thus requiring biomarkers for precise medication to reduce the ineffective treatment. With multiple retrospective clinical studies, more and more candidate prognostic factors have been identified with possible mechanic explanation, including the basic clinical characteristics (e.g., age and gender), molecular features (e.g., PD-L1 expression and tumor mutation burden). After validation in independent patient cohorts with large sample size, several markers have been approved as companion biomarkers. However, validation and combinations of all the possible candidate biomarkers are still challenging to predict the treatment outcomes. In this chapter, we will summarize and introduce the prognostic factors and biomarkers for checkpoint inhibitor-based immunotherapy.

Keywords Biomarker · Diagnosis · Prognosis · Prediction · Immune checkpoint

7.1 Introductions

Mammalian immune system is well known to eliminate the damaged or aberrant cells, but should be monitored by immune checkpoints, which are essential for regulating the duration and magnitude of immune responses, and maintaining self-tolerance (Pardoll 2012). The interaction between immune system and cancer is complicated, and cancer cells can escape immune surveillance through a variety of mechanisms, including overriding natural balance and being identified as "self" for the immune cells (Topalian et al. 2016; de Visser et al. 2006). Therefore, cancer immunotherapy with immune checkpoints inhibitors (ICI) is considered to induce or restore antitumor activity of immune system with an in-depth study on the dual effect of immune system in tumor development (Topalian et al. 2011). Indeed, monoclonal antibodies

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have been produced as targeting the well-studied immune checkpoint blockade proteins to reinvigorate T cell responses at the tumor site, including cytotoxic T lymphocyte antigen 4 (CTLA4), programmed cell death 1 (PD1), and its ligand, PD1 ligand 1 (PDL1) (Boussiotis 2016). Functionally, CTLA4 has significantly strong affinity with CD80 and CD86, leading to blockage of CD80 and CD86 co-stimulation and inhibition of continued T cell activation (Manson et al. 2016), while PD1/PDL1 interaction could reduce T effector cells and increase T regulatory cells (Tregs) as well as T exhausted cells (Boussiotis 2016). Until recently, six ICIs have been proved by Food and Drug Administration of United States (FDA), including one against CTLA4 (i.e., Ipilimumab), two against PD1 (i.e., Nivolumab and Pembrolizumab), and three against PDL1 (i.e., Durvalumab, Atezolizumab, and Avelumab) (Yan et al. 2018). During the past decade, remarkable success in advanced stage cancer treatment with anti-CTLA4 and anti-PD1/PDL1 ICIs has been achieved in multiple types of cancer previously identified as gloomy prognosis according to traditional treatment (Thomas and Hassan 2012; Gogas et al. 2013; Lee et al. 2015; Restifo et al. 2016), thus drastically prolonged overall survival with acceptable adverse events in patients with non-small cell lung cancer (NSCLC) (Gettinger et al. 2015; Gettinger et al. 2016; Hellmann et al. 2017), advanced stage melanoma (Hodi et al. 2010; Wolchok et al. 2013; D'Angelo et al. 2017), head and neck squamous cell cancer (HNSCC), bladder cancer, liver cancer, and ever-growing list of cancers (Hamanishi et al. 2015; Morris et al. 2017; Overman et al. 2017; Gong et al. 2018; Havel et al. 2019).

Ipilimumab (CTLA4 antibody) was first approved by FDA for clinical use in 2011, and has increased twice fold of 10-year survival for metastatic melanoma compared with historical data (Hodi et al. 2010; Ma et al. 2016). Pembrolizumab, Nivolumab, and Avelumab targeting PD1/PDL1, manifested a more optimal survival in different malignancies, with higher response rates and lower risk of adverse drug reactions (ADR) than CTLA4. Notably, though anti-PD1 and anti-PDL1 share almost similar mechanisms, anti-PDL1 therapy may present distinct role from anti-PD1. The subtle difference lies in that PD1 has two ligands, PDL1 and PDL2. PD-L2 is majorly expressed on antigen-presenting cells (APCs), while PDL1 is expressed across many cell types including tumor cells, immune cells, epithelial cells, and endothelial cells (Sharma and Allison 2015; Zou et al. 2016). Thus, anti-PDL1 does not shut off the interaction between PD1 and PDL2, while anti-PD1 cannot block PDL1 binding to CD80, which is expressed on T cells and plays a role in anti-tumor activities (Butte et al. 2007). Moreover, there is growing evidence that combination of anti-PD and anti-CTLA4 antibodies is more synergistic to improve clinical outcome than either agent alone (Hellmann et al. 2017; Larkin et al. 2015; Hodi et al. 2016; Wolchok et al. 2017), presumably with different functional mechanisms. In another hand, such success also encourages the scientists to find more targetable immune checkpoint blockade proteins, such as T cell immunoglobulin and mucin-domain containing (TIM)-3, lymphocyte activation gene (LAG)-3, and T cell immunoreceptor with immunoglobulin and ITIM domains (TIGIT). ICIs against these novel targets are also under development or have been evaluated in clinical trials (Yan et al. 2018; Pauken et al. 2019).

Despite great success achieved with ICI-based immunotherapy in clinical care, only minority of patients can get benefit from such therapy, and the response rates vary among different cancer types (Topalian et al. 2016; Manson et al. 2016; Zou et al. 2016; Gibney et al. 2016; Pitt et al. 2016; Nishino et al. 2017). For instance, the objective response rate of patients treated with CTLA4 inhibitor is slightly over 10%, and that of patients who have received PD1 blockade is elevated but rarely exceeds 40% (Brahmer et al. 2012; Hamid et al. 2013). Recently, more than 20 phase III clinical trials have even failed in expanding indications, including continuous failure of pembrolizumab in second-line liver cancer (KEYNOTE-240), second-line TNBC (three-negative breast cancer) (KEYNOTE-119), and first-line gastric cancer (KEYNOTE-062), similar situation as Nivolumab in first-line glioblasts. The tumor (CheckMate-498) and the first-line liver cancer (CheckMate-459), suggesting a large variation in terms of cancer types.

In another hand, severe ADR of these ICIs has also been noticed. For instance, immune-related adverse events (irAEs) are derived from non-specific immunologic activation by PD1/PDL1 blockade. In general, it is reported that irAEs in cancer patients are more common with anti-CTLA4 (60–85%) than anti-PD1 (16–37%) or anti-PDL1 (12–24%) at standard doses of these drugs (Larkin et al. 2015; Robert et al. 2015; Postow et al. 2018). The ICIs treatment can develop differently severe irAEs, including the most frequent fatigue and possibly fatal inflammatory pneumonitis, and even leading to forced termination of the treatment (Pitt et al. 2016). What is more, disease hyperprogression occurred after receiving ICIs treatment for some patients, more than half of whom increase tumor burden and double in progression pace (Champiat et al. 2017; Kato et al. 2017).

Due to the highly individualized treatment outcomes of ICI-based immunotherapy across different tumor types, predictive biomarkers are urgently required to improve both efficacy and safety of ICI-based immunotherapy for precision medication (Yan et al. 2018). Indeed, some candidates have been put into practice and proved by FDA to indicate the suitable patient subgroups (e.g., PDL1 expression as a biomarker for patient selection) (Garon 2017). In this chapter, we focus on the prognostic factors and their potential as biomarkers for ICI-based immunotherapy, including tumor genomics, tumor microenvironment, host germline genomics, commensal bacteria, and clinical characteristics. These factors have been summarized in Table 7.1.

7.2 Tumor Autonomous Profile

7.2.1 PDL1 Expression

The ligation of highly expressed PDL1 to PD1 is a key strategy for tumor cells to avoid host immune response and subsequent activation of tumor-specific T cells. Upregulation of PDL1 in tumor cells is negatively related to the dynamic immune activities in the tumor microenvironment (Taube et al. 2012; Spranger et al. 2013).

Table 7.1 Factors that predict response to immune checkpoint inhibitor therapy

Factor	Clinical outcome association	Clinical phases	Approved by the FDA	Predictive or prognostic	Tumors	Sample source of biomarkers	Details of approach for biomarkers	Disadvantages
Tumor autonomous profile								
High tumor mutation burden	Positive	Phase 3	Yes	Predictive	Multiple tumor types	Neoplastic tissue	NGS, WES or targeted panel sequencing	No one universal standard
High neoantigen count	Positive	Phase 1	No	Predictive	Melanoma, NSCLC, glioblastoma	Neoplastic tissue	NGS or WES	Low validation rate
Mismatch repair deficiency	Positive	Phase 2 and Phase 3	Yes	Prognostic	Multiple tumor types	Neoplastic tissue	NGS or WES	No one universal standard
High microsatellite instability	Positive	Phase 2	Yes	prognostic	Colorectal cancer	Neoplastic tissue	Multiplex PCR-capillary electrophoresis, IHC	No one universal standard
Copy number variation	Negative	No	No	Both	Multiple tumor types	Neoplastic tissue	NGS, WES or targeted panel sequencing	

(continued)

Table 7.1 (continued)

Factor	Clinical outcome association	Clinical phases	Approved by the FDA	Predictive or prognostic	Tumors	Sample source of biomarkers	Details of approach for biomarkers	Disadvantages
PD-L1 expression	Positive	Phase 3	Yes	Predictive	Multiple tumor types	Neoplastic tissue	NGS or WES	Tumor heterogeneity and lack of uniform evaluation criteria and threshold for assessing positivity
STK11 alteration	Negative	No	No	Predictive	NSCLC	Neoplastic tissue	NGS or WES	
PTEN mutation	Negative	No	No	Predictive	Melanoma and uterine leiomyosarcoma	Neoplastic tissue	NGS or WES	
JAK1/JAK2 mutation	Negative	No	No	Predictive	Melanoma	Neoplastic tissue	NGS or WES	
B2M mutation	Negative	No	No	Predictive	Melanoma	Neoplastic tissue	NGS or WES	
Tumor microenvironment								
Treg cells	Negative	No	No	Both	Renal cell carcinoma	Blood/neoplastic tissue	IHC	
Depletion of myeloid-derived suppressor cells	Positive	Phase 2	No	Both	Non-small cell lung	Blood/neoplastic tissue	IHC	
Density of tumor-infiltrating lymphocytes	Positive	Phase 1 and Phase 2	No	Prognostic	Multiple tumor types	Neoplastic tissue	IHC	

(continued)

Table 7.1 (continued)

Factor	Clinical outcome association	Clinical phases	Approved by the FDA	Predictive or prognostic	Tumors	Sample source of biomarkers	Details of approach for biomarkers	Disadvantages
Systemic noninvasive markers								
Blood neutrophil-to-lymphocyte ratio < 5	Positive	No	No	Prognostic	Melanoma, NSCLC, renal cell carcinoma	Blood	Routine blood test	
Low lactate dehydrogenase level	Positive	No	No	Predictive	Melanoma	Blood	Routine blood test	
Exosomal PD-1/PD-L1 expression	Negative	Underway	No	Predictive	Melanoma	Blood	Liquid biopsy	
cfDNA-derived TMB	Positive	No	No	Predictive	NSCLC	Blood	Liquid biopsy or NGS	
Host-related factors								
MHC class I diversity	Positive	No	No	Predictive	Melanoma, NSCLC	Blood	NGS WES or PCR	
TCR repertoire diversity	Negative	No	No	Predictive	Melanoma	Neoplastic tissue	NGS	
Virus positive	Positive	No	No	Prognostic	Gastric cancer, Merkel cell carcinoma	Blood	Routine blood test	
Clinical features								
Age > 75	Negative	No	No	Predictive	NSCLC		Clinical information	
Female	Negative	No	No	Predictive	Multiple tumor types		Clinical information	

(continued)

Table 7.1 (continued)

Factor	Clinical outcome association	Clinical phases	Approved by the FDA	Predictive or prognostic	Tumors	Sample source of biomarkers	Details of approach for biomarkers	Disadvantages
Gut microbiome								
Gut microbial diversity	Positive	No	No	Predictive	Melanoma	Feces	NGS or PCR	

NSCLC non-small cell lung cancer, *NGS* next-generation sequencing, *IHC* immunohistochemical staining, *PD-L1* programmed cell death 1 ligand 1, *WES* whole-exome sequencing

Theoretically, blocking the interaction of PD1 and PDL1 by through the immune system induced by ICIs can re-identify and kill the tumors cells, which take such PDL1-overexpression strategy to escape from immune surveillance. Based on this hypothesis, PDL1 expression was estimated by immunohistochemistry assay and evaluated as the first impact factor to the ICI response rate in multiple retrospective studies, exhibiting significantly positive association with higher treatment response rates as well as longer overall survival (OS) (Passiglia et al. 2016; Muller et al. 2017; Carbognin et al. 2015; Taube et al. 2014). A meta-analysis was subsequently conducted, containing patients treated with anti-PD1 antibodies in a variety of cancer types. Positive correlation of high PDL1 expression with increased response rate was further confirmed for both Nivolumab and Pembrolizumab. Interestingly, PDL1 on tumor-infiltrating cells may also play an important role in treatment outcome of MPDL3280A (an early used anti-PD1 antibody) (Herbst et al. 2014; Powles et al. 2014). Therefore, PDL1 expression has been considered as one of the well studied and most widely used biomarkers for ICI response, and already approved by US FDA as a companion diagnostic for anti-PD1 treatment for patients with NSCLC (Yan et al. 2018; Havel et al. 2019; Gibney et al. 2016). Moreover, independent perspective clinical trials in NSCLC were conducted for both Nivolumab (CheckMate-026) and Pembrolizumab (KEYNOTE-024), and the inclusion was set as positive PDL1 expressed cells $\geq$5% and $\geq$50%, respectively. Although Pembrolizumab was developed later than Nivolumab, such precise design contributes to the final success of the trials and approval of its usage as first line for NSCLC treatment. However, failure of Nivolumab-based trials as the first-line therapy partially leads to the market's sale of such ICI relegated to the second place in 2018 (Carbone et al. 2017).

Nevertheless, PDL1 expression is not a perfect predictive biomarker of ICI response. For instance, positive correlation between PDL1 expression and treatment response has not been detected in advanced NSCLC and renal cell carcinoma in some studies (Carbone et al. 2017; Brahmer et al. 2015; Motzer et al. 2015). On the contrary, low to no PDL1 expression is detected for many patients who still experience durable clinical benefit (Sunshine and Taube 2015). The contradictory results in these studies may be caused by several reasons, including the technique issue and dynamic molecular factors. First, biopsies from tumor tissues can only reflect a small region, and the overall profile of PDL1 expression may be largely impacted by tumor heterogeneity (Yan et al. 2018). Second, uniform evaluation criteria/methods and threshold for assessing positivity are lacking (Havel et al. 2019). Furthermore, dynamic and inducible characteristic may also lead to contradictory results. For example, PDL1 can be upregulated by IFNγ, and with the progress of treatment, low baseline PDL1 expression may be induced by inflammatory circumstance (Manson et al. 2016; Zou et al. 2016). More importantly, other factors (e.g., tumor mutation burden [TMB]) were reported as independent predictors for ICI response. For instance, PDL1 staining and TMB were independent predictors of response in a study of patients with NSCLC treated with anti-CTLA4 and anti-PD1 therapy, and a multivariate model analysis containing both factors demonstrated improved predictive sensitivity and specificity (Hellmann et al. 2018a). Therefore, PDL1 expression status alone cannot be a sufficient biomarker for making clinical treatment decisions.

7.2.2 Tumor Mutational Loads and Neoantigen

Genomic alterations are one of the hallmarks for all types of cancers, contributing to the aberrant capability of tumor cells (Hanahan and Weinberg 2011). In turn, the more alterations carried by the cancer cells, the more likely they can be recognized by the immune system and be killed after releasing the "brakes" of the immune system by inhibiting the immune checkpoint. Indeed, existing evidences from clinical trials for NSCLC and melanoma have first suggested the positive correlation between high tumor mutational burden (TMB) and improved clinical efficacy of anti-PD1, anti-PDL1, anti-CTLA4 therapies, as well as the combination of PD1 and CTLA4 inhibitors (Snyder et al. 2014; Rizvi et al. 2015; Van Allen et al. 2015; Hugo et al. 2016; Forde et al. 2018; Hellmann et al. 2018b). Thereafter, a significantly strong positive correlation was observed in a pooled analysis across 27 tumor types or subtypes between their TMB and corresponding objective response rate to anti-PD1 (Yarchoan et al. 2017), indicating the biomarker potential of TMB for efficacy of PD1 blockade therapy. Recently, the largest study with clinical and genomic data from 7033 cancer patients was analyzed to evaluate the predictive response of TMB on immunotherapy, confirming that high TMB is significantly associated with better overall survival for patients with 10 cancer types after receiving ICI-based immunotherapy (Samstein et al. 2019). Actually, TMB has been proved as a considerable biomarker for ICI-based immunotherapy. However, TMB belongs to a quantitative rather than an all-or-none index (e.g., mutation in EGFR for the tyrosine kinase inhibitor-based targeted therapy), and thus it is very hard to define a universal threshold of high TMB across multiple cancer types and detection methods (mainly by high throughput of whole-exome sequencing or sequencing for candidate gene panels).

As mechanism, elevated TMB increases the chance of producing new immunogenic antigens, which can be detected and identified as "non-self" or "foreign" by the adoptive immune system (Gibney et al. 2016; Schumacher and Schreiber 2015). Once recognizing antigens (e.g., epitope derived from a pathogen or a tumor), T cells are activated and proliferate to destruct the cells presenting the antigen. Therefore, neoantigens, named and derived from tumor-specific antigens, provide specific targets for anti-tumor activities (Hacohen et al. 2013; van Rooij et al. 2013). Neoantigen recognition with T cells receptor may be competitive binding by PDL1 or other ligands. Not surprisingly, inhibition of immune checkpoints will release the blockade of the immune system and reinvigorate neoantigen recognition. Although accumulating data indicates that clinically efficacious cancer immunotherapies are driven by T cell reactivity against tumor mutation-derived antigens, neoantigens perform no better than TMB in predicting ICI response or survival, probably because neoantigens can only be predicted by bioinformatic method with computational algorithms mainly based on MHC binding affinity so far, resulting in a pretty low validation rate by experiments (Kvistborg et al. 2014; Tran et al. 2015). However, as fast accumulation of experimentally validated neoantigens, more accurate prediction tools are expected to be developed through considering more impact factors. Moreover, although high

correlation of TMB with neoantigens burdens has been observed, outliers have also been indicated probably due to two factors (i.e., intratumor heterogeneity and the strength of antigenicity of the neoantigens). It is reported that patients with both high TMB and low neoantigen intratumor heterogeneity have a better response to ICI-based immunotherapy than those with high TMB alone (McGranahan et al. 2016). For antigenicity strength, it is not necessary for a responder of ICI-based immunotherapy to have high TMB if they carry only a few strong neoantigen, and vice versa. As an example, Merkel cell carcinoma patients induced by polyomavirus have much lower TMB but higher response rate than those induced by ultraviolet, probably because the strong virus-derived antigens presented in formers (Wong et al. 2015; Goh et al. 2016). Therefore, systematical consideration of the tumoral neoantigens is expected to be more practical than TMB alone as biomarkers for ICI-based immunotherapy.

7.2.3 Mismatch Repair Deficiency and Microsatellite Instability

It is well reported that mismatch repair deficiency (dMMR) can induce large number of somatic mutations in tumors, involving loss of function mutations in DNA repair genes (e.g., *BRCA2*, *POLD1*, *POLE,* and *MSH2*) in either germline or somatic level. dMMR commonly exhibits microsatellite instability (MSI) and has been observed in multiple cancer types, especially frequent in colon and gastric cancer. MSI state has been routinely detected in clinical practice by determining signals of several microsatellite or expression status of several mismatch repair genes through immunohistochemistry, which is easier to separate the patients into groups compared with the linear distribution of TMB, efficiently predicting the patients with the good response (Dudley et al. 2016). Indeed, MSI patients have significantly higher response rate and tend to get durable clinical benefit from ICI immunotherapy regardless of the tissue of origin (Rizvi et al. 2015; Hugo et al. 2016; Le et al. 2015). For instance, the rate of immune-related response and progression-free survival for colorectal cancer MSI patients were 40% and 78%, respectively, while 0% and 11% for mismatch repair proficiency colorectal cancers (Le et al. 2015). The positive relationship between TMB and high sensitivity to ICI has further supported that tumors with high MSI state produce a number of neoantigens attribute to the hypermutated phenotype. As mechanism, MSI-positive tumors are a specific subtype of high TMB tumor generated from dMMR. In some respects, the positive relationship dMMR is positively related to improved ICI response benefiting from rising TMB. However, it is worth noting that dMMR-induced mutations are often dominated by subclones, resulting in highly heterogeneous tumors (Alexandrov et al. 2013). Moreover, MSI status detection has been approved by the US FDA as biomarkers for ICI treatment predicting their efficiency without regarding to tumor histology (Havel et al. 2019).

7.2.4 Somatic Copy Number Alterations (SCNA), Structure Variations (SV), and Aneuploidy

SCNAs and SVs are common genomic features in tumor cells that generally indicate poor outcomes in cancer treatment (Merkel and McGuire 1990), and herein also reported to affect ICI responses. According to the analyses on 12 human cancer types from TCGA, tumors with arm level and even aneuploidy of SCNA rather than focal SCNAs tumors exhibit reduced infiltrating immune cells in 10 out of 12 cancer types (Davoli et al. 2017). Subsequently, this finding was validated in another large patient group (Taylor et al. 2018). More importantly, SCNAs state exhibits association with ICI response independent of TMB, and combination of SCNAs and TMB has a higher prediction efficiency for ICI treatment according to independent clinical trials for metastatic melanoma treated with anti-PD1 and anti-CTLA4 ICIs (Davoli et al. 2017; Roh et al. 2017). Mechanistically, the SCNAs and aneuploidy may result in deletion of HLA but needed to be further investigated (Havel et al. 2019). However, it is also hard to set a cutoff for SCNAs and SVs to separate the patients into groups.

7.2.5 Specific Gene Mutations

Several studies have reported that some specific mutated genes can also affect the ability of tumor cells to evade immune surveillance, especially alterations in genes that are essential for immune response. An investigation on comparison of primary with relapsed tumor in four patients with melanoma revealed relapse-specific mutations in *JAK1*, *JAK2*, and *B2M* genes. Nonsense mutation in *JAK* genes results in loss of response to interferon gamma (IFNγ) according to the consequent experimental analysis, while *B2M* mutation can induce loss of major histocompatibility complex I (MHC I) (Zaretsky et al. 2016). Actually, loss of IFNγ signaling is considered as a mechanism to explain ICI resistance (Gao et al. 2016), while several studies have indicated that *B2M* mutations are frequently observed in both primary and relapsed tumors in different cancer types that can result in acquired ICIs resistance (Gettinger et al. 2017; Janjigian et al. 2018; Grasso et al. 2018; Pereira et al. 2017). However, the sample size of these studies is relatively small, requiring independent validation in large patient cohorts to support them as biomarkers for precise usage of ICIs. In another hand, an experimental-based high-throughput CRISPR-CAS9 screening, dysfunction of B2M as well as multiple novel genes were identified to impact ICI response, including APLNR, which is also involved in IFNγ pathway by interacting with JAK1 (Patel et al. 2017). Mechanisms for other screened candidates have not been mentioned, including mutations in *PTPN2* (Manguso et al. 2017) and SWI/SNF complex genes (Pan et al. 2018). Although the role of *B2M* mutation on ICI response was validated in both patients and experiments, clinical evidence is lacking for the rest of the novel mutated genes.

Besides the immune-related genes, well-reported cancer genes were also reported to play a role in ICI-based immunotherapy. In a study for melanoma treatment, combination of anti-CTLA4 and anti-PD1 significantly increases overall survival of patients with *BRAF*^{V600E} mutation compared with use of anti-PD1 alone, while no benefit can be received for patients without such mutation (Wolchok et al. 2017). For lung adenocarcinoma, *STK11/LKB1* alterations were identified as a major driver of primary resistance to PD1 blockade only in patient with *KRAS* mutations (Skoulidis et al. 2018). In addition, loss of PTEN leads to resistance of anti-PD1 therapy through increasing immunosuppressive cytokines and attenuating T cell infiltration/activity (Peng et al. 2016). It is reported that inactive PTEN is the reason for resistance to ICI agents in patients with melanoma and uterine leiomyosarcoma (Peng et al. 2016; George et al. 2017). Similarly, patients with *EGFR* mutation even have a short overall survival by treating with ICIs compared to chemotherapy, possibly due to development of immunosuppressive microenvironment (Borghaei et al. 2015; Rittmeyer et al. 2017). Besides studies have shown that c-MET, a receptor tyrosine kinase, can reduce mobility and recruitment of neutrophils into tumors, drains lymph nodes, and increases effector T cell infiltration by inhibition of c-MET in mouse tumor models, indicating that inhibition of c-MET pathway may improve responses to ICI agents (Glodde et al. 2017).

Discrepancy was also found for some mutated genes, such as *PBRM1* (encode a component of the SWI/SNF complex), and patients with renal cell carcinoma carrying mutation of this gene were more likely to respond to immune checkpoint in one study (Miao et al. 2018), but can be replicated in another independent cohort (McDermott et al. 2018).

Collectively, mutations in certain genes have been identified to be associated positively or negatively with ICI-based immunotherapy. Some mutated genes (e.g., B2M) have been supported by both clinical evidence and experiment explores, exhibiting their biomarker potential. However, independent consistent validations are largely required for most of the mutated genes before widely used for predicting clinical outcomes.

7.3 Tumor Microenvironment

Tumor cells can interact with extracellular matrix, stromal cells, and immune cells in the tumor microenvironment (TME). Tumor-infiltrating lymphocytes (TILs) have been considered to play a crucial role in anticancer immunity, and also a favorable predictor for ICI-based immunotherapy (Ruffini et al. 2009; Brambilla et al. 2016; Reissfelder et al. 2015). It is reported that high level of pre-existing baseline CD8 positive (CD8+) T cells and increasing tumor-infiltrating CD8+ T cells are positively associated with response rate of anti-PD1 therapy (Wei et al. 2017; Zhou et al. 2017). However, there is a gastric cancer study which suggests that cumulative CD8+ T cells are significantly associated with poor survival outcome as well as high levels of PDL1 expression. This result has already marked a tumor microenvironment with immune

tolerance (Thompson et al. 2017). Meanwhile, the activities of effector T cells are augmented and recuperated with the treatment of anti-PD agents (Wei et al. 2017; Zhou et al. 2017). Notably, presence of TILs is associated with TMB, PDL1 overexpression, and dMMR (Nishino et al. 2017; Herbst et al. 2014). Besides increased TILs density followed by the second administration of checkpoint inhibitors was significantly associated with ICI clinical outcomes, instead of the baseline of TILs status (Hamid et al. 2011; Tumeh et al. 2014). In addition, immunoscore, that is, index systematically evaluating T cell infiltration within and around tumor, is regarded as a powerfully prognostic predictor to ICI treatment based on combined assessment of immune characteristics (Mlecnik et al. 2016; Voong et al. 2017).

Both MDSCs (myeloid-derived suppressor cells) and Tregs can lead to dysfunction of T cell and immunosuppression of TME, thus performing deep-going effect on resistance to PD blockades (Kalathil et al. 2013). For example, Tregs may partly block the response rate of anti-PD1 and lead to the sensitivity differences of patients for anti-PD1 therapy (Ngiow et al. 2015), so depletion of Tregs can make ICI more effective (Taylor et al. 2017). Recent studies have shown that chemical inhibition of c-Rel (a subunit NF-κB, a canonical nuclear factor) weakens Treg-mediated immunosuppressive effect and reinforces the response of anti-PD1 therapy agents (Grinberg-Bleyer et al. 2017). MDSCs perform the function of immunosuppressive via limiting T cell activity. Decreasing MDSCs has been proved to restore anti-tumor outcome of anti-PD1 agents (Orillion et al. 2017). IDO (Indoleamine-2, 3-dioxygenase) is regarded as a crucial negative regulator crippling effects of cytotoxic T cell and survival of patients, which is a rate-limiting enzyme which can regulate and control tryptophan catabolism and MDSCs within the TME (Schafer et al. 2016). Tumor growth restraint and improved survival were observed in knockout mice of IDO, which have been administrated with immune checkpoint inhibitors such as anti-CTLA4 or anti-PD1/PDL1, and a great success of tumor rejection has been achieved by combination of IDO suppressants and anti-CTLA4 agents (Holmgaard et al. 2013). Furthermore, clinical trials I/II have found that combination of anti-PD1 and IDO inhibitor can improve the effect of ICI agents and overall survival of patients. Unfortunately, clinical trials phase III failed with increased irAEs and no benefit in 2018, which indicates that it is possible to find a biomarker to separate the good responders.

7.4 Systemic Noninvasive Markers

As a result of routine clinical biomarkers with a minimally invasive manner, the development of biomarkers of ICI response gained from serum or blood is attracting more attention and larger interest of researchers due to noninvasiveness. From the perspective of immune cells, melanoma patients with a relatively high number of eosinophils and lymphocytes exhibit better overall survival when received Pembrolizumab (Weide et al. 2016). Multiple studies have reported that peripheral blood neutrophil-to-lymphocyte ratio (NLR) <5 is related to extend survival of patients receiving anti-CTLA4 and anti-PD1 across various cancer types (Ferrucci et al. 2016;

Bagley et al. 2017; Bilen et al. 2018; Jiang et al. 2018). Besides there are other blood markers associated with ICI response, covering cytokine levels such as IL-6, IL-8, and IL10, number of lymphocytes, clone of T cell clone, level of Treg cells l, amount of circulating monocytes or MDSCs, and activity of lactate dehydrogenase (LDH). Several articles have comprehensively reviewed these and other features (Voong et al. 2017; Buder-Bakhaya and Hassel 2018; Thompson and Menon 2018). In addition, PDL1 is expressed on the surface of extracellular exosomes released from tumor cells. Distinct states of anti-tumor immunity may be reflected by PDL1 of circulating exosome before and on therapy procedure, and exosomal PDL1 has been considered as a predictive biomarker of anti-PD1 treatment. (Chen et al. 2018). A study on 59 patients with anti-CTLA4 treatment demonstrated that upregulated expression of PD1 and CD28 located on exosomes was correlated to improve progression-free survival (Tucci et al. 2018). Therefore, the blood-derived cfDNA is possible to be as a liquid for ICI response biopsy (Thompson and Menon 2018).

In fact, based on 69 patients across 23 different cancer types, the number of mutations determined from blood-derived ctDNA NGS testing was positively related to progression-free survival and overall survival (Khagi et al. 2017). Moreover, based on the retrospective study about cfDNA in two randomized trials, TMB derived from cfDNA is closely related to increased survival in atezolizumab-treated NSCLC patients (Gandara et al. 2018), indicating a noninvasive way to estimate the biomarkers described above.

7.5 Gut Microbiome

The gut microbiome composed innumerable bacteria are useful to maintain host physiology and immune homeostasis (Eberl 2010). Intestinal microbiota disorder is possibly contributed to the development of colorectal cancer as well as extraintestinal cancers (Brennan and Garrett 2016; Loo et al. 2017). Studies have reported that gut microbiota may impact on clinical chemotherapy outcome of tumor patients (Viaud et al. 2013; Iida et al. 2013). Subsequently, following investigations have revealed that diversity of the gut microbiome as well as some specific intestinal bacteria is significantly correlated to ICIs clinical response, but with inconsistency among different cohorts (McQuade et al. 2019). Four independent studies focusing on melanoma (Chaput et al. 2017; Matson et al. 2018; Gopalakrishnan et al. 2018), non-small cell lung cancer (NSCLC), renal clear cell carcinoma (RCC), and urothelial cancer (Routy et al. 2018) have elucidated that some specific gut bacteria are associated with ICI response, which have analyzed the baseline fecal samples and identified the influence of gut bacteria on immune therapy response of patients. Furthermore, bioinformatics analysis of gut microbiome samples of patients has indicated that 30 responding patients with higher diversity and abundance of the Ruminococcaceae family were compared with 13 non-responders (Gopalakrishnan et al. 2018).

Importantly, the study found that the high abundance of Faecalibacterium and other Firmicutes was correlated to a more positive clinical outcome to ipilimumab,

but induced colitis has more frequent occurrence in patients (Chaput et al. 2017). However, it is not clear whether this metric is suitable for other independent cohorts. Nevertheless, it is hard to consider gut microbiome as biomarkers so far, because of its dynamic changes and multi-factor impacted.

7.6 Host-Related Factors and Clinical Features

7.6.1 HLA Class I Molecules and T Cell Receptor

MHC class I, also named as HLA in human, is a necessary prerequisite for presenting antigens to cytotoxic T cells, achieving immune recognition and elimination of non-self cells (Aptsiauri et al. 2007). Low expression of HLA class I molecules has been considered as a common factor for tumor cells of immune escape and also an important determining factor for clinical success of many immunotherapies (Haworth et al. 2015). Consequently, b2-microglobulin (B2M) is a necessary composition of HLA class I molecules for CD8+ T cell recognition, in which deficiency can damage MHC class I molecules, thus providing a candidate strategy to partly solve immunotherapy tolerance of patients (Zaretsky et al. 2016; Patel et al. 2017; Restifo et al. 1996; Wang et al 2016). Loss of function of B2M is associated with impaired HLA class I antigen recognition, leading to acquired tolerance to anti-PD1 therapy (Gettinger et al. 2017). Meanwhile, mutations of B2M, CALR, PDIA3, and TAP1 destructed HLA-1 complex can impact response to anti-PD agents (Pereira et al. 2017). In addition, the clinical outcome of anti-PD is contributed to the diversity of HLA class I. Compared to those with homozygosity at any one of HLA locus, patients with maximally heterozygous HLA-I loci exert better overall survival. Patients with HLA-B44 super type have enlarged survival, but poor outcome in melanoma cohorts is associated with loss of heterozygosity of HLA-B62 or HLA-1 (Chowell et al. 2018). Remarkably, loss of heterozygosity in HLA has been reported to be related to higher neoantigen burden, increased cytotoxicity and PDL1 expression, which indicates that combining multiple biomarkers is more significant to predict the response to ICI therapy (McGranahan et al. 2017).

Besides the diversity of T cell receptor (TCR) is also connected with clinical outcome. T cell repertoire is prone to the formation of more clones and more diversity in responding patients with metastatic melanoma who have received anti-PD1 therapy (Tumeh et al. 2014), but T cell repertoire is opposite for those responders receiving anti-CTLA4 treatment (Postow et al. 2015).

7.6.2 Age, Gender, and Diet

Aging is usually accompanied by confined and dysregulated immune activities with decreased lymphocyte amplification as well as elevated exhaustion of T cells, which has led to increased probability of various diseases and cancer incidence (Fulop et al. 2010; Lee et al. 2016). The study has found overexpression of PD1 on T cells of elder animals, which plays an essential part in PD1 blockades in the aged (Mirza et al. 2010). Other studies have reported that ICIs therapy is remarkably conducive to patients of all ages with NSCLC except for patients over 75 years (Ferrara et al. 2017; Nishijima et al. 2016). On the other hand, anti-PD agents have been reported to be possibly causing hyper-progressive disease during therapy, which is more common in aging patients (Champiat et al. 2017). Therefore, the age at diagnosis is an important factor to influence the efficacy and irAEs of ICI therapy. But more evidences are required to settle this controversial topic with a large sample size and reduced heterogeneity.

The congenital and acquired immunities have varied from different gender since a long time ago, which leads to variation of vulnerability and immune abilities in response to autoimmune diseases and infections between the genders (Fischer et al. 2015; Klein and Flanagan 2016). Interestingly, increasing evidence has shown that gender is also associated with response to ICIs therapy. Review studies show that the efficiency of ICI-based therapies is gender-dependent, and male patients can receive more benefits than female across cancer types (Conforti et al. 2018), and the effect of gender is stronger for patients treated with anti-CTLA-4 agents than those treated with anti-PD1 (Wu et al. 2018).

Healthy diet providing adequate nutrition is of significant importance for preventing human beings from the invasion of pathogens, especially for patients defending tumor progression during the treatment. Unbalanced diet may result in destructed immune function and expedite disease progression, and obesity from unbalanced diet is related to chronic diseases and tumor development (Fang et al. 2017; Quail et al. 2017). Paradoxically, a study shows that obese patients with metastatic melanoma may acquire more benefit from anti-PD therapy than those with normal body mass index (BMI) (McQuade et al. 2018). Interestingly, this correlation is only found in male patients without clear mechanisms. In addition, recent studies discovered that CD8+ T lymphocytes enhance signal transduction of peroxisome proliferator-activated receptor (PPAR)-α, which is involved in fatty acids catabolism when simultaneously encountering hypoxia and hypoglycemia. The efficiency of tumor-infiltrating lymphocytes is significantly improved by increasing fatty acid catabolism, which can postpone tumor progression and synergize with PD1 inhibitors to efficiently boost the response of melanoma immunotherapy (Zhang et al. 2017). It is speculated that dietary and metabolic factors may be associated with the clinical outcome of PD1 inhibitors, but there is still no direct evidence.

7.6.3 Viral Infections

Viral infection may lead to disorders and dysfunction of immune system, which also influence the response of ICI therapy. As described above, patients with terminal Merkel cell carcinoma exhibit remarkable clinical response, which provides a novel direction that virus-infected patients may benefit from anti-PD1 treatment (Nghiem et al. 2016). Theoretically, oncogenic viruses may act as powerful tumor-specific neoantigens, and tumor cells should evade from the immune checkpoint through inducing inhibition of immune system. In fact, upregulation of PDL1 is common in virus-induced Merkel cell carcinoma cells (Wong et al. 2015). Also, recent studies have found that Epstein–Barr virus (EBV)-infected gastric carcinoma has a low mutation burden, but there is overexpression of genes involving in the immune checkpoint blockade pathway and high abundance of lymphocyte infiltration, thus exhibiting an effective response rate to PD1 and PDL1 blockade agents (Janjigian et al. 2018; Panda et al. 2018). Besides there are some CD8+ TILs named bystander T cells that can recognize tumor unrelated epitopes, e.g., EBV, influenza virus, and human cytomegalovirus, which may partly explain the reason why virus positivity promotes the function of host immunity. Besides these bystander CD8+ TILs lack the expression of CD39, though having various phenotypes overlapping with cancer-specific cells, which suggests that the detection of CD39 expression may be applied to pick out the patients with high probability of virus infection (Simoni et al. 2018). There are a number of evidences which supported that oncogenic virus could be a predictive biomarker of potential for response to anti-PD treatment, but ICI treatment clinical trials of more virus-infected patients are urgent.

7.7 Conclusions

Immunotherapy of ICI agents, a revolutionary shift in cancer treatment, is expanding the range of treatment for cancer patients. However, it is not a perfect and versatile solution that there exists that a large proportion of patients demonstrate no response for ICI therapy and develop hyperprogression. Therefore, it is necessary to explore effective predictive and prognostic biomarkers to understand the complex interactions between tumors and immune systems, and to further determine the precise treatment strategies for each patient. Nowadays, the use of biomarker-assisted treatment options and the design of therapeutic combinations of biomarkers have been attracting attentions, which will be the next-epoch-making change and be made more patients benefiting from ICI therapies in cancer treatment.

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Chapter 8
Spatiotemporal Changes in Checkpoint Molecule Expression

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Abstract Immune checkpoint inhibitors (ICIs), particularly PD-1/PD-L1 blockade, have led to therapeutic breakthrough in patients with advanced malignancy, covering the lung, breast, gastrointestinal, head and neck, urinary system, lymphoma, and solid tumor harboring MSI/dMMR. In certain cancer types, the expression level of immune checkpoint molecule will be required if the immune-based approaches are considered, especially the PD-L1 expression. However, in other types, survival benefit has been proven regardless of PD-L1 expression. It raises a question of how to select patients for immune therapy and whether the expression of immune checkpoint molecules will be optimal biomarkers. Before answering this question, a comprehensive map for the expression of immune checkpoint molecules is needed. In this chapter, we describe our current knowledge on the spatiotemporal changes in the expression of checkpoint molecules. We discuss the different frequencies of expression depending on tumor types and stages, the different patterns between primary and metastatic tumors, as well as the change of expression before and after treatment. The expression of PD-L1 has been most studied, but the threshold that separate "positive" and "negative" PD-L1 expressions and the consistency of testing platform remain under debate. Better understanding on the tumor microenvironment and expression of checkpoint molecules will help to identify patients who will benefit from checkpoint blockade therapy.

Keywords Immune checkpoint molecule · Spatiotemporal change · Malignancy · Immunotherapy

Brief introduction of the checkpoint molecules discussed in this chapter

Immune checkpoint molecules play a crucial role in immune regulation of cancer surveillance. Notably, PD-1/PD-L1, PD-L2, and CTLA-4 are the most commonly

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recognized targets for cancer immunotherapy. In this chapter, the expression of several clinical relevant checkpoint molecules will be illustrated: PD-L1, PD-L2, CTLA-4, LAG3, B7-H3, VISTA, TIM-3, IDO, and A2AR.

8.1 PD-L1

PD-L1, also known as CD274 or B7H1, plays a major role in suppressing the adaptive immune system. PD-L1 is normally expressed on activated T cells, plasmacytoid DCs, myeloid DCs, monocytes, lung, vascular endothelium, liver nonparenchymal cells, and placental syncytiotrophoblasts at a low level (Keir et al. 2008). Binding of PD-L1 to PD-1 causes the exhaustion of effector T cells and immune escape of tumor cells, resulting in tumor progression. Accordingly, PD-L1 overexpression has been detected in several tumor types, including melanoma, NSCLC, HNSCC, both on tumor cells and surrounding immune cells.

PD-L1 expression on tumor cells and immune cells is a dynamic process; consequently, both temporal and spatial factors would influence its expression. Theoretically, PD-L1 expression on tumor cells could be secondary to IFN-γ production by tumor-infiltrating T cells or be activated by constitutive oncogene activation (Pardoll 2012; Taube et al. 2012).

8.1.1 Frequency of PD-L1 Expression by Tumor Site and Stage

8.1.1.1 NSCLC

Clinical benefit of ICIs treatment had been demonstrated in patients with advanced NSCLC without actionable driver mutation genes. Based upon the clinical trials, pembrolizumab demonstrated significantly improved efficacy and survival in patients with PD-L1 tumor proportion score (TPS) ≥ 50% (Garon et al. 2015; Herbst et al. 2016), defined as the percentage of tumor cells with membranous PD-L1 expression (Table). PD-L1 expression is now a routine companion diagnostic assay for treatment decision-making.

However, the reported prevalence in clinical trial setting varied, PD-L1 TPS ≥ 50% ranged between 23.2% and 28%, while that for TPS ≥ 10% ranged between 16% and 28.4% (Borghaei et al. 2015; Gettinger et al. 2015).

A large real-world study (Dietel et al. 2018) on the prevalence of PD-L1 expression in advanced NSCLC was reported in 2018. Among 2435 patients with PD-L1 data, 540 (22%) of whom were TPS ≥ 50% and 1256 (52%) were TPS ≥ 1%. 1088 patients in this study were tested negative for both EGFR mutation and ALK alteration, among which the percentage of patients with PD-L1 TPS ≥ 50% and TPS ≥ 1% were 26%

and 53%, respectively. PD-L1 expression was not observed to be correlated with EGFR mutation and ALK alteration in this study, while another study (Lee et al. 2019) reported that PD-L1 expression (TPS ≥ 1%) was significantly associated with wild-type EGFR. But Brown et al. reported that EGFRm NSCLC tumors occasionally expressed PD-L1 and the ratio of PD-L1 positive to PD-L1 negative EGFRm tumors was significantly lower than that of PD-L1 positive to PD-L1 negative EGFR-WT tumors (Brown et al. 2019). The expression of PD-L1 in early stage of NSCLC was little known. Yang et al. (2014, 2016) revealed that the overall frequency of PD-L1 overexpression in stage I was 39.9–56.2% (TPS ≥ 5%), and PD-L1 positive score was associated with higher grade differentiation and vascular invasion. No correlation was found between PD-L1 expression and driver mutation gene status, which included EGFR, KRAS, BRAF, ALK, PI3KCA, and FGFR1. Cooper et al. (2015) reported similar results in early stage NSCLC.

8.1.1.2 SCLC

PD-L1 antibody (atezolizumab) combined with platinum-based chemotherapy has been approved as first-line therapy for patients with extensive stage small cell lung cancer (SCLC) (Horn et al. 2018). PD-L1 testing is not mandatorily based on current available phase III data, which is different from that of NSCLC. The prevalence of PD-L1 protein expression in SCLC varied dramatically from 0% to 71.2% in different studies (Schultheis et al. 2015; Ishii et al. 2015; Zhao et al. 2019) (Table 8.1). Most of the studies reported low level of PD-L1 expression on SCLC tumor cells (Sequist et al. 2016; Antonia et al. 2016). High prevalence of PD-L1 positivity was detected using the antibody from Abcam (Cambridge, United Kingdom) without rigorous validation. Correlation analysis between PD-L1 expression and tumor stage revealed disparate results. Zhao et al. (2019) found the frequency of PD-L1 positivity expression was similar among the different pathologic stages, while Ishii et al. (2015) showed that expression of PD-L1 was significantly higher in patients with limited disease stage than in those with extensive stage. So far, no correlation between PD-L1 expression and treatment response has been established for SCLC.

8.1.1.3 Head and Squamous Cell Cancer (HNSCC)

In HNSCC, tumor-infiltrating lymphocytes and especially T helper 1 cells could activate interferon-mediated signaling and induce expression of PD-L1 on cells in the tumor environment, protecting tumor cells from tumor-directed immunity. Meanwhile, Baruah et al. reported that HPV-positive HNSCCs, but not HPV-negative HNSCCs, regulate PD-L1/2 expression on fibroblasts via TLR9 (Baruah et al. 2019). Moreover, HNSCC tumor cells are known to exhibit high levels of PD-L1 expression, about 57.3–82% of patients in clinical trials were recorded as PD-L1 positive (Mehra et al. 2018; Ferris et al. 2016), with a CPS score ≥ 1% (Table 8.1).

Table 8.1 The frequency of PD-L1 expression by tumor site in non-small cell lung cancer (NSCLC), small cell lung cancer (SCLC), and head and neck squamous cancer (HNSCC)

Type	Stage	Ref.	No. of patients	Prevalence	Cell location	Anti-PD-L1 Antibody (clone)
NSCLC	Advanced	Herbst et al. (2016)	2222	28% (score[a] ≥ 50%), 66% (score ≥ 1%);	TC	22C3[b]
	Advanced	Garon et al. (2015)	824	23.2% (score ≥ 50%), 60.8% (score ≥ 1%);	TC	22C3
	Advanced (nonsquamous)	Borghaei et al. (2015)	582	28.4% (score ≥ 10%), 31% (score ≥ 5%);	TC	28-8[c]
	Advanced (squamous cell)	Brahmer et al. (2015)	272	25.4% (score ≥ 10%), 29.8% (score ≥ 5%);	TC	28-8
	Advanced (squamous cell)	Rizvi et al. (2015)	76	33% (score ≥ 5%)	TC	28-8
	Advanced	Spira et al. (2015)	287	16% (score ≥10%), 37% (score ≥5%)	TC and/or IC	SP142[d]
	Advanced	Dietel et al. (2018)	2435	22% (score ≥ 50%) 52% (score ≥ 1%)	TC	22C3
	Stage I (adenocarcinoma)	Yang et al. (2014)	163	39.9% (score ≥ 5%)	TC	Proteintech Group Inc.
	Stage I (squamous cell)	Yang et al. (2016)	105	56.2% (score ≥ 5%)	TC	Proteintech Group Inc.
SCLC	Pulmonary and extrapulmonary	Schultheis et al. (2015)	92	0% in TC, 18.5% (score ≥ 1%, macrophages), 48% (lymphocyte)	IC	5H1[e]
	All stage	Ishii et al. (2015)	102	Total 71.2%	TC	Abcam, Cambridge, United Kingdom
	Limited stage		41	85.4% (score ≥ 5%)		

(continued)

Table 8.1 (continued)

Type	Stage	Ref.	No. of patients	Prevalence	Cell location	Anti-PD-L1 Antibody (clone)
	Extensive stage		61	62.3% (score ≥ 5%)		
	Extensive stage	Ott et al. (2015)	135	27% (score ≥ 1%)	TC/IC	22C3
	Stage I–IV	Zhao et al. (2019)	205	12.9% (TC ≥ 1%)	TC	22C3
	Limited and extensive stage	Antonia et al. (2016)	213	11.3% (TC ≥ 1%)	TC	28-8
HNSCC	Advanced	Mehra et al. (2018)	192	65% (TPS) 81% (CPS)	TC and IC	22C3
	Recurrent/Metastatic	Baumal et al. (2016)	172	82% (score ≥ 1%)	TC and IC	22C3
	Recurrent/Metastatic	Ferris et al. (2016)	361	57.3% (score ≥ 1%)	TC	28-8

TCs: tumor cells
ICs: macrophages, dendritic cells, and lymphocytes
TC/IC 0,1,2,3: PD-L1 expression on TCs or ICs was scored using immunohistochemistry (IHC) assay into four levels—0 (< 1%), 1 (≥ 1% and < 5%), 2 (≥ 5% and < 10%), and 3 (≥ 10%)
NR: not reported
CPS: a combined positive score (CPS) is defined as the percentage of tumor and mononuclear inflammatory cells within the tumor nests and adjacent supporting stroma expressing PD-L1 at any intensity
[a]Score was calculated as the percentage of neoplastic cells showing membranous staining of PD-L1
[b]22C3: 22C3 anti-PD-L1 antibody. The PD-L1 IHC 22C3 pharmDx is the only companion diagnostic indicated as an aid in identifying patients with NSCLC for treatment with pembrolizumab
[c]28-8: Anti-PD-L1 clone 28-8. The PD-L1 IHC 28-8 pharmDx kit is FDA-approved as a complementary diagnostic and CE-marked as an in vitro diagnostic device for nivolumab therapy
[d]SP142: Anti-PD-L1 clone SP142. VENTANA SP142 immunohistochemistry assay (Ventana Medical Systems) is intended for use in the assessment of the programmed death-ligand 1 (PD-L1) protein in tumor cells and tumor-infiltrating immune cells with atezolizumab therapy
[e]5H1: Noncommercial anti-PD-L1 clone from L. Chen, John Hopkins University, Baltimore, MD

8.1.1.4 Metastatic Melanoma

Melanoma is the first tumor type treated with ICIs that resulted in great improvement on survival time in patients with advanced melanoma. High prevalence of PD-L1 expression in melanoma was believed to contribute to the favorable response. About 50–81% of patients, included those previously treated with systemic therapy or BRAF or MEK inhibitor, were PD-L1 positive as reported in phase III clinical trials (Robert et al. 2015; Ribas et al. 2015; Schachter et al. 2017; Weber et al. 2015). In the CheckMate 066 trial, 418 previously untreated patients with *BRAF* wild-type metastatic melanomas were enrolled. The prevalence of positive PD-L1 was 35.4%, which is lower than that of other trials. Most evidence showed that PD-L1 expression and BRAF mutation are independent in melanoma (O'Malley et al. 2017; Rodić et al. 2015), and it is demonstrated that the expression of PD-L1 is upregulated by IFN-γ depending on p53 mutation in melanoma (Thiem et al. 2019). The frequency of PD-L1 expression by tumor site in metastatic melanoma was summarized in Table 8.2.

8.1.1.5 Urothelial Cancer

Urothelial carcinoma (UC) accounts for approximately 90% of all bladder cancers. Atezolizumab was initially approved by FDA as the initial treatment for patients with advanced UC in 2014 and was granted conditional approval for patients who are not candidates of platinum-based chemotherapy in 2017. As reported in previous trials, about 29.7–53% of patients with advanced urothelial cancers were PD-L1 positive (Balar et al. 2017; Petrylak et al. 2018; Rosenberg et al. 2016), and PD-L1 expression was generally higher in TCs than in ICs (Rijnders et al. 2019) (Table 8.2). A significant increase in PD-L1 expression among tumors was associated with a greater degree of tumor infiltration lymphocytes (TILs), especially in tumor-infiltrating mononuclear cells (Bellmunt et al. 2015). Besides higher PD-L1 expression was significantly associated with advanced tumor stage (Bellmunt et al. 2015; Thompson et al. 2006; Nakanishi et al. 2007).

8.1.1.6 Esophageal and Gastric Cancer

PD-L1 expression was correlated to virus infection, such as Helicobacter pylori (Hp) in gastric cancer (Das et al. 1950). Silver et al. (2016) summarized that the PD-L1 expression was increased after Hp infection. Further whole-genome sequence analyses also revealed frequent gene amplification of PD-L1 in Epstein–Barr virus-associated gastric cancer (EBVaGC) (Wang et al. 2014; Cancer Genome Atlas Research Network 2014). As recorded in several clinical trials, PD-L1 expression has been detected in more than 15.8% of esophageal and gastric cancer samples (Doi et al. 2018; Fuchs et al. 2018; Janjigian et al. 2018; Kang et al. 2017; Kudo et al. 2017; Muro et al. 2016; Shah et al. 2018; Sun et al. 2007). Higher PD-L1 expression in

Table 8.2 The frequency of PD-L1 expression by tumor site in metastatic melanoma, urothelial cancer (UC), and renal cell carcinoma (RCC)

Cancer type	Stage	Ref.	No. of patients	Prevalence	Cell location	Anti-PD-L1 antibody (clone)
Metastatic melanoma	Advanced	Hamid et al. (2017)	540	54.4% (positive[a], TC and IC ≥ 1%)	TC and IC	22C3
	Advanced	Schachter et al. (2017)	834	81% (positive, TC and IC ≥ 1%)	TC and IC	22C3
	Advanced (BRAF wt)	Robert et al. (2015)	418	35.4% (score ≥ 5%)	TC	28-8
	Advanced	Weber (2015)	405	49.6% (score ≥ 5%)	TC	28-8
Urothelial cancer (UC)	Locally advanced/metastatic	Balar et al. (2017)	370	29.7% (score ≥ 10%)	TC	22C3
	Metastatic	Pethylak et al. (2018)	95	53% (score ≥ 5%)	IC	SP142
	Locally advanced/metastatic	Rosenberg et al. (2016)	310	32.3% (score ≥ 5%), 66.8% (score ≥ 1%)	IC	SP142
	Locally advanced/metastatic	Sharma et al. (2016)	78	32% (score ≥ 1%), 18% (score ≥ 5%)	TC	28-8
	UCC with radical cystectomy	Boorjian et al. (2008)	314	12.4% (score ≥ 5%) Ta/T1: 5.1% CIS: 0% T2-4: 94.9% N0: 82.1% N1-2: 18%	TC	5H1

(continued)

Table 8.2 (continued)

Cancer type	Stage	Ref.	No. of patients	Prevalence	Cell location	Anti-PD-L1 antibody (clone)
Renal cell carcinoma (RCC)	Metastatic	Motzer, et al. (2015)	107	27% (score ≥ 5%)	TC	28-8
	Advanced	Motzer et al. (2015)	821	10.4% (score ≥ 5%), 22% (score ≥ 1%)	TC	28-8
	Advanced	Motzer et al. (2018)	1002	24% (score ≥ 1%)	TC	28-8
	Advanced	Bellmunt et al. (2017)	542	31.2% (CPS ≥ 10%)	TC and IC	22C3

TCs: tumor cells
ICs: macrophages, dendritic cells, and lymphocytes
CPS: a combined positive score (CPS) is defined as the percentage of tumor and mononuclear inflammatory cells within the tumor nests and adjacent supporting stroma expressing PD-L1 at any intensity
[a]Positive is defined as ≥1% staining in tumor and adjacent immune cells as assessed by immunohistochemistry using the 22C3 antibody

gastric cancer was significantly correlated to a higher number of lymph node metastasis, larger tumor size, increased depth of invasion (Wu et al. 2006), high densities of TILs, MMR deficiency, and EBV positivity (Kawazoe et al. 2017). The frequency of PD-L1 expression by tumor site in esophageal and gastric cancer was summarized in Table 8.3.

8.1.1.7 Hepatocellular Carcinoma (HCC)

HCC has been suggested to be associated with inflammation and a suppressed immune environment. Higher expression of PD-L1 was found to be correlated with a poorer prognosis in HCC patients and tumors with vascular invasion, whereas no relationship between PD-L1 expression and TNM stage and post-surgery therapy was proved (Gao et al. 2009). Furthermore, an analysis of the clinicopathological features of PD-L1 and its correlation with CD8 + T cells in 304 HCC patients was performed and the results showed that positive expression of PD-L1 was correlated with higher CD8 + T cells infiltration in immune stroma; in addition, higher levels of PD-L1 correlated with higher expression of immune-related genes, enhanced cytolytic activity, and larger proportions of immune/stromal cell infiltration (Liao et al. 2019).

Based on previous clinical trials, both pembrolizumab and nivolumab have been approved by FDA for HCC patients previously treated with sorafenib. The rates of PD-L1 positive patients with advanced hepatocellular carcinoma were about 13% to 42% (Gao et al. 2009; El-Khoueiry et al. 2017; Zhu et al. 2018). The frequency of PD-L1 expression by tumor site in hepatocellular carcinoma was summarized in Table 8.3.

8.1.1.8 Colorectal Cancer (CRC)

In colorectal cancer, patients with microsatellite unstable (MSI-H) primary tumors were associated with increased TILs in tumor, resulting in elevation of PD-L1 expression on tumor cell. Evidence from recent clinical studies demonstrated that MSI-H/dMMR status predicted response to PD-1 blockade (Le et al. 2015; Overman et al. 2018). The reported PD-L1 expression on tumor cells in these two trials that explored the efficacy of nivolumab in patients with CRCs was a positive rate of PD-L1 of 22% and 28%, respectively. The relationship between PD-L1 expression level and MSI status in tumor is disputable. Some studies reported inverse association between PD-L1 expression and MSI-high status (Masugi et al. 2017; Droeser et al. 2013), while others showed that MSI-high colorectal cancers harbored a larger number of PD-L1-expressing myeloid cells in tumor tissue than microsatellite stable (MSS) cancers (Llosa et al. 2015). The frequency of PD-L1 expression by tumor site in colorectal cancer was summarized in Table 8.3.

Table 8.3 The frequency of PD-L1 expression by tumor site in esophageal cancer (EC) and gastric cancer (GC), hepatocellular carcinoma (HCC), pancreatic cancer, and colorectal cancer (CRC)

Cancer type	Stage	Ref.	No. of patients	Prevalence	Cell location	Anti-PD-L1 antibody (clone)
Esophageal cancer (EC) and gastric cancer (GC)	Recurrent/metastatic GC and GI junction	Muro et al. (2016)	39	100%	TC	22C3
	GC	Sun et al. (2007)	102	42.2% (score ≥ 10%)	TC	2H11[a]
	Advanced EC adenocarcinoma	Doi et al. (2018)	83	45%	TC	22C3
	Advanced GC and GI junction	Fuchs et al. (2018)	259	57.1% (CPS ≥ 1)	TC and IC	22C3
	Advanced EC (adenocarcinoma or squamous cell carcinoma)	Shah et al. (2018)	121	47.9%	TC and IC	22C3
	Advanced GC and GI junction	Kang et al. (2017)	493	15.8% (score ≥ 1%)	TC	28-8
	Metastatic esophagogastric cancer	Jianjigian et al. (2018)	160	24.4% (score ≥ 1%)	TC	28-8
Hepatocellular carcinoma (HCC)	Advanced	El-Khoueiry et al. (al. 2017)	174	20% (score ≥ 1%)	TC	28-8
	Advanced	Zhu et al. (2018)	52	13% (score ≥1%) or 42% (CPS ≥ 1)	TC and IC	22C3

(continued)

Table 8.3 (continued)

Cancer type	Stage	Ref.	No. of patients	Prevalence	Cell location	Anti-PD-L1 antibody (clone)
Pancreatic cancer	Stage I–IV	Tessier-Cloutier et al. (2017)	252	12% (score >1%) 7% (score >5%) 5% (score >10%) Cutoff score >10%: pT1 0, pT2 8.3%, pT3 91.7%, pT4 0%; pN0 16.7%, pN1 83.3%	IC	SP142
Colorectal cancer	Stage IV	Le et al. (2015)	119	22% (score ≥ 1%)	TC	28-8
	Stage IV	Overman et al. (2017)	74	28% (score ≥ 1%)	TC	28-8
	Advanced	Lee et al. (2016)	394	5% (score ≥ 1%)	TC	E1L3N

TCs: tumor cells
ICs: macrophages, dendritic cells, and lymphocytes
CPS: a combined positive score (CPS) is defined as the percentage of tumor and mononuclear inflammatory cells within the tumor nests and adjacent supporting stroma expressing PD-L1 at any intensity
[a]2H11, anti-PD-L1 antibody established and provided by the Institute of Biotechnology, Key Laboratory of Clinical Immunology of Jiangsu Province, Suzhou University

Table 8.4 The frequency of PD-L1 expression by tumor site in triple-negative breast cancer (TNBC), cervical cancer, and ovarian cancer

Cancer type	Stage	Ref.	No. of patients	Prevalence	Cell location	Anti-PD-L1 antibody (clone)
Triple-negative breast cancer (TNBC)	Metastatic	Emens et al. (2019)	116	78.4% (score ≥ 1%)	IC	SP142
	Metastatic	Schmid et al. (2018)	899	40.9% (score ≥ 1%)	IC	SP142
	Early stage	Mittendorf et al. (2014)	105	19% (cell surface membrane staining >5%)	IC	5H1
Cervical cancer	Advanced	Chung et al. (2019)	82	83.7% (score ≥ 1%)	TC	22C3
	Advanced	Frenel et al. (2017)	20	100% (score ≥ 1%)	TC	22C3
	Locally advanced	Enwere et al. (2017)	120	87.9% (score ≥ 1%)	TC	E1L3N[a]
Ovarian cancer	Stage I–IV	Hammanishi et al. (2007)	70	68.5% (score ≥ 5%)	TC	27A2

TCs: tumor cells
ICs: macrophages, dendritic cells, and lymphocytes
[a]E1L3N, rabbit monoclonal anti-PD-L1 antibody, Cell Signaling Technology, Danvers, MA, USA

8.1.1.9 Triple-Negative Breast Cancer (TNBC)

PD-L1 expression in breast cancer is rare, but has been reported to be enriched in infiltrating immune cells of hormone-receptor-negative and triple-negative patients (Ali et al. 2015; Wimberly et al. 2015). In the recent report, positive rate of PD-L1 in tumor cells was significantly higher in TNBC than in non-TNBC, and the expression of PD-L1 was strongly correlated with p53 (Zeng et al. 2019). In early stage TNBC, 19% of tumors were identified as PD-L1 positive (Mittendorf et al. 2014). The positive percentage was higher in metastatic TNBC patients, reaching 40.9–78.4% with a cutoff value of 1% in the phase I study of atezolizumab for the treatment of TNBC (Emens et al. 2019). The frequency of PD-L1 expression by tumor site in triple-negative breast cancer was summarized in Table 8.4.

8.1.1.10 Cervical Cancer

Persistent HPV infection has been suggested to be a causative factor in the development of CIN and cervical cancer. In one study conducted by Yang et al. (2013), a direct correlation between the expression of PD-L1 and HPV positivity was confirmed, supporting immune blockade in HR-HPV-related cervical cancer. In a phase

II basket study of pembrolizumab as second-line therapy, 82 pretreated cervical cancer patients (83.7%) with PD-L1 expression ≥1% showed better ORR and longer median duration of response. PD-L1 expression was greatly increased in the dysplastic/invasive squamous cells and surrounding inflammatory cells were HPV infected, with a percentage of positive expression of 80–95%, while no expression was evident in normal cervical epithelia even near to CIN or cancer. The role of virus in driving the PD-L1 expression was speculated and needed further investigation (Mezache et al. 2015). The frequency of PD-L1 expression by tumor site in cervical cancer was summarized in Table 8.4.

8.1.1.11 Thymic Neoplasm

So far, no immune checkpoint inhibitor has been approved for the treatment of thymomas and thymic carcinomas. However, a phase II study evaluated the activity of pembrolizumab in patients with advanced thymic carcinoma and achieved a satisfactory response rate similar to that of NSCLC (Giaccone et al. 2018). Among the 40 patients enrolled in the trial, 25% were PD-L1 positive, and a longer survival time was observed in high-PD-L1 expression cohort. PD-L1 positive rates of ≥ 60% in tumor tissue samples of thymic neoplasm had been reported in retrospective studies (Katsuya et al. 2015; Weissferdt et al. 2017). These rates were similar to a recent report including 36 patients (Bedekovics et al. 2018). The high prevalence of PD-L1 expression may bring light to thymic neoplasm as another ICIs-favored tumor. The frequency of PD-L1 expression by tumor site in thymic neoplasm was summarized in Table 8.5.

8.1.1.12 Hodgkin Lymphoma (HL)

The response rates in unselected patients with relapsed and/or refractory HL treated with Nivolumab were as high as 87%, leading to the FDA approval of nivolumab for this indication in May 2016.

Classic HL tumors are characterized by clonal, multinucleated, malignant Reed–Sternberg cells. Abundant PD-L1 expression in patients with classic HL was mainly observed on the Reed–Sternberg cells. The prevalence of PD-L1-positive CHL varies from 43.5% to 97% (Ansell et al. 2015; Chen et al. 2013) (Table 8.5).

8.1.2 Inter-tumoral Heterogeneity of PD-L1 Expression

The PD-L1 expression on tumor cells varied through the course of tumor progression, either by upregulation of PI3K-Akt kinases or secretion of IFN-γ, suggesting

Table 8.5 The frequency of PD-L1 expression by tumor site in thymic neoplasm, Hodgkin lymphoma (HL), and soft tissue sarcoma

Cancer type	Stage	Ref.	No. of patients	Prevalence	Cell location	Anti-PD-L1 antibody (clone)
Thymic neoplasm	Stage I–IV	Katsuya et al. (2015)	149	70% of thymic cancer 23% of thymoma (score ≥ 1%)	TC	E1L3N
	Stage I–IIIb	Weissferdt et al. (2017)	100	61% (score ≥ 5%)	TC	EPR4877(2)
	Advanced refractory or recurrent thymic carcinomas (III–IVb)	Giaccone et al. (2018)	40	25% (score ≥ 50%)	TC	22C3
Hodgkin lymphoma (HL)	Relapsed or refractory	Ansell et al. (2015)	23	43.5%	RS cell membrane	405.9A11[a]
	Classic HL	Chen et al. (2013)	33	97% positive[b]	RS cell membrane	clone 15[c]
Soft tissue sarcoma	NR	Sandra et al. (2015)	50	12% (score ≥ 1%) in TC 30% in lymphocyte 58% in macrophage	TC and IC	DAKO
	Stage I–IV	Jung et al. (2013)	105	58% in IC	IC	Clone H130[d]

TCs: tumor cells
ICs: macrophages, dendritic cells, and lymphocytes
RS cell: Hodgkin Reed–Sternberg cell
[a]405.9A11, anti-PD-L1 clone 405.9A11, from G, Freeman
[b]Tumor staining for PD-L1 was considered positive if ≥5% of the tumor cell population showed 2+ or 3+ membrane staining
[c]Clone 15, #10084-R015, a rabbit anti-PD-L1 monoclonal antibody, manufactured by Sino Biological, Beijing, China
[d]Clone 130, from Santa Cruz Biotechnology, clone H-130, CA, USA

discrepancies of PD-L1 status between primary and metastatic tumor. Several studies had investigated the longitudinal intra-patient concordance of PD-L1 expression, with various findings in different tumor types.

Pinato et al. (2016) reported that intra-tumoral heterogeneity in the expression of PD ligands is common in NSCLC, while PD-L1 is homogeneously undetectable in primary and metastatic SCLC. Kim et al. (2017) reported the concordance rate of PD-L1 expression using E1L3N antibody between primary and metastatic pulmonary adenocarcinoma in 161 patients with matched metastatic tissues from 146 patients (83.2% regional nodal metastasis) using cutoff values of ≥1% and ≥50% as 80.1% and 90.7%, respectively. For RCC, the discordant rate of positive PD-L1 expression in tumor cells between primary tumor and metastatic surgical excision samples was 20.8% (Callea et al. 2015).

The positive consistency between the primary tumor and metastatic lymph node/distant metastases was only 26% in melanoma patients and the negative expression status similarity was 22%. Even the PD-L1 expression status was not significantly concordant in local recurrence lesion and distant metastases (Madore et al. 2015). In colorectal cancer, more positive PD-L1 status was detected in metastatic lesion rather than that in primary tumors. HB Wang et al. (2017) reported that the prevalence of PD-L1 expression doubled in metastatic lesion (81.8% versus 40.9% in primary tumor) and increased PD-L1 expression was frequently found during the metastatic process. In breast cancer, PD-L1 expression was only detected in 12% of primary tumor and a 71% in paired metastases, while the remaining cases acquired PD-L1 expression in immune cells after metastases (Dill et al. 2017). Another study found that PD-1 was discrepant between primary tumor and metastasis in 50% of the patients, and PD-L1 on tumor cells and immune cells was discrepant in 28.5–40.8% of the patients, respectively (Manson et al. 2019).

The disparity between the primary and metastatic lesions illustrates the necessity of re-biopsy and reevaluation of the PD-L1 status after metastases.

8.1.3 Longitudinal Change of PD-L1 Expression After Treatment Intervention

Dynamic changes of PD-L1 expression after anti-cancer treatment had been reported, although the sample size of the studies was not very large. The majority data showed the PD-L1 expression was enhanced after treatment intervention (including chemotherapy, radiation, target therapy, and immunotherapy), especially in the responded subset (Haratake et al. 2017; Vilain et al. 2017; Katsuya et al. 2016). Positive conversion of PD-L1 and improved expression level were observed in lung cancer patients after chemotherapy or EGFR-TKI resistance (Han et al. 2016). Similar improved trend was also seen in paired tumor sample after immunotherapy, such as the anti-PD-1 treatment in melanoma (Vilain et al. 2017) and non-small lung cancer (Haratake et al. 2017), and Bacillus Calmette Guerin treatment in bladder cancer

(Hashizume et al. 2018). However, decreased expression was reported in one study which is focusing on 45 locally advanced lung cancer patients after the concurrent chemoradiation (Fujimoto et al. 2017).

8.1.4 Factors Influencing the Pathology Assessment Concordance

8.1.4.1 Pathologist Concordance (Intensity Staining and Percentage, TC/IC)

PD-L1 expression is examined by immunohistochemistry (IHC) as other IHC companion diagnostic tests, and the assay needs to be read by specific pathologists after training since the judgement standard is not equivalent (Doroshow et al. 2019). The expression pattern of PD-L1 involves several information, the location of the expression (membranous or cytoplasm), cutoff value for positivity, and expression in different effective cells.

Currently, the assessment of PD-L1 expression in clinical practice mainly contains two parts: (1) the percentage of tumors cells with an intensity of membranous expression and (2) the percentage of immune cells with similar expression. The expression of PD-L1 in both the tumor cells (TC) and immune cells (IC, also called tumor-infiltrating immune cells) should be evaluated separately, and the intensity threshold for positivity needs to be determined in different types of cancer on the basis of previous clinical studies.

A study by Yale University in 2017 investigated the pathologist reading heterogeneity of PD-L1 (Rehman et al. 2017). Compared to the high concordance for PD-L1 expression in TC with an intraclass correlation coefficient of 95%, the concordance of reading in stromal/IC PD-L1 expression is not satisfying and only 27% agreement was seen even by pathologists with training. Besides the quantitative methods for stromal IC were also less concordant, which is raising the questions about the accuracy and ability to score the expression in IC.

8.1.4.2 Score Criteria and Diagnostic Cutoff for Positivity

Two score criteria are now commonly used as evaluable index of PD-L1 companion diagnostic test, tumor proportion score (TPS), and combined positive score (CPS).

The definition of TPS was first introduced in KEYNOTE-024 study (Reck et al. 2016), which demonstrated that checkpoint inhibitor pembrolizumab alone could prolong the efficacy and survival in front-line treatment of advanced NSCLC who had a membranous PD-L1 expression $\geq$50% of the malignant cells, leading to the approval of FDA in this population. However, similar compound nivolumab did not mirror the success neither with a PD-L1 TPS of $\geq$1% nor $\geq$50% (Carbone et al.

2017). TPS is usually divided into different expression levels by the cutoff value of 1–50%.

Compared to TPS, CPS considers the expression on IC in addition to tumor cells. CPS is a numerical value, which is calculated as the number of PD-L1 staining cells of all types divided by the total viable tumor cells and multiplied by 100 (Kulangara and Waldroup 2017). The cutoff value of CPS varies in different cancer types. For example, pembrolizumab is indicated for patient with locally advanced or metastatic urothelial carcinoma whose tumors express PD-L1 CPS $\geq$ 10 (KEYN0TE 052); the CPS $\geq$ 1 is regarded as positive expression in cervical cancer (KEYNOTE 158) and gastric or gastroesophageal junction adenocarcinoma (KEYNOTE 059), while in metastatic and recurrent head and neck squamous cell carcinoma, tumors with CPS $\geq$ 20 exhibit a superior response benefit (KEYNOTE 048).

8.1.4.3 Samples for Testing: Resection Specimen Versus Matched Biopsy

The significance of PD-L1 expression in clinical practice raises another question of the heterogeneity on specimens obtained, whether the PD-L1 expression is concordant in the same samples by resection or biopsy is still conflicting. In the French study (Ilie et al. 2016), the discordance rate reached close to 50% in 160 NSCLC patients, and biopsy specimens were thought to have underestimated the PD-L1 status especially in IC. However, one Japanese study demonstrated a good concordance rate of 92.4% between the resected specimens compared with corresponding small biopsy samples in 79 NSCLC cases (Kitazono et al. 2015). Another Korean study suggested that PD-L1 status in metastatic biopsied sample may be acceptable, based on analysis of 15 cases, the expression between primary resection with metachronous metastatic biopsied specimen shows a good concordance rate of 66.7–86.7% by cutoff value of 1%–50% (Kim et al. 2017).

Moreover, a good concordance and similar prevalence distribution in PD-L1 expression level were found in archival and newly collected tumor samples (Herbst et al. 2019), indicating that direct utilization of the archived tumor block could be used without worrying about inaccurate estimation of response to treatment, which would be convenience for patients.

8.1.4.4 Antibody Clone (DAKO 22C3, DAKO 28-8, Ventana SP142, Ventana SP263)

Several ICIs have been approved by FDA. However, the combination of antibody clone and detection system as companion diagnostic test for patient selection for immunotherapy is not uniform, even for the same class of agent in the same cancer type. The use of different PD-L1 IHC testing platforms and different antibody clones may also influence the concordance rate of PD-L1 expression. For example, in NSCLC, there are three PD-1 inhibitors (pembrolizumab, nivolumab, and

durvalumab) and one PD-L1 inhibitor (atezolizumab) approved by FDA, and the testing assay of PD-L1 expression was based on different antibody clones (DAKO 22C3, Abcam 28-8, SP 263, and SP142, respectively) and detection systems (DAKO, DAKO, Roche, and Ventana Medical Systems, respectively). DAKO 22C3 is the only companion diagnostic assay for pembrolizumab approved by FDA, while others are just approved as complementary (Hersom and Jorgensen 2018). The Blueprint Project (Hersom and Jorgensen 2018), which was initiated to harmonize companion diagnostics across a class of targeted therapies sponsored by FDA-ASCO, found that the three PD-L1 assays (22C3, 28-8, and SP263) were similar in analytic staining performance, while SP142 showed a relatively lower sensitivity in tumor cells. Even among the three similar antibody clones, the interchange of the assay was not allowed as the specific therapy-related PD-L1 cutoff value was established using different predefined staining assays in clinical studies.

Besides the antibodies above, there are other clones that were used in clinical or experimental testing for PD-L1 expression, such as the E1L3N (Cell Signaling Technology, Danvers, MA) and 5H1.

The different antibody clones and platforms may account for the varied prevalence of PD-L1 expression in certain tumor type. Standardized methods and definition of PD-L1 positivity are urgently needed to facilitate the predictive value analysis.

8.2 PD-L2

In large phase 3 trials, the clinical benefit from anti-PD-1 mAbs appears to be independent of PD-L1 expression in some cancers, such as UC and NSCLC (Bellmunt et al. 2017; Kang et al. 2017; Brahmer et al. 2015). This suggests that some other molecular interactions with PD-1 other than PD-L1 may be relevant to predicting clinical responsiveness to ICI treatments.

It has been reported that mPD-L2 (previously called protein AF142780) encodes a polypeptide with 38% amino acid identity to mPD-L1. Ohaegbulam et al. (2015) reported that PD-L2 and PD-1 binding requires less complex conformational changes than direct binding of PD-L1 to PD-1. Moreover, the binding strength of PD-L2 to PD-1 is three times greater than that of PD-L1. In addition, the simultaneous binding of PD-L1 and L2 to PD-1 has been disproven, implying that the two ligands compete to bind to the receptor. Anti-PD-L2 seems to be another promising ICI for cancers. The expression patterns of PD-L2 assessed in different tumor types are summarized in Table 8.6.

PD-L2 is predominantly expressed on dendritic cells, macrophages, and mast cells, as well as some tumor cells (Latchman et al. 2001). Increased expression of PD-L2 has been identified in a few tumor types, including Hodgkin lymphoma, primary mediastinal B cell lymphoma (PMBCL), primary central nervous system (CNS) lymphoma, and primary testicular lymphoma (Panjwani et al. 2018; Tanaka et al. 2018). Patients with classic HL and PMBCL have higher rates of PD-L2 expression than other types of B cell lymphoma. PD-L2 was absent in patients with T cell

Table 8.6 Expression patterns of PD-L2 in different tumor types

Cancer type	Stage	Ref.	No. of pts.	Prevalence	Cell location	Anti-PD-L2 antibody
Hodgkin's lymphoma (HL)	Relapsed or refractory	Ansell et al. (2015)	23	43.5%	RS cell membrane	366C.9E5[a]
	Classic HL	Panjwani et al. (al. 2018)	49	41%	RS cell membrane	Clone D7U8C
	Stage I–IV	Tanaka et al. (2018)	34	24% (IHC > 0%)	RS cell membrane	Clone D7U8C
Barrett's esophagus, esophageal adenocarcinoma, and squamous cell carcinoma	T1-T4	Derks et al. (2015)	354	51.7% in all[b] T1: 24.9% T2: 4.8% T3: 21.2% T4: 0.6%	TC	366C.9E5
	Barrett's esophagus	Derks et al. (2015)	21	42.8%[b]	Epithelial expression	366C.9E5
	Stage I–IV esophageal squamous cell carcinoma	Tanaka et al. (2016)	180	48.3% (pI-II 26.4%; pIII-IV 74.6%)	TC	Clone 176611
	Stage I–IV	Hsieh et al. (2018)	150	42%	TC	329602, BioLegend
Breast cancer	Stage I–III	Baptista et al. (2016)	191	50.8%	TC	Polyclonal antibody from Abcam
Ovarian cancer	Stage I–IV	Junzo (2007)	70	37.1%	TC membrane	Goat anti-PD-L2 polyclonal Abs (R&D Systems, Minneapolis, MN)

(continued)

Table 8.6 (continued)

Cancer type	Stage	Ref.	No. of pts.	Prevalence	Cell location	Anti-PD-L2 antibody
Head and neck cancer	Advanced head and neck squamous cell carcinoma	Mehra (2018)	172	65% (score ≥1%)	TC and IC	Clone 3G2 antibody (Merck & Co., Inc.)
Renal carcinoma	NR	Tanegashima et al. (2019)	29	72.4% (score ≥1%)	TC	#176611, R&D Systems, Inc
	pT stage 1–4 clear cell RCC	Shin et al. (2016)	214	36% (score ≥ 5%)	TC	#176611, R&D Systems, Inc
	pT stage 1–4 papillary RCC	Shin et al. (2016)	201	49.6% (score ≥ 5%)	TC	#176611, R&D Systems, Inc
NSCLC	NR	Tanegashima et al. (2019)	27	85.2% (score ≥ 1%)	TC	#176611, R&D Systems, Inc
Pancreatic cancer	Stage I–IV	Nomi et al. (2007)	51	27% (score ≥ 10%)	TC	MIH18, mouse immunoglobulin G1
Gastric carcinoma	I–IV adenocarcinoma	Xing et al. (2018)	1014 (TMA)	49.9% in TC 20% in IC	TC or IC (cytoplasm)	NBP1–88964, Novus Biologicals
B cell lymphoma	DLBCL	Panjwani et al. (2018)	83	6%	TC	Clone D7U8C
	PMBCL	Panjwani et al. (2018)	14	78%	TC	Clone D7U8C
	PMBCL	Tanaka et al. (2018)	20	45%	TC	Clone D7U8C
	CLL/SLL	Panjwani et al. (2018)	25	4%	TC	Clone D7U8C
	EMZL	Panjwani et al. (2018)	9	11%	TC	Clone D7U8C

(continued)

Table 8.6 (continued)

Cancer type	Stage	Ref.	No. of pts.	Prevalence	Cell location	Anti-PD-L2 antibody
T cell lymphoma	PTCL, NOS	Panjwani et al. (2018)	112	2%	TC	Clone D7U8C
	Anaplastic large cell lymphomas	Panjwani et al. (2018)	15	7%	TC	Clone D7U8C
	AITL	Panjwani et al. (2018)	20	5%	TC	Clone D7U8C
Extranodal NK/T cell lymphomas	Stage I–IV	Panjwani et al. (2018)	95	0	NR	Clone D7U8C
	Stage I–IV	Han L et al. (2014)	30	63.3%	TC and stromal cell	Polyclonal antibody from ZSGB-BIO
Hepatocellular carcinoma	Stage I–IV	Jung et al. (2017)	85	23.5% (score of 3–5)[c]		NR
Colorectal cancer	Stage I–IV	Guo et al. (2018)	348	41% (score of 2–3)[d]	TC	ab200377
	Stage I–IV	Wang et al. (2017)	124	38.7% (score of 2–3)[e]	TC	anti-PD-L2 from Abcam
Melanoma	Stage III–IV	Obeid et al. (2016)	148	25% (score ≥ 5%)	TC	

[a]Clone MEB123.3G2.038 (3G2): generated through immunization of mice with a combination of human PD-L2-Fc (amino acids 20–219) and human PD-L2-His (amino acids 1–219) fusion proteins and was identified by screening supernatants from 446 hybridomas (Merck Research Laboratories)
[b]PD-L2 expression was considered positive when ≥50% of tumor cells on TMAs or ≥10% of tumor cells on whole-tissue slides had moderate–strong PD-L2 staining in the cytoplasm and/or membrane
[c]Score of 3–5: The staining percentage (0 points, <10%; 1 point, 10–50%; and 2 points, >50%) and intensity of PD-Ls (0, no staining; 1, weak staining; 2, moderate staining; and 3, strong staining) in tumor cells were scored, and the overall score for PD-Ls expression was the sum of the scores
[d]A semiquantitative scoring scheme based on the distribution of positive tumor cells and the staining intensity was used to evaluate the expression of PD-L2. A score of 2–3 was designated as high expression
[e]The staining intensity of PD-L2 in tumor cells was assigned with scores 0 (negative), 1(weak), 2 (moderate), and 3 (strong)
TMA: tissue microarray; NR: not reported

lymphoma and myeloid disease (Dail et al. 2016), except extranodal NK/T cell lymphomas (Han et al. 2014).

In solid tumors, Yearley et al. (2017) reported that PD-L2 expression in tumor cells varied quite significantly across tumor types, with the highest expression levels in TNBC and gastric carcinoma, rare-to-low expression in RCC, and moderate expression in bladder, NSCLC, HNSCC, and melanoma. PD-L2 expression was detected with higher frequency in stromal cells including immune cell infiltrate than in tumor cells.

PD-L2 expression generally correlates to PD-L1 expression, which has been observed in TNBC (Baptista et al. 2016), HNSCC (Mehra et al. 2018), NSCLC (Yearley et al. 2017), esophagus squamous cell carcinoma (Hsieh et al. 2018), RCC (Shin et al. 2016), and CRC (Wang et al. 2017).

Jung et al. (2017) reported that PD-L2 expression was related to histological differentiation ($p = 0.002$) in patients with HCC, but not tumor stage. However, a larger sample size is needed to reach this conclusion.

In patients with esophageal carcinoma, positive PD-L2 expression had significantly higher probability of deeper tumor invasion (pT) ($P = 0.0024$), more extensive lymph node involvement (pN) ($P = 0.0005$) and higher pathological stage ($P = 0.0003$) (Tanaka et al. 2016).

Younger age at diagnosis, lymph node positivity, negative estrogen receptor, and recurrence at distant sites were all associated with both PD-L1 and PD-L2 expressions in breast cancer (Baptista et al. 2016).

In patients with ovarian cancer (Hamanishi et al. 2007), neither PD-L1 nor PD-L2 expression is associated with primary tumor status, lymph node metastasis, distant metastasis, histological type, residual tumor status, and chemotherapy.

Interestingly, PD-L2 expression is negatively correlated with lymph node metastasis and tumor stage in patients with CRC (Guo et al. 2018). Moreover, Wang et al. (2017) found that in patients with CRC, PD-L2 positive tumors displayed slight increase with the mucinous histological type. Furthermore, strong membranous expression pattern of PD-L2 is associated with infiltrating ulcerative pathological type.

PD-L2 expression was significantly associated with VEGF ($P = 0.001$) and c-MET ($P = 0.008$) positivity in clear cell RCC and was positively correlated with VEGF expression in papillary RCC (Shin et al. 2016).

BRAF V600E mutation was identified in 47% of the melanoma metastases cases. But no significant correlation has been found between the percent expression of PD-L1 or PD-L2 on tumor cells and BRAF V600E mutation (Obeid et al. 2016).

8.3 Others Checkpoint Inhibitors

8.3.1 CTLA-4

Coreceptor-based immunotherapy is a rapidly developing approach to treat cancer patients. Among those targeted receptors, the immune checkpoint receptor CTLA-4 (cytotoxic T-lymphocyte-associated antigen-4, CD152) is the primary attenuator of adaptive immune responses and the most prominent and extensively investigated molecule in this field. The observation of fatal autoimmunity in CTLA-4 knockout mice resulting from the release of self-reactive T cells illustrated that CTLA-4 plays a crucial role in negatively regulating T cell activation and preserving self-tolerance (Ise et al. 2010). In the cellular level, CTLA-4 is expressed on nonlymphoid cells including placental fibroblasts (Kaufman et al. 1999), cultured muscle cells (Nagaraju et al. 1999), monocytes (Wang et al. 2002), and a variety of leukemia cells. As in tumor tissue, CTLA-4 is frequently overexpressed in a variety of malignancies, such as NSCLC, breast cancer, mesothelioma, melanoma, etc. (Yu et al. 2015; Paulsen et al. 2017; Snyder et al. 2014). Most studies supported that CTLA-4 is mainly expressed in tumor cells. Notably, CTLA-4 expression varied greatly among different types of cancer. Also, different levels of CTLA-4 expression had been reported to be a prognostic factor for survival but current data remain inconclusive. Donnem T and colleague have reported the CTLA-4 expression level by tumor cells in lymph nodes but not primary tumors were a negative prognostic factor in NSCLC patients (Paulsen et al. 2017). This was in line with the observation reported by that in breast cancer (Yu et al. 2015). However, several other studies indicated no significant association with survival in NSCLC patients with different expression levels of CTLA-4 in primary tumor (Deng et al. 2015).

8.3.2 B7-H3

B7-H3 (CD276) is a type I transmembrane protein that belongs to the Ig superfamily and a member of the B7 immunoregulatory molecules. B7-H3 is expressed on many tissues and cell types. In the cellular level, northern blotting showed that B7-H3 mRNA is widely expressed in multiple normal tissues including liver, pancreas, testes, heart, small intestine, and colon tissues. B7-H3 protein expression is constitutively found on some immunity cells including B cells, T cells, monocytes, or NK cells. Of note, numerous studies have described B7-H3 overexpression in human malignancies, including melanoma (Wang et al. 2013), leukemia (Hu et al. 2015), breast (Cong et al. 2017), prostate (Zang et al. 2007), pancreatic (Ingebrigtsen et al. 2012), colorectal (Bin et al. 2014), but the role of B7-H3 has not been well established. In NSCLC, B7-H3 protein expression has been associated with a negative impact on prognosis (Lou et al. 2016; Danilova et al. 2016). B7-H3 expression on lung cancer was associated with a lower number of TILs and with lymph node

metastasis, suggesting a role for B7-H3 in immune evasion and tumorigenesis. Several studies have shown association of high B7-H3 expression in primary tumor with regional nodal metastasis (Arigami et al. 2010) and poor prognosis in breast cancer patients (Cong et al. 2017), while its prognostic value in clinic in patient with CRC is still controversial (Bin et al. 2014; Ingebrigtsen et al. 2014).

8.3.3 LAG-3

LAG-3 is a member of the immunoglobulin superfamily (IgSF) and associated with T cell function (Triebel et al. 1990). In the cellular level, LAG-3 is expressed on cell membranes of B cells, NK cells, TILs, T cells, and dendritic cells (DC) (Grosso et al. 2007; Workman et al. 2009; Kim et al. 2010). Previous studies have reported that LAG-3 was mainly expressed in Hodgkin's lymphoma (HL) (Gandhi et al. 2006), chronic lymphocytic leukemia (CLL) (Kotaskova et al. 2010), multiple myeloma (Camisaschi et al. 2014), breast cancer (Cappello et al. 2003) esophageal squamous cell carcinoma (ESCC) (Zhang et al. 2018), gastric cancer, etc. (Takaya et al. 2015). In ESCC, high level of LAG-3 was reported as an independent prognostic factor which was associated with improved survival (Zhang et al. 2018). In contrary, NSCLC patients with LAG-3 expressed on TILs in tumor tissues had poor prognosis (He et al. 2017). While LAG-3 in residual tissues in patients with triple-negative breast cancer was associated with poor prognosis (Wang et al. 2018).

Several other checkpoint molecules including VISTA, TIM3, IDO, A2AR, and their expression in various tumor types are summarized in Table 8.7.

Table 8.7 Expression patterns of VISTA, TIM-3, IDO, A2AR in various tumor types

Checkpoint molecules	Cancer type	Ref.	No. pts	Prevalence (%)	Cell location
VISTA	NSCLC	Villarroel-Espindola et al. (2018)	758 (TMA)	>99	IC
	Gastric	Boger et al. (2017)	464	83.6	IC
	Melanoma	Kakavand et al. (2017)	16	67	IC
TIM-3	Breast cancer	Burugu et al. (2018)	330	12	IC
	Gastric cancer	Cheng et al. (2015)	52	18.5	IC
IDO	Cervical cancer	Heeren et al. (2018)	71	79	TC
	Breast cancer	Li et al. (2018)	54	50	TC

(continued)

Table 8.7 (continued)

Checkpoint molecules	Cancer type	Ref.	No. pts	Prevalence (%)	Cell location
	Pancreatic cancer	Zhang et al. (2017)	80	59	TC
	Colorectal cancer	Ogawa et al. (2017)	60	40	TC
	ESCC	Jia et al. (2015)	196	56.1	TC
	GBM	Wainwright et al. (2012)	343	21.9	TC
	NSCLC	Ma et al. (2019)	183	79.8	TC
A2AR	NSCLC	Inoue et al. (2017)	642	49.2	TC
	DLBCL	Wang et al. (2019)	65	43.08	IC

TMA, tissue microarray; ESCC, esophageal squamous cell cancer; GBM, glioblastoma multiforme; NSCLC, non-small cell lung cancer; DLBCL, diffuse large B cell lymphoma

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Chapter 9
Functions of Immune Checkpoint Molecules Beyond Immune Evasion

Yaping Zhang and Junke Zheng

Abstract Immune checkpoint molecules, including inhibitory and stimulatory immune checkpoint molecules, are defined as ligand–receptor pairs that exert inhibitory or stimulatory effects on immune responses. Most of the immune checkpoint molecules that have been described so far are expressed on cells of the adaptive immune system, particularly on T cells, and of the innate immune system. They are crucial for maintaining the self-tolerance and modulating the length and magnitude of immune responses of effectors in different tissues to minimize the tissue damage. More and more evidences have shown that inhibitory or stimulatory immune checkpoint molecules are expressed on a sizeable fraction of tumor types. Although the main function of tumor cell-associated immune checkpoint molecules is considered to mediate the immune evasion, it has been reported that the immune checkpoint molecules expressed on tumor cells also play important roles in the maintenance of many malignant behaviors, including self-renewal, epithelial–mesenchymal transition, metastasis, drug resistance, anti-apoptosis, angiogenesis, or enhanced energy metabolisms. In this section, we mainly focus on delineating the roles of the tumor cell-associated immune checkpoint molecules beyond immune evasion, such as PD-L1, PD-1, B7-H3, B7-H4, LILRB1, LILRB2, TIM3, CD47, CD137, and CD70.

Keywords Immune checkpoint · Self-tolerance · Inflammation · Autoimmune disease · Epithelial–mesenchymal transition

9.1 Introduction

The term immune checkpoint refers to a group of inhibitory or stimulatory molecules expressed on immune cells, antigen-presenting cells, tumor cells, or other types of

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cells, which mainly mediate the progress of the adaptive immune system, in particular, T cells and innate immune system. The number of immune checkpoints is increasingly discovered, like PD-1, PDL-1, LAG3, B7-H3, TIM3 (Fig. 9.1). Both inhibitory immune checkpoints and stimulatory immune checkpoints have become prime targets in pharmaceutical research according to their unique roles in immune escape, such as applying for PD-1 and PDL-1 monoclonal antibodies that can reactivate dormant immune responses effectively in many types of tumor (Postow et al. 2015). Over last decade, emerging evidence supports that the blockade of immune checkpoints is the most promising approach in cancer immunotherapy (Topalian et al. 2015). A variety of immune checkpoint molecules are abnormally expressed on different types of tumor, which is called tumor cell-associated immune checkpoint molecules, and are also found to play important roles in tumor cell biology itself, in particular, the induction of epithelial–mesenchymal transition (EMT), acquisition of tumor-initiating potential, unique metabolism network for capacity to metastasize, resistance to apoptosis, antitumor drugs, and higher proliferation requirement that facilitate tumor survival which is summarily shown in Table 1. Here, we will focus

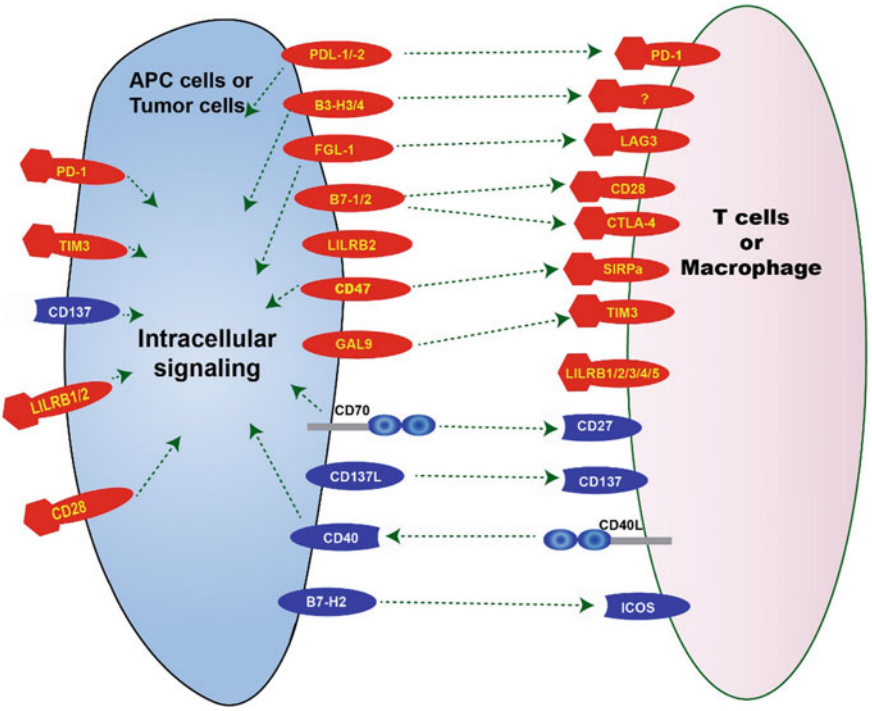

Fig. 9.1 Diagram of inhibitory and stimulatory immune checkpoints between APCs, tumor cells, T cells, and macrophage. Some of these immune checkpoint molecules are not only involved in the immune escape of tumor cells, but also participate in maintaining their malignant behaviors. Red, inhibitory immune checkpoints; blue, stimulatory immune checkpoint

on discussing the specific roles of immune checkpoints in tumor cell biology and their applications in the future.

9.2 PD-L1

PD-L1 (also known as B7-H1 or CD274) is a member of the B7 family that comprises 10 members (Ni and Dong 2017). PD-L1 is a 290 aa type I transmembrane protein encoded by the CD274 gene on mouse chromosome 19 and human chromosome 9. CD274 comprises seven exons, and the first of which is non-coding and contains the 5'-UTR. The next three exons contain the signal sequence, IgV-like domain, and IgC-like domains, respectively. The transmembrane domain and the intracellular domains are contained in the next two exons (exons 5 and 6). The last exon contains intracellular domain residues plus the 3'-UTR. The intracellular domain of PD-L1 is very short with only 30 aa, and highly conserved in all the reported species. There is no known function for the intracellular tail of PD-L1 (Keir et al. 2008). PD-L1 can be expressed on T cells, natural killer cells, macrophages, myeloid dendritic cells, B cells, epithelial cells, vascular endothelial cells, and multiple tumor cells, and serves as one of the ligands for PD-1 involved in immune inhibition. By binding to PD-1 on immune cells, PD-L1 helps tumor cells evade the supervision of the immune system by inhibiting T cell activity and proliferation, facilitating T cell anergy and exhaustion, and inducing activated T cell apoptosis (Chen and Han 2015; Dong et al. 2002; Chen et al. 2012; Butte et al. 2007). Recently, more and more studies indicate that PD-L1 is involved in maintaining the tumor-associated biological features. In current chapter, we mainly summarize the new functions of these immune checkpoint molecules as listed below.

9.2.1 Epithelial–Mesenchymal Transition (EMT)

EMT is one of the critical steps in the early stages of cancer metastasis. Recent researches have established a causal relationship between PD-L1 expression in tumor cells and the induction of EMT. Y. Wang et al. found that PD-L1 could induce EMT and enhance renal cell carcinoma stemness through upregulation of sterol regulatory element-binding protein 1 (SREBP-1c), which is an important transcription factor in lipogenesis (Wang et al. 2015). Yujia Cao's data indicate that upregulation of PD-L1 in skin epithelial cells promotes EMT and accelerates carcinogenesis accompanied with the loss of E-cadherin and elevated expression of the transcription factors Slug and Twist that drive EMT (Cao et al. 2011). Abdullah et al. have demonstrated a bidirectional effect between EMT status and PD-L1 expression especially in claudin-low subtype of breast cancer cells. Induction of EMT in human mammary epithelial cells enhanced PD-L1 expression, which was mainly dependent on the activation of

the PI3K/AKT pathways. Importantly, specific downregulation of PD-L1 in claudin-low breast cancer cells showed reversed phenotypes in EMT as evidenced by CD44 and Vimentin downregulation and CD24 upregulation (Alsuliman et al. 2015).

9.2.2 Acquisition Tumor-Initiating Potential and Increased Proliferation

Given that EMT gives rise to tumor cells with increased tumor-initiating potential, it is not surprising that PD-L1 is associated with the tumor-initiating activities in multiple types of cancers. The level of PD-L1 is highly correlated to $CD133^+$ colorectal tumor-initiating cells which showed the cancer stem cell-like properties such as tumor sphere-forming ability and more tumorigenic in NOD/SCID accompanied with higher levels of other stem cell markers of Oct4 and Sox-2 (Zhi et al. 2015). There was a high significant association of proliferation with PD-L1 expression and the presence of the proliferative marker Ki-67 (Ghebeh et al. 2007). H. Ghebeh et al. reported that the proliferative ability of $PD\text{-}L1^+$ cancer stem cells is significantly enhanced by comparing with the $PD\text{-}L1^-$ counterparts in gastric cancer (Yang et al. 2015). In the mouse model of lung squamous carcinoma with biallelic inactivation of LKB1 and PTEN, the tumor-initiating cells are highly expressed for PD-L1 (Xu et al. 2014).

9.2.3 Resistance to Antitumor Drugs/Apoptosis

Several studies suggest PD-L1 serves as oncogenic protein participating in tumor cell drug resistance and antiapoptotic responses through the PI3K/AKT signaling pathway. Suppression of PD-L1 expression significantly inhibited cell proliferation and increased apoptosis induced by the chemotherapeutic treatment with downregulation of expression of cycle-related genes and antiapoptotic genes (Ishibashi et al. 2016). Black M. et al. also found PD-1/PD-L1 axis led to tumor cell resistance to conventional chemotherapy, and increased metastasis and proliferation through ERK and mTOR pathways (Black et al. 2016). There is a tight correlation between PD-L1 and multidrug resistance 1/P-glycoprotein (MDR1/P-gp) protein levels. Further study indicates the interaction of PD-L1 with PD-1-induced phosphorylation of AKT and ERK, resulting in the activation of PI3K/AKT and MPK/ERK pathways and increased MDR1/P-gp expression in breast cancer cells (Liu et al. 2017). Recent findings from Cao et al. revealed that PD-L1 played a critical in participating the proliferation and glucose metabolism and increased cisplatin-induced apoptosis by ITGB6/STAT3 signaling axis in bladder cancer (Cao et al. 2019). In contrast, other studies also indicated that PD-L1 expression is adversely correlative with the tumorigenicity. And lower expressed PD-L1 in cholangiocarcinoma exhibited tumor cells

with CSC-related features involved in drug resistance, a dormant state in the cell cycle, and reduced reactive oxygen species production (Tamai et al. 2014).

9.2.4 Protection from DNA Damage

Clinically approved antibodies that block the binding of extracellular PD-1 and PD-L1 do not show the expected results all the time in spite of the high level of PD-L1, indicating there are more unknown functions to be addressed. Tu et al. demonstrated that intracellular PD-L1 acts as an RNA-binding protein that regulates the mRNA stability of NBS1, BRCA1, and other DNA damage-related genes. Through competition with the RNA exosome, intracellular PD-L1 protects targeted RNAs from degradation and increased resistance to DNA damage. RNA immunoprecipitation and RNA-seq experiments demonstrated that PD-L1 regulates RNA stability genome wide. Furthermore, they developed a PD-L1 antibody, H1A, which abrogates the interaction of PD-L1 with CMTM6, thereby promoting PD-L1 degradation. Their study suggests that intracellular PD-L1 may be a potential therapeutic target to enhance the efficacy of radiotherapy and chemotherapy in cancer through the inhibition of DNA damage response and repair (Tu 2019).

9.2.5 Switch to Glycolytic Metabolism

Tumor cell-associated PD-L1 expression supports the translation of glycolysis enzymes and promotes this metabolic pathway through AKT/mTOR signaling, which helps tumors to survive in the process of nutrient competition and tumorigenesis (Chang et al. 2015). Qin et al. also found autophagy defect promoted a shift from mitochondrial oxidative phosphorylation to aerobic glycolysis depending on ROS/NF-κb/HIF-1a pathway, which further contributed to the metastasis and chemoresistance and poor prognosis of gastric cancer (Qin et al. 2015). There may exist a connection between PD-L1 expression and glycolysis. It has been reported that PKM2, which catalyzes the final rate-limiting step of glycolysis, regulates the expression of PD-L1 on tumor cells by stimulating HIF-1A transactivation and recruitment of p300 to the hypoxia response elements sites on the PD-L1 promoter (Palsson-McDermott et al. 2017). The high level of lactate in the microenvironment also induced the expression of PD-L1 on human lung cancer by binding to its receptor GPR81 which further enhanced the glycolysis (Feng et al. 2017). The tumor-associated macrophages were also found to increase the expression of PD-L1 promoting the tumor cell glycolysis in non-small cell lung cancer by secreting TNF-A (Jeong et al. 2019).

9.3 PD-1

PD-1 is a 288 amino acid (aa) type I transmembrane protein composed of one immunoglobulin (Ig) superfamily domain, a 20 aa stalk, a transmembrane domain, and an intracellular domain of approximately 95 residues containing an immunoreceptor tyrosine-based inhibitory motif (ITIM) as well as an immunoreceptor tyrosine-based switch motif (ITSM). PD-1 is encoded by the Pdcd1/PDCD1 gene on chromosome 1 in mice and chromosome 2 in humans. In both species, PDCD1 consists of 5 exons. Exon 1 encodes a short signal sequence, whereas exon 2 encodes an Ig domain. The stalk and transmembrane domains are encoded in exon 3, and exon 4 for a short 12 aa sequence that marks the beginning of the cytoplasmic domain. Exon 5 contains the C-terminal intracellular residues and a long 3'-UTR (Keir et al. 2008).

PD-1 is a prominent checkpoint receptor for its tumor-specific immunity and its blocking antibody has shown remarkable efficiency in the treatment of multiple solid tumors. Surprisingly, PD-1, expressed on surface of melanomas cells, triggers an intrinsic downstream effector of mTOR independent of PI3K-AKT signaling to promote tumorigenesis in addition to its role involved in immune evasion (Kleffel et al. 2015).

9.4 B7-H3

B7-H3, also called CD276, is a type I membrane protein with its sequence similarity to the extracellular domain of PD-L1. Human B7-H3 contains a tandem repeat of IgV and IgC domain and a transmembrane domain and a short cytoplasmic tail (Ni and Dong 2017). It is mainly expressed on antigen-presenting cells (APC) to be involved in the inhibition of T cells, although it was initially found to stimulate the T cell response and IFN-R production which was characterized by Chapoval et al. (2001). The function of B7-H3 is still controversial in the immunologic function that may result from binding to different partners. B7-H3 is expressed at low levels in most normal tissues, but is highly expressed on various types of cancers and is significantly associated with poor outcome in patients with lung cancer (Wu et al. 2016), colorectal carcinoma (Fan et al. 2016), breast cancer (Flies et al. 2014), and others. Although B7-H3 has become an interesting target for new immunotherapeutic treatments based on its role in immune evasion, more and more evidence show B7-H3 plays a role in cancer progression beyond immune evasion, including invasion, migration, angiogenesis, and gene regulation via epigenetic modifiers.

9.4.1 B7-H3 in Migration and Invasion

The roles of B7-H3 in cancer progression have been recently investigated. In vitro studies have found that the decreased expression of B7-H3 impairs the cell adhesion to fibronectin and inhibits the migration and Matrigel invasion (Chen et al. 2008). Consistent with this finding, overexpression of B7-H3 in SW480 cell promoted the invasion and metastasis accompanied with the downregulation of E-cadherin and b-catenin but upregulation of N-cadherin and Vimentin expression in colorectal cancer cells by activating the PI3K-AKT pathway and upregulating the expression of Smad1 (Jiang et al. 2016), a transcription factor involved in EMT induction. Tekle C. et al. reported that there was strong connection between high B7-H3 expression and tumor metastasis in primary melanoma cancer cells. More importantly, they found that the metastasis-associated proteins, matrix metalloproteinase 2 (MMP2), signal transducer and activator of transcription 3 (Stat3), and the level of secreted interleukin-8 (IL-8) were reduced in the B7-H3 knockdown cells. MMP2 breaks down extracellular matrix, which allows cells to migrate from the primary tumor to the new loci. STAT3 signaling promotes metastasis via induction of MMP2 expression (Tekle et al. 2012; Kortylewski et al. 2005). Moreover, STAT3 was also found to enhance NF-kb activity in tumors (Grivennikov and Karin 2010), NF-kb is a major transcription factor in control of tumor apoptosis and invasiveness of both pre-neoplastic and malignant cells (Karin 2006). Additional mechanisms proposed by Li et al. suggested that B7-H3 co-localized with the C-X-C chemokine receptor type 4 in gastric cancer cells and induced the phosphorylation of AKT, ERK, and JAK2-STAT3 to promote gastric cancer cell migration and invasion (Li et al. 2017).

9.4.2 B7-H3 in Cell Proliferation

B7-H3 has been found to promote the proliferation of tumor cells in different manners, such as transmembrane, exosomal, or soluble proteins. Zhao X. et al. found the knockdown of B7-H3 in the membrane decreased the tumor growth rate in vivo, but did not affect the cell proliferation in vitro in pancreatic cancer (Zhao et al. 2013). Whereas Wang et al. (2016) showed that B7-H3 expressed in osteosarcoma cells and enhanced proliferation and invasion in vitro. In addition, enhanced proliferation and invasion in vivo were also observed when B7-H3 was highly expressed in cervical cancers (Li et al. 2017). Marimpietri et al. show that B7-H3 plays a role in tumor proliferation via exosomal activity and cell–cell interactions (Marimpietri et al. 2013). Many studies have demonstrated the connections between soluble B7-H3 protein and poor prognosis of patients with different malignant tumors. Pancreatic carcinoma cells secreted B7-H3 to facilitate the migration and invasion of the tumor cells. Xie C. et al. found sB7-H3 promotes the invasion and metastasis of pancreatic carcinoma cell via activating the TLR4/NF-kb/IL-8 and VEGF pathways (Xie et al. 2016).

9.4.3 B7-H3 in Drug Resistance

Recently, other studies also suggest B7-H3 expression on circulating epithelial tumor cells is correlated with their rapid proliferation and resistance to radiotherapy of breast cancer cells (Pizon et al. 2018). Similarly to PD-L1, B7-H3 was also found to promote the expression of SREBP-1, which resulted in aberrant lipid metabolism via SREBP-1/FASN signaling pathway and induced the resistance toward chemotherapy (Luo et al. 2017). Consistent with these findings, the treatment with the combination of an inhibitory B7-3 monoclonal antibody with antitumor small-molecule inhibitors resulted in significantly increased antiproliferative effect in melanoma cells.

9.4.4 B7-H3 in Angiogenesis

Multiple studies have shown B7-H3 is highly expressed in tumor-associated endothelial cells of human lung, breast, colon, renal, bladder, cervix, esophagus, and ovarian cancer, but not in normal angiogenic tissues. Thus, B7-H3 can be used to distinguish physiological and pathological angiogenesis (Seaman et al. 2007). Recently, Seaman S. et al. demonstrated that the cell-surface protein B7-H3 is widely upregulated by multiple tumor types of cancer cells and tumor-infiltrating blood vessels as well. When treated with a conventional chemotherapeutic drug of pyrrolobenzodiazepine together with B7-H3 antibodies, both cancer cells and vasculature were notably reduced, which eventually led to the eradication of cancer growths, metastasis, and improvement of overall survival of tumor recipient mice (Seaman 2017). Also, high level expression of B7-H3 in the tumor vasculature was recently reported to improve the accuracy of breast cancer diagnosis (Bachawal et al. 2015). Xie et al. found that soluble B7-H3 promoted VEGF expression and increased tumor angiogenesis through the activation of toll-like receptor 4 and NF-kb pathway in pancreatic cancer cells. Zhang et al. also indicated B7-H3 was co-localized with Tie2, another angiopoietin-1 receptor in endothelial cells, and enhanced the microvessel formation in tumor microenvironment (Zhang et al. 2017).

9.4.5 B7-H3 in Glycolysis

In human triple-negative breast cancer cell lines, high level of B7-H3 expression is correlative with the increased glycolytic level, while B7-H3 knockdown in tumor cells decreased the glycolysis and increased their susceptibility to the treatment of AKT-mTOR inhibitors (Nunes-Xavier et al. 2016). In addition, B7-H3 expression has been shown to inhibit the activity of the stress-activated transcription factor NRF2 and its downstream target genes of SOD1, SOD2, and PRX3, which further increases the levels of reactive oxygen species-dependent stabilization of HIF1a and

its downstream targets of key enzymes in the glycolytic pathways, such as lactate dehydrogenase A (LDHA) and pyruvate dehydrogenase kinase 1 (PDK1). The upregulation of B7-H3 can lead to markedly increased level of glucose uptake and tumor growth in human breast cancer (Lim et al. 2016).

9.5 B7-H4

B7-H4, also known as B7-S1, B7x, Vtcn1, is another member of the B7 family of immune-regulatory ligands and is considered to be a negative regulator of immune response, although its receptor has not yet been identified. It contains one IgV and one IgC domain and is highly evolutionarily conserved between mouse and human. B7-H4 mRNA is widely expressed in both mouse and human peripheral tissues, while its protein expression is more restricted to stimulated antigen-presenting cells, which suggests post-transcriptional mechanism is strictly regulated. Several studies found that aberrant B7-H4 was expressed on a broad spectrum of cancers and its expression serves as a predict for poor prognosis (Ni and Dong 2017). Despite its existence as type I transmembrane, B7-H4 can also exist in soluble form which is detected at higher levels in the serum from cancer patients. Therefore, soluble B7-H4 may be a valuable biomarker for the predication of the progression and prognosis of patients. However, increasing evidences show that B7-H4 plays an important role in tumorigenesis and progression via different molecule mechanisms. Here, we will focus on the function of B7-H4 in human cancers beyond its role in immune regulation.

B7-H4 protein was detected on the membrane, in the cytosol, and/or in the nucleus in tumor tissues (Zhang et al. 2013). In renal cell carcinoma patients, B7-H4 expression on tumor cell membrane was associated with adverse clinical and pathologic features, including constitutional symptoms, tumor necrosis, and advanced tumor size, stage, and grade. The high B7-H4 expression is adversely correlated with the overall survival of patients. B7-H4 was also found to be expressed on tumor endothelial cells, indicating its important role in tumor-associated angiogenesis (Krambeck et al. 2006). B7-H4, an extensively glycosylated surface transmembrane protein, is overexpressed in breast and ovarian cancers and promotes malignant transformation of epithelial cells. Overexpression of B7-H4 in a human ovarian cancer cell lines enhanced the tumor formation in SCID mice. Consistently, knockdown of B7-H4 expression in breast cancer cell lines results in marked apoptosis (Salceda et al. 2005). In human lung cancer, B7-H4 promotes tumor cell proliferation, invasion and migration, anti-apoptosis, and enhanced cell cycle progression. In vivo, the disruption of B7-H4 expression in tumor cells led to a marked decrease in tumor growth in the immune-compromised mice was observed when the expression of B7-H4 in tumor cells was knocked down (Zhang et al. 2017). The role of B7-H4 in tumorigenesis and oncogenicity was also observed in human pancreatic cancer (Qian et al. 2013) and ovarian cancer (Cheng et al. 2009). Interestingly, Kim H. K. et al. found that B7-H4 depletion significantly downregulated the cAMP/cAMP response element-binding

protein/peroxisome proliferator-activated receptor gamma coactivator 1-alpha signaling pathway, resulting in decreased oxygen consumption rate, ATP production and mitochondrial membrane potential, and reactive oxygen species production. Disruption of B7-H4 expression in Hela cells dramatically activated the JNK/P38 signaling and increased its sensitivity to doxorubicin (Kim et al. 2014).

Jeon Y. K. et al. reported that the expression of cancer cell-associated cytoplasmic B7-H4 can be induced by hypoxia. Further study showed that HIF-1a is bound to a proximal hypoxia response element site of the B7-H4 promoter to promote its transcriptional regulation and to increase cell proliferation accompanying with increased expression of proliferation-related genes including CCNA1, MKI67, and Myc in primary multiple myeloma patients (Jeon et al. 2015). Increased cell proliferation is also observed in human esophageal squamous cell carcinoma cells with high expression level of B7-H4, which may result from the interleukin-6 secretion and its downstream signaling JAK2/STAT3 activation (Chen et al. 2016).

However, Xia et al. reported that B7-H4 serves as a tumor suppressor to enhance the differentiation of murine leukemia-initiating cells and inhibit leukemogenesis by suppressing REST corepressor 2 (RCOR2) to reduce RUNX1 expression through PTEN/ AKT signaling (Baudhuin et al. 2013). These findings indicate that B7-H4 also acts as a transcript factor in cytoplasm in addition to as a membrane ligand (Xia et al. 2017).

9.6 LILRB1

Leukocyte immunoglobulin-like receptor 1 (LILRB1, also known as CD85J, ILT2, LIR1, and MIR7) belongs to LILRBs family, which contains 4 ITIMs in the cytoplasmic site and 4 immunoglobulin domains in the extracellular portion (Borges et al. 1997; Samaridis and Colonna 1997). LILRB1 is widely expressed on variety of cells, such as certain NK cells, monocytes/macrophages, dendritic cells (DCs), eosinophils and basophils, subsets of T cells, B cells (Katz 2006), progenitor mast cells (Tedla et al. 2008), and osteoclasts (Mori et al. 2008). LILRB1 is activated by ligation of different ligands, and it transduces a negative signal that downregulates the immune response and inhibitory effects on NK cells, DC cells, T cells, B cells, monocytes/macrophages, osteoblasts, and other cells. LILRB1 is also expressed on certain cancer cells, including AML cells (especially in monocytic AML cells) (Kang et al. 2015), neoplastic B cells (B cell lymphoma, B cell leukemia, and multiple myeloma cells) (Naji et al. 2012), T cell leukemia and lymphoma cells (Harly et al. 2011), and gastric cancer cells (Zhang et al. 2012). In addition to the role in immune evasion, LILRB1 is involved in the differentiation and growth of gastric cancers (Zhang et al. 2012). In contrast, blocking the binding of LILRB1 on neoplastic B cells with its ligand HLA-G inhibits cell proliferation (Naji et al. 2012). Another study demonstrates blocking of LILRB1 on myeloma or lymphoblastic cells in culture using neutralizing antibodies did not affect cell lysis mediated by NK cells (Heidenreich et al. 2012).

The tumor-promoting effect of other members of LILRBs family, like LILRB3, LILRB4, LILRB5, mainly depends on the immune surveillance. For example, LILRB4 is expressed on myeloid-derived suppressor cells (MDSCs) in human lung cancer patients playing an important role in immune suppression (de Goeje et al. 2015). Recently, Deng et al. found LILRB4 supported acute leukemia cells infiltration into tissues and suppresses T cell activity via ApoE/LILRB4/SHP-2/Upar/Arginase-1 signaling pathways (Deng et al. 2018). LILRB4, which was also expressed on some certain solid organ tumors, such as colorectal carcinoma, pancreatic carcinoma and melanoma, inhibited T cell immunity in vitro (Suciu-Foca et al. 2007; Cortesini 2007). Besides Zhang et al. found that LILRB4 may inhibit NK cell-mediated cytotoxicity to gastric cancer cells (Zhang et al. 2012).

9.7 LILRB2

Leukocyte immunoglobulin-like receptor 2 (LILRB2), also known as inhibitory immunoglobulin-like transcripts (ILT4) and monocyte/macrophage immunoglobulin-like receptor 10 (MIR-10) and CD85D, is a classic type I transmembrane protein with four extracellular tandem Ig-like domains, a short transmembrane region, and a cytoplasmic tail with three ITIMs (Colonna et al. 2000; Wagtmann et al. 1997). LILRB2 is physiologically expressed on monocytes, macrophages, dendritic cells, and granulocytes (Baudhuin et al. 2013; Sloane et al. 2004) to control both innate and adaptive immune response and regulate immune homeostasis and disease progression. In addition to immune cells, LILRB2 is also expressed in other types of cells involved in their biological functions. Most importantly, LILRB2 is highly enriched in a variety of malignant tumor cells to promote their malignant transformation (Gao et al. 2018). Upon the new findings about the roles in tumors, LILRB2 is suggested to be a novel immune checkpoint molecule for cancer eradication. In this section, we will mainly focus on the role of LILRB2 in both physiological and pathological functions beyond immune function.

9.7.1 LILRB2 in Physiological Function

LILRB2 was found to be expressed on many types of cells, including hematopoietic stem cells (HSCs), osteoclast precursor cells, platelets, and neurons. Zheng et al. identified angiopoietin-like proteins served as the ligands to LILRB2 on HSCs, which was considered as orphan ligands because no receptors were identified except LILRB2, and triggered SHP-2 signaling to maintain the self-renewal ability of HSCs (Zheng et al. 2012). However, LILRB2, expressed on osteoclast precursor cells, suppressed osteoclast development even in the presence of osteoclast formation stimulated factors RANKL and M-CSF (Mori et al. 2008). It is found by Kim T. et al. that LILRB2 are receptors for Ab oligomers with high affinity in mediation of the process of

Alzheimer's neuropathology (Kim et al. 2013). Murine paired immunoglobulin-like B (PIRB), the ortholog of human LILRB2, and its ligand ANGPTL2 possess an antithrombotic function by suppressing collagen receptor glycoprotein VI and integrin αIIbβ3-mediated signaling. Moreover, the expression of LILRB2 in neutrophils can also decrease their phagocytotic function and reactive oxygen species production (Baudhuin et al. 2013).

9.7.2 LILRB2 in Malignant Tumor Cell

More and more evidences have shown that LILRB2 is highly enriched in malignant tumor cells from both hematopoietic and solid tumors, including chronic B-cell lymphocytic leukemia (B-CLL) (Colovai et al. 2007), acute myeloid leukemia (AML) (Zheng et al. 2012), non-small cell lung cancer (NSCLC) (Liu et al. 2015; Sun et al. 2008), esophageal cancer (Warnecke-Eberz et al. 2016), pancreatic ductal carcinoma (Carbone et al. 2015), lobular breast cancer (Liu et al. 2014), and Lewis lung cancer (Ma et al. 2011).

By using MLL-AF9-induced mouse AML model, we revealed that PIRB was expressed on neoplastic cells and promoted the development and progression of AML accompanied by the increased infiltration of malignant cells in bone marrow, liver, and spleen (Zheng et al. 2012). In addition, we further found both LILRB2 and its soluble ligand ANGPTL2 were highly expressed in primary NSCLC samples and the levels were adversely related to overall survival. Mechanistically, our study revealed that the autocrined ANGPTL2 could bind to LILRB2 on tumor cells and trigger the downstream SHP2/CaMK1/CREB signaling pathway, resulting in a significant increase in proliferation, colony formation, and migration (Liu et al. 2015). Whereas studies from other groups suggested that LILRB2 promoted the tumor invasion and metastasis in vivo by manipulating extracellular regulated protein kinases (ERK1/2)/vascular endothelial growth factor C(VEGF-C) signaling pathway. Most importantly, Kaplan–Meier survival analysis also indicated that the NSCLC with high expression of LILRB2 had a poor patient's overall survival (Zhang et al. 2015). In human pancreatic ductal adenocarcinoma (PDAC), ANGPTL2/LILRB2 axis is responsible for the EMT and the early metastatic behavior of cells in pancreatic pre-neoplastic lesions (Carbone et al. 2015). LILRB2 has also been identified to be strongly induced by IL-10 in primary ductal and lobular breast cancer. The expression level of LILRB2 in breast cancer was positively correlated with the poor cell differentiation, increased metastasis, and higher grade and reduced overall survival in these patients (Liu et al. 2014).

9.8 TIM3

T cell immunoglobulin and mucin domain 3 (TIM3) belong to the TIM gene family, which includes three members TIM1, TIM3, TIM4 in humans and Tim1-Tim8 in mice. TIM-3 is expressed on Th1, Th17, $CD8^+$ T cells and myeloid cell lineages (Anderson et al. 2007; Hastings et al. 2009; Monney et al. 2002). TIM3 contains an immunoglobulin domain (IgV), single transmembrane domain, and a C-terminal cytoplasmic tail with a conserved tyrosine-based signal motif. Four relevant ligands, including galectin-9 (Gal-9), high mobility group protein B1 (HMGB1), carcinoembryonic antigen cell adhesion molecule 1 (Ceacam-1), and phosphatidylserine (PtdSer), have been identified to serve as a negative regulator of both adaptive and innate immune responses (Du 2017).

TIM3 is expressed on many kinds of tumor and acts as a potential negative prognostic marker based on meta-analysis (Zhang et al. 2017). It has been identified that TIM3 is highly expressed on the surface of AML stem cells, but not on the HSCs fraction of normal bone marrow. The treatment with TIM3 blocking antibody dramatically reduced the leukemic burden and eliminated leukemia stem cells without harming reconstitution of normal human HSCs. Thus, TIM3 serves as a functional marker to target human AML stem cells (Jan et al. 2011; Kikushige et al. 2010). Interestingly, TIM3 and its ligand, Gal-9, constitute an autocrine loop to activate NF-kb and b-catenin signaling pathway to promote self-renewal and development of human AML (Kikushige et al. 2015). However, other groups also found that TIM3, expressed in AML cells, triggered the responses to many growth factors by activating the PI3K/mTOR pathway and enhanced hypoxic-induced glycolysis and pro-angiogenic responses (Prokhorov et al. 2015)

In addition, TIM3 is also highly overexpressed in osteosarcoma tissues compared to adjacent normal tissue. And inhibition of TIM3 impairs its anti-apoptosis and invasion abilities with decreased Snail and Vimentin expression and increased E-cadherin expression (Feng and Guo 2016). In esophageal squamous cell carcinoma, TIM3 facilitated the metastasis by inducing EMT via the AKT/GSK-3B/Snail signaling pathway (Shan et al. 2016).

9.9 CD47

CD47, formerly known as integrin-associated protein (IAP), is a ubiquitously expressed cell membrane protein, which contains a single Ig V-like domain at the N-terminus, five times membrane-spanning segments, and an alternatively spliced cytoplasmic tail, and belongs to the immunoglobulin (Ig) superfamily (Lindberg et al. 1993). The Ig V-like domain of CD47 is required for interaction with several proteins to participate in a variety of biologic processes. By interacting with integrins, CD47 mediates leukocyte motility, adhesion, and migration (Brown et al. 1990; Lindberg et al. 1996). However, CD47 is also involved in the platelet activation when it binds to

thrombospondin (Isenberg et al. 2006). Additionally, CD47 plays a key role in maturation of dendritic cells and is involved in the regulation of apoptotic cell clearance by interacting with SIRPa (Matozaki et al. 2009).

A series of reports showed that CD47 was highly expressed on various types of human tumors compared with that in normal cells,such as acute myeloid leukemia-initiating cells (Majeti et al. 2009), acute lymphoblastic leukemia cells (Chao et al. 2011), non-Hodgkin's lymphoma (NHL) cells (Chao et al. 2010), primary effusion lymphoma (Goto et al. 2014), breast cancer cells (Zhang et al. 2015), bladder cancer cells (Chan et al. 2009), lung cancer cells (Liu et al. 2017), osteosarcoma cancer cells (Xu et al. 2015), hepatocellular carcinoma (Lee et al. 2014), pancreatic ductal adenocarcinoma (Cioffi et al. 2015), gastric cancer (Yoshida et al. 2015), prostate tumor cells, and colon glioblastoma (Willingham et al. 2012). CD47 is considered as a biomarker of cancers and its high expression is an adverse clinical prognostic factor. SIRPa is a molecule expressed on macrophages, while the interaction between the two components of the pair sends a "don't eat me" signal resulting in inhibition of phagocytosis of tumor cells to easily escape from this immunosurveillance (Jaiswal et al. 2009, 2010). Moreover, several studies reported that blocked CD47 with anti-CD47 monoclonal antibody significantly prolonged the survival of engrafted tumor mice and effectively suppressed the tumor growth and tumor cells invasion into other organs (Chao et al. 2010; Goto et al. 2014; Xiao et al. 2015). However, the treatment with anti-CD47 antibody not only induces the phagocytosis of tumor cells by macrophages, but also initiates the antitumor cytotoxic T cell (Tseng et al. 2013) and NK cell (Kim et al. 2008) immune response.

Apart from the roles in immune evasion, tumor cell-associated CD47 has been shown to regulate the tumor apoptosis, angiogenesis, metastasis formation, acquisition of tumor-initiating ability, promote drug resistance, proliferation EMT. In vivo anti-CD47 antibody treatment promoted phagocytosis of cancer stem cells (CSCs). Interestingly, the antibody induced the apoptosis of CSCs even in the absence of macrophages, suggesting a direct antitumor effect of the antibody. It has been demonstrated by several researches that ligation of CD47 by anti-CD47 antibodies, thrombospondins, or CD47 agonist peptide (4N1K) induced type III apoptosis (Bras et al. 2007) in different types of cancer cells depending on different apoptosis pathways (Mateo et al. 1999; Mateo et al. 2002), such as Cdc42/WASP signaling (Mateo et al. 2002), hypoxia-inducible factor (HIF)-1a pathway (Sagawa et al. 2011), c-AMP/PKA pathway (Manna and Frazier 2004), which induced the cancer cell apoptosis characterized by shrinkage, decreased mitochondrial transmembrane potential, phosphatidylserine externalization, and lysosomal permeabilization, but without the biochemical hallmark of nuclear apoptosis (Bras et al. 2007; Mateo et al. 1999). It

also has been found that ligation of CD47 could suppress vascular endothelial growth through breaking the NO signaling pathway by inhibiting the activity of endothelial nitric oxide synthase (eNOS) (Kaur and Roberts 2011).

CD47 is associated with chemotherapy drug resistance, especially in tumor-initiating cells (TICs). Lee T. K. et al. found that CD47 was preferentially expressed in hepatocellular carcinoma TICs. Knockdown of CD47 suppressed the tumor initiation, self-renewal, and metastasis of tumor stem/progenitor cell. Mechanistically, they found that $CD47^+$ hepatocellular carcinoma cells (HCC) preferentially secreted cathepsin S, which regulates liver TICs through the cathepsin S/protease-activated receptor 2 loop. Suppression of CD47 by morpholino approach suppressed growth of HCC in vivo and exerted a chemosensitization effect through blockade of cathepsin S/protease-activated receptor 2 signaling (Lee et al. 2014). Another study from Tan W. et al. found that miR-708/CD47 signaling pathway played an important role in breast cancer stem cells' self-renewal and chemoresistance (Tan 2019).

According to the data from Zhao H. et al., increased CD47 expression correlated with NSCLC clinical staging, lymph node metastasis, and distant metastasis. The downregulation of CD47 significantly inhibited tumor growth and metastasis both in vivo and in vitro. Mechanistically, it indicates that Cdc42 is a downstream mediator of CD47-mediated metastasis (Zhao et al. 2016). CD47 also serves as one of the markers of metastasis-initiating cells within circulating tumor cells in breast cancer patients (Baccelli et al. 2013). Overexpression of CD47 in human prostate cancer cell can facilitate cell metastasis to lung and liver in a mouse model (Rivera et al. 2015).

Treatment of triple-negative MDA-MB-231 breast cancer cell lines with anti-CD47 (B6H12) inhibited the proliferation and asymmetric cell division and suppressed the expression of epidermal growth factor receptor (EGFR) and the stem cell transcription factor KLF4 through enhanced microRNA-7 expression (Kaur et al. 2016). In astrocytoma cells, CD47 was demonstrated to promote the proliferation and survival of tumor cells by binding to the $G\beta\gamma$dimer, which subsequently activated the PI3K/Akt pathway (Sick et al. 2011). Furthermore, CD47 promoted migration and invasion, induced EMT through modulating E-cadherin and N-cadherin in high-grade serous ovarian carcinoma (HGSOC) (Li et al. 2017). All these studies may suggest that CD47 is a functional surface immune molecule regulating the tumor cell fate independent of immune activation.

9.10 CD137

CD137 (also called 4-1BB or TNFRSF9) is a co-stimulatory molecule belonging to the tumor necrosis factor receptor superfamily (TNFRSF). CD137 was identified in 1989 as an inducible gene that was expressed on antigen-primed T cells (Kwon and Weissman 1989). Afterward, it was found to be expressed in activated $CD4^+$ and $CD8^+$ T lymphocytes, dendritic cells (DCs), natural killer cells (NKs), natural killer T cells (NKTs), and mast cells (Vinay and Kwon 2006, 2012; Croft 2009).

CD137 can be activated by binding to its ligand (CD137L or 4-1BBL) and the ligation leads to cytokine induction, prevention of activation-induced cell death, and upregulation of cytotoxic T cell activity. CD137L is expressed by all types of antigen antigen-presenting cells, and it drives signals back into antigen-presenting cells, which promote their activation and differentiation and enhance the secretion of proinflammatory cytokines (Dharmadhikari et al. 2016). Several studies show that in mono or combined therapies for cancers, anti-CD137 antibodies strength antitumor immune response because they can activate or regulate immune subsets in the tumor microenvironment, such as increase the activation, proliferation, and activities of $CD8^+$ T cells, $CD4^+$ T cells, NK cells, and macrophages; increase IFN-gamma production, but inhibit the proliferation and functions of myeloid-derived suppressor cells and regulatory T cells (Chu 2019).

Palma C et al. reported that several T leukemia and B lymphoma cell lines expressed CD137 or CD137L, and soluble CD137L has been found in sera of leukemia patients, which promoted the proliferation and prolonged survival of these tumor cells. In addition, CD137/CD137L signaling opposed the anticancer drug cytotoxic effects and drug resistance, reduced the apoptotic DNA fragmentation, and stimulated the proliferation of doxorubicin-escaped leukemia cells (Palma et al. 2004). CD137 is ectopically expressed by Hodgkin lymphoma, which induces the secretion of IL-13. CD137-induced IL-13 secretion not only facilitates escape from immune surveillance, but also enhances the proliferation of Hodgkin lymphoma cells (Rajendran et al. 2016). Jiang p et al. reported that CD137 promotes the migration of monocytes and macrophages to tumor microenvironment and the differentiation into osteoclasts via upregulating the expression of Fra1. All these processes provide a favorable microenvironment for the colonization and growth of breast cancer cells and metastasis into bone microenvironment. A novel anti-CD137 blocking antibody could efficiently inhibit both bone and lung metastases of breast cancer cells in vivo (Jiang et al. 2019). CD137 stimulation by ligation of its ligand promotes the survival of chronic lymphocytic leukemia (CLL), and this effect was mediated by activation and the nuclear translocation of p52 (a non-canonical NF-κb factor) (Nakaima et al. 2013).

It has been found CD137 expression in activated T cells is regulated by certain transcription factors, such as the activator protein 1(AP-1) and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κb) as well as cytokines (interleukin-2 and interleukin-4) and possibly in other immune cells (Kim et al. 2003, 2011; Pollok et al. 1995; Vinay and Kwon 2011). In another study, hypoxia was shown to enhance the expression of CD137 in activated T cells and this process was mediated by HIF-1a expression (Palazon et al. 2012). While it was showed that NF-kappa B pathway was mainly stimulated by the KRAS-induced secretion of interleukin-1a (IL-1a), which promoted the transcription of CD137 in pancreatic cancer cells (Rielland et al. 2014).

9.11 CD70

CD70 is another member of the TNF superfamily that is found on activated dendritic cells, B cells, T cells, and NK cells. CD70 is also a type II transmembrane receptor of co-stimulatory CD27/CD70 pair of immune checkpoint molecules which play an important role in providing co-stimulation signaling during the activation of functional lymphocytes (Denoeud and Moser 2011).

CD70 is expressed on a broad range of malignancies (Jacobs et al. 2015), such as glioblastomas (GBMs) (Jin et al. 2018), head and neck squamous cell carcinoma (HNSCC) (Park et al. 2018), melanoma (Pich et al. 2016), AML (Riether et al. 2017), chronic myeloid leukemia (Riether 2015), and the high expression level correlates with poor survival (Ge et al. 2017). Ablation of CD70 in primary GBM inhibited tumor migration, growth, and chemoatrractive abilities of monocyte-derived M2 macrophages and decreased CD44 and sex determining region Y-box 2 (SOX2) gene expression, which has been reported for the indication of the stemness of cancer cells (Ge et al. 2017). Interestingly, CD70 plays a complexic role in melanoma metastasis. In vitro and in vivo experiments demonstrated that monomeric CD70 expression inhibited melanoma cell migration, invasion, and pulmonary metastasis. However, the formation of CD70 trimers led to the increase of the invasiveness of melanoma cells and the disappearance of stress fibers and adhesions (Pich et al. 2016). Riether C. et al. found the pair of CD27/CD70 was expressed on AML blasts and stem/progenitor cells. CD70/CD27 signaling in AML cells activates WNT pathway and promotes symmetric cell divisions and proliferation, while all these phenotypes can be reversed by blocking the CD70/CD27 interaction via mAb (Riether et al. 2017). CD70 is upregulated on tyrosine kinase inhibitor resistant CML leukemia stem cells. It seemed that TKIs induced the expression of CD70 that further resulted in the activation of WNT pathway to enhance TKI resistance in a compensatory manner. Combined treatment with TKIs and CD70 blockade effectively eliminated human $CD34^+$ CML initiating cells in xenografts and murine CML model (Riether 2015). Recently, several preclinical studies showed that CAR-T target CD70 induces potent antitumor response in xenograft and syngeneic models without adverse effects in GBMs and HNSCC (Jin et al. 2018; Park et al. 2018). CD70 may be a promising target for tumor therapy since tumor-associated CD70 is involved in the initiation and maintenance of cancer stem cells, drug resistance, and tumor cell proliferation.

9.12 Conclusion

Significant advances have been made in cancer immunotherapy in the last decade since the relatively recent knowledge of the biological consequences of tumor-associated immune checkpoint molecules. Blockade of inhibitory immune checkpoints can positively recover T cell activation and prevent immune escape of cancer cells within the tumor microenvironment, and activation of stimulatory immune checkpoints can augment the effect of immune response. However, targeting those checkpoint molecules according to their roles in maintaining malignant traits in tumor cells may provide us novel therapeutic approaches (Fig. 9.2) (Table 9.1).

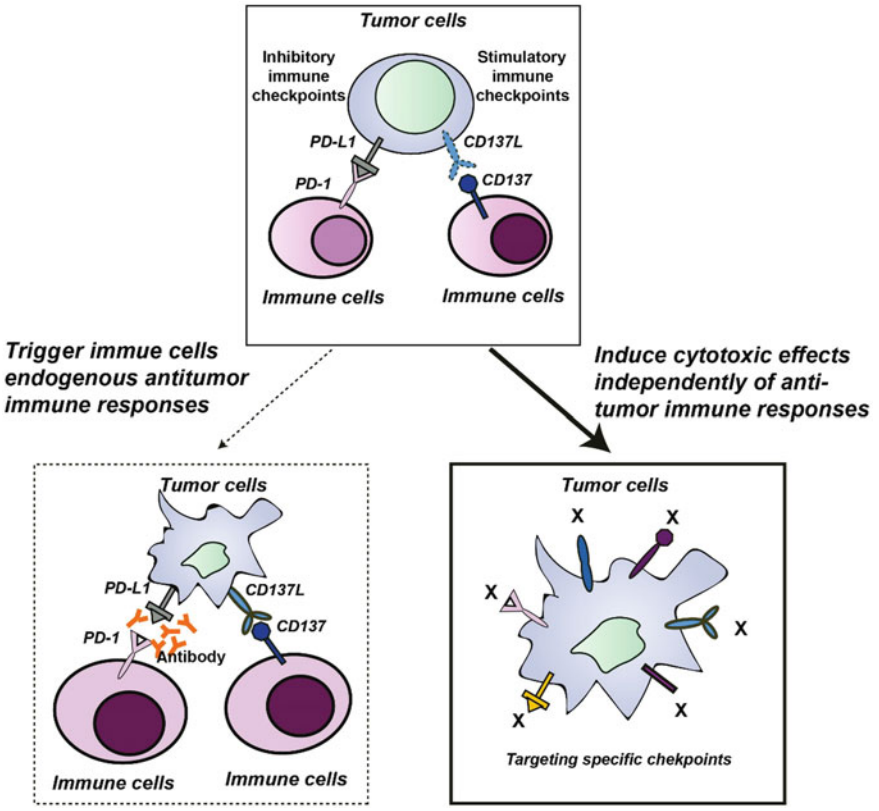

Fig. 9.2 Diagram of traditional and novel approaches for the elimination of tumor cells. Dashed line: immune cells are activated by blocking inhibitory immune checkpoints or activating immune stimulatory checkpoints, which can further result in the death of tumor cells. Bold lines: increasing evidence indicates the possibility of the eradication of tumor cells independent of the canonical immune evasion functions of these immune checkpoints, but via their unique roles in maintaining malignant behaviors

Table 9.1 Malignant traits of tumor cells regulated by immune checkpoint molecules

Malignant traits									
Immune checkpoint molecules	EMT	Increased proliferation	Stemness acquisition	Drug resistance	Anti-apoptosis	DNA repair	Metabolism alert	Tumorigenesis	Angiogenesis
PD-L1	Yes	Yes	Yes	Yes	Yes	Yes	Yes	NA	NA
PD-1	Yes	Yes	Yes	Yes	Yes	NA	Yes	Yes	Yes
B7-H3	Yes	Yes	NA	Yes	NA	NA	Yes	Yes	Yes
B7-H4	Yes	Yes	Yes	Yes	Yes	NA	NA	Yes	NA
LILRB1	NA	Yes	NA	NA	NA	NA	NA	NA	NA
LILRB2	Yes	Yes	Yes	NA	NA	NA	NA	Yes	NA
TIM3	Yes	Yes	Yes	Yes	Yes	NA	Yes	Yes	Yes
CD47	Yes	Yes	Yes	Yes	Yes	NA	NA	Yes	Yes
CD137	NA	Yes	NA	Yes	NA	NA	NA	NA	NA
CD70	NA	Yes	Yes	Yes	Yes	NA	NA	Yes	NA

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Chapter 10
Genetic Alterations and Checkpoint Expression: Mechanisms and Models for Drug Discovery

Shuai Ding, Siqi Li, Shujie Zhang and Yan Li

Abstract In this chapter, we will sketch a story that begins with the breakdown of chromosome homeostasis and genomic stability. Genomic alterations may render tumor cells eternal life at the expense of immunogenicity. Although antitumor immunity can be primed through neoantigens or inflammatory signals, tumor cells have evolved countermeasures to evade immune surveillance and strike back by modulating immune checkpoint related pathways. At present, monoclonal antibody drugs targeting checkpoints like PD-1 and CTLA-4 have significantly prolonged the survival of a variety of cancer patients, and thus have marked a great achievement in the history of antitumor therapy. Nevertheless, this is not the end of the story. As the relationship between genomic alteration and checkpoint expression is being delineated though the advances of preclinical animal models and emerging technologies, novel checkpoint targets are on the way to be discovered.

Keywords Genetic alteration · Checkpoint inhibitor · Oncogenic mutation · Chromosomal aberration · Preclinical mouse model

10.1 Genomic Aberrations, Cancer Neoantigens, and Immune Evasion

10.1.1 Genomic Abnormalities in Cancer Cells

Genomic alterations may have caused the change of protein structure, activity, and abundance, which ultimately lead to uncontrollable growth and malignancy in the context of cancer (Mardis 2017). Genomic variations encompass single nucleotide variation, insertion, or deletion (indels; gain or loss of short segments of chromosomal

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DNA), and structural variant (SVs; genomic rearrangements that affect >50 bp of sequence).

The most common and important inherited sequence variations are single nucleotide polymorphism (SNP), with a minor allele frequency greater than 1% in at least one population (Erichsen and Chanock 2004; Risch 2000). Most SNPs are synonymous and only a very small number of them with high penetrance and detrimental phenotype, such as the XmnI SNP caused β-thalassemia (Badens et al. 2011), or IX F9 SNP caused hemophilia (Simhadri et al. 2017), can be identified with pedigrees analysis. For complex diseases, such as most cancers, the strategy to explore differences in genetic variability is the genome-wide association study (GWAS) between unrelated, diseased, and healthy individuals. More specifically, the strategy contains estimating haplotype frequencies and testing association between a disease and haplotypes of multiple genetic markers inherited as a unit. The rational lies in the fact that the majority of SNPs represent ancestral haplotypes rather than phenotypic effects. Concurrently, throughout molecular cancer epidemiology, each SNP contributes only a tiny amount to the overall risk of developing cancer. Haplotypes and SNPs, inferred by linkage disequilibrium, enable the characterization of genetic features for predicting individuals' inherited susceptibility (Slatkin 2008).

According to a certain estimate, cancers caused by germline mutations represent only 5–10% of all cancer cases (Tung et al. 2016; Skaro et al. 2019). Single nucleotide variant (SNV), another frequently used term referring to a somatic variation in a single nucleotide without any limitations of frequency, could be identified by genomic profiling of cancer cells with comparisons to normal cells (Moncunill et al. 2014). Insertion and deletion, commonly abbreviated "indel", is one of the main events contributing to genetic variation. Indel of intervening nonrepetitive and repetitive DNA is often the result of polymerase slippage errors. Insertion can be anywhere in size from single base-pair inserted into a DNA sequence to a section of one chromosome inserted into another. Deletion can also act the same. Non-3n indels could result in frameshift mutations to create incorrect and/or incomplete proteins, which contribute significantly to driver mutations in oncogenesis. A study of somatic mutations in cancer database further discussed the phenomenon that frameshift mutations are present in tumor suppressor genes in much higher frequencies than those in oncogenes (Yang et al. 2010). Another study estimated more deletions than insertions and more frameshift mutations than in-frame ones in tumor cells based on the COSMIC database. Meanwhile, two studies independently arrived, at the same conclusion with regard to the preferred mutations modes of tumor suppressors and proto-oncogenes (Iengar 2012).

A noteworthy term here is nonsynonymous mutations, referring to a nucleotide substitution that alters the corresponding amino acid in the encoded protein, as a result of a single nucleotide indel during transcription or, occasionally, a nucleotide change based at the third position of a codon. Independent cohort studies demonstrated that higher nonsynonymous SNV burden is closely associated with progression and prognosis of tumors (Kandoth et al. 2013; Rizvi et al. 2015). Notable among these studies are neoantigens generated by nonsynonymous somatic mutations, which will be explained in detail subsequently.

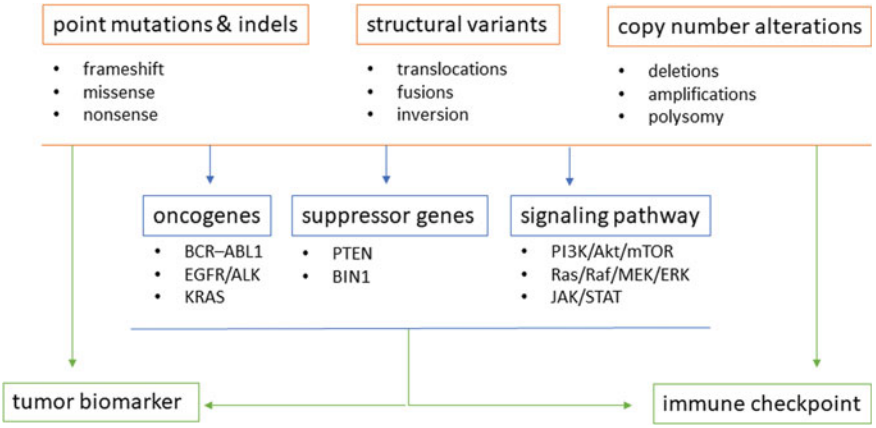

Fig. 10.1 Impacts of genomic alterations and oncogenes to immune checkpoints

SV is defined as genomic alterations include indels, duplications, translocations, and inversions, ranging from submicroscopic sequence variants greater than 50 bp in size, to larger variants cytogenetically visible (Moncunill et al. 2014). Now it is generally acknowledged that SVs can be the driving force in the evolution of human phenotypes, as well as the evolution of cancers. For instance, a higher copy number of the epidermal growth factor receptor (EGFR) has been identified in non-small cell lung cancer (NSCLC). Nonrandom genetic abnormalities, including aneuploidy (gains and losses of whole chromosomes) and structural rearrangements that often result in the formation of chimeric oncogenes (e.g., BCR–ABL1) (Diouf et al. 2011), can be found in the majority of hematologic malignancies (Yoshizato et al. 2017; Anderson et al. 2011). Moreover, as will be discussed in the following parts of this chapter, these modes of alterations also have an impact on immune checkpoints (Fig. 10.1).

10.1.2 Neoantigens Derived from Genomic Abnormalities

Neoantigens are immunogenic protein products of tumor-specific mutations which are likely to activate the immune system and cause the immune system to attack.

The first successful application of neoantigens in personalized cancer immunotherapy hails from Novartis Institutes for BioMedical Research Incorporate. By performing exome sequencing of the tumor tissue biopsies from a patient with advanced cholangiocarcinoma, scientists identified 26 nonsynonymous mutations. Tumor-infiltrating lymphocytes (TILs) containing CD4 T helper cells were able to recognize a mutation in erbb2 interacting protein (ERBB2IP) (substitution mutation A-G) expressed by cholangiocarcinoma cells. After re-injecting T cells with greater

than 95% recognizing abnormal form of ERBB2IP, lesions on the patient eventually reached complete remission and the tumor disappeared (Tran et al. 2014).

The above strategy that largely relies on screening for neoantigens by constructing all candidate gene fragments in tandem, unfortunately, is only suitable for low tumor mutation burden (TMB) cases (Rizvi et al. 2015; Chan et al. 2015; Hause et al. 2018). New techniques would be the key to identify neoantigens in cancers with high TMBs, such as melanoma and NSCLC. By creating NetMHC4.0, Chizu Nonomura et al. recently screened 12066 neoantigen candidates from 1348 nonsynonymous mutations in melanoma and finally identified ARMT1 (SNV mutation C-T) as an efficient neoantigen (Nonomura et al. 2019).

Each nonsynonymous mutation increases the chance of neoantigen formation. A total of 617,354 somatic mutations, predominantly consisting of 398,750 missense mutations had been depicted across 12 major cancer types (Kandoth et al. 2013). However, neoantigens derived from indels have been shown with higher frequency for high-affinity binders and mutant specific bindings, as compared to non-synonymous SNV derived neoantigens (Turajlic et al. 2017). The most frequent mutation in acute myeloid leukemia is 4 bp frameshift insertions in nucleophosmin 1 (NPM1) gene. It causes mutated NPM1 (ΔNPM1) 4 amino acid longer than its wide type counterpart and produces new 11 amino acid (CLAVEEVSLRK) at the C terminal. Based on this reading frame, Dyantha et al. obtained 5 AML-specific peptides by allelic HLA-matching, including two 8-mer peptides (VEEVSLRK and AVEEVSLR), two 9-mer peptides (CLAVEEVSL and AVEEVSLRK), and one 11-mer peptide (CLAVEEVSLRK). They selected CLAVEEVSL as ΔNPM1 derived neoantigen candidate according to its binding capacity and expression distribution, then further confirmed its neoantigenicity by TCR gene transfer and antitumor efficacy testing (van der Lee et al. 2019).

Although Next Generation Sequencing (NGS) can accurately assess the mutational landscape of tumor, its application may compromise when dealing with tumors with SVs and low TMBs such as glioblastoma multiforme, thyroid carcinoma, ovarian serous, malignant pleural mesothelioma and the like (Cristescu et al. 2018; Bueno et al. 2016). This limitation promotes the development of sequencing technology. Mansfield et al. used mate-pair sequencing to detect SVs in malignant pleural mesothelioma, which can reliably detect indels and rearrangements by tiling the whole genome into larger fragments (2–5 kb). 3 peptides (NYLELETTSDF, CYGETYQNI, and NYLETTSDFHF) were proven to cause neoantigens from all inter- or intrachromosomal rearrangements (Mansfield et al. 2019). Besides chromosomal rearrangements, gene fusion-derived neoantigens are also noteworthy. In a cohort of head and neck tumors with low TMBs and minimal immune infiltration, a novel gene fusion that produces a neoantigen has been proved to elicit cytotoxic T cell response by Morris et al. (Yang et al. 2019).

10.1.3 Tumor Immune Escape

Although neoantigens result in improved immune surveillance, tumor can evolve a variety of mechanisms to evade immune recognition and elimination, so-called tumor immune escape. Currently, the mechanisms of tumor immune escape can be broadly summarized in two directions. One is to block the infiltration of antitumor cells; the other is to inhibit the function of antitumor cells.

Tumorigenesis is usually accompanied by expression of abnormal proteins (neoantigens). These neoantigens can be presented to CD8 T cells by tumor cells or antigen-presenting cells (APCs) patrolling around the body, thereby inducing specific CD8 T cell infiltration. However, in order to survive, tumor cells begin to undergo a selection process in which clones that activate the immune system are eliminated, and remaining clones can inhibit the presentation of tumor antigen (Khong and Restifo 2002; Han et al. 2019). The most widely studied mechanism is the absence of neoantigens or MHC class I molecules in tumor immunology (Han et al. 2019; Donawho et al. 2001; Angell et al. 2014; Vesely et al. 2011; Dunn et al. 2002).

In addition to tumor neoantigens, inflammation signals secreted by tumor cells or APCs around tumor also recruit T cells. A large number of studies have shown that oncogenic pathway affects immune system from recognizing and attacking tumor cells through regulation of chemokines. Among them, the up-regulation of the WNT–β-catenin signaling pathway reduces the secretion of CCL4 to inhibit T cell priming and CXCL9/10 to inhibit T cell trafficking, ultimately leading to reduced T cell recruitment (Spranger et al. 2015, 2017; Seiwert et al. 2015). Loss of p53/LKB1 function is reported to be associated with T/NK cell infiltration decrease and T cell dysfunction by upregulating IL-33, CXCL7, and IL-6 or downregulating CCL2, CCL3, CCL4, CCL5, CXCL1, and CXCL2 (Quigley et al. 2015; Koyama et al. 2016; Iannello et al. 2013).

In fact, even though immunogenic tumors bearing high TMB or neoantigenicity are infiltrated by immune cells, they are still capable of evading immune elimination by inhibiting immune function or inducing apoptosis of tumor-killing cells. Typically, tumor cells induce proliferation of myeloid-derived suppressor cells (MDSCs) via multiple inflammatory factors (Marigo et al. 2010; Sade-Feldman et al. 2013; Baert et al. 2019). In addition, macrophages are recruited and secrete IL-10 and CCL22 to promote monocyte PD-L1 expression. Macrophages also inhibit T cell function by regulating influx of regulatory T cells (Treg) (Steidl et al. 2010; Cassetta et al. 2019; Kuang et al. 2009; Curiel et al. 2004). Among these mechanisms, immune checkpoint is one of the most important strategies for preventing excessive immune activation. In addition to inducing expression of PD-L1 on immunosuppressive cells, tumor cells also express PD-L1 molecules to inhibit cytotoxic T cells directly through genomic abnormalities, such as EGFR overexpression and anaplastic lymphoma kinase (ALK) rearrangements as discussed later in this chapter. Accordingly, several immunological checkpoint inhibitors targeting PD-1/PD-L1 or CTLA4 such as nivolumab, atezolizumab, and lpilimumab have been developed with high response rate in different tumor types including melanoma (Sullivan et al. 2019;

Sade-Feldman et al. 2018; Auslander et al. 2018), NSCLC (Remon et al. 2019; Fillon 2018; Mathew et al. 2018), Hodgkin's lymphoma (Ansell et al. 2015; Chen et al. 2017), urothelial cancer (Niglio et al. 2019; Szabados et al. 2018), head and neck squamous cell carcinoma (Migden et al. 2018; Seiwert et al. 2016), and so on.

It is worth noting that intestinal microbiota also affects tumor responses to checkpoint inhibitors. Inspired by the differences in spontaneous antitumor immunity in mice with melanoma harboring distinct commensal microbiota, Sivan et al. identified that *Bifidobacterium* was associated with the antitumor effects, and microbial transplantation of *Bifidobacterium* together with PD-L1 treatment abolished tumor growth (Sivan et al. 2015). Another recent study found an association between specific T cell responses to *B. thetaiotaomicron* or *B. fragilis* and the efficacy of CTLA-4 blockade, and further demonstrated the importance of *Bacteroidales* in the immunostimulatory effects of CTLA-4 blockade (Vetizou et al. 2015).

10.2 Regulation of Checkpoint Expression via Mutations in Oncogenic Signaling Pathways

Genetic alterations in signaling pathways that control cell differentiation, proliferation, and apoptosis are hallmarks of cancer. A recently published study depicted the alteration landscape in 10 main signaling pathways from 33 cancer types. It has been shown that signaling pathways are somatically changed at varying frequencies with varying combinations across different cancer types, denoting the complex interplay and pathway crosstalk (Sanchez-Vega et al. 2018). Notably, these scenarios could also affect checkpoint expression. Although the signaling pathways involved in PD-L1 regulation are complex and only partially understood, in this section we will discuss 3 canonical signaling pathways with regulation of checkpoint related genes and focus on oncogenic driver mutations.

10.2.1 PI3K/Akt/mTOR Pathway

Phosphoinositide 3-kinase (PI3K)/AKT/mammalian target of rapamycin (mTOR) pathway is a major regulator of cell growth, proliferation, metabolism, and tumorigenesis (Engelman et al. 2006). Aberrant activation of the PI3K/AKT/mTOR pathway promotes tumor cell survival and proliferation, through several mechanisms including activation of EGFR, loss of tumor suppressor PTEN function, amplification or mutation of PI3K and AKT, and exposure to carcinogens (LoPiccolo et al. 2008). The first two mechanisms are also found to be related to the expression of PD-L1 as discussed below.

EGFR, as a cell surface receptor, controls cell growth in normal and malignant tissue (Jackson and Ceresa 2017). Attention was first focused on EGFR mutation that

is associated with PD-L1 expression in lung cancer. Further studies observe that PD-L1 positive NSCLC patients received EGFR tyrosine kinase inhibitors (TKIs) have higher response rate and longer median time to tumor progression compared with PD-L1 negative ones (Chen et al. 2015). A closer observation from Ota et al. showed the gene fusion of echinoderm microtubule-associated protein-like 4 and anaplastic lymphoma kinase (EML4–ALK) as another oncoprotein to promote immune evasion by PD-L1 induction. Furthermore, ALK rearrangement and EGFR mutation regulate PD-L1 expression via common downstream signaling pathways mediated by PI3K-AKT and by MEK-ERK (Ota et al. 2015). Another study appended that in NSCLC, PD-L1 overexpression was positively correlated with EGFR expression but inversely correlated with human epidermal growth factor receptor 2 (HER2, also known as ERBB2) expression (Okita et al. 2017). The effect of HER2 on PD-L1 induction is different from EGFR and the mechanism of action still remains to be elucidated.

As a tumor suppressor gene, PTEN participates in preventing the onset and progression of cancers by antagonizing protein tyrosine kinases. Accordingly, PTEN loss could activate AKT/mTOR pathway and subsequently increase PD-L1 expression (Seront et al. 2013). Parsa et al. firstly demonstrated the loss of PTEN following the increased expression of PD-L1 in human glioma (Parsa et al. 2007). Song et al. confirmed this association through downregulation of PTEN expression by RNA interference in colorectal cancer (Song et al. 2013). George et al. furtherly reported that PTEN loss was associated with resistance to anti-PD-1 monotherapy in sarcoma (George et al. 2017). These findings indicate PTEN as a potential mediator of resistance to immune checkpoint therapy.

10.2.2 Ras/Raf/MEK/ERK Pathway

The Ras/Raf/MEK/ERK pathway involving a series of protein kinase cascades, also known as the MAPK/ERK pathway, plays a pivotal role in regulation of cell cycle progression and cell survival response. Under pathological conditions, early studies of colorectal cancer indicate that MAPK signaling pathways are linked to cell adhesion, proliferation, aggressiveness and metastasis, as reviewed by Fang and Richardson (2005). Recent studies have increasing evidence showing the involvement of MAPK/ERK pathway with PD-L1 expression.

Activation of MAPK/ERK pathway is commonly detected in malignant tumors, in which constitutively active EGFR as a result of somatic mutation and/or gene amplification triggers downstream signaling network. Yataro et al. found higher frequency of EGFR mutations in NSCLC cell lines with high PD-L1 expression, and the significant decrease of PD-L1 after MEK inhibitor treatment. MEK inhibitor affects transcriptional machinery of PD-L1 by changing the enhancer activity in the candidate AP-1 binding site (Sumimoto et al. 2016).

In addition, mutations in components of the pathway, such as the KRAS gene, also lead to constitutive activation of the signaling pathway and enhanced PD-L1 production. Chen et al. demonstrated that KRAS mutation-induced PD-L1 expression

through MAPK/ERK signaling in lung adenocarcinoma (Chen et al. 2017). T cell apoptosis regulated by PD-L1 can be reversed by Pembrolizumab or ERK inhibitor. A noteworthy fact is that KRAS mutation is usually exclusive with other oncogenic mutations, such as EGFR and ALK. Coelho et al. demonstrated that oncogenic RAS signaling is efficient to upregulate PD-L1 expression by increasing its mRNA stability via MEK and PI3K pathways (Coelho et al. 2017).

The tumor suppressor genes also participate in these signaling pathways by regulating proto-oncogenes and receptors. Bridging integrator-1 (BIN1) was discovered as early as 1996, encoding a c-Myc-interacting adapter protein with tumor suppressor properties (Sakamuro et al. 1996). A recent study of NSCLS has come up with the conclusion that overexpression of BIN1 could reduce the c-MYC and EGFR-induced PD-L1 expression, even reversed the suppressive immuno-microenvironment in vivo (Wang et al. 2017).

10.2.3 JAK/STAT Pathway

Immune checkpoint blockade releases the brakes on immune responses by restoring T cell function, secreting IFNγ and breaking the tumor microenvironment. IFNγ suppresses the growth of cancer cells through a signaling pathway that requires Janus kinase (JAK) proteins (Nguyen et al. 2000). In fact, JAK/signal transducer and activator of transcription (STAT) pathway are involved in many important processes, such as cell division, cell death, and immunity. A study conducted in head and neck cancer cases suggests that PD-L1 expression is extrinsically regulated by IFNγ and intrinsically regulated via EGFR by JAK2/STAT1 dependent manner (Concha-Benavente et al. 2016).

The mechanism of JAK/STAT signaling is relatively straightforward. However, the biological consequences of signaling transduction are more complicated due to pathway interaction (Heinrich et al. 2003; Shuai 2000). In NSCLC, accumulating evidence indicates that activation of PI3K/AKT and ERK/MAPK result in upregulated PD-L1 expression. However, Hayakawa et al. arrived at the conclusion that AKT/STAT3 pathway is responsible for regulating EGFR-driven PD-L1 expression on NSCLC cells (Abdelhamed et al. 2016). Nakata et al. also suggested that JAK/STAT pathway is involved in EGFR and HER2 regulated PD-L1 expression (Okita et al. 2017). One upstream signal rarely triggers just one signal transduction cascade. This provokes the thinking of combining different signaling inhibitors to block the expression of immune checkpoints on tumor cells.

Nevertheless, the high-frequency oncogenic mutations are not consistently associated with expression of known checkpoint molecules. A new study has shown that high mutational loads and predicted neoepitopes are detected in triple-negative breast cancers (TNBCs) patients. However, PTEN deletion and activating PIK3CA mutation is not linked to the increased PD-L1 expression (Barrett et al. 2018). These data reflect alternative immune evasion mechanisms mediated through unidentified checkpoint molecules in highly mutated tumors with low PD-L1 expression. On the

other hand, limited response to current checkpoint blockades also suggests that other checkpoint molecules may be functionally complementary.

10.3 Regulation of Checkpoint Expression via Chromosomal Aberration

10.3.1 Novel Checkpoints Transcripts and Proteins Created by Gene Fusion

Gene fusions are hybrid genes formed by juxtaposing two separate and distinct genes, which result from structural rearrangements such as translocations and inversions, along with fusion by transcription or splicing (Nacu et al. 2011; Kim et al. 2014; Li et al. 2009; Zhang et al. 2012; Velusamy et al. 2013; Qin et al. 2015). Gene fusions have been widely recognized as key drivers of oncogenic pathways in hematological cancers and sarcomas, and also accompanied by the emergence of novel checkpoints transcripts (Watson et al. 2013; Yoshihara et al. 2015).

The expression of high-aggregate PD-L1 and PD-L2 in certain subsets of tumor cells is not just attributable to the activating of EGFR mutations and AKT-STAT3 pathway as mentioned above. In primary mediastinal large B cell lymphoma (PMBCL), Steidl et al. found that highly recurrent breaks of MHC II transactivator CIITA fused with PD-L1 and PD-L2, result in overexpression of both ligands and downregulation of MHC II expression (Steidl et al. 2011). It is a typical way to ramp up the chimeric transcript expression through juxtapositioning under a strong promoter or enhancer, and exactly that happens in PMBCL. Although overexpression of PD-L1 and PD-L2 has been previously described in PMBCL to be directly linked to copy number gain, the expression levels in cases with translocations obviously exceeded the levels of those cases without.

Another typical form of fusion proteins with oncogenic property has been reported mostly involving tyrosine kinases and transcriptional regulators (Hochhaus et al. 2011; Hantschel et al. 2012; Mitelman et al. 2004). Similar phenomena have also been identified for CTLA-4 immune checkpoint in T cell lymphoma. The fusion gene, which consists of the extracellular region of CTLA-4 and the cytoplasmic region of CD28, could lead to constitutive T cell activation by transforming inhibitory signals into stimulatory signals. Specifically, skin biopsies and peripheral blood samples from 11 patients with mycosis fungoides and Sézary syndrome were tested for somatic genetic alterations by whole-exome sequencing. Aside from recurrent TNFR2 Thr377Ile mutant, two of those patients have been detected with a large deletion on chromosome 2 and CTLA4-CD28 fusion. Subsequently, CTLA4-CD28 fusion has been reported to occur in 58% angioimmunoblastic T cell lymphomas, 23% peripheral T cell lymphomas, 29% extranodal NK/T cell lymphomas in the total number of 115 samples of diverse subtypes, using RT-PCR analysis and Sanger sequencing (Yoo et al. 2016).

Several new high-throughput sequencing and analysis strategies have been proposed to overcome the limitation of current diagnostic resolution and throughput. Heyer et al. established targeted RNAseq by optimizing laboratory and bioinformatic variables using spike-in standards and cell lines, and further verified its effectiveness by comparisons with conventional approaches (Heyer et al. 2019). Tretiakova et al. identified high-frequency fusion candidates in renal cell carcinoma (RCC) and set the FusionPlex accordingly. This platform, allowing identification of both known and novel fusion partners, is recommended to patients under age 50 or when the histologic appearance suggests RCC (Tretiakova et al. 2019). The next questions are whether these data would provide comprehensive assessment, treatment planning, and intervention for patients with certain gene fusions and whether gene fusion with checkpoint could serve as potential therapeutic targets.

10.3.2 Gene Amplification and Polysomy Affect Checkpoints Expression

Gene amplification refers to an increase in the copy number of a restricted chromosomal region, through the formation of extrachromosomal double minutes or intrachromosomal homogeneously staining regions (Chatterjee et al. 1999; Natarajan and Boei 2003). It results in overexpression of the affected genes or further leads to deregulation of its related signaling pathways. Polysomy is defined by the presence of more than two copies of a chromosome in a diploid somatic cell mainly as the result of non-disjunction during meiosis. It also provides an alternative mechanism for certain gene amplification.

Both gene amplification and polysomy serve as genetic mechanisms for activating oncogenes in cancer initiation and progression, as well as for affecting checkpoint expression exploited by tumor cells to avoid immune attack. In Reed-Sternberg cells, copy number alterations in chromosome 9p24.1 increase the abundance of PD-L1 and PD-L2 (Green et al. 2010). The phase 1 trial study of Nivolumab and Pembrolizumab for relapsed and refractory Hodgkin lymphoma has further shown that gene amplification and polysomy are closely associated with primary resistance of checkpoint blockade immunotherapies and incomplete remission. More specifically, fluorescence in situ hybridization (FISH) analyses of tissue biopsied taken on relapsed Hodgkin lymphoma patients identified the polysomy, copy number gain, and amplification of 9p24.1 in 16, 58, and 27% of patients, respectively. By correlating the level of 9p24.1 alteration and PD-L1 expression with treatment responses, Younes et al. found that none of the patients with polysomy achieved a complete remission, and vice versa (Younes et al. 2016).

To tackle the challenges which exist with long-term incomplete remission, it is essential to decipher the message of amplification and polysomy. To date, different methods are available to detect gene amplification. The DNA-based techniques, including PCR and southern blot, and the molecular cytogenetic techniques as FISH

are generally used to identify only copy number alterations of known genes. Alternatively, NGS and dense microarray-based comparative genomic hybridization (aCGH) are two genome-wide technologies that are able to detect also unknown copy number alteration (Przybytkowski et al. 2013; Taboada et al. 2005). FISH is the standard diagnostic assays for polysomy detection. Nuclei with a target to control probe ratio are concrete measurements used in identifying multifocal polysomy (MFP) and unifocal polysomy (UFP).

10.4 Preclinical Mouse Models for Checkpoint Mechanistic Studies and Immunotherapy Evaluations

Cancer treatment traditionally emphasized on blocking the expansion of tumor cells (chemotherapy and radiotherapy) or the initiation of tumor formation (targeting oncogenic pathways and cancer stem cells) (Hanahan and Weinberg 2000). Despite the success of cocktail chemotherapies and targeted therapies in certain tumors, many other tumors did not benefit from these treatments with long-term remissions, not to mention cures (Zitvogel et al. 2016). The relationship between neoplastic cells and host immunity was unraveled first in mouse models of cancer, consequently led to the recent success of immune checkpoint blockade therapy (Leach et al. 1996; Iwai et al. 2005). Mouse models of human cancer have contributed considerably to our knowledge of the dual role of immune system in oncogenesis and tumor progression. In this section, we will briefly discuss the characteristic and appropriate use of mouse models of human cancer for immune checkpoint studies, with a focus on the "humanized" mice for preclinical and personalized evaluation of checkpoint drugs.

10.4.1 Spontaneous and Induced Mouse Tumor Models

Certain inbreed mouse strains demonstrate a characteristic of cancer proneness. Spontaneous cancers, such as leukemia, breast cancer, and hepatoma, develop in selected strains but this process occurs generally in aged mice (Currie et al. 2013). Environmental factors, such as hormone, diet, chemical carcinogen, radiation/UV light, and oncogenic virus, have causal links with specific tumor types and thus are employed to accelerate tumorigenesis in mouse models. For example, skin painting of mice with methylcholanthrene (MCA) induces fibrosarcomas (Zitvogel et al. 2016); exposure to DNA-damaging agent 7, 12-dimethylbenzanthracene (DMBA) plus UV light or phorbol ester 12-O-tetradecanoylphorbol-13-acetate (TPA) induces melanomas (Gali-Muhtasib et al. 2000) or papillomas, which would evolve into squamous cell carcinomas (Abel et al. 2009); a combination of DMBA and hormonal medication of progesterone derivative (Medroxyprogesterone) would induce breast cancer (Schramek et al. 2010); a colitogenic chemical dextran sodium sulfate (DSS)

together with DNA-damaging agent azoxymethane (AOM) drives colon cancer (De Robertis et al. 2011); infection of oncogenic viruses, such as murine leukemia viruses (MLVs), Murine polyomavirus (MPYV), Murine sarcoma virus (MSV), and mouse mammary tumor virus (MMTV), directly causes or promotes the development of various cancers in mice. Although tumors developed in spontaneous and induced mouse tumor models demonstrate complex genetic diversity and resemble the process of tumorigenesis in human, the high heterogenicity with respect to genetic alterations, tumor staging, and antigen diversity made them rather impractical to obtain sufficient numbers for drug screening purpose.

Notwithstanding limitations associated with these models, observations of accelerated tumor growth in carcinogen-induced tumor mice deficient or suppressed for cellular immunity eventually inspired the concept of immunesurveillance and checkpoint. For instance, deficiencies in T lymphocytes or relevant cytokines promoted tumor progression and ploidy in MCA induced tumor mice (Mattarollo et al. 2011). In addition, therapeutic effect of anthracycline-based chemotherapy, which depletes regulatory T cells, is lost upon depletion of CD8 T cells in DMBA and Medroxyprogesterone induced breast tumor mice (Baracco et al. 2016). Data accumulated from carcinogen-induced tumor mouse models delineate the existence and components of antitumor host immunity.

10.4.2 Genetically Engineered Mouse Models (GEMM)

Analysis of patient tumors has yield a plethora of frequent genetic alterations. Precise germline or somatic manipulations of oncogene or tumor suppressor gene in GEMM are employed as powerful tools to validate clinical findings and deepen our understanding of cancer mechanisms. Various approaches have been developed to create GEMMs including transgenic overexpression, targeted gene inactivation or mutation, Cre-loxP recombination system for conditional gene inactivation, combination of Cre-loxP with Tet-On/Off or ER system for inducible gene expression or inactivation, RCAS/TVA viral-based gene delivery for tissue-specific expression of a gene of interest, and CRISPR/Cas9 technology for genetic modifications in somatic cells (Zhang et al. 2011; Day et al. 2015). Currently, genetic engineering modalities are evolving rapidly to achieve temporal and spatial manipulations of oncogene and tumor suppressors so that GEMMs can recapitulate human cancer development better. Indeed, GEMMs nowadays represent the most comprehensive and autochthonous model to cover the entire cancer development from initiation, progression to metastasis in their nature microenvironment. In comparison with spontaneous or induced tumor models, tumor in GEMM occurs approximately synchronous and is driven by defined genomic alterations towards a variety of histiocytic cancer types, thus make it suitable not only for the discovery of basic mechanism but also screening of a number of drug candidates. However, similar to spontaneous or induced tumor models, GEMMs are not feasible for large-scale drug screening or evaluation purpose due to long tumor induction time and expensive longitudinal tomographic evaluation.

Since immune system is intact and coevolved with tumors in GEMMs, these models are naturally used to explore or evaluate tumor immunotherapies. It is common to observe immune cell infiltration into tumor lesions, often accompanied by recruitment of immunoregulatory cells to suppress antitumor responses. By a combination of tumor models with lineage-specific depletion of immune cells or disruption of immune-related gene expression in GEMM, accelerated tumor progression is documented in many reports, supporting the immune surveillance theory. For instance, knocking out perforin compromises tumor antigen-specific CD8 T cell response and promotes tumor growth in multiple GEMMs (Vesely et al. 2011). With regard to checkpoint therapies, it has been reported that inactivation of Myc in transformed T cell acute lymphoblastic lymphoma leads to tumor regression, possibly as a result of downregulation of checkpoint gene CD47 and PD-L1 expression and corresponding activation of T cell immunity (Rakhra et al. 2010; Casey et al. 2016). Despite all the merits mentioned, it should be noted that relatively synchronous expression of oncogene and resulting abundant tumor-initiating cells in GEMMs do not resemble the natural carcinogenesis history in human and may cause difference in antitumor responses. Moreover, species difference between mice and human prevents conventional GEMMs from evaluating and screening preclinical antibodies targeting human checkpoint molecules. This issue could be tackled by humanizing individual targeted checkpoint molecules like CTLA-4 and PD-1, and ideally its counterpart as well.

10.4.3 Tumor Cell Transplant Models

While GEMM and induced tumor models are suitable for fundamental discovery on tumorigenesis, transplantable tumor models provide platforms for large-scale drug screening because of low cost, reproducibility, synchronous growth of tumor and easiness for longitudinal tracking of tumor growth, thus widely applied by pharmaceutical industry to develop chemotherapies and targeted therapies (Fig. 10.2). Syngeneic tumor mouse models have been the first transplantable model developed in this category for over 5 decades. They are created by transplanting immortalized mouse cancer lines or primary tumors from GEMMs or induced tumor models into the same inbred immunocompetent strains. The identical genetic background prevents rapid allogenic responses against transplanted tumor cells but still allows host to mount specific immune responses against tumor antigens. Of note, the first evidence of classical checkpoint inhibitors like CTLA-4, PD-1, and PD-L1 blocking antibodies are described in syngeneic tumor mouse models (Leach et al. 1996; Iwai et al. 2002, 2005), therefore pioneering the preclinical development of checkpoint therapies. Syngeneic models, despite its long history, are undergoing a resurgence as a fast and cost-effective screen method in evaluation and development of novel immunotherapies. Nevertheless, human-mouse species differences and limited cancer types restrict the further application of syngeneic models.

Human tumor cell line derived xenograft into immunodeficient mice were developed in 1980s to fill the gaps between human and mice (Fidler and Hart 1982). This

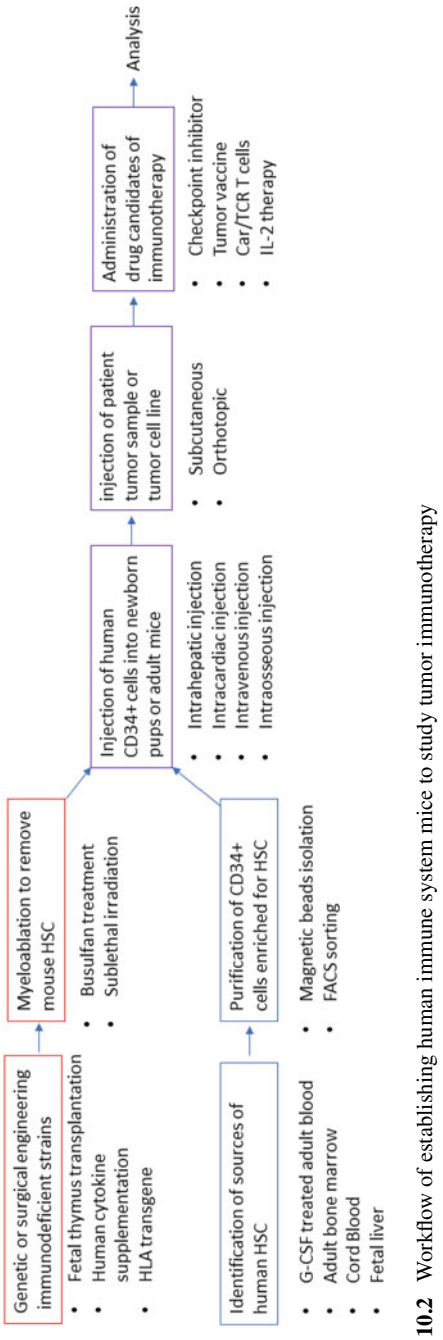

Fig. 10.2 Workflow of establishing human immune system mice to study tumor immunotherapy

model is widely utilized by laboratories and industries to test cytotoxic drug candidates for chemotherapy with success. Similar to syngeneic models transplanted with mouse cell lines, human cell-derived xenograft (CDX) models failed to predict the efficacy of many drug candidates for targeted therapies due to the lack of tumor etiology and heterogeneity (Johnson et al. 2001). As an improvement to CDX models, immunodeficient mice received surgically derived human tumor specimen subcutaneously or orthotopically are generated with intact human tissue microenvironment and diverse tumor heterogeneity. These models successfully predicted the drug responses in human where CDX models failed. In early PDX passages with host immune cells and stromal cells still preserved, data collected from models can parallel clinical outcomes and predict drug targets for second-line treatment (Malaney et al. 2014; Girotti et al. 2015). Despite that, PDX models hold great promise for therapeutic evaluation, restricted patient sample access, low take rate for certain tumor types, decreased prediction power in high passages, and missing host immunity remains critical issues to be overcome with.

10.4.4 Human Immune System Mouse Models

The growing interest in checkpoint inhibitors from pharmaceutical industry requires suitable model for target discovery and preclinical evaluation. One major reason that induced models, GEMM models, and syngeneic models do not translate well into clinical efficacy is the significant differences existing between human and mouse immune system (Mestas and Hughes 2004). The direct consequence of this disparity is that human proteins structural and function may not share sufficient homology with their mouse equivalents, thus making the preclinical evaluation of antibodies targeting human checkpoint molecules impractical with these models. Although humanization of individual checkpoint gene in GEMMs could solve partially this issue, the risk of impaired ligand/receptor interaction and function of downstream cellular signaling still questions the accountability of these models. In human CDX and PDX models, checkpoint molecules such as PD-L1/2 could be found on transplanted human tumor cells but this model does not have human immune effectors responding to checkpoint inhibitors. Transplantation of human PBMCs or enriched immune subsets, such as T cells, into CDX/PDX models could provide targets for checkpoint inhibitors. However, xenografted human T cells from PBMCs become unspecifically activated to attack recipient mice (Li and Di Santo 2019). Hence, it is difficult to separate antitumor responses from graft versus host responses after checkpoint inhibitor treatment in this model.

Human immune system (HIS) mice are developed to solve drawbacks of abovementioned models. HIS mice are referred to as severely immunodeficient mice carrying human immune cell lineages derived from transplanted hematopoietic stem cells. De novo human T cell development from HSC was achieved between 2003 and 2005 when *il2rg* gene inactivation was introduced into NOD/SCID and Balb/c *rag1/2* knockout strains as a result of reduced mouse NK activity against human

graft (Li and Di Santo 2019). In comparison to PBMC mice, human T cells developed in HIS mice are negatively selected on mouse thymus and therefore tolerant to mouse peripheral tissues. Nevertheless, human T cells selected in mouse thymus do not efficiently initiate antigen-specific responses. Several modifications to HIS mice are experimented to boost T cell responses. 1. Transplantation of a piece of human fetal thymus under kidney capsule to facilitate T cell selection under human thymic microenvironment (Melkus et al. 2006). However, human thymus selected T cells recognize mouse tissue as foreign and cause graft versus host disease as in PBMC mice. 2. Enhancement of human dendritic cell engraftment by supplementation of human myeloid cytokines. Mouse myeloid cytokines such as GM-CSF, IL-3 and M-CSF do not act on human cells (Manz 2007). Hence, humanization of these cytokines by hydrodynamic injection, protein injection and genetic engineering considerably improved reconstitution of human antigen-presenting cells (Li et al. 2013, 2016; Rongvaux et al. 2014). Notwithstanding stronger peripheral activation of T cells in these mice, thymic selection of human T cells on mouse MHC context still impairs their function. 3. Human leukocyte antigen (HLA, human equivalent of mouse MHC) transgenic mice. To instruct proper human T cell selection under a mouse environment, common HLA-A, HLA-DR, HLA-DP, and HLA-DQ gene alleles have been introduced via transgene into recipient mouse strains (Shultz et al. 2010; Masse-Ranson et al. 2019). Antigen-specific CD8 T cells and T cell-dependent antibody responses are significantly enhanced in these strains after immunization with model antigens or clinically used vaccines. However, the scarcity of HLA matched donor human HSCs restrict the use of these model from large-scale studies.

Improved HIS mice with functional immune subsets have been applied to assess cellular and checkpoint immunotherapies against transplanted CDX/PDX. Although tumor cells or tissues are allogenic to immune cells in most studies, results from HIS mice generally match clinical findings (Li et al. 2017). Basic and translational studies with HIS mice are more active in laboratories rather than in pharmaceutical industry due to technical challenges and requirements for stable supply of human HSCs. Nevertheless, in recent years, surging number of companies switched from CDX/PDX models and syngeneic models towards HIS CDX/PDX models to screen novel targets for immunotherapy or to evaluate preclinical drug candidates in a physiologically relevant settings (Fig. 10.3).

10.5 Conclusion and Perspective

A comprehensive understanding of genomic alterations needs to take somatic mutations, gene deletions or amplifications, and chromosomal rearrangements into consideration. These alterations, occurred in oncogenes, oncogenic signaling pathways and tumor suppressor genes, lead to the formation of tumors with characteristic biomarkers or immunogenic neoantigens. To escape from neoantigen induced anti-tumor immunity, pathways regulating immune checkpoints are hijacked by tumor cells to induce TIL exhaustion or suppression. Although checkpoint molecules such

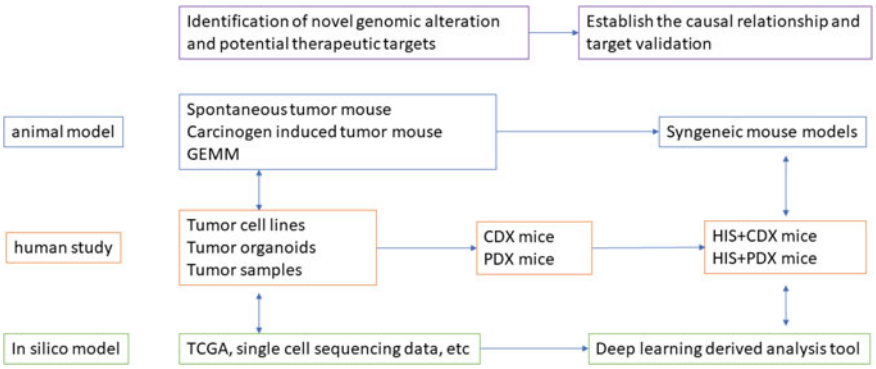

Fig. 10.3 Model systems for the discovery of checkpoint inhibitors

as PD-1 and CTLA-4 have achieved great success in treating several tumors, there are cases that patients do not respond to current checkpoint inhibitors even in the presence of high neoantigen loads and TIL exhaustion, suggesting other checkpoints remain to be explored.

Mouse models of human tumors have supported immune surveillance and escape theory and eventually led to the discovery of checkpoint inhibitors. Emphasis on clinical relevance pressured scientists to develop novel models to faithfully recapitulate natural carcinogenesis process. With respect to economic and ethical concerns to replace, reduce, and refine animal experiments, alternative technologies like organoids and microfluidic chip offer exciting directions to investigate tumor immune cell interaction and evaluate drug response in vitro. Moreover, single-cell sequencing of clinical tumor samples in combination with computational algorithms like deep learning will lead to breakthrough finds to unveil the secrets between genetic alterations and checkpoint expressions.

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Chapter 11
Regulations on Messenger RNA: Wires and Nodes

Jean-Philippe Brosseau

Abstract Somatic cells of an organism virtually share the same DNA but it is the timely expression of specific genes that determine their phenotype and cellular identity. A series of complex molecular machinery allows for the regulated process of RNA transcription, splicing, and translation. In addition, microRNAs and specialized RNA binding proteins can trigger the degradation of mRNAs. Long non-coding RNAs can also regulate mRNA fate in multiple ways. In this chapter, we reviewed the RNA processing mechanisms directly regulating immune checkpoint genes. We also cover RNA-based therapeutic strategies aiming at restoring immunity by targeting immune checkpoint genes.

Keywords Gene transcription · mRNA · Splicing · microRNA · lncRNA

11.1 Basic Gene Expression Mechanism

The dogma of molecular biology (protein-coding gene) indicates that the genes are transcribed into messenger RNAs (mRNAs) which are later translated into proteins. Thus, DNA is the reference, the RNA is the message and the protein holds the function. The transcription step takes place when general transcription factor TATA-binding protein (TBP), transcription factor IID et IIB (TFIID and TFIIB), respectively, bind a promoter transcription sequence (typically a TATA motif) located 30 nucleotides (nt) upstream of the transcription start site. Next, RNA polymerase II and other transcription factors assemble at the promoter site and start transcription until about a hundred of nt pass a Uridine or Guanidine/Uridine rich region.

During the human gene transcription, the nascent pre-mRNA is subject to constitutive RNA maturation. By default, the 5' and 3' end are protected by adding a capping structure and poly-A tail, respectively, and introns are spliced out. For protein-coding genes, the coding region is found within the larger mRNA sequence and hence the

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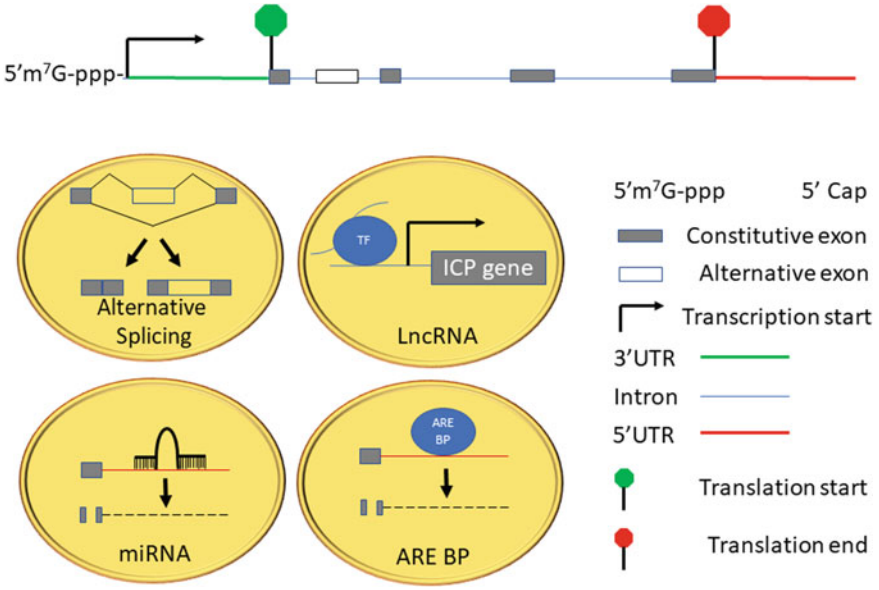

Fig. 11.1 ICP mRNA regulation. ICP genes are transcribed into mRNAs and subjected to potentially many regulated mRNA processing such as alternative splicing, targeting by miRNA, mRNA destabilization by AU-rich element-binding proteins (ARE-BP), and regulation by long non-coding RNA (lncRNAs)

term 5' untranslated region (5'UTR) and 3' untranslated region (3'UTR). These sequences play a regulatory role that may influence the expression level of the final protein. As described in this chapter, cancer immune checkpoint (ICP) genes (like many other genes) are subject to additional layers of regulation at the RNA level to fine-tune their expression level and/or function such as alternative splicing, regulation by endogenous [microRNA (miRNA) and long non-coding RNA (lncRNA)], exogenous RNA (antisense oligonucleotide) acting in trans and mechanism affecting mRNA stability (Fig. 11.1).

11.2 Transcriptional Regulation

11.2.1 ICP Regulated by Transcription Factors

Some transcription factors can be expressed in a tissue- or developmental-specific context. They can bind to a define DNA sequence and/or to protein partners shared by a sub-group of genes. For example, the gene *MYC* is amplified/overexpressed in numerous cancer types and activates a number of pro-tumorigenic factors involved in cell growth and proliferation. Therefore, a unique transcription factor can coordinate

the expression of multiple functionally related genes, and hence a gene expression program.

Surprisingly, the transcriptional control of immune checkpoint (ICP) genes is currently understudied. The best example so far is the transcription factors STAT3, HIF-1a and members of the AP-1 family controlling the expression of PD-L1. Indeed, STAT3 binds to the promoter region of PD-L1 and activated its transcription (Hu et al. 2019; Marzec et al. 2008). The JAK/STAT pathway is an established pathway driving tumorigenesis and immunity. HIF-a is a transcription factor sensitive to the percentage of oxygen. Under hypoxic conditions (low oxygen levels), HIF-a translocates to the nucleus where it binds specific hypoxia response element sequences. It was recently discovered that the gene PD-L1 is under the control of HIF-a (Noman and Chouaib 2014). As explained in Wang et al. (Wang et al. 2018), a hypoxic and lactate rich microenvironment may protect cancer cells from cytotoxic T cells, and hence immunosuppression. Interestingly, CD28 induce AP-1 (Edmead et al. 1996) and the latter is involved in the transcriptional control of CD40L in T cells (Tsytsykova et al. 1996). Finally, AP-1 response elements were identified in the PD-L1 promoter region of PD-L1 (Green et al. 2012; Sumimoto et al. 2016), suggesting that AP-1 family members may orchestrate a regulatory transcriptional network controlling multiple ICPs expression.

11.3 Alternative Splicing

11.3.1 Mechanism Overview

Upon transcription, the nascent RNA is coated with various RNA binding proteins. Consensus splicing sequences are bound by the spliceosome machinery with strong affinity but less well-defined sequences (alternative sequences) can also be occupied. Together with positive and negative splicing regulatory sequences recruiting alternative splicing factors, some of them being expressed in a tissue-specific manner, it results in two or more RNA isoforms for the majority of the multi-exon genes in human (Wang et al. 2008, 2012). The most frequent alternative splicing event in human is the alternative usage of an entire exon (also known as exon cassette or exon skipping). In a protein-coding gene, this can ultimately lead to a protein with a widely different function. For example, skipping of an exon coding for a nuclear localization signal or for a transmembrane domain can drastically alter its cellular localization. Alternative splicing is also used as a way to titer the level of a functional protein. Indeed, exon skipping can lead to a dominant-negative or non-functional protein that lower the overall level of a functional product. In up to a third of the cases, alternative splicing modulates the presence of a premature stop codon, which is a signal for targeted degradation by the non-sense mediated decay pathway, and hence modulation of the overall gene expression (Garneau et al. 2007; Lewis et al. 2003; da Costa et al. 2017). By an analogy to gene expression program where a transcription factor

regulates a define set of functionally related mRNAs, splicing factors coordinate the alternative splicing of certain genes to fine-tune cellular response (Brosseau et al. 2014).

11.3.2 ICP mRNA Regulated by Alternative Splicing

Several ICP genes are regulated by alternative splicing (see Table 11.1). Some of them have the sequence encoding their transmembrane domain subject to alternative splicing, and hence regulating their function by switching from a membrane bound to

Table 11.1 Alternative Splicing regulates the function of ICP genes. The ICP gene, cell type, alternative splicing event (ASE) type and functional impact in vitro is listed

ICP	Cell type	ASE type	Functional impact	References
PD-1	Peripheral blood mononuclear cells	Exon 3 skipping	Create a soluble isoform that blocks PD-L1/PD-1 interactions Activate CD8+ T cells	Nielsen et al. (2005)
PD-L1	Tumor cells	Use of an alternative 5'ss creating a premature stop codon and use of an alternative 3'ss	Inhibit T cells proliferation	Mahoney et al. (2019)
PD-L2	Activated leukocytes	Exon 3 skipping creating a frameshift	Create a soluble isoform with unknown function	He et al. (2004)
CTLA-4	Immature monocytes and dendritic cells	Exon 2 skipping	Create a soluble isoform that inhibits T cell responses	Magistrelli et al. (1999)
CD80	Unstimulated monocytes and B cells	Exon 4 skipping	Create a soluble isoform that inhibits PD-1/PD-L1 pathway	Kakoulidou et al. (2007)
BTLA	splenocytes	Exon 3 skipping	Increase proliferation of splenocytes	Monaghan et al. (2018)
CD40	Epithelial cells	Exon skipping of individual or combined exons 3–8	Dictate the fate between the soluble and the membrane form	Eshel et al. (2008)

a soluble form (Gu et al. 2018). For example, multiple exon skipping generates a short isoform of CD80 that lacks the transmembrane domain. As CD80 is a costimulatory factor, its soluble isoform can still bind to CD28 and activate T cells (Kakoulidou et al. 2007). Similarly, the localization of PD-1 and CTLA-4 is also tightly regulated by alternative splicing of exon encoding transmembrane domain. The short and soluble isoform of PD-1 is produced by skipping exon 3 (PD-1 delta exon 3). Once translated, this shorter isoform can inhibit all three established PD-1 interactions (PD-1 and PD-L1; PD-1 and PD-L2; PD-1 and CD80) (Song et al. 2011), most probably by directly competing with the membrane-bound form to ultimately activate T cells. In some instances, PD-1 delta exon 3 inhibits tumor growth (Elhag et al. 2012). It is therefore not surprising that some patients with elevated levels of PD-1 delta exon 3 have prolonged survival (Sorensen et al. 2016). Similarly, skipping of CTLA-4 exon 2 produces an isoform with exclusively the extracellular domain (Magistrelli et al. 1999). Again, overall survival and response to treatment correlate with the high serum level of CTLA-4 delta exon 2 (Leung et al. 2014).

The ligand PD-L1 and PD-L2 also, produce an alternative splicing isoform missing a transmembrane domain. This time, it is due to the use of an alternative 5' splice site (5'ss) and 3' splice site (3'ss), respectively. This results in a reading frame shift, and hence disruption of the remaining C-terminal protein domain (He et al. 2004; Mahoney et al. 2019). The functional consequences in regards to adaptive immunity and cancer development have not yet been specifically addressed but one can speculate that these soluble isoforms would still be able to signal through their receptor and repress T cell activation, favoring tumor growth. Of note, alternative splicing is not the only mechanism generating soluble form as it can also be the result of proteolytic cleavage (Gu et al. 2018).

11.4 miRNA

11.4.1 miRNA Biogenesis and Mode of Action

In addition to protein-coding genes, the RNA polymerase II also transcribed primary microRNAs (pri-miRNAs) genes. These are typically found within intronic sequences of protein-coding genes or in intergenic sequences. Pri-miRNAs are matured into pre-miRNA by the nuclear enzyme Drosha and subsequently exported into the cytoplasm where it becomes a substrate for Dicer. The resulting double-stranded short RNA oligonucleotides averaging 22 nt can then recruit the miRISC complex, bind to its mRNA targets (typically in the 3'UTR) and ultimately facilitate mRNA degradation and/or translational repression. Importantly, multiple miRNAs can regulate a define mRNAs and conversely, one miRNA can regulate thousands of genes (O'Brien et al. 2018).

11.4.2 ICP Regulated by miRNAs

Although several miRNAs such as miR155, miR-138, and miR-34 are indirectly affecting ICP by targeting their regulators (Dragomir et al. 2018), the focus of this chapter is to present the ICP directly regulated by miRNAs (Fig. 11.2). Multiple miRNAs were reported to directly bind and downregulate the expression of PD-L1 and/or PD-1 (for detailed review see Wang et al. 2017). For example, in non-small cell lung cancer, miR-34 (Cortez et al. 2016) and miR-200 (Chen et al. 2014) level are strongly inversely correlated to PD-L1 and directly suppress it. In gastrointestinal cancer, miR-570 (Guo et al. 2015; Wang et al. 2012, 2013) and miR-138 (Zhang et al. 2017; Zhao et al. 2016) directly target the 3'UTR of PD-L1 resulting in translation repression. The miR-138 family also directly regulates PD-1 in the context of glioma (Wei et al. 2016). miR-28 also targets PD-1, as well as BTLA (Li et al. 2016). Lastly, miR-155 is another miRNA express by T cells that targets multiple ICP [BTLA (Liu et al. 2016) and CTLA-4 (Zhang et al. 2017)]. Altogether, it suggests that the expression of key miRNAs in both cancer cells and T cells is coordinated to promote immune suppression. Finally, by indirectly targeting key ICP regulators, some miRNAs profoundly affect the overall adaptive immunity response (Dragomir et al. 2018).

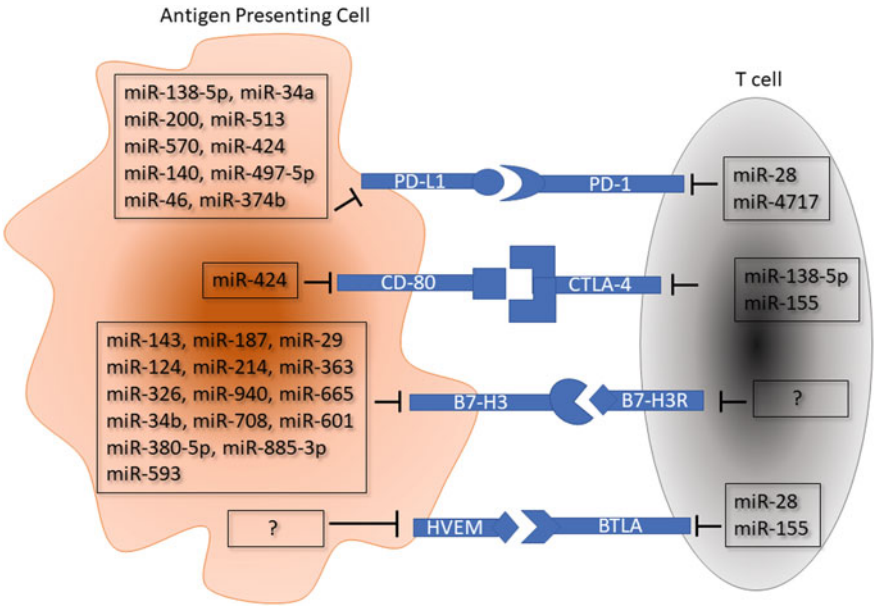

Fig. 11.2 ICP regulation by miRNAs. Schematic representing ICP proteins expressed by antigen-presenting cells and T cells

11.5 mRNA Stability

In addition to miRNAs, other gene expression mechanisms targeting the 3'UTR of mRNA have been reported. Indeed, the stability of about 5% of mRNAs can be controlled by the binding of specific RNA binding proteins (e.g., AUF1, Hu proteins, tristetraprolin, KSRP, ZFP36L1/2) on define AU-rich element (ARE). Subsequently, these protein recruits mRNA degrading machinery or repress translation to ultimately reduce expression of the targeted gene (Otsuka et al. 2019).

11.5.1 ICP Regulated by AU-Rich Element-Binding Proteins

So far, only PD-L1 mRNA was reported to be regulated by an AU-rich element-binding protein (Coelho et al. 2017). Bioinformatic analysis indicated three conserved (ARE) in PD-L1 3'UTR conforming to the consensus sequence of the ARE-binding protein (ARE-BP) tristetraprolin (TTP). As expected, mutating these sequences significantly increase the luciferase signal in an ARE dependent reporter assay. Conversely, modulating TTP levels impact the expression of PD-L1 in an ARE dependent manner (Coelho et al. 2017). Interestingly, knocking down AUF1 does not rescue PD-L1 level, suggesting specificity for TTP among the ARE-BP (Zhu et al. 2019). It remains to be determined if other ICPs are also regulated by TTP.

11.6 LncRNA

With the advent of next generation sequencing, it was realized that transcription is naturally performed much beyond protein-coding genes (Djebali et al. 2012). From there, a new class of RNA molecules called long non-coding RNA (lncRNA) was born. By definition, lncRNA are longer than 200 nucleotides and, are polyadenylated in their 3', have a 5' cap structure, and are spliced in a similar way as mRNA from protein-coding genes. Interestingly, the number of lncRNAs in human is similar to the number of protein-coding genes (Hon et al. 2017). They are typically categorized by either their genomic location (e.g., intronic, intergenic, or antisense to protein-coding gene) or by their mechanism of action (e.g., miRNA sponge, natural antisense transcript, modulation transcription, or translation factors activity) (Elling et al. 2016).

11.6.1 ICP Regulated by LncRNAs

So far, there is only one example of a lncRNA directly regulating an ICP (Kathuria et al. 2018). Knocking down the lncRNA NKX2-1-AS1 results in an increased level of PD-1/PD-L1 pathways, suggesting that NKX2-1-AS1 acts as a negative regulator. Indeed, NKX2-1-AS1 interferes with NKX2-1, which is a direct regulator of PD-L1 transcription (Kathuria et al. 2018). It would be interesting to investigate if NKX2-1-AS1 affects other ICP and whether or not other lncRNA could directly regulate ICP expression.

11.7 RNA-Based Therapeutic Strategies Targeting ICP mRNA Maturation

Antisense oligonucleotides represent a mature class of compound with proven safety and efficacy in clinical trials. Unlike small molecule inhibitors, their design is relatively straightforward. Their mechanism of actions varies and mainly depends on their relative targeted location on a mRNA and the backbone chemistry of the antisense (Brosseau 2018; Crooke 2017; Brosseau et al. 2014).

11.7.1 Down-Regulation of ICP mRNA by RNA-Based Therapeutics

Cleavage and subsequent degradation of the targeted mRNA are achieved by recruiting endogenous enzymatic machinery (e.g., RNase H, RISC). The enzymatic properties of those complexes dependent on the RNA versus DNA character of the resulting duplex and any nucleotide modification in the proximity of the cleavage site (usually not well-tolerated). Thus, partially modified DNA antisense that forms DNA/RNA duplex with a target mRNA induces RNAse H cleavage. Double-stranded siRNAs with sufficient RNA helix character are loaded into the RISC complex and one of the strands is retained (i.e., the guide strand) to direct the degradation of a specific mRNA of complementary sequence.

SiRNAs are routinely used to decipher gene function and not surprisingly, a large number of publications were reported targeting ICP mRNAs. Despite this success, one of the main challenges related to the use of oligonucleotides concerns their delivery to target cells (Juliano 2016). Interestingly, Liang et al. successfully knockdown PD-1 and CTLA-4 in vivo in an orthotopic mouse model of hepatocarcinoma (Liang et al. 2018). Depletion of PD-L1 and/or CTLA-4 increases survival, decreases tumor volume, and enhances the vitality of the T cells.

Prior to the advent of siRNAs, RNase H-dependent antisense was commonly used to knockdown specific mRNAs. This type of antisense was used to target CD80

mRNA in dendritic cells in vitro. Functionally, CD80 knockdown dendritic cells elicit a reduced cytotoxic lymphocyte activity (Liang et al. 2003). Similar strategies targeting B7-H3 were used effectively (Chapoval et al. 2001).

11.7.2 Translation Inhibition of ICP mRNA by RNA-Based Therapeutics

A fully modified antisense oligonucleotide triggers an RNAse H independent mechanism. When design to target the start codon, the resulting duplex bloc translation initiation, and elongation and hence reduce gene expression of a specific mRNA. Indeed, an antisense against the PD-L1 mRNA effectively derepresses downstream cytokine effectors such as IL-2 and interferon gamma (Mazanet and Hughes 2002).

11.7.3 Alternative Splicing Reprogramming of ICP mRNA by RNA-Based Therapeutic

When designing fully modified antisense targeting alternative splice sites or splicing regulatory sequences, one can reprogram the fate of a pre-mRNA. For example, the CTLA-4 delta exon 2 isoform is lacking the B7 ligand-binding domain. Antisense targeting the 3'ss of exon 2 results in exon 2 skipping in T cells, and hence in reduced activation (Mourich et al. 2014). As mentioned previously, the exon 3 of CTLA-4 encodes a transmembrane domain and therefore targeting exon 3 results in the creation of a soluble isoform (Mourich et al. 2014). In theory, this strategy can be applied to alter the immune-modulatory functions of the ICP listed in Table 11.1.

11.7.4 RNA-Based Therapeutic Strategies Targeting miRNA Regulating ICP mRNA

Whereas directly interfering with a miRNA using an antisense (a.k.a antagomir) to ultimately restore the targeted mRNA level is usually desired, the opposite is true for ICP. Indeed, the goal is to decrease ICP expression. Therefore, the strategy is to design a synthetic version of a miRNA (a.k.a miRNA mimetic) and used it to induce ICP mRNA degradation and hence alleviate the immune suppression.

In this sense, miRNA mimetic for several of the miRNA mentioned in Fig. 11.2 was designed and tested for efficacy. MRX34 is one of the miRNA mimetics tested in phase 1 clinical trial for safety (Yang et al. 2012; Bader 2012). Unfortunately, immune-related safety concerns were raised but dexamethasone as premedication has

improved tolerability (Beg et al. 2017). miR-28 is a promising target for immunomodulation as miR-28 mimic can partially restore the exhausted phenotype of PD-1 positive T cells (Li et al. 2016).

Since one miRNA regulate multiple mRNAs, miRNA mimetic can be used to target multiple ICP. For example, miR-138 mimetic targets both PD-1 and CTLA-4 (Smolle et al. 2017). In theory, the ICP mRNA targets could actually be on two different cells, allowing to regulate simultaneously tumor cells and T cells.

11.8 Conclusions

The regulated coordination of gene expression allows the developmental program to take place and maintain homeostasis. The transcriptional regulation of gene expression programs, including the control of ICP genes is known in some details but much less is known about post-transcriptional and alternative processing of ICP genes (Zerdes et al. 2018). Identifying the regulators of ICP mRNAs may culminate into novel therapeutics and/or the determination of the resistance of mechanisms.

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Chapter 12
Folded or Degraded in Endoplasmic Reticulum

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Abstract In consistent with other membrane-bound and secretory proteins, immune checkpoint proteins go through a set of modifications in the endoplasmic reticulum (ER) to acquire their native functional structures before they function at their destinations. There are various ER-resident chaperones and enzymes synergistically regulate and catalyze the glycosylation, folding and transporting of proteins. The whole processing is under the surveillance of ER quality control system which allows the correctly folded proteins to exit from the ER with the help of coat proteinII(COPII) coated vesicles, while retains the rest of terminally misfolded ones in the ER and then eliminates them via ER-associated degradation (ERAD) or ER-to-lysosomes-associated degradation (ERLAD). The dysfunction of the ER causes ER stress which triggers unfolded protein response (UPR) to restore ER proteostasis. Unsolvable prolonged ER stress ultimately results in cell death. This chapter reviews the process that proteins undergo in the ER, and the glycosylation, folding and degradation of immune checkpoint proteins as well as the associated potential immunotherapies to date.

Keywords Immune checkpoint proteins · Endoplasmic reticulum · Glycosylation · ER chaperones · ER enzymes · Protein folding · COPII · Vesicle trafficking · ER proteostasis · ER quality control · ER stress · UPR · ERAD · ERLAD · Immunotherapy

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12.1 Introduction

In eukaryotic cells, approximately one-third of proteins go through a series of modification in the endoplasmic reticulum (ER) before they function in their destinations. It is of significance for ER to provide a stable and pleasant environment for proteins to get their native three-dimensional (3D) structures. A variety of chaperones and enzymes reside in the ER, participating in the targeting, translocation, folding, and trafficking of the protein substrates. Attributing to genetic mutations, transcription or translation mistakes, hostile environment, and other random negative factors, the folding and assembly of polypeptides could be quite error-prone, which requires the ER to dispose of aberrant products rapidly. Generally, the misfolded proteins are instantly eliminated via ER-associated degradation (ERAD). However, when the substrates are too large, generate aggregates, or cannot engage ERAD chaperones, another pathway termed ER-to-lysosomes-associated degradation (ERLAD) emerges to engulf the ERAD-resistant proteins into vesicles and delivers the cargoes to the lysosome for degradation. In some cases, particularly under the circumstance of inflammation or tumors, the intrinsic or extrinsic negative factors such as hypoxia, nutrient deprivation, and oxidative stress incapacitate the efficiency of clearance mechanism of ER and trigger ER stress. In order to restore the homeostasis, the ER initiates the unfolded protein response (UPR), which functions to inhibit the transcription and translation, increase the folding and export rate, facilitate the synthesis of various ER chaperones to promote ERAD and autophagy. All of these activities aim to empty the ER and resolve the stress. However, under prolonged or severe stress, UPR tends to facilitate apoptosis. The dysfunction of ER can lead to a number of human diseases. In contrast, it can be exploited to silence harmful proteins as well.

As we know, most immune checkpoint proteins are membrane-bound proteins which are folded and assembled in the ER before they are transferred to the membranes. However, there has been little research on the modification or degradation of immune checkpoint proteins in the ER to date. This chapter focuses on the mechanism of how proteins get folded or degraded in the ER, which might help us explore the immune checkpoint proteins from a different perspective.

12.2 Protein Folding in the Endoplasmic Reticulum

Notably, most membrane and secreted proteins, including a number of immune checkpoint proteins, are glycoproteins, which are usually precisely modified in the ER with the help of a series of ER-resident chaperones and folding enzymes. The folding process includes signal sequence cleavage, glycosylation, disulfide bonding, pro-isomerization, and oligomerization.

12.2.1 Signal Sequence Cleavage

Once a peptide is synthesized in the ribosomes, it subsequently targets and translocates to the ER for further modification. There are diverse pathways for emerging polypeptide chains to reach their destinations with the help of various cytosolic factors and receptors (Aviram and Schuldiner 2017; Ast and Schuldiner 2013). Generally, most nascent polypeptides are recognized and targeted with the help of their signal sequences and enter the ER lumen through the SEC61 translocon complex channel (Gorlich and Rapoport 1993; Gorlich et al. 1992). The hydrophobic segment of the protein activates the Sec61 channel, allowing the chain to cross the lipid bilayer (Voorhees and Hegde 2016; Voorhees 2014). The signal peptidase complex then cleaves the amino-terminal signal sequence co-translationally or post-translationally (Braakman and Hebert 2013). Otherwise, mislocalized proteins (MLPs) in the cytosol are quickly degraded by proteasomes, whereas the proteins trapped in the Sec61 translocon are eliminated by the membrane protease ZMPSTE24 (Fregno and Molinari 2019). Notably, the specific signal sequences direct protein targeting and affect the translocation efficiency, the opportunity of signal peptide cleavage and the maturation process after cleavage (Hegde and Bernstein 2006).

12.2.1.1 Signal Sequence Cleavage of Immune Checkpoint Proteins

Cytotoxic T-lymphocyte antigen 4 (CTLA-4) is a membrane-bound glycoprotein highly expressed on activated T cells, and it negatively regulates the immune response. As previously reported, CTLA-4 polymorphisms have been found to confer susceptibility to type 1 diabetes, thyroid disease, and several other autoimmune disorders. Particularly, a common T17A polymorphism in the signal peptide of CTLA-4 is associated with the risk of autoimmune diseases. This polymorphism contributes to the failure of glycosylation of the said protein receptor in the ER although it could be targeted and cleaved correctly, which eventually resulting in the down-regulation of CTLA-4 expression on the cell surface (Anjos et al. 2002). Although no similar findings are reported in studies on programmed cell death protein-1 (PD-1) and programmed death ligand-1 (PD-L1), there are reasons to believe that this would be the case for these two proteins as well.

12.2.2 Protein Glycosylation

Glycosylation is of significance for the maturation of most membrane-bound and secretory proteins because it is involved in protein folding (Shental-Bechor and Levy 2008), transporting (Vagin et al. 2009) and functioning (Ferris et al. 2014; Wang and Kaufman 2016). It is believed that the modification initiates in the ER

and is further processed in the Golgi apparatus, involving a series of highly efficient machineries. Furthermore, the vital roles that glycosylation plays in the activation of the immune system are increasingly underscored in the literature (Wolfert and Boons 2013). Usually, the glycosylation process can be divided into N-linked glycosylation and O-mannosylation.

12.2.2.1 N-Linked Glycosylation

Glycosylation is a prevalent and highly conserved post-translational modification in eukaryotic cells. Canonical protein N-glycosylation includes the following two phases: (1) synthesis of the lipid-linked oligosaccharide (LLO) donor and (2) transfer of carbohydrates to nascent polypeptides (Ferris et al. 2014; Breitling and Aebi 2013).

In the first phase, glycans start assembling along with dolichol (Dol) on the cytoplasmic face of the ER, forming the intermediate dolichyl pyrophosphate-linked $Man_5GlcNAc_2$-PP-Dol, which is then translocated to the lumen face of the ER to be conveniently assembled (Braakman and Hebert 2013; Xu and Ng 2015). Sequentially, the activated hetero-oligosaccharyl transferase (OST) complex transfers the oligosaccharide comprising three glucoses, nine mannoses, and two N-acetyl glucosamines ($Glc_3Man_9GlcNAc_2$) from the LLO to selected asparagine residues in the Asn-X-Ser/Thr sequence (Kelleher and Gilmore 2006). Usually, this modification occurs in the early stage of the folding process, during translocation, either co-translationally or post-translationally. There are two catalytic subunits of the OST complex, known as STT3A and STT3B. The former mostly acts co-translationally, whereas the latter functions post-translationally, maximizing the efficiency of N-glycosylation (Ruiz-Canada et al. 2009).

12.2.2.2 O-Mannosylation

There is another type of glycosylation carried out by mannose in the ER, defined as O-mannosylation, which seems to conduct significant activities correlated to the protein quality control system (Xu and Ng 2015; Xu et al. 2013). This modification was first observed in yeast cell walls (Sentandreu and Northcote 1968). Quite different from the traditional hetero-oligomeric structure of the N-linked oligosaccharide, the conformation of O-mannosylation comprises only a single mannose, which can be selectively attached to Ser or Thr residues on the peptide chain in the ER and further elongated once transported to the Golgi apparatus (Haselbeck 1983). Interestingly, studies have shown that in yeast, O-mannosylation participated in preventing proteins from complete folding and in inducing unfolded substrates to give up failed folding attempts. This keeps them soluble to be degraded via the ER-associated degradation (ERAD) pathway (Xu et al. 2013). Although it is still unknown whether this unfolded protein O-mannosylation activity exists in mammals, the O-mannosylation machinery is conserved in mammals (Xu and Ng 2015; Praissman and Wells 2014).

12.2.2.3 N-Glycan Trimming

Once the nascent protein is attached to a sugar moiety, it is sequentially recognized and modulated by corresponding glucosidases to remove the glucoses from N-glycan. The deglycosylation process provides a series of precise mechanisms that are vital for glycoproteins to fold correctly.

First, glucosidase I (GS-I) removes the outermost glucose residue from the A-branch of the N-glycan immediately after the translocation and glycosylation of the peptides to trigger the trimming cascade (Hubbard and Robbins 1979). Simultaneously, this trimming step can effectively reduce the activity of OST (Helenius and Aebi 2004). Second, the second glucose residue is cleaved by glucosidase II (GS-II), resulting in the formation of $Glc_1Man_9GlcNAc_2$. The monoglycosylated state facilitates the incompletely folded glycoprotein to be recognized by calnexin (CNX) and calreticulin (CRT) to enter the CNX/CRT cycle" (Ou et al. 1993; Hammond et al. 1994).

The ER lectin-like chaperones CNX and CRT, along with the oxidoreductase ERp57 (Oliver et al. 1999) and the peptidyl-prolyl cis-trans isomerase (PPIase) cyclophilin B (CypB) (Kozlov et al. 2010; Jansen et al. 2012), transiently interact with the glycoprotein to enhance their folding efficiency (Ou et al. 1993). At this point, removal of the innermost glucose of the N-glycan by GS-II makes the polypeptide unglycosylated; the protein is released and is free to fold. Simultaneously, the quality control sensor UDP-glucose: glycoprotein glucosyltransferase (UGGT) detects the folding state of the protein. The fully and correctly folded substrate dissociates from the CNX/CRT cycle and undergoes further maturation. Alternatively, when UGGT recognizes abnormal proteins, it re-glycosylates the substrates and returns them to the CNX/CRT cycle (Caramelo et al. 2003; Caramelo and Parodi 2008). The process is likely to be repeated until the glycoprotein maintains a proper three-dimensional structure or is degraded by the ER quality control system (Caramelo and Parodi 2008). It is believed that the substrate-specific recognition of UGGT, which has a preference for a partially structured non-native form, can also influence the interaction between CNX and the substrate (Taylor et al. 2003; Solda et al. 2007; Trombetta and Helenius 2000). Thus, the folding process is mainly associated with the three terminal glucoses of the N-glycan and a series of modifications occurring upon them. Moreover, it is the remaining $Man_9GlcNAc_2$ glycan that degrades misfolded proteins via the quality control system (Xu and Ng 2015).

12.2.2.4 Glycosylation of Immune Checkpoint Proteins

Glycosylation is tightly associated with immune activation, including antigen modification, presentation, and T cell priming (Wolfert and Boons 2013). However, there is not much research on the role played by glycosylation in immune checkpoint proteins.

PD-L1 is a major immune checkpoint protein widely observed in diverse malignancies because it protects cancer cells from immune surveillance. PD-L1 is a

membrane-bound glycoprotein capable of combining with its receptor PD-1 to silence the immune activities of T cells. Notably, a recent study showed that N-glycosylation of PD-L1 had a significant influence on its immune functions (Li et al. 2016).

The following four glycosylation sites appear on the extracellular domain of the PD-L1 protein: N35, N192, N200, and N219. The extensive glycosylation of N192, N200, and N219 (but not N35) contributes to the stabilization of the PD-L1 protein. The site where glycogen synthase kinase 3β (GSK3β) phosphorylates PD-L1 is extremely close to the residues N192, N200, and N219. PD-L1 can easily resist the phosphorylation of GSK3β when fully glycosylated. Otherwise, the unglycosylated form of PD-L1 is targeted by GSK3β, facilitating the proteasome-related degradation of PD-L1 via β-transducin repeats-containing proteins (β-TrCP) (Li et al. 2016). Similarly, it is suggested that metformin-activated AMP kinase (AMPK) can target PD-L1 and phosphorylate serine 195 of PD-L1 before N-glycan trimming. Serine 195 is between the glycosylation sites N192 and N200, making this phosphorylation likely to interfere sterically with the normal folding process of PD-L1, induce an excessive mannose trimming of N-glycan and facilitate ERAD pathway recognition (Cha 2018).

PD-1 is expressed on T cells and contains an immunoglobulin variable-type (V-type) region with a complementary determining region. Murine PD-1 contains the following four putative glycosylation sites: three in the immunoglobulin V-type domain (N16, N41, and N83) and one in CDR1. In view of these three sites residing in the distal end of PD-1, far from the binding surface, it is suggested that the glycosylation sites of PD-1 have little effect on the interaction of PD-1 with PD-L1. Unexpectedly, glycosylation of these sites in vivo determines the orientation of PD-1 on the cell surface, and thus, they are indirectly connected to the association of PD-1 with PD-L1. Glycosylation of the fourth site, N25 within CDR1, which is suggested to be of significance in ligand binding, is likely to influence PD-1 local structure and affinity for PD-L1 (Zhang et al. 2004).

CTLA-4, another widely used checkpoint protein, contains the following two N-linked glycosylation sites on its extracellular region: N78 and N110. N78 contributes to shielding the hydrophobic patch against aggregation (Metzler et al. 1997). No O-glycosylation site has yet been found in CTLA-4. CTLA-4 is a homodimer, and its dimerization is associated with intermolecular disulfide bonding at the C122 residue (Linsley 1995). However, with the mutation of C122, CTLA-4 still maintains dimerization and remains fully functional with the help of aberrant N-glycosylation in the Golgi apparatus. Surprisingly, although CTLA-4 fails to dimerize in the absence of both disulfide bonding at C122 and N-glycosylation, its monomer still folds correctly.

CD80 (B7-1) and CD86 (B7-2) are the ligands of CTLA-4, which is mostly expressed on antigen-presenting cells (APC) but also on T cells. Once a T cell is activated, its CD86 expression is rapidly up-regulated. However, it has been demonstrated that CD86 produced by activated T cells has an extremely low affinity for CTLA-4 compared with the affinity of CD86 produced by APCs, probably because of hypoglycosylation of CD86 modified in T cells (Hollsberg et al. 1997).

T cell immunoglobulin- and mucin-domain-containing-3 protein (TIM-3) is a novel immune checkpoint protein expressed on the surface of differentiated TH1 cells, CD8+ T cells and some macrophages. As a type I membrane protein, TIM-3 is widely glycosylated, with both O-linked and N-linked glycosylation sites on the extracellular N-terminal domain. The binding candidate of TIM-3, CEACAM1, is proposed to induce the glycosylation of TIM-3 and stabilize the ligand protein (Das et al. 2017).

12.2.3 ER Chaperones

The folding process is the most error-prone maturation step of nascent peptides. Abundant chaperones in the ER work actively to facilitate proper folding and protect proteins from aggregation. In general, chaperones in the ER are divided into the following two parts: the glycan-independent and glycan-dependent chaperone system (Braakman and Hebert 2013). The former system is ubiquitous and generally interacts directly with substrates, whereas the latter is quite less common compared with the former, is mostly ER-specific and facilitates the modification of hydrophilic glycans. ER chaperones work quite promiscuously rather than specifically interacting with a single partner, and they are synergistically dedicated to ordering the folding environment in the ER (Jansen et al. 2012). In this study, we have briefly reviewed and highlighted several main kinds of ER chaperones.

12.2.3.1 Glycan-Independent Chaperones

Immunoglobulin-binding protein (BiP, also known as glucose-regulated protein 78, GRP78), belonging to the heat-shock protein 70 (Hsp70) family, is the most popular chaperone of ER residents. The chaperone contains the following two functional sections: an amino-terminal for binding to the nucleus (nucleus binding domain, NBD) and a carboxylic terminal for binding the substrates (substrate-binding domain, SBD), with a cap to facilitate the combination (Braakman and Hebert 2013; Flynn et al. 1991). BiP can directly interact with an immature polypeptide by recognizing its exposed hydrophobic segments with the help of ERdj5, which is a co-factor of the Hsp40-family, and nucleotide exchange factors (NEFs) in an ATP-dependent process. With a switch to the ATP- or ADP-bound status, the affinity of BiP to its substrate can change from low to high (Blond-Elguindi et al. 1993). Once the unfolded, aggregation-prone substrate is combined with BiP, it becomes soluble, which begins the processes such as translocation, maturation, and ERAD pathway (Kabani et al. 2003; Brodsky et al. 1995; Alder et al. 2005; Hendershot 2004). Numerous co-operators of BiP have been found, indicating that BiP is a multifunctional protein worth further exploration (Jansen et al. 2012).

GRP94, a 94-kDa ER protein homologous to the Hsp90 family, is a classical ER chaperone. Besides its association with BiP, its combination with CypB via a

C-terminal acidic tail has been recently reported (Jansen et al. 2012). In addition, GRP94 can interact with immature immunoglobulin chains after the substrates bind to BiP (Melnick et al. 1994). GRP94 can also work with OS-9, which along with Hrd1-SEL1L, contributes to the degradation of aberrant proteins via the ER quality control system (Christianson et al. 2008; Dersh et al. 2014).

12.2.3.2 Glycan-Dependent Chaperones

CNX, a type I membrane-bound molecular protein (Schrag et al. 2001), is extensively studied as a typical ER glycan-dependent chaperone. CRT is the soluble paralogue of CNX. There are two functional domains defined in CNX: a lectin-like domain and an extended arm named the P domain, which directly interacts with its co-factors ERp57 and CypB (Jansen et al. 2012; Pollock et al. 2004). This complex specifically targets the carbohydrate of monoglycosylated proteins trimmed by glucosidase II. When the complex is assembled with its target, glucosidase II removes the terminal glucose from the glycan. Then, the fully folded glycoprotein dissociates from the chaperone (Hebert et al. 1995). However, incompletely folded proteins detected by UGGT1 are reglycosylated, and they return to the CNX/CTR cycle until misfolded proteins terminally enter the ERAD pathway (Molinari et al. 2005). The CNX/CRT cycle plays a crucial role in the folding period for preventing the aggregation of aberrant proteins, promoting the formation of a disulfide bond with ERp57 and pro-isomerization with CypB (Oliver et al. 1999; Kozlov et al. 2010; Hebert et al. 1996). Additionally, CNX and BiP are responsible for retaining abnormal, unassembled proteins in the ER for proper folding or degradation (Popescu et al. 2005; Rajagopalan et al. 1994). All these mechanisms assist in stabilizing the folding process (Braakman and Hebert 2013).

Malectin is a lectin newly found in the ER. Notably, it exclusively recognizes the $Glc_2Man_9GlcNAc_2$ moiety (Schallus et al. 2008), with a tendency to combine with aberrant peptides, stopping their pathway and sending the chains to the ERAD pathway (Chen et al. 2011; Galli et al. 2011). However, the mechanism by which the lectin accurately targets the aberrant peptide chains in the early stages of glycosylation without affecting the normal proteins remains unknown (Braakman and Hebert 2013).

12.2.4 ER Folding Enzymes

Abundant ER enzymes that facilitate proper folding have been discovered, and these are divided into two groups according to their functions: protein disulfide isomerases (PDIs) or oxidoreductases and PPIases.

12.2.4.1 Protein Disulfide Isomerases (PDIs) or Oxidoreductases

Compared with the highly reduced cytoplasmic environment, the environment in the ER is quite more oxidizing, which is suitable for disulfide bond formation and oxidative protein folding. ER-resident PDIs are the first discovered oxidoreductases considered to catalyze this process (Ellgaard and Ruddock 2005). PDI contains four thioredoxin-like repeats, two of which have oxidoreductase activities, whereas the others can catalyze the formation of hydrophobic and non-covalent disulfide bonds (Wallis and Freedman 2013). As oxidoreductases, PDIs may catalyze both directions of the reaction depending on different environments in the ER (Braakman and Hebert 2013). Remarkably, these enzymes can distinguish different folding states of proteins. PDIs are also involved in the conformational forming of peptides, preventing aggregation in nascent peptides (Wallis and Freedman 2013).

ERp57, a mammalian PDI homologue, exclusively combines with the P domain of CXN (Oliver et al. 1999; Pollock et al. 2004). The PPIase CypB interacts with CXN on the same domain (Kozlov et al. 2010; Jansen et al. 2012). ERp57, along with CXN/CRT and/or CypB, operates the glycosylation-dependent quality control of protein folding in the ER (Oliver et al. 1997). ERp72 is also a PDI homologue associated with the modulation of the ERAD pathway. Similar to ERp57, interaction between ERp72 and CypB has been detected, and this complex is likely to promote the folding of immunoglobulin G (Jansen et al. 2012). ERdj5, another widely studied human PDI homologue, is a member of the Hsp 40 family. As a typical co-operator of BiP, ERdj5 functions as a reductase and destroys the disulfide bonds of aberrant peptides, facilitating the retro-translocation of substrates and leading to ERAD along with BiP and ERAD-enhancing α-mannosidase-like protein (EDEM) (Ushioda et al. 2008). Accordingly, the deficiency of ERdj5 leads to ER retention (Dong et al. 2008).

ER oxidoreductin 1-α (Ero1-α) is identified as another ER-resident oxidoreductase playing an essential role in protein-folding machineries (Pollard et al. 1998). Remarkably, Ero1-α modulates and balances ER redox homeostasis efficiently in collaboration with PDI (Appenzeller-Herzog et al. 2008; Appenzeller-Herzog et al. 2010; Araki et al. 2013). Intriguingly, PDI is claimed to be oxidized by Ero1-α and to generate mixed-disulfide complexes together with Ero1-α. The PDI homologue Mpd2p is also a substrate of Ero1 (Frand and Kaiser 1999). Cys(94)–Cys(131) and Cys(99)–Cys(104) disulfide bonds are the major sites where the Ero1-PDI pathway catalyzes the folding of oxidative substrates (Araki and Nagata 2011). Notably, the correlation between up-regulated Ero1-α and tumors has also been noticed, which is likely to be a predictive factor of poor prognosis (Tanaka et al. 2015; Kukita et al. 2015; Kutomi et al. 2013). Additionally, Ero1 is proposed to catalyze the formation of disulfide bonds via HIF-1α under hypoxic conditions (May et al. 2005; Gess et al. 2003).

12.2.4.2 Peptidyl-Prolyl Cis-Trans Isomerases (PPIases)

It is prevalent that trans-proline residues are present in nascent polypeptides for the combination of ribosomes. Therefore, isomerization is necessary for generating cis-prolines in mature proteins with the help of PPIases, also known as immunophilins. The transformation from trans to cis is reversible during modification and depends on the conditions. Proline isomerization is associated with the rate-limiting refolding of some denatured proteins (Lang et al. 1987). Because secretory proteins are much larger, it is hypothesized that the modifications initiate simultaneously in multiple folding domains (Braakman and Hebert 2013).

Cyclophilins and FK506-binding proteins (FKBPs) are proposed to be the two main subfamilies of PPIases according to their different specific inhibitions by immunosuppressive drugs cyclosporine A and FK506, respectively. CypB interacts with the P domain of CXN, CRT, and calmegin, sharing the same binding site with ERp57 (Kozlov et al. 2010; Jansen et al. 2012). CypB is a target for cyclosporine A to suppress the degradation of misfolded proteins, which facilitates the elimination of misfolded proteins containing cis-proline via ERAD of luminal substrates (Bernasconi 2010). FKBPs are a large family, localized in different organelles, participating in diverse cellular activities besides protein folding (Somarelli et al. 2008).

12.2.5 ICP Protein-Related Folding Process

The intrinsic disulfide bond of PD-L1 is of significance in protein folding and translocation (Schwartz et al. 2002; Tanaka et al. 2017). A recent study showed that the ER-resident oxidoreductase Ero1-α participated in the post-translational regulation of PD-L1 via catalysis of oxidative folding, thus promoting the expression of oxidized PD-L1 proteins. Accordingly, knockdown of Ero1-α induced the down-regulation of PD-1 and PD-L1, which sequentially led to the apoptosis of Jurkat leukemia T cells. Conversely, Ero1-α promotes the translation of PD-L1 via stimulation of HIF-1α under hypoxic conditions. Furthermore, over-expression of Ero1-α was detected in triple-negative breast cancer concurrently with PD-L1 expression, indicating that Ero1-α could be a potential target of tumor immunotherapy.

The FK506-binding protein 51, a member of the FKBP family encoded by the FKBP5 gene, is involved in radioresistance in malignant melanoma (Romano et al. 2010). FKBP51 contains a C-terminal tetratricopeptide repeat (TPR) motif and two N-terminal FK506 binding domains (Somarelli et al. 2008). The level of a spliced isoform of FKBP51 lacking the TPR motif FKBP51 s was recently found to be elevated in the PBMCs of patients with melanoma, and a novel bidirectional regulation between FKBP51 s and PD-L1 in certain melanoma cell lines was proposed (Romano et al. 2015). The interaction between PD-1 and PD-L1 induced the up-regulation of FKBP51, which sequentially produced the spliced isoform FKBP51s. Simultaneously, FKB51s catalyzed the folding of PD-L1 as a PPIase, which in turn facilitated

an increase in the level of PD-L1; however, FKBP51 did not have the same effect. In addition, the expression of FKBP51 was also increased in glioblastoma, which was indicated to promote protein folding and thus result in the up-regulation of PD-L1 (D'Arrigo et al. 2017).

In the ER, a nascent CTLA-4 monomer forms two intra-disulfide bridges. The one between the C21 and C92 residues contributes to the classical immunoglobulin folding and the binding motif for B7 and the other contributes to the interaction between C48 and C66, the function of which is still unknown. There is an inter-disulfide bridge in the extracellular domain at C122 that links two CTLA-4 monomers (Linsley 1995). However, CTLA-4 can still maintain a dimeric state by removing the disulfide bond at C122, and dimerization of CTLA-4 seems unnecessary for its interaction with B7 (Linsley 1995).

12.3 Protein Export from the ER

Nascent polypeptides processed in the ER go through a set of intricate machineries including signal sequence cleavage, glycosylation, disulfide bonding, pro-isomerization, and oligomerization to gain proper conformation. Figure 12.1 shows the process that proteins undergo in the ER. Once the native functional form has been inspected by the ER quality control system, the protein obtains admission to Golgi for further modification. And this is assisted by the intracellular ER-derived vesicle trafficking mechanism (Lord et al. 2013).

12.3.1 Cargo Assembly and Egress

The synthesis of vesicle starts with the stepwise assembly of the five highly conserved core COPII proteins (Matsuoka et al. 1998). The formation of the vesicle is GTP-dependent (Barlowe et al. 1994). Sec12 is a guanine nucleotide exchange factor that is responsible for activating GTP to ensure that the small GTPase Sar1 residing in cytoplasm (Nakano et al. 1988; Barlowe and Schekman 1993) inserts into the membranes with its N-terminal amphipathic alpha helix, inducing the deformation of the nearby lipid bilayer (Bielli et al. 2005; Lee et al. 2005). Simultaneously, the recruitment of Sec23/Sec24 heterodimer is triggered by Sar1, given that GTPase-activating protein Sec23 can be stimulated and directly bind to Sar1 (Yoshihisa et al. 1993; Bi et al. 2002). A recent study showed that a site-specific O-GlcNAcylation of SEC23A plays the role of regulating the secretion of vesicles (Cox et al. 2018). Sec24 has a variety of homologues with diverse independent binding sites, which enables the vesicles to capture numerous cargo proteins (Miller et al. 2003; Mancias and Goldberg 2008). SNARE proteins which catalyze the vesicle fusion at the destination are selectively added to the coat via interaction with Sec24. At this point, the cargo enters the corresponding vesicle and waits for export. Sequentially, the Sec13/Sec31

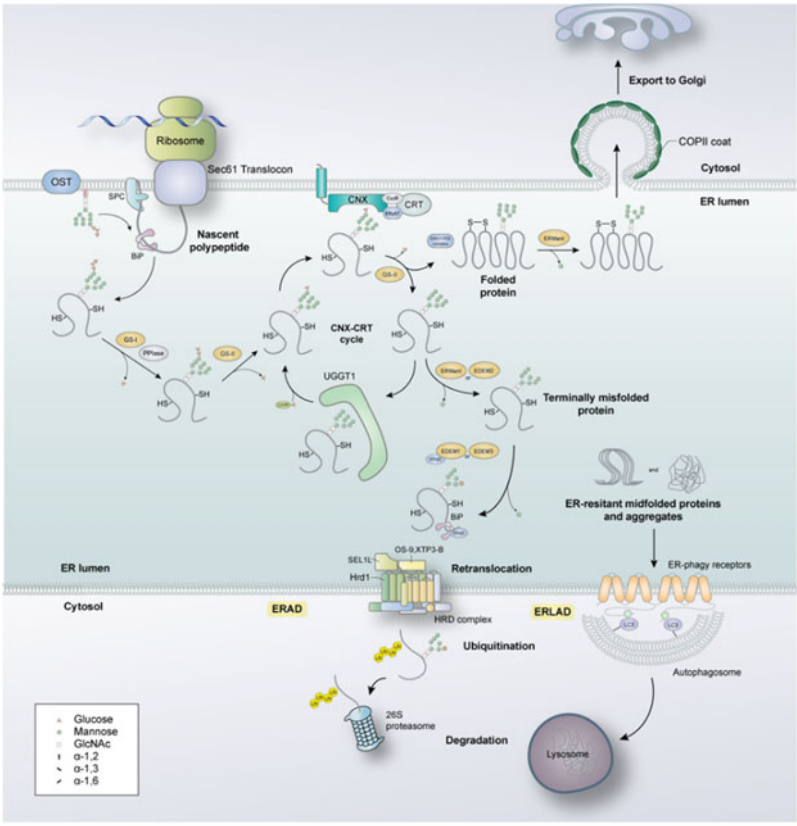


Fig. 12.1 The process that proteins undergo in the ER. Nascent polypeptides enter the ER lumen via the SEC61 translocon complex with the N-terminal signal sequence cleaved by the signal peptidase complex(SPC) co-translationally or post-translationally. Simultaneously, the oligosaccharyl transferase (OST) complex transfers the oligosaccharide ($Glc_3Man_9GlcNAc_2$) from LLO donor to the polypeptides. The N-glycan trimming is catalyzed by two glucosidases GS-I and GS-II, which sequentially remove glucoses to generate the structure of $Glc_1Man_9GlcNAc_2$. The monoglucosylated proteins enter the "CNX/CRT cycle", where GS-II removes the innermost glucose of the N-glycan and thus the proteins are free to fold. The quality control sensor, UDP-glucose: glycoprotein glucosyltransferase (UGGT) detects the folding state of the protein, which allows the fully folded substrates to dissociate from CNX/CRT cycle and be transferred to Golgi via COPII-coated vesicles. Otherwise, UGGT re-glucosylates the substrates and sends them back to the CNX/CRT cycle. The terminally misfolded proteins are released from the cycle and enter ER-associated degradation (ERAD). After a serious of mannose trimming catalyzed by ERManI, EDEM1-3, the $Man_{5\text{-}7}GlcNAc_2$ structure can be targeted by lectin-like receptors OS-9 and XTP3-B which facilitate the misfolded substrates to be retranslocated through HRD complex to the cytosol. Eventually, the substrates are ubiquitinated and then degraded by the proteasome. Additionally, the ERAD-resistant misfolded proteins and aggregates which cannot cross the ER membrane are eliminated through ER-to-lysosomes-associated degradation (ERLAD), which are engulfed by autophagosomes and delivered to the lysosome for clearance ultimately. The green sphere with the red edge in the glycan structure represents the α-1,6-linked mannose that can be targeted by ERAD chaperones. BiP, immunoglobulin-binding protein (also known as GRP78); G-I, glucosidase I; G-II, glucosidase II; ERManI, ER mannosidase I; EDEM, ER degradation-enhancing α-mannosidase-like protein; CNX, calnexin; CRT, calreticulin; CypB, Cyclophilin B; PDI, protein disulfide isomerases

complex is recruited to form the outer layer of the vesicle by Sar1-Sec23/Sec24 complex which further induces membrane deformation (Bi et al. 2007; Copic et al. 2012). It is also indicated that the activated Sar1 is responsible for the detachment of vesicles from the ER via Sar1-mediated GTP hydrolysis (Bielli et al. 2005). Sec16, a distinct membrane-bound scaffold protein residing discretely on transitional ER (tER) membrane (Hughes et al. 2009), is likely to prevent Sec23 and Sec31 from being stimulated by GTPase activity of Sar1 (Ivan et al. 2008) and directly interact with Sec24 to reduce GTPase activity, thereby regulating the normal release rate of COPII vesicles (Kung et al. 2012). The GTP-dependent process of assembly and disassembly of COPII and monoubiquitylation of Sec31 are considered to modulate the coat size of large cargo (Jin et al. 2012; Hutchings and Zanetti 2019), and some accessories (such as TANGO1 and cTAGE5), participating in the transportation of cargoes which are too bulky to be shipped via the classical COPII vesicle (Saito et al. 2009; Malhotra and Erlmann 2011) Moreover, it is recently proposed that some distinct subdomains in COPII proteins exert their functions within each ER exit site with the joint effort of SEC16 and TANGO1/cTAGE5/Sec12 complex (Saito and Maeda 2019; Maeda et al. 2019). After the coat assembly is finished and the proper cargo is loaded, the vesicle buds from the ER to the next destination. The main structure of COPII-coated vesicle is shown in Fig. 12.2.

12.3.2 Cargo Transport

The COPII-coated vesicles loaded with cargo proteins leave from a particular domain termed transitional ER (tER) to the next stop defined as ER-Golgi intermediate compartment (ERGIC). ERGIC is described as a unique independent intermediate near the tER, sharing no typical characteristics with ER or cis-Golgi (Schweizer et al. 1991), which acts not only as a staging post concentrating the newcomers, but also as a supervisor re-sorting the retrograde and anterograde cargoes (Ben-Tekaya et al. 2005). Studies found that COPII was in charge of the route from the ER to ERGIC, without the participation of microtubules. While, COPI, another coat complex, was recruited onto ERGIC where it sequentially coupled with COPII and took responsibility for transporting the retrograde and anterograde proteins to ER and Golgi, respectively (Aridor et al. 1995). The vesicles with no residents would be sent back to the ER. The COPI-dependent transport went along microtubules with the help of a microtubule plus end-directed motor kinesin to the ER (Lippincott-Schwartz et al. 1995) and the minus-end-directed motor complex dynein/dynactin to cis-Golgi (Presley et al. 1997). ARF proteins were proposed to regulate the process of vesicular traffic (D'Souza-Schorey and Chavrier 2006). The cargo with correct assembly undergoing re-sorting then finally arrives at Golgi apparatus for further modification.

Based on the mechanism elucidated above, it seems that COPII-mediated transport is likely to implicate in protein disorder-associated diseases or therapies. However, the vesicle trafficking of the ICP-related proteins is seldom studied up to now.

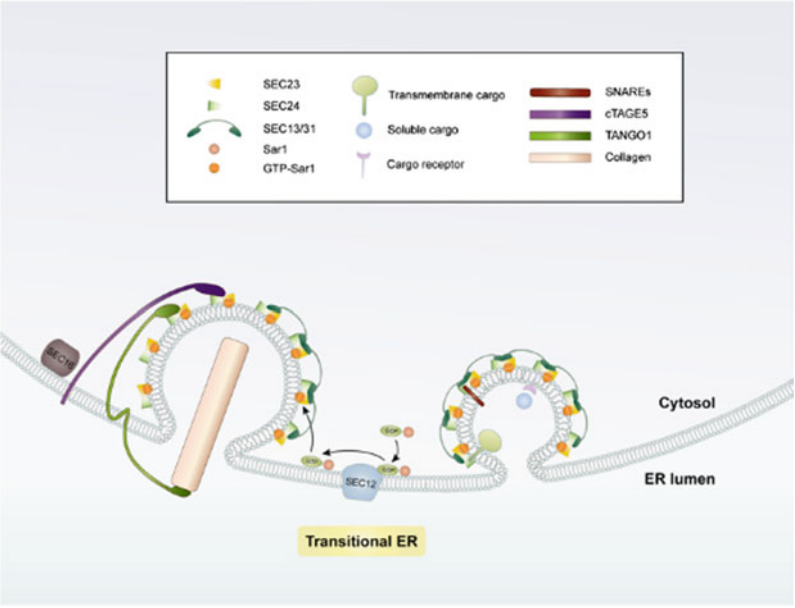


Fig. 12.2 Protein export via COPII-coated vesicle. The correctly folded proteins checked by ER quality control system destine for the Golgi with the help of COPII-coated vesicles. The small GTPase Sar1 in cytoplasm activated by Sec12 inserts into the ER membrane to deform the nearby lipid bilayer and triggers the recruitment of Sec23/Sec24 heterodimer. Sec23 can be stimulated and directly bind to Sar1 and Sec24 is responsible for the capture of cargo proteins. The SNARE proteins which catalyze the vesicle fusion at the destination are selectively added to the coat via the interaction with Sec24. Subsequently, the Sec13/Sec31 complex is recruited to form the outer layer of the vesicle by Sar1-Sec23/Sec24 complex, which further induces the scission of the membrane. Sec16, a membrane-bound scaffold protein residing discretely on transitional ER (tER) membrane regulates the assembly of the vesicle. Some accessories, like TANGO1 and cTAGE5, are considered to modulate the coat size for large cargo like collagen. The COPII-coated vesicles loaded with cargo proteins leave from a particular domain termed transitional ER (tER) to the next stop

12.4 Proteostasis and Quality Control in the ER

Considering the numerous folding and processing of proteins in the ER, it is error-prone for proteins folding and acquiring native structures, owing to genetic mutations, transcription or translation mistakes, unsuitable environment or other random negative factors. Thusly, it is critical for the ER to maintain proteostasis. Thanks to a sophisticated quality control system, the ER makes a balance via exporting native folded proteins while degrading aberrant proteins in virtue of ER-associated degradation (ERAD) or ER-to-lysosome-associated degradation (ERLAD). Once the ER fails to maintain proteostasis, ER stress arises and sequentially unfolded protein response (UPR) occurs to relieve stress and stabilize the ER. However, sustained unfolded protein response can also result in cell apoptosis.

12.4.1 ER Stress

Various intrinsic and extrinsic perturbations can induce ER stress, including accelerated synthesis or deficient export of polypeptides, aberrant degradation via proteasomes or autophagy, hostile environment (such as energy deprivation), oxidative stress, dysregulated calcium levels and so on. Specifically, tumor microenvironment (TME) is likely to initiate ER stress in malignant cells and tumor-infiltrating leukocytes. Additionally, sustained and severe ER stress is constantly associated with cell death which induces tumor progression and (Mohamed et al. 2017; Cubillos-Ruiz et al. 2017) chemoresistance.

12.4.2 Unfolded Protein Response (UPR)

Under the condition of ER stress, unfolded protein response (UPR) is triggered by three classical UPR mediators, including inositol-requiring enzyme 1α (IRE1α), pancreatic ER kinase (PKR)-like ER kinase (PERK), and activating transcription factor 6 α (ATF6α). All the sensors constitutively bind to the ER chaperone BiP with their luminal domain in a monomeric and inactive form. Once the environment in the ER becomes unstable, the dissociation of BiP activates the sensors and initiates the corresponding signal pathways (Bertolotti et al. 2000; Shen et al. 2002).

12.4.2.1 Ire-1

IRE1α is a type I transmembrane protein encoded by ERN1 gene. Once BiP binds misfolded proteins, IRE1α initiates to dimerize and stimulate its endoribonuclease domain, specifically leading to the cleavage of a 26-nucleotides fraction from X-box binding protein 1 (XBP1) mRNA in the cytosol (Yoshida et al. 2001). The newly generated XBP-1s acts as a highly activated transcription factor, shifting to the nucleus and activating the transcription of substrates associated with diverse ER quality control molecules (Shoulders et al. 2013). Additionally, IRE1α can reduce the protein-folding load through inducing the turnover of many protein synthesis-related mRNAs, which is termed (Hollien et al. 2009). As regulated Ire1-dependent decay (RIDD). However, when ER stress remains unsolved, the overactive IRE1 takes part in the cleavage of other microRNAs, subsequently inducing cell death (Ghosh et al. 2014; Lerner et al. 2012; Upton et al. 2012).

12.4.2.2 PERK

PERK is also a type I transmembrane protein with a kinase activity in lumen. The dissociation of BiP activates PERK, which in turn phosphorylates eIf2α. P-eIf2α

attenuates the global mRNA translation to enable the cell to resolve the stress (Harding et al. 1999). However, p-eIF2α activates the translation of a transcription factor known as ATF4, which in turn activates the transcription factor C/EBP homologous protein (CHOP). The two factors heterodimerize and synergistically initiate a set of gene transcription encoding protein synthesis and autophagy (B'Chir et al. 2013; Han et al. 2013). Moreover, the excessive and chronic activation of ATF4 and CHOP results in oxidative stress and even cell death (Han et al. 2013).

12.4.2.3 ATF6α

Unlike IRE-1 and PERK, ATF6α is a type II transmembrane monomer. After being released from BiP, ATF6α is transferred to the Golgi apparatus via binding to COPII vesicles, where it is cleaved to acquire an active soluble fragment which is in turn translocated to the nucleus (Shen et al. 2002; Schindler and Schekman 2009). The newly generated fragment of ATF6α functions as a transcription factor, initiating the transcription of various gene encoding proteins, such as ER quality control proteins and ER chaperones, to resolve ER stress. PERK deficiency can inhibit the activation of ATF6α, thereby hindering the transcriptional induction of ER quality control (Adachi et al. 2008).

UPR helps the ER to maintain homeostasis under acute aberrant stress in virtue of the rapid reduction of protein synthesis, the up-regulation of protein export, ERAD, and autophagy. Nevertheless, UPR can result in cell death under chronic ER stress.

12.4.3 ERAD

The folding process of nascent polypeptides in the ER is under strict surveillance by the ER quality control mechanism. The newly synthesized peptides which do not enter the ER are degraded by proteasomes in the cytosol (Fregno and Molinari 2019), and the ones stuck in the ER membrane are wiped out by membrane protease ZMPSTE24 (Ast et al. 2016). As for the peptides that get into the ER successfully yet fail to fold correctly, most of them are instantly detected and degraded via the ER-associated degradation (ERAD). The rest of aberrant proteins which form aggregates, or cannot be recognized by ERAD chaperones, or too bulky to be retranslocated back to cytosol are degraded by lysosomes termed ER-to-lysosomes-associated degradation ERLAD (Fregno and Molinari 2019). ERAD is a sophisticated mechanism in charge of the clearance of misfolded, unassembled and toxic proteins, involving the recognition, targeting, retranslation, extraction, ubiquitination, and degradation by 26S proteasome.

The glycosylated polypeptides attain their native three-dimensional structure during the calnexin/calreticulin cycle. The proteins that fail to fold is terminally disassociated from the cycle and trimmed by mannosidases progressively for ERAD. ER degradation-enhancing alpha-mannosidase-like proteins (EMDMs) are responsible

for the cleavage of mannoses. EDEM2 is considered to catalyze the first mannose trimming from the glycan and trigger the ERAD mechanism (Ninagawa et al. 2014). ER mannosidase I (ERManI) was reported to participate in this step as well, yet recent study indicated the distinct contribution of ERManI in vitro and in vivo (Gonzalez et al. 1999; Avezov et al. 2008) and suggested the possibility of functional redundancy (Ninagawa et al. 2014). ERManI is even proposed to have other localization besides ER (Pan et al. 2011; Benyair et al. 2015). Subsequently, EDEM1 and EDEM3 progressively remove mannoses from the glycan. The activities of EDEMs are rate-limiting to guarantee that the nascent proteins have enough time for folding. OS-9 and XTP3-B act as lectin-like receptors and target the substrates with terminal α-1,6-linked mannose moieties of $Man_{5\text{-}7}GlcNAc_2$ structure with their mannose-6-phosphate receptor homology (MRH) domains (Xu and Ng 2015). Besides, EDEM1 interacts with ER-resident oxidoreductase ERdj5, which can remove disulfide bonds inside the aberrant substrates and make the retro-translocation easier (Ushioda et al. 2008).

Additionally, the assembly of substrates can affect the recognition of ERAD. For example, the folding of the subunits of multimeric complexes can occur before the formation of the quaternary structure (Copeland et al. 1988) or after the oligomerization (Bonifacino et al. 1990). It is still unclear whether the assembly promotes the stability of substrates or the capacity for transporting. The prolonged ER retention may initiate the recognition of ERAD, yet whether the assembly can shield the hydrophobic patches of the unfolded peptides from being exposed and recognized as degradation signals (Shenkman et al. 2000; Fra et al. 1993) remains to be elucidated.

The multifunctional ER-resident chaperone BiP is extensively involved in the recognition, retention of the ERAD substrates. BiP targets the substrates to the retranslocation complexes by recognizing its hydrophobic patches (Knittler et al. 1995; Vembar and Brodsky 2008; Zhang et al. 2001; Schmitz et al. 1995). In regard to the non-glycosylated substrates, BiP binds the substrates and interacts with derlin-1 and HERP which contains a ubiquitin domain and is likely to retranslocate substrates and combine with the proteasome as the component of Hrd1 complex (Okuda-Shimizu and Hendershot 2007; Schulze et al. 2005). BiP interacts with ER chaperone GRP94, which can also bind OS-9 with Hrd1-SEL1 (Christianson et al. 2008; Dersh et al. 2014).

Given that both misfolded proteins and native folding intermediates may share similar incompletely folded structures, it is essential to distinguish ERAD substrates from folding intermediates. Evidence suggested that a glycan "timer" monitors the folding process, and that the aberrantly prolonged folding results in the excessive mannose trimming can be recognized by ERAD chaperones (Helenius and Aebi 2004). As for non-glycosylated proteins, it is probable that a novel ER membrane protein complex, Slp1-Emp65, will work on it. With Slp1 SUN domain direct targeting and releasing the substrates, Slp1-Emp65 serves to transiently protect soluble unfolded proteins and folding-intermediate proteins from being degraded by ERAD (Zhang et al. 2017).

Since there is no device in the ER for degradation, the substrates must be targeted to the ER membrane for retranslocation in the first step. No exclusive retrotranslocon is

responsible for the activity probably because the specific substrate to the corresponding channels varies considerably. Sec61, E3 ligases like Hrd1 and Doa10, derlin-1 and Cdc48 (p97) are demonstrated as the components of retrotransloction complexes. The concurrent function of chaperone is proposed to ensure more efficient substrate degradation (Vembar and Brodsky 2008). The aberrant proteins sequentially undergo ubiquitylation, extraction, deubiquitylation, and degradation by proteasome.

Defective ERAD mechanism leads to the rapid accumulation of aberrant proteins and ER stress. In contrast, overactive ERAD can also result in cellular dysfunctions. ERAD tuning is another mechanism in charge of ERAD activity, which can wipe out surplus ERAD factors via LC3-I coated vesicles to maintain homeostasis (Bernasconi and Molinari 2011).

12.4.4 ER-to-Lysosomes-Associated Degradation (ERLAD)

Most of the misfolded or unassembled proteins will undergo ERAD for degradation. However, emerging evidence shows that there are alternative clearance pathways to degrade proteins that are too bulky to across the ER membrane, or insoluble aggregates that cannot be recognized by ERAD chaperones. It is indicated that these ERAD-resistant proteins are degraded by lysosome in virtue of kinds of vesicles. Accordingly, a novel conception known as ER-to-lysosomes-associated degradation (ERLAD) is proposed to elucidate the situation. ERLAD involves the autophagic and non-autophagic pathways (Fregno and Molinari 2019; De Leonibus et al. 2019).

Intriguingly, studies indicate that EDEM1 which catalyzes ERAD plays a part in the novel vesicle trafficking carrying aberrant proteins from the ER to the cytoplasm cytosol, where they are recognized by the autophagic receptors and subsequently engulfed into autophagosomes for further clearance (Zuber et al. 2007; Le Fourn et al. 2013). In addition, ER-associated compartments (ERACs) are characterized by the distinct compartment adjacent to the ER involving in the segregation of insoluble proteins (Kamhi-Nesher et al. 2001; Fu and Sztul 2009; Huyer et al. 2004).

A study showed that cystic fibrosis transmembrane conductance regulator (CFTR) proteins can be transferred to ERACs in the event of sustained over-expression of CFTR; the aggresomes are subsequently devoured by autophagosomes and delivered to the lysosome (Fu and Sztul 2009). It is reported that ERACs serve to stabilize the ER via separating aggregated proteins (Huyer et al. 2004). Indeed, CFTR can assist the formation of cytoplasmic and juxtanuclear aggresomes and can be ubiquitylated by HRD1 to ensure their degradation through ERLAD and ERAD, respectively. This corroborates the co-existence of the ERAD and ERLAD pathways (Fu and Sztul 2009; Farinha and Amaral 2005). The aggregates in the cytoplasm are rapidly decorated with LC3 in the double-membrane autophagosomes and are delivered to the lysosomes.

Aside from being transferred to cytosol and engulfed by autophagosomes, the proteasome-resistant misfolded proteins can also be disposed in other ways. The autophagy of ER (ER-phagy) is characterized by the sequestering of ER subdomains

into autophagosomes to ensure the clearance of the lysosome. To date, six ER-resident membrane-bound proteins have been characterized as ER-phagy receptors, including FAM134B (Khaminets et al. 2015), SEC62 (Fumagalli et al. 2016), RTN3 (Grumati 2017), CCPG1 (Smith et al. 2018), ATL3 (Chen et al. 2019), and TEX264 (An et al. 2019; Chino et al. 2019). These receptors can directly bind LC3 in the cytosol to initiate lysosome degradation-related vesicle trafficking. These proteins also promote the elimination of particular ER components under the perturbation of environment. Moreover, SEC62 can induce the degradation of ER chaperones and folding enzymes (except from ERAD molecules) in response to ER stress (Fumagalli et al. 2016). In some cases, ERLAD clients can specifically activate corresponding LC3-binding proteins and trigger the ER capture by autophagosomes (Smith et al. 2018; Forrester 2019). Notably, it is indicated that A1-antitrypsin Z (ATZ) can be segregated with the participation of ER lectin calnexin and FAM134B. ATZ is then transported via a LC3 lipidation-associated vesicle and distinct from autophagosomes, which is made up of ER-derived single membrane. Thanks to the concerted effort of SNARE, the vesicle is terminally delivered to the lysosome for degradation (Fregno 2018).

Previous study indicates that ectopic procollagen binds to COPII subunits and LC3 after entering the ER exit sites (ERES). Instead of being transferred to Golgi, the vesicle is rapidly devoured by the nearby lysosomes (Omari et al. 2018). Likewise, another study implied that under the stimulation of nutrient deprivation or aggregates-related ER stress, COPII components SEC24C-Sec23, together with LC3 segregated aberrant aggregates and ER subdomains into a specific vesicle at ER-phagy sites (ERPHS), which is different from the canonical ER exit sites where COPII-coated vesicles budded. Additionally, it was demonstrated that SEC24C is also responsible for the clearance of FAM134B and RTN3 (Cui et al. 2019).

ERLAD provides an alternative approach to the ER to cope with ERAD-resistant proteins, so as to enable the efficiency of the ER to dispose of aberrant proteins. Therefore, UPR, ERAD, and ERLAD are complementary to one another to maintain proteostasis in the ER quality control system. Notably, the extracting of proteins from Golgi and endosomes for proteasome degradation in the endosome and Golgi-associated degradation pathway (EGAD (Schmidt et al. 2019)) has been identified, which further complicates the regulation of cellular homeostasis.

12.4.5 ICP Protein-Related ER Quality Control

PD-L1 is a membrane-bound protein which acquires extensively glycosylated structure in the ER. Glycogen synthase kinase 3 (GSK3) is identified as a key point modulated by the proteasome upon ER stress (Fabre et al. 2019). Generally, native glycosylated PD-L1 can resist the binding of GSK3β, since the phosphorylation sites of GSK3β on PD-L1 is quite close to the sites of glycosylation. There is an E3 ligase β-TrCP motif next to GSK3β phosphorylation sites on PD-L1 protein, from which GSK3β can get access to activating β-TrCP (Ding et al. 2007). Once the unfolded

PD-L1 is recognized by GSK3β, it is subsequently ubiquitinated by β-TrCP and degraded by proteasome ultimately (Li et al. 2016).

A recent study revealed that metformin promotes ERAD-associated degradation of PD-L1 (Cha 2018). For starters, metformin activates AMP-activated protein kinase (AMPK), which in turn directly interacts with PD-L1 inside the ER lumen and phosphorylates S195 of PD-L1. The phosphorylation occurs before N-glycan trimming and subsequently induces excessive mannose trimming of PD-L1. The glycan attached on misfolded PD-L1 can be recognized by a series of ERAD chaperones, such as SEL1L, HRD1, ERLEC1, and OS9. As an E3 ligase, HRD1 combines with PD-L1 to ubiquitylate the protein, eventually leading to PD-L1 degradation by the proteasome under the ERAD mechanism.

Sigma1 is a small-molecule modulator exerting various functions. Abundant in the ER of tumor cells, Sigma1 is involved in the processing and transferring of secretory proteins to maintain ER homeostasis (Schrock et al. 2013; Kim and Maher 2017). It is proposed that Sigma1 can directly bind PD-L1 in the ER and positively regulate the expression of PD-L1. Consistently, IPAG, the inhibitor of Sigma1, can significantly suppress IFNγ-induced PD-L1. It is suggested that IPAG induces a novel selective autophagy of PD-L1, which is different from the bulk macroautophagy or ER stress-associated autophagy. Consequently, IPAG-treated PD-L1 is segregated in the autophagosome and then is degraded by the lysosome (Maher et al. 2018). Some reported regulation of PD-L1 is shown in Fig. 12.3.

12.5 Other ICP Protein-Related Regulations in the ER

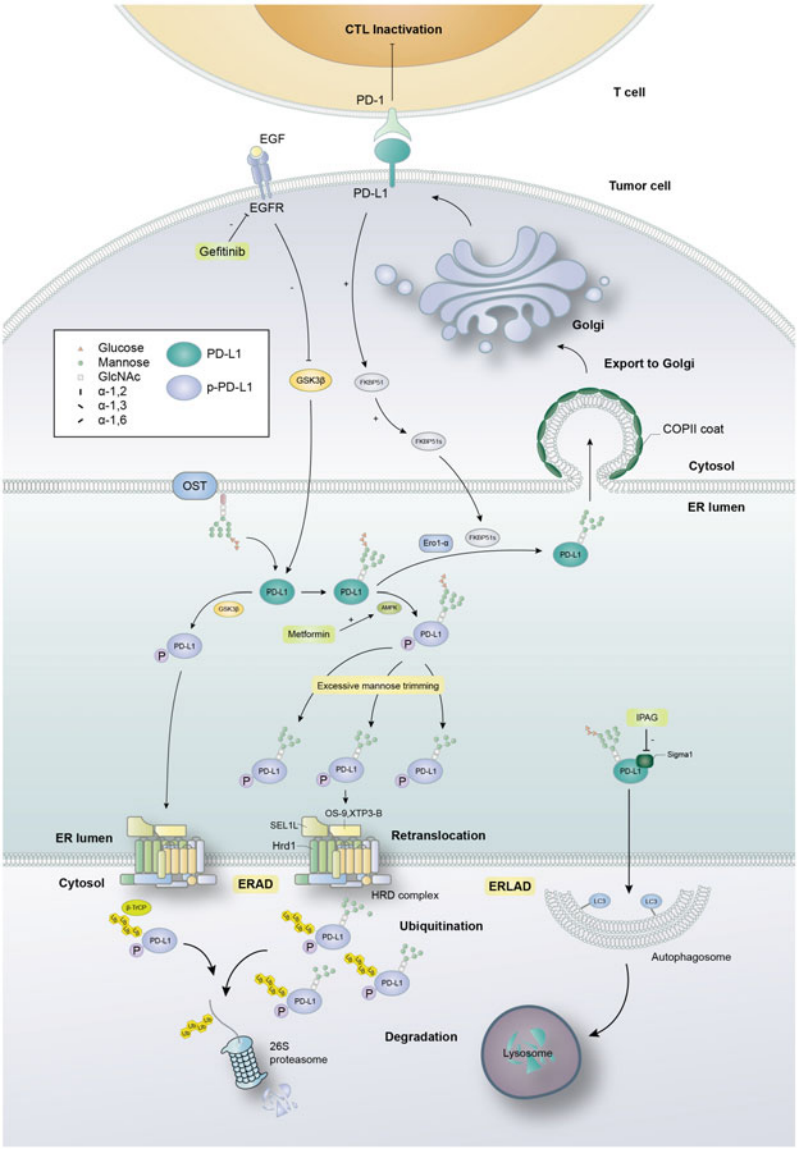
According to recent studies, PD-L1 is modified in a membraneless organelle termed TIS granules. TIS granules are made up of the extensively expressed RNA-binding protein TIS11B, which is tightly intertwined with ER to makes up a novel subcellular compartment—the TIGER domain. The distinct domain facilitates 3'UTR-mediated protein–protein interactions to increase the expression PD-L1 (Ma and Mayr 2018).

12.6 Potential Novel Therapies

To date, most of the immune drugs work through targeting the immune checking point proteins on the cell surface. Nevertheless, it is put forward that if the proteins can be blocked at source. Here, we introduce some therapeutic strategies in light of the interfering of protein folding to facilitate degradation before the proteins being transferred to the membrane. The combination with traditional anti-tumor immunotherapies might further improve therapeutic efficacy and safety.

Metformin is likely to promote the degradation of PD-L1 via ERAD, which activates CTL against malignant cells by suppressing the combination between PD-L1 and PD-1. Consistently, PD-L1 expression is down-regulated in the tumor tissues

of breast cancer patients, in comparison with those without metformin. The in vivo experiments of mouse models with various cancers indicated that the combination therapy of metformin and CTLA4 blockade is likely to be more efficient than the drugs used alone (Cha 2018).



◀**Fig. 12.3 The folding and degradation of PD-L1 in the ER** Once enter the ER, the nascent PD-L1 protein is glycosylated and folded stepwise to acquire the native functional structure before transferred to Golgi. The ER-resident oxidoreductase Ero1-α (Tanaka et al. 2017), as well as a spliced isoform of PPIase FKBP51 termed FKBP51s (D'Arrigo et al. 2017), are demonstrated to catalyze the folding of PD-L1. At the same time, the interaction of PD-1 and PD-L1 induces the up-regulation of FKBP51, which is sequentially spliced into FKBP51s to catalyze PD-L1 (D'Arrigo et al. 2017). Fully glycosylated PD-L1 can easily resist the phosphorylation of GSK3β, yet the non-glycosylation form of PD-L1 is phosphorylated by GSK3β, which contributes to the proteasome-related degradation of PD-L1 via an E3 ligase β-TrCP. The epidermal growth factor (EGF) up-regulates PD-L1 through inactivating GSK3β. In consistent, The EGFR inhibitor Gefitinib can reduce the binding between PD-L1 and PD-1 via GSK3β (Li et al. 2016). Likewise, metformin-activated AMPK can phosphorylate PD-L1 at Serine 195 before N-glycan trimming, which is likely to induce excessive mannose trimming of N-glycan and elimination via ER-associated degradation (ERAD) (Cha 2018). A small-molecule modulator Sigma1 can directly bind and regulate PD-L1 in the ER. IPAG, the inhibitor of Sigma1, significantly suppresses IFNγ-induced PD-L1 probably in virtue of inducing the segregation of PD-L1 in the autophagosome and degradation by the lysosome (Maher et al. 2018). P-PD-L1,phosphorylated PD-L1

Glycogen synthase kinase 3β (GSK3β) functions to destabilize PD-L1 via inducing the ubiquitylation of PD-L1. In contrast, epidermal growth factor (EGF) up-regulates PD-L1 expression through inactivating GSK3β. Given the remarkably high expression of EGFR and PD-L1 in basal-like breast cancer (BLBC), a novel immunotherapy is proposed to combine EGFR inhibitor gefitinib with PD-1 blockade to inhibit the binding between PD-L1 and PD-1 (Li et al. 2016).

12.7 Conclusions

Once a nascent polypeptide is synthesized, the first organelle it encounters is the ER, where the peptide undergoes a series of modifications to acquire its native functional three-dimensional structure. Although the activities in the ER are exquisitely regulated with numerous ER-resident chaperones and enzymes, it is still the most error-prone step during the maturation of proteins, which brings the significance of a sophisticated surveillance system for ER quality control. Glycosylation plays a major role in this quality control system via directing the proper folding and facilitating the degradation of misfolded proteins, aiming to maintain the fidelity of products. On one hand, the dysfunction of protein modification and the accumulation of aberrant proteins cause diverse human diseases. On the other hand, the misfolding and misassembly of proteins and abnormal ER activities can also facilitate to eliminate harmful proteins and cells. Since most of the immune checkpoint proteins are glycosylated, folded, and transported or degraded in the ER, it would be helpful to have a better understanding of the mechanism of how it works in the ER. This chapter systematically reviews the modified process of proteins and the studies associated with the regulation of ICP proteins in the ER. Furthermore, it seems to provide a

promising perspective to find out more efficient immunotherapies and is worthy of further study.

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Chapter 13
Regulation of Cancer Immune Checkpoint: Mono- and Poly-Ubiquitination: Tags for Fate

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Abstract The antagonism, stalemate and compromise between the immune system and tumor cells is closely associated with tumor development and progression. In recent years, tumor immunotherapy has made continuous breakthroughs. It has become an important approach for cancer treatment, improving the survival and prognosis of more and more tumor patients. Further investigating the mechanism of tumor immune regulation, and exploring tumor immunotherapy targets with high specificity and wide applicability will provide researchers and clinicians with favorable weapons towards cancer. Ubiquitination affects protein fate through influencing the activity, stability and location of target protein. The regulation of substrate protein fate by ubiquitination is involved in cell cycle, apoptosis, transcriptional regulation, DNA repair, immune response, protein degradation and quality control. E3 ubiquitin ligase selectively recruits specific protein substrates through specific protein-protein interactions to determine the specificity of the overall ubiquitin modification reaction. Immune-checkpoint inhibitory pathway is an important mechanism for tumor cells to evade immune killing, which can inhibit T cell activity. Blocking the immune checkpoints and activating T cells through targeting the negative regulatory factors of T cell activation and removing the "brake" of T lymphocytes can enhance T cells immune response against tumors. Therefore, blocking the immune checkpoint is one of the methods to enhance the activity of T cells, and it is also a hot target for the development of anti-tumor drugs in recent years, whose inhibitors have shown good effect in specific tumor treatment. Ubiquitination, as one of the most important post-translational modification of proteins, also modulates the expression, intracellular trafficking, subcellular and membranous location of immune checkpoints, regulating the immune surveillance of T cells to tumors.

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13.1 Introduction

Ubiquitin (UB) is a highly conserved small molecular weight protein with a molecular weight of 8.5 kD and 76 amino acid residues, which widely exists in eukaryotic cells (Ganoth et al. 2013). The main function of ubiquitin is to participate in the selective degradation of most proteins in eukaryotic cells (Isaksson et al. 1996). In addition, ubiquitin also plays an important role in various cell life activities, such as signal transduction, immune response, transcriptional translation (Isaksson et al. 1996). Ubiquitin is covalently modified to the substrate by a three-step enzymatic cascade reaction (Pickart 2001). First, the C-terminal glycine residue of ubiquitin forms a thioester bond with the cysteine residue of ubiquitin activing enzyme (E1) in the case of ATP energy. Then the activated ubiquitin is transferred from the ubiquitin activating enzyme to the ubiquitin binding enzyme (E2). Finally, catalyzed by ubiquitin ligase (E3), ubiquitin molecules were transferred from E2 to the substrate molecules to modify the substrate molecules (Fig. 13.1). The lysine residues

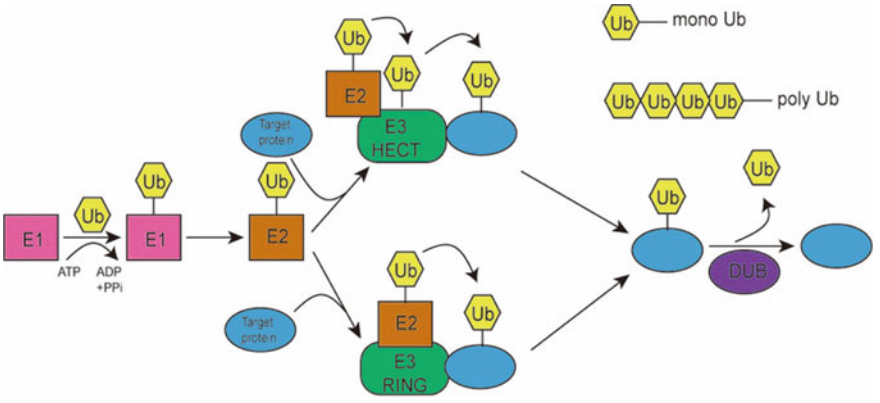

Fig. 13.1 The reaction process of protein ubiquitin modification. Ubiquitin (Ub) is attached to its target protein by the sequential action of E1, E2, and E3 enzymes. The activating enzyme E1first activates ubiquitin in an ATP-dependent reaction by forming a thioester bond at its active-site cysteine with the COOH-terminus of ubiquitin, which is transferred to the active site cysteine of the conjugating enzyme E2. The third step is catalyzed by either an E2 with the help of an E3 (RING-finger) or directly by an E3 (HECT-domain), leading to the transfer of ubiquitin to an epsilon amino group of a lysine residue on the target protein forming an isopeptide bond with the C-terminal glycine of ubiquitin. A single ubiquitin can be attached to proteins (Mono-Ubquitination) at a single and/or multiple lysine residues on the target protein. Alternatively, multiple ubiquitins can be attached to one another forming poly Ub chains (Poly Ubiquitination), typically mainly via lysine 48 or 63 linkages. Deubiquitinating enzymes (DUBs) Ubiquitin is required for removing ubiquitin from target proteins

of the substrate protein can be modified by either a single ubiquitin molecule (mono-ubiquitination) or a ubiquitin chain (poly-ubiquitination). There were two types of poly-ubiquitination: one is called linear ubiquitination, in which a glycine residue at the C-terminal of a ubiquitin molecule forms a peptide bond with a methionine residue at the N-terminal of another ubiquitin molecule. A number of ubiquitin molecules are connected in series by end to end. The other is called nonlinear ubiquitination, in which a glycine residue at the C-terminal of one ubiquitin molecule forms a peptide bond with a lysine residue inside another ubiquitin molecule, and multiple ubiquitin molecules are cross-linked in turn.

Two E1, 40 E2 and 600 E3 have been found in mammalian cells, and the enzymes involved in ubiquitination are summarized below. There are two E1 in mammalian cells: Uba1 (also known as Ube1) and Uba6. Among them, Uba1 is the most well-known and highly conserved in eukaryotic cells, which is mostly found in the protein degradation pathway. Uba1 inhibitors can cause significant changes in cell function, such as the anti-tumor effect of Largazole and its derivatives (Ungermannova et al. 2012). Recent studies have shown that Uba1 is vital to intracellular homeostasis and neurodegeneration, suggesting its great potential as a therapeutic target for a range of neurodegenerative diseases (Groen and Gillingwater 2015). Uba6 is expressed in many human cell lines and tissues, but its function is poorly understood. Research has revealed that the specific substrates of Uba6 are mostly related to cell structure and movement, while the specific substrates of Uba1 are related to some cell metabolic pathways (Zhao et al. 2012; Liu et al. 2017). E2 is not only a carrier of ubiquitin, it has two functions: transmercaptan (transfer of thioesters to sulfhydryl groups) and ammonolysis (transfer of thioesters to amino groups). E2 exists mainly in the form of E2 ~ UB yoke complexes for ready reaction. In addition, E2 can also directly regulate the activity of ubiquitin-related enzymes (Stewart et al. 2016). For example, the activity of the deubiquitination enzyme OTUB1, which specializes in the hydrolysis of Lys48 polyubiquitin chains, is enhanced by the interaction with free E2 (Wiener et al. 2013). E3 is a huge family, with more than 600 E3 discovered. There are three main types of structural features: RING (really interesting new gene, also u-box E3), HECT (homologous to E6AP c-terminus) and RBR (ring-in-between-RING). RING E3 can be subdivided into monomolecular RING E3 and multi-subunit E3 complexes, such as SCF, APC/C, Cullin 2/Elongin B/C/VHL, which all belong to multi-subunit E3 complexes (Smit and Sixma 2014; Liu et al. 2015).

The ubiquitin proteasome pathway (UPP) is an ATP-dependent, highly specific and selective protein degradation pathway. UPP including ubiquitin, ub-activating enzyme E1, Ub-conjugating enzyme E2, Ub-ligating enzyme E3, 26S proteasome and deubiquitinating enzymes DUBs. With the participation of ATP, ubiquitin molecule is activated by a series of reactions mediated of ubiquitin initiating enzyme, covalently binds to the target protein, and completes the single ubiquitin transformation. Ubiquitination is similar to phosphorylation in that it is a reversible process, maintaining in equilibrium by ubiquitinating and deubiquitinating enzymes. Single ubiquitination does not degrade the target gene, but only plays a regulatory role. When at least four activated ubiquitin chains are attached to the target protein to form

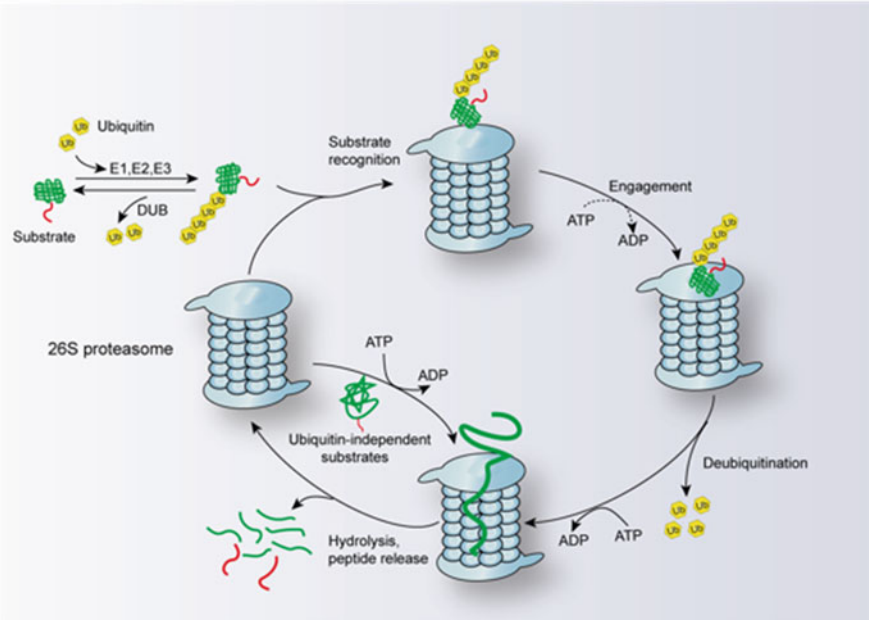

Fig. 13.2 The abridged general view of the reaction process of protein ubiquitination and subsequent protein destruction in 26S proteasome

a polyubiquitin chain, the substrate is transported to the 26S proteasome for degradation (Hurley and Stenmark 2011), while the polyubiquitin chain is decomposed into single ubiquitin molecule under the action of ubiquitin recirculation enzyme for recycling, which is the classic pathway of UPP (Fig. 13.2).

The role of deubiquitinase is to hydrolyze the ubiquitin on the target protein, deubiquitinize the protein that has been ubiquitinated, dissociate the ubiquitin chain into a single ubiquitin molecule, participate in the ubiquitin process again (Amerik and Hochstrasser 2004) (Fig. 13.2). Ubiquitination can ubiquitinate the target protein, which can be degraded by proteasome. The deubiquitination enzyme can dissociate the ubiquitin from the ubiquitinated target protein and prevent the protein from being degraded. Human genes encode about 100 deubiquitination enzymes (Reyes-Turcu et al. 2009). DUBs can be classified into six types according to its structure and function: Ubiquitin c-terminal hydrolase family (UCHs), ovarian tumor-associated protease family (OTUs), ubiquitin specific protease family (USPs), Josephin domain protein family (MJD) and JAB1/MPN/Mov34 protease family (JAMM), and the newly discovered single-cell chemoattractor protein-induced protein family. The first four families of deubiquitination proteinases are cysteine proteinases, while the JAMM family is zinc metalloproteinases. The process of deubiquitination is very precise and orderly. It is involved in a variety of important life activities, including cell cycle regulation, gene transcription, kinase activation, protein degradation, DNA

repair (Amerik and Hochstrasser 2004). The abnormal expression of deubiquitinase can lead to a variety of diseases, including cancer and neurodegenerative diseases.

13.2 Ubiquitination of Membrane Proteins

It has been found that ubiquitination can not only target the receptor and promote its degradation in proteasome, but also play an important role in receptor internalization and its subsequent transport (Hurley and Stenmark 2011). The internalization and endocytic transport of a variety of membrane receptors is mediated by mono-ubiquitination and poly-ubiquitination (Nathan and Lehner 2009). For example, the yeast peptides transporters Ste6 and pheromone factor receptor Ste2 in yeast undergo mono-ubiquitination, which can regulate membrane uptake and degradation in vivo. The yeast factor receptor (Ste3) is not only mono-ubiquitination but also poly-ubiquitination, which can accelerate the degradation of the receptor. Ubiquitin itself has 7 lysine residues (K), located at sites 6, 11, 27, 29, 33, 48 and 63, respectively. These lysine residues connect to ubiquitin to form different ubiquitin chains. The substrate protein is of poly-ubiquitination after connecting to ubiquitin chain, such as k48-polyubiquitin chain and k63-polyubiquitin chain. The response varied when mediated by poly-ubiquitination at different sites, among which the k48-polyubiquitin chain usually mediates the degradation of substrate proteins into the 26S proteasome, while the k63-polyubiquitin chain is mainly involved in non-proteasome degradation functions, such as DNA damage repair, transduction regulation and receptor internalization and transport.

13.2.1 EGFR

Ligand-induced transmembrane receptor activation activates a number of intracellular signaling pathways that regulate key cellular biological processes such as cell proliferation, differentiation, migration, and survival. Therefore, space and time regulation of receptor pathway is very important to cell biology function. One regulatory mechanism is the ubiquitination of the receptor, which ensures the timely termination of the receptor pathway by promoting receptor internalization and its lysosomal degradation. It has been established that mono-ubiquitination of the cargo protein is sufficient for endosomal sorting complexes required for transport (ESCRT)-mediated sorting to the multivesicular body (MVB), which represents a key step before lysosomal degradation (Fig. 13.4) (Hurley and Stenmark 2011). Moreover, yeast vacuoles separation research prompts that K63 ubiquitin chain modification can furtherly increase the efficiency of the above process, perhaps because K63 ubiquitin chain adopted an open configuration to increase its affinity to ubiquitin-interacting domains. A recent mass spectrometry study suggests that more than 50% of EGFR-linked ubiquitin is in the form of a K63 ubiquitin chain (Sigismund et al. 2013). And several

experiments of overexpressing K63-ubiquitin mutation clearly showed that the internalization and transport of TrkA (nerve growth factor receptor) and MHCI (major histocompatibility complex I) also depend on the K63-ubiquitin chains mediated ubiquitination.

Due to the core role of receptor tyrosine kinases (RTKs) in cell biological functions, the research on the ubiquitination modification of RTK is also the most adequate, especially for EGFR, which has already become a hot molecule in the field of ubiquitination research. When binding to the ligand, EGFR is rapidly activated and ubiquitinated, and the major E3 ligase is the Cbl (cas-br-mecotropic energy conversion sequence) RING-type E3 ubiquitin ligase. Cbl can be directly recruited to the phosphoric tyrosine residues (tyr1045-p) in the activated EGFR intracellular segment, or indirectly recruited by adaptor GRB2 (growth factor receptor-bound protein 2) to the cell membrane to promote ubiquitination of activated EGFR (Kozer et al. 2014; Ahmad et al. 2014). Mono-ubiquitination and K63 poly-ubiquitination are the main types of ubiquitination of EGFR and the concentration of ligand or the degree of ubiquitination can affect the pathway of EGFR internalization (Fig. 13.3) (Hurley and Stenmark 2011). For example, when EGF is at a low concentration, the degree of ubiquitination of EGFR is difficult to detect, and EGFR is mainly internalized by clathrin-mediating. However, when EGF is at a high concentration, a large amount of EGFR is in the ubiquitinated state and it mainly occurs clathrin independent but lipid raft dependent internalization. Differences in internalization patterns also determine the fate of EGFR and the duration of signaling pathways. When EGFR is internalized by clathrin, it is not transported to the lysosome but is recirculated to the cell membrane, thus prolongs and enhances the EGFR signaling pathway (Ahmad et al. 2014), whereas clathrin-independent EGFR is more easily transported to lysosomes for degradation (Sigismund et al. 2013; Ahmad et al. 2014). However, some studies have shown that even at low EGF concentrations, EGFR ubiquitination can be detected and promote EGFR to be transport to clathrin-coated pits (Ibach et al. 2015). Although the specific regulatory mechanism and effect of ubiquitination on EGFR have not been fully clarified, almost all experimental data show that ubiquitination can affect the degree of EGFR internalization, regulate the fate of EGFR through the internalization pathway, and thus change the whole EGFR signaling pathway.

13.2.2 GPCR (G Protein Coupled Receptors)

It was reported that ubiquitination occurs in a variety of GPCR, and plays an important role in receptor transport, especially in some typical mammalian GPCR, such as β2-adrenergic receptor (β2-AR) and chemokine receptor 4 (CXCR4), V2 vasopressin receptors, protease activated receptor 2 (PAR2) and speed shock peptide receptor type 1 (Shenoy 2007; Marchese and Trejo 2013; Miranda and Sorkin 2007). Ubiquitination is not necessary for receptor internalization but plays an essential role in the process of targeting the internalized receptor into lysosomes or proteasome for degradation (Xiao and Shenoy 2011; Shenoy et al. 2008, 2001; Bhandari et al.

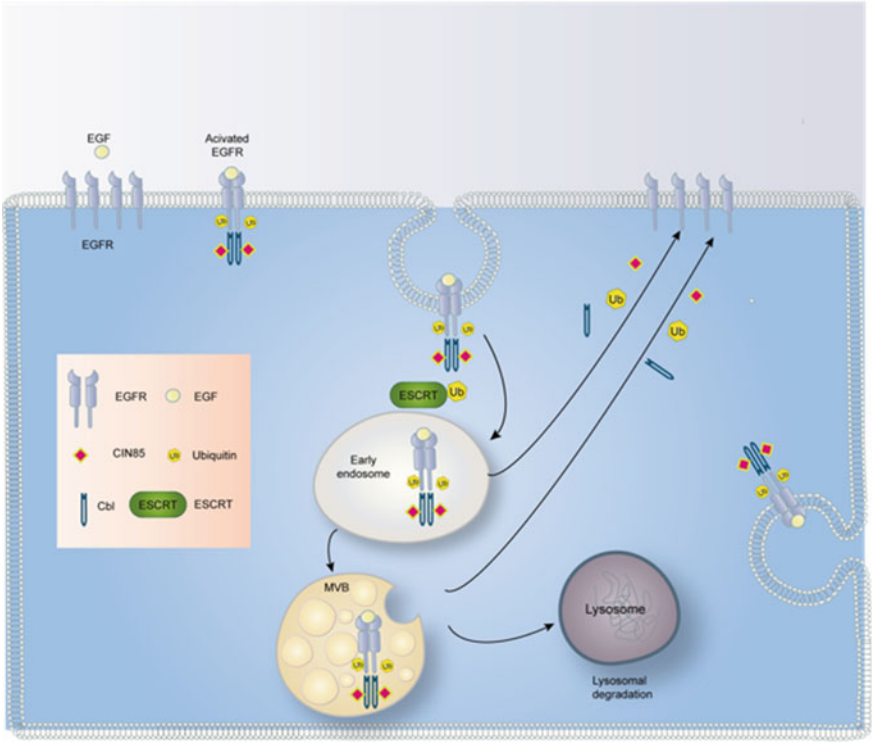

Fig. 13.3 The schematic diagram of EGFR internalization. After binding to EGF, the homodimerization and autophosphorylation of EGFR recruits and interacts with Cbl/CIN85 complex through the variant SH2 domain of Cbls. Ubiquitinated EGFR and CIN85 mediated by Cbls is transported from early endosomes to MVBs and then targets to lysosome for degradation. A certain proportion of EGFR will be deubiquitinated by DUBs and recycled back to the cell membrane

2007; Marchese and Benovic 2001; Martin et al. 2003; Hasdemir et al. 2009; Cottrell et al. 2006). It was found that GPCR was transported to lysosome for degradation through classical ESCRT (endosomal sorting complex required for transport) pathway (Fig. 13.4). ESCRT pathway contains four different proteins, namely ESCRT-0, ESCRT-I, ESCRT-II and ESCRT III and subsidiary factors such as cage-like protein and vacuole separation protein 4(VPS4) containing AAA-ATPase (Fig. 13.4). They function in an orderly and coordinated manner to sorting the ubiquitin substrate to the multi-vesicular body (MVB). ESCRT-0 is composed of hepatocyte growth factor-regulated tyrosine kinase substrate (HRS) and signal-transducing adaptor molecule (STAM) and the both subunits contain ubiquitin-binding domain (UBD) at the ubiquitin interaction site, which can recognize and recruit ubiquitin receptors into the ESCRT pathway and bind cage proteins. ESCRT-I and ESCRT-II can interact with ubiquitin receptors with their UBD while ESCRT-III lack of UBD cannot bind to ubiquitin receptors and mainly function in the division of lumen vesicles (Fig. 13.4). GPCRs are modified by ubiquitination after ligand activation, then the receptors are

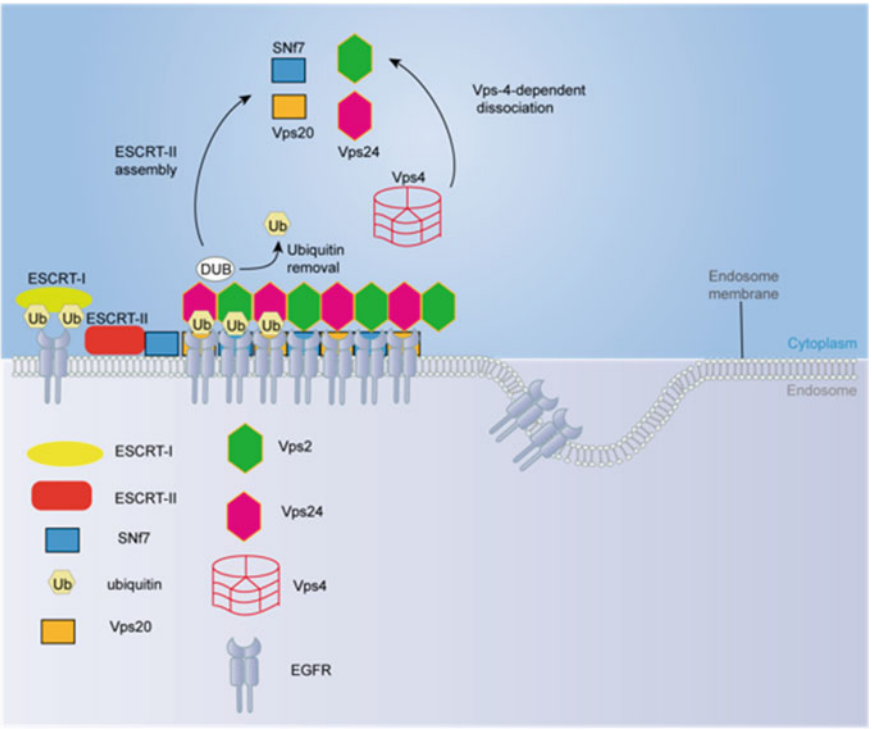

Fig. 13.4 A model of ESCRT-dependent EGFR sorting. The UBC-like domain of Vps23, the UBC-like domain of ESCRT-I binds to multi-ubiquitinated EGFR at the membrane of MVBs, which recruits ESCRT-II. The interaction of ESCRT-I and ESCRT-II then putatively directs the assembly of ESCRT-III complex (which is composed of Vps20, Snf7, Vps2, and Vps24) to the appropriate MVB membrane. ESCRT-III recruits DUBs to remove Ub from EGFRs, resulting in MVB's "swallow" of cargo proteins. Vps4 is responsible for the disassembly and release of the entire MVB sorting machinery, which allows the ESCRT machinery to recycle back into the cytoplasm for MVB sorting of other proteins or further rounds

internalized and transported to early endosome and MVB successively. GPCRs are deubiquitinated before sorting into MVBs. The deubiquitinated GPCRs are transport into the luminal vesicles of MVBs in the regulation of ESCRT-III and the AAA-ATPase VPS4, ensuring ESCRT pathway recycling. The process described above is the classic ESCRT pathway (Hislop and von Zastrow 2011; Alonso and Friedman 2013; Dores and Trejo 2012). The lysosomal sorting of some GPCR through ESCRT pathway depends on ubiquitination such as CXCR4 (Marchese and Benovic 2001) (Fig. 13.5a), β2AR (Shenoy et al. 2001) (Fig. 13.5b), and PAR2 (Hasdemir et al. 2009) while the endocytic trafficking of PAR1 (Dores et al. 2012) (Fig. 13.5c) and δ-receptor (Tanowitz and Von Zastrow 2002) remained unchanged after ubiquitination sites mutation. This suggests that ubiquitination and ESCRT mechanisms are not necessary for some GPCR sorting to lysosomal for degradation, and other mechanisms may be involved in regulating receptor degradation.

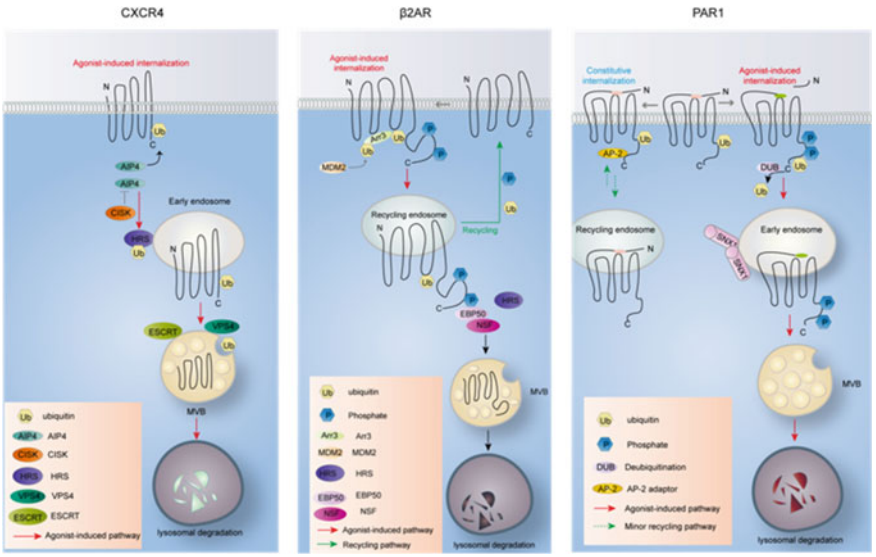

Fig. 13.5 **a** Activated CXCR4 is ubiquitinated at the plasma membrane by the E3 ubiquitin ligase AIP4. Ubiquitinated CXCR4 interacts with HRS and AIP4 mediates ubiquitination of HRS induces CXCR4 activation and the MVB sorting. CISK inhibits AIP4 activity and thereby blocks endosomal sorting of CXCR4. VPS4 also regulates the ubiquitination status of CXCR4 and MVB sorting. **b** Agonist induces rapid phosphorylation and ubiquitination of the β2AR and MDM2-mediated ubiquitination of arrestin-3 increases the recruitment of activated β2AR, which leads to β2AR internalization. After internalized, β2AR are dephosphorylated and rapidly recycled back to the plasma membrane with the help of various protein including EBP50/NHERF, NSF, and HRS. After sustain agonist treatment, activated β2AR targets to MVB-lysosome pathway for degradation. **c** PAR1 displays constitutive and agonist-induced internalization, in which process ubiquitination play different roles. Constitutive internalization of PAR1 requires AP-2 and is negatively regulated by ubiquitination. Activated PAR1 is phosphorylated, rapidly internalized, and sorted from MVB to lysosomes through a SNX1-dependent pathway

13.3 The Main Immune Checkpoints

At present, tumor immunotherapy is booming. The Cancer Research Institute (CRI) in New York published the "global trends in tumor immunotherapy" survey in October 2018, which showed that there are 417 targets of tumor immunotherapy in the clinical Research stage worldwide. Compared with 2017, the number of global tumor immunotherapy programs increased by 67% in 2018, the number of targets increased by 50%, and the number of companies and institutions with clinical development programs increased by 42%. In addition, 50% of global research projects focus on the top 48 targets. It was reported that the top15 tumor immune-related targets CD19.

PD-1, PD-L1, HER2, STAT3, CTLA-4, NY-ESO-1, BCMA, IDO, Neoantigen, MUC1, CSFIR, WT1, CD20 and CD47. At present, the targets of immune regulation mainly fall into three categories: T-cell immune checkpoint, tumor tissue immune

checkpoint and immune environment regulatory target. Cytotoxic T lymphocyte-associated antigen-4 (CTLA-4) and programmed cell death protein 1 (PD-1) are the most classic and most commonly-used T-cell immune checkpoints clinically. Antibody drugs developed for blocking CTLA-4 and PD-1 have shown good therapeutic effects in the clinical treatment of melanoma and lung cancer. In addition, there were other T cell immune-checkpoint such as lymphocyte activation gene 3 (LAG-3), T-cell immunoglobulinmucin 3 (TIM-3), and V-domain Ig suppressor of T cell activation (VISTA) and so on. PD-L1, also known as CD274 and B7H1, was first discovered by Chinese Scientist Professor Lieping Chen's research group. The expression of PD-L1 can be detected in various tumor tissues, and the tumor microenvironment can induce the expression of PD-L1 in tumor cells, which is conducive to the occurrence and growth of tumors and induces the inactivation of anti-tumor T cells. PD-L1 is the ligand of PD-1, and its binding to PD-1 will lead to the tyrosine phosphorylation of the intracellular domain of PD-1and recruit the tyrosine phosphatase SHP-2, thus reducing the phosphorylation of the T cell receptor (TCR) signaling pathway and the activation signal of the downstream of the TCR pathway as well as the activation of T cells and the generation of cytokines (Chamoto et al. 2017). Therefore, the inhibition of the PD-1/PD-L1 pathway will accelerate and strengthen the autoimmunity (Chikuma 2016). The regulation of immune environment refers to the regulation of immune cells such as macrophages, antigen presenting cells and natural killer cells in the tumor microenvironment and the interaction between immune molecules such as IL-2, IL-10 and transforming growth factor β (TGF-β), so as to activate the immune response of anti-tumor cells.

13.4 PD-1/PD-L1

Programmed cell death protein (PD-1), also known as PDCD1 and CD279, is a kind of I transmembrane protein encoded by genes PDCD1, consisting of 288 amino acid residues and belonging to B7-CD28 receptor superfamily (Keir et al. 2008; Carreno and Collins 2002). Its structure includes four parts: IgV, transmembrane region, immunoreceptor tyrosine-based inhibitory motifs (ITIM), and immunoreceptor tyrosine-based switch motifs (ITSM) (Keir et al. 2008). It is expressed on the surface of a variety of immune cells, such as bone marrow cells, dendritic cells, natural killer cells (NK), monocytes, $CD4^{-}CD8^{-}$thymus cells, regulatory T cells, B cells and antigen presenting cells (Keir et al. 2008). Under normal physiological conditions, T cells do not express a large amount of PD-1. When T cells are exposed to antigen stimulation for a long time, the expression of PD-1 is up-regulated and activated T cells can further induce other cells to overexpress PD-L1 by releasing cytokines such as Interferon-r (IFN-r) and interleukin (Chamoto et al. 2017; Hashimoto et al. 2018). Binding of PD-L1to PD-1 results in phosphorylation of ITIM and ITSM in the intracellular domain of PD-1, which recruits the tyrosine phosphatases SHP-1 and SHP-2. These phosphatases can dephosphorylate several key proteins in the T

cell antigen receptor (TCR) signaling pathway and inhibit the downstream signaling pathways of TCR, such as PI3K/AKT/mTOR, RAS/MEK/ERK, c-myc and so on. Then the transcription of related genes is inhibited, and the cell cycle progress of T cells is impeded, as well as the expression of related proteins (Seliger 2019). These will inhibit the proliferation and differentiation of T cells and the production of cytokines.

13.4.1 The Poly-Ubiquitination of PD-1

There have been many studies on the regulation of transcription level of PD-1, while the regulation of protein level is relatively small. A recent study reveals that the surface PD-1 of activated T cells undergoes internalization, subsequent ubiquitination and proteasome degradation and the E3 ligase mediating Lys48-linked poly-ubiquitination of PD-1 is FBXO38 (Serman and Gack 2019; Meng et al. 2018) (Fig. 13.6). The researchers first find that the cell surface PD-1 level of both human

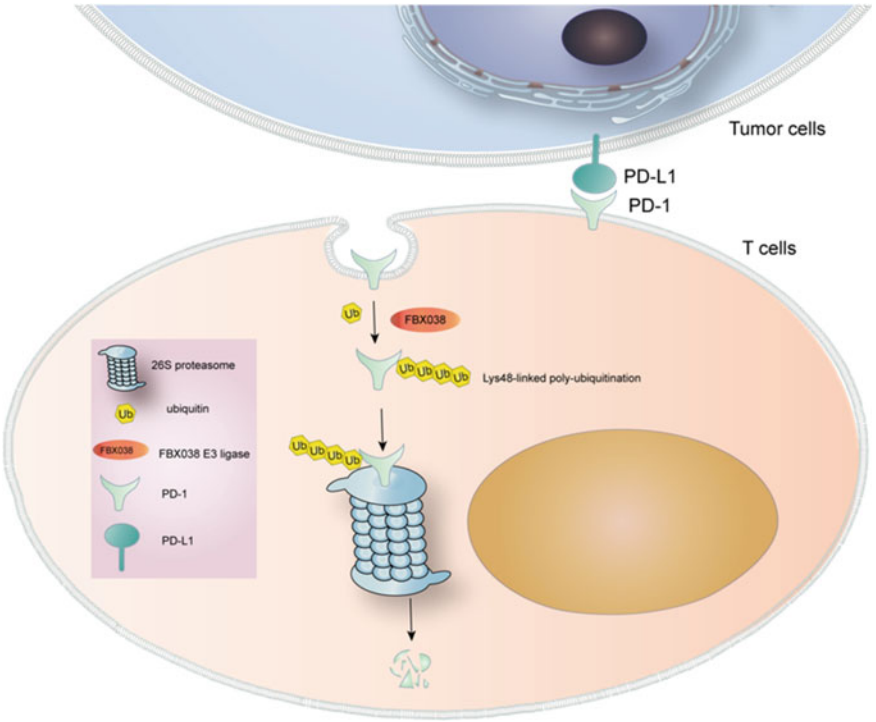

Fig. 13.6 Poly-ubiquitination of PD-1. The surface PD-1 undergoes internalization, subsequent ubiquitination and proteasome degradation in activated T cells. FBXO38 is an E3 ligase of PD-1 that mediates Lys48-linked poly-ubiquitination and subsequent proteasomal degradation of PD-1

and mouse T cells reaches maximum after two days of stimulation and then begin to decline towards basal level, while the PD-1 mRNA expression is only slightly downregulated. Then the rescue experiments suggest the reduction of PD-1 in protein level is due to poly-ubiquitination mediated proteasome degradation other than lysosomal degradation. FBXO38, the E3 ligase of PD-1 is first identified as protein binding to PD-1 through GST-pull down and mass spectrometry experiments and then confirmed by reciprocal co-immunoprecipitation experiments of exogenous or endogenous protein. Moreover, depletion of FBXO38 through knockdown or knockout significantly downregulates ubiquitination of PD-1 whereas ectopic expression FBXO38 in Jurkat cells obviously increases PD-1 poly-ubiquitination, suggesting that FBXO38 can directly mediate PD-1 poly-ubiquitination of PD-1. Furthermore, the result of mutagenesis data of Lys233 and Lys48 or Lys63 mutation on Ub demonstrate that FBXO38 mediates Lys48-linked poly-ubiquitination at the Lys233 site of PD-L1. In addition, the intrinsic role of FBXO38 in the anti-tumour immunity of $CD8^+$ T cells is confirmed in an adoptive T cell transfer therapy against mouse melanoma experiment, which show that FBXO38 can enhance anti-tumour immunity by downregulating membranous PD-1 expression of activated T cells. Interestingly, IL-2 administration rescues reduction of FBXO38 transcription in T cells and causes PD-1 downregulation in the tumour microenvironment, which maybe explain the anti-tumor function of IL-2. Taken together, Lys48-linked poly-ubiquitination has been found as the first protein modification after translation of PD-1 which can regulate the PD-1 protein level on the surface of activated T cells and anti-tumour immunity, therefore FBXO38 has been identified as the specific E3 ubiquitin ligase of PD-1 (Meng et al. 2018). These findings identify poly-ubiquitination as a crucial node for modulating PD-1 stability, and establish FBXO38 E3 ligase as a promising clinical potential target towards enhancing tumour-specific immunity (Meng et al. 2018).

As described above, the ubiquitination level of protein achieves dynamic equilibrium under the co-regulation of ubiquitinase and deubiquitinase. Screening deubiquitinating enzyme special for PD-1can help to find a new approach to decreasing PD-1 protein expression and enhancing anti-tumor immunity. In previous study, PD-1 was only found in immune cells including T cells and B cells, while it was reported recently that PD-1 was also expressed in cancer cells, such as melanoma, liver cancer cell and non-small cell lung cancer cells (Yao et al. 2018). Interestingly, skin and liver cancer cell-intrinsic PD-1 promotes tumorigenesis while blockade PD-1 in non-small cell lung cancer accelerates tumor growth and development (Yao et al. 2018; Kleffel et al. 2015; Li et al. 2017; Du et al. 2018). In Melanoma and liver cancer, PD-1 was found to promote tumor growth even in the absence of functional adaptive immune system, which involved the increased phosphorylation of ribosomal protein S6 (RPS6) and eIF4E as effectors of mammalian target of rapamycin (mTOR) signaling (Kleffel et al. 2015; Li et al. 2017). The different function of PD-1 in T cells and tumor cells prompts that how ubiquitination regulates the expression and function of PD-1 in T cells and whether cancer cell intrinsic PD-1 undergoes mono-ubiquitination and subsequent ESCRT-mediated lysosomal degradation or poly-ubiquitination and subsequent special E3 ligase mediated

proteasome degradation. Future in-depth investigations on ubiquitination of tumor-intrinsic PD-1 may provide additional insights into the unexpected effects of checkpoint blockade therapies and benefit the development of more effective combinatory immunotherapies.

13.4.2 The Poly- and Mono-Ubiquitination of PD-L1

As a kind of membranous protein, PD-L1 undergoes glycosylation in Golgi and then trafficking to cell surface while PD-L1 without glycosylation has been found to facilitating the phosphorylation of PD-L1 by GSK3β (Fig. 13.7), a multifunctional switch that mediates the directs phosphorylation of a wide range of substrates (Wang et al. 2018; Li et al. 2016). Furthermore, phosphorylation modification initiates the binding with E3 ligase, which destabilizes proteins in proteasome (Wang et al. 2018). It has been reported that both mono-ubiquitination and poly-ubiquitination play important role in regulating PD-L1 expression, membrane location and function (Stringer and Piper 2011; Horita et al. 2017; Yao et al. 2019; Lim et al. 2016). Deubiquitinating enzymes CSN5, a subunit of COP9 signalosome has been identified as the special DUB for PD-L1 (Fig. 13.7), which stabilizes PD-L1 and helps cancer cells to evade immune surveillance (Lim et al. 2016). The researchers first found that macrophages secreted inflammatory cytokines including TNF-α, IL-6, IL-8 and IL-1ra (IL-1 receptor antagonists) increased PD-L1 protein level but did not affect PD-L1 mRNA and the above inflammatory cytokines selectively upregulated PD-L1 protein expression but not other immune inhibitory ligands in tumor cells, which implying that tumor microenvironment can regulate PD-L1 through post-translational modification. Among the four inflammatory cytokines, only TNF-α could increase PD-L1 expression in protein level similar to MG132, a special inhibitor for proteasome, which suggested that TNF-α stabilized PD-L1 and promoted tumor growth. Because there are many downstream signaling pathway regulated by TNF-α, several special inhibitors including BAY11-7082, SB203580, PD98059, LY294002, U0126, rapamycin, and nutlin against various signaling pathway were applied to decrease the upregulated PD-L1 expression induced by TNF-α and the result indicated that only BAY11-7082, the special inhibitor for IKKβ could abolish TNF-α-stimulated PD-L1 increase, prompting IKKβ kinase activity and NF-κB may regulate TNF-α-stimulated PD-L1 stabilization. Then knock-out experiments showed that TNF-α was failed to enhance PD-L1 expression in protein level without p65 activation. Moreover, unlike IFNγ upregulates PD-L1 mRNA, p65 activation-TNF-α affects PD-L1 expression in post-translational modification level. Through analysis of PD-L1 binding proteins and a PCR array for deubiquitinating enzymes that responded to TNF-α, CSN5 was confirmed as the protein required for PD-L1stabilization and binding strongly to PD-L1. Then PD-L1 was immunoprecipitation and detected with K48-ubquitination antibody and the results indicated that MG132-induced PD-L1 ubiquitination level was abolished by CSN5 as well as TNF-α treatment. Taken together, it demonstrated that TNF-α upregulated CSN5 to reducing K48-linked PD-L1 ubiquitination and to

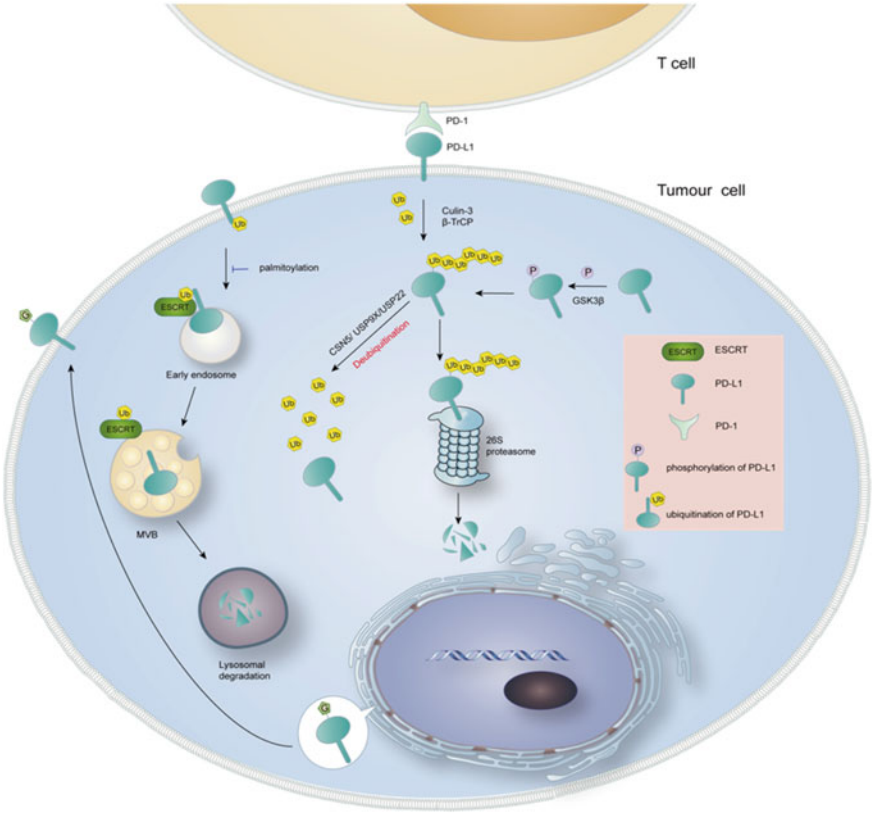

Fig. 13.7 The mono and poly-ubiquitination of PD-L1. N-glycosylation of PD-L1 extracellular domain occurs in the lumen of endoplasmic reticulum (ER), and this modification facilitates the trafficking of PD-L1to cell surface. Glycosylation also inhibits phosphorylation by GSK3β, and thereby blocking the poly-ubiquitination by β-TrCP. Deubiquitination by CSN5, USP9X or USP22 also protects PD-L1 from degradation in proteasome. In addition to poly-ubiquitination, PD-L1 also undergoes mono-ubiquitination and consequent ESCRT-mediated MVB-lysosome pathway, which leads to PD-L1 destruction in lysosome. This process is suppressed by palmitoylation

inhibiting PD-L1 degradation in proteasome, which required p65 to increase transcriptional activation of CSN5 (Lim et al. 2016). In addition, curcumin, which can inhibit CSN5-associated kinase activity as well as NF-κB activity was suggested to attenuating immunosuppression and has potential to application in combination treatment for cancers that are associated with inflammatory diseases (Lim et al. 2016). Besides CNS5, USP9X and USP22 also identified as DUB for PD-L1 (Fig. 13.7) (Jingjing et al. 2018; Huang et al. 2019).

In addition to poly-ubiquitination, it was reported that PD-L1 may be also mono-ubiquitinated, which has been repeatedly verified in a recent study on PD-L1 palmitoylation (Yao et al. 2019). Interestingly, it was demonstrated that

de-pamitoylation-induced mono-ubiquitination of PD-L1 was sufficient for endosomal sorting complexes required for transport (ESCRT)-mediated sorting to the multivesicular body (MVB) (Fig. 13.7), which represents a key step before lysosomal degradation, thus mono-ubiquitinated PD-L1 was trafficked to lysosome for degradation while poly-ubiquitinated of PD-L1 was transported to proteasome for degradation (Stringer and Piper 2011; Horita et al. 2017; Yao et al. 2019). However, it remains unknown about the ubiquitin specific protease for PD-L1 and how it regulates PD-L1 expression and function. Therefore, more detailed and deeper investigation on PD-L1 ubiquitination may help design better biomarkers and more efficacious therapeutic approaches towards cancer immune evasion.

13.5 LAG-3/MHCII

13.5.1 LAG-3

Lymphocyte activation gene-3 (LAG-3, CD223) is a member of the immunoglobulin superfamily, distributed in activated T lymphocytes, NK cells and dendritic cells with a high affinity for MHC-II (Triebel et al. 1990). LAG-3 gene is located in human chromosome 12, closely related to CD4 (Li et al. 2004). As a negative immune regulator, it has the function of maintaining internal stability and participating in immune regulation, and regulating the development of tumor (Workman et al. 2002a; Maeda et al. 2019). Most LAG-3 is expressed in the cell membrane in the form of dimer, and mature LAG-3 can rupture in the cell membrane for the soluble part p54 with a relative molecular weight of 54KD and the transmembrane-cytoplasmic part p16 with a molecular weight of 16KD (Li et al. 2004). The process of LAG-3 molecular rupture from the cell surface to a soluble molecule is regulated by the transmembrane matrix metalloproteases AM AD10 and AM AD17, and TCR signaling pathway plays an important role in these two regulatory modes (Li et al. 2007). The broken process of LAG-3 from the cell membrane play a regulatory role in LAG-3 molecular function as follows: Firstly, it is important for signal transduction in MHC-II positive cells, secondly, fracture of LAG-3 could undermine the binding of MHC-II to CD4 and even TCR due to the high affinity between LAG-3 and MHC-II (Workman et al. 2002a, b). T_{reg} cells are subsets of T cells with regulatory functions, and it was found that the T_{reg} cell surface markers CD49b and LAG-3 were expressed in human and mouse T_{reg} cells as the surface markers (Gagliani et al. 2013). The discovery of CD49b and LAG-3 makes it possible to track T_{reg} cells in vivo, and to purify T_{reg} cells for cell therapy (Gagliani et al. 2013). Because of the selective inhibition of LAG-3 on the proliferation of antigen-specific T cells in the pancreas, LAG-3 can be used as a new surrogate marker for the progression of typeIdiabetes, and the detection of LAG-3 molecules may be a new method for evaluating the efficacy of targeted immunotherapy of T cells (Bettini et al. 2011; Zhang et al. 2005; Delmastro et al. 2012). The expression of LAG-3 on the surface of tumor infiltrating $CD8^+$ T cells

is up-regulated, and the inhibition of LAG-3 plays an important role in the cellular immune response, suggesting that blocking the expression of LAG-3 enhances anti-tumor immunity (Grosso et al. 2007). Moreover, the expression of LAG-3 promotes melanoma proliferation and the inhibitor blocking LAG-3-MHC-II interaction can be used for the treatment of melanoma (Hemon et al. 2011). In spite of the key role of LAG-3 expression for cells within the tumor microenvironment as described above, our understanding of the regulation of the LAG-3 protein, especially of its posttranslational modification such as ubiquitination is very limited. Further understanding of the regulation mechanisms about LAG-3 posttranslational modification is required for elucidating the role of LAG-3 in immune response and developing related new targets for enhancing immune surveillance.

13.5.2 The Poly-Ubiquitination of MHC II

MHC II molecule, the ligand for LAG-3, is mainly expressed in antigen presenting cells, such as dendritic cells, macrophages and B cells. It undergoes synthesis in endoplasmic reticulum and then it is transported to Golgi apparatus and intracellular endocytosis system, where it is combined to antigen peptide and forms MHC-II-peptide complex (Cho and Roche 2013). Finally, the MHC-II-peptide complex is transferred to the cell surface for recognition of $CD4^+$ T cells, thus playing a key role in the development, activation and tolerance of $CD4^+$ T cells (Ishikawa et al. 2014).

It is established that MARCH1, an E3 ubiquitin ligase, plays a critical role in the ubiquitination of MHC-II on the surface of dendritic cells (Fig. 13.8) (Walseng et al. 2010; Shin et al. 2006; Wilson et al. 2018). MARCH1, containing about 289 amino acids with relative molecular mass of 32KD, is mainly expressed in secondary lymphoid tissue, lymph nodes and spleen of follicular B cells, and has high expression in antigen presenting cells, B cells (Drake 2018) and DCs (Ohmura-Hoshino et al. 2009; Bauer et al. 2017). MARCH1 can mediate ubiquitination of transferrin receptor TFRC, CD86 (B7-2), FAS and MHC-II protein (HLA-DRα and HLA-DRβ) (Fig. 13.8), sorting them to lysosome for degradation through the multivesicular bodies (MVBs) (Bauer et al. 2017; Tze et al. 2011; Buschow et al. 2009; Furuta et al. 2013). HLA-DRa/β are heterodimeric, mainly luminal proteins with one TM-helix each and a short cytosolictail. In contrast, the E2-binding RINGv domains of MARCH-1/8 are located in the cytoplasm. Thus, interaction between HLA-DRa/β and MARCH-1/8 can in principle be mediated by the TM regions, the luminal loop, and/or the cytosolic linker between RINGv and TM1 of MARCH-1or MARCH-8 (Bauer et al. 2017) (Fig. 13.8). Of note, a related study demonstrates that Ub chain length plays a key role in determining the intracellular fate of ubiquitinated membrane proteins, which can explain that why immature DCs accumulate MHC-II in late endosomes and lysosomes whereas B cells restore MHC-II at the plasma membrane (Ma et al. 2012). The sole reason can be attributed to the longer Ub chains conjugated to MHC-II in DCs (Ma et al. 2012). Mono-ubiquitination is insufficient for internalization and MVBs-lysosomal delivery of surface MHC-II in DCs and

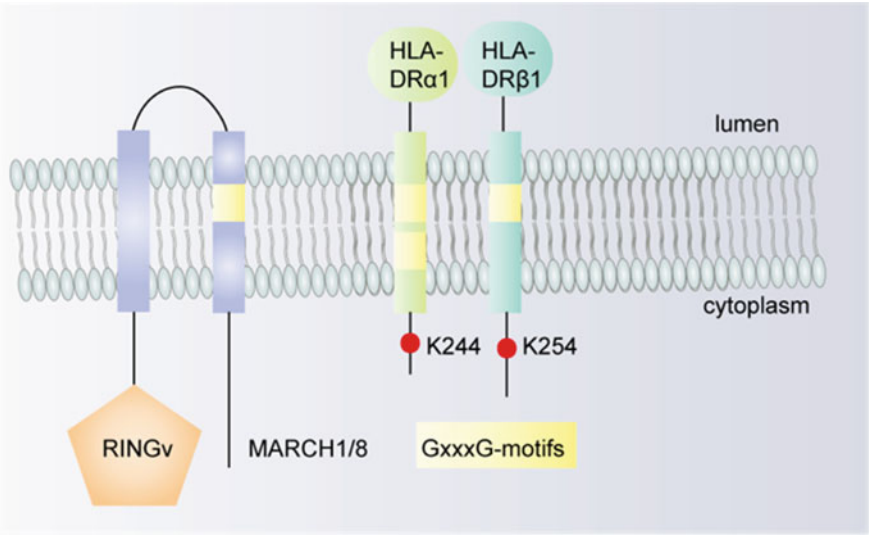

Fig. 13.8 Schematic illustration of GxxxG-motifs in the TM helices of MARCH-1,8 and its ubiquitination target HLA-DRα1/β1, a heterodimeric MHC-II molecule. GxxxG motifs in TM helices are shown in yellow, lysines that are ubiquitinated in the cytosolic tails of HLA-DRα1/β1 are highlighted and marked with a red asterisk and labelled accordingly

reducing the MHC-II Ub chain length in immature DCs produced a localization pattern similar with B lymphocytes: predominantly cell surface (Ma et al. 2012). It has been reported that MARCH1 downregulated MHC-II expression by increasing its ubiquitination in the course of organ dysfunction syndrome (MODS), playing key role in regulating the maturity condition of DCs and subsequently initiating adaptive immune response (Ohmura-Hoshino et al. 2009). Therefore, MARCH1 can be used as a therapeutic target to reduce the clinical mortality of MODS. The specific mechanism about how MARCH1-mediated ubiqiutination regulates MHC-II expression and antigen presenting in DCs in MODS remains to be further in-depth study. Toll role protein (Tollip) can induce a decline in MARCH1 expression and restore the MHC-II expression, so MARCH1 may be a kind of new target for Tollip (Bourgeois-Daigneault et al. 2013). Although MARCH1 is limitedly expressed in secondary lymphatic organs (Bartee et al. 2004), it can be induced or suppressed by different stimuli. For example, it can be regulated by interleukin 10 (IL-10) in human primary monocytes and mouse B cells (Tze et al. 2011). Therefore, MARCH1 can tune the immunosuppressive effect mediated by MHC-II antigen-presenting route induced by IL-10. Inhibition of MARCH1-mediated ubiquitination and degradation of MHC-II induced by IL-10, CD83 can upregulate MHC-II and CD86(B7-2) expression in DCs (Tze et al. 2011). The maturation DCs inhibits induced by LPS suppresses MARCH1 expression, allowing that the newly synthesized MHC molecules can display on the cell surface (Tze et al. 2011).

Similarly, it is found that MHC-II expression on cell surface and its degradation are dynamically regulated in germinal centers (GC) B cells and the fluctuations in B cell membranous MHCII levels are dependent on MARCH1 mediated-ubiquitination (Matsuki et al. 2007; Bannard et al. 2016). MARCH1 expression in centroblasts downregulates surface MHC-II levels, whereas CD83 in centrocytes rescues the reduction of MHC-II expression (Bannard et al. 2016). Interestingly, it has been reported that in thymic epithelial cells (TECs), the related ligase MARCH8 other than MARCH1 is identified as the major E3 ligase responsible for regulating MHC-II trafficking and degradation (Fig. 13.9) (Bauer et al. 2017; Liu et al. 2016; von Rohrscheidt et al. 2016). Moreover, it is demonstrated that MARCH8 activity is regulated by CD83, with critical consequences for $CD4^+$ T cell selection (Liu et al. 2016). The mechanism about how CD83 specifically controls MARCH 8 remains unknown. The furthermore study on distinct cell-intrinsic roles for MARCH 1 and MARCH 8 in DCs and TECs is required to mining mechanism about ubiquitination-mediated regulation of MHC-II expression and function.

Notably, a research about the role of MARCH1-mediated MHC-II ubiquitination in vivo suggests that MARCH1 deficiency causes falling in the number of thymus-derived regulatory T cells (Treg cells) in mice and abrogation of MHC-II ubiquitination also significantly decreases the number of thymic Treg cells (Oh et al. 2013). Meanwhile, DCs deficient in MARCH1 or MHCII ubiquitination both cannot generate antigen-specific T_{reg} cells in vivo and in vitro, in spite of increasing capacity for antigen presentation consistent with the increased surface MHC-II (Oh et al. 2013). The study hints that MARCH1-mediated MHC-II ubiquitination in DCs is indispensable for proper production of naturally occurring T_{reg} cells, suggesting the role of MHC-II ubiquitination in balancing immunogenic and regulatory T cell development (Oh et al. 2013).

In addition, it is found that Salmonella infection induces poly-ubiquitination of MHC-II and leads to removal of mature and peptide loaded MHC-II-peptide complex dimers from the cell surface (Bayer-Santos et al. 2016; Lapaque et al. 2009). While ubiquitination of MHC-II cannot be detected when cells is challenged with Salmonella strain carrying mutation in ssaV, implicating Salmonella T3SS-2 effector proteins in the process and Salmonella encodes a SPI-2 effector protein that directly ubiquitinates MHC-II (Lapaque et al. 2009). Then, ubiquitination of Lys225 in MHC-II induced by Salmonella is confirmed, which clarify the reduction in MHC-II expression seen upon infection as ubiquitination functions as a signal for endocytosis (Lapaque et al. 2009). Furthermore, a recent research indicates that the effector SteD is required and sufficient for the above process (Fig. 13.9). It is observed that SteD is localized to the Golgi network and vesicles containing the E3 ubiquitin ligase MARCH8 and mature MHC-II in Mel Juso cells (Fig. 13.9) (Bauer et al. 2017; Bayer-Santos et al. 2016). SteD induces MARCH8-dependent ubiquitination and reduction of membranous mature MHC-II through mediating the binding of MARCH8 to MHC-II (Bayer-Santos et al. 2016). Infection of Salmonella in dendritic cells caused SteD-dependent depletion of cell membranous MHC-II, the co-stimulatory molecule B7.2, and suppression of T cell activation (Bayer-Santos et al. 2016). Thus, SteD is

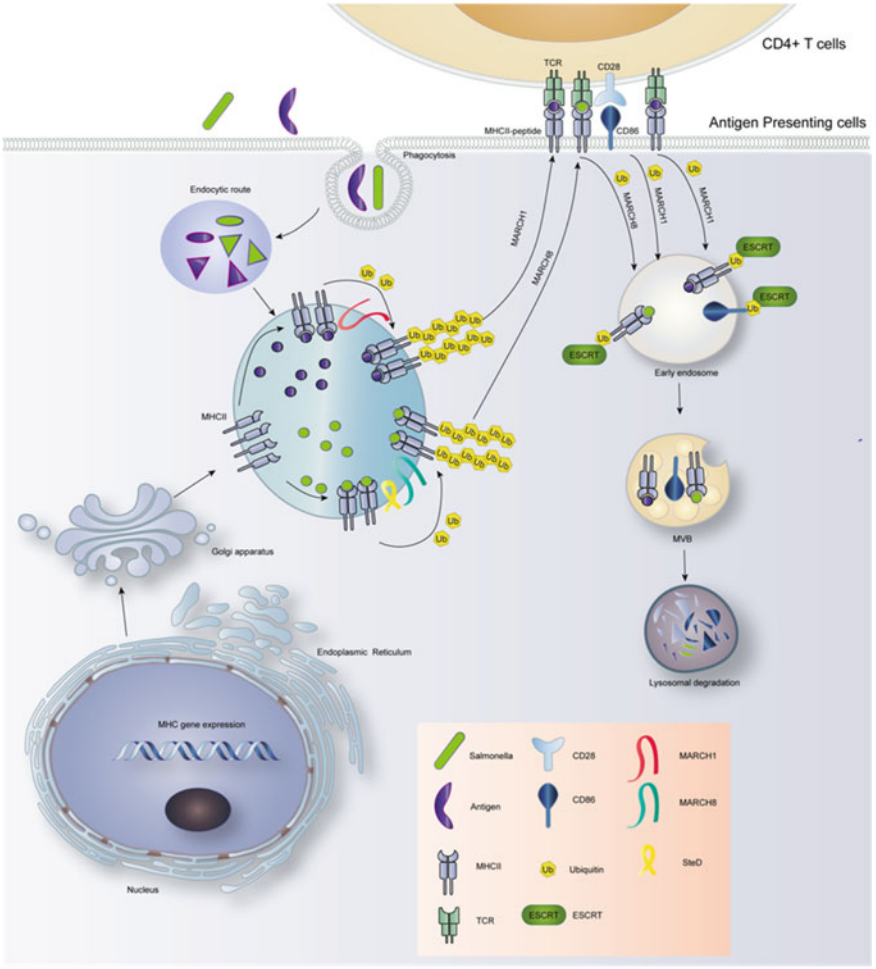

Fig. 13.9 Ubiquitination occurs at various steps in MHC-II antigen presentation pathway. Surface expression of the MHC-II-peptide complex along with co-stimulatory molecule CD86 is controlled by poly-ubiquitination carried out by the E3 ligase MARCH1. TCR interaction with cognate MHC-II-peptide complex results in activation of the T cell, which tightly controlled by a number of E3 ubiquitin ligases. DCs control the intracellular traffic of peptide–MHC II complexes by regulating the ubiquitination of MHC II. In resting or "immature" DCs, MARCH1-mediated MHC II are targeted to MVB-lysosome pathway for degradation, but upon pathogen-induced "maturation," ubiquitination is down-regulated and MHC II can accumulate on the plasma membrane of mature DCs. Besides MARCH1 as the E3 ligase, SteD caused MARCH8-dependent ubiquitination and depletion of surface MCH-II-peptide in Salmonella-infected Mel Juso cells

an adaptor of MARCH8 and MHC-II, mediating MHC-II ubiquitination and expression falling and thereby inhibiting T cell activation and function (Bayer-Santos et al. 2016). These findings uncover a mechanism by which the pathogen can influence the initiation of adaptive immune responses. However, even though more than ten years has passed since the first finding about MARCH-1 as a physiological E3 ligase of MHC-II, pathological relevance of MARCHs remains obscure and MARCH's recognition mode seems to be more complicated than expected (Ishido and Kajikawa 2019). Structural analyses of MARCHs using new biology technology as well as cell biological experiments combined with careful mutational analysis must be carried on.

13.6 CTLA-4/CD86/CD80

CTLA-4 (Cytotoxic T lymphocyte associate protein-4) gene is located in the long arm of chromosome 2 (2q33) and is mainly expressed on the surface of activated T cells. It is highly homologous with the co-stimulating molecular receptor (CD28) on the surface of T cells (Peach et al. 1994). Both CTLA-4 and CD28 are members of the immunoglobulin superfamily and bind to the same ligands CD86 (B7-2) and CD80 (B7-1) (Peach et al. 1994; Azuma et al. 1993; Fernandez-Ruiz et al. 1995). Contrary to the function of CD28, CTLA-4 binds to the B7 molecule and inhibits T cell activation (Azuma et al. 1993). The main T cells expressing CTLA-4 are regulatory T cells (T_{reg}), a class of T cells that negatively regulate cellular immunity (Selby et al. 2013). T_{regs} in the tumor microenvironment are with high expression of CTLA-4 (Selby et al. 2013). CTLA-4 targeting antibodies may mediate the antigen-dependent phagocytosis (ADCP) of macrophages or the antibody-dependent cytotoxic cell (ADCC) of natural killer cells to eliminate T_{reg} cells in the tumor microenvironment, thus potentiating anti-tumor effect (Tang et al. 2018).

The mechanism of CTLA-4 function has not been fully elucidated, which may contain the following several aspects: firstly, playing the role of competitive: CTLA-4 has a high affinity with B7 and binds to B7 on antigen presenting cell (APC) surface in competition with CD28, blocking CD28 and B7 signaling pathway and preventing CD28 from promoting T cell activation (Rowshanravan et al. 2018). Secondly, inhibiting the production of IL-2 to achieve a negative regulatory effect (Appleman et al. 2000). Thirdly, blocking T cells cycle from G phase to S phase, thus inhibiting their proliferation and activation (Patil et al. 2017). CTLA-4 interferes with TCR signal by interacting with PP2A and SHP2 (Lee et al. 1998). Meanwhile, CTLA-4 binds to PI3K, leading to AKT phosphorylation, pro-apoptotic factor BAD inactivation, and up-regulation of anti-apoptotic factors Bcl-xl and Bcl-2, playing a key role in immune tolerance (Wei et al. 2007). It is reported that AP-2 mediates the clathrin-dependent internalization of CTLA-4 from the cell surface to endosomal and lysosomal compartments (Schneider et al. 1999; Schneider and Rudd 2014). As described above, CTLA-4 is preferentially expressed in Th2 cells, whose differentiation depends on the transcriptional regulator GATA3. Interestingly, GATA3 is

regulated by phosphorylation and ubiquitination and the posttranslational modification of GATA3 modulated CTLA-4 expression (Gibson et al. 2013). It is detected that GATA3 and CTLA-4 are both overexpressed in Sezary syndrome (SS), a T-cell malignancy characterized by Th2 cytokine skewing and impaired T-cell responses. In consistence with that increased poly-ubiquitinated and activated GATA3 observed in SS cells, it is demonstrated that blocking proteasome degradation of GATA3 leads to upregulation of GATA3 and CTLA-4, resulting in inhibition of T-cell responses. Targeting this pathway may be beneficial in SS and other CTLA-4-overexpressing T-cell neoplasms (Gibson et al. 2013). In addition, CD86 is ubiquitinated by MARCH1and then targets to MVB-Lysosome degradation pathway (Moffat et al. 2013).

13.7 CD226/CD112/CD155/Tigit

CD226, belonging to the immunoglobulin superfamily, is a I transmembrane glycoprotein widely expressed in various types of immune cells (Sanchez-Correa 2019). CD226 molecules are widely expressed in various immune cells, such as NK cells, T lymphocytes, and monocytes/platelets (Sanchez-Correa 2019). Various cytokines, including IL-1α, IL-1β, IL-2, IL-3, TNF-α and IL-15 (Hromadnikova et al. 2013; Fujii et al. 2018; Xu and Jin 2010), can upregulate the expression of CD226, and super-antigen SEA and SEB (Zhang et al. 2006) also have the same effect on CD226. While TGF-β downregulates the expression CD226 expression (Jin et al. 1989). The ligands for CD226 molecular are mainly PVR/CD155 and PRR2/nectin-2/CD112 which are both highly located on the surface of various tumor cells (Bottino et al. 2003; Pende et al. 2006). CD155 is not only a polio-specific receptor and but also highly expressed in tumor tissues (Gao et al. 2017). CD112 is the receptor of herpes simplex virus, which is largely expressed in $CD34^+$ hematopoietic progenitor cells and $CD33^+CD14^+$ myelo-mononuclear cell line while limitedly expressed in megakaryocytes (Pende et al. 2006). The immunosuppressive molecule TIGIT can bind to CD112 and CD155 in competition with CD226 (Solomon and Garrido-Laguna 2018; Shibuya et al. 2003). The extracellular segment of TIGIT contains an immunoglobulin (Ig) V-like domain, and the intracellular segment contains an immunoreceptor tyrosine-based inhibitory motif (ITIM) which can mediate negative regulatory signal transduction after binding to the receptor (Dougall et al. 2017; Manes and Pober 2011). TIGIT is preferentially expressed in regulatory T cells (T_{reg}), activated and memorized T cells, and has been shown to promote the secretion of IL-10, thereby modulating DCs and effector T cells (Stein et al. 2017). CD226 promotes the adhesion of NK cells to tumor cells by binding to receptor immunoglobulin-like structure, enhancing T cells cytotoxic activity towards tumor cells (Martinet and Smyth 2015).

Taken together, the activating receptor CD226 can be detected on several immune cell surface including NK cells, monocytes, and different T lymphocyte subsets ($CD4^+$ and $CD8^+$, NKT and γδ T cells) and CD226 interacts with both Nectin2 and PVR (Tahara-Hanaoka et al. 2005; Seth et al. 2009). CD226 on NK cell and

cytotoxic $CD8^+$T lymphocytes (CTL) surface contributes to the recognition and killing of transformed and virus-infected cells. The expression of both Nectin2 and CD155 is upregulated on tumor and virus-infected cells, leading to an increased CD226-mediated recognition. Therefore, investigation on the molecular regulation mechanisms of CD226 must be carried on to potentiate new approach for anti-tumor and anti-viral immune responses.

However, little is known about the posttranslational modification related regulation of the above four immune checkpoints except some findings as follows. It is established that posttranslational modification of CD155, namely SUMOylation, regulates its intracellular localization (Zitti et al. 2017). In addition, it is reported recently that CD112 is ubiquitinated, which is responsible for its proteasomal degradation and protein retention in intracellular organelles (Molfetta et al. 2019) (Fig. 13.10). Inhibiting ubiquitination of CD112 upregulates CD112 expression on tumor cell surface, resulting in increased susceptibility of tumor cells to NK-mediated cytolysis (Molfetta et al. 2019). The study firstly demonstrates that ubiquitination has the dominant function on regulating the cell surface expression of CD112, affecting protein stability and thereby impairing tumor cell recognition by NK cells and contributing to tumor evasion. Interestingly, it is likely that the neo-synthetized CD112 is ubiquitinated before trafficking to cell surface and then the ubiquitinated CD112 either subjected to proteasome for degradation or retained intracellularly (Molfetta et al. 2019). Given that there is no more research progress recently in ubiquitination modification of CD226, CD155 and TIGIT, it remains to be determined whether or which posttranslational modification contributes to their intracellular trafficking and expression.

13.8 CD47/SIRPα

CD47 is a protein that is widely expressed in normal cells, acting as a marker of "ego", preventing the body's own cells from being swallowed by immune cells (Weiskopf 2017). While CD47 is often overexpressed in some tumor cells, allowing them to escape the immune system (Weiskopf et al. 2016). SIRPα, the receptor corresponding to CD47 is an inhibitory immune receptor expressed in myeloid cells. As an important immune checkpoint, the CD47/SIRPα plays an important role in maintaining the body's own stability and clearing tumor cells (Zhang et al. 2019). Studies have shown that blocking interactions between CD47/SIRPα can promote the eradication of tumor cells by phagocytes including macrophages and neutrophils (Legrand et al. 2011). In addition, targeting the CD47/SIRPα may also promote the function of antigen presenting cells to stimulate anti-tumor immune response mediating by adaptive T cells (Xu et al. 2019). Therefore, CD47/SIRPα is a promising innate immune checkpoint in the field of tumor therapy. A recent research (unpublished) suggested that CD47 can be ubiquitinated by Cul4A-DDB1 E3 ligase and then be targeted to proteasome for degradation. CRLs (Cullin-RING E3 ubiquitin ligases) are the largest E3 ligase family in eukaryotes, which are responsible

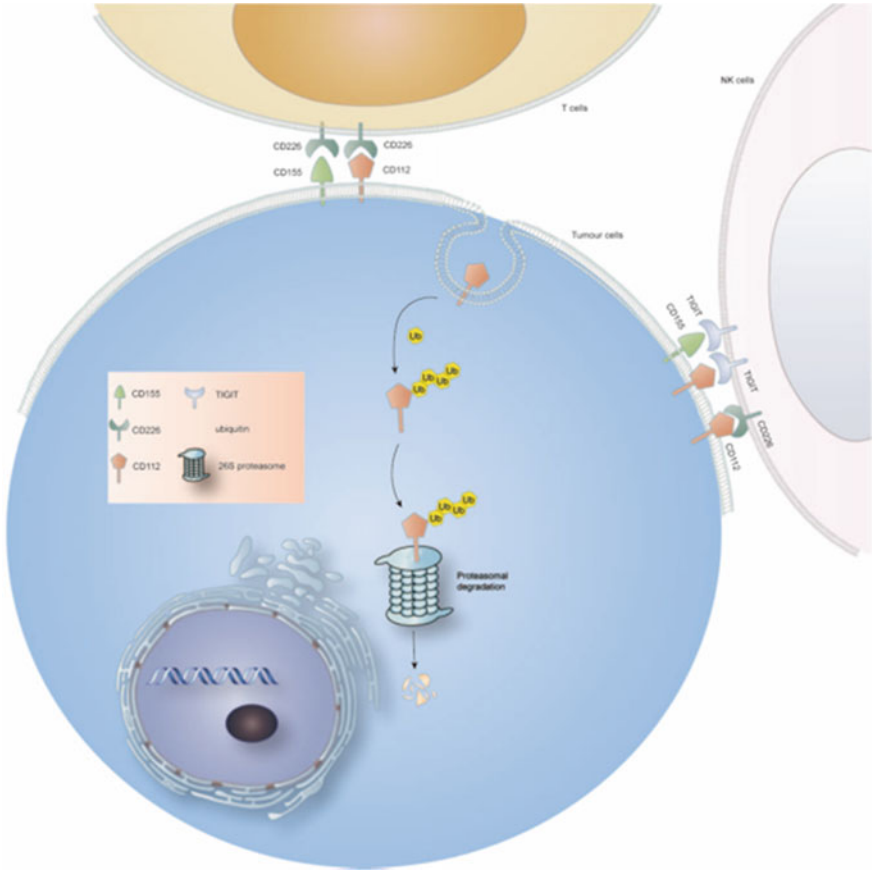

Fig. 13.10 The Ubiquitin-proteasome pathway regulates CD112 expression and impairs NK cell recognition and killing. CD112 is subjected to ubiquitination, which is responsible for CD112 proteasomal degradation and protein retention in intracellular compartments. The inhibition of ubiquitination pathway promotes upregulation of CD112 surface expression on tumor cells that results in increased target cell susceptibility to NK-mediated cytolysis

for the ubiquitination of a wide range of substrates involved in the regulation of cell cycle, signal transduction and transcriptional regulation, DNA damage response, genomic integrity, tumor suppression and embryonic development. CRL4 E3 ubiquitin ligase, as one member of CRLs family, consists of a RING finger domain protein, cullin4 (CUL4) scaffold protein and DDB1–CUL4 associated substrate receptors. The CUL4 subfamily includes two members, CUL4A and CUL4B, which share extensively sequence identity and functional redundancy. In the research of CD47 posttranslational regulation, they found that CD47 was ubiquitinated and transported to proteasome for degradation and CD47 was interacted with CUl4A and DDB1. In spite of the above preliminary results, it remains to determine of the detailed uniquitination-mediated regulatory mechanism of CD47 as well as SIRPα.

13.9 Conclusion

Ubiquitin-mediated immune regulation function is not only regulating autoimmune diseases and preventing pathogen microbial infection, but also affecting the body's anti-tumor immune response. Immune effector cells, especially $CD8^+$ cytotoxic T cells secreting IFN-γ (IFN-γ^+ CTL) and $CD4^+$ Th1 cells secreting IFN-γ (IFN-γ^+ $CD4^+$ T cells), is sharp edge of immune system against tumor cells. E3 ubiquitin ligase MDM2, as a product of oncogene, mediates ubiquitination-depenednt degradation and functional inactivation of tumor suppressor p53 and the expression of MDM2 protein is up-regulated in a variety of human cancers. However, MDM2 also has the function of regulating T cell activation independent of p53. USP15 is a deubiquitination enzyme of MDM2 that is highly expressed in a variety of melanoma and colorectal cancer cell lines. Knockdown of USP15 in malignant cells leads to spontaneous ubiquitination and proteasomal degradation of MDM2, accompanied by up-regulation of p53 and its target genes and an increase in cancer cells apoptosis.

The volume of B16 melanocytes in $Usp15^{-/-}$ mice was significantly smaller than in WT mice, while the proportion of IFN-γ^+ $CD4^+$ T cells and $CD8^+$ effector T cells in the tumor site was correspondingly higher, indicating that inhibition of USP15 not only promotes apoptosis of cancer cells, but also promotes T cell-mediated anti-tumor immunity.

Ubiquitination is a reversible reaction and the ubiquitin chains can be cut off and then be removed by deubiquitinating enzymes (DUBs). The human genome encodes about 100 kinds of DUB genes. Similar to E3 ubiquitin ligase, DUBs have substrate specificity to a certain extent and this property is affected by a number of factors. Firstly, in addition to catalytic domains, DUBs typically include domains for interactions among different proteins that allow them to bind to specific target proteins. Secondly, some DUBs have a preference for specific ubiquitin branches, such as the ubiquitin chains linked by K48 or K63, so the combination specificity of target proteins and ubiquitin connections can determine the substrates identified by DUBs. Thirdly, DUBs vary greatly in expression and subcellular localization, giving them complex functions in the body. A variety of E3 ubiquitin ligases are closely related to immune function, including lymphocyte development and activation, immune tolerance formation and innate immune function regulation. In particular, ubiquitination of K63 linkage is an important molecular mechanism for activating NF-κB signaling pathway.

Ubiquitination, as a post-translational modification of proteins, has a profound impact on the function of immune cells and the prevention and treatment of diseases by regulating protein degradation, signaling pathway transduction and other biological processes. Although the field of ubiquitination regulation of inflammatory signaling has developed rapidly, with the discovery of new members of various ubiquitin systems and the disclosure of new mechanisms, many important scientific questions remain to be answered, such as how E2 and E3 recognizes each other, how to select substrates, and how to catalyze the formation of specific types of ubiquitin chains. In addition, little is known about the mechanisms of coordination and balance

between DUB and ubiquitin ligase to allowing cells function normally. Future studies should be focus on identifying key ubiquitin-modifying enzymes that regulate immune response and revealing the molecular mechanism of their regulation, thus providing new ideas for understanding the occurrence and development of human diseases and new strategies for disease diagnosis and treatment.

Protein ubiquitination is a kind of post-translational modification of proteins that widely exists in eukaryotic cells, firstly discovered in studying the mechanism of protein degradation. A growing number of evidence indicates that ubiquitination and de-ubiquitination play a crucial regulatory role in innate and adaptive immunity by adjusting the function of the different types of cells in the immune system, affecting a variety of disease such as autoimmune diseases, infectious diseases and malignant tumor development.

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Chapter 14
Lysosome as the Black Hole for Checkpoint Molecules

Huanbin Wang, Xue Han and Jie Xu

Abstract Lysosomes, as digestive organelles full of hydrolases, have complex functions and play an important role in cellular physiological and pathological processes. In normal physiological conditions, lysosomes can sense the nutritional state and be responsible for recycling raw materials to provide nutrients, affecting cell signaling pathways and regulating cell proliferation. Lysosomes are related to many diseases and associated with metastasis and drug resistance of tumors. In recent years, much attention has been paid to the tumor immunotherapy especially immune checkpoint blockade therapy. Accumulating data suggest that lysosomes may serve as a major destruction for immune checkpoint molecules, and secretory lysosomes can temporarily store immune checkpoint proteins. Once activated, the compounds contained in secretory lysosomes are released to the surface of cell membrane rapidly. Inhibitions of lysosomes can overcome the chemoresistance of some tumors and enhance the efficacy of immunotherapy.

Keywords Lysosome · Cancer cell · Cancer therapy · Immune checkpoint

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14.1 Introduction

14.1.1 History of Lysosomes

Lysosome, a word derived from the two Greek words—'lysis' (destruction) and 'soma' (body), is a digestive vesicle filled with varies hydrolases and surrounded by lipid protein membrane. Lysosomes were first discovered by Christian de Duve in 1955, who won the Nobel Prize in Physiology or Medicine. The lipoprotein membrane organelles are produced from the Golgi and endoplasmic reticulum (ER) network. These digestive sacs involve two classes of proteins that are necessary for their functions: soluble hydrolases inside and integral lysosomal membrane proteins. Similar to most proteins, they are produced in the endoplasmic reticulum (ER) and transported to the Golgi apparatus. Before targeting the destination, proteins receive a mannose6-phosphate tag in the Golgi (Repnik et al. 2013). The vacuolar pH is kept at 4.5–5.0 because ATPase (V-ATPase) pumps protons into the lysosome to create an acidic compartment (Davidson and Vander Heiden 2017). For more than 60 hydrolases that hydrolyze macromolecules including lipases, glycosidases, phosphatases, and sulfatases (Davidson and Vander Heiden 2017), they are sensitive to the low pH, and function optimally in the acid conditions present within this organelle (Saftig et al. 2010). The Lysosomes are like waste recycling stations in cells, collecting intracellular components that have been isolated by endocytosis, phagocytosis, autophagy, etc. Then, lysosomes digest these structures into nutrition that can be reused for retaining cellular homeostasis and supporting complex biological functions. What's more, emerging evidence suggests that some of the hydrolases keep activity when released from lysosomes. This extra digestive enzyme function can affect many aspects of cell biology. Some studies suggest that the lysosomes can also motivate transcriptional programs and impact cells in hydrolases independent ways (Settembre et al. 2011, 2012, 2013).

14.1.2 Functions of Lysosome

Over the past few decades, research on the molecular regulation of lysosomes has advanced quickly, and our knowledge on lysosomes has been largely extended. It is elucidated by the discovery that lysosomes play a critical role in fundamental cellular processes, such as protein secretion, macromolecule degradation, endocytic receptor recycling, energy metabolism, and complex cell signaling (Fig. 14.1) (Appelqvist et al. 2013). In addition to digesting macromolecules into nutrients for cell activity, lysosomes are associated with cell proliferation, cancer cell death, cancer therapy, drug resistance, and immune checkpoints (Fig. 14.2).

The lysosomal membrane proteins are diverse and play important roles in cellular processes. The primary membrane proteins contain Amino acid (AA) transporters,

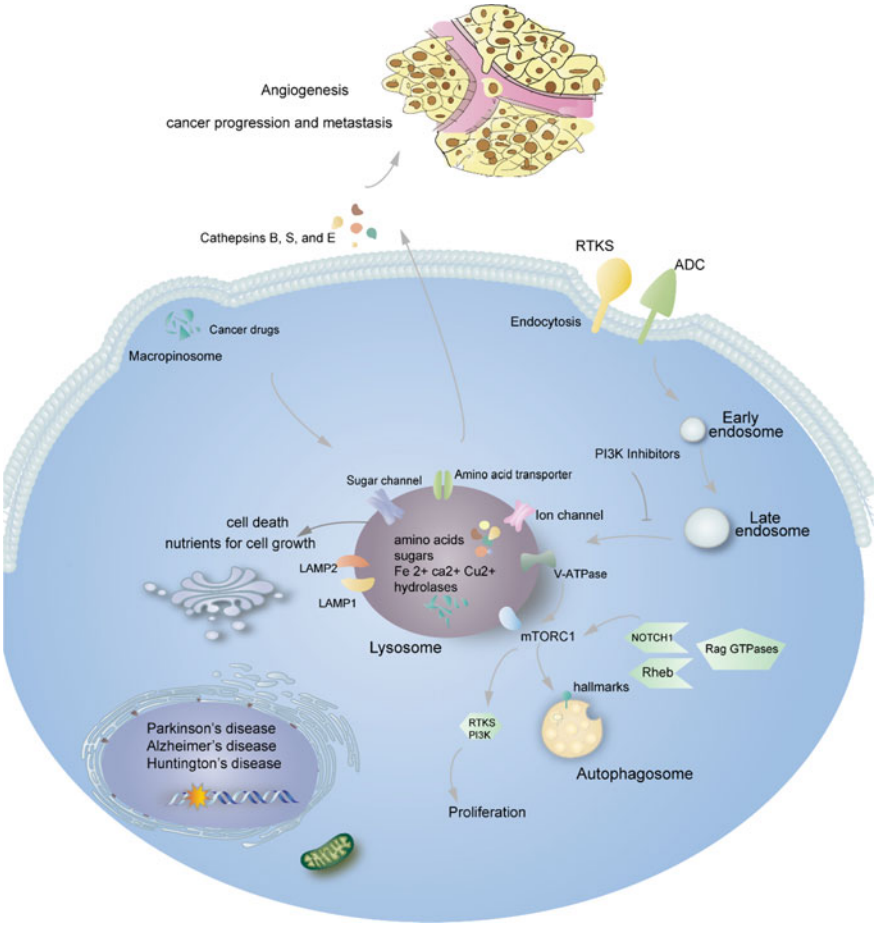

Fig. 14.1 An overview of lysosome functions. Lysosomes play a critical role in fundamental cellular processes: protein secretion, macromolecule degradation, endocytic receptor recycling, energy metabolism, and complex cell signaling. The relationships with different signaling pathways have been shown by arrows. In addition, lysosomes contribute to cancer cell proliferation, maintain genome stability, and provide energy and nutrient, involved in tumor metastasis, invasion, and angiogenesis

sugar channels, and ion channels; AA transporters include lysosomal amino acid transporter 1 homologue (LAAT-1; also known as PQLC2), SLC38A9, LYAAT-1 (also known SLC36A1), and SNAT7 (also known as SLC38A7); Sugar channels include spindlin (SPIN), and ion channels include mucolipin 1 (MCOLN1, also known as TRPML1). These membrane proteins maintain storage of amino acids, sugars, and ions, such as Cu^{+}, Fe^{+}, and Ca^{2+}, within the lysosomal lumen. V-ATPase located on the lysosome membrane is associated with lysosomal lumen acidizing and nutrient sensing. Lysosomal enzymes accumulated in lysosomal lumen digest cellular

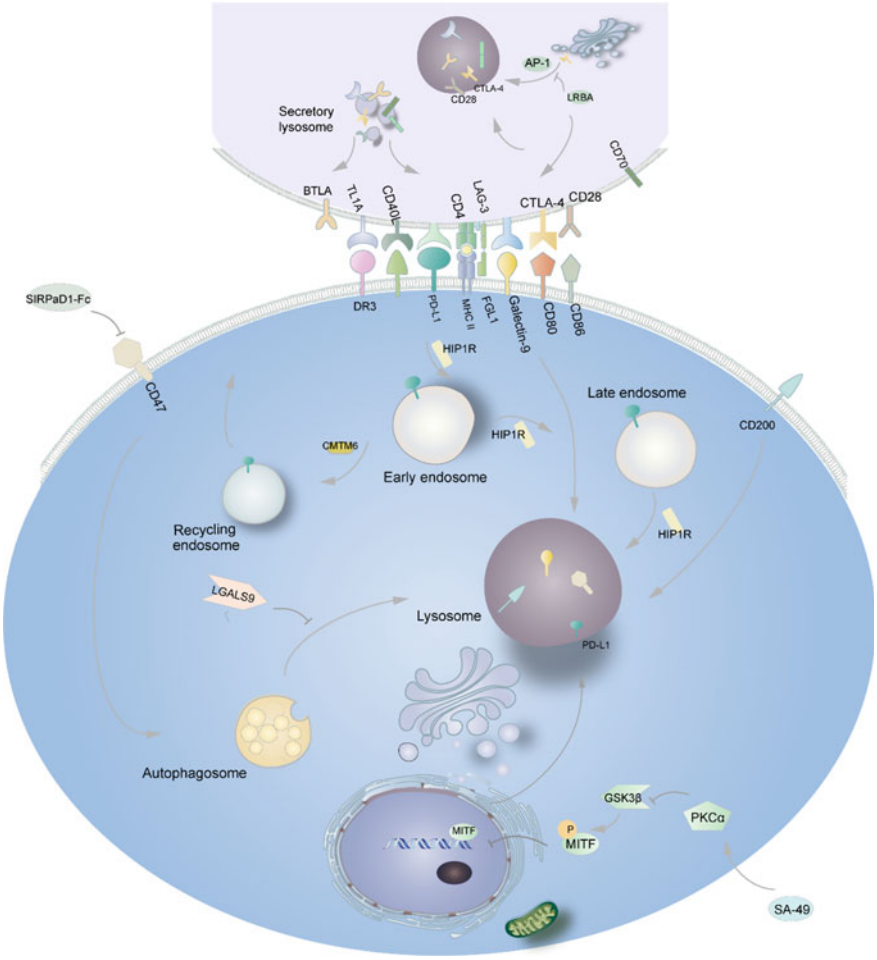

Fig. 14.2 An overview of lysosomal posttranslational regulation of immune checkpoints. Immune checkpoints are degraded through lysosomes; however, some of them are stored in secretory lysosomes temporarily and transported to the membrane immediately when the signal stimuluses are released. Their relationships with different signaling pathways have been shown by arrows

macromolecules into recycling building blocks (Lawrence and Zoncu 2019). These specific proteins make lysosome an important organelle for energy and nutrition supply in cells.

Lysosomes can influence the growth factor signaling pathway by digesting certain important proteins such as cell surface receptors and signal transduction mediators (Davidson and Vander Heiden 2017). Endocytosis is divided into clathrin-mediated endocytosis (CME) and clathrin-independent endocytosis (CIE). Receptor tyrosine kinases (RTKs) are primarily located on the cell membrane surface and regulated by cell vesicular trafficking through endocytosis and lysosome digestion. Studies

have confirmed that lysosome trafficking limits proliferative signals emanating from the RTKs (Goh and Sorkin 2013; Mellman and Yarden 2013). CIE can promote cell anchorage-independent growth and proliferation by increasing PDGFR signaling (Schmees et al. 2012); CIE also controls human umbilical vein endothelial cell proliferation by internalizing syndecan 4–driven receptor, which affects FGFR levels principally (Elfenbein 2012).

PI3K signaling can influence Rab proteins and enhance endomembrane trafficking (Wheeler et al. 2015). In the presence of PI3K inhibitors, late endosomes cannot fuse with lysosomes to form true mature lysosomes, NOTCH1 signaling is increased and activates mTORC1 (mechanistic target of rapamycin complex 1) directly in the lysosome (Mousavi et al. 2003; Luzio et al. 2003; Liu et al. 2016; Dibble and Cantley 2015; Hales et al. 2014). Lysosomes influence cell growth and proliferative signals through mTORC1 localization in an amino acid–dependent manner, and mTORC1 integrates amino acid availability with proliferative signals downstream of RTKs and PI3K (Laplante and Sabatini 2012). When cells in the contexts of amino acid or glucose starvation, nutrient deficiency can promote lysosomal biogenesis through activating the transcriptional network; lysosomes recruit and activate the master growth regulator mTORC1 protein kinase, then accelerate cell and organism growth by triggering the signal proteins in anabolic pathways (Perera and Zoncu 2016; Sancak et al. 2010).

Lysosomes also have a close relation with several human diseases. Gene mutation breaks lysosomal digestive function, leading to the accumulation of lipids, amino acids, ions, and other substances in lysosome cavity. Approximately 50 genes encoding lysosomal associated proteins, such as luminal hydrolases and membrane permeases trigger a family of diseases known as lysosomal storage disorders (Ballabio and Gieselmann 2009). Lysosome dysfunction has been related to the pathogenesis and progression of neurodegenerative diseases, for example: Parkinson's disease, Alzheimer's disease, and Huntington's disease. Besides neurodegeneration, storage of numerous abnormal metabolites in the lumen makes lysosomes lose the normal function of material recycling and communication via physical membrane contact sites (MCS) with other organelles, such as fusing with autophagosomes. The dysfunction leads to metabolic imbalance and severe growth retardation in cellular level and organismal levels (Shen and Mizushima 2014).

As the understanding of lysosomal metabolic regulatory functions deepens, recent researches revealed that lysosomes are involved in the process of cancer occurrence, progression, and treatment. Although the microenvironment is poor in cancer cells, autophagy–lysosome system enhances the function to retain the efficient nutrient scavenging and growth (Perera and Bardeesy 2015). In KRAS-driven malignancies and pancreatic ductal adenocarcinoma cells, lysosomes degrade the components recycling from extracell and intracell, or from the micropinocytosis. Varies hydrolases break down the serum albumin and macromolecules into amino acids, sugar and other nutrient to supply materials for cancer cells growth (Perera and Bardeesy 2015; Mancias et al. 2014; Commisso et al. 2013; Guo et al. 2011; Perera et al. 2015; Yang et al. 2011; Davidson et al. 2017). In addition, the autophagy–lysosome pathway has a close relationship with the hallmarks of cancer, such as evading immune

surveillance, escaping cell death pathways, and deregulating metabolism (Hanahan and Weinberg 2011). Targeting lysosomes has great therapeutic potential in cancer because lysosome triggers apoptotic and cell death pathways, as well as cytoprotective autophagy. What's more, lysosomes are pivotal in cancer drug resistance by sequestering cancer drugs in their acidic environment, resulting in a blunting of the drugs' effects (Gotink et al. 2011).

In general, lysosomes could be considered an Achilles' heel of cancer cells, therefore, several studies have devised pharmacological interventions to manipulate lysosomal nutrient sensing and autophagy in cancer cells. Lysosome has gradually become a new breakthrough in targeting tumor cell therapy. Hydroxychloroquine and its derivatives targeting the low pH of lysosome lumen are being tested in several clinical trials, and these inhibitors are effective either alone or in combination with standard chemotherapeutics (Piao and Amaravadi 2016). Antibody–drug conjugates (ADC) is a new effective treatment method that has been widely used in cancer cells in recent years. ADCs with noncleavable linkers kill antigen-positive cells following a stepwise process of antibody binding. ADCs are internalized into the endolysosomal pathway, then lysosome breaks down ADC into amino acid-linker-warhead and the catabolite exit from lysosome to trigger intracellular downstream signals in cytoplasm, leading to antigen-positive cells death (Rock et al. 2015). Lysosome plays an irreplaceable role in the process of ADCs metabolism, on this basis, additional evidence invented novel phosphate modified cathepsin B linkers which can improve aqueous solubility and enhance payload scope of ADCs (Kern et al. 2016).

14.2 Lysosomes Play a Crucial Role in Cancer Cell Biology

14.2.1 Lysosomes Contribute to Cancer Cell Proliferation, Maintaining Genome Stability, and Providing Energy and Nutrient

An important prominent feature of cancer cells is the rapid growth without restriction. Compared with normal cells, the continuous growth of cancer cells requires a large amount of energy and nutrient. Therefore, lysosome, as an important workstation for nutrient recycling, plays a very important role in supporting the rapid proliferation of cancer cells. Lysosomes provide materials for macromolecular synthesis, and autophagy also plays a critical role in promoting progression of some tumors. Emerging evidence suggests that lysosomes can regulate cell transcription and influence cell biological behavior independently of the release of hydrolytic enzymes (Settembre et al. 2011, 2012, 2013). The quantity of lysosomes and the activity of hydrolases between the cancer cells and normal tissues are obviously different, and the function of lysosomes is elevated in many tumor cells. In fact, the phenomenon that lysosome changing in cancer has been found for decades, which might be a hint for cancer therapies development. In addition to infinite proliferation, cancer cells have specific

hallmarks related to disorder of lysosomal functions (Hanahan and Weinberg 2011; Levine and Kroemer 2008; Kimmelman 2011; White 2015; Kirkegaard and Jaattela 2009).

Lysosome plays a key role in the supply of nutrients and energy to cancer cells. Lysosomes can collect extracellular macromolecules that enter cells through endocytosis, phagocytosis, and micropinocytosis, as well as intracellular materials scavenged through autophagy–lysosome fusing. All the endogenous or exogenous macromolecules are degraded by hydrolases and transformed into lipid, amino acids, and other energy materials in lysosomes. Lysosomes provide nutrients as metabolic precursors for the synthesis of new cell mass. The process of taking in macromolecules, such as endocytosis, phagocytosis, micropinocytosis, and autophagy, is much more active in cancer cells compared with normal cells. It is reported that they are precisely regulated by growth factor signaling and mTORC1 pathway (Commisso et al. 2013; Haigler et al. 1979; Mosesson et al. 2008). Autophagy is regulated by a number of genes. Mice lacking Atg5 or Atg7 die from perinatal amino acid starvation, and this study further underscores the essential function of autophagy–lysosome pathways for catabolism and providing nutrients during metabolic stress (Kuma et al. 2004).

A core function of lysosome is to sense nutrients in cells. Nutrient sensing mechanisms are subtle and complex. Although cytoplasmic proteins are reported to sense some amino acids (Wang 2015), more amino acids sensing is thought to occur in lysosomes involving the mTORC1 proteins. Researchers have demonstrated that mTORC1 inhibition is necessary for autophagy and macro-pinosome to provide amino acids and support growth of some tumors (Palm et al. 2015), because the activation of mTORC1 suppresses autophagy and macropinocytosis. Rag GTPases are mediated by amino acid, and mTORC1 complex localize in lysosomal membranes when Rag GTPases are recruited. Rheb protein and Rag GTPases promote the activation of mTORC1, on the other hand, mTORC1 activation is also related to the mechanisms of lysosomal V-ATPase activity. Glutamine, leucine, and arginine are proposed to be sensed by SLC38A9.1 and influence the localization of mTORC1 (Wang 2015; Rebsamen et al. 2015).

14.2.2 Lysosomes Are Involved in Tumor Metastasis, Invasion, and Angiogenesis

Besides the basic functions of degrading macromolecules and providing nutrition for cell mass synthesis, lysosomes are associated with cancer progression and metastasis. Cathepsin proteases contain three subgroups: cysteine cathepsins, serine cathepsins, and aspartic cathepsins within the lysosome lumen. Emerging data suggests that cathepsins B, S, and E are all concerned with cancer progression and metastasis in various cancer types (Withana et al. 2012; Small et al. 2013; Keliher et al. 2013). Lysosomal membrane proteins are crucial for retaining low-pH in lysosome lumen, and many research groups focusing on the lysosomal membrane proteins suggest that

membrane proteins have indispensable functions and thus may also have potential to be targets for cancer therapeutics. Lysosome-associated membrane protein 1 (LAMP-1) and LAMP-2 are the most abundant proteins on membrane. According to the reports that LAMP-1 is abundant on the cell surface of highly metastatic tumor cells, especially in metastatic colon cancer cells, which suggests that lysosomal membrane protein is important in cell–cell adhesion and migration (Furuta et al. 2001).

Compared with normal cells, lysosomes reveal obvious changes in organelle volume, compartments composition, cellular distribution, and hydrolases activity in cancer cells (Nishimura et al. 1998; Gocheva et al. 2006). Lysosomes in cancer cells are more sensitive to cell death due to weaker lysosomal membranes than normal cells. All the changes in lysosomal functions accelerate invasive growth, angiogenesis, and drug resistance (Fehrenbacher and Jaattela 2005). In the initiation of angiogenesis, lysosomal sacs are secreted into extracellular interstitial through exocytosis, and the vacuolar granules contain lysosomal cathepsins which can degrade basement membrane components at physiological PH. Lysosomes influence endothelial cell migration. Rab4a and Rab11a gene deletion lead to a dysfunction of endosome-to-plasma membrane, and the deletions inhibit the migration of endothelial cells by preventing the recycling of VEGFR2 (Jopling et al. 2014). A further understanding of lysosomal function in cancer cells is essential to detect how treatment with CQ, HCQ, and other lysosomal drugs will affect both cancer and noncancer cells and how to impact the efficacy of these drugs to treat cancer.

14.2.3 Lysosomes Influence Tumor Microenvironment

V-ATPase proteins located in lysosomal membrane possess vital functions of creating the acidic pH within lysosome lumen. Emerging data suggest that V-ATPase proteins regulate cellular endocytosis and disturb the tumor microenvironment by proton extrusion into the extracellular matrix (Dettmer et al. 2006). The acid microenvironment in the extracellular medium or the interstitial tissue promotes the activity of hydrolases, and hydrolases degrade the normal construction more easily. Inhibitors of V-ATPase proteins reduce cancer cells metastasis and prevent cancer cells from expanding and developing by impairing the acidic microenvironment around the tumor (Fais et al. 2007). V-ATPase proteins have been reported to be associated with mTORC1 activation, autophagy, and E2F1-mediated lysosomal trafficking in tumor malignancy. Cancer cells pharmacological treatment targeting V-ATPase proteins is crucial for restricting tumor metastasis, especially for the tumor high expression of E2F1 (Meo-Evoli et al. 2015).

14.3 Lysosomes and Cell Immunity

Innate immunity and acquired immunity are essential in the development of tumor. It is found that lysosomes and autophagy influence the immune modulation. Lysosomes play an important role in the processing of tumor antigens and antigen presentation (Mah and Ryan 2012; Munz 2010). Hydrolases released by lysosomes (also termed lytic granules) impact the function of innate and adaptive immune system in the extracellular matrix. Clotting factors and chemo attractants that affect immune responses are regulated by secretory lysosomes (Blott and Griffiths 2002). The process of lysosomal secretion is controlled by many molecules in immune cells and melanocytes, dysfunction of these proteins leads to many diseases such as Che´diak–Higashi syndrome (Barbosa et al. 1996; Nagle et al. 1996), Griscelli syndrome type 2 (Klein et al. 1994; Menasche et al. 2000), and Hermansky–Pudlak syndrome type 2 (Dell'Angelica et al. 1999; Jung et al. 2006). Human diseases reveal the necessary biological function of secretory lysosomes in both pigmentation and immunity.

Studies have implicated that molecules related to lysosomal trafficking control immune system. The endosomal adaptor protein p14 plays a crucial role in endosomal biogenesis. P14 is characterized as a modulator to confine mitogen-activated protein kinase (MAPK) signaling in late endosomes previously. The distribution of late endosomes was severely perturbed in p14 deficient cells, this protein is critical for the function of neutrophils, B cells, cytotoxic T-cells, and melanocytes (Bohn et al. 2007). As previously mentioned, the mTORC1 protein kinases mediate nutrient sensing. These components play important roles in cellular metabolism during an immune response, and emerging data suggest that the localization of mTOR during asymmetric T-cell division can have a significant impact on cell fate and influence T-cell differentiation (Puleston 2014).

A role of autophagy function in immune response to cancer has been studied in detail. Dysfunction of autophagy leads to restraining of NF-Kb pathways, promoting cell tumorigenesis and inducing cell death. Autophagy deficiency results in p62 accumulation, and p62 amassing with subsequent ROS production downstream facilitate an oxidative environment that inhibits local dendritic cell activation and impairs immune responses to resistant tumor (Lau et al. 2010; Saitoh and Akira 2010).

Referring to DC functions and the development of novel approaches for immune modulation with nanoparticles, recent data indicated that although supposedly inert PS nanoparticles did not influence DC activation or CD4+ T-cell stimulating capacity, 20 nm (but not 1,000 nm) PS particles interfere lysosomal compartment and cause dampening of antigen degradation. These studies remind that lysosomes as well as the related components are closely associated with immune system moderation (Seydoux et al. 2014).

14.4 Lysosomal Posttranslational Regulation of Immune Checkpoints

14.4.1 PD1/PD-L1 PD-L2

Cancer immune therapies, especially immune checkpoint blockade (ICB) therapy (Lawrence and Zoncu 2019) and chimeric antigen receptor (CAR)-expressing T-cells (Owada et al. 2010), have been in the spotlight of cancer research. The ICB therapy targeting programmed cell death 1 (PD-1) and its ligand programmed death ligand 1 (PD-L1; also known as B7-H1 and CD274) have exhibited substantial clinical benefits in different cancers (Daniel et al. 1998). The expression of PD-L1, a type I transmembrane protein containing two extracellular immunoglobulin (Ig)-like domains and a short cytoplasmic domain, protects cancer cells from T-cell-mediated immune surveillance (Zerdes et al. 2018). The interaction between PD-L1 on tumor cells and the PD-1 receptor on T-cells leads to suppression on the tumor-killing activity of T-cells and secretion of inflammatory cytokines, representing a pivotal mechanism for immune evasion of tumor cells (Zhang et al. 2017).

Over the past few years, researches about the posttranslational regulation of PD1/PD-L1 have advanced quickly. Surface membrane PD-L1 molecules stored in both degradative and unconventional secretory lysosomes. PD-L1 maintains continuous internalization, proteolysis, and recirculation to the membrane through lysosomal traffic. CMTM6 specifically protects PD-L1 from being broken down by lysosomes through transporting the molecules into recycling endosomes. CMTM6 participates in the recycle between plasma membrane and intracellular endosomes without disturbing antigen presentation via MHC class (Burr et al. 2017). On the contrary, SA-49 increased the translocation of PD-L1 to lysosome for proteolysis. SA-49 activated PKCα then inhibited the activation of GSK3β and induced MITF nuclear translocation. Biogenesis of lysosome is enhanced and the degradation of PD-L1 is trigged in cancer cells (Zhang et al. 2019). HIP1R has been reported as a natural regulator of PD-L1 lysosomal degradation, and the functions of HIP1R relied on two sequence stretches one involved in the interaction with PD-L1 and for the sorting to the lysosome. On the basis of the 'binding–sorting' model derived from the molecular roles of HIP1R, PD-LYSO is designed as a peptide for targeting PD-L1 to lysosomes in cancer cells (Wang et al. 2019). The EBV latency III program elevates the number of PD-L1 in surface membrane by damping actin export and promoting PD-L1 to be contained in secretory lysosomes. C-Myc influenced the immunogenicity of transformed B cells by controlling the export of secretory lysosomes to plasma membrane (Durand-Panteix et al. 2012).

14.4.2 CD28 CTLA-4/CD80 CD86

CD28 and cytolytic T lymphocyte-associated Ag-4 (CTLA-4) binding to common ligands CD80 and CD86 are expressed differently on T-cells (Linsley 1995; Thompson 1995; Bluestone 1995). They are different in the distribution of expression: CD28 is expressed on the surface of inactivated and activated T-cells; while CTLA-4 is expressed only on activated T-cells. It has been reported that CD28 enhances the T-cell response while CTLA-4 negatively regulates the activation process (Brunet et al. 1987; Lindsten et al. 1993; Schwartz 1992; Jenkins 1994; Thompson and Allison 1997; Walunas et al. 1994; Krummel and Allison 1995). The expression of CTLA-4 is associated with lysosomal degradation. The increasing CTLA-4 synthesis results in an increase of CTLA-4/AP-1 binding and a subsequent increasing accumulation of CTLA-4 in the lysosomal compartments. This phenomenon supports that AP-1 appears to play a crucial role in transportation of excess receptor from the Golgi to the lysosomal compartments for degradation (Schneider et al. 1999). Studies suggest that LRBA competed with AP-1 for binding motif sites to prevent CTLA-4 from being transported to lysosomes for proteolysis. LRBA plays a pivotal immunoregulatory role by blocking the shuttling of CTLA-4 from the Golgi to the lysosomal compartments (Lo 2015). Secretory lysosomes are reported to contain CTLA-4. Upon TCR stimulation, the surface membrane of CTLA-4 is upregulated by releasing the secrete lysosomes containing CTLA-4 molecules (Iida et al. 2000). CD28 can increase lymphokine gene transcription, mRNA stability, and the longevity of the T-cell response by binding CD80/CD86 on presenting cells, preventing nonresponsiveness anergy to antigenic challenge (Noel et al. 1996). The binding can rescue T-cells from TCR-induced apoptosis (Boise et al. 1995; Radvanyi et al. 1996). Studies also suggest that CD28 can be endocytosed and degraded by lysosomes. Therefore, chloroquine or hydroxychloroquine and other inexpensive drugs that inhibit lysosomal degradation may merit investigation as therapies for tumor immunotherapy.

14.4.3 TIM-3/Galectin-9

Tim-3 is considered as a biomarker of dysfunctional pDCs and may negatively regulate IFN-a. Intracellular Tim-3 distributed in the cytoplasm before activation. Once activated, Tim-3 accumulated at the surface membrane and interfered the levels of TLR9 and IRF7. Tim-3 is related to lysosomal degradation by disturbing the TLR signaling and the recruitment of IRF7 and p85 (Schwartz et al. 2017). Exogenous galectin-9 is reported to induce nonapoptotic death in PC-3 cells. Cell death is triggered by the atypical ubiquitination and accumulation of ubiquitinated proteins in lysosomes. Data suggested that galectin-9 is associated with endocytosis and destined to the lysosomal compartment in PC-3 cells (Itoh et al. 2019). LGALS9 acts as a lysosomal inhibitor that restrains autophagosome-lysosome fusion, leading to autophagosome accumulation, excessive lysosomal swelling and cell death in

KRAS mutant CRC. Recombinant LGALS9/Galectin-9 (rLGALS9) shows sensitive therapeutic effect in this CRC therapy (Wiersma et al. 2015).

14.4.4 CD70

CD70 is a TNF-related transmembrane molecule expressed by mature dendritic cells (DCs), which present antigens to T-cells via major histocompatibility complex (MHC) molecules. CD70 is transported to MHC class II compartments (MIICs) and reveals co-localization with MHC class II molecules in late endosomal vesicles. When a DC cell contacts with an antigen-specific CD4+ T-cell, MIICs containing MHC class II and CD70 are transported to the immune synapse. T-cell activation via the antigen receptor and CD70-mediated co-stimulation are synchronized, the transportation of MHC class II to MIICs is dependent on the chaperone known as invariant chain (Ii); CD70 was also found to be transported to late endosomes and/or lysosomes directing by li in an MHC class-II-independent complex. MHC class II and CD70 are conveyed from Golgi to MIICs and stored in secretory lysosomes and coordinated their delivery to CD4+ T-cells when T-cells are activated (Zwart et al. 2010).

14.4.5 CD200

Studies suggested that CD200–CD200R is associated with the maintenance of microglia in a relatively quiescent state under resting conditions (Lyons et al. 2007). Besides, the initial observations found that it reduces markers of activation and inflammatory cytokine production, CD200 ligand-receptor interaction has more complex effects on microglial activation by promoting the lysosomal activity synchronously (Lyons et al. 2017).

14.4.6 CD47

Some cancers are treated with antitumor agents. Specific antibodies and fusion proteins work by blocking the CD47–SIRPa signaling, such as a CD47 targeting fusion protein SIRPaD1-Fc. The CD47 antibody fusion protein regulates macrophages by enhancing the phagocytic and cytotoxic activities. SIRPaD1-Fc can also trigger the improvement of the autophagic flux, eliciting formation and accumulation of autophagosomes, fusion of autophagosomes with lysosomes, and degradation of autophagosomes in lysosomes. Therefore, targeting both CD47 and autophagy in NSCLC xenograft models simultaneously reveals higher anticancer ability with

recruitment of macrophages, activated caspase-3, and overproduction of ROS in tumor cells (Zhang et al. 2017).

14.4.7 CD40

Some data revealed that secretory vesicles can be divided into at least two categories: specialized secretory vesicles that lack lysosomal markers (e.g., RANTES storage vesicles and CXCR3/1-storing granules) (Catalfamo et al. 2004; Gasser et al. 2006) and "secretory lysosomes" (SL), which naive T-cells do not possess, but activated T-cells do (Blott and Griffiths 2002). As explained earlier, CTLA-4 (CD152) is contained in SLs, upon being stimulated by antigen recognition, CTLA-4 is transported to the contact site between T-cells and APCs (Linsley et al. 1996); Fas ligand is a member of the TNF family, also stored in SLs in CD4+ and CD8+ activated T-cells. Upon T-cell receptor (TCR) signaling activated, Fas ligand is released to the cell surface (Bossi and Griffiths 1999). Another tumor necrosis factor (TNF) family member CD40 ligand (CD40L; CD154), is a transmembrane protein expressed on the surface of activated CD4+ T-cells. As an essential cytokine for both humoral immunity and cellular immunity, CD40L can activate the function of B cells, dendritic cells (DCs), and macrophages (van Kooten and Banchereau 2000). It has been established that preformed CD40L is stored in SLs and colocalizes more strongly with FasL than with CTLA-4 (Koguchi et al. 2007).

14.4.8 TL1A/DR3

DR3 is one of the least characterized cell death receptors and multiple potential ligands have been suggested for DR3 including Tweak and TNF-like1a (TL1A, also known as TNFSF15 and VEGI). TL1A has a function of being a T-cell co-stimulator, which can facilitate T-cell proliferation and proinflammatory cytokine production when binds to DR3 as its cognate receptor (Migone et al. 2002; Meylan et al. 2008; Marsters et al. 1998). TL1A is proposed to be stored in the secret lysosomes. Antimitotic chemotherapeutic agents induce apoptosis requiring D3R, mitotic arrested by these agents induces lysosome-dependent secretion of the DR3 ligand, TL1A. Binding of TL1A with DR3 stimulates the formation of Fas-associated death domain (FADD) containing and caspase-8-containing death-inducing signaling complex (DISC), which subsequently activates apoptosis in cells that express DR3 (Qi et al. 2018). Lysosomes participate in the regulation by controling the TL1A molecule.

14.4.9 BTLA

Coinhibitory molecules play pivotal roles in regulating immune responses by inhibiting proliferation and cytokine production of T-cells in vitro and in vivo. Similar to CTLA-4, BTLA is identified as a third coinhibitory molecule, distributing on the lymphoid-specific cell surface (Watanabe et al. 2003). Engagement of BTLA and HVEM impairs the T-cell functions (Watts 2005; Sedy et al. 2005; Gonzalez et al. 2005; Murphy et al. 2006) Emerging data advanced that BTLA is localized mainly in the Golgi apparatus and lysosomes, and has less expression on the cell surface. In CD4 T-cells, BTLA also stores in lysosomes. Lysosomes are considered as conveyers to carry proteins to the cell surface (Blott and Griffiths 2002). Secretory lysosomes existing in most of hematopoietic cells affect not only secretory proteins, such as perforin, granzyme A, and histamine, but also transmembrane proteins which have specific effector functions (Stinchcombe and Griffiths 1999).

14.4.10 MHC Class II/ LAG-3/CD4

MHC class II-peptide complexes can be produced by late endosomes and lysosomes. During the DC differentiation, the complexes are critically regulated to coordinate antigen acquisition and inflammatory stimuli with formation of TCR ligands (Inaba et al. 2000). CD4 binds to MHC class II molecules and facilitates T-cell activation. Oppositely, the CD4-related transmembrane protein LAG-3 (lymphocyte activation gene-3, CD223) binds to the same ligand but restrains T-cell proliferation. It is reported that the vast majority of CD4 localize on the cell surface, while nearly half of the cellular content of LAG-3 is retained in intracellular compartments under the resting state, then degraded within the lysosomal compartments. Upon stimulation, the majority of LAG-3 translocates rapidly to the cell surface without degradation in the lysosomes. Recent results clearly indicate that LAG-3 trafficking from lysosomal compartments to the cell surface is dependent on the cytoplasmic domain through protein kinase C signaling in activated T-cells. Comparing to CD4 goes through early/recycling endosomes and secretory lysosomes, LAG-3 contained in the secrete lysosomes closing to microtubule organizing center and recycling endosomes may facilitate its rapid translocation to the cell surface during T-cell activation and help to mitigate T-cell activation (Bae et al. 2014; Woo et al. 2010). Lag-3 is regulated by lysosomes and recent research indicated that FGL1 is an MHC Class II-Independent major high-affinity ligand of LAG-3. As an inhibiting ligand for LAG-3, FGL1 reveals a new mechanism of immune evasion (Wang 2019).

14.5 Targeting Lysosomes for Tumor Treatment and Cancer Immunotherapy

14.5.1 Inhibitors of Lysosomes

Lysosomes play a pivotal role in numerous intracellular endosomal trafficking pathways. Chemotherapeutics such as sunitinib are segregated by lysosomal sequestration leading to drug resistance. Studies proposed that lysosomes are critical for the mechanism of drug activation, and inhibitions of lysosomal function are necessary for enhancing the curative effect of the chemotherapeutics (Gotink et al. 2011). Further research is needed to figure out the difference of lysosomal transport channels between the normal and cancer settings. A full understanding of lysosomes will help to prevent the development of chemotherapy resistance due to lysosome sequestration, and an in-depth study of lysosomal consequences contained with chemotherapeutics suggests more effective lysosomal targeting methods (Xu and Ren 2015).

Currently, there are five major categories of agents that target the lysosome in cancer: end stage of autophagy, vacuolar H^+ -ATPase, ASM, lysosomal hydrolases, and HPS70. The majority of these agents are still being investigated in the preclinical research process except hydroxychloroquine (HCQ), which is widely tested in many clinical trials combining other anticancer therapies. HCQ can displace other combined drugs from the lysosome to the cytosol or nucleus, improving their intracellular bioavailability. However, many cancer drugs are weak because of accumulation in lysosomes without the effect of autophagy inhibition (Fu et al. 2014). The autophagy–lysosome pathway is closely related to not only deregulating metabolism but also the hallmarks of cancer including escaping cell death pathways and evading immune surveillance.

Bafilomycin A1 is used to block distal autophagic flux at low nanomolar concentrations as a prototypical V-ATPase inhibitor; ASM cleaves sphingomyelin to ceramide and sphingosine in lysosomes upon cellular stress. The activity of ASM is usually lower in cancer cells than in normal cells. Therefore, cancer cells show high susceptibility to ASM targeting therapy, leading to higher sphingomyelin levels (Smith and Schuchman 2008). ASM modulators are cationic amphiphilic drugs, such as clorpromazine, CQ, and amiodarone. These modulators reduce AMS activity and lead to higher levels of sphingomyelin, then interfere the normal function of the lysosomal membrane and induce tumor cell death (Saftig and Sandhoff 2013). Cathepsins localized inside are associated with tumor progression and metastasis, and the release of cathepsin from intracell to outside is characteristic of cancer oncogenic. Cathepsin inhibitors have been developed for cancer therapy, such as inhibitors of cathepsins D, B, K, E, S, and L (Maynadier et al. 2013; Kos et al. 2014; Duong et al. 2014; Lankelma et al. 2010; Tsai et al. 2014). Cathepsins inhibitors are considered as effective targets for cancer drugs, but still not in the clinical trials stage. HSP70 is referred to as a heat-inducible protein, an evolutionarily conserved chaperone protein, it plays a significant role in promoting cancer cells by maintaining lysosome

integrity and promoting the metastasis of cancer cells, increasing the survival of cancer cells. High expression of HSP70 is common in many cancers and is closely related to the prognosis of cancer patients. PES is a HSP70 modulator blocking the interactions between HSP70 and p53, triggering apoptosis by disturbing autophagy and accumulating indigestible autophagosomes (Wu et al. 2010; Granato et al. 2013).

14.5.2 Lysosomal Inhibitor or Combining with Other Drugs for Cancer Therapy

It has been proposed that lysosomal membrane permeabilization (LMP) is a complex process with different outcomes depending on the levels of permeabilization: limited lysosomal enzyme release can result in lysosomal cell death and apoptosis, whereas extensive lysosomal enzyme release can result in necrosis. This type of necrotic about cell death and lysosomal cell death are of great importance in cancer therapy, because the apoptotic machinery of cancer cells is commonly mutated, leading to protection from cell death by the classical apoptotic pathways. Many stimuli can cause the release of cathepsins from the lysosomal lumen into the cytosol to induce LMP. Reactive oxygen species (ROS) may be the most commonly encountered within the tumor microenvironment and activate phospholipases A2 causing alteration of lysosome membranes by degrading membrane phospholipids (Kurz et al. 2008). Accumulation of sphingosine and ceramide converted from sphingomyelin in lysosomes can also induce LMP (Zeidan and Hannun 2010; LeGendre et al. 2015). However, the accurate quantification of LMP is both biologically and technically challenging. In contrast, there are some molecules known to protect lysosomal membranes against permeabilization, such as the heat shock protein 70 (HSP70), LAMP-1, and LAMP-2 (Cesen et al. 2012), which modulate many lysosomal proteins. HSP70 is expressed in many tumor types and can specifically bind to bis monoacylglycero phosphate in the lysosome lumen. This binding activates ASM to break down the lipid sphingomyelin. It has been suggested that increasing ASM activity supports lysosomal integrity, thus inhibition of ASM in cancer cells would increase lysosomal LMP and lead to cell death (Saftig and Sandhoff 2013; Cuervo and Wong 2014). The lysosomal inhibitors can profoundly affect tumor cells and provide effective strategies to cancer therapy including lysosome-targeted therapies and combined application to avoid and overcome drug resistance of tumor. Lysosomes not only directly affect the proliferation and development of cancer cells, but also play an important role in the regulation of immune checkpoints. Therefore, targeting regulators of lysosomes can fuse the drug therapy at immune checkpoints, increase the anticancer effect and reduce drug resistance.

14.6 Conclusion

In recent years, cancer incidence and mortality rate remain high worldwide. According to the 2018 research statistics, there were an estimated 18.1 million new cancer cases and 9.6 million cancer deaths in 2018 (Bray et al. 2018), and the number of cancer patients in the United States will reach 20 million by 2030 (Miller 2019). Tumor treatments include surgery, radiation therapy, chemotherapy, and immunotherapy. Due to the clinical effect being remarkable, much attention has been paid to the checkpoint blockade therapy. The immune-related research of checkpoint pathways has become a hot spot. Checkpoint blocking treatment of drug development for cancer treatment provides new methods. However, some problems also exist, such as drug resistance, low efficiency, and lack of effective biomarkers as a guide. The exploration of the intricate regulation mechanism of the immune checkpoint proteins has important clinical significance.

Lysosome is not only an organelle filled with acid hydrolysis enzyme, it is essential for cell biology. Lysosomes can feel nutrient pressure regulating cell proliferation, not only associated with the diseases of nervous system, also with the proliferation of cancer cells, nutrition deficiency, drug resistance, tumor microenvironment, and so on (Mah and Ryan 2012; Munz 2010). Numerous of lysosome inhibitors are already in clinical trials to treat diseases including cancer.

Lysosomes can reduce the expression of immune checkpoint proteins and impair the immunosuppressive pathway signal through digestion and degradation. Secreted lysosomes can accelerate the immune checkpoint protein translocation to the plasma membrane in short order and enhance the function. Focusing on the specific exploration of these regulatory mechanisms is conducive to better finding solutions to chemotherapy and immunotherapy resistance.

Perhaps a combination of lysosomal inhibitors with chemotherapy or immune checkpoint inhibitors would yield better cancer treatment results. Therefore, the regulation of lysosomal pathway at immune checkpoints deserves further and more detailed study.

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Chapter 15
Phosphorylation: A Fast Switch For Checkpoint Signaling

Yiting Wang, Ping Wang and Jie Xu

Abstract Checkpoint signaling involves a variety of upstream and downstream factors that participate in the regulation of checkpoint expression, activation, and degradation. During the process, phosphorylation plays a critical role. Phosphorylation is one of the most well-documented post-translational modifications of proteins. Of note, the importance of phosphorylation has been emphasized in aspects of cell activities, including proliferation, metabolism, and differentiation. Here we summarize how phosphorylation of specific molecules affects the immune activities with preference in tumor immunity. Of course, immune checkpoints are given extra attention in this book. There are many common pathways that are involved in signaling of different checkpoints. Some of them are integrated and presented as common activities in the early part of this chapter, especially those associated with PD-1/PD-L1 and CTLA-4, because investigations concerning them are particularly abundant and variant. Their distinct regulation is supplementarily discussed in their respective section. As for checkpoints that are so far not well explored, their related phosphorylation modulations are listed separately in the later part. We hope to provide a clear and systematic view of the phosphorylation-modulated immune signaling.

Keywords Phosphorylation · Tyrosine kinase · PTM · Cancer immunity

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15.1 Introduction

15.1.1 Phosphorylation as a Modification

As one of the most well-explored post-translational modifications (PTM), phosphorylation orchestrates a wide range of cellular activities including cell growth, differentiation, and apoptosis (Singh et al. 2017). The history of phosphorylation can be traced back to 1906, when Phoebus Levene reported the discovery of phosphorylated vitellin (Levene 1906). The process of transferring phosphate from ATP to proteins, enzymatic phosphorylation, was discovered by Burnett and Kennedy in 1954 (Burnett and Kennedy 1954). Fischer and Krebs got Nobel Prize in 1955 for their contribution in revealing phosphorylation as a reversible regulatory mechanism of great importance (Fischer and Krebs 1955). Phosphoproteins later became a popular field of research. Around 30% of all proteins in eukaryotes can be phosphorylated and thousands of distinct phosphorylation sites were discovered (Cohen 2000). With more discoveries found in its involvement in almost all cellular processes, phosphorylation got more and more attention (Cohen 2002). The balance of phosphorylation and dephosphorylation was described as yin and yang, intermediated by protein kinases and phosphatases (Hunter 1995). Protein kinases and phosphatases, encoded by 2–5% human genome, were estimated to be the largest protein family in cells (Ubersax and Ferrell 2007). The process of protein phosphorylation often occurs on certain amino acids, namely, threonine, serine, and tyrosine (Humphrey et al. 2015). Among these three sites, tyrosine kinases are the most important group in regulating cell growth and differentiation, while the serine/threonine kinases constitute a larger group.

Proteins can be phosphorylated at multiple sites (Loughrey Chen et al. 2002) or a single site (Dajani et al. 2001), therefore leading to conformational changes and subsequent phosphorylation events, including affinity changes for other proteins or degradation by ubiquitin–proteasome complex. Many proteins can be phosphorylated by different kinases. Similarly, kinases may phosphorylate multiple proteins. The complex network of enzymatic phosphorylation plays a crucial role in cell cascade response to stimulations. While proteomic studies revealed increasing importance of phosphorylation, especially on key regulatory proteins (Humphrey et al. 2015), relation and cooperation between phosphorylation and other PTMs such as glycosylation and ubiquitination attract more and more attention. For example, function of beta3 integrin family in aggregating platelets to form clots is based on phosphorylation and O-linked N-acetylglucosamine (O-GlcNAc) modification and on Ser, Thr, or Tyr and their interplay in the cytoplasmic domain of the beta3 subunit (Ahmad et al. 2006). In addition, phosphorylation on a six-amino acid sequence found in both beta-catenin and the NF-kappaB regulatory protein IkappaBalpha, targets both proteins for ubiquitination (Orford et al. 1997). These interplays between PTMs adds more complexity to the understanding of phosphorylation, not to mention abnormal phosphorylation of proteins causes protein alteration in structure and in function, leading to disease conditions (Ingram 1995; Samelson et al. 1986; Fairbanks et al.

1983). A thorough insight into phosphorylation is yet to be obtained; more efforts should be and will be made in the future.

15.1.2 Implications of Phosphorylation in Cancer

The past decades have witnessed explosive discovery in cancer, the most diversified and notorious human disease (Hanahan and Weinberg 2000). Among them are the deranged phosphorylation and dephosphorylation of a wide range of proteins, altering cancer cells' growth, proliferation and metabolism vastly (Hanahan and Weinberg 2000; Appella and Anderson 2001). The balance of activation and inactivation of many key kinases is delicately maintained by phosphorylation, and deregulations of these processes result in interrupted signal transduction and metabolism (Radivojac et al. 2008). Some mutations of phosphorylation sites or phosphatases are so far acknowledged as the cause and the result of cancer (Lim 2005). The diversified manifestation of cancer and cancer cell reprogramming may be directly related to PTMs, including phosphorylation which regulates proteins rapidly and reversibly. Growing studies in this field may provide potential targets for treatments (Gorini et al. 2014). Notably, protein kinases are the most promising targets for blocking phosphorylation and treat cancer. A new field of kinome study, which focuses on demonstrating the complex network of intracellular kinases, has aroused recently (Tuettenberg et al. 2016). Target-based drugs that block protein kinases activities have been developed like ATP analogs (Fischer et al. 2003) and monoclonal antibodies. Considering the crosstalk among different kinases and pathways, application of kinase blockers still needs more evidence.

15.1.3 Phosphorylation and Coinhibitory Receptors

When studying the function of immune system, a certain group of receptors was found on the surface of lymphocytes and other cells. These receptors are transmembrane glycoproteins with and endocellular tyrosine-based inhibitory motifs that can be phosphorylated by phosphatases and transduce negative signals to inhibit the action of activating receptors; therefore, these receptors were termed coinhibitory receptors (Sinclair 1999). Validated coinhibitory receptors are also called immune checkpoints, which have been used to develop strategies for cancer therapies. The enthusiasm towards immune checkpoints cannot be extravagated, as the landscape of cancer therapy is dramatically changed due to development of immunotherapies. Drugs targeting PD-L1 and CTLA4 have been widely applied to treat human malignancies. The phosphorylation of the tyrosine-based inhibitory motifs undoubtedly catches researchers' attention. Ligation of PD-L1 on cancer cells with PD-1 on lymphocytes causes phosphorylation of immunoreceptor tyrosine-based switch motifs,

and then blocks T cell receptor signaling, leading to inhibition of T cell proliferation, cytokine production, and cytolytic function (Folkl and Bienzle 2010). Similar phosphorylation switches were found on other immune checkpoints and potentially served as a criterion to acknowledge new immune checkpoints. Phosphorylation not only occurs right on immune checkpoints to regulate immune activities, but also modulates immune functions and immune checkpoints in other ways. This chapter will summarize the complex role that phosphorylation plays in cancer immune responses, from cell level to molecular level, and comb through the immune signaling mediated by phosphorylation from upstream to downstream. Furthermore, phosphorylation closely related to immune checkpoints will be particularly explained and discussed. This chapter will present a whole scene of phosphorylation in cancer immunity.

15.2 Phosphorylation Modulates Interaction Between Cancer Cells and Immune Cells

15.2.1 Phosphorylation Alters Cancer Cells' Immunophenotype and Phenotype

Major histocompatibility complex (MHC) class I molecules serve a critical role in immunity by presenting a broad range of peptides generated by proteasomal degradation of intracellular proteins. These peptides presented on cell surface by MHC I on cell surface can be recognized by T cells. Peptides containing post-translational modifications contribute to the repertoire of MHC-binding peptides and represent potential targets for T cell recognition (Engelhard et al. 2006). Phosphorylated peptides are called phosphopeptides which can be specifically recognized from their unphosphorylated form. As a hallmark of malignant transformation, deregulation of protein kinases and deranged phosphorylation causes a differential display of phosphopeptides on cancer cells (Blume-Jensen and Hunter 2001; Evan and Vousden 2001), providing an immunological signature of "transformed self". By solving crystal structures of 4 phosphopeptide-HLA-A2 complexes, Mohammed and colleagues revealed that deranged phosphorylation drastically increased peptide binging affinity for HLA-A2, creating potential neoantigens and affecting the antigenic identity of presented epitopes (Mohammed et al. 2008). More evidence of binding studies indicated that the TCR interaction with an MHC-bound phosphopeptide was both epitope-specific and absolutely dependent upon phosphorylation status (Mohammed et al. 2017).

Another way phosphorylation modulates cancer cells is closely related to the PD-1 molecule. Blockers of programmed cell death 1 (PD-1) have been used in cancer treatment to inhibit PD-1 on immune cells to intrigue immune response to cancer cells. However, evidence have shown that PD-1 expressed on cancer cells can promote cancer progression in a way that is not associated with cancer immunity. In cancer cells, PD-1 binds eukaryotic initiation factor 4E and ribosomal protein S6,

thus promoting their phosphorylation and further enhancing cancer progression (Li et al. 2017). In addition, it was demonstrated that in breast cancer cells, the PD-1/PD-L1 interaction enhanced phosphorylation of Akt and ERK, resulting in the activation of PI3K/AKT and MAPK/ERK pathways and increased MDR1/P-gp expression. The interaction at the same time increased survival of doxorubicin treated breast cancer cells, suggesting that inhibition of PD-1/PD-L1 may strengthen the efficacy of chemotherapy in a non-immunologic way (Liu et al. 2017a, b).

15.2.2 Phosphorylation Regulates Immune Cells Behaviors

15.2.2.1 Macrophages

Macrophages are important components of the tumor microenvironment and potential candidates for immunotherapy. Phosphorylation of key molecules modulates macrophages of different groups in different ways, including the infiltration into tumor microenvironments, the mechanism of macrophage induced EMT, and even differentiation of tumor-associated macrophages.

In tumor microenvironment, plasminogen activator inhibitor-1 (PAI-1) was shown to induce phosphorylation of focal adhesion kinase (FAK) at Tyr(925) and further mediate the infiltration of macrophages into melanoma (Thapa et al. 2014).

Tumor-associated macrophages (TAMs) can be divided into two groups: M1 macrophages, which are basically antitumor immune cells, and M2 macrophages, which are closely involved in tumor progression and invasion. In lung adenocarcinoma, Fucosyltransferase IV (FUT4) and its synthetic cancer sugar antigen Lewis Y (LeY), usually elevated in various solid tumors, were promoted by M2 macrophages. FUT4/LeY was indispensable in M2 macrophages-mediated cytoskeletal remodeling and EMT. Actually, FUT4/LeY mediates the mechanism that M2 indirectly promotes phosphorylation of Ezrin, and the M2-induced EMT (Wang et al. 2017a, b). The differentiation and sometimes transformation of M1 and M2 are critical in the microenvironment and its interaction with tumors. Mechanistically, the polarization of M1 macrophages was shown to be suppressed by phosphorylation of signal transducer and activator of transcription 1 (STAT1), while M2 polarization was reported to be promoted by increasing STAT6 phosphorylation (Yao et al. 2014), as shown in Fig. 15.1.

Modulation of TAMs proves to be promising new strategies for optimizing immunotherapy. In a recent study, glycocalyx-mimicking nanoparticles (GNPs), which can be internalized by TAMs were shown to reverse the M2 macrophages to M1 macrophages. With upregulation of IL-12 production and decrease on suppressive molecules such as IL-10, arginase 1, and CCL22, these macrophages were rebuilt with antitumor capacity. Thus, the efficacy of PD-L1 blockage therapy was enhanced (Zhang et al. 2018a, b, c).

AXL is a member of the TAM (Tyro-3, Axl, and Mer) receptor tyrosine kinase family in Glioblastoma (GBM), which would lead to apoptosis of mesenchymal, but

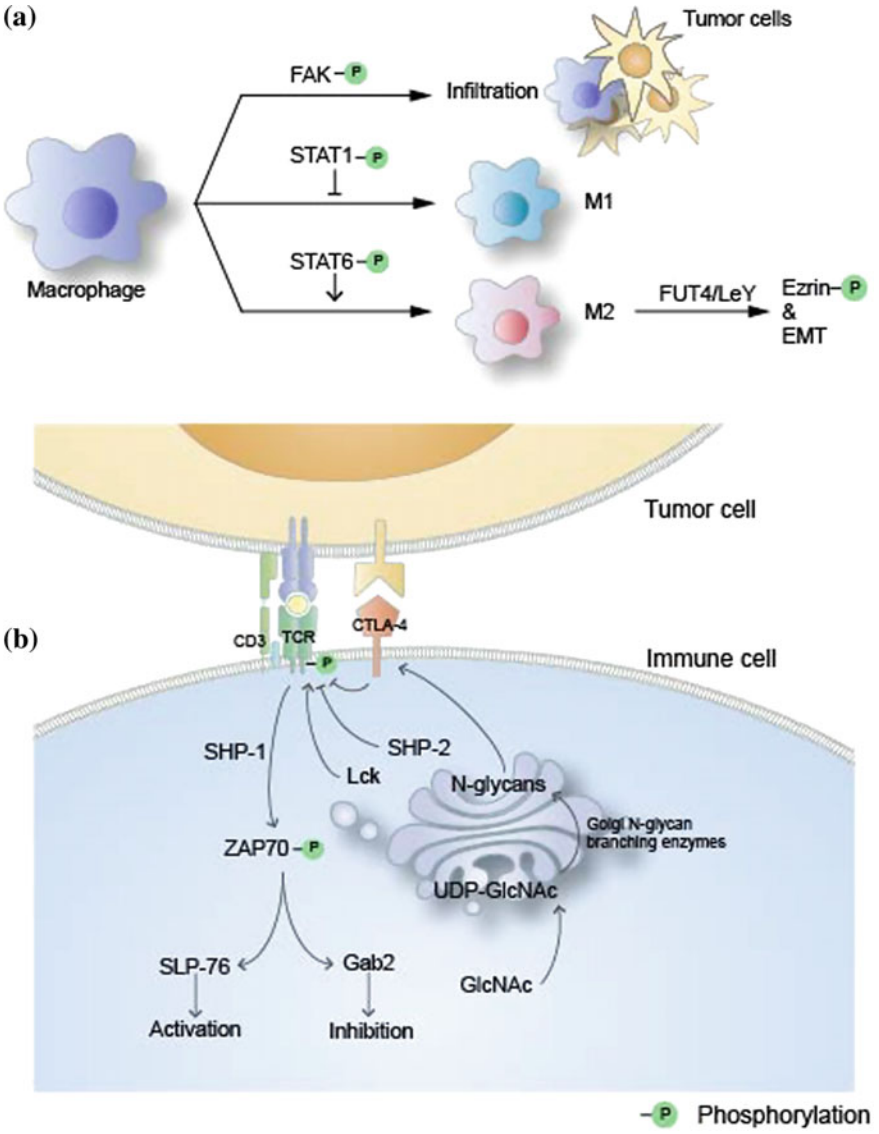

Fig. 15.1 **a** Phosphorylation of FAK mediates the infiltration of macrophages; the polarization of M1 macrophages is suppressed by phosphorylation of STAT1, while M2 polarization is promoted STAT6 phosphorylation. **b** CTLA colligation with TCR inhibits immune signaling through inhibiting tyrosine phosphorylation of TCRzeta and downstream protein signaling effectors, and GlcNAc enhances CTLA expression and inactivates T cells

not proneural, glioma sphere cultures (GSC) when knocked down. A known ligand of other TAM receptors usually secreted by tumor-associated macrophages/microglia, protein S (PROS1), was shown to induce phosphorylation of AXL (pAXL), which could further activate NF-kB in mesenchymal GSC. While a small molecule inhibitor of AXL could reverse the effect. Notably, treatment of a PD-L1 blocking antibody, nivolumab, drastically increased infiltration of macrophages/microglia and activation of AXL, indicating a promising add-on effect on immunotherapy by activating AXL (Sadahiro et al. 2018).

15.2.2.2 DCs

Dendritic cells serve to present antigens in tumor microenvironment and are important components in tumor immunity. Therapeutic strategies using antigen-loaded, cytokine-matured human DCs to treat cancer have been proposed and investigated, indicating larger needs in clarifying the mechanism of antigen-presenting in tumor microenvironment.

Interaction between IgM Abs with cell surface molecules was shown to activate cells in vitro and in vivo. Strong antitumor responses can be stimulated by cross-linking a human B7-DC (PD-L2)-specific IgM Ab with DCs. Ab-activated DC upregulated expression of cytokine and chemokine genes through PI3K-dependent phosphorylation of AKT together with mobilization of NF-kappaB (Radhakrishnan et al. 2007). Interestingly, the inhibition of cancer cell apoptosis by Insulin-like growth factors (IGFs) is also related to DCs. Evidence suggested that IGFs could suppress DCs' maturation, antigen-presenting abilities, and the ability to activate antigen-specific CD8(+) T cell. Increased IGF-1 and IGF-2 concentrations were found in advanced-stage ovarian carcinoma patients in their ascites than early-stage patients. Also, increased secretion of IL-10 and TNF-alpha was observed in OGF-treated DCs, accompanied with decreased phosphorylation of ERK1/2 and reduced dephosphorylation of p38. Consequently, the maturation of DCs in the ascites was significantly intervened. Blockade of IGFs could rescue DCs' maturation and antigen-presenting ability through elevating ERK1/2 phosphorylation and p38 dephosphorylation, indicating a potential target for cancer immunotherapy (Huang et al. 2015).

DC vaccination, the method of injecting antigen-loaded, cytokine-matured DCs into human body to treat cancer, is a novel research area for cancer therapy. Based on previous findings that Th17 infiltration into ovarian tumors is positively associated with better outcome, DC vaccines were developed to promote maturation and infiltration of Th17. Observations of the test showed reduced expression of CTLA-4, PD-1, and Foxp3 following activation with IL-15/p38 inhibitor-treated DC. Further results provided evidence that the modulation of p38 MAPK signaling in DCs is associated with increased phosphorylation of ERK 1/2 MAPK (Cannon et al. 2013). Another vital function of antigen-presenting cells is providing costimulatory molecules to fully initiate T cells activation and thus antitumor responses through amplification and differentiation of cytotoxic T lymphocytes (CTLs). 4-1BBL/4-1BB is a pair of

costimulatory ligand and receptor, playing an important role in the costimulation of CTLs. Later chapter will specifically discuss this pair of costimulatory molecules. DCs transduced with recombinant adenovirus encoding truncated PSMA (tPSMA) and m4-1BBL were shown to decrease apoptosis of CTLs and promote T lymphocyte activation and cytotoxicity through higher expression anti-apoptotic protein of Bcl-xL and phosphorylation of P38, enhanced NF-kappaB activation, as well as more IFN-gamma production. This provides an innovative strategy for DC vaccines to treat tumor (Youlin et al. 2013). Another study focusing on Th17 activation pointed out the role of p38 MAPK signaling in DC was associated with decreased level of PD-L1 and increased phosphorylation of ERK 1/2 MAPK, allowing more insights into the development of DC vaccines to activate Th17 in ovarian cancer patients (Cannon et al. 2013).

15.2.2.3 B Cells

Growing evidence is suggesting an involvement of B cells in cancer immunity. In murine models, decreasing or depleting B cells leads to decreased tumor growth (Brodt and Gordon 1978; Gordon et al. 1982) and at the same time, increased CTLs activity (Qin et al. 1998; Shah et al. 2005). Further analysis revealed B cells' role in converting T cells to CD4(+)CD25(+)FoxP3(+) T regs in addition to inhibiting T cell mediated tumor immunity. Mechanistically, Breg phenotype was shown to display increased inhibitory ligand expression and enhanced phosphorylation of STAT3, together with induction of IL-10 and TGF-beta. Breg induced immune suppression may affect a variety of immune cells including T effector cells, NK cells, myeloid-derived suppressor cells (MDSC) and/or tumor-associated macrophages (Schwartz et al. 2016).

In hepatocellular carcinoma (HCC), a subset of B cells with high level of PD-1 was shown to display a unique CD5(+)CD24(∓)CD27(hi/+)CD38(dim) phenotype rather than conventional regulatory B cell phenotype CD24(hi)CD38(hi). In inducing these B cells in HCC environment, TLR4 mediated Vcl6 upregulation was indispensable, which can be abolished by IL4-elicited STAT6 phosphorylation, indicating another way of modulating immune cell differentiation by phosphorylation (Xiao et al. 2016).

15.2.2.4 Tregs

Regulatory T cell (Treg) is a subgroup of T cells that regulates other T cells to obtain homeostasis of immune elimination and tolerance in physical statues. Tregs prevent auto-reactive T effector cells (Teff) from destructing normal tissue. However, as a crucial component of tumor microenvironment, Tregs are shown to inhibit immune responses toward cancer cells and contribute to the immune-suppressive microenvironment. In order to reverse the immune suppression of cancer, efforts have been made to figure out the mechanism behind Tres' inhibitory function. Besides Treg-associated molecules such as CTLA-4 and GITR, which will be discussed in detail

in later paragraphs, kinase activities are attracting more and more attention from researchers. Although, out knowledge about the complex signaling kinases in Tregs so far are basically limited and still based on single kinase cascades (Huynh et al. 2015; Ulges et al. 2015). Tuettunberg and colleagues applied a phosphorylation-based kinome array to perform kinome profiling in human Tregs in different stages, compared to Teffs. Analysis revealed an altered pattern of CD-28-dependent kinases in activated Tregs, compared to resting Tregs. Furthermore, distinct kinases such as EGFR or CK2 are significantly upregulated in activating Tregs but not in Teffs. A pattern of kinase activation has been proposed to be used for definition of the activation and function of Tregs. Hopefully, further investigation of kinome profiles could bring about a better understanding about Tregs in TME and provide potential targets and strategies for immunotherapies (Tuettenberg et al. 2016).

15.3 Phosphorylation of Checkpoint Related Factors

15.3.1 Phosphorylation and Immune Cell Receptor Signaling

Blockade of the PD-1 signaling pathway inhibited SHP-2 phosphorylation and restored the IFN-gamma-producing function of HL-infiltrating T cells. According to these results, deficient cellular immunity observed in HL patients can be explained by "T cell exhaustion," which is led by the activation of PD-1/PD-L1 signaling pathway (Yamamoto et al. 2008).

15.3.1.1 TCR Signaling

T cell activation is initiated by the binding of agonist peptide-MHC (pMHC) with T cell receptors (TCRs). The signaling of TCR starts with TCR phosphorylation by the Src family tyrosine kinase Lck, and then is transduced by an intracellular phosphorylation cascade, leading to reorganization of the cytoskeleton and organelles, transcriptional changes, and cell proliferation (Brownlie and Zamoyska 2013). Studies concerning the modulation of TCR signaling by phosphorylation have uncovered some key molecules involved in TCR signaling, especially coinhibitory factors and costimulatory factors (Granier et al. 2017).

It was acknowledged that cytotoxic T lymphocyte antigen-4 (CTLA-4) ligation inhibits TCR signaling more than two decades ago. As first, CTLA-4 was found to associate with the TCR complex zeta-chain (TCRzeta) in primary T cells. By reconstitution in 293 transfectants of CTLA-4, the association with TCRzeta was found to be enhanced by p56(lck)-induced tyrosine phosphorylation of TCRzeta. While SHP-2 was expressed to abolish the p56(lck)-inducible TCRzeta-CTLA-4 interaction by dephosphorylating TCRzeta bound to CTLA-4 (Lee et al. 1998). Consistently, more evidence have shown that CTLA colligation with TCR cut down on downstream

protein tyrosine phosphorylation of signaling effectors and inhibited extracellular signal-regulated kinase 1/2 activation. But on the contrary, TCR zeta-chain phosphorylation and subsequent zeta-associated protein of 70 kDa (ZAP-70) tyrosine kinase recruitment were later shown not significantly affected by CTLA-4 engagement, the association of p56(lck) with ZAP-70 was inhibited. Additionally, CTLA-4 ligation caused the selective inhibition of CD3-mediated phosphorylation of the positive regulatory ZAP-70 Y319 site. The major phosphatase activity was attributed to Src homology 2 domain-containing tyrosine phosphatase 1(SHP1), a protein tyrosine phosphatase that has been shown to be a negative regulator of multiple signaling pathways in hemopoietic cells. (Guntermann and Alexander 2002) Another study proposed that Grb-associated binder 2 (Gab2), in addition to CTLA, plays a role of a signaling crossroad of activation or inhibition, based on phosphorylation of either Src homology 2 (SH2) domain-containing leukocyte phosphoprotein of 76 kDa (SLP-76) or Gab2 by ZAP-70 (Saito and Yamasaki 2003). Glucosamine (GlcN), like N-acetylglucosamine (GlcNAc), is salvaged into the hexosamine pathway and is converted to UDP-GlcNAc. Golgi N-glycan branching enzymes produce N-glycans, using UDP-GlcNAc as a substrate, which attach to the T cell receptor (TCR) and cytotoxic T-lymphocyte antigen-4 (CTLA-4). Glucosamine treatment increased the number of T cells expressing beta1, 6GlcNAc-branched N-glycans, with reduced ZAP-70 phosphorylation and enhanced CTLA-4 expression. Glucosamine treatment reduced the number of activated T cells from both the human primary and Jurkat cells and the dermatitis-induced mice (Chen et al. 2013), as illustrated in Fig. 15.1.

As for another immunoinhibitory receptor, programmed death-1 (PD-1), the inhibition of T cell proliferation, cytokine production, and cytolytic function by PD-1 ligation on lymphocytes is based on modulation of phosphorylation of immunoreceptor tyrosine-based switch motifs (Folkl and Bienzle 2010). It was reported that PD-1 signaling inhibits TCR-mediated phosphorylation of ZAP70 and association with CD3zata decreases PKCtheta activation loop phosphorylation, leading to decreased IL-2 production (Sheppard et al. 2004). A mathematical simulation model was built to describe the inhibitory effect of PD-1 on the early activation of TCR and CD28 at the level of phosphorylation of their cytoplasmic domain. The model revealed that ZAP-70 and SLP76 were significantly inhibited, mediated by PD-1 mediated lck inhibition (Arulraj and Barik 2018). In another experiment, researchers blocked PD-L1 and PD-L2 and observed elevated level of Akt(ser(473)) phosphorylation following TCR activation (Henson et al. 2012).

15.3.1.2 BCR Signaling

Certain inhibitory factors not only affect TCR signaling, but also influence B cell receptor (BCR) signaling. It was shown that PD-1 can inhibit BCR signaling by recruiting SHP-2 to its phosphotyrosine and dephosphorylating key signal transducers of BCR signaling (Okazaki et al. 2001). In another study, ZAP-70 phosphorylation was found to be negatively relative to expression of PD-1 while key B cell receptor

signaling kinases such as phosphorylation of SYK and LYN was independent of PD-1 expression (Grzywnowicz et al. 2015).

15.3.2 Phosphorylation in Signal Transduction Pathways

As T cells are the main group of lymphocytes that eliminate cancer cells, a majority of research looked into the mechanism behind their inhibition. On accounting to the phosphorylation of signaling pathways in T cells, there are some common pathways that play critical roles, including Akt/PI3K and ERK/MAPK pathways.

Sometimes, the two pathways are mutually affected. Vdelta2 gammadelta T cells are thought to be inhibitory factors to alphabeta T cells and blockage of CD86/CTLA4 could downregulate the suppressive effect. When pretreated with TLR2 ligands, Vdelta2 T cells were detected with increased phosphorylation of MAPKs, Akt, and NK-kappaB. At the same time, immune-suppressive capacity was partly abolished, as inhibitory molecules on co-cultured responder T cells were down-regulated and phosphorylation of Akt and NF-kappaB was restored (Peters et al. 2014).

15.3.2.1 Akt/PI3K Signaling Axis

Akt pathway is often related to proliferation of cells. In T cells, activation of PI3K/Akt pathway usually indicates increased T cell activation and proliferation.

Decades ago, it was revealed that leukocyte activation is followed by enhancement of adhesion to the extracellular matrix due to the activation of beta 1 integrin receptors. Early studies pointed out that CD28 deficiency was associated with phosphatidylinositol (PI) 3-kinase (PI3K) in integrin activation and the process was mediated by Cbl tyrosine phosphorylation (Zell et al. 1998). The replication capacity of memory T cells relies on the function of telomerase, which can be upregulated by costimulatory signaling molecule CD28. However, CD8(+) T cells are sometimes deprived of CD28 expression after repeated activation. Furthermore, The defect in CD28 expression was found to be related to decrease in Ser(473) phosphorylation of Akt, which can induce telomerase activity by phosphorylating human telomerase reverse transcriptase (Plunkett et al. 2007). Phosphatase and tensin homolog (PTEN) is a key molecule in the PI3K/Akt signaling axis. Phosphorylation of PTEN in the Ser380-Thr382-Thr383 cluster within the C-terminal regulatory domain is induced by casein kinase 2 (CK2) during TCR/CD3- and CD28-mediated stimulation. So that, PTEN is stabilized and overexpressed while PTEN phosphatase activity is diminished. This effect can be inhibited by PD-1 through CK2 inhibition (Patsoukis et al. 2013). As an upstream factor to PI3K/AKT pathway, the human epidermal growth factor receptor 3 (HER3) activates PI3K/AKT pathway by phosphorylating the downstream AKT targets murine double minute 2, X-linked inhibitor of apoptosis (XIAP), and forkhead box O1 (FOXO1). Anti-HER3 Abs could represent a new option for immunotherapy of pancreatic and triple-negative breast cancers (Lazrek et al. 2013).

Chimeric antigen receptors (CARs) link an antigen recognition domain to intracellular signaling domains to redirect T cell specificity and function. The T cells constructed with CARs expression on its surface to treat cancer are called CAR-T. Akt phosphorylation is also involved in regulating the effect of CAR-T therapy. In hepatocellular carcinoma (HCC), ligation of PD-1/PD-L1 would cause T cell exhaustion and it was proposed that disruption of PD-1 protected the GPC3-CAR-T cells. Evidently, the phosphorylation level of Akt and the expression of Bcl-xL are significantly increased in PD-1 deficient GPC3-CAR-T cells, together with enhanced antitumor activity (Guo et al. 2018).

PD-1 signaling inhibits Akt phosphorylation by preventing CD28-mediated activation of phosphatidylinositol 3-kinase (PI3K). In contrast, CTLA-4-mediated inhibition of Akt phosphorylation is sensitive to okadaic acid, providing direct evidence that PP2A plays a prominent role in mediating CTLA-4 suppression of T cell activation. Moreover, PD-1 ligation showed stronger effect of suppressing CD3/CD28-induced events in the T cell transcriptional profile, compared with CTLA-4, suggesting that CTLA-4 and PD-1 inhibit T cell activation through distinct and potentially synergistic mechanisms (Parry et al. 2005), as schematically shown in Fig. 15.2. The signaling of CTLA-4(CD152), was further illustrated to stimulated Protein Kinase B(PKB/AKT) through PI3K, and AKT was activated via phosphorylation at threonine 308 and serine 473 in pro-inflammatory lymphocytes expressing the cognate chemokine receptor CCR5. Activated AKT induced cytoskeleton rearrangements, mediating migration and optimal localization of T cells, as such T cells could effectively function in proper place (Knieke et al. 2012).

As mentioned before, GlcN may suppress TCR signaling and promote CTLA-4 expression, while actually, it may also enhance apoptotic pathways. Supportively, inhibition of PI3K/Akt and NF-kappaB phosphorylation was detected upon GlcN treatment, together with increasing expression of FasL activation caspases, particularly caspase-3 (Chen et al. 2013). Interestingly, the T cell anergy induced by coinhibitory factor, CTLA 4, was found to be accompanied with activated PI 3-K and protein kinase B (PKB/AKT). Taken together, T cell non-responsiveness was induced under the same condition when T cell apoptosis was inhibited, suggesting a more complicated mechanism behind T cell suppression. The effect was shown to be PI3K and PKB/Akt dependent (Schneider et al. 2008). Nuclear factor kappaB (NF-kappaB) is a downstream activating molecule to many pathways. A study concerning the regulation of alphabeta T cells by Vdelta2 T cells that were mentioned above in Akt signaling also pointed out the involvement of NF-kB phosphorylation (Peters et al. 2014).

Another pair of molecules, CD137 and its ligand, CD137L, are expressed on activated T cells and antigen-presenting cells, respectively. Antigen with dual costimulation through CD137 and CD134 induces powerful CD8 T cell responses. These effector T cells are endowed with an intrinsic survival program resulting in their accumulation in vivo (Lee et al. 2009). Ligation of CD137L promotes phosphorylation of Akt and p70S6 kinase, indicating a reverse effect evoked by CD137L in

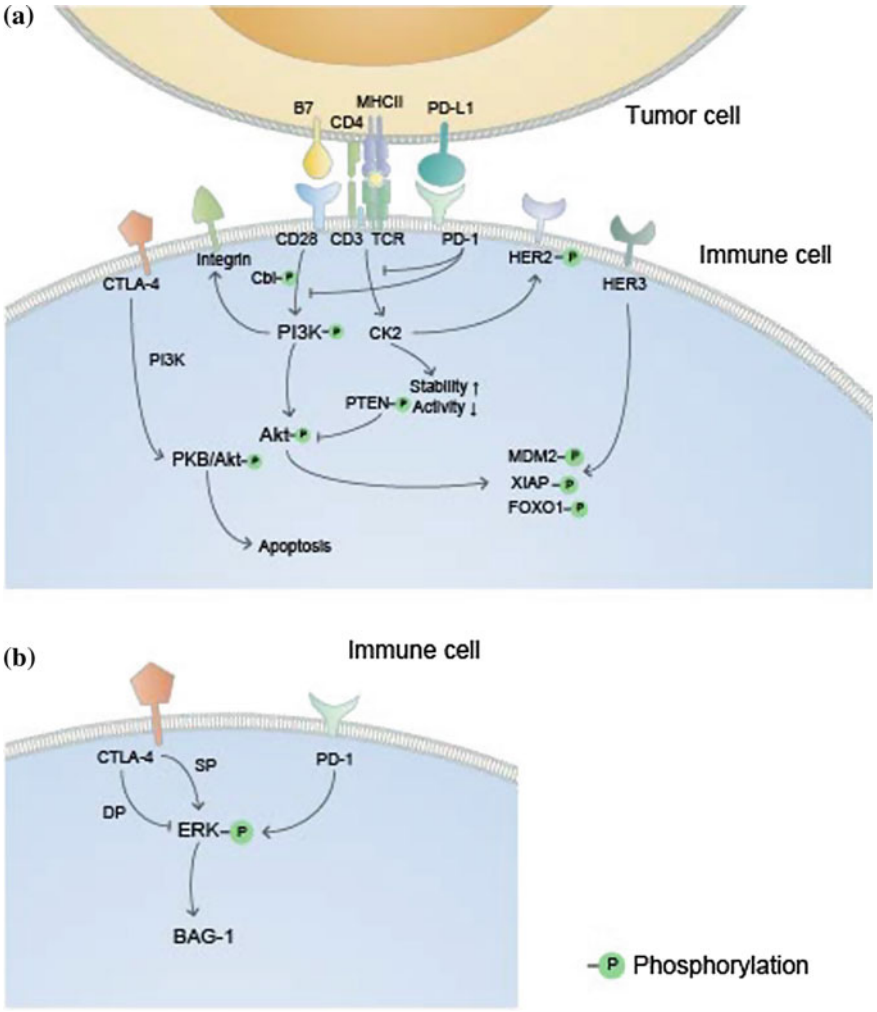

Fig. 15.2 **a** Phosphorylation of Akt/PI3K and downstream factors are delicately associated with a variety of membrane signaling molecules, including CTLA-4, CD28, PD-1, and HER2/3, transducing outside signals into immune cells and consequently modulating cell activities. **b** CTLA-4 and PD-1 are related phosphorylation of ERK and cause further signal transduction

regulating macrophages (Kim et al. 2009). Later, CD137L and Cd 137 were found to be aberrantly expressed on tumor cells, especially hematopoietic malignancies and they promoted tumor growth, where Akt phosphorylation was found to be involved (Kamijo et al. 2018).

15.3.2.2 ERK/MAPK Signaling Axis

Extracellular signal-regulated kinase (ERK) signaling pathway is involved in the "outside-in" transduction of a variety of extracellular signals. When it comes to cancer immunity, the star checkpoint proteins, PD-L1 and CTLA-4, are both associated with phosphorylation of ERK pathway. Figure 15.2 describes the pathways and their correlation with ERK.

In non-small cell lung cancer (NSCLC), high expression of programmed cell death 1 ligand 1 (PD-L1) expression is associated with poor outcomes and tyrosine phosphorylation may be involved. However, failure of tyrosine kinase inhibitor (TKI) treatments was surprisingly discovered, together with increased Bcl-2-associated athanogene-1 (BAG-1) expression. Further evidence suggested that ERK phosphorylation intermediated the induction of BAG-1 transcription by PD-L1. Therefore, the researchers anticipated that a combination of TKI and ERK inhibitors may be a novel treatment to NSCLC (Lin et al. 2017). Another study generated T cell blasts by treating peripheral blood mononuclear cells (PBMCs) with phytohemagglutinin (PHA) to induce PD-1 expression and used exosomal PD-L1 to treat them. Results showed a dose-dependent pattern by which ERK phosphorylation and NF-κB activation of T cells were inhibited. Moreover, IL-2 secretion induced by PHA was also suppressed. This experiment provided solid evidence of how PD-1/PD-L1 ligation influenced ERK phosphorylation and further inhibited T cell activation (Yang et al. 2018).

It is not surprising that CTLA-4 is also associated with ERK phosphorylation. In an investigation focusing on the involvement of CTLA-4 in activation of CD4(+)CD8(+) double-positive (DP) and CD4(+)CD8(-) and CD4(-)CD8(+) single-positive (SP) thymocytes, CTLA-4 was shown to regulate DP thymocytes positively and SP thymocytes negatively. Evidence suggests that blockage of CTLA-4 suppressed ERK phosphorylation in DP while promoted ERK phosphorylation in SP (Kwon et al. 2004). Very recently, a surprising finding was reported that anti-CTLA-4 treatment may induce PD-L1 expression in NSCLC cells. The study revealed that the activation of EGFR and ERK may participate in the induction of PD-L1 (Zhang et al. 2019).

TIGIT is a commonly expressed coinhibitory molecule in follicular lymphoma (FL) that suppresses infiltrating T cells' function. Significantly decreased phosphorylation of ERK was detected in CD8(+) FL T cells, together with lower secretion of INF-gamma, while TCR proximal signaling (p-CD3zeta, p-SLP76) were intact (Josefsson et al. 2018).

15.3.3 STAT: Multifunctional Factor Phosphorylated to Regulate Immune Response

15.3.3.1 STAT Family Phosphorylation Involved in Checkpoints Modulation

Serine phosphorylation has generally been considered indispensable for full transcriptional activity of signal transducers and activators of transcriptions (STAT) proteins. The regulation of PD-L1 expression by STAT family, especially STAT3 and STAT1, has been widely investigated and acknowledged, as demonstrated in Fig. 15.3.

The correlation between STAT3 phosphorylation and PD-L1 upregulation was established gradually in recent years and considered to be a potential strategy for optimizing immunotherapy. In Classical Hodgkin lymphoma (cHL) and primary mediastinal large B cell lymphoma (MLBCL), chromosome 9p24.1/JAK2 amplification specifically increased JAK2 expression. When JAK2 inhibitor was used, reduced phosphorylation of JAK2, STAT1, STAT3, and STAT6 was detected, accompanied by decreased expression of PD-L1 (Hao et al. 2014). In human Epithelial Ovarian Cancer (EOC) cells, IL-27 may modulate other immune-regulatory molecules involved in EOC progression, including Indoleamine 2,3-dioxygenase (IDO) and Programmed Death-Ligand (PD-L1). IDO and PD-L1 were not constitutively expressed by EOC cells in vitro, but IL-27 increased their expression through STAT1 and STAT3 tyrosine phosphorylation (Carbotti et al. 2015). Enhancement of PD-L1, together with other immune inhibitory factors, including GM-CSF receptor (GM-CSF-R) and indoleamine 2,3-dioxygenase (IDO) were found co-expressed on a group of immune inhibitory cells in liver, known as Liver myeloid-derived suppressor cells (L-MDSCs). In these cells, high level of STAT3 activation was also demonstrated. L-MDSCs have been reported to expand in response to granulocyte-macrophages colony-stimulating factor (GM-CSF) and suppress antitumor immunity in liver metastases. Small molecules were exploited in animal models to inhibit JAK2 and STAT3, showing drastic suppression on IDO and PD-L1 expression in L-MDSCs. Conclusively, the GM-CSF/JAK2/STAT3 axis was proposed in L-MDSCs to induce intrahepatic immunosuppressive tumor microenvironment (Thorn et al. 2016). Later, Janus kinase 2 (JAK2) was shown in another investigation to cause phosphorylation of STAT3 and STAT5, leading to PD-L1 promoter activity and PD-L1 expression. Additionally, JAK2(V617F)-mutant cells exhibited higher level of PD-L1, which can be abolished by JAK2 inhibitors. JAK2(V617F) myeloproliferative neoplasms (MPNs) isolated from patients were xenografted to murine models, showing that constitutive JAK2/STAT3/STAT5 activation, mainly in monocytes, megakaryocytes, and platelets, caused PD-L1-mediated immune escape by reducing T cell activation, metabolic activity, and cell cycle progression (Prestipino et al. 2018). Moreover, JAK/STAT activation was revealed to intermediate the autocrine type I IFN-induced PD-L1 expression, where constitutive STAT1 phosphorylation was detected (Xiao et al. 2018).

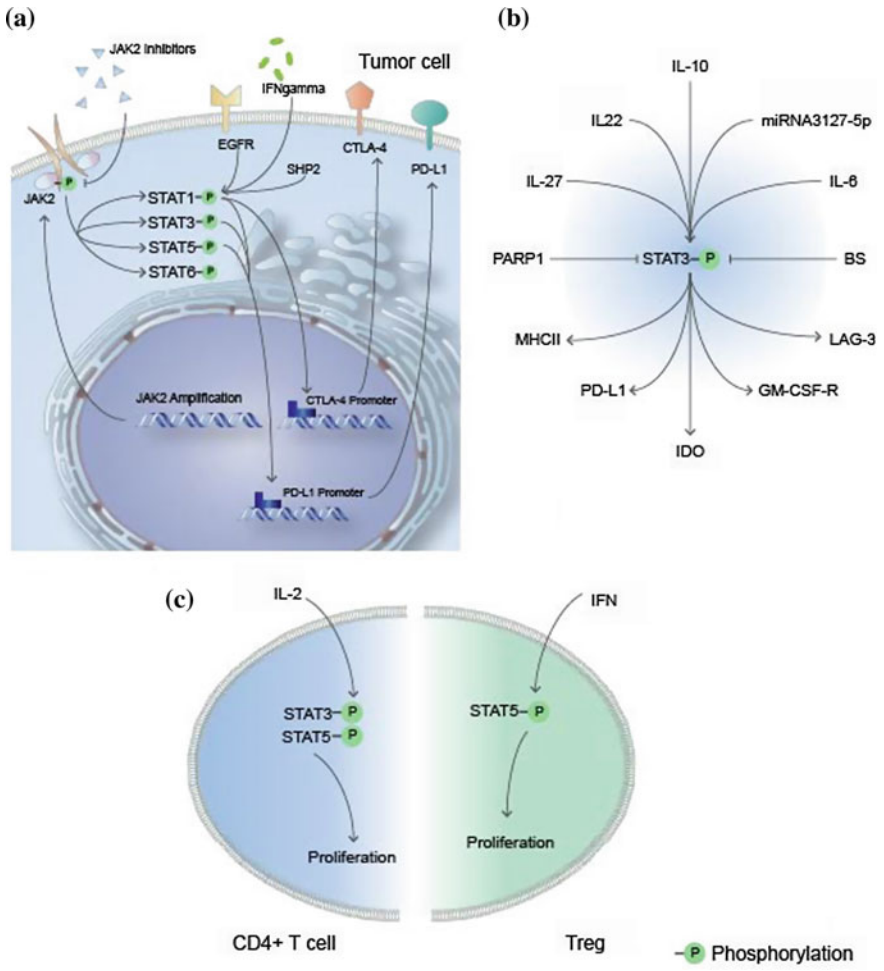

Fig. 15.3 **a** Phosphorylation of STAT proteins, stimulated by extracellular signal reactions, results in overexpression of PD-L1 and/or CTLA-4 through promotion of promoters. Meanwhile, JAK2 amplification and phosphorylation are involved in phosphorylation of STAT proteins. **b** STAT3, known as a key factor in PD-L1 expression, plays a central role in the regulation of a variety of factors. **c** Phosphorylation of STAT proteins leads to different subsequent events in CD4+ T cells and in Tregs

Some researches revealed that STAT3 mediates the regulation of PD-L1 by cytokines. The phosphorylation of STAT was found to be consistent with IL-10 pathway activation, together with upregulation of PD-L1 in monocytes in bladder cancer. But IL-10 was unable to independently cause PD-L1 upregulation, suggesting a novel way of immunosuppression in bladder cancer by promoting PD-L1 expression (Wang et al. 2017a, b). Actually, it was reported that IL-10 secreted by T cells could induce upregulation of PD-L1 and MHC class II on MDSCs in tumor microenvironment of

cancer patients and this effect was mediated by phosphorylation of STAT3. Interestingly, the receptors of these two ligands, PD-1 and LAG-3 were found upregulated on T cells at the same time, proposing a crosstalk between MDSCs and T cells in tumor microenvironment (Pinton et al. 2016). IL-6 was found to correlate with STAT3 phosphorylation that reduced IL-6 secretion could downregulate STAT phosphorylation and further inhibit PD-L1 expression. Factors that activate STAT3 phosphorylation, such as recombinant soluble DLL1 and Notch activator oxaliplatin consistently increase PD-L1 expression (Hildebrand et al. 2018). Further investigation figured out that STAT3 was phosphorylated on Tyr705 to induce PD-L1 upregulation (Liu et al. 2018). Another study detected PD-L1 upregulation in IL-22 treated cells, which can be abrogated by STAT3 siRNA. Notably, mutations of STAT3 promoted phosphorylation (p.D427H, E616G, p.E616K, and p.E696K) and transcription activity of itself. Among them, p.E616K induced programmed cell death-ligand 1 (PD-L1) expression by robust binding of activated STAT3 to the PD-L1 gene promoter (Song et al. 2018).

STAT3 was known to be a common pathway of how many factors affect PD-L1 expression. Actually, it is a central factor in modulating a variety of molecules, as shown in Fig. 15.3. An inverse correlation between PARP1 and PD-L1 was also observed in clinical ovarian cancer samples. Mechanistically, PARP1 poly(ADP-ribosyl)ates STAT3 and subsequently promotes STAT3 dephosphorylation, resulting in reduced transcriptional activity of STAT3 and expression of PD-L1 (Ding et al. 2019). A less known up-regulator of PD-L1, miRNA3127-5p, was found to induce STAT phosphorylation through autophagy suppression, because autophagy retained pSTAT3 into the nucleus in miRNA-3127-5p knocked cells (Tang et al. 2018). A novel thioredoxin reductase inhibitor, butaselen (BS), was shown to suppress oncogenesis in a wide range of human cancer cell lines. To look into the mechanism behind this phenomenon, animal model was used to show that BS can elevate the percentage of CD4(−)CD8(+) T lymphocytes and the secretion of downstream cytokines, where PD-L1 expression was down-regulated on tumor cells. Later, it was found in cell experiments that STAT3 phosphorylation was inhibited by BS, on which the PD-L1 upregulation relied (Zou et al. 2018).

With sufficient evidence, PD-L1 is widely acknowledged as a STAT3-dependent target gene. A study on a murine model Head and neck squamous cell carcinoma (HNSCC) proposed a strategy of combining PD-L1 blockage with inhibition of STAT3, which hopefully restrains compensatory overexpression of PD-L1 after immunotherapy (Bu et al. 2017). In glioblastoma, glioblastoma (GBM)-derived stem cells (GSCs) was thought to induce immunosuppression. Further study showed that phosphorylation of STAT 3 was found to be associated with the formation of immune-suppressive microenvironment caused by GSC-derived exosomes (GDEs), specifically upregulation of PD-L1 (Gabrusiewicz et al. 2018). Moreover, STAT1 knockdown significantly reduced EGF-mediated PD-L1 expression, and ruxolitinib, a JAK1/JAK2 inhibitor, significantly inhibited STAT1 phosphorylation to reduce the IFNr-mediated PD-L1 axis. These results indicate that EGF exacerbates PD-L1 by increasing the protein levels of STAT1 to enforce the IFNr-JAK1/2-mediated

signaling axis in selected EGFR-positive cancers. The inhibition of EGFR by afatinib significantly reduced PD-L1 and may be a potential strategy for enhancing immunotherapeutic efficacy (Cheng et al. 2018).

STAT1 was shown to share a similar function with STAT3 in promoting PD-L1 expression. Selective expression of the programmed death-ligand 1 (PD-L1) was observed on CD44(+) cells compared with CD44(−) cells and was associated with constitutive phosphorylation of STAT3 on CD44(+) cells. Importantly, inhibition of STAT3 decreased expression of PD-L1 on CD44(+) cells. IFN-gamma treatment preferentially induced even further PD-L1 expression on CD44(+) cells and was associated with enhanced IFN-gamma receptor expression and phosphorylation of STAT1 (Lee et al. 2016).

Patients with hypomorphic mutations in STAT3 and patients with hypermorphic mutations in STAT1 share several clinical and cellular phenotypes. Two cohorts based on these mutations were collected and analyzed. Similarly, differentiation of Th17 cells was impaired and STAT1 was hyper-phosphorylated in response to cytokine stimulation. Furthermore, STAT1-dependent PD-L1 upregulation, which was known to inhibit Th17 differentiation in mouse models, was markedly enhanced as well. Defects in Th17 differentiation could be partially overcome in vitro via PD-L1 inhibition and in a mouse model of STAT3 loss-of-function by crossing them with PD-1 knockout mice (Zhang et al. 2017). In PCa cells, SHP2 was shown to upregulate HLA-ABC and PD-L1 expression via STAT1 phosphorylation (Liu et al. 2017a, b). The phosphorylation of STAT1 and an increase in total STAT1 were also observed in the AsPC-1 cells when stimulated by chemotherapy agents, while JAK2 inhibitor could attenuate the effect, suggesting a role of JAK2/STAT1 pathway in cancer immune escape caused by chemotherapy agents (Doi et al. 2017). In addition, IFN-gamma induced PD-L1 upregulation was shown to be related to STAT1 phosphorylation in an inhibitory test (Xu et al. 2018). On the other hand, Stimulation of OVISE, OVTOKO, OV2944-HM-1 (HM-1), and CT26 cell lines with IFN-gamma induced STAT1 phosphorylation and PD-L1 expression. SOCS-1 gene was later shown to potently inhibit this effect, presenting a similar outcome to application of JAK inhibitor I. Intratumoral injection of Adenovirus-mediated SOCS-1 gene delivery (AdSOCS-1) proved to be an effective antitumor strategy in vitro and in vivo, but the mechanism is not quite clear (Nakagawa et al. 2018). In another study applying an IFN-gamma inhibitor, Apigenin PD-L1 expression by MDA-MB-468 and 4T1 cells was inhibited, which was associated with reduced phosphorylation of STAT1. The phosphorylation of STAT1 was early and transient at Tyr701 and sustained at Ser727 (Coombs et al. 2016).

Similarly, CTLA-4 expression was also proved to be promoted through JAK1/2-dependent phosphorylation of STAT1 by INF-gamma. Mechanistically, phosphorylated STAT1 binds to and activates a specific gamma-activated sequence on the promoter of CTLA-4 and therefore opens local DNA through histone acetylation. These results may account for the mechanism behind the activated IFN-gamma-response gene expression, including CTLA-4, upon anti-CTLA-4 treatment in patients with melanoma (Mo et al. 2018), as shown in Fig. 15.3.

STAT5 phosphorylation was found to upregulate expression of cytotoxic T lymphocyte antigen-4 (CTLA-4), FoxP3 and CD25 and the anti-apoptotic protein Bcl-2 in Tregs in autoimmune liver diseases (Jeffery et al. 2017). Interestingly, STAT5 phosphorylation was identified in human anti-pig response in aortic transplant (Li et al. 2013). But its role in cancer immunity remains unknown.

15.3.3.2 STAT Modulates Cancer Immunology in Other Ways

STAT family has wide connections with a lot of upstream and downstream pathways that play important roles in immune regulations. Although STAT phosphorylation was thought to be positively related to the expression of coinhibitory molecules like PD-L1, implicating tumor suppression effect, some other pathways to be discussed below may put forward different perspectives toward the role of STAT in immunity.

Cytokines regulate immune cells' functions by modulating proliferation, activation, and expression of a variety of proteins and their regulations were found to be related to phosphorylation of STAT family. T cell proliferation can be activated upon IL-2 stimulation, which was thought to be mediated by phosphorylation of STAT3 and STAT5 but the response was abandoned by TGF-beta, which can be secreted by many types of tumor cells. Further investigation proposed IL-15 to restore the IL-2 response and STAT phosphorylation, leading to enhanced proliferation of T cells (Campbell et al. 2001). It seems like STAT phosphorylation could support T cell response in this way, but later it was revealed that the combination of interferon 2 and IL-2Ralpha would activate Treg and promote its proliferation through the phosphorylation of STAT5, showing the immunosuppressive effect of STAT phosphorylation (Wu and Xue 2008) (Fig. 15.3). To make things more complicated, with a broad spectrum of dampened pathways including low phosphorylation of CD3zeta, SLP76, Erk1/2, AKT, or S6 and lower calcium flux, it was reported that STAT phosphorylation triggered by interferons, IL2 or IL6, showed variations between Tregs and conventional CD4(+) T cells in magnitude or choice of preferential STAT activation but no general Treg signaling defect (Yan et al. 2015). Even though, Innovative drugs targeting Tregs were passionately developed. A tLyp1 peptide-conjugated hybrid nanoparticle was designed to enhance the effect of imatinib in downregulating Treg cell suppression through inhibition of STAT3 and STAT5 phosphorylation. Combination of this nanoparticle drug with present immunotherapy agents was proposed to magnify positive outcomes. Promisingly, prolonged survival rate, enhanced tumor inhibition, reduced intratumoral Treg cells, and elevated intratumoral CD8(+) T cells against tumor were observed when combined with checkpoint-blockade by using anti-CTLA-4 antibody (Ou et al. 2018).

It is necessary to highlight some T cells that were shown to be distinct in relevance with tumor immune response. Defects of invariant natural killer T cells (iNKT) have been acknowledged in human and mouse cancers, numerically and functionally, resulting in a defect in IFN production in several malignancies. Normally, iNKT cells recognize glycolipids presented on CD1d molecules by dendritic and related cells,

leading to their activation and thereby regulating immune reactions. In prostate cancer, CD1d expressed by cancer cells partially activated iNKT cells, but inhibited the IL-12-induced STAT4 phosphorylation in a cell–cell contact dependent but CD1d-independent manner. Importantly, this defect could be reversed by the addition of both IL-12 and the exogenous CD1d ligand alpha-galactosylceramide, but not by IL-12 alone, both in vivo and in vitro (Nowak et al. 2010). In tumor-infiltrating T cells (TILs) in follicular lymphoma (FL) tumors, phosphorylation of STAT6 and STAT3 induced by IL-4-, IL-10-, and IL-21 was largely reduced, compared to other non-Hodgkin lymphoma TILs. By combining phosphoprotein-specific flow cytometry with several T cell markers, a signature of nonresponsive T cells was identified. These cells with CD4(+)CD45RO(+)CD62L(−) were also observed with differential expression of the inhibitory receptor PD-1. Further, the suppression signal was though to be received through PD-1, providing a possible route to combine PD-1 blockade with immunotherapy in patients with FL (Myklebust et al. 2013). In TILs in breast cancers, STAT1-Y701 phosphorylation was linked to positive outcome. Actually, not only tyrosine phosphorylation but also expression level were confirmed to be prognostic factors, as elevated expression of STAT1 target genes and markers were consistently detected (Tymoszuk et al. 2014). The natural killer (NK) group 2D (NKG2D) receptor, which displays on mouse and human NK cells, activates CD8(+) T cells and small subsets of other T cells. NKG2D(+)CD8(+) T cells play critical roles in both innate and adaptive immunity upon engagement with NKG2D ligands to eliminate tumor and infected cells. The CD28 activation was found to sustain activation of the tyrosine kinase Lck, which recruited and triggered Janus kinase/STAT3 signaling to phosphorylate STAT3, and in turn increases NKG2D expression. So, NKG2D induction on CD8(+) T cells exerts cytolytic activity against target tumor cells in vitro, as well as significantly improves the antitumor therapeutic effects in vivo in an NKG2D-dependent manner (Hu et al. 2016).

Interestingly, a series of studies found that STAT phosphorylation in cancerous cells was associated with the formation of cancer immunosuppression. In four out of five SCLC cells, phosphorylation of STAT1/3 and expression of surface HLA class I antigen and TAP1 and TAP2 mRNA were triggered by IL-27. The one cell line resistant to IL-27, NCI-H146 showed responses to IFN-gamma, with upregulation of HLA class I as well as PD-L1. The distinction of NCI-H146 was further investigated, showing a low level of IL-27RA and GP130 receptor chains, the latter is shared in IL-27R and IL-6R complexes (Carbotti et al. 2017). The induction of STAT phosphorylation by IL-27 was then proved in more cancer cell line of different tissue origins. Consistently, IL27 leads to STAT1 phosphorylation and recapitulates an IFN-gamma-like response in the microarray analyses, with upregulation of genes involved in antiviral defense, antigen presentation, and immune suppression (Rolvering et al. 2018). Similarly, chronic lymphocytic leukemia (CLL) patients treated with ibrutinib displayed reduced interleukin (IL)-10 production, which was also linked to suppression of STAT3 phosphorylation (Kondo et al. 2018).

15.3.4 EGFR Phosphorylation Promotes PD-L1 Expression

Epidermal growth factor receptor (EGFG) is a well-known membrane protein that transduces growth signal and promotes cell growth. Gefitinib, an inhibitor of EGFR, was used to restrain growth of cancer cells in patients by suppressing downstream MAPK signaling. It has been acknowledged before the usage of gefitinib has influence on expression of PD-L1. EGFR tyrosine kinases phosphorylation inactivates GSK3beta to stabilize PD-L1 in breast cancer. Gefitinib, on the contrary, destabilizes PD-L1, so as to enhance antitumor immunity, as shown in syngeneic mouse models (Li et al. 2016a, b). Consistently, another study focusing on PTMs of PD-L1 found out that treatment of the epidermal growth factor (EGF) would induce tyrosine phosphorylation, together with acetylation and ubiquitination of PD-L1 (Horita et al. 2017).

Blockade of PD-L1 has been exploited clinically as a therapy to BSCLC. However, the efficacy is limited. Recent analysis has pointed out that patients with EGFR mutations tend to be less effective to anti-PD-L1 treatment. By comparing PD-L1 expression in cancer cells before and after acquisition of resistance to EGFR tyrosine kinase inhibitor (EGFR-TKI), it was demonstrated that PD-L1 expression was positively related to EGFR tyrosine kinase phosphorylation. Furthermore, after acquisition of resistance to EGFR-TKI, EGFR drastically promotes PD-L1 expression (Suda et al. 2017). In addition, another study in NSCLC cells revealed association between the EGFR-induced PD-L1 expression and phosphorylation of AKT and ERK, with increased protein levels of phospho-IkappaBalpha (p-IkappaBalpha) and hypoxia-inducible factor-1alpha (HIF-1alpha). Additionally, ectopic expression or depletion of EGFR mutants and treatment with EGFR pathway inhibitors targeting MEK/ERK, PI3K/AKT, mTOR/S6, IkappaBalpha, and HIF-1alpha indicated strong accordance among protein levels of PD-L1, p-IkappaBalpha, and HIF-1alpha in NSCLC cells. Moreover, immunohistochemical analysis revealed obviously increased protein levels of p-IkappaBalpha, HIF-1alpha, and PD-L1 in NSCLC tissues with EGFR mutants compared with tissues carrying WT EGFR. Clinical NSCLC tissues with either p-IkappaBalpha or HIF-1alpha positive staining were more likely to possess elevated PD-L1 expression compared with tissues scored negative for both p-IkappaBalpha and HIF-1alpha (Guo et al. 2019). The regulation of PD-L1 by EGFR, with present evidence, is more complicated than it was thought to be.

15.3.5 PD-L1 Expression Is Regulated Through Phosphorylation of MTOR Pathways

Both TUSC2 and rapamycin decreased p70 and SK6 phosphorylation, suggesting that TUSC2 and rapamycin share the same mTOR target. Results have shown that the downregulation of PD-L1 expression was mediated by inhibition of mTOR pathway in NSCLC cells. In addition, the IFN-gamma-induced PD-L1 overexpression was

shown to be abolished by overexpression of TUSC2, indicating a add-on effect of TUSC2 to immune checkpoint-blockade therapy (Cao et al. 2017).

15.3.6 Phosphorylation of Cell Cycle Regulators and Immune Modulation

The molecular control system of cell cycle, majorly including cyclins and cyclin-dependent kinases (CDKs) are regulated by a variety of signaling pathways, maintaining a cellular adaptation to environmental changes. Here listed are some claimed interaction between cell cycle control factors and immune activities.

PD-1 expressed on T cells is known to be an inhibitory factor in activation and proliferation of T cells. It was hypothesized that PD-1 ligation intervenes the molecular control of cell cycle. Supportively, PD-1 was found to block cell cycle progression through the G (Singh et al. 2017) phase by suppressing transcription of SKP2 through PI3K/AKT and MEK/ERK signaling. SKP2 encodes a component of the ubiquitin ligase SCF, and SCF degrades p27(kip1), an inhibitor of CDKs. Thus, in T cells stimulated through PD-1, Cdks were not activated, and two critical Cdk substrates were not phosphorylated. In a word, PD-1 signaling inhibits CDK phosphorylation. On top of that, retinoblastoma (RB) gene product thus E2F target genes were consequently suppressed. PD-1 also inhibited phosphorylation of the transcription factor Smad3. Taken together, these events lead to increased expression of cell cycle checkpoint protein and decreased Cdk-activating phosphatase (Patsoukis et al. 2012). CDK8 was found to be related to NK cell activities that may promote immunotherapy. Specifically, CDK8 phosphorylates STAT1 on S727, which inhibits NK cell cytotoxicity (Putz et al. 2014).

On the other hand, cell cycle associated factors may regulate the expression of PD-L1 as well. Inhibition of CDK4 and CDK6 (hereafter CDK4/6) in vivo increases PD-L1 protein levels by impeding cyclin D-CDK4-mediated phosphorylation of speckle-type POZ protein (SPOP) and thereby promoting SPOP degradation by the anaphase-promoting complex activator FZR1. Loss-of-function mutations in SPOP compromise ubiquitination-mediated PD-L1 degradation, leading to increased PD-L1 levels and reduced numbers of tumor-infiltrating lymphocytes in mouse tumors and in primary human prostate cancer specimens. Notably, combining CDK4/6 inhibitor treatment with anti-PD-1 immunotherapy enhances tumor regression and markedly improves overall survival rates in mouse tumor models (Zhang et al. 2018a, b, c). It was just mentioned above how PD-1 suppressed RB gene products, but most recently, RB was found to cut down on the expression of PD-L1 after radiotherapy. Mechanistically, RB interacts with NFkappaBprotein p65, which relies on CDK4/6-mediated serine-249/threonine-252 (S249/T252) phosphorylation of RB. Knockdown of RB or Inhibition of CDK4/6 could significantly upregulate PD-L1 expression, while expression of an RB-derived S249/T252 phosphorylation-mimetic

peptide suppresses radiotherapy-induced upregulation of PD-L1 and augments therapeutic efficacy of radiation in vivo. These findings suggest that RB-NF-kappaB axis can be exploited to overcome cancer immune evasion triggered by conventional or targeted therapies (Jin et al. 2019).

15.3.7 Phosphorylation of Some Pathways Regulates PD-L1 Expression at RNA Level

Some other pathways are shown to regulate PD-L1 expression at RNA level. For example, RAS signaling was reported to modulate the AU-rich element-binding protein tristetraprolin (TTP) through downstream MEK signaling. In this way, TTP was phosphorylated and inhibited by MK2, which consequently stabilized PD-L1 mRNA and enhanced PD-L1 expression (Coelho et al. 2017). Up to date, a potent, clinical compound that inhibits eIF4E phosphorylation, eFT508, was detected to suppress PD-L1 translational capacity in tumor cells, restraining the aggressive and metastatic characteristics of MYC(Tg); KRAS(G12D) tumors (Xu et al. 2019). Immune checkpoint modulation at RNA level is barely explored and documented, leaving an unknown area where treasure may be buried.

15.4 Switches of PD-1/PD-L1: Phosphorylation and More

Phosphorylation as a PTM, directly regulates PD-1/PD-L1 on different levels. PD-L1 as a typical type of membrane protein, transduces extracellular signals through its tyrosine kinase phosphorylation. Recent studies suggested that glycosylation, phosphorylation, ubiquitination, sumoylation, and acetylation play important roles in the regulation of PD-L1 protein stability (Wang et al. 2018), translocation and protein–protein interactions (Hsu et al. 2018). PTMs of PD-1/PD-L1 are drawing accumulating attention, and among them, phosphorylation is quite important.

15.4.1 Phosphorylation of PD-1/PD-L1 Determines

15.4.1.1 PD-1 Phosphorylation: Orientation of Immune Cells

Two decades ago, it was first revealed that human PD-1 tyrosine phosphorylation, putatively on its cytoplasmic tail signal transduction motif, stimulated by TPA on Jurkat cells, regulates activation and differentiation of T-lymphocytes (Vibhakar et al. 1997).

A research focusing on spinal cord injury (SCI) revealed that macrophage/microglial polarization towards M1 phenotype was inhibited by PD-1 signaling (Yao et al. 2014). To identify the effect of PD-1 on macrophage polarization, a PD-1(−/−) murine model was built and zymosan was used to induce inflammation. As a result, PD-1(−/−) mice displayed with severe peritonitis and more abundant infiltration of M1 macrophages, especially monocyte chemotactic protein-1 (MCP-1) compared with wild-type ones. Mechanistically, phosphorylation of STAT1 and NF-kappaB p65 was found boosted while phosphorylation of STAT6 was suppressed. Furthermore, when treated with zymosan, the recruitment of SHP-2 to PD-1 receptor/ligand was inhibited through tyrosine phosphorylation of PD-1, so that M1 type cytokine secretion was diminished. The presence of PD-1 seems to inhibit macrophage polarization towards M1 phenotype instead of M2 phenotype (Chen et al. 2016).

After all, the role of PD-1/PD-L1 on immune cell development in tumor is of more importance and yet not well understood.

15.4.1.2 PD-L1 Phosphorylation: Stabilization or Destabilization

The stabilization of proteins is closely related to PTMs. Unproper phosphorylation or dephosphorylation may cause abnormal folding, transporting or eliminating of protein molecules.

Glycogen synthase kinase 3beta (GSK3beta), which is known to facilitate E3 ligase recognition to a wide range of proteins (Frame and Cohen 2001), can interact with PD-L1 and result in its phosphorylation-dependent proteasome degradation by the E3 ligase beta-TrCP. The process is closely related to another PTM, glycosylation (Li et al. 2016a, b). Further study on the interaction between GSK3β and PD-L1 revealed that PD-L1 is phosphorylated by GSK3β at two sites of T180 and S184 which further leads to ubiquitination and degradation of PD-L1 in the cytoplasm (Gu et al. 2019). Another study proposed a new way of cooperation between phosphorylation and glycosylation. PD-L1 phosphorylation on S195, which takes place in the ER, induces abnormal glycosylation, leading to disruption of PD-L1 transportation. Accumulation in ER happens then, followed by ER-associated protein degradation (ERAD) (Cha et al. 2018). Figure 15.4 schematically describes this mechanism.

15.4.1.3 PD-1 Phosphorylation: SHP-1 or SHP-2?

As a fast molecule switch, phosphorylation od PD-L1 is deeply involved in downstream signaling activities.

As mentioned above, PD-1 and CTLA-4 inhibit PI3K/AKT activation via different routes. Actually, the suppression is dependent upon the immunoreceptor tyrosine-based switch motif (ITSM) located in its cytoplasmic tail (Parry et al. 2005).

A very typical and widely recognized downstream event of PD-1 tyrosine phosphorylation is recruitment of Src homology region 2-containing protein tyrosine

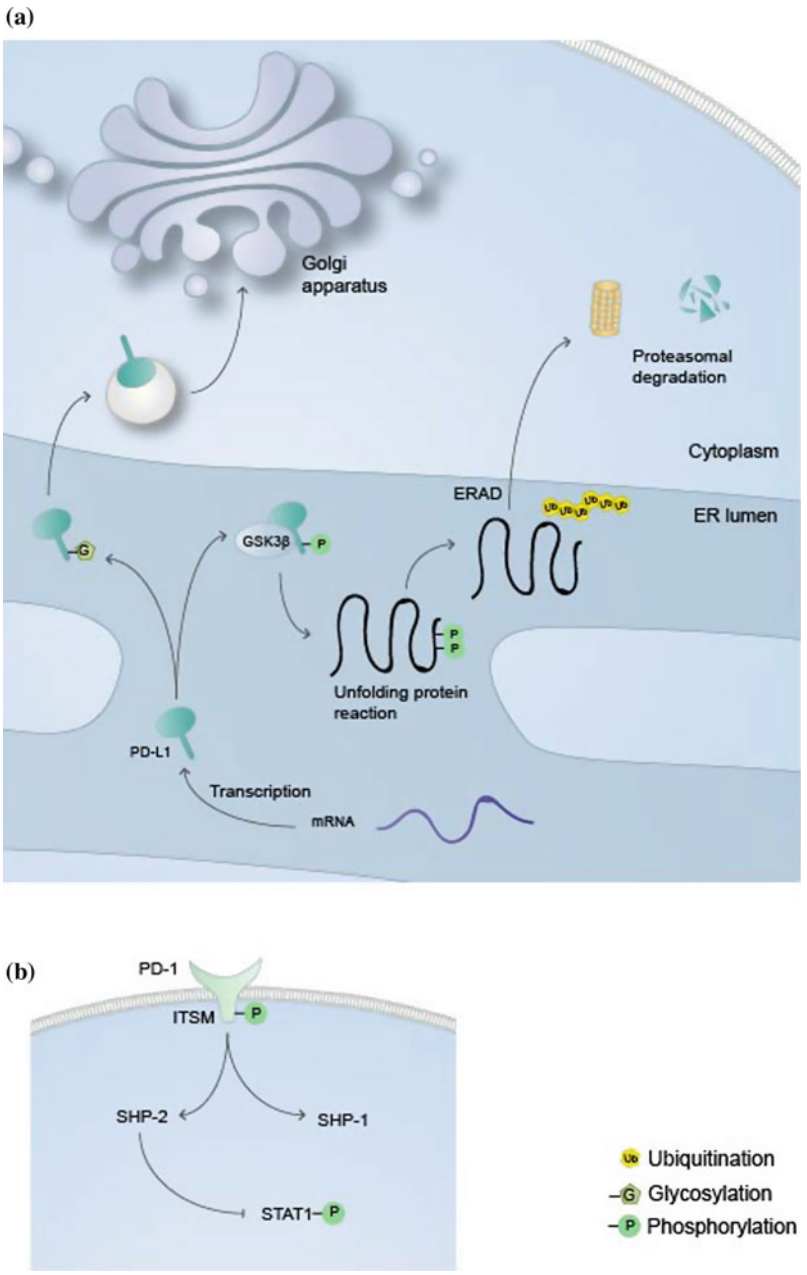

Fig. 15.4 **a** The process of PD-L1 ubiquitination initiates from phosphorylation in ER, which intervenes glycosylation of PD-L1, as proper glycosylation fundamentally determines transportation and localization of this membrane protein; phosphorylated PD-L1 is accumulated in ER and thus ubiquitinated and degraded through ERAD, as schematically shown. **b** Downstream factors recruited by PD-1 ITSM motif, especially SHP-1 and SHP-2, bring about dramatically diverse events

phosphatase (SHP)-1 and SHP-2 (Riley 2009). The C-terminal ITSM and the N-terminal immunoreceptor tyrosine-based inhibitory motif (ITIM) of PD-1 was compared by constructing phosphorylated peptide accordingly. As a result, the phosphorylated peptide containing ITSM motif was shown to be a docking site for both SHP-2 and SHP-1, while the other one only associated with SHP-2 (Sheppard et al. 2004). SHP-1 and SHP-2 each contain two SH2 domains, a classic protein tyrosine phosphatases (PTPs) domain and a C-terminal tail harboring tyrosine phosphorylation sites (Pao et al. 2007). They activate the signal transduction pathways of several growth factors and cytokines (Liu et al. 2017a, b). The dephosphorylation activity of SHP proteins is negatively regulated by their own SH2-binding domain. When SHP proteins are gathered, one SHP encounters another SH2-binding motif on a cytoplasmic tail, and the attached SHP molecules are reactivated to dephosphorylate nearby sequences recognized by their PTP domain (Tsui et al. 2006). Many receptors including PD-1 recruit both SHP-1 and SHP-2, leading to drastically different outcomes and in vivo functions. Thus, the cis factors that recruit SHP-1 and SHP-2 to a receptor are likely to be similar, but the downstream targets of SHP-1 and SHP-2 are likely to be different (Pao et al. 2007).

It was confirmed later that the recruitment and phosphorylation of SHP-1 and SHP-2 mediated the PD-1/PD-L1-induced inhibition of T lymphocytes proliferation (Zhao et al. 2015). Moreover, the role of PD-1 in modulating macrophage polarization was also linked to the phosphorylation and recruitment of SHP-2 (Chen et al. 2016). Interestingly, another study seemed to bring about an inhibitory effect of SHP-2 on the expression of phosphorylated STAT1. In addition, SHP-2 depletion also gave rise to human leukocyte antigen (HLA)-ABC and programmed death-ligand 1 (PD-L1), but this effect could be abolished by pretreatment of JAK2 inhibitor or MEK inhibitor. Together, these connections pointed out a relation between SHP-2 depletion and T cell activation, which was further confirmed by coculture of allogeneic healthy donor peripheral blood monocytes (PBMC) with SHP2 siRNA pretreated PCa cell lines (Liu et al. 2017a, b).

15.4.2 Other Modifications Closely Related to Phosphorylation

15.4.2.1 Glycosylation: Followed by Ubiquitination

The phosphorylation-mediated glycosylation is closely related to ubiquitination and degradation of PD-L1, which is summarized before (Wang et al. 2018) and also above in the stabilization part of PD-L1. Here is the detailed process. The story started with the antitumor effect of metformin by inducing endoplasmic reticulum-associated degradation of PD-L1 (Cha et al. 2018). Mechanistically, AMPK is stimulated by metformin and binds directly to PD-L1. The AMPK-PD-L1 complex, colocalizing

the ER marker (HSP90B1) but not the Golgi marker (TNG46), is then phosphorylated on S195 within the consensus phosphorylation motif (LXRXXSXXXL) of AMPK80 on the extracellular domain of PD-L1 (Cha et al. 2018). Thereafter, the mannose trimming of PD-L1 is defected, thereby causing excessive mannose-rich glycan attachments without the precursor glycan (Glc3Man9GlcNAc2) or trimmed Man8GlcNAc2, leading to its ER retention. Consequently, deficiency of proper glycosylation triggers the unfolding protein response (UPR) and bindings of the components of the endoplasmic reticulum-associated protein degradation (ERAD) complex for ubiquitination and retro-translocation from the ER into the cytoplasm and subsequently degraded by the cytoplasmic proteasome (Cha et al. 2018). Further evidence supplemented the role of ERAD E3 ligase HRD1 in PD-L1 degradation. Tests on patients' tissue samples confirmed the effect of downregulating PD-L1 by metformin (Cha et al. 2018).

Repeated mention and reference of this single research concerning phosphorylation-dependent, glycosylation-related ubiquitination and degradation of PD-L1 reflects the importance of this study, and urgent lack of other studies in this field. PTM related stabilization and degradation of immune checkpoints is a Pandora Box to open, which may bring about as many hopes as challenges.

15.4.2.2 Acetylation: More to Explore

In addition to phosphorylation and glycosylation as discussed above, Horita and colleagues reported that PD-L1 is also subjected to acetylation, upon EGF stimulation (Hsu et al. 2018). The induction of PD-L1 tyrosine phosphorylation and acetylation by EGFR signaling is mild but significant, though no more exploration was performed towards the effect of PD-L1 acetylation (Horita et al. 2017).

However, histone acetylation, a well-documented epigenetic modification, was somehow given attention on regulation of PD-1/PD-L1 in tumor immunity. Histone deacetylases (HDAC) have been targeted in the treatment of some hematologic malignancies and gained clinical success before (Khan and Tomasi 2008; Woods et al. 2015). The effect was shown to be related to not only direct tumor cell toxicity but also immune modulation. Specifically, antitumor immune response was boosted by inhibiting HDAC, especially in melanoma (Woods et al. 2013). Continuous work by Woods and colleagues further revealed that class I HDAC inhibitors upregulated the expression of PD-L1 and, to a lesser degree, PD-L2 in melanomas. A variety of HDAC inhibitors screened with human and murine cell lines and patient tumors show effect of increasing expression of the two ligands. In vivo experiment, combining HDAC inhibitors and PD-1 blockers turned out to be positive and promising, with suppressed tumor growth and enhanced survival rate (Woods et al. 2015).

Very recently, two reports brought better insights into the epigenetic modification of PD-L1. One of them emphasized that epithelial to mesenchymal transition (EMT) was reported to be correlated with PD-L1 upregulation. Instead of depending on promoter CpG methylation, the EMT-associated overexpression of PD-L1 was more

likely to be caused by post-translational histone modifications, specifically acetylation (Darvin et al. 2019). The other one, on the other hand, reported that inhibiting HDAC3 stimulated histone acetylation and thus initiated recruitment of bromodomain protein BRD4 at the promoter region of PD-L1 gene, leading to activation of its transcription in B cell lymphoma. An indirect way of HDAC inhibition-stimulated PD-L1 transcription was by reducing DNA methyltransferase 1 protein levels on dendritic cells in the tumor microenvironment. Again, combination of HDAC3 inhibition and PD-L1 blockade was suggested to provide powerful suppression on tumor growth, supported by syngeneic murine lymphoma model experiment (Deng et al. 2019).

15.5 Phosphorylation and CTLA-4: Where and How?

CTLA-4 (CD152), a high-avidity receptor for CD80 and CD86, is a powerful regulator of T cell activation. Researches on its tyrosine phosphorylation can be dated back to 1997. Nowadays, it has been used as a target in ICBT, displaying promising though limited clinical benefits.

15.5.1 Tyrosine Phosphorylation of CTLA-4 and Downstream Events

Back in 1996, a wide range of T cell activities was reported to be regulated by CTLA-4, including IL-2 production, cell proliferation, and transition from G0/G1 to S phase of the cell cycle. Accordingly, the mechanism behind was thought to be related to tyrosine phosphorylation, which could be promoted by phorbol ester phorbol 12-myrislate 13-acetate (PMA) (Chambers and Allison 1996). Over the years, the topic of tyrosine phosphorylation in CTLA-4 regulation has been pushed forward so far, that understanding of the mechanism becomes more and more complicated.

15.5.1.1 Localization of CTLA-4: Closely Related to Phosphorylation

In 1997, two reports both reported that CTLA-4 phosphorylation determined its localization and signal transduction. The transportation and recycle of CTLA-4 between intracellular vesicles and cell-surface was reported to be regulated by Y165 phosphorylation. The mu2 subunit of AP-2, the clathrin-associated complex found in plasma membrane-associated coated pits interacted with the cytoplasmic tail of CTLA-4, and the interaction was preferential when residue 165Y was non-phosphorylated. Thus, clathrin-mediated endocytosis of CTLA-4 was induced, decreasing inhibitory signal

transduction (Shiratori et al. 1997). Notably, the interaction between mu2 and CTLA-4 was dependent on sequence 161TTGVY in CTLA-4, instead of the N-terminal, a previously identified SH2 binding motif, 165YVKM. Mu2 interacted preferentially with CTLA-4 when residue 165Y was non-phosphorylated, whereas a PI3 kinase SH2 domain interacted preferentially when 165Y was phosphorylated (Bradshaw et al. 1997). Moreover, tyrosine kinases responsible for CTLA-4 phosphorylation were investigated (Saito 1998).

In addition to its endocytosis, endocellular localization was also detected to be regulated by phosphorylation. CTLA-4 resides primarily and mostly in the trans-Golgi network (TGN), where the effect of Src family was tested by transfecting Lck or Fyn, resulting in activated recruitment of PI3K and further pathways. These results suggested an endocellular signaling potency of CTLA-4 (Hu et al. 2001). The phosphorylation-dependent transportation and signaling function are demonstrated in Fig. 15.5.

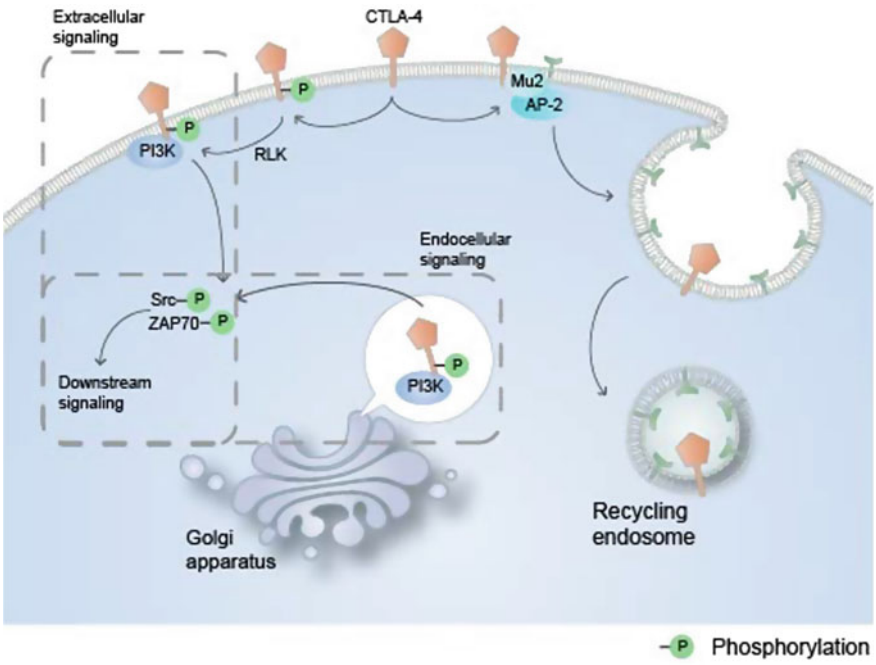

Fig. 15.5 Localization of CTLA-4 is closely related to phosphorylation. Phosphorylated CTLA-4, on the membrane or in the plasma, functionally transduces signals, while dephosphorylated CTLA-4 is silenced and shelved in recycling endosome

15.5.1.2 Tyrosine Kinases: Where and How Do They Interact with CTLA-4?

Further studies focusing on the activating phosphorylation suggested that the activation and transportation of CTLA-4 may be regulated through distinct pathways. A series of tyrosine kinases including Fyn, Lyn, and Lck were detected. The interaction of CTLA-4 with these tyrosine kinases are mostly located on Y165 and Y182. Importantly, the SHP-2 association with CTLA-4 on its SH2 domain was reported to dependent on Fyn (Miyatake et al. 1998). Rlk (resting lymphocyte kinase) was detected to phosphorylate CTLA-4 at the YVKM motif. Consistently, Rlk was known to promote the binding of the SH2 domains of PI 3-kinase to the receptor (Schneider et al. 1998). Another report revealed association between Src kinases Fyn and Lck, and CTLA-4 phosphorylation. Transfection of Fyn or Lck into Jurkat cells promoted CTLA-4 phosphorylation on Y201 and Y218, and thus accumulation of CTLA-4 on cell surface (Chuang et al. 1999). Strangely, they detected recruitment of the tyrosine phosphatase SHP-2, but not phosphatidylinositol 3-kinase upon CTLA-4 phosphorylation induced by Fyn. While Lck-induced phosphorylation of CD28 resulted in the recruitment of phosphatidylinositol 3-kinase, but not SHP-2 (Chuang et al. 1999).

As investigation in CTLA-4 signaling went on, different views of CTLA signaling were proposed. To figure out the functional motif of CTLA-4, various mutants of it was transfected into murine T cell clones. As a recult, the membrane-proximal region of CTLA-4 instead of the YVKM motif was required for CTLA-4 to mediated the suppression of T cell activation (Nakaseko et al. 1999). Moreover, the phosphorylation of Y165 and Y182 was confirmed to pursue cell surface retention of CTLA-4, though through ZAP-70 instead of Src kinases. More importantly, its relevance to T cell suppression was denied (Baroja et al. 2000). Consistently, the phosphorylation of CTLA-4 tyrosines in YVKM and YFIP motif was later denied the effect of recruiting SHP-2. Instead, an indirect association between CTLA-4 and SHP-2 through PI3K pathway was anticipated (Schneider and Rudd 2000). Meanwhile, another signaling factor, JAK2, was claimed to directly associate with a box 1-like motif in the cytoplasmic tail of CTLA-4 and phosphorylate Y165 residue in HUT 78 T cell line (Chikuma et al. 2000). Another twist occurred on the role of YVKM phosphorylation in CTLA-4 function, when an optimal CTLA-4 function in blocking TCRzeta or combined TCRzeta/CD28 signaling was induced by the presence of YVKM. Schneider and colleagues claimed that, former studies neglected the difference between application of soluble and immobilized antibodies, leading to controversial results (Schneider et al. 2001). More results provide evidence that SHP-2 domain-containing tyrosine phosphatase 1 (TP1), a protein tyrosine phosphatase, is a negative regulator of multiple signaling pathways in hemopoietic cells (Guntermann and Alexander 2002).

In recent years, researcher found some other characteristics about CTLA-phosphorylation. One study on crystal structure of CTLA-4 revealed that the binding of B7-2, though not highly specific, may trigger CTLA-4 tyrosine phosphorylation (Yu et al. 2011). Another study investigated the role of PAG in T cell transformation. While PAG depletion enhanced Src kinase activity and augmented proximal

T cell receptor signaling as expected, the T cell activity was not restored. Further study revealed a Fyn-dependent hyper-phosphorylation of CTLA-4 behind this phenomenon. Hyper-phosphorylated CTLA-4 caused re-localization of SHP-1 to lipid rafts, which is possibly responsible for the non-responsiveness of T cells (Smida et al. 2013).

15.5.2 Other Pathways Phosphorylated that Regulates CTLA-4

15.5.2.1 CTLA-4, and CD28

Very early studies have concluded that CTLA-4 ligation regulates T cell activation by inhibiting the T cell receptor-mediated signals. Later, findings propose that the major impact of CTLA-4 ligation is inhibition of signals mediated by CD28 (Olsson et al. 1999). The association between CD28 and CTLA-4 was soon widely investigated. It was reported that the cytoplasmic domains of both CTLA-4 and CD28 can associate with members of the PP2A family of serine/threonine phosphatases, suggesting a novel mechanism for modulating the intracellular signal transduction pathways associated with cell activation (Chuang et al. 2000). Furthermore, it was proposed that regulatory subunit of PP2A (PP2AA) also interacts with the cytoplasmic tail of CTLA-4. Interestingly, TCR ligation induces tyrosine phosphorylation of PP2AA and its dissociation from CTLA-4 when colligated. The association between PP2AA and CTLA-4 involves a conserved three-lysine motif in the juxtamembrane portion of the cytoplasmic tail of CTLA-4. Mutations of these lysine residues prevent the binding of PP2AA and enhance the inhibition of IL-2 gene transcription by CTLA-4, indicating that PP2A represses CTLA-4 function (Baroja et al. 2002).

In addition, it was reported that, a potent analog of Thal, immunomodulatory drug (IMiD), which triggers tyrosine phosphorylation of CD28 on T cells partially overcomes the inhibitory effect of CTLA-4 (LeBlanc et al. 2004).

15.5.2.2 CTLA-4 and Cbl-B Function

The Casitas-B-lineage lymphoma (Cbl) family of proteins consists of an N-terminal tyrosine kinase binding (TKB) domain that encompasses a variant SH2 domain, a RING finger, a C-terminal proline-rich region with potential tyrosine phosphorylation sites, and a ubiquitin-associated domain (UBA). It is now understood that Cbl functions as an E3 ubiquitin ligase with a RING finger that recruits ubiquitin-conjugating enzymes (E2) and a TKB domain that recognizes target proteins for ubiquitin conjugation (Liu et al. 2014; Thien and Langdon 2005). Gene targeting in mice has shown that Cbl-b is involved in pivotal events of lymphocyte activation

(Bachmaier et al. 2000; Chiang et al. 2000), indicating a critical role of Cbl-b in the maintenance of a balance between immunity and tolerance.

Previous studies found that, c-cbl was rapidly tyrosine phosphorylated, when stimulating signal was transduced through TCR. C-cble was also shown to associate with SH2/SH3 domain-containing adaptors such as Grb2, Crk, and Crk-L, which was involved in guanine nucleotide exchange factors specific for the Ras family. The SH2/SH3-containing protein Vav also contains a guanine nucleotide exchange factor domain, and Vav has a crucial role in thymocyte development and activation of peripheral T cells following stimulation through the TCR. Not surprisingly, the interaction between Vav and c-Cbl was soon detected in murine T cells. However, this interaction seems to be promoted by CTLA-4 deficiency, as phosphorylation level of c-Cbl was lifted (Marengere et al. 1997).

In a study focusing on CD28 related integrin interaction, it was revealed that CD28-related surface antigen CTLA-4 also blocked integrin interaction, accompanied with intact PI3K activation. However, CD28 mutation could impair cbl phosphorylation together with PI3K activation, as reported (Zell et al. 1998). Years later, it was found that transforming growth factor-beta (TGF-beta) secretion, CTLA-4 levels, Cbl-b level were associated with immune hyporesponsiveness. Rapid phosphorylation of ERK was shown to correlates with increased levels of CTLA-4 and Cbl-b (Leng et al. 2006). Another group of researchers came to conclusion that CD28 and CTLA-4 signaling control Cbl-b protein expression, which is critical for T cell activation and tolerance induction (Li et al. 2004; Zhang et al. 2002). Later, mechanism was proposed that SHP-1 was recruited by CD3 stimulation to prevent cbl-b tyrosine phosphorylation (Xiao et al. 2015).

15.5.2.3 Downstream Activating Pathways and Cytokine Production of CTLA-4

The PI3K/AKT pathway is an important downstream signal transduction route for CTLA4, as already discussed earlier under the title of PI3K/AKT signaling (Chen et al. 2013; Knieke et al. 2012; Schneider et al. 2008). The same is the ERK pathway (Kwon et al. 2004). In this part, some distinct downstream activities will be discussed.

The inhibitory effect of CTLA-4 on T cell activity was earlier mentioned to be mediated by T cell receptor (TCR)/CD3 and CD28 costimulation, which was widely documented. Additionally, a study revealed the role of the adhesion molecule lymphocyte function-associated antigen-1 (LFA-1) in murine CD4+ T cells, which was related to CTLA-4. Results show that, except for CD3/CD28 costimulation, CTLA-induced downregulation of IL-2 was also induced by costimulated by anti-CD3 and anti-LFA-1 monoclonal antibodies (mAbs). Moreover, it was discovered that, CTLA-4 engagement negatively affects Ca^{2+} mobilization and NF-AT activation, which could be induced by LFA-1 engagement alone. Phospholipase C (PLC) gamma1 phosphorylation was also dampened within minutes after CTLA-4 engagement (Gatta et al. 2002).

Innovatively, a similarity between CTLa-4 and Itch deficient mice was proposed, based on a massive reduction in the overall ubiquitination of proteins induced by CTLA deficiency. Mechanistically, it was suggested that CTLA-4 signaling caused de-phosphorylation and therefore activation of the ubiquitin ligase Itch and enhanced ubiquitination of the Itch target molecule JunB. Supportively, CTLA-4-mediated inhibitory effect, presented by mRNA accumulation of IFN-gamma and IL-4, was found completely abolished by knockdown of Itch (Hoff et al. 2010).

15.6 Phosphorylation and Other Immune Checkpoints

15.6.1 T Cell Immunoglobulin Mucin 3 (TIM-3)

TIM-3 is a T(H)1-specific type 1 membrane protein that belongs to the TIM family. It is involved in the regulation of CD4+ T cells, specifically the proliferation and tolerance induction of T(H)1 cells. Its ligand galectin-9 (Gal-9) can bind to the extracellular domain of TIM-3 and cause apoptosis of T(H)1 cells. Unlike other TIM family members like TIM-1, which is expressed in renal epithelia and cancer, TIM-3 is so far limited to be expressed in neuronal or T cells, presenting excellent specificity to be potential therapeutic target (van de Weyer et al. 2006).

15.6.1.1 Phosphorylation of TIM-3

By cloning TIM-3 in from a liver carcinoma cell line, a highly conserved tyrosine in the intracellular tail, Y265 was identified. TIM-3 was then discovered to be phosphorylated on Y265 in vivo by the interleukin inducible T cell kinase (ITK), a kinase which is located in close proximity of the TIM genes on the allergy susceptibility locus 5q33.3. Binding of Gal-9 was later shown to cause a series events via phosphorylation of Y265. Surrounding the Y265 site is a conserved SH2 biding domain, making up to a functional signaling motif that may be a target site for pharmacological intervention (van de Weyer et al. 2006). Later, the downstream activities of phosphorylated TIM-3 were revealed. T cell anergy was demonstrated to be caused by TIM-3 phosphorylation on its cytoplasmic tail via secretion of Bat3 and possible recruitment of Fyn (Davidson et al. 2007).

15.6.1.2 Phosphorylation and TIM-3

Downstream pathways of TIM-3 were demonstrated in many different aspects. The ligand of TIM-3, Gal-9 was shown to have dual effects. On the one hand, its binding to TIM-3 was shown to induce T cell apoptosis and tolerance, acknowledged to be a potential target for treating autoimmune diseases (Chou et al. 2009; Koguchi et al.

2006; Zhu et al. 2005). On the other hand, the ligation of TIM-3 was shown to activate innate immune response that could enhance pro-inflammatory (Anderson et al. 2007) and antitumor effect (Nagahara et al. 2008; Nobumoto et al. 2009). The dual effect was then proposed to be caused by the two distinct carbohydrate recognition domains (CRD) in the N- and C-terminal regions (Gal-9-N and Gal-9-C), which was shown to have different effects on T cells. By creating recombinant Gal-9 (Gal-9-NC) and homodimers containing either the NCRD (Gal-9-N) or the CCRD (Gal-9-C), Li and colleagues testify their hypothesis. Although these constructs have mutual effects of activating DCs and inducing T cell death, the Gal-9-N was much more prone to induce DCs activation and the Gal-9-C was more effective in causing T cell death. Specially, immune activation induced by Gal-9-N was proved to be activated by testing related pathways including enhanced phosphorylation of p38 and Akt and production of TNF-alpha and IL-6. Moreover, degradation of ikappaBalpha was significantly promoted by Gal-9-N but not Gal-9-C in both T cells and DCs (Li et al. 2011).

TIM-3 was also shown to have crosstalk with other checkpoint molecules. For example, mutual expression of TIM-3 with LAG3, CTLA4, and FOXP3 makes Treg cells highly efficient suppressors of Teff cells. While Tregs without TIM-3 expression suppress Th1 cells but not Th17 cells. It was further revealed that decreased STAT-3 expression and phosphorylation and reduced gene expression of IL10, EBI3, GZMB, PRF1, IL1Ralpha, and CCR6 were found in Th17 cells that were not efficiently suppressed (Gautron et al. 2014). In addition, TIM-3 was reported to have opposite effect with PD-1 on the regulation of ribosomal protein S6 (pS6). TIM-3 was shown to increase phosphorylated pS6, while PD-1 reduced it. Accordingly, TIM-3 expression did not necessarily suppressed T cell redundancy, providing more insight into anti-PD-1 immunotherapy (Li et al. 2016a, b). Indeed, in HNSCC, crosstalk between PD-1 and TIM-3 was emphasized. By blocking PD-1, upregulation of TIM-3 was detected, supporting compensatory downstream signaling, dependent on PI3K/AKT signaling, which potentially enabling escape from ICBT (Shayan et al. 2017).

TIM-3 was found highly expressed in osteosarcoma tissue. An osteosarcoma cell line MG-63 was transfected with Tim-3 siRNA. Results showed that intervention in TIM-3 expression led to inhibited proliferation and metastasis through decreased Snail and vimentin expression, increased E-cadherin level, and an increase in NF-kB p65 phosphorylation (Feng and Guo 2016). In vivo experiment in TIM-3 KO mice proved the connection between tumor progression and TIM-3 expression in liver cancer. Mechanistically, the hepatocyte-Tim-3 receptor was found to activate NF-kappaB phosphorylation, which in turn stimulated IL-6 secretion and STAT3 phosphorylation (Zhang et al. 2018a, b, c). In AML, TIM-3 was found to be over-expressed in Vgamma9Vdelta2 T cells, which can be further promoted by IL-21 treatment. Blocking Tim-3 increased the proliferation and the STAT phosphorylation in Vgamma9Vdelta2 T cells in response to IL-21, providing rationale to combine blockage of TIM-3 and treatment of IL-21 as a novel anti-AML strategy (Wu et al. 2019).

TIM-3's role was meanwhile investigated in other immune cells. The role of TIM-3/Gal-9 interaction was also shown to phosphorylate Erk1/2 in human mast

cells, leading to increased apoptosis, reduced degranulation, but enhance cytokine production at the same time (Kojima et al. 2014). Macrophage polarization was also shown to be modulated by TIM-3 via inhibiting phosphorylation of IRF3, a TLR-4 downstream transcriptional factor (Jiang et al. 2015).

15.6.2 Killer Cell Inhibitory Receptors (KIRs)

Killer immunoglobulin-like receptors (KIRs) are a family of regulatory cell surface molecules expressed on natural killer (NK) cells and memory T cell subsets. Their ability to prevent the formation of an activation platform and to inhibit NK cell activation is the basis of the missing self-model of NK cell function (Henel et al. 2006).

15.6.2.1 Phosphorylation of KIRs

Natural cytotoxicity and antibody-dependent cell cytotoxicity of NK cells and CD3/TCR dependent cytotoxicity of T cells can be blocked by recognition of class I MHC molecules on target cells by KIRs. The inhibitory effect was found to be dependent on phosphorylation of the cytoplasmic tail of KIR and subsequent recruitment of SHP-1. In vitro assay testified this mechanism and revealed that phosphorylation of KIR cytoplasmic tail was mediated by Lck. Further results suggested that PLC-gamma phosphorylation may be the subsequent even of recruited SHP1 (Cho et al. 1999). Another study revealed that the KIRs, also known as Ly49s in mise, once bind to ligands, are phosphorylated on its cytoplasmic immunoreceptor tyrosine-based inhibitory motifs (ITIMs), where SHP-1 and SHP-2 were recruited. Tus, cellular activation was induced by dephosphorylation of a series of critical substrates. On the contrary, some KIRs without the ITIM harbor a charged residue in their transmembrane domains that can interact with DAP12 signal transduction chain. DAP-12 possessed a cytoplasmic immunoreceptor tyrosine-based activation motif (ITAM) that can transduce signal into cells. Engagement of DAP12 with KIR leads to phosphorylation of DAP12 and activation of other kay substrates, including the Syk tyrosine kinase, phospholipase C, and c-Cbl. Consequently, MAPK cascade is induced by DAP12 activation, leading to degranulation and production of cytokines and chemokines (McVicar and Burshtyn 2001). In another study, the subsequent signal transduction of KIRs and killer cell activating receptor-associated protein (KARAP)/DAP12 was investigated. The signal transduced by KIR and KARAP/DAP12 further phosphorylated both JNK and ERK, but only JNK was activated in absence of KARAP/DAP12. It was further revealed that KARAP/DAP12 could promote both cytotoxicity and IFN-gamma production without TCR-derived signals. Evidently, KARAP/DAP12 converted KIR from a costimulatory molecule into a stimulatory molecule, proposing more problems to be proved and explored (Snyder et al. 2004).

15.6.2.2 Phosphorylation and KIRs

It was reported that expression of KIRs in renal cell carcinoma (RCC) infiltrating lymphocytes (TILs) inhibited the antitumor CD8(+) T cell lysis (Guerra et al. 2000). Later, the same research group investigated the impact of KIR CD158a on early steps of T cell activation. Results showed that during the TCR signal transduction, engagement of CD158a inhibited phosphorylation of ZAP-70 and LAD, lipid raft coalescence, and TCR/CD3 accumulation at the CTL/tumor cell interface. Furthermore, with CD158a intervening TCR signaling, the guanine exchange factor Vav was not phosphorylated, and no actin cytoskeleton rearrangement was observed (Guerra et al. 2002). More evidence supported that KIR is involved in TCR signaling. Engagement of KIR2DL2 by the ligand human leukocyte antigen (HLA)-Cw3 did not affect conjugate formation between CD4(+)KIR2DL2(+) T cells and superantigen-pulsed target cells or the development of mature immune synapses with lipid rafts. KIR2DL2 and the corresponding HLA-C ligand were initially recruited to the peripheral supramolecular activation cluster (pSMAC). Consequently, KIR2DL2 engagement did not inhibit the phosphorylation of early signaling proteins and T-cell-receptor (TCR)-mediated cytotoxicity or granule exocytosis. After 15–30 min, KIR2DL2 moved to the central supramolecular activation cluster (cSMAC), colocalizing with CD3. TCR synapses dissociated, and phosphorylated PLC-gamma1, Vav1, and ERK1/2 were reduced 90 min after stimulation. Gene array studies documented that the inhibition of late signaling events by KIR2DL2 affected transcriptional gene activation. Taken together, these results suggested that KIRs on memory T cells operate to uncouple effector functions by modifying the transcriptional profile while leaving granule exocytosis unabated (Henel et al. 2006).

15.6.3 CD137 (4-1BB)

CD137 was first identified in 1993, when Schwarz and colleagues isolated a full-length cDNA from activated human T cell leukemia virus type 1-transformed human T lymphocytes and performed sequence analysis to identify it as a new member of the human nerve-growth-factor receptor/tumor necrosis-factor receptor family and as the potential human homolog of the murine sequence, 4-1BB. As a feature of this family, this gene encodes three cysteine-rich motifs in the extracellular domain. Also, a transmembrane region and a short N-terminal cytoplasmic portion were identified that contain potential phosphorylation sites (Schwarz et al. 1993).

Later, 4-1BB was identified as a costimulation factor. The ligand for 4-1BB (4-1BBL; also called CD137L) was shown to express mainly on activated APCs such as dendritic cells, B cells, and macrophages, so as to activate T cells (Kim et al. 2011). It was reported to repress Smad2 phosphorylation induced by TGF-beta1, a immune-suppressing factor. In addition, the effect of 4-1BB was shown to be promoted by IL-12 but counteracted by IL-4 (Kim et al. 2005). Co-engagement of 4-1BB with TCR was shown to increase phosphorylation of signaling molecules

such as CD3epsilon, CD3zeta, Lck, the linker for activation of T cells, and SH2 domain-containing leukocyte phosphoprotein of 76 kDa (SLP-76). Other display of T cell activation induced by 4-1BB included redistribution of membrane molecules to create crosslink and functional domains. Thus activating TCR signaling pathways in CD8+ T cells (Nam et al. 2005). The Tumor Necrosis Factor (TNF) Receptor-associated factor-1 (TRAF1) adaptor protein plays an important role in lymphocyte survival and it has been shown to be phosphorylated on Serine 139, consequently inhibiting NF-kappaB activation downstream of TNFR2 when expressed in HeLa cells. Phosphorylated TRAF 1 was shown to coordinate signaling events downstream of 4-1BB including degradation of IkappaBalpha (Oussa et al. 2013).

On top of inducing T cell activation and expansion via 4-1BB ligation, it was detected that engagement of 4-1BBL can deliver a reverse signal into cells. In macrophages expressing 4-1BBL on its surface, transmembrane protein 126A (TMEM126A) was found to bind to 4-1BBL, leading to the production of pro-inflammatory cytokines via phosphorylation of Erk, p38 MAPK and Jnk (Kim et al. 2011). In addition, CD137L reverse signaling-induced apoptosis occurred via activation of the intrinsic pathway and depended on phosphorylation of JNK in NSCLC, indicating that CD137L has potential as a novel therapeutic target (Qian et al. 2015).

4-1BB is also widely used as a costimulatory factor expressed on CAR-T cells to promote treat efficacy, the same is CD28. Evidently, CD28/CD3zeta CARs were shown to act faster and larger magnitude changes in protein phosphorylation, which correlated with a Teff-like phenotype and function. In contrast, 4-1BB/CD3zeta CAR-T cells preferentially expressed T cell memory-associated genes and exhibited sustained antitumor activity against established tumors in vivo. The difference of CAR-T efficacy is largely dependent on the costimulatory factors designed on cell surface. Thus, it was proposed that tailoring CAR design based on signal strength may lead to improved clinical efficacy and reduced toxicity (Salter et al. 2018).

15.6.4 Glucocorticoid-Induced Tumor Necrosis Factor Receptor (GITR)

Glucocorticoid-induced tumor necrosis factor receptor (GITR, also calledTNFRSF18) is a member of the tumor necrosis factor receptor superfamily. It was initially identified as a glucocorticoid-responsive gene in a murine hybridoma T cell line (Nocentini et al. 1997). GITR is expressed on macrophages, B cells (Ji et al. 2004; Ronchetti et al. 2004; Shimizu et al. 2002; Shin et al. 2002), and NK cells (Shin et al. 2002; Hanabuchi et al. 2006), whereas its cognate ligand (GITRL) is constitutively expressed on antigen-presenting cells, such as dendritic cells and B cells (Mackay and Kalled 2002; Tuyaerts et al. 2007).

GITR is widely investigated in infection and inflammatory diseases. GITR stimulation also enhanced anti-CD3-induced ERK phosphorylation, suggesting that GITR

is involved in MAPK-pathway activation. Meanwhile, it was detected that Treg proliferation was also triggered by the GITR co-stimulus. Interestingly, Treg cell proliferation was found to be paralleled by the loss of the anergic phenotype and suppressor activity. Nevertheless, unstimulated GITR(−/−) CD4+ CD25+ and GITR(+/+) CD4+ CD25+ cells were equally able to exert suppressor activity on CD4+ CD25− responder cells (Ronchetti et al. 2004). On the other hand, GITR expression on CD8+ T cells was reported to be down-regulated by a known JNK-specific inhibitor, SP600125, which limited JNK phosphorylation (Chattopadhyay and Chakraborty 2009).

For a period, the role of GITR in NK cells was controversial (Hanabuchi et al. 2006; Baltz et al. 2007). Then Liu and colleagues reported that ligation of GITR suppressed NK cell proliferation in response to IL-15. GITR activation also suppressed pro-inflammatory cytokine secretion and increased NK cell apoptosis, possibly via blocked phosphorylation of Stat5 and Akt. Further results indicated that increased apoptosis was independent of the Fas-FasL pathway, but Bcl-XL and phospho-Bad protein expressions were diminished, suggesting involvement of the mitochondrial apoptosis pathway (Liu et al. 2008).

Bioinformatic analysis of mouse GITR identified four N-glycosylation sites, four serine phosphorylation sites, one threonine phosphorylation site, and one tyrosine phosphorylation site, providing a basis for the further expression and functional study of mouse GITR protein (Shen et al. 2014).

15.6.5 Lymphocyte Activation Gene-3 (LAG-3; CD223)

Lymphocyte activation gene-3 (LAG-3; CD223) is a structural homolog of CD4 and it binds to MHC class II molecules. Previous research indicated that signaling mediated by LAG-3 inhibits proliferation and activation of T cells and NK cells (Byun et al. 2007; Workman et al. 2002), and LAG-3 serves as a key surface molecule for the function of regulatory T cells (Huang et al. 2004).

It was shown years ago that engagement of class II molecules by both its natural ligand LAG-3 and class II mAb induces rapid protein phosphorylation of phospholipase Cgamma2 (PLCgamma2) and p72syk as well as activation of PI3K/Akt, p42/44 ERK, and p38 mitogen-activated protein kinase pathways. Studies using inhibitors demonstrate that these three pathways are all important in inducing the maturation process of LAG-3-stimulated DCs. When class II molecules were ligated with LAG-3 versus specific antibody, differences in the phosphorylation pattern of c-Akt were observed (Andreae et al. 2003).

Differential subcellular localization is a feature of LAG-3. It was shown that LAG-3 was expressed on the surface of activated T cells, whole in unstimulated T cells, the majority of LAG-3 is retained in intracellular compartments. The translocation was thought to be modulated through interaction with PKC on its cytoplasmic domain. However, the two predicted phosphorylation site in this domain was not proved to be phosphorylated in this process (Bae et al. 2014).

15.6.6 Programmed Death-Ligand 2 (PD-L2)

The B7 family member programmed death-ligand 2 (PD-L2) has been implicated in both positive and negative regulation of T cell activity. PD-L2 was shown to have overlapping functions with PD-L1 (Latchman et al. 2001). PD-L2 shares common receptor with PD-L1. The inhibitory effect of PD-L2 is also transduced through PD-1, displaying as inhibited proliferation, IL-2 production, and INF-gamma production. It was shown that PD-L2 inhibited anti-CD3-induced AKT phosphorylation within minutes and ERK phosphorylation after hours. Further, Anti-CD3 mAb + PD-L2 stimulation also increased the level of SHP-2 associated with the PD-1 receptor (Saunders et al. 2005).

15.6.7 B and T Lymphocyte Attenuator (BTLA)

B and T lymphocyte attenuator (BTLA), an immunoglobulin domain-containing glycoprotein with two immunoreceptor tyrosine-based inhibitory motifs is induced during activation and remains expressed on T helper type 1 (T(H)1) but not T(H)2 cells. BTLA was shown to block B and T cell signaling through its tyrosine phosphorylation and association with SHP-1 and SHP-2. At the same time, IL-2 production is attenuated. Tests on BTLA KO mice detected enhanced immune responses. The ligand of BTLA, a peripheral homolog of B7 family, B7x, has already been identified. BTLA was found to be similar with other receptors on T cells like PD-1 and CTLA-4 (Watanabe et al. 2003). In addition, the similarity between BTLA and PD-L1 was then discovered in that they are both regulated by phosphorylation of STAT. Specifically, phosphorylated STAT3 (pSTAT3) was found to have association with BTLA expression. Specially, Cells with BTLA expression also presents high expression of other checkpoint molecules in patients with diffuse large B cell lymphoma (DLBCL), suggesting a prognostic value of BTLA expression. Meanwhile, blocking BTLA together with other checkpoints may provide better efficacy in treatment of DLBCL (Quan et al. 2018).

15.6.8 V-Domain Immunoglobulin (Ig) Suppressor of T Cell Activation (VISTA)

V-domain Immunoglobulin (Ig) Suppressor of T Cell Activation (VISTA) is one of the inhibitory actors (including TIM-3, LAG-3 and so on) that has been under evaluation to be potential therapeutic target in cancer ICBT (Granier et al. 2017). VISTA was shown to intermediate the induction of Tregs from naive CD4+ T cells

by CD71. In the process, AKT phosphorylation was though to be involved, but the relation between VISTA and subsequent signaling pathways is unknown (Shahbaz et al. 2018).

15.7 Discussion

In this chapter, we have summarized the role of phosphorylation in many aspects. As a fast switch, phosphorylation is deeply involved in signal transduction and protein modulation in the process of immune activities.

Phosphorylation is involved in the formation of neoantigens expressed on the surface of cancer cells, which is the initiation of immune responses. Many types of immune cells are influenced through phosphorylating key regulators. TCR or BCR recognition of antigens is the start point of adaptive immune response. But in tumor environment, these signaling pathways are interrupted. Aberrant phosphorylation or de-phosphorylation of the key factors in subsequent pathways are responsible for the immune repression. Two pathways participated in the regulation of immune cell activities are PI3K/AKT pathways and ERK/MAPK pathways. The association between T cell proliferation and activation between phosphorylation of these pathways is confirmed by many studies over the years.

STAT family proteins have vital roles in immune modulation. Phosphorylated STAT was shown to correlate expression of immune checkpoints, including PD-L1, CTLA-4, and BTLA. Not to mention other routes in immune reaction involves STAT family. EGFR and mTOR are both involved in regulation of PD-L1 expression through phosphorylation. Moreover, translational regulation of PD-L1 was shown to be regulated by phosphorylation. Cell cycle control molecules, when phosphorylated, interact with immune responses as well.

Many immune checkpoints were found to have a cytoplasmic tail which harbors sites for tyrosine phosphorylation. Many tyrosine phosphatases including Lck, Fyn are involved in the phosphorylation of the cytoplasmic domain of many checkpoint molecules, leading to recruitment of SHP-1 and SHP-2 and their respective subsequent events. Tyrosine phosphorylation of PD-L1 determines polarization and differentiation of some immune cells and stabilization of itself. Further, PD-L1 phosphorylation has cooperation with its glycosylation and ubiquitination, as well as acetylation. When it comes to CTLA-4 a similar tyrosine kinase domain was identified to induce downstream events. Localization of CTLA was shown to closely correlate with its phosphorylation. But accumulation and function of tyrosine kinases and their interaction with CTLA-4 are more complicated. CTLA further regulates cbl expression and function. Its function in inhibiting T cell function is thought to be closely related to CD28 activation. Many other checkpoints are discussed. Among them, TIM-3 and KIRs are better investigated, but LAG-3 and VISTA are already designed as therapeutic targets and the drugs are under evaluation. PD-L2 are given more and more attention these years for their overlapping function with PD-L1.

BTLA presents similar characteristics with PD-L1 and CTLA-4. GITR has potential effect of restoring NK cell activity in diminishing cancer cells.

In summary, phosphorylation is a common switch for signaling pathways, and many of them play a vital role in immune modulation. Many types of immune checkpoint molecules are found to transduce extracellular signals into cells through phosphorylation of their cytoplasmic tails, which recruit phosphatases and subsequent factors. Further, immune cell proliferation and activation, production and secretion of cytokines, and immune checkpoints themselves are modulated.

Accumulating knowledge of the complicated and intercrossed signaling network underlying immune checkpoint regulation brings about enormous novel strategies to fight against the immune-suppressive environment of tumors. Inhibitors of downstream effectors directly inhibit phosphorylation and signal transduction. Crosstalk between phosphorylation and checkpoint expression suggest combined treatment of ICBT with involved regulators, which can suppress compensatory expression of checkpoint molecules, and enhance the efficacy of ICBT. Correlation and involvement of checkpoints with antigen receptors are also better considered when designing CAR-T strategies. Formation of neoantigens by phosphorylation provides potential application in tumor vaccines.

Although ICBT has been clinically used all over the world, many areas of phosphorylation in immune modulation remain unexplored. The blockage of tyrosine phosphorylation of checkpoints may be a promising treatment if properly designed, but the effect on other pathways are yet to be clarified. The regulation of molecules except for PD-L1 and CTLA-4 are far from enough to achieve solid understanding of immune checkpoints and their modulations. Different molecules may have far different qualities due to their tissue specificity and distinct subsequent pathways.

After all, phosphorylation is a fast and transient modification. Its effects on signaling pathways are quite variable, adding to the difficulty and complexity in researching. But on the other side, its transiency adds more advantages to its value of developing effective and distinctive therapies.

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Chapter 16
Palmitoylation as a Signal for Delivery

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Abstract The ligands and receptors in immune checkpoint signaling are typically transmembrane proteins, which may be regulated by palmitoylation as a reversible lipid modification. Our recent work demonstrated that palmitoylation reduces the lysosomal degradation of PD-L1 trafficking and may present a new therapeutic target. To facilitate future investigations on palmitoylation and immune checkpoints, here we summarize the molecular roles of palmitoylation on protein stability, trafficking, membrane association, and protein—protein interaction. The biological effects of palmitoylation are exemplified by well-studied substrates such as Ras, EGFR, and Wnt proteins. Finally, the strategies for targeting protein palmitoylation are discussed to facilitate future translational studies.

Keywords Palmitoylation · Protein modification · Protein localization · Cancer therapy

16.1 Introduction

16.1.1 Lipid Post-translational Modifications

In recent years, more and more attention has been paid to phenotypes and protein functions rather than merely genotypes. This has aroused growing enthusiasm

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towards protein regulatory mechanisms, among them is post-translational modifications (PTMs), whose role in regulating protein functions in diseases, including neuron degenerations and cancers, has been vastly recognized (Adams et al. 2011). As one of the most important PTM, protein lipidation, especially fatty acylation of proteins, is not only a PTM but also a co-translational modification. With the attachment of different fatty acyl groups onto protein subsets, localization, activation, interaction and stability of a set of important proteins are vastly influenced, leading to a series of cellular activities. Some of them promote cancer progression while some others contribute to retrieve normal cell behaviors (Hannoush 2015).

16.1.2 Palmitoylation

Two commonly recognized forms of fatty acylation are palmitoylation and myristoylation. They respectively attach saturated fatty acids of 16 and 14 carbon length onto specific amino acid residues by forming an ester (O-acylation), thioester (S-acylation) or amide bonds (N-acylation). Palmitoylation is typically reversible due to the nature of thioester bond, and thus palmitoylation is more often mentioned as S-palmitoylation (Hannoush 2015). Palmitoylation is typically indispensable for some membrane proteins to be delivered, localized, and to interact with other proteins (Van Itallie et al. 2005). On the other hand, the process of depalmitoylation, meaning the detachment of palmityl group from a protein, affects protein trafficking, stability and functions in an opposite manner to palmitoylation (Wang et al. 2015).

16.1.3 Palmitoylation in Cancer

As progresses in the field of cancer genome research proceed, there still remain a lot of unsolved problems and unknown areas. The flourishing immunotherapy brings to us no fewer obstacles than hopes in the future, let along the growing knowledge of the complexity of tumor microenvironment. Researches of palmitoylation and other modifications of proteins marked the discovery of a novel territory of cancer regulation. In order to attain a comprehensive view of palmitoylation, modulations of proteins are reviewed in this chapter, providing promising ideas to be considered in developing targeting drugs in the future.

16.2 Palmitoylation Regulates Protein Trafficking and Localization

The most well-known function of palmitoylation is mediating protein localization, as illustrated in Fig. 16.1. In a large portion of human mammary gland tumors, protein tyrosine kinase 6 (PTK6), not expressed in normal mammary gland, was detected to localize flexibly due to an absence of amino-terminal myristoylation/palmitoylation

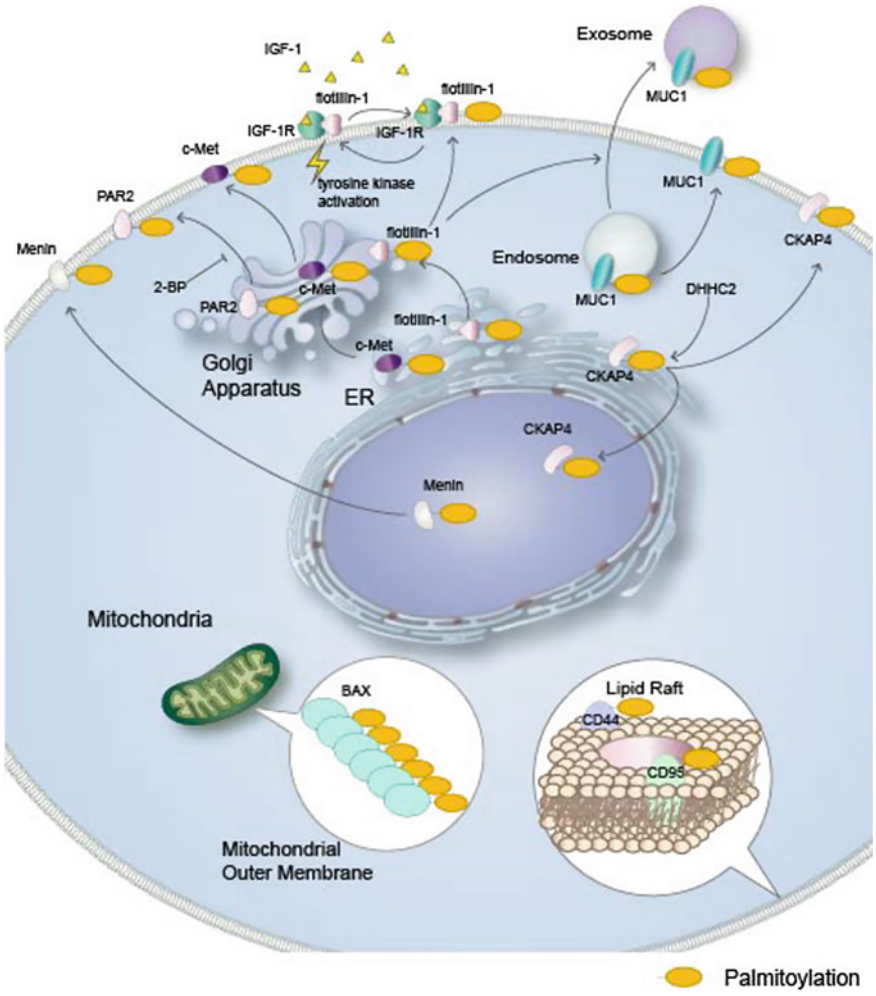

Fig. 16.1 A complex network of protein transportation regulated by palmitoylation is schematically presented. The network not only includes protein traveling between organelles, but also gives attention to protein localization among sub-domains within membrane, which is acknowledged to be crucial for membrane proteins to function properly

(Brauer and Tyner 2010). Similarly, mutation of the palmitoylation site of Ring finger protein 11 (RNF11) interrupted its movement from compartments of the early secretory pathway (Santonico et al. 2010). More transportations processes are regulated by palmitoylation as follows.

16.2.1 Routes of Protein Navigation and Distribution

16.2.1.1 From Nucleus to Plasma Membrane

Transcription factors are canonically nuclear proteins. For example, Menin, as a tumor suppressor in endocrine organs, is often mutated in multiple endocrine neoplasia type I. Different views of it have derived though, that it also serves as an oncogenic factor in mixed lineage leukemia and that a small fraction of it is likely associated with cell membrane fractions through palmitoylation of its serine residues (He et al. 2016). Further investigations may lead to a better understanding of the tissue-specific function of Menin.

16.2.1.2 From Cytoplasm to Plasma Membrane

Palmitoylation is mainly involved in the routine transportation of membrane proteins to the plasma membrane after their synthesis and modification in the endoplasmic reticulum (ER) and Golgi apparatus. It was reported that c-Met, a receptor tyrosine kinase, was stably palmitoylated in ER; after transportation to Golgi, the palmitoylation played a crucial role in targeting c-Met to the plasma membrane by mediating its Golgi exit. Involved in both mitogenic and motogenic activities, c-Met is a notorious cancer-driver; the findings above suggest a potential target for blocking these cancers (Coleman et al. 2016). The Insulin-like Growth Factor 1 (IGF-1) pathway promotes cancer cell proliferation and the mechanism is controlled by flotillin-1, where palmitoylation deeply corelates. Firstly, flotillin-1 is palmitoylated at Cys34 in the ER, which is a predisposition of the ER egress and PM localization of flotillin-1 and Insulin-like Growth Factor 1 Receptor (IGF-1R); furthermore, the IGF-1 interacts with flotillin-1 in depalmitoylation and repalmitoylation of flotillin-1, sustaining the tyrosine kinase activation of IGF-1R (Jang et al. 2015). Wnt family plays a critical role in tumorigenesis, and their cell membrane navigation requires palmitoylation. Palmitoylated Wnt proteins were shuttled by a conserved protein Wntless (Wls) in intrahepatic cholangiocarcinoma (ICC) (Shi et al. 2018) The significance of palmitoylation in Wnt pathways is further emphasized in later parts.

MUC1 is a mucin-like transmembrane protein expressed on the apical side of the polarized epithelial cell; in conditions of epithelial cancer, MUC1 localization can be messed up. Interestingly, MUC1 can be trafficked to endosomes and released to exosomes, both of which are related to palmitoylation. Flotillin mentioned above as a mediator of IGF-1R localization, seems to play a no less important role in

the lipid raft-associated pathway of MUC1 releasing to exosome (Hanisch et al. 2012). After endocytosis to endosomes, S-palmitoylation at the boundary between transmembrane and endoplasmic site of MUC1 was reported to be the most important procedure that drove MUC1 recycling back to the cell surface (Kinlough et al. 2006). In prostate cancer cells, protease-activated receptor-2 (PAR2) is not only localized on the cell surface but also desensitized and stabilized by its palmitoylation in Golgi apparatus, contributing to normal function and life cycle of this G-protein coupled receptor. Consistently, blocking of palmitoylation by 2-bromopalmitate (2-BP) leads to insufficient and unstable expression of PAR2 (Adams et al. 2011).

Cytoskeleton-associated protein 4 (CKAP4) is revealed to be palmitoylated by DHHC2, so that it can be targeted to the cell surface as well as nuclear, inhibiting tumor growth. In this picture, DHHC2 is likely a tumor suppressor (Planey et al. 2009).

16.2.1.3 Sub-domain Modulation Within Membranes

Palmitoylation helps to localize proteins not only to membranes but also within membranes. Scrupulously concentrated and segregated membrane molecules build up astonishingly variable sub-domains on the cell surface, comprising tremendous cell activity units both structurally and functionally. Some domains enriched in saturated lipids are likely to hide drug targets and lead to drug resistance consequently. Inhibition of fatty acid synthase (FASN), the main producer of palmitates, was reported to change the nature of tumor cell membrane, resensitize anti-tumor drugs and even kill tumor cells directly, with minimal toxicity to normal cells (Buckley et al. 2017).

Typically, lipid rafts are highly organized microdomains on the cell surface; with rich components of sphingolipids, cholesterol, and gangliosides, these domains can be easily distinguished from surrounding membrane, providing platforms and hubs for assembly of signaling complexes. CD95 death receptor is one of them. The accumulation of CD95 to lipid rafts depends on S-palmitoylation in a reversible manner, then it is activated by its innate ligand or a set of other stimulators including some chemotherapy drugs (Gajate and Mollinedo 2015). A similar mechanism has been proved to the recruitment of CD44 to rafts in breast cancers, indicating a new drug target of metastasis (Babina et al. 2014). According to another study of breast cancer, curcumin was used as an inhibitor of Integrin β4, so as to intervene its lipid raft affiliation and signaling transduction (Coleman et al. 2015). The palmitoylated CD151 is required for the formation of the complex of CD151 with laminin-binding integrins. Then the complex would be navigated into tetraspanin-enriched microdomains, which are critical for HPV16 endocytosis, a high-risk initiation of cervical cancer (Scheffer et al. 2013).

An antimalarial and anticancer agent, dihydroartemisinin, was reported to induce transferrin receptor-1 palmitoylation and interaction with caveolin-1, with which lipid rafts was concerned (Ba et al. 2012).

Another kind of membrane domains is called structured membrane microdomains (SMDs). Neurotensin receptor-1(NSTR-1), involved in mitogenic signaling in breast

cancer, failed to mediate downstream ERK 1/2 phosphorylation because of diminished localization to SMDs after its palmitoylation sites were mutated, suggesting a novel target of cancer treatment (Heakal et al. 2011).

16.2.1.4 Mitochondria Membrane Localization

BCL-2-associated X (BAX) is a traditionally recognized protein for its role of mediating programmed cell death by inducing the release of cytochrome c from mitochondria to cytosol. Mechanistically, the mitochondrial translocation of BAX was confirmed to rely on palmitoylation of the protein, which is the predisposition of BAX oligomerization and its regulation on mitochondrial outer membrane (MOM) permeability. Reduced caspase activity and apoptosis were observed when palmitoylation was inhibited. On the contrary, increasing palmitoyl transferases resulted in increased BAX S-palmitoylation and enhanced apoptotic activity (Frohlich et al. 2014).

16.2.2 Typical Protein Trafficking Directed by Palmitoylation

Protein membrane localization that involves palmitoylation is much more than mentioned above. Actually, some of the proteins are discovered to support similar functions or mediate common pathways. To provide a clearer understanding of these groups of proteins, regulations of protein localization by palmitoylation are grouped by mutual pathways or similar functions in this section.

16.2.2.1 Junction/Adhesion Protein Localization

Cell junction and adhesion are firmly regulated by membrane proteins and are closely related to tumor cell migration and cancer metastasis. Study on lung cancer cells shows that junctional adhesion molecule C (JAM-C) was palmitoylated to be targeted to tight junctions on the cell surface, so as to prevent trans-well migration (Aramsangtienchai et al. 2017). The claudin family is known to be involved in maintaining the integrity of the tight junction. Several potential palmitoylation sites were predicted for claudin-1, -3 and -4, suggesting novel anti-cancer targets (Butt et al. 2012). Furthermore, it has been revealed that palmitoylation of claudin-14 prepared it for efficient localization into tight junctions and normal functions (Van Itallie et al. 2005).

Scribble (SCRIB) protein is localized to cell-cell junctions, maintaining epithelial cell polarity and suppressing tumorigenesis. Under circumstances of cancer, SCRIB is commonly amplified but unproperly localized and its tumor suppressor effect is also disrupted. Researches have addressed this alteration mechanistically that SCRIB palmitoylation mediated by ZDHHC7 plays a key role in targeting

SCRIB to its authentic location and fulfilling its function (Chen et al. 2016). Not surprisingly, SCRIB palmitoylation was revealed to be regulated by the epithelial-to-mesenchymal transcription factor (EMT-TF) Snail. Actually, the palmitoylation cycle regulators were targeted by a program of transcriptions induced by Snail, inhibiting many protein acyltransferases and promoting thioesterases including protein acyl thioesterase 2 (APT2). Consequently, SCRIB palmitoylation cycle was disturbed and SCRIB was unproperly localized, resulting in insufficient membrane expression and elevated malignancy (Hernandez et al. 2017).

Binding of cell integrin to basement membrane proteins including laminin332 (laminin-5) plays a critical role in both cell adhesion and migration and thus it may either restrain cell metastasis or promote it, depending on specific integrin receptors for laminin-332. Interacting with integrin alpha3beta1, laminin-332 contributes to rapid cancer invasion; while interaction with integrin alpha6beta4 strengthens cell attachment (Zevian et al. 2011). Furthermore, palmitoylation is compulsory to form an intact complex of CD151 with laminin-binding integrins, as mentioned above (Scheffer et al. 2013).

Interestingly, the protein acyltransferase DHHC3 was detected to be responsible for palmitoylation of integrin beta4 and alpha6, ensuring their functional localization to the cell membrane. In addition to that, their stability was also shown to be affected, which will be discussed in later parts about protein degradation (Sharma et al. 2012).

Depalmitoylation is also important in regulating protein activities. As reported, the melanoma cell adhesion molecule (MCAM) can be palmitoylated at residue cysteine 590, which can also be depalmitoylated by Wnt5a. Its depalmitoylation is sufficient to promote cell invasion (Wang et al. 2015).

16.2.2.2 Ras Protein Localization

The famous cancer driver protein Ras family, including H-Ras, N-Ras, K-Ras4A and K-Ras4B, belongs to a class of proteins named small GTPase. Their abnormal activation and expression enormously contribute to cancer proliferation and malignancy (Castellano and Santos 2011; Hobbs et al. 2016; Lin et al. 2017). It has been long since three of the four Ras proteins were discovered to be palmitoylated, which is required for their membrane affiliation, and the CAAX motif was recognized as the palmitoylated site (Hancock et al. 1989).

The transportation of N-Ras and H-Ras to the PM typically represents a reversible palmitoylation and depalmitoylation cycle that governs intracellular localization and function of them (Brock et al. 2016). After being palmitoylated in the Golgi, these two proteins could be stably anchored in the membrane until they are packed and transferred to the cell surface through vesicles. At some point, they are depalmitoylated and then released from the membrane, recycled to Golgi where another round of transportation is reinitiated (Lin et al. 2017). Additionally, DHHC9 was found to be predominantly responsible for H-Ras palmitoylation in cortical neurons, and

DHHC9 was shown to be inhibited by microRNA-134 (Chai et al. 2013). By reducing H-Ras palmitoylation, H-rev107 was reported to suppress RAS signaling and downstream molecules (Wang et al. 2014).

K-Ras4A and K-Ras4B are both encoded by the KRAS gene, but they are quite different in many ways. Among four Ras proteins, K-Ras4A featured to behold a hybrid membrane-targeting motif, a site of palmitoylation as well as a bipartite polybasic region at its C-terminus. Each of the above is sufficient to target K-Ras4A to the PM, making it much more efficient of K-Ras4A delivery (Tsai et al. 2015). This isoform-specific difference goes on. N-Arachidonoyl Dopamine (NADA) blocks the PM association as well as tumorigenetic transformation of K-Ras4A, but not that of K-Ras4B. Even more, NADA restores N-Ras from cell membrane to Golgi, which was proved to be palmitoylation-dependent (Wu et al. 2017). Mutations of the palmitoylation site significantly derived K-Ras4A of its oncogenic effect in leukemia in mice (Zhao et al. 2015).

Interestingly, a dual role of K-Ras4A was proposed. In a state that K-Ras4A is only farnesylated, it acts more like K-Ras4B, binding with calmodulin and associates with a range of cancers including colorectal, lung and pancreatic ductal adenocarcinomas. However, when K-Ras4A is both farnesylated and palmitoylated, it appears to mimic N-Ras in association with melanoma and acute myeloid leukemia. H-Ras, with two palmitoylation sites, performs distinct functions between singly and doubly palmitoylated states (Nussinov et al. 2016). But the H-Ras-specific additional Cys184 palmitoylation site does not account for the distinguished function between H-Ras and N-Ras (Yong et al. 2011).

Appearing to be a promising anti-cancer target, inhibition of Ras palmitoylation became a hot spot in cancer research (Cox et al. 2015). With palmitoyl acyltransferases (PATs) being identified, systematic knowledge of PAT family proteins was established shortly after. Although first detected in yeast, they are now widely acknowledged in human cells including cancer cells. PATs such as DHHC9, are featured by a highly conserved ~50 residue DHHC-CRD (cysteine-rich domain) where the transferase active site may be located (Roth et al. 2002). Usually, 2-bromopalmitate (2-BP) is regularly used as PAT blocker to inhibit palmitoylation in vitro. Results have revealed that Ras proteins could be retained to Golgi, accelerating cell death with subsequent pathways (Garant et al. 2016). But because of its non-selectivity, high level of cellular toxicity remains to be a huge obstacle in front of 2-BP usage (Davda et al. 2013). Further efforts may be put on delivery systems that could target drugs directly to Ras-driven cancer cells (Tamanoi and Lu 2013), yet it still holds promise to develop palmitoylation inhibitors selectively targeting individual DHHC PATs involved in Ras localization (Lin et al. 2017).

On the other side of the coin is depalmitoylation. Early efforts on this topic discovered protein depalmitoylase, PPT1 (palmitoyl protein thioesterase 1) (Tamanoi and Lu 2013) with minor potential and later, acyl protein thioesterase 1 (APT1), as a promising target, for its role in regulating in vitro depalmitoylation of H-Ras and Gαi(inhibitory G-protein α subunit) (Duncan and Gilman 1998). Soon APT1 inhibitors were developed and palmostatin B (Palm B) was among the first ones (Dekker et al. 2010). With updated technologies, competitive inhibiting small

molecule ML348 was discovered for APT1 and ML349 for APT2, respectively and selectively (Adibekian et al. 2012). For now, failures of applying farnesyltransferase inhibitors (FTIs) as anti-Ras drugs have not put out the last flame of targeting the membrane association of Ras; rather, more selective and effective methods are in urgent need (Cox et al. 2015).

RalA and RalB are Ras-like small GTPases that were identified to co-influence in RAS oncogene-driven cancers. Their distinct CAAX motif PTMs distract their localization to the PM with a lack of Ras converting CAAX endopeptidase 1 (RCE1), providing potential strategies for anti-Ras therapies (Gentry et al. 2015).

16.2.2.3 Steroid Receptor Localization

For tumors associated with hormones like breast cancer and ovarian cancer, the regulation of sex steroid receptors (SRs) has an obvious and deep influence on both cancer progression and sometimes therapeutic effects. As a classic type of membrane receptor, SRs are localized at the PMs of cells, so as to bind with extracellular steroid hormones and induce intracellular kinase cascades. Researches have proved the necessity of palmitoylation for SRs to be properly localized and functional (Le Romancer et al. 2011). The DHHC-7 and -21 were singled out to be selectively associated with membrane SR palmitoylation and localization (Pedram et al. 2012).

Take estrogen receptor (ER) as an example. Widely researched in breast cancer, estrogen receptor α (ER α) localized at the PM is known to be palmitoylated at residue Cys447 by PAT (Acconcia et al. 2004). Evidence suggested that this kind of PTM mediated the affiliation of ERα36 to the PM (Soltysik and Czekaj 2015). Moreover, Depalmitoylation of ER α induced by 17β-estrodiol (E2) enables further kinase reaction that promotes cell proliferation and intervenes its association with caveolin; while palmitoylation site mutations blocks these effects (Galluzzo et al. 2008; Song et al. 2004). Heat shock protein (Hsp27) was reported to promote estrogen receptor α (ERα) palmitoylation (Razandi et al. 2010). The other type of ER, ER β was also shown to be localized to the cell membrane (Pedram et al. 2007). Contrary to ER α, depalmitoylation induced by E2 promotes its interaction with caveolin-1, leading to an utterly different outcome. The p38-dependent apoptotic cascades were also claimed to involve ER β palmitoylation (Galluzzo et al. 2007).

Some other receptors were also found to be regulated by palmitoylation-dependent mechanisms. Binding to human chorionic gonadotropin (hCG), luteinizing hormone (LH) receptors were detected to aggregate within nanoscale only if it was palmitoylated (Wolf-Ringwall et al. 2011). As for prostate cancer, androgen receptor 8 (AR8) was investigated and testified to localize on the PM via palmitoylation of two cysteine residues within its C-terminal sequence (Yang et al. 2011).

16.3 Signaling Transduction and Interference by Palmitoylation

Except for the position of proteins, palmitoylation also participates in the activation and signaling pathways. Specifically, the complexity of signaling pathways includes a variety of proteins. Examples are listed below, while specific pathways weigh significantly among others, like EGFR, Wnt and Hedgehog pathway. Of importance, endocytic Ca^{+} flux may also be affected by palmitoylation.

According to a research in prostate cancer, downstream signaling of a versatile tumor promoter, Src kinase family, depends on palmitoylation modification on its SH4 domain (Cai et al. 2011). Palmitoylation was also recognized to be competitive inhibition of the phosphorylation site by PI4KIIa (Wang et al. 2017). Palmitoylated proteins located on the surface of exosomes secreted by breast cancer cells were assumed to facilitate the activation of NF-κB (Chow et al. 2014).

Though not seemed like a promising target for Ras inhibition, PPT1 was later identified as a key molecule modulating the transformation of another antimalarial, dimeric quinacrines (DQ). Interestingly, DQ potentially inhibits mTOR and autophagy, while the signaling could be blocked by an accumulation of palmitoylated proteins (Rebecca et al. 2017).

16.3.1 Palmitoylation of EGFR Affects Signaling Activation

Epidermal growth factor receptor (EGFR) has been proved to be an oncogenic receptor and its targeting anticancer drugs have been well developed like gefitinib. Normally, EGFR is activated as a tyrosine kinase by its extracellular ligands binding to its extracellular structure. But in EGFR-driven cancers, constant activation independent of ligands was found and closely related to intracellular modification, which was revealed to be FASN-dependent palmitoylation (Bollu et al. 2015). Specifically, lack of palmitoylation suspends EGFR continued activation and sensitizes cells to EGFR tyrosine kinase inhibitors (Runkle et al. 2016), as illustrated in Fig. 16.2. Additionally, PAT DHHC20 promotes gefitinib-induced cell death in K-Ras and EGFR mutant cell lines (Kharbanda et al. 2017). There is another group of EGFR named as mitochondria EGFR (mtEGFR). It is activated by plasma membrane EGFR via synthesized palmitate, which leads to elevated mitochondrial fusion and enhanced cancer vitality (Bollu et al. 2014). Deeper and wider knowledge about the regulation of EGFR indicates not only novel therapeutic targets but also strategies towards resistance of EGFR-targeted drugs.

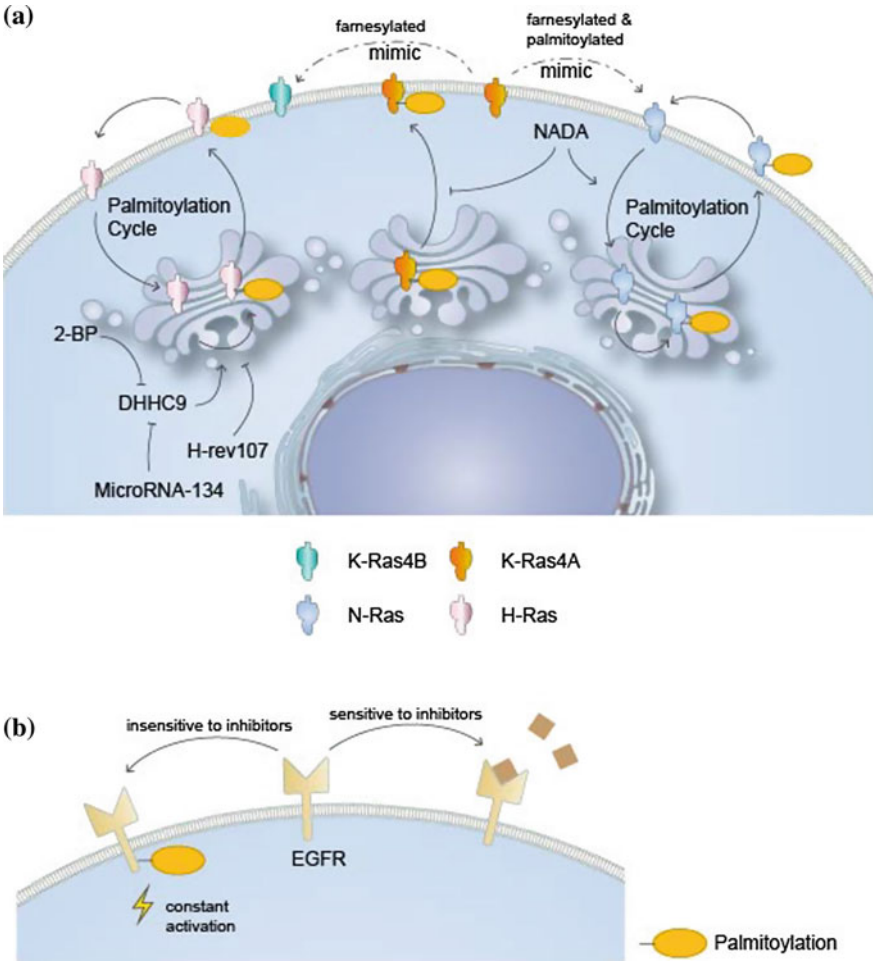

Fig. 16.2 **a** Ras family proteins, categorized into 4 different kinds, are regulated by palmitoylation in their distinct ways. N-Ras and H-Ras palmitoylation cycle are demonstrated. K-Ras4A is capable to mimic other Ras proteins by palmitoylation and/or farnesylation. **b** Lack of palmitoylation suspends EGFR continued activation and sensitizes cells to EGFR tyrosine kinase inhibitors

16.3.2 Wnt Palmitoylation Regulates Downstream Pathways

The Wnt pathways transfer an exogenous or autogenous signal to intracellular activities. Their roles in carcinogenesis are widely acknowledged. Wnt-1, palmitoylated by FASN, was assumed to contribute to the stabilization of β-catenin as well as a subsequent signaling pathway. Consistently, overexpression of FASN led to enhanced tumor proliferation and ameliorated apoptosis, likely via regulation of the Wnt pathway (Fiorentino et al. 2008).

When it comes to Wnt palmitoylation, a membrane-bound O-acyl transferase, Porcupine (PORCN), is unneglectable. It catalyzes the palmitoylation of Wnt proteins and thus modulating a sequence of Wnt activities, including Wnt secretion, signaling and its binding to Wls, which shuttles Wnt proteins to the PM, indicating an independent target for inhibiting cancer cells (Covey et al. 2012).

Efforts and achievements have been made in developing inhibitory molecules to PORCN. For example, LGK974 was discovered, and its inhibitory effect towards PORCN was testified in vitro and in vivo (Liu et al. 2013). Additionally, PORCN inhibitor IWP2 was proved to efficiently cut down on palmitoylation-dependent Wnt secretion, which further held back the epithelial transition of mesenchymal in cultured cells (Schwab et al. 2018). Years later, direct inhibition of PORCN was attained by a novel compound named Compound 62. Compound 62 was obtained from a scaffold hybridization strategy from two known porcupine inhibitor classes by Dong. And colleagues, and it displays outstanding biological stability so far. The discovery of Compound 62 has shed lights on a potential anti-cancer treatment (Dong et al. 2015). Meanwhile, another group of researchers, Xu and colleagues identified another leading compound 59, with the same strategy. In vivo experiments exhibited excellent bioavailability in the rat (Xu et al. 2016). More evidence supported the effectiveness of Wnt inhibition and safety of in vivo application (Proffitt et al. 2013).

16.3.3 Hedgehog Pathways Functioned by Palmitoylation

The cell-cell signaling pathway governed by hedgehog family plays a critical role in animal development, while deficiency or misregulation is likely to result in cancer or congenital diseases. Canonically, Hedgehog signaling is turned off by Patched, a membrane tumor suppressor; yet downstream signals are stimulated by its secreted ligands by disturbing its interaction with Patched, where a palmitate-dependent two-pronged arm was demonstrated (Tukachinsky et al. 2016). Although commonly overexpressed and involved in the aetiology of many tumors, Sonic Hedgehog pathways will not be properly functional, unless hedgehog is palmitoylated at the N-terminus by Hedgehog acyltransferase (Hhat). Hhat belongs to the MBOAT family and is located in multiple subcellular membranes. Inhibition of Hhat was evidenced to restrain the growth of pancreatic ductal adenocarcinoma (PDAC) cell line (Konitsiotis et al. 2014).

16.3.4 Intracellular Ca^{2+} Flux Regulated by Palmitoylation

The cellular reservoir of Ca^{2+}, mainly in the endoplasmic reticulum (ER), is crucial for a wide range of cell physiological activities. Mitochondria metabolism is regulated by Ca^{2+} flux from the ER to the mitochondria-associated membrane (MAM).

Cancer cells are frequently observed with altered mitochondria metabolism, leading to elevated oxidative stress which further results in chemotherapy resistance and tumor growth. The intimate contact between the ER and the MAM brings sufficient and efficient Ca^{2+} flux. It is reported to be intermediated by the redox-sensitive oxidoreductase TMX1, which is located on the MAM in a large amount. To maintain this contact and productive mitochondria activity, thioredoxin motif and palmitoylation of TMX1 were necessary (Raturi et al. 2016).

Calnexin is another member on the MAM, and it is palmitoylated and then accumulated here. Further results uncovered an interaction between palmitoylated calnexin and the sarcoendoplasmic reticulum (SR) Ca^{2+} transport ATPase (SERCA) 2b, and this interaction modulates the ER-mitochondria Ca^{2+} crosstalk. Especially, under circumstances of ER stress, calnexin goes through a rapid depalmitoylation, drifting it away from its role as Ca^{2+} signaling modulator to acting as a chaperone in ER quality control compartment (ERQC). In a word, the switch of stress or rest determines the palmitoylation as well as function in Ca^{2+} signaling (Lynes et al. 2013).

Unlike the two above, the selenoprotein K (Selk) is mainly located on the ER, together with the PAT DHHC6. Interestingly, Selk and DHHC6 both contain a predicted Src-homology 3 (SH3) domain, a sign of their mutual function in regulating palmitoylation. Selk was revealed to participate in the palmitoylation and degradation of some ER proteins (Polo et al. 2016). Actually, the complex of DHHC/Selk, forged by SH3/SH3 binding domain interactions, regulates the ER Ca^{2+} flux by palmitoylating inositol 1,4,5-triphosphate receptor (IP3R) (Fredericks et al. 2014).

16.4 Protein Interaction and Metabolism Regulated by Palmitoylation

16.4.1 Palmitoylation Regulates Nuclear Activities

TEA domain (TEAD) transcription factors modulate the transcriptional output and activation of the Hippo pathway, playing an important role in limiting organ size and controlling differentiation. The palmitoylation of TEAD was demonstrated to be necessary for its cooperation with coactivators YAP and TAZ and their binding to the Vgll4 tumor suppressor (Chan et al. 2016), as shown in Fig. 16.3. Another publication provides more evidence by decoding the folding and structure modulation of TEAD by palmitoylation, providing a feasible target for inhibiting the Hippo pathway (Noland et al. 2016).

The transcription factor Snail was already mentioned in the above discussion about junction protein localization, that it contributes to reprogramming transcription to drive EMT in invasive tumors. Evidence suggested involvement of Snail overexpression in the S-palmitoylation cycle of some proteins, indicating a novel route of Snail's tumorigenic effects (Hernandez et al. 2016).

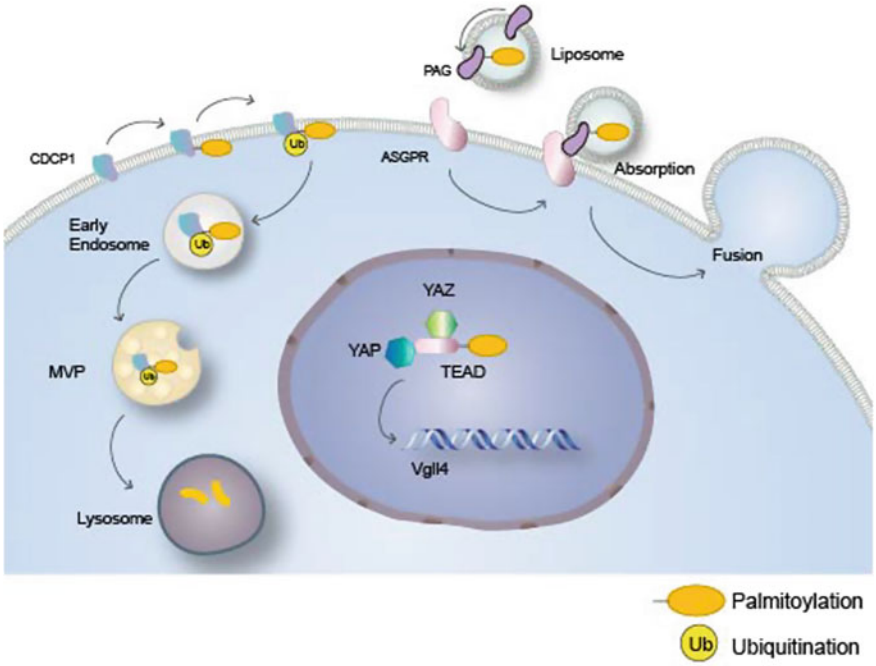

Fig. 16.3 In the cell nuclear of this figure is a schematic demonstration of TEAD cooperation with YAP and TAZ, which is dependent on palmitoylation, thus enabling their binding to the Vgll4 tumor suppressor. The left part shows that palmitoylation of CDCP1 leads to proteasome-mediated degradation and deficiency of membrane expression. In this figure it is also described that liposomal delivery system requires PAG palmitoylation so that the delivery could be targeted to ASGPR

A series of pathways including the phosphatidylinositol-3 kinase-related kinases and p53 are activated in response to DNA damage, arresting cell cycle for DNA repair or apoptosis. The response could be disturbed when protein palmitoylation was inhibited, which further compromised DNA damage-induced p53 and Atm activation. When zDHHC16, a protein acyltransferase with zinc-finger and Asp-His-His-Cys domains (zDHHC), is knocked out, a similar effect of disrupting DNA damage response occurs (Cao et al. 2016).

16.4.2 Palmitoylation Regulates Protein Structure and Stability

Significant reduction of tubulin palmitoylation, as well as mRNA expression, were induced by FASN blocker TVB-3166 or TVB-3664, which led to the disruption of microtubule organization and cell vitality in tumor cells (Heuer et al. 2017).

Ring finger protein 11 (RNF11), a small RING E3-ligase, is commonly overexpressed in human prostate, colon, and invasive breast cancers. It was reported to be S-palmitoylated on Cys4, and the mutation of this site could impair the in vivo ubiquitination (Santonico et al. 2010).

A cancer promoter, named CDCP1 (CUB domain-containing protein 1), functions properly only when expressed on cell surface. Palmitoylation of it leads to proteasome-mediated degradation and deficiency of membrane expression, as illustrated in Fig. 16.3. Interestingly, the relocalization instead of degradation can be achieved by disrupting its palmitoylation or EGF treatment, suggesting a protein stabilizing and membrane recycling role of EGFR (Adams et al. 2015). More evidence was produced that mutations of a palmitoylation motif (C689,690G) strongly defected CDCP1 function (Kollmorgen et al. 2012). The role of DHHC3 in localizing integrin alpha6beta4 has already been talked about above, but an additional association between them was detected by DHHC3 knockdown, which gave rise to enhanced degradation of alpha6beta4 (Sharma et al. 2012).

16.4.3 Palmitoylation Regulates Membrane Transportation

Recent proteomic studies have revealed correlations between the Big Potassium (BK) channels with various proteins (Zhou et al. 2012). Some of these interactions provide further insight into the role that BK channels play in cancers, especially in brain tumors (Ge et al. 2014). Evidence has revealed the critical role of S0-S1 linker palmitoylation in the control of BK channel cell surface expression and function (Jeffries et al. 2010). In hepatocellular carcinoma (HCC), it was found that the liposomal delivery system requires arabinogalactan (PAG) palmitoylation so that the delivery could be targeted to asialoglycoprotein receptors (ASGPR) (Shah et al. 2014), as vividly demonstrated in Fig. 16.3.

Prospectively, researches deepened the understanding of the regulation of palmitoylation in drug sensitivity and tumor therapy. Palmitoylation of the Tat-doxorubicin conjugate was shown to promote its anti-cancer activity (Zhang et al. 2014).

16.5 Palmitoylation Concerning Immune Response

16.5.1 Palmitoylation Affects Immune Cell Functions

16.5.1.1 T Cell Functions Regulated by Palmitoylation

In T cell signaling, several molecules are S-palmitoylated, such as Src family signaling kinases LCK (Resh 2006; Paige et al. 1993; Shenoy-Scaria et al. 1993) and FYN (Timson Gauen et al. 1996; van't Hof and Resh 1999; Shenoy-Scaria et al. 1994),

transmembrane adaptor LAT (linker for activation of T cells) (Wange 2000; Zhang et al. 1998), and co-receptors CD4 (Crise and Rose 1992; Balamuth et al. 2004) and CD8 (Arcaro et al. 2000). These molecules are modified to participate in TCR signaling functionally (Resh 2006). Concerning the Src family, their S-palmitoylation kinases are involved in critical signaling pathways in acquired immunity. Signaling cascades are then initiated by their interaction with raft-associating plasma membrane immunoreceptors (Liang et al. 2001; Kabouridis et al. 1997).

16.5.1.2 B Cell Functions Regulated by Palmitoylation

B cell antigen receptor (BCR) signaling is transduced by its colligation with the CD19/CD21/CD81 coreceptor complex. Evidently, selective, rapid and reversible palmitoylation of tetraspanins CD81 is induced by ligation of the BCR and the CD19/CD21/CD81 complex, which functions to stabilizing the BCR in sphingolipid- and cholesterol-rich membrane microdomains termed lipid rafts, therefore signaling is amplified and prolonged (Cherukuri et al. 2004).

In a study identifying novel palmitoylated proteins in B lymphocytes, CD20 and CD23 (low-affinity immunoglobulin epsilon Fc receptor) are outstandingly focused and confirmed, suggesting potential effective/potential therapeutic targets for hematological malignancies, autoimmune diseases and allergic disorders (Ivaldi et al. 2012).

16.5.1.3 Dendritic Cell Function Regulated by Palmitoylation

In dendritic cells, S-palmitoylation occurs on several cell surface receptors that are responsible for antigen uptake or dendritic cell activation during virus infections such as CD36 (Urban et al. 2001) and the interferon α/β receptor (Claudinon et al. 2009), respectively.

16.5.2 Palmitoylation Regulates Other Immune Pathways

16.5.2.1 Fas/FasL Palmitoylation Regulates Apoptosis

Palmitoylation at a membrane proximal cysteine residue enables Fas to localize to lipid raft microdomains and induce apoptosis in cell lines (Cruz et al. 2016). On the other hand, FasL palmitoylation, which occurs within its transmembrane domain, is critical for efficient FasL-mediated killing and FasL processing (Guardiola-Serrano et al. 2010), as shown in Fig. 16.4.

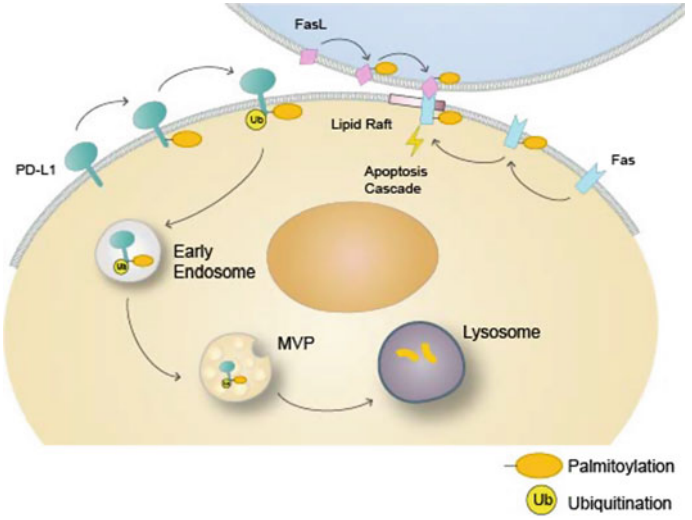

Fig. 16.4 This figure combines the regulation of PD-L1 and Fas by palmitoylation. Palmitoylation of PD-L1 subsequently induces ubiquitination of PD-L1 on its endocellular domain, which further leads to its endocytosis and degradation. On the other hand, palmitoylation of Fas helps localize itself into lipid raft, where Fas can interact with palmitoylated FasL and induce apoptosis

16.5.2.2 The Palmitoylated TRAPs Regulates Innate Immune Pathways Bidirectionally

By palmitoylation, a subset of the TRAP family, namely the palmitoylated TRAPs (pTRAPs), are targeted to lipid rafts, tetraspanin-enriched microdomains, and protein microclusters in membranes. Their scaffolds cause spatiotemporal variation of membrane signaling kinases, especially Src and Syk family members, as well as Csk, and other effectors, including Fc receptor and pattern recognition receptor signaling (Curson et al. 2018).

16.5.3 Palmitoylation of Immune Checkpoints

Researches on palmitoylation of immune checkpoints are still at an early stage. But increasingly, the importance of PTM including palmitoylation of checkpoints are revealed. Recently, PD-L1 is shown to be palmitoylated by a covalent attachment of palmitic acid (a 16 C saturate fatty acid) to its cysteine residue at 272 for stability, and further promote breast cancer progression (Yang et al. 2019). ZDHHC9 is associated with palmitoylation of PD-L1. Mutation (C272A) of PD-L1 or decrease of ZDHHC9 abolishes palmitoylation, and thus reduces cell surface distribution upon INF-γ treatment, sensitizes breast cancer cells to T-cell killing and inhibits tumor growth in mice (Yang et al. 2019; Gu et al. 2018). Latest results have linked PD-L1

palmitoylation with its storage and stabilization, where its ubiquitination is blocked. Palmitoyltransferase ZDHHC3 (DHHC3) is identified as the main acetyltransferase responsible for the palmitoylation of PD-L1. By suppressing DHHC3, or inhibiting palmitoylation with 2-BP, antitumor immune activities are restored in vitro and in mice bearing MC38 tumor cells. Accordingly, a competitive inhibitor of PD-L1 palmitoylation is designed to decrease PD-L1 level and enhance immune clearance of tumor cells, suggesting a novel strategy for immunotherapies (Yao et al. 2019). Recent results indicate a promising prospect of immune checkpoints palmitoylation, although investigations on other immune checkpoints are still awaited.

16.6 Discussion

We have combed through the role of palmitoylation in cell activities, especially in cancer progression. Protein trafficking and localization are firmly regulated in many aspects, including transportation and anchoring to plasma membrane and redistribution within the membrane to microdomains. Functional units of the membrane also count on protein palmitoylation of membrane proteins so that these signaling factors can be gathered in subdomains like lipid rafts for efficient signal transduction. Some junction proteins that affect cell adhesion and cancer cell migration, are regulated by palmitoylation modification. Ras proteins palmitoylation as an anti-cancer target has been widely focused and will potentially produce a powerful weapon to Ras-driven cancers. So far, palmitoylation of Ras isoforms and their complex functions still awaits deeper investigation. Steroid receptors and their palmitoylation have predominant influence over certain types of cancers. Several signaling pathways were then discussed. Ligand-independent activation of EGFR requires palmitoylation modification, which promotes cancer progression. Wnt pathway seems to have bidirectional regulatory crosstalk with palmitoylation. Hedgehog pathway promotes cancer growth, where Hhat plays an important role. ER Ca^{2+} flux concerning mitochondria metabolism is modulated by palmitoylation of some proteins located on the ER and the MAM. The regulation of the Hippo pathway by palmitoylation of TEAD was proved, and some other proteins are palmitoylated in cell nuclear. Though, membrane proteins are the main battle field where palmitoylation proves its value. Membrane transportation of some ions and even drugs can be modulated by membrane protein palmitoylation, proposing more questions to be considered when targeting cancer cells. Moreover, palmitoylation regulates the stability and degradation of some proteins that are involved in cancer activities. Typically, Recent results have discovered this effect on the superstar molecule PD-L1, whose ubiquitination is intervened by palmitoylation. Other immune-associated activities including immune cell vitality, Fas/Fas-L pathways, and innate immune responses are also closely related to palmitoylations of key proteins. It is unpredictable how enormous the effect of palmitoylation has on all aspect of cell modulation unless more efforts are made towards the

discovery of novel protein targets that could be palmitoylated and therefore regulated. In the field of PTM, palmitoylations of immune checkpoints are especially worth investigating, due to lack of knowledge and present progress.

The PAT with distinct DHHCs were widely researched in terms of different proteins they palmitoylate, but so far, we are still in lack of effective and selective strategy to restrain protein palmitoylation. Usage of 2-BP is limited because of its severe cell toxicity; maybe newer delivery system or other congenerous molecules could be developed to deal with the dilemma. Speaking of the Wnt pathway, inhibition of PORCN seems more promising, for that several compounds have been identified, introducing possible strategies in searching for PAT inhibitors. FASN cannot be neglected in the palmitoylation of many proteins including EGFR and Wnt. The present failure of targeting protein palmitoylation is not a sign of the failure of this strategy, but a reality that better methods need to be figured out. In order to achieve the goal, a global understanding and consideration of palmitoylation's impact and crosstalk between enzymes in cells are always meaningful, and still on its way.

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Chapter 17
Methodology for Detecting Protein Palmitoylation

Haojie Lu and Caiyun Fang

Abstract It is well established that palmitoylation plays a key role in the regulation of immune checkpoints, but the technical challenges in detecting protein palmitoylation have significantly prohibited further researches in this field. Till now, different approaches have been proposed, such as mutagenesis, antibody-based methods, bioinformatic prediction, "palmitate-centric" approaches, and "cysteine-centric" approaches. Of specific importance, high-throughput methods that allow the unbiased discovery of palmitoylation in the whole proteome should be further improved and employed. This chapter will summarize the methodological progresses for detecting protein palmitoylation, aiming to facilitate future researches in the lipid modification of immune checkpoint proteins.

Keywords Palmitoylation · Mutagenesis · Mass spectrometry · Antibody-based detection · Metabolic labeling

To date, a variety of methods to study protein palmitoylation have been developed, thereby significantly facilitating the identification and functional study of palmitoylated proteins. These techniques include the following.

17.1 Mutagenesis

Mutagenesis of the potentially palmitoylated cysteine residue to alanine or serine is the standard method for confirming sites of palmitoylation and for investigating the effects of loss of palmitoylation on protein subcellular localization or functions (Qi et al. 2013). As for these two substitutes, serine has very similar structure to cysteine

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than alanine so that it can maintain the size and properties of the putative palmitoylated protein after substituting serine for cysteine, but serine is more hydrophilic than cysteine and might also cause unwanted side chain effects (Nagano et al. 1999).

17.2 Antibody-Based Methods

For a long time, no commercial antibodies for detecting protein palmitoylation seriously hamper the comprehensive survey of protein S-palmitoylation. Until now, there are two reports about protein palmitoylation antibodies, an antibody specific to palmitoylated PSD-95 (Fukata et al. 2013) and a pan antipalmitoylation antibody (Fang et al. 2016), although their performance should be further improved.

17.3 Prediction Software and Database of Protein Palmitoylation

Several software programs have been developed to predict protein palmitoylation, such as CSS-Palm (CSS: clustering and scoring strategy) (Zhou et al. 2006), incremental feature selection (IFS)-Palm (Hu et al. 2011), weight, amino acid composition and position-specific scoring (WAP)-Palm (Shi et al. 2013), PalmPred (Kumari et al. 2014), and so on. In 2015, a SwissPalm database was launched (Blanc et al. 2015, 2019), which is a comprehensive database of protein palmitoylation, integrating palmitoylation prediction, topology data, species homologues, and proteomic data, and available at https://swisspalm.org/.

17.4 "Palmitate-Centric" Approaches

17.4.1 Metabolic Labeling with Radioactive Palmitic Acids

Using radioactive-isotope-labeled palmitic acids (e.g., ^{3}H-, ^{14}C-, and ^{125}I-palmitic acids) to label proteins metabolically, followed by immunoprecipitation of the selected protein and detection of the incorporated fatty acid by autoradiography, is the earliest reported and classical method to detect protein palmitoylation (Veit et al. 2008; Swarthout et al. 2005). However, low sensitivity is the most obvious disadvantage of this technique, as well as requiring long exposure time (counting in days) and the use of radioactive material.

17.4.2 Metabolic Labeling with Non-radioactive Derivatives of Palmitic Acids

To solve the problem of low sensitivity of radioactive palmitic acids, bio-orthogonal palmitic acid probes containing a terminal azido or alkynyl group have been developed, such as 17-octadecynoic acid (17ODYA) or alk-16. Combined with click chemistry, this technique not only allows for detection of the protein of interest (based on fluorescence or chemiluminescence) but also for affinity pull-down of the cellular pool of labeled proteins (usually based on a biotin–streptavidin interaction) and global analysis of protein S-acylation when combined with mass spectrometry (MS)-based proteomics (Charron et al. 2009; Martin and Cravatt 2009; Yount et al. 2010). Gao and Hannoush (2014a, b) used the click chemistry-based method to determine the subcellular localization of palmitoylated proteins by immunofluorescence when combined with proximity ligation assay. By using click chemistry-based labeling in the pulse-chase mode, the dynamics of protein palmitoylation can also be revealed (Zhang et al. 2010; Martin et al. 2011).

Compared with radiolabeled palmitic acid probes, these bio-orthogonal probes have high detection sensitivity and are more convenient to handle. In addition, the click chemistry-based assay has high specificity, because the alkyne group introduced in the analog of palmitic acid is not normally found in cells. However, they can only detect those proteins that undergo palmitoylation during the period of the metabolic labeling of cells. Furthermore, the metabolic pathway in eukaryotes is extremely complicated, so the introduction of palmitic acid analog may interfere with global metabolism status and disrupt normal cell processes. For example, the palmitic acid analog can be incorporated at *S*-, *N*-, and *O*-palmitoylation sites alike (Gao and Hannoush 2014a, b). Although 17ODYA (alk-16) is preferentially used to mimic palmitoylation of proteins, it can also be incorporated with low efficiency at *N*-myristoylation sites of proteins. In addition, those proteins bearing the glycosylphosphatidylinositol (GPI) anchor, which are not *S*-palmitoylated, can also be labeled with the palmitic acid analog (Jones et al. 2012; Wright et al. 2014).

17.5 "Cysteine-Centric" Approaches

17.5.1 Acyl-Biotin Exchange

Acyl-biotin exchange (ABE) method relies on in vitro exchange of thioester-linked palmitate to a derivative of biotin, in which three main steps are involved as follows: (1) irreversible blockage of all the free cysteines in proteins by alkylation, most often with *N*-ethylmaleimide; (2) selective cleavage of the thioester bonds existing in palmitoylated proteins to release palmitoyl moieties using neutral hydroxylamine solution; and (3) capture of the proteins with newly exposed thiol groups using

sulfhydryl-reactive biotin (such as biotin-HPDP) and subsequent streptavidin pull-down (Drisdel and Green 2004; Wan et al. 2007; Drisdel et al. 2006). The target proteins can be eluted with agents that reduce the disulfide bond between the protein and biotin-HPDP, such as β-mercaptoethanol, DTT, or TCEP, followed by being separated by SDS-PAGE and visualized by gel staining or immunoblotting, or identified by mass spectrometry.

17.5.2 ABE-Derived Methods

As an alternative to biotinylation, the newly exposed protein thiol groups in hydroxylamine-treated cell lysates can also be directly captured by sulfhydryl-reactive solid phase carriers, such as thiopropyl Sepharose beads (Acyl-RAC assay) (Forrester et al. 2011; Kumar et al. 2016) or magnetic microspheres (Zhang et al. 2018). In addition, various weights of PEG (5 or 10 kDa) are also used to substitute S-acyl groups in palmitoylated proteins instead of biotin, known as acyl-PEG exchange (APE) assay (Howie et al. 2014; Percher et al. 2016). In this method, PEGylation leads to a mass shift that can be observed by SDS-PAGE and western blotting, so it can not only quantify the number of S-acyl groups, but also determine the relative abundance of each palmitoylated form and non-palmitoylated form.

The cysteine-centric approaches are not involved in metabolic labeling, and thus they can be applied to all kind of protein samples from animal models and other biological samples, including cells, tissues, and body fluids. However, these methods have relatively high false-positive rate. On the one hand, the capture of proteins is based on the selective cleavage of thioester bonds in these methods; thus, proteins bearing a thioester linkage with compounds other than fatty acyl residues, such as ubiquitin in the E2 ubiquitin conjugase Ubc1 and many enzymes involved in lipid synthesis (Roth et al. 2006a, b), can also be picked up as false positives for palmitoylation. On the other hand, incomplete blockage of free thiol groups can bring false positives. These methods are indirect methods due to the hydroxylamine treatment step, which removes the lipidation from cysteines and therefore obscures which form of lipidation (palmitoylation or other acyl groups) is occurring on the cysteine residues. In addition, it can only investigate the static palmitoylome in samples.

To quantify the aberrations in protein palmitoylation, different quantitative proteomics have been applied together with the above enrichment methods, such as whole animal stable isotope labeling of mammals (SILAM) (Wan et al. 2013), $^{16}O/^{18}O$ labeling (Morrison et al. 2015), stable isotope labeling by amino acids in cells (SILAC) (Serwa et al. 2015), isotope-coded affinity tag (ICAT) (Zhang et al. 2008), isobaric tags for relative and absolute quantification (iTRAQ) (Hemsley et al. 2013), and so on. Recently, a quantitative method, named as cysteine-stable isotope labeling in cell culture (cysteine-SILAC), has been developed to quantitatively analyze protein palmitoylation (Zhang et al. 2018).

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Chapter 18
Checkpoints Under Traffic Control: From and to Organelles

Shouyan Deng, Xiaolin Zhou and Jie Xu

Abstract Immune checkpoints are variegated stimulatory and inhibitory signals that are fundamental in immune homeostasis. The regulative molecules for immune checkpoints include programmed cell death protein 1 (PD1), programmed death-ligand 1 or 2 (PD-L1 or PD-L2), cytotoxic T-lymphocyte-associated protein 4 (CTLA-4), and so on. While the immune checkpoint molecules have gained soaring attention in recent years, the trafficking of them has been rarely studied. Since all of the discovered immune checkpoint molecules are transmembrane domain (TMD) proteins, they share similar pathophysiological characteristics which make studies about their trafficking and associated disorders resembled. PD-L1 is one of the most classic immune checkpoint molecules, and anti-PD1 monoantibodies have shown promising immunotherapeutic effects. PD-L1 trafficking has been particularly studied, the key regulators of which include metformin, chemokine-like factor-like MARVEL transmembrane domain-containing family member (CMTM), Huntingtin-interacting protein 1-related (HIP1R), exosomes, ALIX, polyI:C, and various post-translational modifications. Here, we focus on the checkpoints under traffic control, counting PD-L1, CTLA-4, lymphocyte-activation gene 3 (LAG-3), killer immunoglobulin-like receptors (KIRs), CD70, CD94, and attempt to shed light on the potentials of drug targets based on these findings and look forward to further studies in combinatorial therapeutic regimens in the meantime.

Keywords Immune checkpoints · TMD proteins · Trafficking · Exosomal PD-L1 · Combinatorial immunotherapy

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18.1 Transmembrane Domain Proteins Trafficking and Associated Disorders

18.1.1 Immune Checkpoints and Trafficking of Transmembrane Domain Proteins

Immune checkpoints refer to a wide variety of stimulatory and inhibitory signals that keep immune responses in balance, and many of them are regulated by small surface molecules, including programmed cell death protein 1 (PD1), programmed death-ligand 1 or 2 (PD-L1 or PD-L2), cytotoxic T-lymphocyte-associated protein 4 (CTLA-4), lymphocyte-activation gene 3 (LAG-3), V-domain immunoglobulin suppressor of T cell activation (VISTA), CD27, CD70, CD94, CD137, Siglec-15, T cell immunoglobulin mucin 3 (TIM-3), killer immunoglobulin-like receptors (KIRs), and so forth (Dal Bello et al. 2017). Although studies about immune checkpoint molecules are numerous, only few of them focus on trafficking. Since all discovered immune checkpoint molecules are transmembrane domain (TMD) proteins of no exception, first of all we have to introduce the trafficking of TMD proteins.

The trafficking of different intracellular cargoes is essential for the maintenance of homeostasis in cellular environment. Endoplasmic reticulum (ER) serves as the protein quality control center, and the cargoes can be transported from ER to golgi apparatus (GA); from GA to ER, plasma membrane, endosomes/lysosomes; and from plasma membrane to endosomes and then to lysosomes or back to GA. Through different pathways of intracellular trafficking, the TMD proteins are finely transported and manipulated, carrying out proper functions. However, once the intracellular trafficking becomes chaotic, the cellular dysfunctions and subsequent disorders occur.

18.1.2 TMD Proteins Trafficking and Association with Disorders

TMD proteins are mostly integral proteins that form proteinaceous "bridges" that connect various cellular compartments by spanning the lipid bilayers, functioning for communication and molecular flow between the different compartments separated by such impermeable lipid barrier (Christian and Screenivasan 2003). TMD proteins therefore perform miscellaneous physiological functions, such as initiation of intracellular signals in response to ligand binding, activating a complex intracellular signal network, which eventually augments the gene transcription (Gargalionis et al. 2014). Classic examples of TMD proteins are the G protein-coupled receptors (GPCRs), receptor tyrosine kinase (RTK), and immune checkpoint molecules.

Despite the diverse functions, the TMD proteins are all synthesized and assembled in the ER before being transported into GA (Borel and Simon 1996). The ER serves

as the sorting center of the proteins that makes sure they are properly folded (Swanton and Bulleid 2003; Rutishauser and Spiess 2002). Under normal circumstances, the misfolded proteins would subsequently be transported to lysosomes or proteasomes to be eradicated (Meacham et al. 2001). The disorganized degradation results in accumulation of misfolded TMD proteins in intracellular compartments, contributing to cell dysfunction. Archetypal diseases caused by accumulated misfolded proteins owing to interfered protein degradation are Alzheimer's disease (Caporaso et al. 1994) and Parkinson's disease (Dauer and Przedborski 2003), the TMD proteins of which are β-amyloid precursor protein and α-synuclein, respectively.

GPCRs are the largest and most functionally diverse family of cell surface receptors (Bunnett and Cottrell 2010). Characterized by their heterotrimeric structures, they share the same properties: they are a class of TMD proteins coupled with GDP/GTP and undergo conformational changes to transduce the upstream extracellular signals into downstream intracellular cascades to regulate gene expression.

As a kind of TMP proteins, GPCRs require proper protein quality control as well. When the trafficking becomes problematic, whether caused by disturbed post-translational modifications such as phosphorylation, ubiquitination, or glycosylation that affect the endocytosis and recycling of the GPCRs, the signal pathways will be disordered (Marchese and Benovic 2001). Since GPCRs play significant roles in all of the human body systems, especially the nervous, cardiovascular, and gastrointestinal systems (Hanyaloglu and von Zastrow 2008), the dysfunctions of the GPCRs would generally lead to multisystemic disorders, with McCune-Albright syndrome as a typical instance, and only the mosaicism can survive (Boyce et al. 1993).

Epidermal growth factor receptor (EGFR) family is a genre of RTK with fundamental roles in normal development and physiological functions of epithelial cells (Lemmon and Schlessinger 2010). Considering the general function of EGFR is stimulating cell growth, differentiation, and motility (Tan et al. 2016), hyperactivation of EGFR pathways is instrumental in tumor growth and invasion, making itself predictive for tumor prognosis (Mendelsohn and Baselga 2006). The upregulation and downregulation of EGFR signals are mediated by the ligand-induced endocytosis and EGFR intracellular trafficking (Eden et al. 2012). The degradation of EGFR demands ubiquitination (Roepstorff et al. 2009), a process that transports EGFR from the cell membrane into endosomes, which is then recognized by the endosomal sorting complex required for transport (ESCRT) and moved into multivesicular endosomes (MVEs), and finally the MVEs fuse with the lysosomes to eliminate EGFR (Raiborg and Stenmark 2009; Henne et al. 2011).

The traditional immunotherapy that targets EGFR consists of a large family of monoantibodies known as RTK inhibitors (Lemmon and Schlessinger 2010). Imatinib is one of the most classic drugs and first-generation RTK inhibitors in chronic myeloid leukemia (CML) (Laurence et al. 2016). The applications of the RTK inhibitors are diverse, whose main targets are non-small cell lung cancer (NSCLC) (Che et al. 2015), colorectal cancer (CRC) (Feng et al. 2010), CML, etc. Taking the growing resistance to RTK inhibitors into account by far, regulators of EGFR trafficking are gaining increasingly greater attention.

Take the roles of Sprouty2 (SPRY2) and mitogen-inducible gene 6 (MIG6) in EGFR trafficking for instance (Walsh and Lazzara 2013). Both of them are regulators of EGFR endocytosis and recycling, and SPRY2 can promote EGFR stability and recycling through enhancing extracellular signal-regulated kinase (ERK) phosphorylation; MIG6 inhibits EGFR activity and augments internalization of EGFR on the other hand. SPRY2 mutations impair the related ERK phosphorylation and endocytosis, which reduce surface EGFR expression, and consequently the target cells become less responsive to RTK inhibitors. Such endocytosis-associated EGFR mutations elucidate the reason for RTK inhibitors resistance in several cancers, especially in NSCLC and CRC (Che et al. 2015; Feng et al. 2010).

18.2 PD1 and PD-L1 Trafficking and Their Implications in Combinatorial Immunotherapy

18.2.1 Introduction to PD1 and PD-L1

PD1, a kind of cell surface receptor, one of the most classic immune checkpoint molecules, is a type of TMD proteins in essence that becomes activated when being interacted with PD-L1 and PD-L2, leading to reduced T cell proliferation, cytokine production, and T cell cytolysis through diminished cell growth factors and survival signals (Freeman et al. 2000; Latchman et al. 2001; Rodig et al. 2003). PD-L1 expression is in a relatively balanced level under physiological homeostasis in vivo, but in the case of cancers, the tumors have overexpression of the ligands to evade the immune system, which favors tumorigenesis and invasiveness, making themselves less susceptible to specific $CD8^+$ T cell-mediated lysis (Iwai et al. 2002). The anti-PD1 monoantibodies, including nivolumab, pembrolizumab, pidilizumab, MPDL-3280A, etc., can effectively block the PD1/PD-L1 interaction, which have statistically proved to have impressive clinical efficacy in several tumors, such as NSCLC, CRC, and head and neck cancer (Hui 2014; Ribas 2014; Seiwert et al. 2014). On account of the fact that such immunotherapy merely blocks the PD1/PD-L1 interaction after the cell surface expression of the molecules, the recycling and re-expression of the immune checkpoint molecules can reduce the efficacy of immunotherapy to a large extent. Recent studies have attempted to reduce the PD-L1 expression intrinsically by interfering with its intracellular transport in light of the recent research results about the TMD proteins trafficking. While PD1 trafficking has been rarely studied till now, several articles have shed light on the significance of PD-L1 trafficking modulations.

18.2.2 PD-L1 Trafficking: From ER to GA

The PD-L1 trafficking from ER to GA is actually demonstrated by a kind of outstanding hypoglycemic drug to control type II diabetes mellitus—metformin. Metformin has been currently recognized as a multifunctional immune–metabolic adjuvant for conventional immunotherapy (Sara Verdura et al. 2019), which possesses antitumor activity in various cancer types (Evans et al. 2005). Such antitumor activity is connected with enhanced functionality of T cells (Pereira et al. 2018), suppressed tumor microenvironment (Ding et al. 2015; Kunisada et al. 2017), changed gut microbiome composition (Forslund et al. 2015; Wu et al. 2017; de la Cuesta-Zuluaga et al. 2017; Kyriachenko et al. 2019; Pollak 2017; Shin et al. 2014; Lee et al. 2018), and reduced surface PD-L1 level (Eikawa et al. 2015). Research reveals that metformin might retard the ER to GA trafficking of PD-L1 to decrease surface PD-L1 expression and boost the cytotoxic T lymphocytes (CTL) activity ultimately (Eikawa et al. 2015). Studies have indicated that probably the main effector of such antitumor activity is AMP-activated kinase (AMPK), the activation of which is induced by metformin. AMPK modulates the phosphorylation and abnormal glycosylation of PD-L1. The N-glycosylation of PD-L1 is an essential post-translational modification, manipulating folding, intracellular transport, and the subsequent PD-L1 functions (Li et al. 2016), which is performed in both ER and GA. Under normal circumstances, the glycosylation remodeling in GA is the next step of precursor glycan attached to the NXT motif of glycoprotein trim in ER (Breitling and Aebi 2013). Nevertheless, when the glycan structure is modified wrongly or the protein turns out to be misfolded, the intracellular PD-L1 will be recognized by the ER-associated protein degradation complex (composed of various components involved in substrate recognition, ubiquitination, and retro-translocation), and degradation in the cytoplasmic proteasome happens thereafter (Ferris et al. 2014; Xu and Ng 2015). By altering the PD-L1 glycan structure, AMPK enhances PD-L1 degradation and eventually decreases tumor surface PD-L1 expression, which blocks the immune inhibitory signaling by attenuating the PD1/PD-L1 interaction (Fig. 18.1). Through interfering with the ER to GA trafficking, drugs such as metformin are likely to have antitumor potential by strengthening cellular immunity in the tumor microenvironment (Cha et al. 2018).

18.2.3 PD-L1 Trafficking: From Cell Membrane to Recycling Endosomes

Once PD-L1 has expressed on the cell membrane, it can be transported into the recycling endosomes to evade degradation. Such pathway has been illustrated clearly by a ubiquitously expressed protein chemokine-like factor-like MARVEL transmembrane domain containing family member 6 (CMTM6), which binds and colocalizes PD-L1, and CMTM6 can maintain the PD-L1 surface expression by preventing the

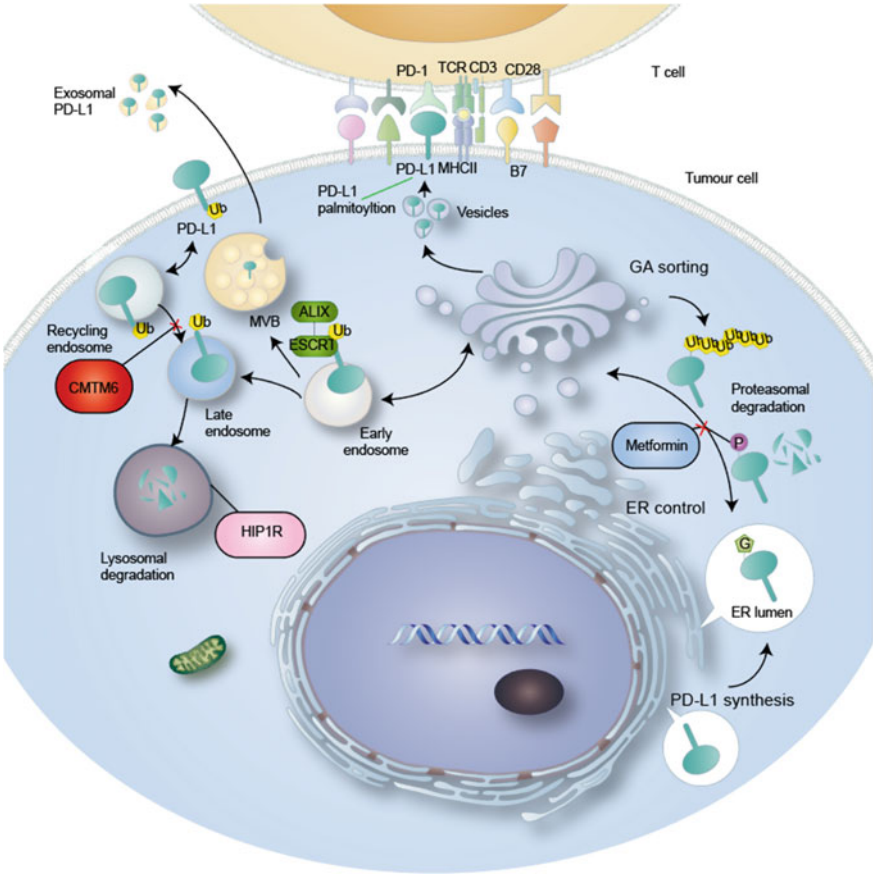

Fig. 18.1 Trafficking of PD-L1 in cancer cells

lysosome-mediated degradation (Burr et al. 2017). CMTM6 derives from a family of proteins encoded primarily by two distinct gene clusters on chromosome 16 (CMTM1-4) and chromosome 3 (CMTM6-8) (Han et al. 2003). Research data has evinced that the CMTM6 is not required for the trafficking of PD-L1 from ER to GA or to cell surface, but per se regulates the PD-L1 expression through an interferon (IFN) γ-independent pathway (Burr et al. 2017). The endocytosed PD-L1 would undergo degradation in lysosomes instead of being recycled to the cell membrane under the CMTM6-depleted circumstances, which suggests that CMTM6 promotes the expression of PD-L1 by enhancing the recycling of PD-L1 from plasma membrane into recycling endosomes (Burr et al. 2017), and the PD-L1 once expressed on the cell surface can be recycled and expressed again. The studies have demonstrated that CMTM6 is responsible for the downregulation of PD-L1 polyubiquitination (a type of post-translational modification necessary for degradation), which prevents PD-L1

from intracellular degradation (Burr et al. 2017). Therefore, blocking such recycling by inhibiting the CMTM6 can significantly minimize surface PD-L1 expression.

18.2.4 Huntingtin-Interacting Protein 1-Related (HIP1R) and PD-L1 Trafficking

The removal of intracellular PD-L1 involves several protein degradation systems, including designed proteolysis targeting chimeras (PROTACs) (Bondeson et al. 2015; Sakamoto et al. 2001), tag-based degradation system (dTAG) (Nabet et al. 2018), and lysosome-targeting molecules (Bauer et al. 2010; Fan et al. 2014). The lysosomal pathway of the PD-L1 degradation is highly associated with the expression of Huntingtin-interacting protein 1-related (HIP1R), a gene that plays its part in the cellular logistics and is one of the most significant negative regulators of the surface PD-L1 expression (Gottfried et al. 2010; Jain et al. 2008). Numerous experimental results have revealed that HIP1R does not affect the transcription, translation, or any other parts of PD-L1 protein trafficking but the lysosome-dependent degradation (Huanbing et al. 2018), for the incubation of selective lysosomal inhibitors in contrast to the inhibitors for autophagy or proteasomes statistically counteracts the downregulation of PD-L1 by HIP1R. Such regulation seems to be attributed to a di-leucine sorting signal (Leu-Leu, LL/ Leu-IIe, LI) motif of the HIP1R sequence— supporting data has shown the contributing roles played by Nef-LL (Greenberg et al. 1998) and CLN3-LI (Kyttala et al. 2005) motif in the upregulation of lysosome-dependent degradation for various TMD proteins, and such mechanism might apply to HIP1R and PD-L1 lysosomal degradation as well. The HIP1R-associated immunotherapy that downregulates the surface PD-L1 expression can aid the traditional monoantibody therapy theoretically thereafter.

18.2.5 Exosomal PD-L1

Exosomes (size between 30 and 150 nm) are described as small extracellular vesicles (EVs) of multivesicular endosomal origin, which are formed from inward luminal budding of membrane, and are released into extracellular environment after fusion of multivesicular bodies (MVB) with cell membrane (Van der Pol et al. 2012; Kalra et al. 2016; Zaborowski et al. 2015; Hurley 2015). Exosomes share a great number of common characteristics with other EVs (Corrado et al. 2013), but distinguishable from them by their size, morphology, and surface protein profiles (Van der Pol et al. 2012; Cocucci et al. 2008; Gyorgy et al. 2011), which carry membrane-tethered molecules between different cells. Recent studies have demonstrated that exosomes play various roles in different human body systems under physiological and pathological situations, including cardiovascular diseases (Leroyer et al. 2007; Emanueli

et al. 2015), neurodegenerative disorders (Danzer et al. 2012), liver inflammation (Masyuk et al. 2013), cancer progression (Abd Elmageed et al. 2014; Zhang et al. 2015), etc. Till now, the linkage between exosomes and PD-L1 has been hardly studied and the exact effects of the exosomal PD-L1 remain controversial till now. Although generally only the surface PD-L1 is considered to be effective since various coreceptors on the cell surface are crucial for the PD1-PD-L1 interaction, several articles have concluded that the tumor-expressed exosomal PD-L1 is associated with reduced T cell activity and therefore stronger immunosuppression in the tumor microenvironment, making it easier for cancer cells to evade the immune system (Becker et al. 2016; Haderk 2017; Yi et al. 2018). With the application of exosome secretion inhibitors, GW4869 (Li et al. 2013), or by the knockdown of gene Rab27a which controls the exosome secretion (Ostrowski et al. 2010; Bobrie et al. 2012), marked tumor growth suppression is seen (Yi et al. 2018). Moreover, in metastatic melanoma, the level of circulating exosomal PD-L1 positively correlates with that of IFN-γ (Gang et al. 2018), which indicates that when the PD-L1 are transported in the MVBs, they are negative regulators of the immune system to help tumor cells escape immune surveillance. Furthermore, in patients with chronic lymphoproliferative leukemia (CLL), rather than directly present or deliver PD-L1 to the plasma membrane surface of monocytes, the tumor-derived exosomes seem to induce the overexpression of PD-L1 in monocytes by promoting endosomal toll-like receptor 7/8 (TLR7/8) signaling (Haderk 2017). Some data has shown that the exosomal PD-L1 shares the same membrane topology as the cell surface PD-L1 (Gang et al. 2018), which explains why the exosomal PD-L1 also binds PD-1 and takes effect, and it is further supported by the studies that the inhibition of exosomal pd-l1 contributes to systemic and enduring antitumor immunity (Poggio et al. 2019), but it is still not absolutely determined till now.

18.2.6 ALIX and PD-L1 Trafficking

The results of the study about the connection between ALIX and PD-L1 expression are contradictory to other studies about exosomal PD-L1, and PD-L1 in its exosomal form is considered nonfunctional in this study—when PD-L1 is in a limited amount, increase in exosomal PD-L1 level inevitably leads to minimized surface PD-L1 expression, upregulating immune reactions finally (Monypenny et al. 2018). ALIX is one of proteins associated with the ESCRT, which inhibits the EGFR activity and enhances exosomal PD-L1 biogenesis (Bissig and Gruenberg 2014; Carlton 2010). Several studies have revealed a positive correlation between EGFR mutation and surface PD-L1 expression in NSCLC (Akbay et al. 2013; Murillo et al. 2014), and while targeting the wild-type EGFR has little effect on PD-L1 expression, drugs aiming at the mutant EGFR greatly restore the immune ability in their counterparts, which suggests that PD-L1 expression depends upon the kinase activity of EGFR (Akbay et al. 2013; Azuma et al. 2014). ALIX is regarded as a negative regulator of EGFR, and it can upregulate the exosomal PD-L1 level by manipulating the cargo

sorting in addition to exosome biogenesis (Monypenny et al. 2018). When ALIX is in absence, PD-L1 becomes mis-secreted with upregulated EGFR activity, and the lower PD-L1 exosomal packaging is compensated with higher PD-L1 surface presentation, which directly interacts with PD1 to reinforce immunosuppression in tumor microenvironment. Therefore, the maintenance of normal ALIX level has great therapeutic promise in combinatorial immunotherapy as well.

18.2.7 The Regulative Role of PolyI: C in CTL Responses and PD-L1 Trafficking

Apart from different patterns and regulative molecules of PD-L1 trafficking, a recent study has demonstrated the association between polyI:C and PD-L1 trafficking linked by CD40 (Aditi et al. 2016). PolyI:C is a synthetic double-strand RNA mimetic immunostimulant and has been applied for immunotherapy solely or in combinatorial immunotherapy for tumor models (Caskey et al. 2011; Pradere et al. 2014). Several studies have unveiled that when polyI:C and anti-PD1 monoantibodies are combined together, the treatment efficacy is outstanding (Pulko et al. 2009; Nagato et al. 2014; Boes and Meyer-Wentrup 2015; Salmon et al. 2016). Some research data shows a difference between the immune response of polyI:C-matured dendritic cells (DCs) and the LPS-matured DCs, and blockade of PD-L1 restores T cell proliferation in the former rather than the latter (Aditi et al. 2016). Additionally, the data suggests that the difference is caused by the selective trafficking of PD-L1 to the cell surface in polyI:C-matured DCs, which is induced by the interactions between DCs and T cells via CD40 (Aditi et al. 2016). The combinatorial immunotherapy of anti-PD1 monoantibodies and polyI:C is therefore favorable. Nevertheless, the exact pathways of PD-L1 trafficking involved in such reactions have not been clearly clarified, and therefore further study about such interaction remains to be carried out.

18.2.8 Post-translational Modifications in PD-L1 Trafficking

Aside from various molecules that influence the PD-L1 trafficking, different post-translational modifications of PD-L1 molecules are critical for the trafficking as well, especially glycosylation, phosphorylation, palmitoylation, and ubiquitination (Eikawa et al. 2015; Li et al. 2016; Yang et al. 2019; Zhang et al. 2018; Lim et al. 2016).

Just as mentioned above, glycosylation occurs in both ER and GA and is essential for the normal trafficking of PD-L1, enhancing the stability of PD-L1. Besides the mentioned information, studies have disclosed that the half-life of glycosylated PD-L1 is four times longer than the non-glycosylated form, which is mainly attributed to evasion from the degradation in 26S proteasome (Li et al. 2016). The glycosylation

of PD-L1 interferes with the phosphorylation of enzymes involved in ubiquitination, preventing PD-L1 from being recognized by ubiquitin E3 ligase (Frame and Cohen 2001). Meanwhile, antibodies that target the β-1,3-N-acetylglucosaminyltransferase 3 (B3GNT3, a type II transmembrane protein) in GA (the organelle responsible for the PD-L1 glycosylation) has shown the ability to reduce PD-L1 expression in breast cancer cells (Li et al. 2018).

PD-L1 phosphorylation is another key modification for trafficking. On the contrary of glycosylation, phosphorylation of PD-L1 ^{195}S on the extracellular domain disturbs PD-L1 trafficking from ER to GA, trapping the misfolded PD-L1 in the ER (Eikawa et al. 2015). The ER-trapped PD-L1 will be recognized by endoplasmic-reticulum-associated protein degradation complex (ERADC) and undergo subsequent eradication there, which is supported by the intracellular accumulation of phosphorylated PD-L1 after ERADC knockdown (Eikawa et al. 2015). Palmitoylation of PD-L1 is a relatively new but crucial post-translational modification. Recent research utilizes mouse model to reveal that palmitoylation stabilizes PD-L1 and increases its surface expression through assisting GA to plasma membrane trafficking, aiding tumor growth (Yang et al. 2019). The main enzyme responsible for PD-L1 palmitoylation is palmitoyltransferase ZDHHC3 (DHHC3), and silencing of DHHC3 or inhibition of PD-L1 palmitoylation via 2-bromopalmitate boosts antitumor immunity. The decline in palmitoylation reduces PD-L1 trafficking to the plasma membrane and sensitizes cancer cells to T cell-dependent immune reactions. The PD-L1 palmitoylation inhibitors might take unignorable roles in new combinatorial immunotherapeutic regimens toward cancers (Yao et al. 2019).

Ubiquitination involves the trafficking of PD-L1 to proteasome and is principal for degradation of PD-L1 there. It is regulated by sundry molecules. For instance, TNF-α uses COP9 signalosome 5 (C5N5), a deubiquitinase, to remove the initial ubiquitination to maintain PD-L1 surface expression (Lim et al. 2016). In most cases, ubiquitination of PD-L1 is held in a relatively homeostatic level, but once any associated regulators become disordered, the balance will break. Downregulation of ubiquitination would increase the recycling and surface expression of PD-L1, just as the stated example CMTM6 (Burr et al. 2017). One famous illustration is speckle-type POZ protein (SPOP), and cullin 3-SPOP E3 ligase is the pivot of PD-L1 polyubiquitination and degradation, which is stabilized by cyclin D-dependent CDK4. Inhibition of CDK4/6 would increase the surface PD-L1 expression to a large extent and promote the therapeutic effect of anti-PD1 therapy, for the level of surface PD-L1 expression and anti-PD1 therapeutic efficacy is positively correlated. The respective CDK4/6 inhibitor palbociclib has already shown therapeutic benefits in mouse models in combination with anti-PD1 immunotherapy (Zhang et al. 2018).

18.3 Other Immune Checkpoint Trafficking

18.3.1 CTLA-4 Trafficking

CTLA-4 is the second best studied immune checkpoint molecule in trafficking. CTLA-4 downregulates immune responses by competing with T cell surface receptor CD28 to binding the respective ligand B7 (CD80/86) to prevent excessive T cell activation (Sansom 2000). While most of CD28 is expressed on the plasma membrane, the majority of CTLA-4 exists in vesicles in intracellular compartments, including trans GA (Leung et al. 1995; Valk et al. 2006; Mead et al. 2005), endosomes, secretory granules (Linsley et al. 1996), and lysosomal vesicles (Iida et al. 2000), which is generally ascribed to the effect of clathrin adaptor protein complex AP2 (Leung et al. 1995; Linsley et al. 1996; Zhang and Allison 1997; Chuang et al. 1997; Shiratori et al. 1997; Schneider et al. 1999). Once CTLA-4 vesicles fuse with cell membrane, surface CTLA-4 will undergo endocytosis—a process mediated by tyrosine-based motif (YVKM) in CTLA-4 and clathrin adaptor protein complex AP2—and similar to other TMD proteins; after endocytosis, CTLA-4 can be recycled to cell membrane or transported to lysosomes for degradation (Khailaie et al. 2018). Therefore, any molecules or modifications that affect such trafficking will greatly influence CTLA-4 expression on cell surface (Fig. 18.2).

The influencing factors of CTLA-4 trafficking have been elucidated via a model dividing the trafficking process into CTLA-4 synthesis, recycling, internalization, and degradation separately (Khailaie et al. 2018). The experiments applying such model evince that without new CTLA-4 formation, the intracellularly stored CTLA-4 can maintain the surface CTLA-4 expression level through recycling for a while, but CTLA-4 synthesis is the main impact on its trafficking to the cell surface in the long term, which is the determining factor for the surface CTLA-4 expression (up to 50%). Meanwhile, CTLA-4 synthesis does not change the subcellular CTLA-4 distribution greatly; for the CTLA-4, distribution will almost return to the previous steady state after each alteration in the level of CTLA-4 synthesis. Statistics have also revealed that CTLA-4 is mostly degraded in lysosomes; for the half-life of surface, CTLA-4 is around 3.3 h in average, but blockade of lysosomal degradation elongates the half-life of surface CTLA-4 for about 5.5 h (Khailaie et al. 2018).

Experiments have demonstrated that several molecules can control the CTLA-4 trafficking (Sansom 2015). One of the co-localizers of CTLA-4 in endosomal vesicles, lipopolysaccharide-responsive and beige-like anchor protein (LRBA), is critical for the trafficking. LRBA has been considered to free CTLA-4 from degradation and enhances CTLA-4 recycling. A diminishment in CTLA-4 amount has been detected in patients with LRBA mutations (Sansom 2015; Lo et al. 2015), the main mechanism of which is elucidated that reduced CTLA-4 recycling cannot meet the demand to induce sufficient immune inhibition. Apart from LRBA, T cell receptor-interacting molecule (TRIM) is of vital importance as well, which has been regarded as a chaperone in CTLA-4 trafficking that promotes surface CTLA-4 expression (Valk et al. 2006). Furthermore, GTPase ADP ribosylation factor-1 (ARF-1) and

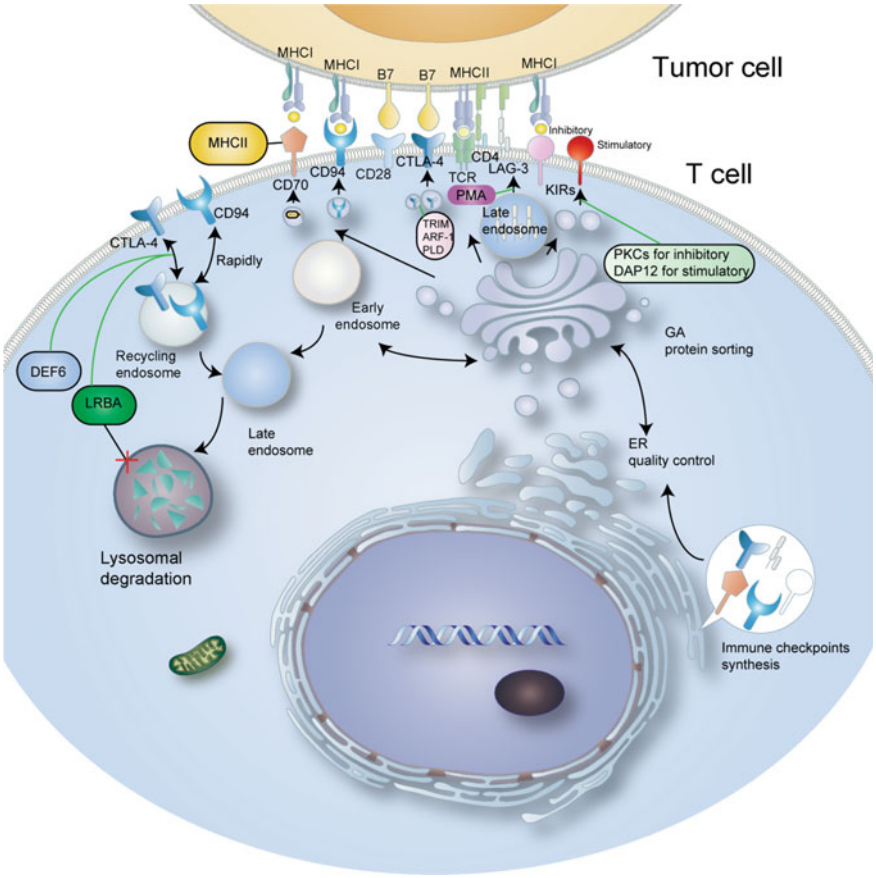

Fig. 18.2 Trafficking of other immune checkpoint molecules

phospholipase D (PLD) can also regulate the amount of CTLA-4 released to cell surface through enhancing the budding of CTLA-4 vesicles in GA (Mead et al. 2005). Apart from that, a recent published study has revealed that the different expressions in FDCP6 homolog (DEF6), a unique guanine nucleotide exchange factor (GEF) which is mostly expressed in T cells and NK cells (Becart and Altman 2009), contribute to the recycling of CTLA-4 in the form of endosomes through interactions with small GTPases (essential for vesicular transportations, especially RAB11), which also enhances surface CTLA-4 expression ultimately (Serwas et al. 2019).

Post-translational modifications are equally significant in CTLA-4 trafficking. Defective N-glycosylation of CTLA-4, induced by substitution of threonine 17 to alanine 17 in the signal peptide, results in retention of misfolded protein in ER and reduced surface CTLA-4 expression (Anjos et al. 2002).

Any steps or molecules involved in CTLA-4 trafficking will greatly alter the surface expression, and it is indicated that regulators of CTLA-4 trafficking can be possibly useful in combinatorial immunotherapies.

18.3.2 LAG-3 Trafficking

Several investigations have focused on another immune checkpoint molecule, LAG-3. LAG-3 belongs to the Ig superfamily and is expressed on activated T cells and invariant natural killer (NK) cells (Triebel et al. 1990), which is served as a binder to MHC class II molecules and is considered as a competitor of CD4 (Huard et al. 1995; 1997). After LAG-3-MHC class II binding, T cells will receive negative regulative signals and cytotoxic activities (especially CD3/TCR activation) will be attenuated (Woo et al. 2010). Recent studies scrutinized the intracellular trafficking and the trafficking to plasma membrane of LAG-3 through Jurkat cells.

Normally, LAG-3 subsists in late endosomes and secretory lysosomes in unstimulated T cells, which is translocated to the surface of T cells more rapidly than CD4 upon activation (Bae et al. 2014). By overexpressing LAG-3-EGFP in Jurkat cells, antibodies that recognize subcellular organelle markers unveiled that LAG-3-EGFP was mostly colocalized with cathepsin D (lysosome marker) and LAMP-2 (late endosome and lysosome marker) rather than Rab5A (early endosome and cellular membrane marker), demonstrating that the majority of LAG-3 are stored in late endosomes and lysosomes in quiesced T cells. Upon stimulation, the LAG-3 will be transported from late endosomes and lysosomes to cell surface, and this process requires cytoplasmic domain without the EP motif—the LAG-3 that possesses EP motif manifests reduced translocation to cell surface for it would not be activated to be transported to cell surface upon stimulation (Li et al. 2007).

Certain molecules have shown their unignorable significance in LAG-3 trafficking as well. Ammonium chloride can inhibit lysosomal enzyme activity to increase surface LAG-3 expression. In addition, protein kinase C (PKC) is essential for LAG-3 translocation to plasma membrane. Phorbol 12-myristate 13-acetate (PMA), an activator of PKC, has proved to augment surface LAG-3 expression by influencing trafficking; for the PKC, inhibitors do not change the total LAG-3 amount but only the distribution (Li et al. 2007).

And there is no exception for LAG-3—any molecules that disturb the trafficking from late endosomes or lysosomes to the cell surface can be beneficial in the combinatorial immunotherapy.

18.3.3 KIRs Trafficking

The trafficking of another kind of immune checkpoint molecules, KIRs, has also been probed. KIRs refer to a kind of transmembrane glycoproteins expressed on T

cells and NK cells that contain two or three extracellular Ig-like domains (Steffens et al. 1998). There are stimulatory KIRs and inhibitory KIRs, and both of them bind to class I MHC molecules on target cells. Studies have suggested that the trafficking of inhibitory and stimulatory KIRs are differently regulated (Chwae et al. 2008; Mulrooney et al. 2013).

For the inhibitory KIRs, they possess immunoreceptor tyrosine-based inhibition motifs (ITIMs) in their cytoplasmic tails to carry out immunoinhibitory functions (Gumperz and Parham 1995; Moretta et al. 1996). The phosphorylation of the ITIMs dephosphorylates the downstream activating molecules to downregulate immune reactions, especially target cell lysis and cytokine release (Lanier 1998). Research data has demonstrated that various PKCs can upregulate surface KIRs expression through phosphorylation of the amino acid sequences in the cytoplasmic motifs evidenced by the Jurkat cells (Chwae et al. 2007). The recycling process of KIRs can be divided into two steps, the endocytosis and the exocytosis, and the amino acid sequences responsible for the regulation of each of them are distinct. While the endocytosis-associated PKC activation depends upon Y-based motif, the acidic region, PKC-phosphorylatable S^{415}, the acidic cluster sorting motif-like region, and the L-based motif-like region, exocytosis-associated PKC activation is related to PKC-phosphorylatable T^{407} residue. Through such phosphorylation, the conventional PKCs can enhance recycling of KIRs from sorting endosomes and maturation from ER to GA trafficking process, and PKCδ promotes KIRs trafficking to plasma membrane through lytic granules ultimately (Chwae et al. 2007).

The stimulatory KIRs have lesser affinity for HLA ligands than their inhibitory counterparts (Stewart et al. 2005; Biassoni 1997; Chewning et al. 2007). They greatly rely on adapter molecules, such as DNAX activation protein DAP10 and DAP12, to covey the stimulatory signals, for immunoreceptor tyrosine-based activation motifs (ITAMs) are absent in their cytoplasmic motifs (Snyder et al. 2003, 2004). Existing studies have pointed out that DAP12 contributes to KIRs trafficking to the cell surface and DAP12 knockdown is consistent with diminished surface KIRs expression (De Rham et al. 2007). It is proposed that DAP12 promotes the maturation of KIRs through enhancing post-translational glycosylation, which is necessary for the trafficking from ER to GA, stabilizes the KIRs expressed on the cell surface, and prevents the internalized KIRs from being transported to or degraded in lysosomes (Snyder et al. 2004). Meanwhile, DAP10 can increase KIRs trafficking to the plasma membrane and attenuates the degradation in lysosomes and proteasomes (Burgess et al. 2006; Park et al. 2011).

Although stimulatory and inhibitory KIRs have opposite effects on immune signals, the trafficking of them are regulated quite differently, and the corresponding potential therapeutic targets do not overlap with each other.

18.3.4 CD94/NKG2A Trafficking

Another immune checkpoint molecule, CD94/NKG2, shares several characteristics with KIRs—it is also mainly expressed on NK cells and subsets of T cells; it is composed of a family of receptors that some of them upregulate immune responses and the others on the contrary; it interacts with MHC class I molecules as well (Borrego et al. 2006; Colonna et al. 2000). And it is the trafficking (especially endocytosis) of CD94/NKG2A, a receptor in the family that transmits inhibitory signal, that has been focused on.

CD94/NKG2A has the classic ITIM in its cytoplasmic tail. After binding with MHC class I molecules, phosphorylation of ITIM leads to dephosphorylation of the downstream activating cascade, resulting in reduced immune reactions (Sanni et al. 2004). CD94/NKG2A has a unique pathway for endocytosis—CD94/NKG2A endocytosis is macropinocytosis-like, and many traits of it, such as amiloride sensitivity, Rac1 dependency, colocalization with internalized dextran and LY, are consistent with micropinocytosis (Madhan et al. 2008). Nevertheless, the independence from actin, clathrin, dynamin, or lipid rafts makes the endocytosis of CD94/NKG2A distinct from macropinocytosis (Madhan et al. 2008). CD94/NKG2A can be recycled back to the cell surface rapidly. After endocytosis into early endosomes, CD94/NKG2A is directly recycled to the cell surface without being internalized into late endosomes, lysosomes, or recycling endosomes. This trafficking mechanism explains why the surface CD94/NKG2A expression is almost constant (Colonna et al. 2000). Further studies into the unique endocytosis of CD94/NKG2A are essential for the understanding of related immune reactions and prospective drug targets.

18.3.5 CD70 Trafficking

CD70, the ligand of CD27 (a costimulatory receptor in the formation of effector and memory T cells), is a homotrimeric transmembrane molecule mostly expressed on B cells, T cells, and DCs in association with TNF to reinforce immune responses (Goodwin et al. 1993; Tesselaar et al. 1997; Oshima et al. 1998). Since CD70 lacks the tyrosine- or leucine-based sorting motifs for intracellular trafficking to specific compartments, CD70 is delivered to plasma membrane by default in cells without MHC class II molecules presenting system (Anna et al. 2007). But in professional antigen-presenting cells (APCs), CD70 is delivered specifically to immunological synapse (the contact region between a mature DC and a naïve T cell (Friedl et al. 2005)) in vesicles with MHC class II molecules simultaneously, which is supposed to be associated with chaperones containing sorting motifs (Tesselaar et al. 1997).

In professional APCs, the intracellular distribution of CD70 highly resembles that of MHC class II molecules (Anna et al. 2007). CD70 can be detected mainly on plasma membrane, in early and late endosomes, lysosomes, and GA, mostly matching the distribution of MHC class II molecules (Pierre et al. 1997; Turley et al. 2000).

Late endocytic vesicles defined as MHC class II compartments (MIIC) contain most of the CD70, and the majority of CD70 is resided inside the compartments instead of staying on the membrane, which is regulated by the professional antigen-presenting mechanism mastered by the class II transactivator (CIITA) (Chang and Flavell 1995). The MIIC containing CD70 is transported along microtubules to the immunological synapse and is finely regulated by the dynein–dynactin motor proteins (Wubbolts et al. 1999). The whole trafficking process of CD70 is shared by MHC class II molecule trafficking, and such association is critical for further study.

18.4 Conclusions and Discussions

Since trafficking is of vital importance in the expression of the TMD proteins, regulations of any step in the trafficking can be effective in the regulation of surface TMD proteins' expression. The immune checkpoint molecules are just as other TMD proteins that whether the augmentation of their degradation or the inhibition of the extracellular secretion can be beneficial in the combinatorial immunotherapy; in other words, any techniques that can downregulate the expression of the inhibitory immune signals in tumor microenvironment have their own therapeutic potential, whether by promoting the degradation of surface immune checkpoint molecules through the lysosomal/proteasomal pathway or decreasing their expression by interfering the ER to GA or the plasma membrane to the recycling endosomes trafficking.

The most important immune checkpoint molecule is PD1/PD-L1, but the exosomes in PD-L1 are especially intriguing and the effects are not absolutely determined. The exosomal PD-L1 pathway is still quite mysterious, and the actual effect remains to be seen. Although from our perspective, the exosomal PD-L1 does not possess the ability to interact with PD-L1 to carry out the normal PD1-PD-L1 interactive effects as the surface PD-L1 does, studies do suggest that the exosomal PD-L1 share the same kind of surface topology as the surface one and the inhibition of exosomal pd-l1 apparently reinforces immune reactions. But the studies about ALIX are controversial to such result, suggesting that upregulating exosomal PD-L1 decreases the surface PD-L1 expression and reduces immunosuppression. The exosomal PD-L1 certainly requires further studies and it is worth looking deeper into it.

As the anti-PD1 monoantibodies' immunotherapy has become increasingly noticeable since its emergence, the trend for studies about it has never been more zealous. Although great efforts have been put into perfecting such immunotherapy, the main focus is synthesizing better monoantibodies which possess greater specificity to minimize the adverse effects and better efficacy in combining the surface PD1 molecules. However, mechanism exists for recycling of the surface PD1 in tumor to escape the effects of anti-PD1 antibodies after interactions. In contrast to the monoantibody therapy, drugs and substances that disrupt the trafficking directly affects the expression level of PD-L1 from the very beginning, waiving the evasion by the preceding mechanism.

The principles of such combinatorial immunotherapy might not only apply to PD1/PD-L1 immunotherapy but immunotherapy involving other immune checkpoint molecules. Since the studies of CTLA-4, LAG-3, KIRs, CD94/NKG2A, and CD70 have already existed, the respective interference with the trafficking process might also be helpful in combinatorial immunotherapy. Apart from these checkpoint molecules, others such as CD27, TIM-3, VISTA, etc. have their values of investigations and potential of therapeutic target as well. This area is relatively promising in light of the current studies.

To wrap up, substances and drugs that disrupt the normal intracellular trafficking of the immune checkpoint molecules can attenuate the immunosuppression induced by the negative immune signals and therefore acquire therapeutic potential; the accurate effects of certain pathways (the exosome pathway of PD-L1 especially) and the trafficking processes of immune checkpoint molecules demand further explorations; such combinatorial therapy does not only apply to the PD1 immunotherapy but may serve as a therapeutic principle for all the combinatorial tumor immunotherapies. We look forward for further studies and investigations into this area.

Conflicts of Interest The authors have indicated that they have no conflicts of interest with regard to the content of this article.

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Chapter 19
Exosome and Secretion: Action On?

Ye Hu, Rui Zhang and Gang Chen

Abstract Originally treated as part of a cellular waste, extracellular vesicles (EVs) are being shown to possess a vast variety of functions, of which exosome is the most studied one. Most cells, such as tumor cells, immunocytes, and fibroblasts can secrete exosomes, especially under certain stresses the amount is much higher, and the contents of exosome represent the status of the donor cells and the tumor microenvironment. As crucial transporters for cells' content exchange, much attention has been raised in the utilities of exosomes to suppress immune response, and to modify a microenvironment favorable for cancer progression. Exosomal immune checkpoints, such as programmed cell death ligand 1 (PD-L1), contribute to immunosuppression and are associated with anti-PD-1 response. Many forms of soluble immune checkpoint receptors have also been shown to influence efficacy mediated by their therapeutic antibodies. Therefore, targeting pro-tumorous exosomes may achieve antitumor effect supplementary to existing therapies. Exosome, itself natural liposome-like structure, allows it to be a potential drug delivery tool.

Keywords Tumor-derived exosome · Exosomal PD-L1 · Soluble immune checkpoint receptors · miRNA

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19.1 The Introduction of Exosome in Cancer Immunology

Mammalian cells synthesize and release heterogeneous EVs which can be generally subclassified as exosomes (30–120 nm in diameter), microvesicles (MVs, or ectosomes or microparticles, 0.1–1.0 μm), and apoptotic bodies (0.8–5.0 μm), each differing in their biogenesis, composition, and biological functions from others (Han et al. 2019) (Fig. 19.1). Exosomes are considered to be endosomally derived, while MVs bud from the surface of plasma membrane. EVs are circular pieces of membranes that incorporate various bioactive molecules including membrane receptors, proteins, mRNAs, microRNAs (miRNAs), and organelles, and thus potentially affect target cells by transferring their cargo or by receptor-to-ligand interaction. Endosomally-derived vesicles was first reported in transferrin recycling Rat reticulocyte endocytoses and then releases transferrin through multivesicular endosomes or multivesicular bodies (MVBs), which were described as often in 250–300 nm diameter but range from 120 to 800 nm diameter and may be irregularly shaped, into the surrounding microenvironment via fusion with plasma membrane of the parental cell. The MVBs intracellular passage does not enter into lysosome (Pan and Johnstone 1983; Harding et al. 1983).

The biogenesis of exosomes includes three distinct steps, begin with the development of endocytic vesicles by inward budding of the plasma membrane, then the generation of MVBs by invagination of the endosomal membrane, and at last, the fusion of plasma membrane with MVBs and subsequent release of the vesicular components, named exosomes (Batista et al. 2011) (Fig. 19.1). Exosomes and other EVs differ in size, density, morphology, marker expression, and rely on specific enzymes for their biogenesis. Vital enzymes in their generation include budding of intravesicular vesicles promoter—NSMASE2 (aka SMPD3), and fusion of the MVB

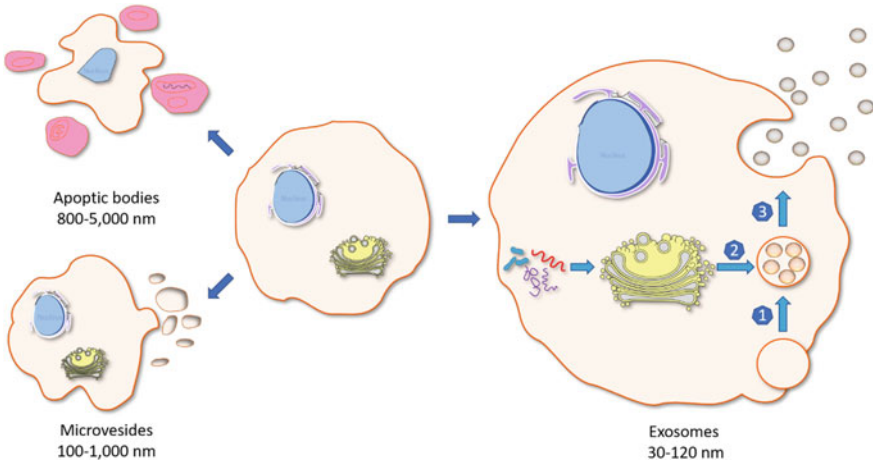

Fig. 19.1 The biogenesis of exosomes

to the plasma membrane catalyzer—RAB27A (Ostrowski et al. 2010; Kosaka et al. 2010). Genetic manipulation of these enzymes provides an opportunity to dissect the role of exosomes in vivo. Various exosome biomarkers have been identified, for example, TSG101, Ras-related protein Rab-11B (RAB11B), CD63, charged multivesicular body protein 2a (CHMP2A), and CD81 proteins, as well as lipids, including cholesterol, ceramide, sphingomyelin, and phosphatidylserine (Dickens et al. 2017; Colombo et al. 2014; Raposo and Stoorvogel 2013).

The isolation of pure exosomes is vital to uncover their action mechanisms and for further application in biomedical sciences. Since they are tiny and the isolation is challenging, a variety of techniques have been developed to facilitate the isolation. Successful isolation of exosomes can be achieved in a variety of ways like ultracentrifugation, ultrafiltration, chromatography, affinity capture on antibody-coupled magnetic beads, and polymer-based precipitation (Peterson et al. 2015). Practically, the size difference between cells, subpopulations of EVs, and proteins allow them to be separated and isolated by centrifugation. Ultrafiltration is rapid but hard to get rid of contaminating proteins, which can be improved by combining with ultracentrifugation. Membranes are used to filtrate cells and large EVs, and then ultracentrifugation can further separate exosomes from proteins (Alvarez et al. 2012). Aiming to purify exosome isolations, immuno-affinity purification (IP) techniques have been adopted that can selectively capture desired exosomes from a complex population basing on certain surface markers. IP techniques are rapid, convenient, and compatible with regular laboratory equipment, during which process anti-CD63, anti-CD9, and anti-CD81 antibodies are typically used (Tauro et al. 2012).

Exosomes released from tumor cells actively lead to tumor progression and metastasis (Chiodoni et al. 2019). It has been shown by studies that exosome released from cancer cells can be stimulated under different stresses, which also cause the alteration of exosome's content. Oxidative and heat stresses have been shown to induce the exosome generation from leukemia/lymphoma T and B cell lines (Hedlund et al. 2011) and hypoxic environment has been reported to effectively augment tumor-derived exosomes (TEX) shedding from breast cancer cells (Wang et al. 2014). Moreover, sublethal doses of a number of chemotherapeutic drugs, such as proteasome inhibitors and genotoxic drugs, enhance exosome release in various cancer models. As such, multiple myeloma (MM) cells can enhance the release of nanovesicles receiving bortezomib or melphalan treatment (Lehmann et al. 2008; Yu et al. 2006).

Tumor-suppressor gene p53 has been described in stress-induced exosome secretion. In this regard, irradiation of prostate cancer cells trigged an amplified secretion of exosome-like vesicles by p53 activation (Lehmann et al. 2008). Furthermore, a p53-inducible gene product, the tumor-suppressor-activated pathway 6 (TSAP6), was found to regulate exosome trafficking and secretion in cells undergoing DNA damage (Yu et al. 2006; Amzallag et al. 2004). The fact that exosome secretion is reported to be severely restrained in TSAP6-deficient mice can further support these observations (Lespagnol et al. 2008). Moreover, increased exosome secretion in tumor cells can be observed in a senescent phenotype (Borrelli et al. 2018), which can pose protumorigenic effects in pre-malignant recipient cells. Senescence-associated secretory

phenotype (SASP) can be enhanced either by inactivation of p53 or gain of oncogenic RAS (Coppe et al. 2008).

EVs are implicated in multiply processes, such as tumor cells proliferation, chemoresistance, immune escape, metabolic reprogramming, metastatic enhancement, and angiogenesis. Herein, we will mostly focus on how exosomes affect cancer immunology.

19.2 The Role of TEX in Cancer Immunology (Detailed in Fig. 19.2)

By generating an immunosuppressive microenvironment, TEX is beneficial for tumor growth and distant dissemination. Studies have been showing the mechanisms and targets of immune suppression by TEX are diverse (Graner et al. 2018), while some reporting TEX is immune activator. Both success of immune-based therapies and active endogenous antitumor immune responses rely on effector cells that directly target and kill cancer cells, such as cytotoxic T lymphocytes (CTLs) and natural killer (NK) cells. We will herein discuss how TEX contributes to those cancer-related immunocytes' function.

19.2.1 TEX Suppresses T Cell Functions

Tumor cells actively release immunosuppressive MVs into the microenvironment, disrupting T cell immunosurveillance. Tumor-derived MVs harvested in suspension

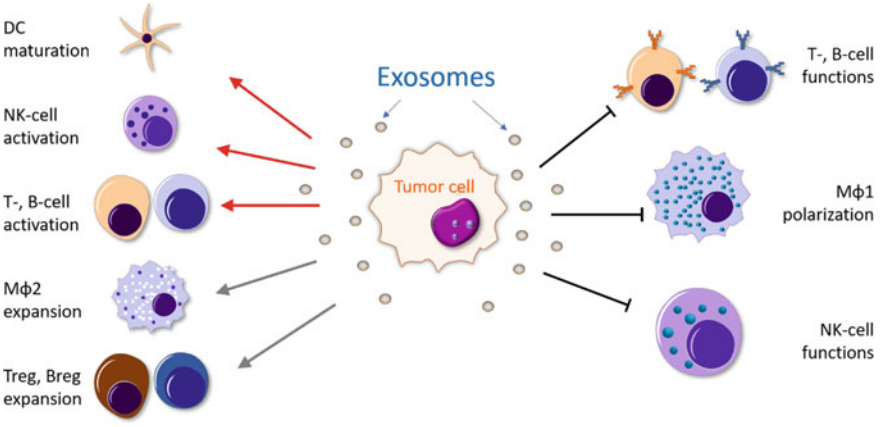

Fig. 19.2 The roles of tumor-derived exosomes in cancer immunology

of head and neck squamous cell carcinoma (SCCHN) cell line, PCI-13, with combination of size-exclusion chromatography and ultracentrifugation methods, were variably enriched in major histocompatibility complex (MHC) class I. And those MVs suppressed signaling and expansion, and induced apoptosis of activated CTLs, while enhancing the proliferation and suppressor activity of CD4+ CD25+ FOXP3+ Treg cells (Wieckowski et al. 2009), leading to immune suppression to allow tumor escape. Melanoma cell line-derived exosomes are able to alter the metabolic function of CTL cell line, CTLL-2, by transferring mRNA/miRNA contents. Notably, the delivery of mRNA/miRNA loaded exosome happened within a short time frame. Four of the top 20 mRNAs found within B16F0 exosomes (such as Cmtm4, Wsb2, Ptpn14, and Fam168b) were detected in CTLL-2 cells in 30 min. Another cluster of genes was upregulated at 4 h upon exosome treatment, which contained several gene targets involved with cellular metabolism. CMTM4 mRNA is upregulated in TEX exposed CTLL-2 cells, and this is potentially significant given its role in PD-L1 trafficking (Mezzadra et al. 2017).

Exosomes isolated from the ascites of ovarian cancer (OVCA) patients are identified, which can be internalized rapidly by T cells, and inhibited various T cell response endpoints like translocation of NFAT and NFκB into the nucleus, upregulation of CD69 and CD107a, release of cytokines, and elevated cell proliferation. However, T cell viability was not affected and T cell arrest was transient. After removal of the immunosuppressive exosomes, T cell regained their activation potential within 24 h (Shenoy et al. 2018).

However, TEX can activate CTL clones after processing by antigen-presenting cell (APC) expressing the correct MHC haplotype (Andre et al. 2002). TEX comprises cancer-related antigens that may initiate an immune response using dendritic cell (DC) as intermediaries. Melanoma-associated antigen (MAGE) was enriched in TEX from malignant ascites of patients with melanoma, and it can be recognized by T cells (MART-1). These TEX, once delivered to DCs, facilitated in vitro cross-presentation of the antigen, and response of a CTL clone, leading to an efficient in vitro antitumor cellular activation, as monitored by the quantity of interferon (IFN)-γ produced, and by the induction of specific cancer cell lysis (Andre et al. 2002; Wolfers et al. 2001).

19.2.2 Mechanisms of TEX Influencing NK Cells and DCs

TEX can regulate the functions of NK cells, by affecting the differentiation of precursors to mature antigen-presenting cells. NKG2D (activating natural killer group 2 member D) ligand on tumor cells triggers cytotoxic activity in NK cells through interacting with NKG2D (Bauer et al. 1999; Rincon-Orozco et al. 2005). TEX NKG2D is loaded with many other functional molecules including death receptor ligands, MHC class I/II, adhesion molecules, etc. (Whiteside 2013). Therefore, the alteration of the immune response by NKG2DL containing TEX is also impacted by other coexisting molecules within the exosome (Clayton et al. 2008). NKG2D interacts with several kinds of ligands. For instance, the NKG2DL in humans includes MICA\B and

UL16-binding proteins 1–6 (ULBP1–6), exosomal MICA*008 decreased NKG2D expression on NK cell and suppressed its cytotoxicity (Ashiru et al. 2010). Exosomal ULBP3 was also reported to bring down NKG2D on primary NK cells and restrain NK cell-mediated elimination of MICA-expressing target cells (Fernandez-Messina et al. 2010). Nevertheless, human DCs can secrete exosomal NKG2DL to directly enhance NK cells' function ex vivo (Viaud et al. 2009).

The other extracellular presence of NKG2DL is soluble form, which can downregulate NKG2D on NK cells or T cells, leading to suppressed cytotoxicity. Soluble MICA (sMICA) downregulated NKG2D by enhancing its endocytosis and then degradation, leading to a reduced NKG2D expression on tumor-infiltrating T cells (Groh et al. 2002). Similarly, sULBP was found to downregulate NKG2D on NK cells (Fernandez-Messina et al. 2010).

Moreover, heat shock protein 70 (Hsp70) on tumor cell surfaces is a recognized ligand for the NK cell receptor CD94 and can enhance the cytotoxicity of NK cells against Hsp70-positive tumor target cells (Gross et al. 2003). By contrast, TEX surface Hsp70 will cause reduction of NK CD94, and in this case TEX functions as systemic decoy for NK cells (Hedlund et al. 2011). In addition, transforming growth factor β (TGF-β), acting as a component of TEX (Graner et al. 2009), has also been shown to be an immune suppressor of NK cells (Szczepanski et al. 2011). Fetal liver mesenchymal stem cell (mSC)-derived exosomes contain several immunomodulatory molecules—latency-associated peptide (LAP), TGFβ, and thrombospondin 1 (TSP1), through stimulating the downstream TGF-β/Smad2/3 signaling, inhibiting proliferation, activation, and cytotoxicity of NK cells (Fan et al. 2019).

In normal human cells, inhibition of exosome secretion reduces removal of harmful nuclear DNA, causes the stacking of nuclear DNA, and thus activates cGAS-STING pathway and causes type I IFN production from those cells (Takahashi et al. 2017), indicating exosome secretion is vital for maintaining parental cell's homeostasis. Exosomal double-stranded (Ds) DNA secreted from irradiated mouse breast cancer cells could be transferred to DCs and upregulate DCs' surface costimulatory molecules, leading to the STING-dependent activation of type I IFNs (Diamond et al. 2018). The same phenomenon has been observed under the antitumor agent topotecan, an inhibitor of topoisomerase I treatment (Kitai et al. 2017). Collectively, these findings indicate that dsDNA associated with TEXs induces type I IFN production directly from cancer cells or indirectly through the DCs stimulation. Type I IFNs are known to play a crucial role in cancer progression through the promotion of anti-cancer immune responses, (Medrano et al. 2017; Zitvogel et al. 2015) such as directly activate NK cell-mediated functions, increase perforin-dependent cytotoxicity (Nguyen et al. 2002), and induce TNF-related apoptosis-inducing ligand (TRAIL) expression. In addition, with the coordinated action of IL-12, type I IFNs greatly promote NK cell-mediated IFN-γ production.

19.2.3 Mechanisms of TEX Influencing Macrophages

Preclinical and clinical studies indicate that tumor-associated macrophages (TAMs) provide important pro-tumorigenic and survival factors (Noy and Pollard 2014). TEX stimulates the macrophage infiltration and polarization in remote site for establishment of premetastatic niche. After injection of breast TEXs into mice, the amount of macrophage is shown to be increased in axillary lymph nodes, with CD206 positive M2 macrophages much more detected than NOS2 positive M1 macrophages (Piao et al. 2018). Regarding macrophages, toll-like receptor (TLR) signaling is vital for pro-inflammatory cytokines, miRNAs, and other components secretion, greatly enhancing inflammation and favoring cancer development. Through TLR on macrophages, breast TEXs activate NF-κB and induce secretion of G-CSF, TNF-α, IL-6, and CCL2, while genetic depletion of TLR2 or MyD88, a vital signaling adaptor of the NF-κB pathway, completely abrogates this effect (Chow et al. 2014). TEX can also induce monocytes to release immune-modifying factors. TEX in chronic lymphocytic leukemia (CLL) can enhance monocytes to secrete cytokines IL-6, CCL2, and CCL4, and express PD-L1 (Haderk et al. 2017). HY4, a noncoding Y RNA enriched in exosomes of CLL patient plasma can achieve the same effects on monocytes as TEX, resulting in cancer-associated inflammation and potential immune evasion via PD-L1 upregulation.

Under hypoxic stress, TEX enhances oxidative phosphorylation in bone marrow-derived macrophages through transfer of let-7a miRNA and subsequent suppression of the insulin-Akt-mTOR signaling pathway, leading to M2 polarization (Park et al. 2019).

However, macrophage receiving TEX miRNAs also have antitumor effects, as in the case where murine breast cancer was treated with EGCG (epigallocatechin gallate), a component of green tea extract with known anti-cancer properties (Jang et al. 2013). EGCG enhances TEX-derived miR-16, which is an important regulator of CHUK/IKK α (inhibitor of nuclear factor kappa-B kinase subunit alpha) complex. Delivery of these TEX to macrophage is favorable for M1 phenotype cytokines secretion. MiR-16 causes reduction of CHUK/IKK α complex and subsequent accumulation of I-κB, thus preventing NF-κB activation and M2 phenotype cytokines production (Hagemann et al. 2009; Lawrence 2009).

19.2.4 Other Immune Cell Lineages

Other immune cells are also subject to the impact of EVs produced by tumor cells. B cells primed by hepatocellular carcinoma (HCC) cell line-derived exosome strongly expressed T cell immunoglobulin-1(TIM-1) protein and were endowed with suppressive activity against CD8+ T cells. A major portion of TIM-1 is expressed by B cells and serves as a marker for Breg cells (Jang et al. 2013). HCC-exosome has high level of HMGB1, which activates B cells and promotes TIM-1^{+}Breg cell expansion

through the TLR2/4 and mitogen-activated protein kinase (MAPK) signaling pathways (Yan et al. 2012, 2018). TIM-1+ Breg cells secrete the highest proportion of IL-10 among all types of B cells.

19.3 Exosome and Cancer Immune Checkpoints (Fig. 19.3)

19.3.1 Exosomal PD-L1

Tumor cells evade the immune surveillance through increasing surface expression of PD-L1, which interacts with PD-1 on T cells (Dong et al. 2002; Chen and Han 2015), contributing to dephosphorylation of the T cell receptor as well as its co-receptor CD28 through Shp2 phosphatase, thus inhibiting antigen-driven activation of T cells (Graner et al. 2018; Hui et al. 2017). Therapeutic antibodies of PD-L1 and PD-1 block this interaction, and therefore can reactivate the antitumor immune effect (Chen and Mellman 2017). Both anti-PD-1 and PD-L1 antibodies have shown remarkable promise in curing tumors, such as renal cell carcinoma and metastatic melanoma (Chen and Han 2015; Ribas et al. 2016; Topalian et al. 2016). Unfortunately, a major portion of patients have low response rate (Ribas et al. 2016; Zaretsky et al. 2016), requiring deep understanding of PD-L1-mediated immune evasion to predict patient response and promote treatment efficacy.

PD-L1 is found on the surface of EVs, and more interestingly EV PD-L1 levels have been related with cancer progression and response to immunotherapy (Yang et al. 2018; Theodoraki et al. 2018; Ricklefs et al. 2018; Chen et al. 2018). Chen et al. reported that metastatic melanomas released EVs, with exosomes being the major form, bearing PD-L1 on their surface. Treatment with IFN-γ can enhance PD-L1 level on these vesicles, leading to CTLs dysfunction and tumor growth acceleration.

The evolvement of systemic exosomal PD-L1 along the course of anti-PD-1 therapy has predictive value for cancer prognosis. The amplitudes of the increase in systemic exosomal PD-L1 during early stages of pembrolizumab treatment, indicating the adaptive response of the tumor cells to T cell re-invigoration, can stratify clinical responders from non-responders. However, circulating exosomal PD-L1 before and on treatment may reflect different states of antitumor immunity. High pretreatment level may signify the "exhaustion" of patient T cells to a turning point, by which they are unable to be re-invigorated by the anti-PD-1 treatment. For the on-treatment patients, on the contrary, a rise in the level of exosomal PD-L1, correlating proportionally with the T cell re-invigoration, would represent a strong antitumor immunity, thereby a favorable prognosis. Therefore, TEX PD-L1 may serve as a predictor for anti-PD-1 therapy efficacy (Chen et al. 2018).

PD-L1 from TEX imposes systemic immunosuppression through inhibiting T cell activation in the draining lymph node. Wild-type tumor cells grow slower with exposure to exosomal PD-L1-deficient tumor cells, which are injected simultaneously at a distant site. Inhibition of exosomal PD-L1 induces systemic antitumor immunity,

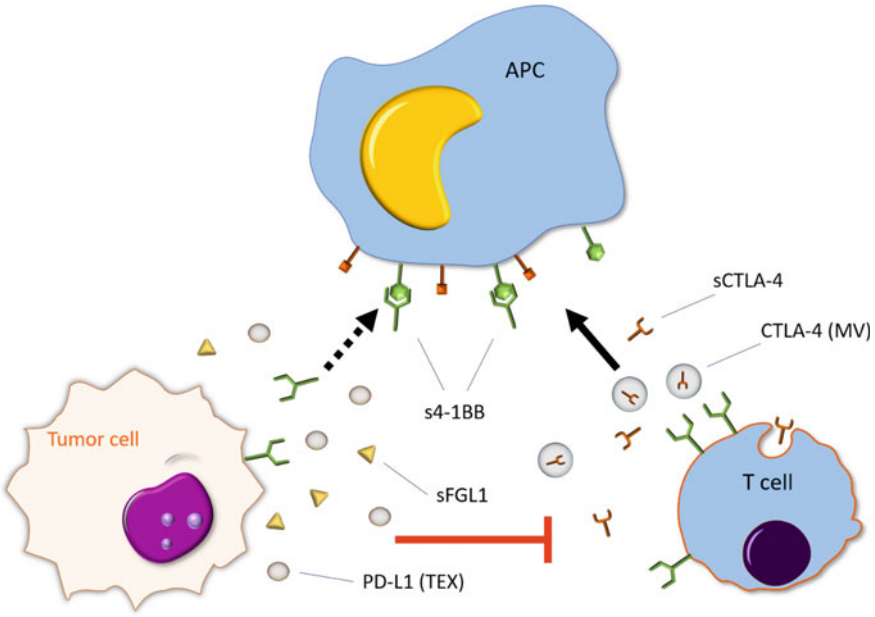

Fig. 19.3 Cross talk among tumor cells, APCs and T cells

even in models resistant to anti-PD-L1 antibodies (Poggio et al. 2019). Systemically introduced exosomal PD-L1 rescues growth of tumors unable to secrete their own. Anti-PD-L1 antibodies work additively, not redundantly, with exosomal PD-L1 blockade to suppress tumor growth.

Not only PD-L1 proteins, but also PD-L1 mRNA can be detected in exosomes. Researchers have found that PD-L1 mRNA contained exosome was more enriched in periodontitis patients than control subjects. Exosomal PD-L1 mRNA level in saliva correlates with the severity/stage of periodontitis, and can potentially be used to distinguish periodontitis from the healthy (Yu et al. 2019).

Yang et al. revealed in cancer cell lines, exosomal PD-L1 significantly suppressed CD3/CD28-driven ERK phosphorylation and NF-κB stimulation of T cells in a dose-dependent manner, as well as PHA-induced interleukin-2 (IL-2) secretion, leading to T cell dysfunction. Exosomes are capable of transferring functional PD-L1 to other cells. Exosomal PD-L1 detected on the surface of target cells is able to bind to PD-1 and mount an immunosuppressive effect. Suppression of exosome secretion from 4T1 tumor cells by either pharmacological inhibitor GW4869 treatment or Rab27a knocks down remarkably restrained tumor growth, and hence the antitumor effect is relatively superior than anti-PD-L1 therapeutic treatment. Combination of inhibition of exosome secretion and anti-PD-L1 antibody treatment achieved much stronger tumor suppression.

Researchers also reported exosomal release of PD-L1 occurs at the expense of surface PD-L1 levels. ALIX, as endosomal sorting complexes required for transporting (ESCRT)-associated protein, is the regulator of both PD-L1 surface presentation and EGFR activity in basal-like breast cancer (BLBC) cells. Besides locating on cell surface, PD-L1 also exits in the limiting membrane as well as intraluminal vesicle (ILVs) of CD63-positive MVBs in HCC1954 cells after IFNγ treatment. ILVs are the intracellular precursors of extracellular vesicles. Failure of PD-L1 incorporation in ILV leads to defective exosomal packaging, and following MVB-PM (plasma membrane) fusion thereby enhances cell surface PD-L1. In $ALIX^{KD}$ cells, a higher ratio of PD-L1 was observed at the limiting membrane of MVBs, compared within the endosomal lumen, resulting in prolonged and enhanced stimulation-induced EGFR activity as well as defective PD-L1 exosomal release, and its promoted redistribution to the cell surface, which implies an enhanced immunosuppressive phenotype (Monypenny et al. 2018).

19.3.2 Soluble Immune Checkpoint Receptors

The immunomodulatory interactions of cytotoxic T lymphocyte antigen-4 (CTLA-4), 4-1BB and PD-1 on T cells with their corresponding ligands on APCs or tumor cells have been extensively studied. Antibodies such as anti-CTLA-4 (ipilimumab) and anti-PD-1 (nivolumab) that counter these ligand–receptor interactions have shown clinical improvements in patients with solid tumors or autoimmune diseases. Still, a large portion of patient is unresponsive to these therapies and the full immunomodulatory mechanisms of these ligand–receptor interactions have not been resolved. More researchers are trying to uncover alternatively spliced soluble isoforms of these receptors to amplify efficacy mediated by their therapeutic antibodies.

19.3.2.1 Soluble PD-L1 as a Biomarker in Patients Treated with Checkpoint Inhibitors

Soluble PD-L1 (sPD-L1) may serve as a putative predictive biomarker for disease outcome and patient stratification under some circumstances. For instance, sPD-L1 may be a marker of systemic inflammation in pancreatic cancer (Kruger et al. 2017). sPD-L1 level in sera correlates with aggressiveness of renal cell carcinoma (RCC) and predicts survival in patients with MM or diffuse large B cell lymphoma (Wang et al. 2015; Rossille et al. 2014; Frigola et al. 2011).

SPD-L1 detected in the sera of RCC patients may cause systemic immunosuppression, facilitating tumor progression and resulting in poor prognosis (Frigola et al. 2011). Higher preoperative sPD-L1 levels were associated with poor clinical characteristics, including larger tumor volume, later stage, higher grade, and more tumor necrosis. Twice of sPD-L1 levels was related with a 41% increased risk of death. SPD-L1 was also detected in the cell supernatants of some PD-L1-positive RCC cell

lines. Those sPD-L1s retained receptor-binding domain was indicated by protein sequencing and was able to trigger pro-apoptotic signals in T cells (Frigola et al. 2011).

Multiple splice variants of PD-L1 have been identified and elevated sPD-L1 was observed in sera of patients with metastatic melanoma compared with healthy. High pretreatment sPD-L1 levels are correlated with rapid deterioration under anti-CTLA-4 or anti-PD1-based therapy, perhaps due to enhancing aberrant splicing activities in tumor cells, large tumor burden, or a diminishing antitumor immune effect, which are not easy to treat with a checkpoint blockade. Rise in sPD-L1 after 5 months of treatment rather than early alterations of sPD-L1 levels correlated with partial responses when receiving anti-CTLA-4 or anti-PD-1 therapy (Zhou et al. 2017).

Soluble PD-L1 harbors potential utility for antitumor therapy by blocking PD-1/B7-H1 pathway in murine model. In preclinical models of hepatocarcinoma (HCC), intramuscular injection of a plasmid encoding sPD-1 was reported to enhance lysis of tumor cells and extend overall survival of tumor-bearing mice (He et al. 2005).

19.3.2.2 Soluble CTLA4

The transmembrane isoform of CTLA-4 (Tm-CTLA-4) receptor plays a critical role in downregulating the immune response and sustaining the immune homeostasis. Alternatively spliced mRNA of the CTLA-4 gene that lacks exon 3 is found in human, mouse, and rat immune cells (Magistrelli et al. 1999; Oaks et al. 2000). When sCTLA-4 was first described, it was considered as a product of resting T cells, with its manufacture being cut down following T cell activation (Oaks et al. 2000). However, recently researchers have shown human T cells release more sCTLA-4 under physiological stimuli such as peptide immunogens or other recall antigens, indicating that sCTLA-4 may have functions relevant to ongoing immune responses (Ward et al. 2013). Antagonism of sCTLA-4 by isoform-specific monoclonal antibody (mAb) could remarkably stimulate antigen-dependent immune effects in a range of experimental systems in vitro and in vivo, as is shown in enhanced cell proliferation and pro-inflammatory cytokine levels like IFN-γ. Furthermore, in a melanoma-bearing B16F10 mouse model, isoform-selective anti-sCTLA-4 mAb treatment can achieve similar effect as panCTLA-4 mAb on the reduction of lung metastases, demonstrating soluble isoform is capable of modulating the overall outcome (Ward et al. 2013).

The extracellular domain of sCTLA-4, similar to that of the integral membrane isoform, has the MYPPY motif that can bind to the CD28-shared CD80/CD86 ligands on APCs. In a mixed lymphocyte response, recombinant sCTLA-4 showed immunomodulatory effect on inhibiting cell proliferation in a dose-dependent manner (Oaks et al. 2000).

19.3.2.3 Other Soluble Immune Checkpoints

Soluble 4-1BB can restrain over-zealous immune responses by acting in a negative feedback loop, as shown in animal models to suppress development of type I diabetes (Kachapati et al. 2013). Moreover, human renal, lung, melanoma, and hepatocellular tumor cell lines can generate s4-1BB under hypoxic stress, causing 4-1BB ligand engagement, thus blocking its costimulatory effect (Labiano et al. 2016).

Lymphocyte-activation gene 3 (LAG-3) is an immune suppressive receptor, with major MHC-II as a canonical ligand. Wang et al. demonstrated that fibrinogen-like protein 1 (FGL1), secreted by liver, acted as the major LAG-3 ligand independent of MHC-II. Inhibition of antigen-specific T cell activation was observed by FGL1-LAG-3 interaction, ablation of which enhances T cell response in mice. Tumor cells generated excessive FGL1, and increased plasma FGL1 was correlated with stronger resistance to anti-PD-1/PD-L1 therapy and a poor outcome in patients (Wang et al. 2019).

19.4 Immunocytes-Derived Exosomes in Cancer Immunology

Not only tumor cells, but also a variety of immune cells are able to release exosomes, such as T cells, DCs, macrophages, B cells, and mast cells (Skokos et al. 2003). Immunocytes-derived exosomes are shown to modulate tumor microenvironment and affect cancer outcome.

19.4.1 T Cell-Derived Exosomes

T cell-derived exosomes contain heterogeneous components, targeting divergent cells, and perform distinct types of function. Using proteomic approach, researchers were able to show activated T cells can secrete exosomes that contain signaling components associated with RAS, such as ZAP70, RAP1, RASGRP1, and AKT, and these vesicles can lead to ERK phosphorylation in mast cells (Azoulay-Alfaguter and Mor 2018). Activated T cells can release EVs which promote proliferation of autologous resting CD8 T cells (Wahlgren et al. 2012). T cells were activated and released exosomes after interaction with antigen-bearing DCs. In return, T cells could enhance the protective roles of DCs via transfer of exosomal DNA, and may modulate the immune system when encountering threats (Torralba et al. 2018). On the other hand, T cell-derived exosomes can prevent autoimmune damage through bioactive FasL and TRAIL, which eliminated activated T cells (Monleon et al. 2001).

Fibroblastic tumor stroma consisting of mesenchymal stem cells (MSCs) and cancer-associated fibroblasts (CAFs) promotes the invasion and metastasis of cancer

cells. EVs derived from activated CD8+ T cell could disrupt fibroblastic stroma-mediated tumor growth, as evidenced from activated $CD8^+$ T cells in healthy mice transiently secreting cytotoxic EVs which causes significant inhibition of invasive and metastatic properties of tumor through apoptotic elimination of mesenchymal tumor stromal cells. EV-releasing $CD8^+$ T cells infiltrate in neovascular areas with high mesenchymal cell density, and tumor MSCs preferentially engulf $CD8^+$ T cell-derived EVs than other cell populations like tumor cells. Thereby, $CD8^+$ T cells can prevent cancer progression via EV-mediated depletion of mesenchymal tumor stromal cells besides their conventional direct cytotoxicity (Seo et al. 2018).

Follicular helper T cells (Tfh cells) secreted exosomes correlate with occurrence and progression of antibody-mediated rejection (AMR) in renal transplantation. Tfh cell-derived exosomes could promote the proliferation and differentiation of B cells and may play a critical role in the development of AMR after renal transplantation. Analysis of the peripheral blood from 42 kidney transplant patients indicated that CTLA-4 level of CD4+ CXCR5+ exosomes was significantly lower in AMR group than that in non-AMR group (Yang et al. 2019).

19.4.2 NK Cell-Derived Exosomes

NK cells in tumor microenvironment could attenuate cancer progression through their exosomes. NK cell-derived exosomes performed cell-killing activity targeting cancer cells through the cytotoxicity factors, including perforin, granulysin, and granzymes A and B, which were able to activate caspase pathways in tumor cells, as well as blocking caspase inhibitors (Jong et al. 2017). NK cell-derived exosomes contain tumor-suppressive miR-186, which downregulates certain oncogenic proteins, for example, the mitotic kinase aurora kinase A (AUKRA) and N-myc proto-oncogene protein. TGF-β1 could inhibit the levels of miR-186 in NK cells, rendering NK cells inactive. Restoration of miR-186 levels increases the cell-killing capabilities of NK cells, resulting in decreased tumor burden and prolonged survival in neuroblastoma (Schmittgen 2019).

19.4.3 DC-Derived Exosomes

DC is the key player in antigen-presenting process, its EVs are in charge of intercellular communicators in adaptive immunity. DCs and DC-derived exosomes have several similarities. Like DCs, their exosomes express functional MHC-peptide complexes, T cell stimulatory factors, and other components that interact with other immune cells. After activated by T cells, DCs exhibited a capacity for antigen-specific T cell activation through exosomes (Lindenbergh et al. 2019). Accumulated evidence has shown that DC-derived exosomes could facilitate immune cell-dependent cancer therapy (Pitt et al. 2016). Given the component of DCs in modulating immune

responses, a majority of studies focused on the effect of DC-derived exosomes against tumor progression, the potential immune-modifying function, and feasibility and safety of application (Chen et al. 2018a, b; Pitt et al. 2014). DC-derived exosomes can limit cancer cells via activation of naïve T cells and NK cells (Gao and Jiang 2018).

19.4.4 Macrophage-Derived Exosomes (MDE)

Clinical and experimental evidence has shown that tumor-associated macrophages induce cancer initiation and progression. MDE has been shown to accelerate colorectal cancer (CRC) cells' migration and invasion through its miRNA contents including miR-21-5p and miR-155-5p. Both miR-21-5p and miR-155-5p downregulate expression of BRG1, which is crucial for the CRC metastasis (Lan et al. 2019). After activated by IL-4, macrophage can enhance invasiveness of breast cancer cells via the Mef2c-b-catenin pathway through transferring miR-233. MiR-223 antisense oligonucleotide reduced the expression of miR-223 in macrophages, thus depressing the invasiveness of the co-cultured breast cancer cells (Yang et al. 2011).

Hypoxic epithelial ovarian cancer (EOC) cells induced macrophages into a TAM-like phenotype, which then deliver exosomes to the co-cultivated EOC cells, enhancing the malignant phenotype and drug resistant of EOC cells via the PTEN-PI3K/AKT pathway (Zhu et al. 2019).

19.4.5 Other Immunocytes-Derived Exosomes

B cell-derived exosomes mediate part of B cell's functions, including antigen-presenting capacities, MHC-restricted antigen recognition, and induction of different types of immune responses as well. In lymph nodes, B cell-derived exosomes were found to act reciprocally with CD169+ macrophages and further interrupted the spread of viruses or tumor cells (Saito et al. 2015). B cell-derived exosomes promoted T cell response, and those effects were independent of B cell presence or B cell-secreted antibody (Saunderson and McLellan 2017).

Myeloid-derived suppressor cells (MDSCs), known as an immune suppressor, are generated from immature myeloid cells under certain conditions. MDSC-derived exosomes contained different cargos in accordance with the immunosuppressive activity (Geis-Asteggiante et al. 2018). It was also demonstrated that MDSCs are present in cancer patients, inhibiting antitumor immunity and scrambling anti-cancer immunotherapies. MDSC-derived exosomes, which were primed by tumor milieu, could also promote oncogenesis (Burke et al. 2014).

19.5 Exosome as Biomarker and Vaccine for Cancer Progression

19.5.1 Liquid Biopsy

TEX delivers malignant signals in a variety of forms, including nucleic acids, such as messenger RNA (mRNA) and miRNAs, or proteins like chemokines, cytokines, growth factors, or angiogenic and immunomodulatory molecules (Chiodoni et al. 2019). Measuring exosomal contents would be noninvasive, however, a promising way to detect cancer occurrence and monitor tumor progression. The problem is exosomal-specific proteins are in very low abundance, and thus a large amount of serum or culture medium is needed to enrich sufficient exosome to conduct the proteomics or western blot measurement. Thereby, exosome protein profile is still in the starting stage. Genomic profile of the exosome, including miRNA, mRNA, long noncoding RNA (lncRNA), and mitochondrial RNA, being amplified by PCR to increase quantity seems to be excellent candidate biomarkers to subclassify tumor types.

19.5.2 Exosomal miRNA (Detailed in Table 19.1)

In recent years, much of the research on cancer blood biomarkers has shifted from protein-based to nucleic acid-based molecules (Chiodoni et al. 2019). For example, Fang et al. reported that hepatoma cells produced high levels of miR-103 and release it in exosomes to induce tumor metastasis, indicating exosome miR-103 can be used as a predictive marker for cancer progression (Fang et al. 2018). Shi et al. found exosome-derived miR-638 was significantly decreased in serum of HCC patients with advanced disease, such as at later TNM stage (III/IV) or with larger tumor size (>5 cm) (Shi et al. 2018). Moreover, several lncRNAs, like lnc-h19, lnc-sox2ot, and lncRNA-ARSR, have been investigated in circulating exosomes and closely related with tumor stage and overall survival of patients (Fang et al. 2018; Shi et al. 2018; Qu et al. 2016; Zhao et al. 2018; Lin et al. 2018; Conigliaro et al. 2015). Several studies have implicated exosome-derived miRNAs as potential biomarkers for detection of CRC occurrence and monitor recurrence. The serum levels of exosomal miRNAs, such as miR-1224-5p, miR-1229, miR-21, miR-223, miR-150, and let-7a, are much higher in CRC patients than healthy, dropping after tumor resection (Ruiz-Lopez et al. 2018). Exosomal miRNAs such as miR-19a, miR-18a, and miR-100 may be useful to detect the recurrence of CRC (Komatsu et al. 2014; Matsumura et al. 2015; Cha et al. 2015). These findings suggest that exosomal RNA molecules detected from circulation or other sources can serve as biomarkers to evaluate cancer occurrence and progression, with potentially high sensitivity and specificity.

Table 19.1 TEX miRNAs regulate immune cells' response

miRNA name	Target immune cell	Activation/suppression
miR-23a (Berchem et al. 2016)	NK cell	Suppression
miR-362-5p (Yang et al. 2015; Wu et al. 2015; Ni et al. 2016)	NK cell	Suppression
miR-24-3p (Ye et al. 2014)	T cell	Suppression
miR-891a (Ye et al. 2014)	T cell	Suppression
miR-106a-5p (Ye et al. 2014)	T cell	Suppression
miR-20a-5p (Ye et al. 2014)	T cell	Suppression
miR-1908 (Ye et al. 2014)	T cell	Suppression
miR-21 (Fabbri et al. 2012; Hsieh et al. 2018)	Macrophage	Activation/suppression
miR-214 (Yang et al. 2015)	T cell	Suppression
miR-146a-5p (Cheng et al. 2019)	T cell	Suppression
miR-212-3p (Ding et al. 2015)	Dendritic cells	Suppression
miR-203 (Zhou et al. 2014)	Dendritic cells	Suppression
miR-29a (Fabbri et al. 2012)	Macrophage/Dendritic cells	Suppression
miR-16 (Jang et al. 2013)	Macrophage (M2)	Activation
miR-222-3p (Ying et al. 2016)	Macrophage (M2)	Activation

19.5.2.1 TEX miRNAs and T Cells

Bland et al. found out that the tumor cell line B16F0 can deliver mRNA/miRNA loaded exosomes to CTLs and alter their metabolic function and IFN-γ production. TEX from nasopharyngeal carcinoma was reported to contain high levels of miR-106a-5p, miR-1908, miR-24-3p, miR-891a, and miR-20a-5p, yielding almost 20 targets linked to the MAPK1 pathway for potential downregulation. The net effect on T cells was a shift from Th1 and Th17 phenotypes to Th2 and Treg phenotypes, through suppression of ERK/STAT1/STAT3 phosphorylation (Ye et al. 2014). Evaluating overexpressed tumor miRNAs from patients with non-small cell lung cancer, breast cancer, pancreatic cancer or HCC, the ubiquitous miR-21 and miR-214 were shown to be consistently upregulated in tissue and in plasma exosomes/microvesicles. The same phenomenon was observed in murine sarcoma and lung cancer models, where miR-214 was enriched in MVs and downregulated PTEN in T cells, favoring Treg cell's expansion (Yin et al. 2014; Walsh et al. 2006), but runs somewhat contradictory to other reports concerning the role of PTEN in Treg maintenance (Shrestha et al. 2015; Sharma et al. 2015).

19.5.2.2 TEX miRNAs and NK Cells

In lung cancer and leukemia, TEX-derived miR-23a was found to decrease the level of LAMP1 (lysosome-associated membrane glycoprotein 1)/CD107a (Berchem et al. 2016), which is an NK cell activation marker signifying lymphocytes degranulation (Cohnen et al. 2013). As the TEX also deliver TGFβ that inhibits NKG2D expression, they were generally considered as NK cell inhibitors. TEX-derived miR-362-5p has distinct effects depending on tumor cells themselves (Yang et al. 2015; Wu et al. 2015; Ni et al. 2016), but seems to be crucial in enhancing NK cell response via downregulation of CYLD, a suppressor of NF-κB signaling (Ni et al. 2015). However, the overabundance of the miRNA could lead to overstimulation resulting in hypo-responsiveness (Shifrin et al. 2014).

19.5.2.3 TEX MiRNAs and Monocytes

It was shown TEX-pulsed DCs could supply antigens to T cells and promote effector T cell's response. The context in the immune system is likely a critical factor to determine TEX involvement of immune stimulation versus immune suppression (Kunigelis and Graner 2015). Researchers revealed an inhibition of stimulatory capacity in immature DCs when exposed to the human pancreatic cancer cell line-Panc-1 TEX (Zhou et al. 2014), which can deliver miR-203 to DCs and decrease TLR4 expression and downstream cytokines like TNFα and IL12. The changes of the cytokines in the tumor microenvironment can influence both T cells and B cells interacting with DCs.

A few years ago, Fabbri et al. revealed that TEX from non-small cell lung carcinoma (NSCLC) transfers miR-29a and miR-21 to macrophage existing in the tumor microenvironment (Fabbri et al. 2012). These miRNAs bound and enhanced TLR8 (murine TLR7) as ligands, activating the NF-κB pathway and resulting in production of IL6 and TFNα, creating a prometastatic inflammatory microenvironment. TLR7/8 belongs to intracellular TLRs subset, existing in endosomal and other vesicular membranes, and mediates innate immune reactions against multiple pathogens (Cervantes et al. 2012; Challagundla et al. 2015). MiR-21 from neuroblastoma TEX could also trigger TLR8 in monocytes, which led to upregulation of miR-155 in those cells, and the latter miRNA could then be sent back to the cancer cells via exosomes, resulting in downregulation of telomeric repeat binding factor 1 (TERF1). TERF1 is a telomerase inhibitor, downregulation of which increases cisplatin resistance in neuroblastoma cancer cells (Guo et al. 2009). Thus, the cross-interaction through exosomal miRNAs in the microenvironment is generally beneficial for the tumor.

19.5.3 Clinical Potential of Exosomes

The major role of TEX is to create an adaptive microenvironment for cancer cells to grow; however, several studies showed that EVs can inhibit cancer progression,

either by direct effect of the EV-transported protein and nucleic acid contents or through antigen presentation to immunocytes. Tumor cells can deliver some of the same antigens through exosomes as the ones presenting on the surface (Chiodoni et al. 2018, 2019). For instance, DCs under the influence of rat glioblastoma cell-derived exosomes can trigger a strong antitumor reaction and dramatically prolong median survival in glioblastoma-bearing rats when used in combination with α-galactosylceramide (Liu et al. 2017). Given the relative longevity of EVs within the circulation, modification of those antitumor ones creates the potential to design new tools for cancer therapy.

The exosome liposome-like structure allows them to be loaded with various drugs. Exosomes are considered as a new generation of a natural nanoscale delivery system. Hemopurifier® is currently being accessed for its efficacy on seizing exosomes released by cancer cell lines or released in biofluids from cancer patients (Marleau et al. 2012). Researchers are studying a refined biomimetic nanostructure to deliver doxorubicin to breast cancer patient, by re-engineering immuno-exosome with a synthetic liposome (Rayamajhi et al. 2019).

Indeed, exosomes derived from different types of cells present different signaling molecules, and thereby have a great potential for targeted drug therapy (Xu et al. 2016). In a mouse breast cancer model, treatment with human-specific anti-CD9 or anti-CD63 antibodies inhibited metastasis to the lungs, lymph nodes, and thoracic cavity via the depletion of circulating EVs. EVs incubated with the targeted antibodies were preferentially internalized by macrophages and might be further eliminated by macrophages (Nishida-Aoki et al. 2017). Phase I clinical trial for advanced CRC has been performed using ascites-derived exosomes and granulocyte-macrophage colony-stimulating factor (GM-CSF). Combination of those two components efficiently enhances antitumor cytotoxic T cell response as a safe and feasible immunotherapy of advanced CRC (Dai et al. 2008).

19.6 CAF-Derived Exosome

The tumor microenvironment comprises tumor cells, nontumor cellular, and noncellular components such as fibroblasts, inflammatory cells, lymphocytes extracellular matrix, blood vessels, and signaling pathways. This dynamic context contributes to tumorigenesis through complex interactions of these elements. One of the main components of tumor microenvironment is CAFs. The interaction of tumor cells and CAFs has been reported to promote cancer progression (Alguacil-Nunez et al. 2018). Exosomes can induce normal fibroblast differentiation into CAFs through TGFβ signaling (Ringuette Goulet et al. 2018). CAFs then pose pro-tumor feedback to induce epithelial–mesenchymal transition of bladder cancer cells via paracrine IL-6 signaling (Goulet et al. 2019).

Next-generation sequencing and bioinformatics study on primary human normal and CAFs from nine paired normal colorectal mucosa and cancer tissues displayed

significant differences between the ncRNA component and enrichment within exosomes of the normal and CAFs. NcRNA regulatory factors are specifically detected in CAF-generated exosomes, indicating a specific interaction between CAFs and CRC cells (Herrera et al. 2018).

Exosomal miRNAs were profiled from paired patient-derived normal fibroblasts and CAFs, from an ongoing prospective biomarker study. In vitro CAFs exosomes are delivered to CRC cells, with a subsequent increase in cellular miRNA levels, influencing tumor cell proliferation and chemoresistance. An exosomal CAF signature composed of miRNAs 21, 215, 181a, 329, 199b, and 382 was identified. Of these, miR-21 showed highest abundance in CAF exosomes. In an orthotopic CRC murine model, co-injection with miR-21-overexpressing fibroblasts led to increased liver metastases than with control fibroblasts (Bhome et al. 2017).

CAFs constitute the majority of the tumor bulk of pancreatic ductal adenocarcinomas (PDACs). CAFs exposed to chemotherapy have an active role in regulating the survival and proliferation of cancer cells through exosome secretion. Gemcitabine increases the secretion of both miR-146a and Snail in pancreatic CAF exosomes. Blocking CAF exosome secretion inhibited PDAC tumor cell survival (Richards et al. 2017). Exosomal miR-196a derived from CAFs confers cisplatin resistance in head and neck cancer through targeting CDKN1B and ING5 (Qin et al. 2019). In addition, loss of exosomal miR-3188 in CAFs leads to HNC progression (Wang et al. 2019).

Fibroblast growth factor 2(FGF2)-FGFR1 signaling regulates generation of leukemia-protective exosomes from bone marrow stromal cells. It was demonstrated that bone marrow stromal cells deliver exosome FGF2 to leukemia cells, protecting leukemia cells from tyrosine kinase inhibitors (TKIs). Expression of FGF2 and its receptor, FGFR1, are both enhanced in a subgroup of stromal cell lines and primary AML stroma. Activated FGF2/FGFR1 signaling can further enhance exosome secretion. Inhibiting FGFR cuts off stromal autocrine growth and remarkably suppresses secretion of FGF2-containing exosomes, contributing to compromised stromal guard of leukemia cells. In addition, Fgf2 −/− mice transplanted with retroviral BCR-ABL acute leukemia had prolonged survival compared with Fgf2 +/+ mice given TKI. Therefore, suppression of FGFR can downregulate stromal function, inhibit exosome secretion, and serve as a therapeutic target to conquer TKIs resistance (Javidi-Sharifi et al. 2019).

19.7 Conclusions

Tumor cells can develop a variety of mechanisms, including transferring TEX to evade and subvert the immune system for their survival. EVs represent a diverse category of cellular releasing products present in multiple types of biofluids and cell culture media. Exosomal contents directly reflect the metabolic state of the cells from which they originate. PD-L1 can be transferred to multiple cell types including tumor cells, macrophages, and DCs through PD-L1-containing exosomes

in the tumor microenvironment, indicating a systemic regulatory role of exosomal PD-L1. Exosomal PD-L1 represents an unexplored therapeutic target, which could overcome resistance to current immune checkpoint inhibitors. The general pattern is that TEX deliver miRNAs to immune cells that ultimately lead to situations that benefit the cancer. Administration of therapeutic antibody effectively inhibits EV-induced tumor metastasis and that the removal of EVs could be a novel cancer treatment (Nishida-Aoki et al. 2017).

Though our understanding of EVs continues to grow, it is far from complete. Experimental data accumulated since decades ago evidently suggests that EVs play pivotal roles for some, if not all, cancer hallmarks. Until now, the field of EV research has drawn mounting interest from scientists and physicians, with growing number of investigators dissecting on the critical role of EVs in cancer biology, and thereby requires more transparent reporting and documenting to streamline interpretation and enable replication of experiments. EV-TRACK, a crowdsourcing knowledge-base (http://evtrack.org), is recently built to improve centralization of EV biology and relevant methodology to help reviewers, authors, editors, and funders to fulfill experimental guidelines and increase research reproducibility (Van Deun et al. 2017; Consortium et al. 2017). Vesiclepedia (http://www.microvesicles.org) is an established web-based compendium of components including RNA, lipids, proteins, and metabolites transported by EVs from both published and unpublished researches, with the input currently from 1254 EV investigations, consisting of 38,146 RNA entries, 349,988 protein entries, and 639 lipid/metabolite entries (Pathan et al. 2019). There are also alternative or supplementary initiatives to characterize EVs, for example, ExoCarta and EVpedia, two typical web domains that help researchers to promptly upload proteomic lists of identified proteins of the EVs being investigated (Mathivanan and Simpson 2009; Kim et al. 2015). Widespread implementation of those knowledgebases by the EV scientific community is believed to facilitate the success of the exosome research in the long run.

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Chapter 20
Macromolecules and Antibody-Based Drugs

Xiao-Dong Su and Yao Shuai

Abstract Macromolecule drugs particularly antibody drugs are very powerful therapies developing rapidly in the recent 20 years, providing hopes for many patients diagnosed with "incurable" diseases in the past. They also provide more effective and less side effects for many afflicting diseases, and greatly improve the survival rate and life quality of patients. In the last two decades, the proportion of US Food and Drug Administration (FDA) approved macromolecules and antibody drugs are increasing quickly, especially after the discovery of immune checkpoints. To crown all, the 2017 Nobel prize in physiology or medicine was given to immunotherapy. In this chapter, we would like to summarize the current situation of macromolecule and antibody drugs, and what effort scientists and pharmaceutical industry have made to discover and manufacture better antibody drugs.

Keywords Macromolecule drugs · Antibody drugs · Immunotherapy · Immune checkpoint · Engineered antibody-based drugs

20.1 History and Development of Macromolecule Drugs

Macromolecule drugs, also known as biologics, are characterized by their large molecular weight—comparing to chemical drugs. FDA classified a wide range of products into biological products, such as vaccines, blood and blood components, allergenics, somatic cells, gene therapy, tissues, and recombinant therapeutic proteins (Biological Product Definitions 2019). In this chapter, we are talking about recombinant therapeutic proteins, especially engineered antibodies.

Early therapeutic proteins are mainly separated directly from human or specific animal plasma, body fluid, or tissues. However, during the past half-century, more and more recombinant therapeutic proteins have been emerging due to the in-depth understanding of protein structure, function, and disease-related molecular medicine,

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especially the great progress in biotechnology of protein production (Fig. 20.1). The most famous example is perhaps insulin. It was first isolated from cows and pigs in the 1920s by Banting and Best in Canada. From then on, insulin was officially used for clinical diabetes treatment (Rosenfeld 2002). In 1965, functional bovine insulin was successfully synthesized by chemical methods for the first time in China (邹承鲁 2015). Until 1982, the genetic-engineered human insulin expressed in Escherichia coli was approved by the FDA and was recorded as the first recombinant therapeutic protein (Johnson 1983). From then on, new protein drugs came out sporadically every year (Table 20.1), such as the first recombinant enzyme drugs Activase (alteplase) used for heart attack (approved by FDA in 1987), and another outstanding example of recombinant protein drugs is monoclonal antibodies (MAbs) Herceptin (trastuzumab) targeting at HER2 overexpressed in breast cancer (approved by FDA in 1998). Excellent performance and surprising efficacy of protein drugs have spurred the birth of a large number of new protein drugs and biosimilars.

Macromolecule drugs generally have good target specificity and little side effects. At present, the indications of clinical protein drugs are mainly cancer, autoimmune diseases, as well as nervous system diseases, eye diseases, respiratory diseases, and so on. With new discoveries of disease mechanisms and related signaling pathways, more and more therapeutic targets have been identified. The development of macromolecular drugs with high efficacy and low side effects is mainly dependent on selection of good targets. Star target examples are CTLA4 and PD-1/PD-L1 for cancer treatment, TNF-α for autoimmune diseases, and traditional dominant targets such as CD20, HER2, VEGF/VEGFR, etc.

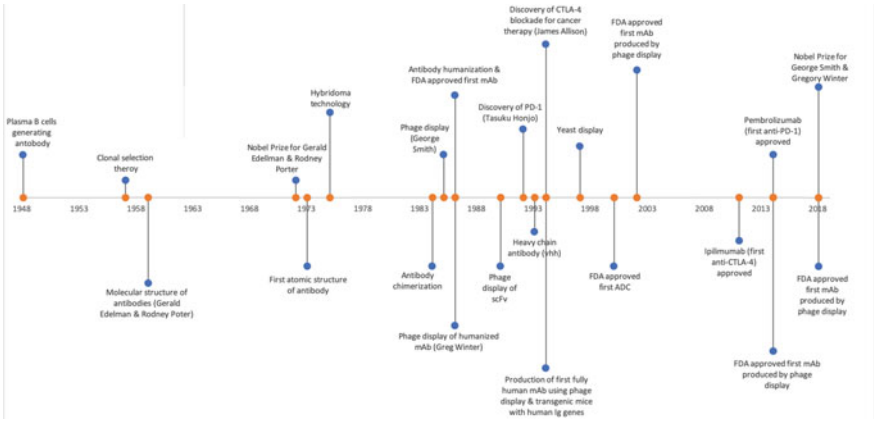

Fig. 20.1 Milestones of antibodies drugs

Table 20.1 FDA-approved antibody and antibody-related drugs

Number	INN	Commercial name	Company	Approval data	Target	Approved major indications	Type	Sequence derived	Dosing route	Expression system	MoA
1	Muromonab-CD3	Orthoclone OKT3	Ortho Biotech (J&J)	1986-6-19; withdrawn 2011	CD3E (CD3ε)	OTR	Murine IgG2a	Mouse hybridoma	i.v.	Hybridoma	Blocks function of T-cell expressed CD3; reverses graft rejection
2	Abciximab	ReoPro	Centocor (now J&J)/ Lilly	1994-12-22	ITGA2B/ ITGB3 (gPIIb/ IIIa)	CVD	Chimeric FAb fragment	Mouse hybridoma	i.v.	Sp2/0	Binds and antagonizes receptor; inhibits platelet aggregation
3	Rituximab	Rituxan	Biogen/Idec/ Genentech	1997-11-26	MS4A1 (CD20)	NHL, RA	Chimeric IgG1	Mouse hybridoma	i.v.	CHO	Depletes target cells by ADCC, CDC, and inducing apoptosis
4	Daclizumab	Zenapax	Biogen/ Abbott (PDL/ Roche)	1997-12-10; withdrawn 2009 reapproved 2016 for MS, withdrawn 2018	IL2RA (IL-2Rα; CD25)	OTR	Humanized IgG1	Mouse hybridoma	s.c.	Sp2/0	Binds and antagonizes receptor

(continued)

Table 20.1 (continued)

Number	INN	Commercial name	Company	Approval data	Target	Approved major indications	Type	Sequence derived	Dosing route	Expression system	MoA
5	Infliximab	Remicade	Centocor (now J&J)	1998-08-24	TNF (TNF-α)	CRD, RA	Chimeric IgG1	Mouse hybridoma	i.v.	CSC-Ps0006	Binds soluble and trans-membrane forms of TNF; inhibits binding to TNFR; depletes TNF-positive cells
6	Palivizumab	Synagis	MedImmune	1998-06-19	RSV F-protein	RSV infection	Humanized IgG1	Mouse hybridoma	i.m.	NS0	Binds and neutralizes RSV; inhibits viral fusion and replication
7	Trastuzumab	Herceptin	Genentech	1998-09-25	ERBB2 (HER2)	Breast cancer	Humanized IgG1	Mouse hybridoma	i.v.	CHO	Inhibits tumor cell growth in vitro and in vivo; depletes target cells by ADCC
8	Etanercept	Enbrel	Immunex (now Amgen)	1998-11-02	TNF (TNF-α)	RA	P75-TNFR-Fc fusion	Fc fusion	s.c.		
9	Basiliximab	Simulect	Novartis	1998-12-05	IL2RA (IL-2Rα; CD25)	OTR	Chimeric IgG1	Mouse hybridoma	i.v.	NS0	Binds and antagonizes receptor

(continued)

Table 20.1 (continued)

Number	INN	Commercial name	Company	Approval data	Target	Approved major indications	Type	Sequence derived	Dosing route	Expression system	MoA
10	Gemtuzumab ozogamicin	Mylotarg	Wyeth (now Pfizer)	2000-5-7; withdrawn 2010 re-approved 2017	CD33	Leukemia	Humanized IgG4-ADC	Mouse hybridoma	i.v.	NS0	The cytotoxin, calicheamicin, induces dsDNA breaks, leading to cell cycle arrest and apoptosis
11	Alemtuzumab	Campath-1H	Genzyme	2001-5-7; withdrawn 2012	CD52	Leukemia	Humanized IgG1	Rat hybridoma	i.v.	CHO	Depletes target cells by ADCC and CDC
12	Ibritumomab tiuxetan	Zevalin	Biogen/Idec	2002-02-19	MS4A1 (CD20)	NHL	Murine IgG1 radio-conjugate (Y-90, In-111)	Mouse hybridoma	i.v.	CHO	Radiation from 90Y induces cellular damage
13	Adalimumab	Humira	CAT, Abbott	2002-12-31	TNF (TNF-α)	RA, CRD	Human IgG1	Human antibody phage library	s.c.	CHO	Binds soluble and trans-membrane forms of TNF; inhibits binding to TNFR; depletes TNF-expressing cells in presence of complement

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Table 20.1 (continued)

Number	INN	Commercial name	Company	Approval data	Target	Approved major indications	Type	Sequence derived	Dosing route	Expression system	MoA
14	Alefacept	Amevive	Biogen	2003-01-30	CD2	Psoriasis	CD58 (LFA-3)-Fc fusion	Fc fusion	i.m.		
15	Omalizumab	Xolair	Genentech	2003-06-20	IGES (IgE)	Asthma	Humanized IgG1	Mouse hybridoma	s.c.	CHO	Binds and neutralizes IgE; reduces degranulation of mast cells and basophils; downregulation of receptor (FcεRI)
16	Tositumomab-I131	Bexxar	Corixa	2003-6-27; withdrawn 2014	MS4A1 (CD20)	NHL	Murine IgG2a radio-conjugate (I-131)	Mouse hybridoma	i.v.	Hybridoma	
17	Efalizumab	Raptiva	Genentech	2003-10-27; withdrawn 2009	ITGAL (CD11A)	Psoriasis	Humanized IgG1	Mouse hybridoma	s.c.	CHO	

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Table 20.1 (continued)

Number	INN	Commercial name	Company	Approval data	Target	Approved major indications	Type	Sequence derived	Dosing route	Expression system	MoA
18	Cetuximab	Erbitux	ImClone/BMS	2004-02-12	EGFR	CRC	Chimeric IgG1	Mouse hybridoma	i.v.	Sp2/0	Binds and antagonizes receptor; inhibits cell proliferation; induces apoptosis; sensitizes cells to chemotherapy and radiotherapy; decreases VEGFA production; depletes target cells by ADCC
19	Bevacizumab	Avastin	Genentech	2004-02-26	VEGFA	CRC	Humanized IgG1	Mouse hybridoma	i.v.	CHO	Binds and neutralizes ligand; reduces microvascular growth, and inhibits metastatic disease progression in mouse xenografts

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Table 20.1 (continued)

Number	INN	Commercial name	Company	Approval data	Target	Approved major indications	Type	Sequence derived	Dosing route	Expression system	MoA
20	Natalizumab	Tysabri	Biogen/Elan	2004-11-23	ITGA4(α4 integrin)	MS	Humanized IgG4	Hybridoma	i.v.	Murine myeloma	Binds and antagonizes receptors; inhibits leukocyte adhesion
21	Abatacept	Orencia	BMS	2005-12-23	CD80/CD86	RA	CTLA4-Fc fusion	Fc fusion	s.c.		
22	Ranibizumab	Lucentis	Genentech Novartis	2006-06-30	VEGFA	Wet AMD	Humanized Fab fragment	Hybridoma	i.v.t	E. coli	Binds and neutralizes ligand; reduces endothelial cell proliferation, vascular leakage and new blood vessel formation

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Table 20.1 (continued)

Number	INN	Commercial name	Company	Approval data	Target	Approved major indications	Type	Sequence derived	Dosing route	Expression system	MoA
23	Panitumumab	Vectibix	Amgen	2006-09-27	EGFR	Colorectal cancer	Human IgG2	TG XenoMouse	i.v.	CHO	Binds, antagonizes, and downregulates receptor; inhibits cell proliferation; induces apoptosis; decreases pro-inflammatory cytokine and VEGF production
24	Eculizumab	Soliris	Alexion Pharmaceuticals	2007-03-16	C5	PNH	Humanized hybrid engineered IgG2/4	Mouse hybridoma	i.v.	NS0	Binds C5 and inhibits its cleavage, thus preventing the generation of the terminal complement complex C5b–9
25	Rilonacept	Arcalyst	Regeneron	2008-02-27	IL1A (IL-1α), IL1B (IL-1β), IL1RN (IL-1RA)	CAPS, MWS	IL-1R & IL-1AP-in-line Fc fusion	Fc fusion	s.c.		
26	Romiplostim	Nplate	Amgen	2008-08-22	MPL (TPO-R)	Thrombocytopenia	Fc-peptide fusion	Peptide phage library	s.c.		

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Table 20.1 (continued)

Number	INN	Commercial name	Company	Approval data	Target	Approved major indications	Type	Sequence derived	Dosing route	Expression system	MoA
27	Golimumab	Simponi	Centocor/J&J	2009-04-23	TNF (TNF-α)	RA	Human IgG1	HuMAb TG mouse	s.c.	Sp2/0	Binds soluble and trans-membrane forms of TNF; inhibits binding to TNFR
28	Ustekinumab	Stelara	Centocor/J&J	2009-09-25	IL12B (p40 subunit of IL-12 and IL-23)	Psoriasis	Human IgG1	HuMAb TG mouse	i.v. or s.c.	Sp2/0	
29	Catumaxomab	Removab	Fresenius/ Trion	EU only 4/23/ 09; withdrawn 2017	EPCAM, CD3E	Malignant ascites	Rat IgG2b-mouse IgG2a hybrid bispecific IgG	Mouse and rat hybridomas	Intra-peritoneal	Rat–Mouse hybrid hybridoma	
30	Certolizumab pegol	Cimzia	UCB/ Schwartz	2009-05-14	TNF (TNF-α)	RA	PEGylated humanized FAb fragment	Mouse hybridoma	s.c.	E. coli	Binds soluble and trans-membrane forms of TNF; inhibits binding to TNFR
31	Canakinumab	Ilaris	Novartis	2009-06-19	IL1B (IL-1β)	CAPS	Human IgG1	HuMAb TG mouse	s.c.	Sp2/0	Binds and neutralizes ligand
32	Ofatumumab	Arzerra	GenMab/ Novartis	2009-10-26	MS4A1 (CD20)	CLL	Human IgG1	HuMAb TG mouse	i.v.	NS0	Depletes target cells by CDC and ADCC

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Table 20.1 (continued)

Number	INN	Commercial name	Company	Approval data	Target	Approved major indications	Type	Sequence derived	Dosing route	Expression system	MoA
33	Tocilizumab	Actemra	Roche/ Chugai/ Genentech	2010-01-09	IL6R (CD126)	MCD; RA	Humanized IgG1	Hybridoma	i.v. or s.c.	CHO	Binds to membrane-bound and soluble forms of IL-6R and inhibits IL-6 binding
34	Denosumab	Prolia/Xgeva	Amgen/GSK	2010-06-01	TNFSF11 (RANK-ligand)	Osteoporosis, Bone cancer	Human IgG2	TG XenoMouse	s.c.	CHO	Binds and neutralizes ligand; decreases bone resorption and increases mass and strength of some bones
35	Belimumab	Benlysta	GSK/HGS	2011-03-09	TNFSF13B (soluble BLyS)	SLE	Human IgG 1	Human antibody phage library	i.v.	NS0	Binds and neutralizes ligand; inhibits survival of B lymphocytes
36	Ipilimumab	Yervoy	Medarex/ BMS	2011-03-25	CTLA4	Melanoma	Human IgG 1	HuMAb TG mouse	i.v.	CHO	Binds and antagonizes receptor; augments T lymphocyte activation and proliferation

(continued)

Table 20.1 (continued)

Number	INN	Commercial name	Company	Approval data	Target	Approved major indications	Type	Sequence derived	Dosing route	Expression system	MoA
37	Belatacept	Nuloji	BMS	2011-06-11	CD80/CD86	OTR	CTLA-4 Fc fusion	Fc fusion	i.v.		
38	Brentuximab vedotin	Adcetris	Seattle Genetics/ Takeda/ Millenium	2011-08-19	TNFRSF8 (CD30)	Hodgkin's lymphoma	Chimeric IgG1 ADC	Mouse hybridoma	i.v.	CHO	The cytotoxin, MMAE, disrupts microtubules, leading to cell cycle arrest and apoptosis; depletes target cells by ADCP
39	Aflibercept	Eylea	Bayer-Schering/ Regeneron	2011-11-18	VEGFA	Wet AMD	VEGF-R-Fc fusion	Fc fusion	i.v.		
40	Ziv-aflibercept	Zaltrap	Sanofi/ Regeneron	2012-06-08	VEGFA	MCRC	VEGFR-Fc fusion protein Trap	Fc fusion	i.v.		

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Table 20.1 (continued)

Number	INN	Commercial name	Company	Approval data	Target	Approved major indications	Type	Sequence derived	Dosing route	Expression system	MoA
41	Pertuzumab	Perjeta	Genentech	2012-08-03	ERBB2 (HER2)	Breast cancer	Humanized IgG1	Mouse hybridoma	i.v.	CHO	Binds and antagonizes receptor; arrests cell proliferation; induces apoptosis; depletes target cells by ADCC; augments antitumor activity of trastuzumab in xenograft models
42	Raxibacumab	Abthrax	GSK; Human Genome Sciences	2012-12-14	Bacillus anthracis PA toxin	Anthrax biodefense	Human IgG1	Human antibody phage library	i.v.	NS0	Binds and neutralizes antigen; prevents intracellular entry of anthrax lethal factor and oedema factor

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Table 20.1 (continued)

Number	INN	Commercial name	Company	Approval data	Target	Approved major indications	Type	Sequence derived	Dosing route	Expression system	MoA
43	Trastuzumab emtansine	Kadcyla	Roche/ Genentech	2013-02-23	ERBB2 (HER2)	Breast cancer	Humanized IgG ADC	Mouse hybridoma	i.v.	CHO	The cytotoxin, DM1, disrupts the microtubule network in cells leading to cell cycle arrest and apoptosis; inhibits HER2 signaling; depletes target cells by ADCC; inhibits shedding of HER2
44	Obinutuzumab	Gazyva	Roche/ Genentech/ Biogen	2013-11-01	MS4A1 (CD20)	CLL	Humanized IgG1-low fucose	Mouse hybridoma	i.v.	CHO	Depletes target cells by ADCC, CDC, ADCP and inducing apoptosis; reduced fucosylation for increased ADCC
45	Eftrenonacog alfa	Alprolix	Biogen IDEC/ Biovitrum	2014-03-28	Factor substitute	Hemophilia B	Monomeric Factor IX Fc fusion protein	Fc fusion	i.v.	CHO	Substitute coagulation factor IX

(continued)

Table 20.1 (continued)

Number	INN	Commercial name	Company	Approval data	Target	Approved major indications	Type	Sequence derived	Dosing route	Expression system	MoA
46	Ramucirumab	Cyramza	Lilly/Dyax	2014-04-22	KDR (VEGFR-2)	Gastric cancer	Human IgG1	Human antibody phage library	i.v.	NS0	Binds and antagonizes receptor; inhibits ligand-induced proliferation and endothelial cell migration; inhibits angiogenesis in an animal model
47	Siltuximab	Sylvant	Janssen R&D/ J&J	2014-04-23	IL6	MCD	Chimeric IgG1	Mouse hybridoma	i.v.	CHO	Binds and neutralizes ligand
48	Vedolizumab	Entyvio	Takeda/ Millenium	2014-05-20	ITGA4/ ITGB7 (α4β7 integrin)	CDR	Humanized IgG1	Mouse hybridoma	i.v.	CHO	Binds and antagonizes receptor; inhibits leukocyte adhesion
49	Efmoroctocog alfa	Eloctate	Biogen Idec/ SOBI	2014-06-06	Factor substitute	Hemophilia A	Monomeric Fc domain-deleted F-VIII fusion	Fc fusion	i.v.	CHO	Substitute coagulation factor IX

(continued)

Table 20.1 (continued)

Number	INN	Commercial name	Company	Approval data	Target	Approved major indications	Type	Sequence derived	Dosing route	Expression system	MoA
50	Pembroli-zumab	Keytruda	Merck	2014-09-04	PDCD1 (PD-1)	Melanoma	Humanized IgG4	Mouse hybridoma	i.v.	CHO	Binds and antagonizes receptor; releases inhibition of the immune response, including the antitumor response
51	Dulaglutide	Trulicity	Eli Lilly	2014-09-18	GLP1R (agonist)	Type 2 diabetes	GLP-1 – Fc fusion	Fc fusion	s.c.		
52	Blinatumomab	Blincyto	Amgen (Micromet)	2014-11-14	CD19, CD3E	B-cell ALL	BiTE	Mouse hybridoma	c.i.v	CHO	Mediates formation of a T lymphocyte–tumor cell synapse that results in tumor cell lysis
53	Nivolumab	Opdivo	BMS	2014-12-03	PDCD1 (PD-1)	Melanoma	Human IgG4	HuMAb TG mouse	i.v.	CHO	Binds and antagonizes receptor; releases inhibition of the immune response, including the antitumor response

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Table 20.1 (continued)

Number	INN	Commercial name	Company	Approval data	Target	Approved major indications	Type	Sequence derived	Dosing route	Expression system	MoA
54	Secukinumab	Cosentyx	Novartis	2014-12-22	IL17A	Plaque psoriasis	Human IgG1	HuMAb TG mouse	s.c.	CHO	Binds and neutralizes ligand
55	Dinutuximab	Unituxin	United Technologies/ NCI	2015-01-21	GD2	Neuro-blastoma	Chimeric IgG1	Mouse	i.v.	Sp2/0	Depletes target cells by ADCC and CDC
56	Alirocumab	Praluent	Sanofi/ Regeneron	2015-03-10	PCSK9	High cholesterol	Human IgG1	VelocImmune TG mouse	s.c.	CHO	Binds and neutralizes ligand; lowers LDL-C levels
57	Evolocumab	Repatha	Amgen	2015-07-24	PCSK9	High cholesterol	Human IgG1	TG XenoMouse	s.c.	CHO	Binds and neutralizes ligand; lowers LDL-C levels
58	Idarucizumab	Praxbind	Boerhinger Ingelheim	2015-08-27	Dabigatran	Drug Reversal	Humanized Fab fragment	Mouse hybridoma	i.v.	CHO	Binds and neutralizes anticoagulant activity of dabigatran
59	Asfotase alfa	Strensiq	Alexion (from Enobia)	2015-10-16	Factor substitute	Hypopho-sphatasia	TNSALP-Fc fusion peptide	enzyme/Fc fusion	s.c.	CHO	Replace TNSALP enzyme and reduces the enzyme substrate levels
60	Mepolizumab	Nucala	GSK	2015-10-23	IL5	COPD	Humanized IgG1	Mouse hybridoma	s.c.	CHO	Binds and neutralizes ligand

(continued)

Table 20.1 (continued)

Number	INN	Commercial name	Company	Approval data	Target	Approved major indications	Type	Sequence derived	Dosing route	Expression system	MoA
61	Daratumumab	Darzalex	Janssen R&D (J&J)/Genmab	2015-11-06	CD38	MM	Human IgG 1	HuMAb TG mouse	i.v.	CHO	Depletes target cells by CDC, ADCC, ADCP and inducing apoptosis
62	Necitumumab	Portrazza	Lilly/ ImClone/ Dyax	2015-11-16	EGFR	Squamous NSCLC	Human IgG1	Human antibody phage library	i.v.	Sp2/0	Binds, antagonizes and induces internalization and degradation of receptor; depletes target cells by ADCC; increases sensitivity to chemotherapy (in in vivo models)
63	Elotuzumab	Empliciti	BMS/ Abbvie (from PDL)	2015-11-24	SLAMF7	MM	Humanized IgG	Mouse hybridoma	i.v.	NS0	Activates NK cells through SLAMF7 pathway and Fc receptors; depletes target cells by ADCC

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Table 20.1 (continued)

Number	INN	Commercial name	Company	Approval data	Target	Approved major indications	Type	Sequence derived	Dosing route	Expression system	MoA
64	Obiltoxaximab	Anthim	Elusys Therapeutics	2015-11-30	Bacillus anthracis PA toxin	Anthraxbio-defense	Chimeric IgG	Mouse hybridoma	i.v.	NS0	Binds and neutralizes antigen; prevents intracellular entry of anthrax lethal factor and oedema factor
65	Ixekizumab	Taltz	Eli Lilly	2016-03-21	IL17A	Psoriasis; PsA	Humanized IgG4	Mouse hybridoma	s.c.	a recombinant mammalian cell line	Binds and neutralizes ligand
66	Reslizumab	Cinqair	Teva Ception/ Cephalon	2016-03-22	IL5	Eosinophilic asthma	Humanized IgG4	Rat hybridoma	i.v.	NS0	Binds and neutralizes ligand
67	Atezolizumab	Tecentriq	Roche/ Genentech	2016-03-23	CD274 (PD-L1, B7-H1)	Bladder cancer	Humanized IgG1	Mouse hybridoma	i.v.	CHO	Binds and neutralizes ligand; releases inhibition of the immune response, including the antitumor response, without inducing ADCC

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Table 20.1 (continued)

Number	INN	Commercial name	Company	Approval data	Target	Approved major indications	Type	Sequence derived	Dosing route	Expression system	MoA
68	Olaratumab	Lartruvo	Lilly/ImClone	2016-05-18	PDGFRA	Soft tissue sarcoma	Human IgG1	UltimAb TG mouse	i.v.	CHO	Binds and antagonizes receptor; in vitro and in vivo antitumor activity
69	Bezlotoxumab	Zinplava	Medarex/ MBL/Merck	2016-5-27 withdrawn 2018	Clostridium difficile B toxin	CDAD	Human IgG 1	HuMAb TG mouse	i.v.	CHO	Binds and neutralizes toxin B
70	Brodalumab	Siliq	Valeant/ AstraZeneca	2016-10-19	IL17RA	Psoriasis	Human IgG2	TG XenoMouse	s.c.	CHO	Binds and antagonizes receptor
71	Avelumab	Bavencio	Pfizer/Merck KGaA (EMD Serono)/ Dyax	2016-10-22	CD274 (PD-L1, B7-H1)	Merkel cell carcinoma	Human IgG 1	Human antibody phage library	i.v.	CHO	Binds and neutralizes ligand; releases inhibition of the immune response, including the antitumor immune response; depletes target cells by ADCC
72	Dupilumab	Dupixent	Regeneron/ Sanofi	2017-02-15	IL4R	Atopic dermatitis	Human IgG4 S/P	VelocImmune TG mouse	s.c.	CHO	Binds and antagonizes receptor

(continued)

Table 20.1 (continued)

Number	INN	Commercial name	Company	Approval data	Target	Approved major indications	Type	Sequence derived	Dosing route	Expression system	MoA
73	Ocrelizumab	Ocrevus	Roche/Biogen	2017-02-23	MS4A1 (CD20)	Primary, progressing MS	Humanized IgG1	Mouse hybridoma	i.v.	CHO	Depletes B lymphocytes by ADCC and CDC
74	Durvalumab	Imfinzi	AstraZeneca (MedImmune)/ Celgene	2017-03-28	CD274 (PD-L1, B7-H1)	Metastatic urothelial carcinoma	Human IgG1	TG XenoMouse	i.v.	CHO	Binds and neutralizes ligand; releases inhibition of immune response; Fc-engineered to attenuate effector functions
75	Sarilumab	Kevzara	Regeneron Pharmaceuticals, Sanofi	2017-03-28	IL-6R	RA	Human IgG1	HuMAb TG mouse	s.c.	CHO	Binds to membrane-bound and soluble forms of IL-6R and inhibits IL-6 binding
76	Guselkumab	Tremfya	MorphoSys, Johnson & Johnson	2017-05-01	IL-23 p19	Plaque psoriasis	Human IgG1		s.c.	a mammalian cell line	Binds and neutralizes ligand

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Table 20.1 (continued)

Number	INN	Commercial name	Company	Approval data	Target	Approved major indications	Type	Sequence derived	Dosing route	Expression system	MoA
77	Inotuzumab ozogamicin	Besponsa	Pfizer, UCB	2017-05-22	CD22	Relapsed or refractory acute lymphoblastic leukemia (ALL)	Humanized IgG4-ADC		i.v.	CHO	The cytotoxin, calicheamicin, induces dsDNA breaks, leading to cell cycle arrest and apoptosis
78	Benralizumab	Fasenra	MedImmune, AstraZeneca, Kyowa Hakko Kirin, Lonza	2017-07-13	IL-5Rα	Asthma	Humanized IgG1 glycol-engineered		s.c.	CHO	Binds and antagonizes receptor; depletes target cells by ADCC; afucosylated for increased ADCC
79	Emicizumab	Hemlibra	Chugai, Roche	2017-08-17	Coagulation factors IXa and X	Hemophilia A	Humanized bispecific IgG4		i.v.	CHO	Binds coagulation factors IXa and X, mimicking the cofactor function of coagulation factor VIII
80	Ibalizumab-uiyk	Trogarzo	Theratechnologies, Inc	2017-11-14	CD4	HIV	Humanized IgG4		i.v.	NS0	Binds CD4 and inhibits HIV-1 post-attachment

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Table 20.1 (continued)

Number	INN	Commercial name	Company	Approval data	Target	Approved major indications	Type	Sequence derived	Dosing route	Expression system	MoA
81	Tildrakizumab	Ilumya	Sun Pharma	2017-11-16	IL-23 p19	Plaque psoriasis	Humanized IgG1		s.c.	CHO	Binds and neutralizes ligand
82	Burosumab-twza	Crysvita	KRN23; Kyowa Hakko Kirin Co. Ltd, Ultragenyx Pharmaceuti-cal Inc.	2018-03-06	FGF23	x-linked hypophos-phatemia (XLH)	Human IgG1		s.c.	CHO	Binds to and inhibits the biological activity of FGF23 restoring renal phosphate reabsorption and increasing the serum concentration of 1,25 dihydroxy vitamin D.
83	Erenumab-aooe	Aimovig	Novartis	2018-03-20	CGRPR	migraine	Human IgG2		s.c.	CHO	Binds and antagonizes receptor
84	Mogamuli-zumab-kpkc	Poteligeo	Kyowa Hakko Kirin	2018-04-17	CCR4	non-Hodgkin lymphoma	Humanized IgG1	Mouse hybridoma	i.v.	CHO	Depletes target cells by ADCC, afucosylated for increased ADCC

(continued)

Table 20.1 (continued)

Number	INN	Commercial name	Company	Approval data	Target	Approved major indications	Type	Sequence derived	Dosing route	Expression system	MoA
85	Lanadelumab	Takhzyro	Shire	2018-05-17	KLKB1	Types I and II hereditary angioedema	Human IgG1		s.c.	CHO	Binds plasma kallikrein and inhibits its proteolytic activity
86	Moxetumomab pasudotox-tdfk	Lumoxiti	AstraZeneca	2018-08-08	CD22	Hairy cell leukemia (HCL)	Mouse IgG1 dsFv-PE38	Phage display	i.v.	E. coli	CD22-directed cytotoxin results in ADP-ribosylation of elongation factor 2, inhibition of protein synthesis, and apoptotic cell death
87	Fremane-zumab-vfrm	Ajovy	Teva Pharma-ceuticals	2018-08-23	CGRPR	Migraine	Humanized IgG2		s.c.	CHO	Binds to calcitonin gene-related peptide (CGRP) ligand and blocks its binding to the receptor

(continued)

Table 20.1 (continued)

Number	INN	Commercial name	Company	Approval data	Target	Approved major indications	Type	Sequence derived	Dosing route	Expression system	MoA
88	Galcane-zumab-gnlm	Emgality	Eli Lilly and Company	2018-09-13	CGRPR	non-small cell lung cancer	Humanized IgG4		s.c.	CHO	Binds to calcitonin gene-related peptide (CGRP) ligand and blocks its binding to the receptor
89	Cemiplimab-rwlc	Libtayo	Sanofi	2018-09-14	PD-1	cutaneous squamous cell carcinoma (CSCC)	Human IgG4		i.v.	CHO	Binds and antagonizes receptor; releases inhibition of the immune response, including the antitumor response
90	Emapalumab-lzs gemapalumab-lzsg	Gamifant	Novimmune/ Swedish Orphan Biovitrum AB	2018-09-27	IFN	primary hemophago-cytic lymph histiocytosis (HLH)	Human IgG 1		i.v.	CHO	Binds and neutralizes interferon gamma (IFNγ)

(continued)

Table 20.1 (continued)

Number	INN	Commercial name	Company	Approval data	Target	Approved major indications	Type	Sequence derived	Dosing route	Expression system	MoA
	Ravulizumab	Ultomiris	Alexion	2018-09-28	Complement 5	Paroxysmal nocturnal hemoglobinuria (PNH)	Humanized IgG2/4		i.v.	CHO	Binds C5 and inhibits its cleavage, thus preventing the generation of the terminal complement complex C5b–9
91	Caplacizumab-yhdp	Cablivi	Sanofi	2018-11-20	von Willebrand factor (VWF)	Acquired thrombotic thrombocytopenic purpura (aTTP)	Bivalent nanobody		i.v. or s.c.	E. coli	Binds VWF and inhibits VWF-mediated platelet adhesion and platelet consumption
92	Romosozumab-aqqg	Evenity	Amgen/UCB Pharma	2018-12-21	Sclerostin	Osteoporosis in postmenopausal women at high risk of fracture	Humanized IgG2		s.c.	CHO	Inhibits sclerostin and increases bone formation

(continued)

Table 20.1 (continued)

Number	INN	Commercial name	Company	Approval data	Target	Approved major indications	Type	Sequence derived	Dosing route	Expression system	MoA
93	Risankizumab-rzaa	Skyrizi	Abbvie, Boehringer Ingelheim	2019-02-06	IL-23 p19	Plaque psoriasis	Humanized IgG1		s.c.	a mammalian cell line	Binds and neutralizes ligand and inhibits the release of pro-inflammatory cytokines and chemokines
94	Polatuzumab vedotin-piiq	Polivy	Roche	2019-04-09	CD79b	Relapsed or refractory diffuse large B-cell lymphoma	Humanized IgG1-ADC		i.v.	CHO	Depletes target cells by ADC. MMAE binds to microtubules and kills dividing cells by inhibiting cell division and inducing apoptosis

Abbreviations: ADC, antibody–drug conjugate; AMD, Age-related macular degeneration; ATL, adult T-cell leukemia/lymphoma; BiTE, bispecific T-cell engager; BlyS, B-lymphocyte stimulator; C5, complement component C5; CAPS, Cryopyrin-associated periodic syndrome; CCR4, C-C motif receptor-4; CD, cluster of differentiation; CDAD, clostridium difficile-associated disease; CLL, chronic lymphocytic leukemia; COPD, chronic obstructive pulmonary disease; CRC, colorectal cancer; CRD, Crohn's disease; CTLA4, cytotoxic T-lymphocyte-associated protein-4; CVD, cardiovascular disease; EGFR, epidermal growth factor receptor; ERBB2, erb-b2 receptor tyrosine kinase 2; F-VIII, Factor VIII; Fab, fragment, antigen-binding; Fc, fragment, crystallizable; GD2, disialoganglioside-2; GLP-1R, glucagon-like peptide-1 receptor; I-131, Iodine-131 (radioactive); HER2, human epidermal growth factor receptor-2; Ig, immunoglobulin; IL, interleukin; KDR, kinase insert domain receptor; LFA, lymphocyte-associated antigen; MCD, multicentric Castleman's disease; MCRC, metastatic colorectal cancer; MM, multiple myeloma; MPL, myeloproliferative leukemia virus oncogene; MS, multiple sclerosis; MWS, Muckle–Wells syndrome; ND, not disclosed; NHL, non-Hodgkin lymphoma; NSCLC, non-small cell lung cancer; OTR, organ transplant rejection; PA, protective antigen; PCSK9, proprotein convertase subtilisin/kexin type 9; PDCD1, programmed cell death 1; PDGFR, platelet-derived growth factor receptor; PD-L1, programmed cell death protein ligand-1; PEG, poly-ethylene-glycol; PNH, paroxysmal nocturnal hemoglobinuria; PsA, psoriatic arthritis; RA, rheumatoid arthritis; RANK, receptor activator of nuclear factor kappa-B; RR-MS, relapsing-remitting multiple sclerosis; RSV, respiratory syncytial virus; SC, subcutaneous; SLAMF7, signaling lymphocytic activation molecule family member 7; SLE, systemic lupus erythematosus; S/P, mutations in hinge of IgG4; TG, transgenic (humanized); TNALP, tissue-nonspecific alkaline phosphatase; TNF, tissue necrosis factor; TPO-R, thrombopoietin receptor; VEGF, vascular endothelial growth factor

20.2 Development and Application of Antibody-Related Drugs

Antibody drugs are exciting therapeutic protein drugs. They generally have advantages of striking specificity, stable structure, long circulating plasma half-life, remarkable curative effect, and low unexpected clinical risks (Carter and Lazar 2018; Lau and Dunn 2018; Albericio 2019). In addition, they are relatively easy to carry out gene manipulation (Hudson and Souriau 2003), giving scientists and pharmaceutical industries ample room to design and optimize antibody-based drugs.

Over the past half-century, the number of antibody-based drugs has increased exponentially. Six of the top 10 global drug sales in 2018 are therapeutic antibodies, and the global market for therapeutic antibodies has reached 123.2 billion US dollars (EvaluatePharma 2019). The two recently highly anticipated anti-PD-1 therapeutic antibodies (Keytruda and Opdivo) have ranked among the top 10 in global sales in only 5 years. Such astonishing achievements attribute to advances in lots of disciplines such as immunology and oncology, and most importantly, technology breakthroughs in antibody engineering.

20.2.1 Humanization of Antibody Drugs

With the determination of antibody structure and the development of hybridoma technology in the 1970s, the mechanism of antibody function has become more and more clear, and gradually the production progress of monoclonal antibody became effective and reliable. People start to believe that antibodies would become the "silver bullets", because their antigen specificity and diversity evolved by natural selection make them great potential as specific targeting reagents. Since then, in order to make the antibody into a real therapeutic drug as soon as possible, scientists and pharmaceutical industries have overcome a lot of unexpected difficulties making various modifications on antibody proteins and developing many production methods.

Conventionally, hybridoma technology was used to derive murine monoclonal antibodies (suffix-omab). First, antibody-producing B cells were isolated from repeatedly immunized mice, and then fused with myeloma cells to obtain immortalized hybridoma cells which would last to produce a specific type of monoclonal antibodies (Nelson et al. 2000). However, due to the xenogeneic nature of murine antibodies, sensitization was regularly observed among patients treated with murine antibodies. In addition, elimination by patient's immune system led to a short in vivo half-life of murine antibodies. In the clinical feedback of cancer patients in the 1980s, although the murine antibodies were not as effective as expected, many patients were still in continuous remission. What hampered the therapeutic effect of murine antibodies? In further researches, evidences show that murine antibodies quite easily provoke human anti-mouse immune response, circulate a short half-life, limitedly penetrate into the tumor sites and inadequately recruit of host effector functions.

Besides, there were also lack of highly specific tumor antigens (Stern and Herrmann 2005).

Chimeric (suffix-ximab) and humanized antibodies (suffix-zumab) were designed through engineering murine antibodies to remove immunogenic content and to increase immunologic efficiency. Chimeric antibodies are obtained by genetically fusing murine antigen recognition domains (variable domains of both heavy and light chains) and human Ig Fc domains. Humanized antibodies further minimize the murine portion of antibodies, retaining only the murine complementarity-determining regions (CDRs), and replacing the whole Ig framework with the human homologues (Riechmann et al. 1988; Wu et al. 1999). Thanks to the human frameworks, chimeric antibodies and humanized antibodies, as expected, are less likely to be cleared by human immune system, more guaranteed to stimulate effector cells, and have a half-life comparable to that of fully human antibodies (Stern and Herrmann 2005). Their promising specificity and human-like antibody advantages are destined to make the new types of MAbs very popular drugs. For example, the landmark Rituxan® (Rituximab), the first chimeric CD20 MAb drug approved by FDA (1997), is still a very popular non-Hodgkin's lymphoma (NHL) treatment drug (the fifth largest global sales in 2018, $7.414 billion). But Zenapax® (Daclizumab), a humanized antibody approved in the same year, is a regrettable example. It was first approved to treat organ transplant rejection (OTR), but eventually withdrawn from the market in 2009. In 2016, it was reapproved (under Zinbryta®) for relapsing forms of multiple sclerosis (MS), but was soon withdrawn in 2018 because of safety issues.

In recent years, the new monoclonal antibody drugs are mainly humanized and completely human-like. The realization of fully human monoclonal antibodies (suffix-umab) benefits from two biotechnologies, one is phage display and the other is transgenic Ig humanized mice (Baca et al. 1997; He et al. 2002). For example, two now powerful antibodies durvalumab and avelumab, durvalumab is obtained from XenoMouse® (Kucherlapati et al. 2000; Nastri et al. 2017; Queva et al. 2014) (transgenic mice genetically engineered with a "humanized" humoral immune system), and avelumab is screened out of phage display technology (Nastri et al. 2017).

As of June 2019, FDA had approved 83 antibody drugs, 44 of them have been approved in the past 5 years (about 20% of the newly approved drugs in the past 5 years by FDA, 44 of 213), and four new antibody drugs in the first half of 2019 (including monoclonal antibodies, bispecific antibodies, antibody fragments, antibody–drug conjugates, etc.) (Fig. 20.2). Oncology and autoimmune diseases are the most important therapeutic areas of antibody drugs. For a long time, market share of the two areas is comparable. With the emergence of anti-PD-1/PD-L1 antibodies, the number of anti-cancer antibodies and anti-autoimmune disease drugs in 2018 has widened the gap (including those in clinical trials). From the target point of view, TNF-alpha is still a worthy star target, because of its widespread expression in autoimmune diseases. The development of cancer immunity corresponds to the production of anti-cancer antibody drugs such as PD-1/PD-L1 with broad-spectrum indications (Tang et al. 2018; Mullard 2019; Lagasse 2017). Most of the traditional targets are the cluster differentiation (CD) proteins, markers on cancer cell's surface, receptors and inflammatory factors, which make most of the targeted antibodies require

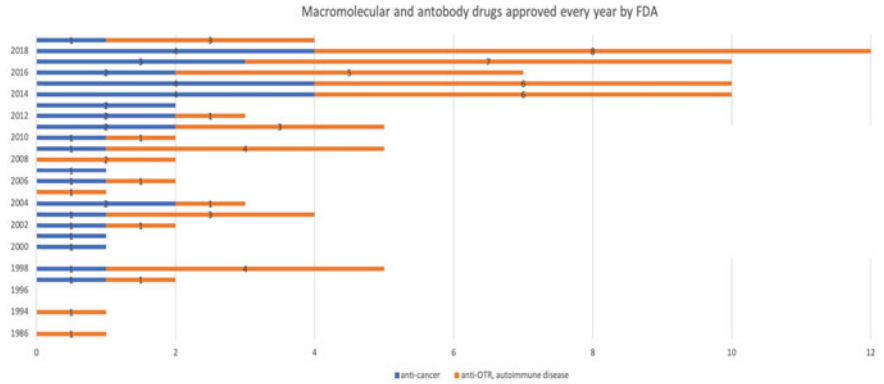

Fig. 20.2 Macromolecular and antibody drugs approved every year by FDA

antibody-dependent cellular cytotoxicity (ADCC) to enhance immune system to kill cancer cells, and therefore IgG1 is the main subclass; immune checkpoint antibodies (anti-PD-1/PD-L1, anti-CTLA4) mainly rely on removing inhibitory pathways that block effective antitumor T-cell responses, so most of them are IgG4 subclass.

20.2.2 Antibody–Drug Conjugates (ADCs)

Antibody drugs have two key basic attributes, one is specific affinity with target antigens, and the other is triggering ADCC [or antibody-dependent cellular phagocytosis (ADCP), complement-dependent cytotoxicity (CDC)] (Clynes et al. 2000; Gul and van Egmond 2015; Introna and Golay 2009). With these two attributes, naked antibodies can be used for targeted therapy. The traditional classical design of therapeutic antibodies is based on this idea. There are many attempts to further augment the effector functions on the basis of existing naked antibodies, one of which is antibody–drug conjugates (ADC). ADCs chemically couple antibodies with different cytotoxic molecules through various linkers. Cytotoxic molecules augment the lethality of antibodies to tumors. Antibodies reduce unnecessary killing of cytotoxic molecules against non-targets. They complement each other to achieve better therapeutic effect.

20.2.2.1 Antibody Conjugated to Chemical Drugs or Immunotoxins

The earliest ADCs are covalent conjugations of antibodies and chemotherapeutic drugs. These chemotherapeutic drugs were often too toxic to be used as drugs alone, but they could be used as warheads on cancer-specific antibodies to cause strong

killing of targeted cancer cells. Common drugs of these kinds are calicheamicins, esperamicins, auristatins, and maytansines (Beck et al. 2017).

Calicheamicin and esperamicin are DNA-destroying biotoxins isolated from bacteria in the 1980s (Shor et al. 2015). The first FDA-approved ADC (2000), Mylotarg® (gemtuzumab ozogamicin), is a calicheamicin-based ADC. Gemtuzumab is an anti-CD33 antibody, calicheamicins act as warheads and are coupled to random lysines of gemtuzumab via cleavable hydrazone linkers (Hamann et al. 2002). Gemtuzumab ozogamicin is used to treat acute myeloid leukemia (AML). It was once withdrawn from the market in 2010 because of its fatal toxicity. But after optimizing the treatment dose, it substantially benefits AML patients and was eventually reapproved in 2017.

Maytansines and auristatins are two kinds of molecules that prevent microtubules assembly. The former is separated from plants and the latter is synthetic analogs of dolastatin 10. They and their derivatives have good water solubility, long half-life, and limited immunogenicity, making them the most commonly used drugs for ADCs in clinical trials currently (Beck et al. 2017). Monomethyl auristatin E (MMAE) and MMAF are the two most frequently used auristatin derivatives. Adcetris® (Brentuximab vedotin) is one well-known anti-CD30 MMAE-conjugated ADC, treating Hodgkin's lymphoma (HD). The naked IgG brentuximab has already been able to inhibit the growth of HD cell lines in vitro, and could further improve survival rate after attaching 4 MMAE to hinge cystines of brentuximab via protease-cleavable linker (Senter and Sievers 2012). Kadcyla® is a successful maytansine-based ADC attaching 3–4 maytansinoid derivatives (DM1) to the random lysines of Herceptin (already a first-line drug for breast cancer) through a non-cleavable thioether linker. It can effectively improve survival and reduce side effects (Lambert and Chari 2014).

There have been five ADCs approved for the market: Mylotarg® (Gemtuzumab ozogamicin, anti-CD33, approved in 2000), Adcetris® (Brentuximab vedotin, anti-CD30, 2011), Kadcyla® (Trastuzumab emtansine, anti-HER2, 2013), Besponsa® (Inotuzumab ozogamicin, anti-CD20, 2017), and Lumoxiti® (Moxetumomab pasudotox-tdfk, anti-CD22, 2018). They are all ADCs made up of the above drugs. The recently approved Lumoxiti® is an ADC expressed in Escherichia coli. It is composed of disulfide-linked heavy-chain variable (VH) and light-chain variable (VL) domains of the murine anti-CD22 monoclonal antibody fused to PE38. After binding CD22 on B-cell membrane, it results in ADP-ribosylation of elongation factor 2, inhibition of protein synthesis, and apoptotic cell death (Kreitman and Pastan 2015).

Over time and technological advances, some new toxins have also been used in ADCs and have reached clinical trials. Such as pyrrolobenzodiazepine (PBD)—cross-linking DNA and blocking cell division, camptothecin analogs (SN-38 and DX-8951f)—inhibition of DNA topoisomerase 1 (TOP1), and some novel auristatin derivatives (Chari et al. 2014).

20.2.2.2 Antibody Fusing with Human Proteins

Around the beginning of the twenty-first century, advances in genetic engineering and expression systems made antibody fusion proteins possible and soon available. One of the major expected functions of recombinant antibodies is delivering important cargos to designated tissues. In addition to drugs, many therapeutic potential proteins, such as enzymes (Whyte 2017), hormone (Glaesner et al. 2010), cytokines (Bowles and Weiner 2005), etc. are fused to the delivery vehicles (Lagasse 2017).

Taking cytokines for example, these small proteins serve as immunomodulating targets and play very important roles in cell death signaling pathways. Cytokines such as interleukin-2 (IL2), granulocyte/macrophage colony-stimulating factor (GM-CSF), and interleukin-12 (IL12) were fused to antibodies in order to achieve targeted delivery of cytokines to tumors or specific tissues, which can reduce the side effects of cytokine system administration and effectively activate protective immune response (Penichet and Morrison 2001). Many of these fusion immune cytokines have entered clinical trials, most of which are fusion of cytokine and Fv domains, and some are fusion with IgG (Neri and Sondel 2016).

Another fusion format is fusing therapeutic proteins and Fc domains. The main purpose of this operation is to prolong the half-life of therapeutic proteins by using the FcRn-mediated recycling of the Fc and/or to extra trigger ADCC (Saxena and Wu 2016; Strohl 2015). Eftrenonacog alfa (Alprolix™) comprising human coagulation factor IX (FIX) covalently linked to the Fc domain of human IgG1 is an approved drug (2014) for hemophilia B. Fc domain extends FIX half-life, permitting prolonged treatment intervals (Hoy 2017). Asfotase alfa (Strensiq®, approved in 2015) is a first-in-class drug for hypophosphatasia, composed of catalytically active homodimeric soluble TNSALP (tissue-nonspecific isoenzyme of alkaline phosphatase) domain fused to Fc domain of human IgG1. It is a substitute for loss-function enzymes to reduce harmful accumulated substrate levels.

20.2.3 Engineered Antibodies: From IgG to Different Formats Attempt

Antibody engineering and producing have undergone several generations of design and optimization (Fig. 20.3) (Lo 2004). Since the discovery of Y-shape structure of immunoglobulin (mainly IgG), many modifications have been attempted upon Y-shape framework, including amino acid mutations(Hudson and Souriau 2003), humanization(Apgar 2016), Fc engineering(Liu et al. 2017), and ADC (Beck et al. 2017) which aim for higher affinity (Lippow et al. 2007), lower immunogenicity (Hwang and Foote 2005), stronger effector function (Lazar et al. 2006), and better therapeutic efficacy (mentioned in the previous sections) (Carter 2006). Besides there have emerged many innovative formats through gene manipulation, pursuing minimal size, multi-specificity, extended performance, economical production, or other

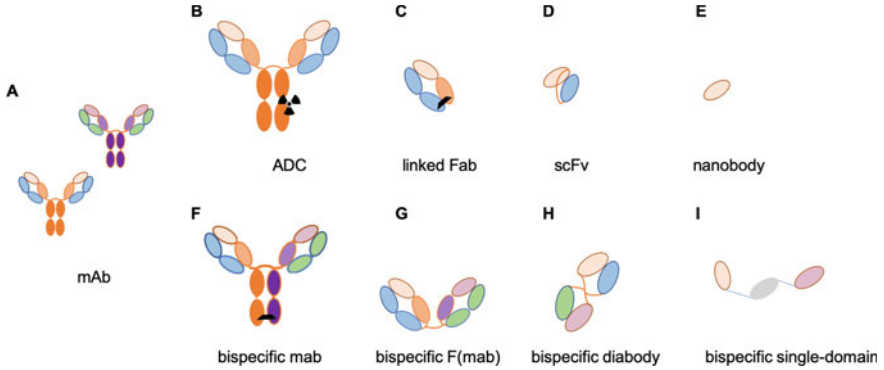

Fig. 20.3 Antibodies engineering. **A** Monoclonal antibody, **B** antibody–drug conjugates, **C** C-terminal interaction enforced Fab, **D** single-chain variable fragments, variable domains of heavy and light chains linked by flexible peptide, **E** single variable domain of heavy chain, VHH or nanobody, **F** bispecific monoclonal antibody, constant region interaction enforced, **G** bispecific F(MAb), two Fabs linked by flexible peptide or chemical bonds, **H** bispecific diabody, two variable fragments linked by flexible peptides, **I** bispecific single domain, two single variable domain of heavy chain linked by peptides or Ig family domain

benefits. Most manipulations retain variable fragments (Fv) to ensure full antigen-binding capacity. These kinds of format are particularly useful in applications where epitope binding is sufficient for the desired effect including therapeutic applications such as virus neutralization or receptor blocking.

20.2.3.1 Single-Chain Fv and Its Therapeutic Applications

Traditional monoclonal antibodies were derived from small mammals, hybridomas, or human immunocytes, which is very laborious and time-consuming. The drawbacks have led several research groups to investigate new format antibodies that could be gene manipulated easily and quickly, and expressed in more economical expression systems (Ahmad 2012). Single-chain variable fragment (scFv) is a marvelous strategy along this direction, containing VL and VH linked in tandem by a flexible peptide linker that covalently binds C-terminal of VL and N-terminal of VH (or vice versa). This design successfully circumvents undesirable bindings of IgG constant regions. As a single-chain fragment, it solves Fv fragment reassociation problem, guaranteeing correct VH and VL molar matching and functional folding (Bird et al. 1988). At the same time, scFv, as the smallest immunoglobulin antigen-binding unit, can also be used as a building block for other forms of recombinant antibodies, such as Lumoxiti mentioned above, which replaces polypeptide linker with disulfide bond, or bi-/multi-valent antibodies, or bispecific antibodies, which will be described later (Weisser and Hall 2009).

The scFv could be conveniently expressed in many kinds of host: mammalian cells (Ho et al. 2006), yeast (Chao et al. 2006), insect cells (Choo et al. 2002), plant

(Stöger et al. 2000), and particularly in E. coli (Jurado et al. 2002). Comparing to other expression systems, E. coli is much simpler and faster to conduct large-scale screening and production. ScFv could be expressed as soluble protein in periplasm or as inclusion body in cytoplasm (Skerra and Pluckthun 1988). The former strategy requires secreting signal peptide added in front of scFv, and the latter requires refolding in vitro. Both strategies have their own advantages and disadvantages.

When scFv is expressed on a bacterial phage surface, a very powerful technology is born, which is called phage display (McCafferty et al. 1990). By fusing scFv with the gene III protein (a protein on phage tip, responsible for attachment to bacteria), scFv can be displayed on the phage surface and maintain the ability of antigen binding. With the aid of the small physical size of phages as well as scFv, and each phage expressing a unique kind of antibody, a potential giant antibody clone library can be constructed quickly (Vaughan et al. 1996), which facilitates the screening and isolation of the desired antibodies. Then the selected phage can quickly infect bacteria host, from which the desired antibody gene can be enriched and amplified, and eventually antibody could be produced abrogating hybridomas and immunization. Such in vitro screening above, which mimics natural selection, has played an important role in immunological research and antibody production, including several drugs already on the market, and 2017 Nobel prizes in chemistry have been awarded to the phage display technology.

The most successful clinical application of scFv so far is the approval of chimeric antigen receptor (CAR) T-cell therapy in 2018 (CAR 2018). CAR T-cell therapy employs patients' autologous T-cells to kill tumor cells and promote immune surveillance (June and Sadelain 2018). First, isolated patient's T-cells were transfected CARs in vitro, which is a fusion of a specific tumor antigen-derived scFv, a spacer, a transmembrane domain, two costimulatory endodomains (e.g., CD28 or 4-1BB) and a CDζ. Then, after ex vivo proliferation, genetic-engineered CAR T-cells were reinfused to the patients. Once infused, CAR T-cells engraft and undergo extensive proliferation in the patient, not only to eliminate tumor cells, but also to prevent the tumor recurrence (Kalos 2011; Maude et al. 2014). Kymriah and Yescarta, two CAR T-cell therapies approved in past 2 years, both are made up of murine anti-CD19 scFv CARs and indicated for blood cancers (Kymriah for ALL and DLBCL (Novaritis 2019), Yescarta for DLBCL (Mullard 2018)). Though it remains a challenge for CAR T-cell to treat solid tumor, this therapy is a very promising treatment for broader indications (Novaritis 2019).

In any clinical applications, one common demand for scFv is stability (Wörn and PluÈckthun 2001). It is prolonged circulating half-life that guarantees therapeutic function. Many efforts such as amino acid mutations, introducing new covalent bonds and interface modifications, have been made to engineer scFv to enhance stability and specificity. The scFv and its engineered formats still remain potential medicine candidates.

20.2.3.2 Nanobody and Its Therapeutic Applications

As early as the 1960s, scientists found that single antibody heavy chain or light chain still had antigen-binding ability, the affinity and stability of single variable domain were not as good as paired VH and VL (Harmsen and De Haard 2007). Not until the discovery of a kind of naturally occurring heavy-chain homodimer immunoglobulins in the 1990s, which were found in the blood of camel (Desmyter et al. 1996) (similar light-chain-devoid immunoglobulins were also found in sharks later Stanfield et al. 2004), did it spur the engineering on single domain antibodies. Unlike conventional antibodies, these camelid-IgGs lack light chains and CH1 domains, but nevertheless still possess antigen-binding diversity and specificity. Their variable domains (also known as VHH or nanobody) have thus become a hot engineering antibody moiety.

Fortunately, camelid VHH sequences are highly similar to human VHs (Muyldermans et al. 2001), and therefore they are easy to be humanized and expected to generate slight immunogenicity in treatments. VHH is only half the size as scFv, but small size is a double-edged sword. On the one hand, it is easier to penetrate tumors, but on the other hand, it is also faster to be eliminated in patients' blood. One approach is fusing human Fc domain to VHH in order to increase its serum longevity by neonatal Fc receptor (FcRn)-based protection. Another strategy is introducing an extra nanobody and hence expand molecular size to prolong half-life (Chanier and Chames 2019). Structures of camelid and shark antibodies reveal that their CDR3 loops are longer than normal Fv, which contribute a lot to their favorable features. This extended region covering the lipophilic site, where normally binds to a light chain in Fv, endows its single variable domain high stability (Stanfield et al. 2004) and allows the binding to non-conventional epitopes such as protein clefts (De Genst et al. 2006). And like scFv, nanobodies also have good modularity to construct new format antibodies.

Cablivi (Caplacizumab-yhdp) (FDA 2019) is a bivalent nanobody drug approved by both EMA (2018.8.31) and FDA (2019.2.6) for acquired thrombotic thrombocytopenic purpura (aTTP), consisting of two identical anti-von Willebrand factor (vWF) humanized nanobody linked by a three-alanine linker. It is produced in Escherichia coli by recombinant DNA technology and has an approximate molecular weight of 28 kDa. Unlike traditional antibodies, which kill targets, Cablivi prevents the interaction between vWF and platelets, thereby reducing both vWF-mediated platelet adhesion and platelet consumption.

20.2.3.3 Other Formats

As mentioned above, many alternative antibody formats are constructed using scFv or nanobody as building blocks, spanning molecular size range, valency, and specificity. Most common linkers are still flexible short peptides or covalent bonds. Bi-/multivalent formats combine two or more copies of the same specific antigen-binding fragment, while bi-/multi-specific formats join two or more specific antigen-binding fragments in one or more copies (Beck et al. 2010).

It has been more than 50 years since the first bispecific antibody was developed. Most significant function of bispecific antibodies is in T-cell redirection, where one specificity recognizes makers on tumor cells and the other targets CD3E on T-cells, thereby the T-cells were redirected to tumors. Blincyto (Blinatumomab, approved in 2014) belongs to this kind of drugs, called bispecific T-cell engager (BiTE) (Strohl 2018). It is a bispecific CD19-directed CD3 T-cell engager indicated for the treatment of Philadelphia chromosome-negative relapsed or refractory B-cell precursor acute lymphoblastic leukemia (ALL). Hemlibra (Emicizumab-kxwh) is a humanized IgG4 bispecific antibody (approved in 2017) binding factor IXa and factor X and mimicking the cofactor function of coagulation factor VIII. Hemlibra bridges activated factor IX and factor X to restore the function of missing activated factor VIII that is needed for effective hemostasis.

There exist some drawbacks of these bi-/multi-specific formats, one significant limitation is fixed ratio of antigen-binding components, restricting flexible dose administration when compared to mixed antibodies combination administration. Correspondingly, the antibody mixture therapy also has problem of vague ratio.

20.3 Antibody-Based Drugs and Immunotherapy

During the past three decades, our insight into immunology and oncology has become more and more clear and comprehensive. Tumor cells could escape the attack of the immune system by turning on immunosuppression and many critical immune checkpoint pathways are identified. Therefore, how to harness the individual immune system to fight against cancer becomes a constant attempt. And indeed, many immunotherapy options have come into clinical reality. Immunotherapy could be divided into many types, including cytokines which use interferons and interleukins to enhance immune-fighting against tumor (Capuron et al. 2002); therapeutic antibodies which highlight cancer cells for immune system (Weiner 2015); treatment vaccines to boost immune response (Rosenberg et al. 2004); adoptive cell transfer which, like CAR T-cell, improves the natural ability of individuals' T-cells; and checkpoint inhibitors which "release brakes" of immune-attacking. In this section, we would focus on those antibody-targeting checkpoints.

20.3.1 Innovation and Breakthrough

Science named cancer immunotherapy as 2013 "Breakthrough of the Year" for quite accomplishments of CAR T-cell and immunomodulatory antibodies drugs (Couzin-Frankel 2013). In 2017, the 3-year overall survival with combined nivolumab (Opdivo, anti-PD1 mAb drug approved in 2014) and ipilimumab (Yervoy, anti-CTLA4 mAb drug approved in 2011) in advanced melanoma was reported 58% (Wolchok et al. 2017). It is a marvelous increase comparing with that of 12% in

chemotherapy and shows strong evidence of immunotherapy potential (Kvistborg and Yewdell 2018).

The clue of employing immune system to conquer cancer could be traced back to a surgeon William Coley in the 1890s who attempted on stimulating patients' immune system to cure cancer by injecting bacteria into tumors (McCarthy 2006). Nearly one century later, the monoclonal antibody-targeting tumor-associated proteins are approved intermittently. These antibodies (described above) eliminated tumor cells by directly killing them or triggering ADCC, or plus ADC. In addition to them, many recombinant cytokines were also approved to active immune system. At the beginning of this century, immune checkpoints showed potent role in tumor progression, pushing cancer immunotherapy to a new generation—immune checkpoint blockade therapy (ICB, or checkpoint inhibitor therapy, ICI) (Sharma and Allison 2015). The first checkpoint blockade antibody drug targets at cytotoxic T-lymphocyte-associated protein 4 (CTLA-4), which was demonstrated to arrest T-cell activation and proliferation. Several years later, programmed cell death 1 (PD-1) receptor on T-cells with its ligand programmed cell death ligand 1 (PD-L1) on tumor cells, which were dominated regulators in immunosuppression and became the most popular targets in checkpoint blockade drugs screening.

Anti-checkpoint antibodies function differently from other antibody drugs in the past. First and obviously, their targets are no longer markers of cancer cells, but molecules that regulate immune system (more specifically, T-cell responses), and further their duty is cleaning those obstructs that inhibit effective antitumor T-cell responses, other than directly killing the tumor cells by triggering immune attack, giving this class of antibodies the ability to apply in a wide range of cancers including lung cancer, melanoma, breast cancer, lymphoma, and head and neck cancer (Topalian et al. 2016).

As of September 2018, active clinical trials testing anti-PD1/L1 agents have amounted to at least 2250, which is a dramatic increase from 1 in 2006 (Tang et al. 2018). As expected, anti-PD1/L1 therapy did show remarkable result and thus was associated with higher objective response rates (ORR), progression-free survival (PFS), and lower rate adverse events (AEs). Nivolumab was an impressive success in treating melanoma, giving higher ORR (32% versus 11%) and lower rate of grade 3–4 AEs (9% versus 31%) when compared with chemotherapy and are significantly better 1-year OS rate (73% vs. 42%), median PFS (5.1 months versus 2.2 months), and ORR (40% versus 14%) than dacarbazine (Robert et al. 2015). What is more impressive is their survival curves indicate that most patients would survive longer after the treatment (Topalian 2019).

Despite existing and potential clinical benefits, the ICI still faces some problems. Though many common cancers are demonstrated associating with immunosuppression, ICI response rates also are influenced by tumor microenvironments and patients' immune systems and thus vary with different tumor types and individuals (Topalian et al. 2015). Among the current active anti-PD1/L1 trials, 1716 trials combine CTLA-4 or other therapies like chemotherapy and radiotherapy (Tang et al. 2018). Besides, ICI has alternative chances: diagnosis and analysis of patients' tumor mutations and

presence of toxic T-cells with corresponding immune checkpoints. ICI remains a long way to go but still a promising therapy.

20.3.2 Immune Checkpoints and Their Antibodies

Immune checkpoints refer to the intrinsic regulatory molecules of immune system, which are evolved to maintain self-tolerance and prevent collateral damage to innocent tissues during the immune response. Tumors take advantage of this mechanism and play innocent through building microenvironments to avoid immune surveillance and attack, especially regulating certain immune checkpoint pathways (Topalian et al. 2015). So far, six immune checkpoints and their respective ligands have been identified, which are receptors expressed on immune-activated cells and ligand proteins on cancer or myeloid cells (Fig. 20.4). Many antibodies are designed to target them in order to block their respective interactions and thus cut the immune inhibitory pathways.

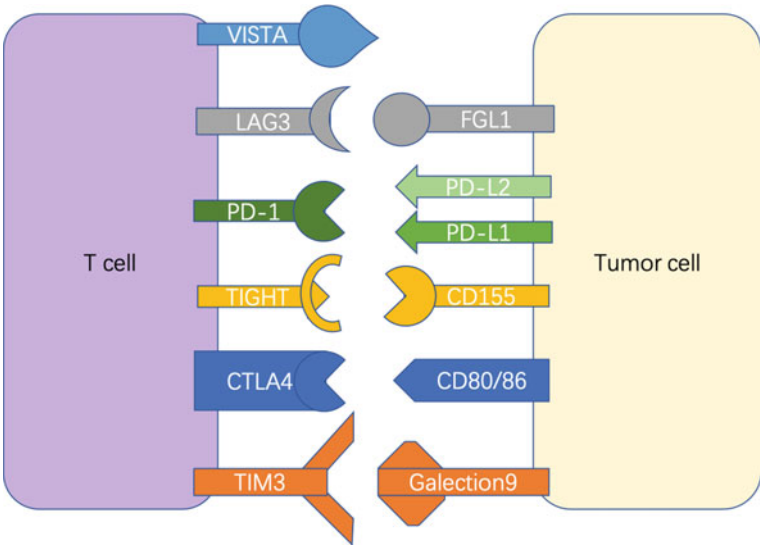

Fig. 20.4 Identified immune checkpoints. Abbreviations: PD-1: programmed cell death protein 1; PD-L1: programmed cell death 1 ligand 1TIGHT: T-cell immunoglobulin and ITIM domain; TIM-3: T-cell immunoglobulin and mucin-domain containing-3; LAG-3: lymphocyte activation gene 3 protein; FGL1: fibrinogen-like protein1; VISTA: V-domain Ig suppressor of T-cell activation

20.3.2.1 CTLA-4

CTLA-4, also known as CD152, is a pivotal checkpoint molecule in ICI history. It shares high homology with the synergistic stimulatory molecule receptor CD28, which is expressed constitutively on the surface of T-cells. Resting T-cells are initially activated by the engagement of T-cell receptor (TCR) and the binding of costimulatory CD28 to its ligands. Both of CD28's two ligands CD80 (B7-1) and CD86 (B7-2) are also ones to CTLA-4 and present on antigen-presenting cells rather than tumor cells. At a later stage of T-cell's activation, CTLA-4 translocates to cell surface where it reverses the stimulatory pathways to inhibitory ones and arrests activated T-cell responses. Immunology and preclinical studies show two mechanisms of CTLA-4 inhibitory signaling. One is directly reducing stimulatory signals in T-cells through outcompeting CD28 for binding to CD80 and CD86 or recruiting phosphatases to block TCR and CD28 signal transduction (Rudd et al. 2009). The other one is cell-extrinsic depletion of ligands where CTLA-4 removes CD86 from antigen-presenting cells by a process of trans-endocytosis and results in impaired T-cell response (Qureshi et al. 2011).

CTLA-4 contains an Ig-V-like extracellular domain and a cytoplasmic tail quite similar to CD28. Its pathway is a significant component of regulatory CD4+ T-cell (Treg)-suppressive function to maintain immune homeostasis, though the mechanism remains somewhat unclear (Walker 2017). Antibodies blocking CTLA-4 could mediate this suppressive pathway and reconstruct immune homeostasis. The first and only approved anit-CTLA-4 antibody drug is Yervoy (ipilimumab), which remarkably increases the survival rate of metastatic melanoma patients comparing with chemotherapy (Hodi et al. 2010). However, CTLA-4 blockade does not meet expected clinical response rate and is frequently accompanied by toxicities including enterocolitis, inflammatory hepatitis, and dermatitis (Postow et al. 2018). In addition, two other drugs Tremelimumab and AGEN1884 are now in the clinical stage and the rest are mostly in the early clinical stage.

20.3.2.2 PD-1

Of current clinical interest is the PD-1/PD-L1(-L2) blocking. PD-1 (also known as CD279) is a receptor expressed on CD4-CD8- thymocytes, and CD4+ CD8+ T-cells and selectively other activated T-cells. It interacts with two ligands, PD-L1 (CD274), which are present both on immune cells and many nonimmune cells upon exposure to pro-inflammatory cytokines, and PD-L2 (CD273) which are mainly expressed on antigen-presenting cells. Notably, PD-L1 is also expressed on the surface of various cancer cells and noncancer cells in tumor stoma, including squamous cell carcinoma of the head and neck, melanoma, and carcinomas of the brain, thyroid, thymus, esophagus, lung, breast, gastrointestinal tract, colorectum, liver, pancreas, kidney, adrenal cortex, bladder, urothelium, ovary, and skin (Wang et al. 2016). Therefore, the PD-1/PD-L1 axis attracts considerable attention in ICI.

PD-1 and its two ligands are single-pass type I transmembrane proteins belonging to immunoglobulin superfamily. PD-1 has only one extracellular domain (Ig V-like domain) and a long cytoplasmic tail containing immunoreceptor tyrosine-based inhibition motif (ITIM) and an immunoreceptor tyrosine-based switch motif (ITSM), while its ligands bear two extracellular domains (an Ig-V-like domain and an Ig-C-like domain) and a short cytoplasmic tail without knowing signaling motifs. Following the extracellular domain of PD-1 engaged by its ligands, the cytoplasmic ITIM and ITSM motifs were phosphorylated, which results in recruitment of protein tyrosine phosphatase PTPN11/SHP-2 that mediates dephosphorylation of key TCR proximal signaling molecules and thus cut off T-cell activation (Fife and Pauken 2011). Those tumors expressing PD-L1 exploit this pathway to attenuate antitumor immunity and facilitate tumor survival.

Clinical data show that PD-L1 is the dominant inhibitory ligand of PD-1 in tumor microenvironment (Sun et al. 2018), where antitumor T-cells are repeatedly exposed to tumor antigens, leading to PD-L1 expression on target cells and continuous PD-1-induced T-cell exhaustion (Pardoll 2012). Antibodies blocking PD-1/PD-L1 interaction have been developed rapidly during last few years. Currently, six anti-PD-1/L1 antibodies are approved for 14 indications. The first two anti-PD-1 antibodies nivolumab and pembrolizumab treating melanoma were under accelerated approval in 2014. After then, a wide range of cancer indications of them have been approved, such as melanoma, NSCLC, HNC, HD, and urothelial carcinoma (UC). Later, the first anti-PD-L1 antibody atezolizumab was approved for UC and NSCLC in 2016, followed by avelumab for UC and Merkel cell carcinoma, and durvalumab for bladder cancer in 2017. Another anti-PD-1 antibody, cemiplimab treating cutaneous squamous cell carcinoma (CSCC), was approved in 2018. Besides there were two new anti-PD-1 antibodies approved by China National Medical Products Administration (NMPA), one is toripalimab, approved in 2018 December for the treatment of metastatic melanoma with an ORR of 17.3% in Chinese patients (Keam 2019). 30 ¥/mg renders it the most economical anti-PD-1 antibody in the world, which costs less than a third of pembrolizumab per year. Another one is sintilimab approved in 2019 February with objective response up to 80% in Hodgkin's lymphoma (Chinese patients) (Shi et al. 2019).

Dozens of anti-PD-1/L1 antibodies wait in line for the market. As mentioned above, thousands of active clinical trials test anti-PD-1/L1 agents, among them, the top five most-studied cancers are lung cancer (254 trials), melanoma (139 trials), breast cancer (106 trials), lymphoma (99 trials), and head and neck cancer (72 trials) (Tang et al. 2018). Since PD-1 and CTLA-4 are the two dominant checkpoints, anti-CTLA4 antibodies are the most common agents employed in PD-1/L1 combination therapies (339 from 1716) (Tang et al. 2018). Indeed, nivolumab and ipilimumab combination achieves 58% response rate in melanoma, which is an extraordinary clinical benefit (Wolchok et al. 2017).

20.3.2.3 TIGHT

T-cell immunoglobulin and ITIM domain (TIGIT) is a single-pass type I membrane receptor with an extracellular Ig-V-like domain and an ITIM domain in cytoplasmic tail. Its expression level on Treg and NK cells would upregulate after activation. It has a high-affinity ligand CD155 [poliovirus receptor (PVR)] present on dendritic cells and a low-affinity ligand CD112 (PVRL2) expressed on tumor and immune cells in tumor microenvironment (Yu et al. 2009). Following the binding with CD155, secretion of IL10 increases and secretion of IL12 decreases, and by promoting the generation of mature immunoregulatory dendritic cells, T-cell activation is suppressed (Yu et al. 2009). TIGHT and PD-1 co-over-express on tumor-infiltrating lymphocytes and co-blockade lead to enhanced CD8+ T-cell effector function, resulting in tumor clearance (Johnston et al. 2015).

20.3.2.4 TIM-3

T-cell immunoglobulin and mucin-domain containing-3 (Tim-3), also known as Hepatitis A virus cellular receptor 2 (HAVCR2), is a co-inhibitory receptor that is expressed on IFN-γ-producing T-cells, FoxP3+ Treg cells, and innate immune cells (macrophages and dendritic cells). It belongs to TIM family cell surface receptor proteins, containing an extracellular Ig-V-like domain and a cytoplasmic tail that interacts with many TCR complex. TIM-3 has many ligands such as Galectin-9, Ceacam1, HMGB1, and PtdSer (Anderson et al. 2016).

Accumulated evidences show that high-level TIM-3 is associated with T-cell dysfunction and could regulate T-cell exhaustion in tumor-infiltrating leukocytes (TILs) (Das et al. 2017; Anderson 2014). Blockade of TIM-3 would increase production of IL-2, IL-20 and TNF, IFN-c and restore the proliferation of T-cell, which is similar to PD-1 blockade. TIM-3 co-expression with PD-1 on CD8+ T-cells exhibits the most severe exhausted phenotype, and in this model, TIM-3 and PD-1 blockade combination results in higher efficiency and greater tumor regression.

20.3.2.5 LAG-3

LAG-3 (Lymphocyte activation gene 3 protein) is a LAG3-family protein on activated T-cells, NK cells. This inhibitory receptor consists of four extracellular Ig-like domains (one Ig-V-like and three Ig-C-like domains) and a cytoplasmic KIEELE motif for downstream signaling. Interaction of LAG-3 and major histocompatibility complex class II (MHC-II) which are selectively recognized by LAG-3 would negatively modulate T-cell functions. Fibrinogen-like protein1 (FGL1), thought to be a major functional ligand of LAG-3, is highly produced in cancer cells. It inhibits antigen-specific T-cell activation following binding to LAG-3 (Anderson et al. 2016). There are several anti-LAG-3 antibody drugs under clinical accessing. Relatlimab is one developed to treat melanoma, and now a reagent in phase II/III trails alone

or in combination with other immune checkpoint inhibitors such as nivolumab and ipilimumab.

20.3.2.6 VISTA

V-domain Ig suppressor of T-cell activation (VISTA) is both an immune regulatory receptor and ligand (Wang et al. 2011; Nowak et al. 2017). Just as its name, it inhibits T-cell proliferation and cytokine production. VISTA belongs to B7 family, sharing high similarity with PD-1 and CTLA-4 [thus bearing another name: Programmed death-1 homolog (PD-1H)], and its extracellular domain is homologous to PD-L1/L2. VISTA is constitutively expressed on several hematopoietic cell subsets. Upon expressing on antigen-presenting cells, it is likely to function as a co-inhibitory ligand to suppress T-cell responses. Meanwhile, VISTA also acts as a co-inhibitory receptor for CD4+ T-cells to suppress T-cell responses to antigen. In addition, VISTA highly exists on tumor-infiltrating lymphocytes. Its blockade results in increased activated dendritic cells in tumor microenvironment and its combination with the vaccine effectively arrests the growth of established tumors (Le Mercier et al. 2014). Significant suppression on tumor growth in tumor models renders VISTA a promising checkpoint, although its ligands/receptors are not identified yet.

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Chapter 21
Mechanisms Inspired Targeting Peptides

Yunsheng Yuan

Abstract Peptides, as a large group of molecules, are composed of amino acid residues and can be divided into linear or cyclic peptides according to the structure. Over 13,000 molecules of natural peptides have been found and many of them have been well studied. In artificial peptide libraries, the number of peptide diversity could be up to 1×10^{13}. Peptides have more complex structures and higher affinity to target proteins comparing with small molecular compounds. Recently, the development of targeting cancer immune checkpoint (CIP) inhibitors is having a very important role in tumor therapy. Peptides targeting ligands or receptors in CIP have been designed based on three-dimensional structures of target proteins or directly selected by random peptide libraries in biological display systems. Most of these targeting peptides work as inhibitors of protein–protein interaction and improve CD8+ cytotoxic T-lymphocyte (CTL) activation in the tumor microenvironment, for example, PKHB1, Ar5Y4 and TPP1. Peptides could be designed to regulate CIP protein degradation in vivo, such as PD-LYSO and PD-PALM. Besides its use in developing therapeutic drugs for targeting CIP, targeting peptides could be used in drug's targeted delivery and diagnosis in tumor immune therapy.

Keywords Peptides · Targeted protein degradation · Targeted delivery · Peptidic inhibitors · Random peptide libraries

21.1 Peptide Biogenesis and Function

Peptides are ubiquitously produced in different species and involved in several important biological processes of life, including inhibition of microorganisms, modulation of development and growth, or regulation of signal transduction (Sewald and Hans-Dieter 2009). It is well known that many peptides work as neurotransmitters in the

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cardiovascular, and central and peripheral nervous systems. For example, opioid peptides are neurotransmitters whose level in the body is associated with emotion control and a sense of pain in both the central and peripheral nervous systems. Recent studies have revealed that endogenous opioid peptides were also involved in myocardium repair in the heart and activation of immune system (Sinova et al. 2019; Liu et al. 2008). Unlike neurotransmitter peptides, antimicrobial peptides (AMPs) are one of the biggest groups of natural peptides, and over 2500 molecules have been identified (de la Fuente-Nunez et al. 2017). The AMPs database (DBAASP) has collected the information of more than 13,000 AMP monomers (Pirtskhalava et al. 2016; Gogoladze et al. 2014). Natural AMPs have different molecular weight and structure, but they share characteristics, such as many positive charge amino acid residues (Arg and Lys), making their molecules with a positive charge, and consisting of about 50% hydrophobic amino acids. AMPs are oldest components of the innate immune system during long-term biological evolution history, and they have been found in almost all tissues and organs of the human body (Lei et al. 2019). In fact, AMPs have more physiological functions besides host-defense against pathogens by killing microorganisms. AMPs also modulate the immune system to induce tumor cells apoptosis and death by stimulating the release of chemokines and activating immune cells in both innate and adaptive immune systems (de la Fuente-Nunez et al. 2017). The synthesis of peptides in the organisms can be divided into two biological processes. One biosynthesis pathway belongs to enzyme's dependence, e.g., glutathione (GSH) synthesis. The synthesis of GSH includes two ATP-requiring enzymatic steps. The first step is formation of γ-glutamylcysteine (γ-GC) from glutamate and cysteine, and this process is catalyzed by γ-glutamylcysteine synthetase (GCL). The second step is the formation of GSH from γ-GC and glycine and catalyzed by GSH synthetase (GS) (Fig. 21.1a) (Lu 2013). Another type of peptide biosynthesis depends on peptide precursor digestion by classical protein synthesis and maturation process. For example, opioid neuropeptide is coded with prodynorphin (PDYN) gene in the human (Noda et al. 1982; Horikawa et al. 1983). PDYN is translated to a pro-protein called as proenkephalin-B preproprotein which can produce six mature peptides by digestion. These peptides include opioid neuropeptide, β-neoendorphin, dynorphin, leumorphin, rimorphin, and leu-enkephalin (Fig. 21.1b) (Sukhov et al. 1995).

In general, peptides specifically bind to receptors or target molecules to work as agonists, antagonists, or signal peptides of proteins. Based on peptides' physiological and biochemical function and intrinsic properties, they could be selected as starting molecules to develop new drugs or targeting peptides which are parts of targeted drugs according to their usage in therapeutic development (Fosgerau and Hoffmann 2015). So far, more than 70 peptide drugs have been used to treat different disorders, such as GnRH and GLP-1 receptor agonists (Lau and Dunn 2018). Tumor therapy strategies are always hot points in academic institutions and the pharmaceutical industry, and studies of cancer targeting peptides focus on the tumor homing peptides targeting receptors over-expressed on the tumor cells surface. Cancer immune therapy received special attention and gets a great approach when inhibitors of PD1/PDL1 were approved by FDA to treat 14 types of cancer (Abril-Rodriguez and Ribas 2017). The peptides targeting immune checkpoints play a very important role in the field

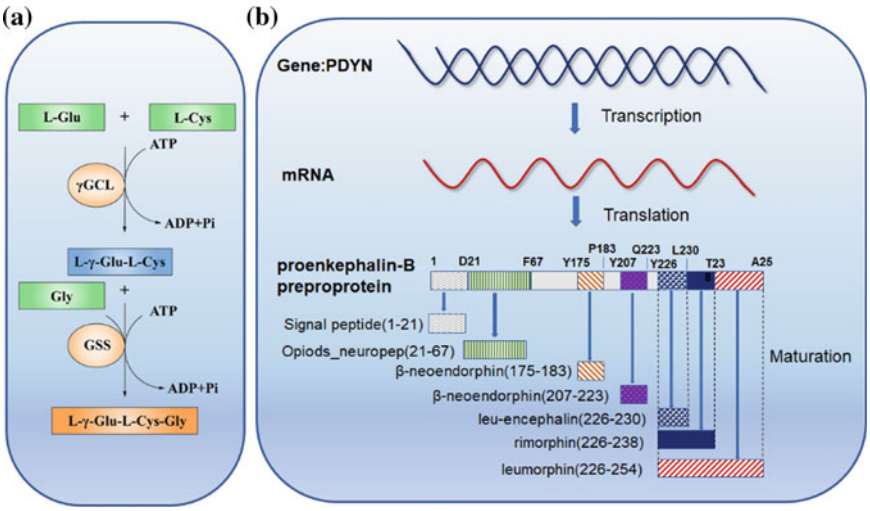

Fig. 21.1 Two processes of peptide biosynthesis in organisms. **a** Schematic diagram of GSH biosynthesis; **b** bioprocess of PDYN transcription, translation, and maturation

of cancer targeting peptides, for example, PD-1/PDL1 (Li et al. 2016). The targeting peptide has a higher specific affinity to target proteins than small molecules with lower molecular weight than bio-macromolecules, but most natural peptides cannot be directly developed into drugs due to their low affinity and short half-life in the body. Targeting peptide's maturation and evolution, and design or screening are necessary to study in new drug candidates.

21.2 Targeting Peptides Design

21.2.1 Targeting Peptides Design Based on the Protein Structure of Immune Checkpoints

Currently, more three-dimensional structures of protein have been modeled, and more than 140,000 structures of proteins had been deposed in the protein data bank (PDB, http://www.rcsb.org). Computational docking technologies based on protein structures have been broadly used to design new small compounds for new drug discovery. Although peptides differ from small molecules on both size and structure, this difference can be convenient to use in the development of protein–peptide docking methods (Fig. 21.2) (Ciemny et al. 2018). High-quality protein structure data from X-ray crystallography, NMR spectroscopy, and cryo-electron microscopy (cryo-EM) is very useful for molecular docking (Kruger et al. 2017). The novel targeting peptides could be directly designed based on the structure of the target protein

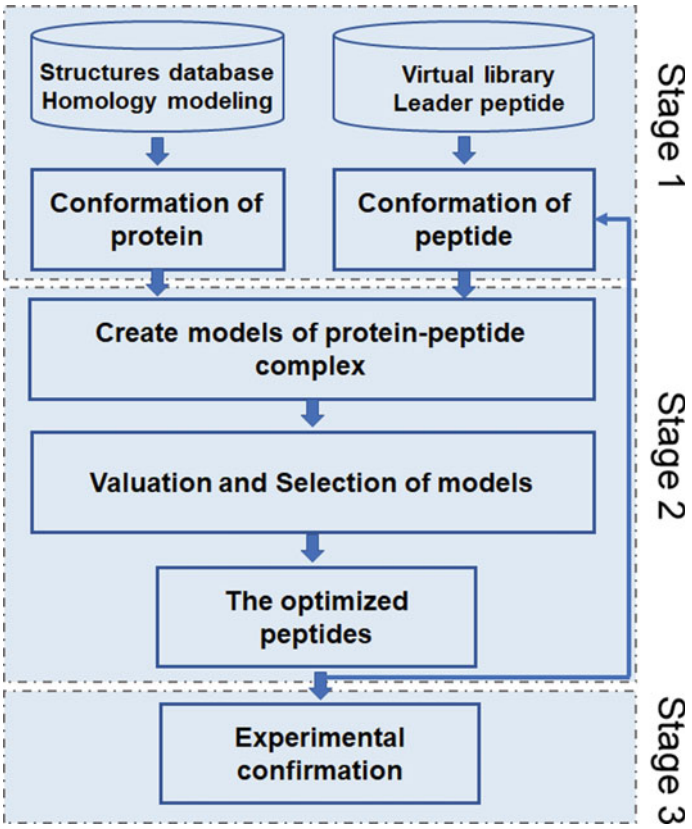

Fig. 21.2 Flowchart for targeting peptide design based on protein structures

when the experimental structural model of a protein with peptide ligand complexes is available (Ciemny et al. 2018). The peptide binding sites in this protein could be well defined. The protein structure without bound peptide ligands could also be used for molecular docking computation, but the flexibility of the target protein should be considered, especially if the ligand binding induces potential conformational changes in the protein. Sometimes, the structure of the target protein in humans is not available. A target protein homologous structure might be used for molecular docking after assessing the similarity of these proteins. If those proteins have a poor similarity of sequences (<40%), an initial model of the complex would be prepared. Accuracy of the resolution in the root means square deviation (RMSD) from the experimental structure influences the results of molecular docking (Park et al. 2018). FlexPepDoc (Alam et al. 2017), DynaDock (Antes 2010), and PepCrawler (Donsky and Wolfson 2011) are excellent tools in this field.

ICP ligands and receptors play a central role in tumor immune therapy and autoimmune diseases, so their complex with bound ligands or inhibitors has been modeled and deposed in PDB, for example, human PD-L1 (PDB code:3BIS, 5JDR)

(Lin et al. 2008), PD1/PD-L1 complex (PDB code:3BIK, 4ZQK) (Zak et al. 2015), CTLA4 (mouse, PDB code:1DQT) (Ostrov et al. 2000), CTLA4/B7-2 complex (PDB code:1I85) (Schwartz et al. 2001). Key pockets for molecular docking in the PD-L1 structure are A121, D122, Y123, Y56, and R113; these have been identified based on structure data of PD1/PD-L1 complex (Fig. 21.2). Several peptides targeted PD-L1 have been developed to block PD-L1 activity in the body (Magiera-Mularz et al. 2017; Li et al. 2018; Shindo et al. 2017). Linear peptides usually are used in protein–peptide docking as leader peptides which could be from experiment of peptide's library selection or computational design (Li et al. 2016). A scoring process should predict protein–peptide binding modes and their binding affinities. Several tools could be explored to score those models and evaluate targeting peptides to optimize peptide's affinity. Typical programs are X-score (Pencheva et al. 2010; Wang et al. 2002), Hotlig (Wang et al. 2013), and Chemscore (Eldridge et al. 1997).

21.2.2 Targeting Peptides Design Based on Protein Interaction in ICP

Protein–protein interactions (PPIs) have a crucial role in several cellular processes, and the range of human PPI is about 650,000 distinct pair-wise interactions (Bruzzoni-Giovanelli et al. 2018). Ligands binding to receptors on the surface of cells also belong to PPI. Therefore, the development of drugs targeting PPIs is considered an important field (Shin et al. 2017). Strategies for screening small molecules are hard to use when seeking inhibitors of PPIs because the PPI interface usually involves large, flat, and featureless surfaces ranging from 800 to 3000 A^2, which is bigger than protein pockets for small molecules. Based on the structure of the protein–protein complex, peptides derived from the domain of protein-binding epitopes in PPIs can be used as leader peptides for the design of PPI inhibitors (Pelay-Gimeno et al. 2015). The modified peptide sequences for improving biological functions are defined as peptidomimetics, which include all the artificial peptides that mimic the binding characteristics of natural peptide precursors. Several linear peptides targeting ICP proteins have been developed. For example, three peptides target carcinoembryonic antigen-related cell adhesion molecule 1(CEACAM1), a suppressor of active T cells, were identified as having an interaction site in CEACAM1/CEACAM1 (Skubitz and Skubitz 2011).

Cyclic peptides are often found in natural products, such as cyclosporine A and Gramicidin S (Gang et al. 2018). Cyclization of peptidomimetics can mimic secondary structure in proteins, the loop, or the turn structure of β-sheets, resulting in increased affinity for binding (Gang et al. 2018; Dougherty et al. 2017). On the other hand, cyclic peptides are more stable because the structure could resist proteases in the body. Over hundred macrocyclic peptides targeting PD1/PDL1 interaction were designed by Bristol-Myers Squibb (Shaabani et al. 2018). Peptide-57 and peptide-71 are two representative molecules, and half-maximal inhibitory concentration (IC50)

of two peptides is less than 10 nm (Magiera-Mularz et al. 2017). The structures of the complexes PD-L1/peptide-71 and PD-L1/peptide-57 in X-ray crystallography show that the cores of both peptides bind at the interface site of PD-L1, which consists of the PD1 binding site of PD-L1 (Magiera-Mularz et al. 2017). The binding surfaces of such peptides with PD-L1 partially overlap with epitopes of the anti-PD-L1 antibody. So the surfaces of PPIs or epitopes of antibody inhibitor-binding proteins could serve as templates for the design of new peptides targeting PPIs.

21.3 Targeting Peptides Selection Strategies

21.3.1 Biological Display System

The biological display system is used to discover novel targeting peptides, and it is one of the most efficient tools for peptide's high-throughput screening. The biological display system includes phage, bacteria, yeast, mammalian cells, Mrna, and ribosome (Liu et al. 2017). To select targeting peptides, the biological display system shares similar procedures, including library preparation, target protein or cell preparation, binding and elution, and enrichment and DNA sequencing (Fig. 21.3). The peptide library and structure of target protein are initial material for peptide screening, and the quality of the library is evaluated by peptide diversity and entities number. Structures of protein play an important role in the stage of peptides binding to proteins. A phage-display system is a typical tool in the biological display system, and it has been

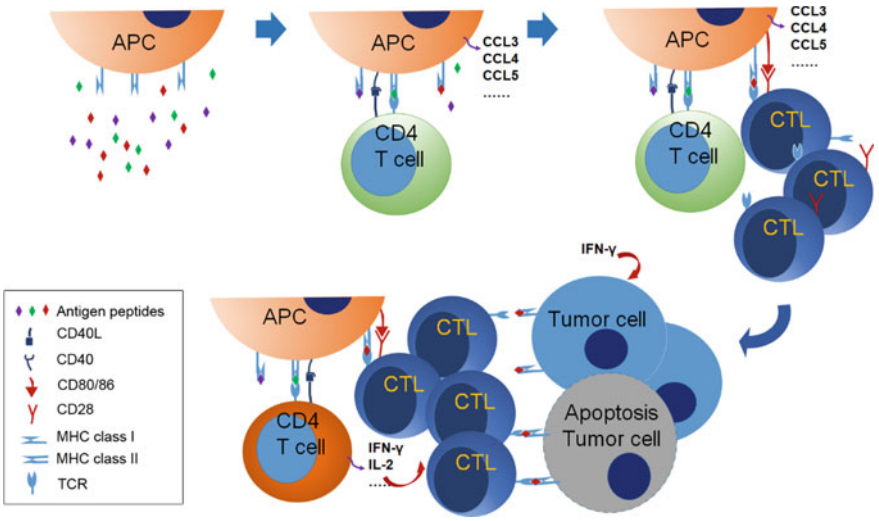

Fig. 21.3 The biological display system procedure

successfully used to targeting peptide selection or evolution (Saw and Song 2019). Several types of peptide libraries for the phage-display system, such as random peptide libraries, have been commercialized, and new investigators can easily get leader peptides after following the screening protocol. If someone is planning to create a peptide library for peptide evolution or optimization, they have to produce the library in their laboratory.

So far, several ICP peptide targeted proteins have been identified using a biological display system. For example, TPP1, a targeting peptide for PD-L1, was selected from a random peptide library with the bacteria display system. After eight rounds of screening, the consensus sequence "CWCWR" was identified. Then, the focused library with the format X5CWCWRX5 was constructed and used for the next 13 cycles screening for improving peptide's affinity binding to PD-L1. Finally, 20 peptide candidates were obtained, and affinity evaluation and specificity of targeting peptides were performed with in vitro or in vivo methods. The KD value of the binding between TPP-1 and PD-L1 was 9.467×10^{-8} mol/L (Li et al. 2018).

21.3.2 Synthesis Peptide Library and Peptide Microarray

Peptides in the biological display system usually are limited by the system biosynthesis. Although the phage-display system gets some approaches by molecular biotechnology methods and could display cyclic peptides on the surfaces of phages, peptides in the libraries are only composed of natural L-amino acids (Liu et al. 2019). Unlike the biological display system, synthesis peptide libraries with chemical methods could offer more possibilities. For example, unnatural amino acids could be involved in peptide synthesis in the libraries, and the types of libraries included are linear peptides, branch peptides, cyclic peptides, or macrocyclic peptides (Liu et al. 2017). Peptides in the synthesis peptide library usually were conjugated with different scaffold materials, such as beads or solid surfaces. Currently, at least three types of synthesis libraries, including one-bead one-compound (OBOC) peptide library, peptide microarray, and PNA-encoded peptide library have been developed and used to select targeting peptide. The design of the OBOC peptide library is very important for screening efficiency. In contrast with phage-display peptide library, the OBOC library is synthesized on solid phase and contains several types of structures of peptides, such as a linear peptide, cyclic peptide, branched peptide, or glycopeptide (Lam et al. 2003). The screening process is similar to bacterial display. Positive beads could be collected by sorting, and chemical decoding of targeting peptides is performed with mass spectrometry (MS) (Paulick et al. 2006) or Edman microsequencing if tag consists of α-amino acids (Liu and Lam 2001). Several integrin-targeting peptide receptors [e.g., PB (Tang et al. 2019), LXW64 (Wang et al. 2016), pM2 (Mikawa et al. 2004)] have been screened out from OBOC libraries.

Peptide microarray development started 20 years ago, and it has been commercialized for the past decade. The concept of peptide microarray is the same as the DNA array. Although different strategies reported could be used to prepare the peptide

microarray, in situ synthesis of peptide and in situ immobilization are widely used in peptide microarray preparation (Szymczak et al. 2018). After peptide synthesis, the peptides are printed on the surface of the solid chip and immobilized by physical adsorption, chemical reaction, or biological interaction. This method could produce low-density arrays with 300 spots on 10 mm × 10 mm slides. The density of peptides spots on the chip made with the in situ synthesis approaches is up to 9000 spots on standard 75.4 mm × 25 mm slides (Szymczak et al. 2018). Comparing with other peptide libraries, the peptide microarray only has a small number of peptides for use during screening. Therefore, this technology could be suitable for rapid ligand optimization (Liu et al. 2017).

21.4 ICP Regulated by Targeting Peptide Modulation of Tumor Microenvironments

21.4.1 Peptides Targeting Tumor-Associated Macrophages

Solid tumors contain tumor cells and noncancerous cells including fibroblasts, endothelial cells, innate, and adaptive immune cells. Noncancerous cells and matrix components in the tumor are called tumor environment (TME). TME is involved in the tumor progress, metastasis, and efficacy of therapy. It is well known that inflammatory cells and macrophages are recruited in solid tumors, and these cells produce many cytokines and chemokines to modulate the tumor environment. Macrophages derived from monocytes could be stimulated to M1-polarized or M2-polarized macrophage in the tissue. M1 macrophages are classical activated macrophages, and M1 macrophages can directly kill and clear tumor cells via NO and phagocytosis, respectively. M1 macrophages also recruit T cells, neutrophil, and express proinflammatory cytokines such as TNF-α, IL-1β, and NOS2. Unlike M1 macrophages, M2 macrophages reside in tissues, regulate immune homeostasis in normal tissues, and help to repair injured tissues. Macrophages in the tumor are called tumor-associated macrophages (TAMs) whose functions are to secrete PD-L1, IL-1RA, IL-10, and TGFβ, and these cytokines directly suppress CD8+ cytotoxic T-lymphocyte (CTL) activation.

Accumulated evidences have shown that the number of TAMs in the solid tumors correlates with the stage of tumor and poor prognoses in several tumors, such as breast cancer (Zhang et al. 2018; Tiainen et al. 2015), pancreatic cancer (Atanasov et al. 2018), head and neck cancer (Hu et al. 2016), and glioma (Hambardzumyan et al. 2016). The peptides targeting TAM are very useful in the development of targeting TAM drugs or delivery tools. For example, the peptides targeting CD47 have been identified. The CD47/ signal regulatory protein-α(SIRPα) signaling axis works as an innate immune checkpoint in tumors. CD47 is broadly expressed on the membrane of all cell types and interacts with SIRPα, a receptor on the macrophage, to inhibit phagocytosis activation (Matlung et al. 2017). The targeting peptide, which was

named PKHB1, could target CD47 and induce chronic lymphocytic leukemia (CLL) cell apoptosis by CD47 signal stimulation and upregulating tyrosine-783 phosphorylation at phospholipase C gamma-1 (PLCγ1) (Martinez-Torres et al. 2015). Another peptide, C5D5.1, is designed to block CD47/ SIRPα interaction and presented significant inhibition of neutrophil transmigration; however, the function of this peptide in regulating TAM is unclear (Liu et al. 2004). Peptides binding to receptors of TAM could also be used as targeting peptides for drug precision delivery. UNO and hBD3, two peptides targeting M2 macrophages, can specifically bind to CD206 and CCR2 on macrophages, respectively (Scodeller et al. 2017; Jin et al. 2010). M2pep is a typical peptide targeting TAM (Cieslewicz et al. 2013), and M2pep-coated nanoparticles can accurately reach M2 macrophages and control TAM survival in the tumor xenografts (Pang et al. 2019).

21.4.2 Peptides Targeting Tumor Invasion CD4+ T Cells

CD4+ T lymphocytes develop in the thymus and can be classified into different functional subsets based on functions. Among them, CD4+ T-helper1 (Th1) and CD4+ T-helper2 (Th2) lymphocyte subsets have a very important role in regulating anti-tumor response through secreting cytokines to induce tumor apoptosis, activating CTL response, recruiting NK cells, and macrophages (Kennedy and Celis 2008). The anti-tumor peptide vaccine is a new strategy to upregulate CTL response and kill tumor cells. CD4+ T cells can recognize the antigen peptides from antigen-presenting cells (APCs), which include macrophage and dendritic cell; therefore, CD4+ T cells produce cytokines to stimulate CTL proliferation and activation (Yarchoan et al. 2017). In a mouse melanoma model, several peptides targeting CD4+ T cells can significantly bind to T cell receptors on the surface of CD4+ T cells and stimulate CD4+ T cells anti-tumor activity. For example, tyrosinase-related protein 1 (Trp1) peptide (Trp1113-127) can improve IFN-γ production and co-stimulates tumor-infiltrating T cells (TIL) reactive with OX40/CD40 (Kumai et al. 2017). Thus, the mechanism of peptides targeting CD4+ T cells, peptide vaccine contains self-activation, immune cell recruitment, stimulation, and anti-tumor cytokines production (Melssen and Slingluff 2017; Calvo Tardon et al. 2019) (Fig. 21.4).

21.5 Peptides Targeting and Regulating ICP Pathways

21.5.1 Peptides Targeting PD1/PDL1 Pathway

PD1/PDL1 signal pathway plays a critical role in the regulation of peripheral tolerance by limiting the self-reactive T cell proliferation and cytotoxic function in the tissue (Salmaninejad et al. 2019). Overexpression of PDL1 in tumor cells is involved

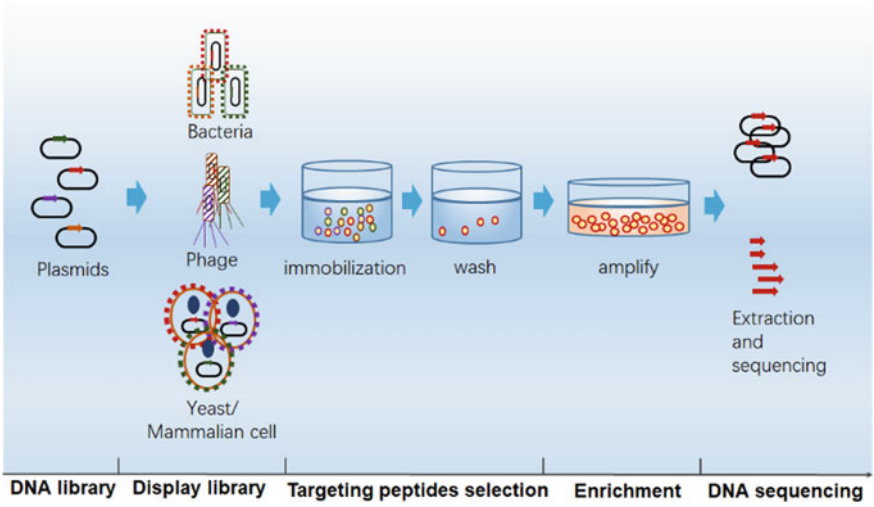

Fig. 21.4 Tumor antigen peptide targets CD4+ T cells for recruiting and activating CTL cells in the tumor microenvironment. Antigen peptides are presented on MHC class II in antigen-presenting cells (APCs) and recognized by the T cell receptor (TCR) on the surface of CD4+ T cells. Interaction of antigen-TCR stimulates CD4+ T cells to produce cytokines, and CD40L-CD40 interaction upregulates APC expressing CD80, CD86, and MHC class I. Interaction of APCs and CD4+ T cells is necessary to recruit CTL and co-stimulate CTL activation in the tumor microenvironment. CD4+ T cells also improve CTL proliferation by IL-2 and recognition of tumor cells via IFN-γ

in the tumor cell immunologic escape and invasion. PD1/PDL1 inhibitors are successfully developed and used for treating various types of tumors, such as non-small cell lung cancer (NSCLC), melanoma, renal cell carcinoma, urothelial carcinoma, colorectal cancer, cervical cancer, hepatocellular carcinoma, and Hodgkin lymphoma (Shergold et al. 2019). Currently, five monoclonal antibodies have been approved by the FDA, two anti-PD1 antibodies (pembrolizumab and nivolumab), and three anti-PDL1 antibodies (atezolizumab, avelumab, and durvalumab) (Abril-Rodriguez and Ribas 2017). PD1/PDL1 interaction is a good target for designing or screening targeting peptides. Peptide inhibitors can overcome some of the issues with antibodies, including high production costs, potential immunogenicity, and limitation of tumor penetration (Araste et al. 2018). Various peptides targeting PDL1 have been reported (Fig. 21.5). Ar5Y4 (Li et al. 2016) and TPP1 (Li et al. 2018), two linear peptides, can block PD1/PDL1 interaction in vitro or in vivo, and TPP1 shows high efficacy of anti-tumor in mice model. Bristol-Myers Squibb has reported over hundreds of cyclic peptides, which have good efficiency in inhibiting PD1/PDL1 by binding to PDL1 (Magiera-Mularz et al. 2017; Shaabani et al. 2018).

Unlike those peptides disturbing interaction between PDL1 and PD1, a new strategy is to consider the stability of PDL1 in the tumor. PD-LYSO peptide was designed based on Huntingtin-interacting protein 1–related (HIP1R) interaction with PDL1 and regulation of PDL1 degradation. PD-LYSO peptide contains HIP1R/PDL1

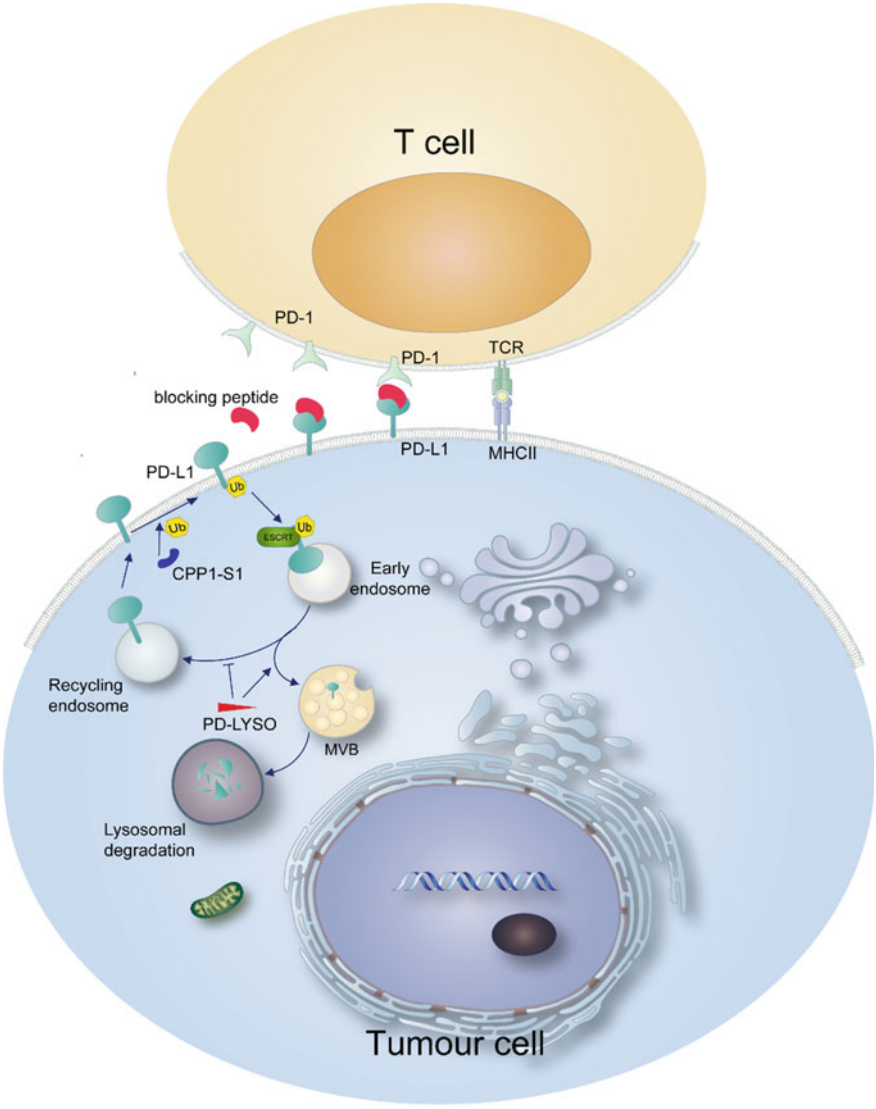

Fig. 21.5 Mechanism of peptides targeting PD1/PDL1 pathway

interaction sequence ($HIP1R_{784\text{-}807}$) and HIP1R lysosomal sorting signal sequence ($HIP1R_{966\text{-}979}$). It can efficiently improve PDL1 degradation in the cytosol of tumor cells by blocking the PDL1 recycling pathway in the endosome (Figure) (Wang et al. 2019), because palmitoylation of PDL1 significantly influences PDL1 ubiquitination and inhibits PDL1 degradation in the tumor. Targeting PDL1 palmitoylation peptide (called PD-PALM) can inhibit the PDL1 palmitoylation process by disturbing in

DHHC3 acetyltransferase recognizing PDL1. It can change the level of PDL1 palmitoylation and markedly improve PDL1 ubiquitination and degradation in tumor cells (Fig. 21.5). PD-PALM peptide presents good efficiencies of anti-tumor both in vitro and in vivo experiments. These studies give us a new perspective to extend strategy in the design of targeting immune checkpoint peptides.

21.5.2 Peptides Targeting CTLA4 Pathway

Cytotoxic T-lymphocyte antigen-4(CTLA-4) is a critical negative regulator of T cell activation and is also a receptor of the immune checkpoint. Unlike PD1, CTLA-4 and CD28 share two ligands, CD80 and CD86, which were expressed on the surface of APCs (Rowshanravan et al. 2018). CD80 is a dominant ligand to CD28, and CD28/CD80 interaction stimulates T cell's activation in conjunction with TCR signal. In contrast, CTLA-4 has a higher affinity with CD80/86 binding receptors, and both ligands tend to bind to CTLA-4 and suppress T cell's proliferation (O'Day et al. 2007). So far, two anti-CTLA-4 antibodies (called as ipilimumab and tremelimumab) have been developed and evaluated in several advanced stage cancer trials (O'Day et al. 2007). CD80-CAP, targeting the peptide of CTLA-4 pathway, has been designed based on the sequence of the interface in the CD80/CTLA-4 complex. It can bind to CTLA-4, inhibit T cell activation, and attenuate collagen-induced arthritis (CIA) in a mouse model (Srinivasan et al. 2005). A recent study has found that LTX315, an anti-tumor peptide, can reprogram TME, downregulate immunosuppressive Tregs, and increase anti-tumor Th1 and CTLA-4 level. Although LTX315 is not directly targeting the CTLA-4 signal pathway, it can improve tumor sensitive to immunotherapy with CTLA-4 inhibitor (Yamazaki et al. 2016).

21.6 Conclusion

Since the FDA has approved the Inhibitors of PD1/PDL1, ICP pathways have become a group of critical targets in the tumor therapy. Biologists and researchers have tried to find new ICP receptors or ICP regulators. Several new ICP pathways were reported, for example, fibrinogen-like protein 1(FGL1)/LAG3 (Wang et al. 2019), osteopontin/CD44 (Klement et al. 2018), NKG2A/HLA-E (Andre et al. 2018; van Montfoort et al. 2018; Haanen and Cerundolo 2018), and P-selectin glycoprotein ligand-1(PSGL-1) (Tinoco et al. 2016, 2017). Most targeting ICP peptide studies are in the preclinical stage besides LTX315, which is now in the stage of clinic trails. ICP targeting peptide development is limited by peptide design and leader peptide structures, which are usually linear peptides or simple second structures. AMPs might be a good resource in leader peptides because AMPs belong to an ancient and diverse group of molecules. It is also a part of the innate immune system and regulator of the adaptive immune system in humans. Unlike the random peptide library, AMPs

library has only hundreds of entities and cannot satisfy the requirements of high-throughput screening in ICP targeting peptide selection. Integration of multiple bio-information technologies, e.g., artificial intelligence (Wang et al. 2019) and virtual screening (Duffy et al. 2015), might overcome the limitation of AMPs entities' library. Because immune checkpoints are composed of a set of cost stimulatory and inhibitory factors, immune checkpoint targeting peptides can exert more functions in combination treatment or diagnosis of in vivo images in tumor immunotherapy in the future.

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Chapter 22
Small Molecular Immune Modulators as Anticancer Agents

Yongxin Han, Li Zhu, Wei Wu, Hui Zhang, Wei Hu, Liguang Dai and Yanqing Yang

Abstract After decades of intense effort, immune checkpoint inhibitors have been conclusively demonstrated to be effective in cancer treatments and thus are revolutionizing the concepts in the treatment of cancers. Immuno-oncology has arrived and will play a key role in cancer treatment in the foreseeable future. However, efforts to find novel methods to improve the immune response to cancer have not ceased. Small-molecule approaches offer inherent advantages over biologic immunotherapies since they can cross cell membranes, penetrate into tumor tissue and tumor microenvironment more easily, and are amenable to be finely controlled than biological agents, which may help reduce immune-related adverse events seen with biologic therapies and provide more flexibility for the combination use with other therapies and superior clinical benefit. On the one hand, small-molecule therapies can modulate the immune response to cancer by restoring the antitumor immunity, promoting more effective cytotoxic lymphocyte responses, and regulating tumor microenvironment, either directly or epigenetically. On the other hand, the combination of different mechanisms of small molecules with antibodies and other biologics demonstrated admirable synergistic effect in clinical settings for cancer treatment and may expand antibodies' usefulness for broader clinical applications. This chapter provides an overview of small-molecule immunotherapeutic approaches either as monotherapy or in combination for the treatment of cancer.

Keywords Small molecules · Cytotoxic lymphocyte responses · Drug screening · Cancer immunotherapy · Combination therapy

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22.1 Introduction

The successful development and the durable anticancer efficacy of immune checkpoint inhibitors, such as anti-PD-1/PD-L1 antibodies, are revolutionizing the concepts in the treatment of cancers. The success of these biologics in the clinic, the autoimmune side effects, and their intrinsic limitations have inspired much effort for the discovery and development of small molecules that act on extracellular and intracellular targets affecting immune-modulatory pathways in cancers. Compared with biologics, small molecules may offer the convenience of oral administration, the ability to cross cell membrane and physiological barriers such as the blood–brain barrier (BBB), and access tumor/tumor microenvironment with greater exposure. Small molecules' bioavailability is more amenable for fine control, which may enable them to avoid some of the immune-associated adverse events associated with long-lasting antibodies and cell-based biologic therapies. In addition, the combination of different mechanisms of small molecules with antibodies and other biologics demonstrated admirable synergistic effect in clinical settings for cancer treatment and may expand antibodies' usefulness for broader clinical applications. This chapter provides an overview of small-molecule immunotherapeutic approaches either as monotherapy or in combination for the treatment of cancer.

22.2 Small-Molecule Immune Checkpoint Inhibitors

22.2.1 PD-1/PD-L1 Inhibitors

PD-1/PD-L1 signaling pathway is currently one of the most actively explored fields in cancer treatment. PD-L1 is mainly expressed in cancer cells and PD-1 is mainly expressed on T-cells. Cancer cells escape immune attack through the interaction of PD-L1 with PD-1 receptor on T-cells. The interaction of PD-L1 and PD-1 activates the PD-L1/PD-1 pathway and subsequently inhibited T-cell's killing ability against cancer cells and resulted in immune escape (Sun et al. 2018).

Antibodies targeting PD-1 and PD-L1, such as nivolumab, pembrolizumab, and atezolizumab have provided unprecedented clinical benefit in several types of cancers, including melanoma, non-small cell lung cancer (NSCLC), renal cell carcinoma (RCC), Hodgkin's lymphoma, and microsatellite instable-high (MSI-H) or mismatch repair-deficient (dMMR) solid tumors. Due to the innate and acquired resistance caused by multiple regulation mechanisms in tumor microenvironment, such as TAM, and IDO, only a small portion of patients can benefit from anti-PD-1 and anti-PD-L1 antibodies (Poggio et al. 2019). It has been recently noted that tumor cell-intrinsic PD-L1 clearly contributes to cancer stemness, EMT, tumor invasion, and chemoresistance in multiple tumor types (Dong et al. 2018; Escors et al. 2018; Chen et al. 2016). Antibodies can only have effect on the tumor cells with membrane surface PD-L1. The tumor cells having low or only cytoplasma PD-L1 tend to resist

and show limited effect against PD-1/PD-L1 antibodies. PD-1/PD-L1 antibodies have long half-life and special care is needed once immune-related adverse events (irAEs) occur (Hwang et al. 2016; Naidoo et al. 2015). The limitation to plasma PD-L1, irAEs, high manufacturing cost, and potential immunogenicity made it appealing to explore small-molecule PD-L1 inhibitors. The success of PD-1/PD-L1 antibodies and the advantage of small molecule in cell penetration, oral administration, and amenable dose tuning have inspired the discovery and development of small-molecule PD-L1 inhibitors, and a number of small molecules have been developed at different stages of preclinical and clinical studies (Table 22.1).

The reported immunomodulators targeting the PD-1/PD-L1 signaling pathway before 2015 mainly focused on benzenesulfonamide derivatives (compound **1, 2, 3** in Fig. 22.1), peptidomimetics and macrocyclic peptides (see Fig. 22.1 for representative structures) (Weinmann 2016; Zarganes-Tzitzikas et al. 2016).

CA-170, a small-molecule blocking PD-1/PD-L1 interaction is the first orally bioavailable small-molecule immune checkpoint inhibitor entering into clinical study (WO2015033301A1 2015; WO2015033299A1 2015). This molecule, reported as a

Table 22.1 Small-molecule PD-L1 inhibitors in preclinical and clinical development

Drug	Drug developer	Development status
CA-170	Aurigene/Curis	Phase II: lymphoma, solid tumors (India) Phase I: advanced solid tumors or lymphomas (USA, Korea, Spain, UK) NCT02812875
PD-1/PD-L1 small molecule	BMS	Preclinical, USA
INCB86550	Incyte	Phase I: solid tumor (USA), NCT03762447
CCX4503	ChemoCentryx, Inc.	Preclinical, USA
MAX-101219	Guangzhou MaxiNovel Pharma	Preclinical, China
JBI-426	Jubilant Biosys	Preclinical, India
PD-1/PD-L1 small molecule	Guangzhou Wellhealth BioPharmaceutical	Preclinical, China
IMM-H008	Adlai Nortye/Institute of Materia Medica of Beijing—CAMS&PUMC	Preclinical, China
IMM-H010	Tianjin Chase Sun Pharm/Institute of Materia Medica of Beijing—CAMS&PUMC	Preclinical, China
CS-17938	Chipscreen biosciences	Preclinical, China
PD-1/PD-L1 small molecule	Arising International LLC	Preclinical, USA
PD-1/PD-L1 small molecule	Polaris Pharmaceutical Inc	Preclinical, USA

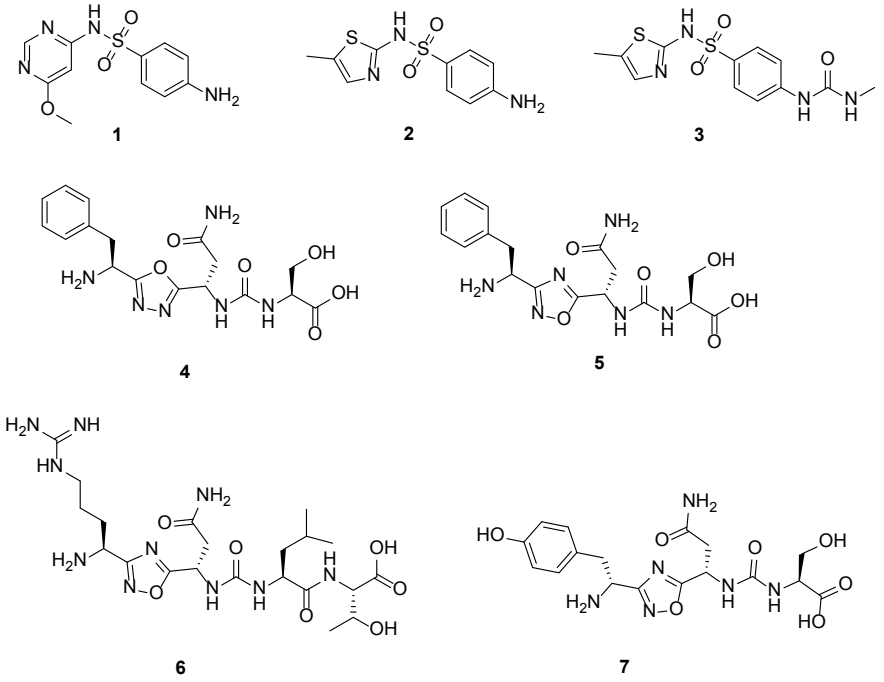


Fig. 22.1 Representative structures of benzenesulfonamide and peptidomimetics PD-1/PD-L1 inhibitors

dual VISTA and PD-L1 antagonist, was discovered by Aurigene and co-developed with Curis, and is currently in a phase II study for treatment of lymphoma and solid tumors in India [CTRL/2017/12/01102]. The structure of CA-170 is not disclosed yet. Phase I study showed that CA-170 is well tolerated with the maximum tolerated dose (MTD) at 1200 mg BID (NCT02812875) (Bang et al. 2018). The serious irAEs, reported with antibody ICIs which are intractable due to long half-life and strong target occupancy of mAbs in the sustained target inhibition (Naidoo et al. 2015; Sasikumar et al. 2013), are milder and reversible with CA-170 treatment. This is possibly due to relatively quicker drug elimination after dose interruption. CA-170 demonstrated an overall clinical benefit rate (CBR) at 59.5% in the phase II study, and the overall clinical response rate for non-small cell lung cancer (NSCLC) and Hodgkin's lymphoma (HL) patients reached 70% and 77.8%, respectively (Radhakrishnan et al. 2018). One HL patient's tumor decreased 57% after 60 days at 400 mg/day dose, and another head and neck cancer patient's tumor decreased by 48% after 30 consecutive days of administration at 400 mg/d dosage. The CBR rate at 400 mg/day dosage is comparable to PD-1/PD-L1 antibodies (Carretero-Gonzalez et al. 2018). Even though higher proportion of patients developed irAEs at 400 mg dosage, the immune-related hematological events either as fatal or prolonged duration as reported with antibodies (Wright and Brown 2017; Atwal et al. 2017) have

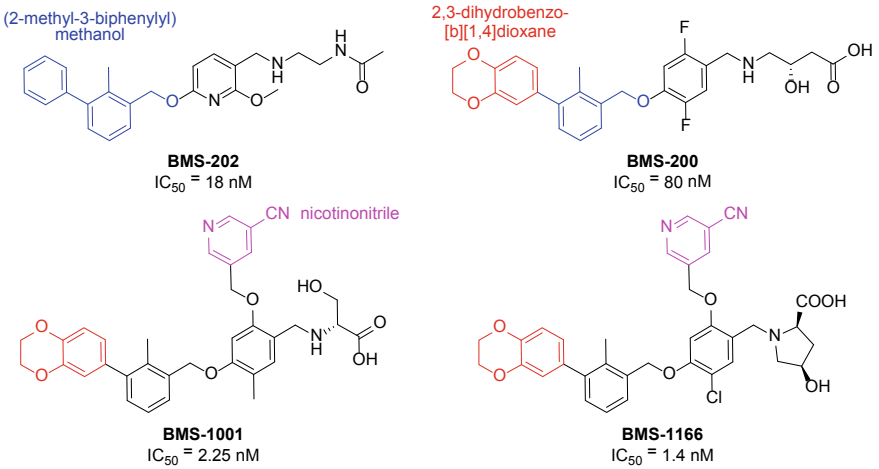


Fig. 22.2 Structures of BMS small-molecule PD-1/PD-L1 inhibitors (Chupak and Zheng 2015; BMS patents)

been reversible and of shorter duration. Recently, Jacek Plewka and Tad A. Holak (Musielak et al. 2019) demonstrated through NMR binding assay, HTRF, and cell-based activation assays that there is no direct binding between CA-170 and PD-L1. This implies that CA-170 may act through other T-cell activating pathway.

The first non-peptide inhibitor targeting PD-1/PD-L1 interaction was discovered by Bristol-Myers Squibb (BMS) and was based on (2-methyl-3-biphenylyl)methanol scaffold (Fig. 22.2) (Chupak and Zheng 2015; Abdel-Magid 2015). One year after the release of BMS's patent (Chupak and Zheng 2015), Tad A. Holak's group at Jagiellonian University elucidated the binding mode of this class of molecules using ^{15}N-labeled PD-L1 and PD-1 and the "SAR-by-NMR" approach and AIDA assay (Zak et al. 2016). This study showed that BMS's compounds bind to PD-L1 and dissociate the human PD-1/PD-L1 complex.

Furthermore, by evaluating the relative affinity of BMS-202 toward PD-L1 and PD-L2 using differential scanning fluorimetry (DSF), they found that both compounds bind specifically only to PD-L1, but not PD-L2. The co-crystal structure of BMS-202/PD-L1 complex illustrated that two PD-L1 dimers form an asymmetric unit with one BMS-202 molecule located at the interface of each dimer. BMS-202 inserts deeply into a cylindrical, hydrophobic pocket formed at the interface of the PD-L1 dimer. It was further illustrated that BMS-202 induced hPD-L1 dimerization in solution. Tad A. Holak's research elucidated the molecular mechanism of PD-L1 dimerization and how PD-L1 inhibitor blocked PD-L1/PD-1 interaction, which is very valuable for the rational design of PD-L1 inhibitors. Between March 2015 and August 2019, BMS has successively filed six PD-L1 inhibitor patents (Chupak and Zheng 2015; BMS patents). Using the 2,3-dihydrobenzo-[*b*][1,4]dioxane class of compounds, BMS-200, BMS-1001, and BMS-1166 (Fig. 22.2), Tad A. Holak's group solved the co-crystal structure and elucidated the binding mode of such compounds

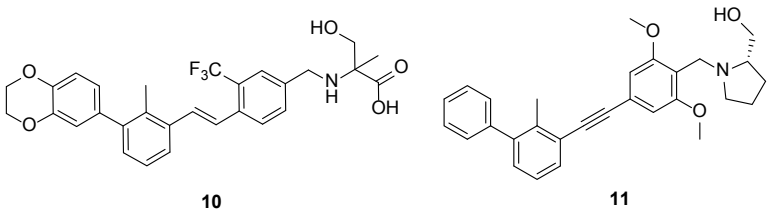


Fig. 22.3 MaxiNovel's representative compounds

with PD-L1 (Guzik et al. 2017; Skalniak et al. 2017). Two optimized compounds, BMS-1001 and BMS-1166, showed improved PD-1/PD-L1 inhibition potency at IC_{50} 2.25 nM and 1.4 nM, respectively, by HTRF binding assay and demonstrated low toxicity toward Jurkat T-cells with EC_{50} values at 33.4 and 40.5 μM (Skalniak et al. 2017). The nicotinonitrile group induced the formation of a sub-pocket comprised of $_{B}$Arg113, $_{B}$Tyr123, $_{B}$Arg125, and $_{A}$Asp61, and resulted in the improved potency (Guzik et al. 2019).

Guangzhou MaxiNovel Pharmaceuticals applied for the patents of a series of PD-1/PD-L1 inhibition molecules based on BMS's methyl biphenyl scaffold with the replacement of benzyl ether moiety using ethenyl or ethynyl linkage (Fig. 22.3) (Guangzhou MaxiNovel patents). MaxiNovel-27 exhibited improved oral bioavailability in mice at 52.3%. As reported in 2019 AACR meeting, MaxiNovel's oral PD-L1 inhibitors demonstrated comparable tumor growth inhibition as durvalumab, however, exhibited higher $CD8^{+}$/Treg ratio than durvalumab ($p < 0.01$) in the MC38 model (Maxinovel's orally active PD-l1 inhibitor demonstrating similar efficacy to PD-L1 antibody durvalumab).

A number of other companies reported their small-molecule PD-L1 programs based on biphenyl pharmacophore but without much information (see Fig. 22.4 for representative structures and patent information). Incyte Corporation has also reported a dozen of patents based on BMS's biaryl moiety since 2017 (Incyte patents) and launched a phase I clinical study using INCB86550 for treatment of solid tumors (NCT03762447). Based on the similar pharmacophore, Feng Zhiqiang et al. reported a series of compounds with the introduction of a bromine group into the methyl biphenyl scaffold (Feng Zhiqiang patents). Two compounds, IMM-H010 and IMM-H008, were licensed to Tianjin Chase Sun Pharma and Adlai Nortye, respectively, for further development. ChemoCentryx discovered that 4-phenyl-2,3-dihydro-1*H*-inden-1-ol derivatives are potent PD-L1 inhibitors that can block the PD-1/PD-L1 interaction (ChemoCentryx patents). The leading compound, CCX4503, exhibited comparable in vivo antitumor efficacy to PD-L1 antibodies in an animal model study (Vilalta Colomer et al. 2018). Gong Ping from Shenyang Pharmaceutical University disclosed a series of [1,2,4]triazolo[4,3-*a*]pyridines-based PD-L1 inhibitors, with SYPHU-A22 exhibiting 92.3 nM of IC_{50} using HTRF binding assay (Qin et al. 2019). Dömling A. from the University of Groningen in the Netherlands reported a few novel scaffolds as PD-1/PD-L1 axis inhibitors with the potency in the range 0.001–1000 μM (IC_{50}) by NMR binding assay (Dömling A. patents). Guangzhou

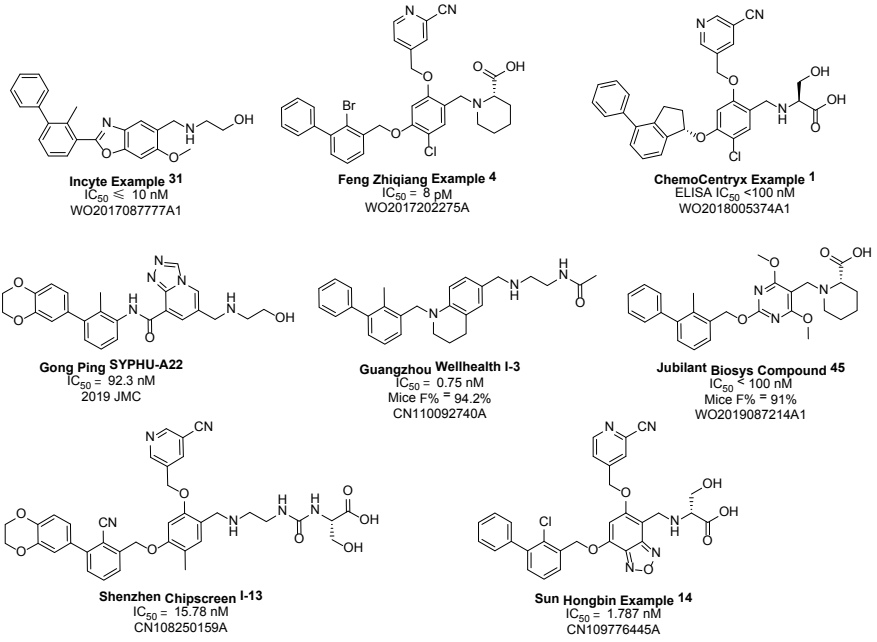


Fig. 22.4 Representative structures from Incyte, Feng Zhiqiang, and ChemoCentryx etc.

Wellhealth BioPharmaceutical filed a series of patents in 2019 and some compounds demonstrated much improved oral bioavailability up to 94.2% in mice (Guangzhou Wellhealth BioPharmaceutical patents). Shenzhen Chipscreen Biosciences (Shenzhen Chipscreen Biosciences patent), Jubilant Biosys (Jubilant Biosys patent), and Sun H.B. from China Pharmaceutical University (Sun Hongbin patent) are also developing small-molecule PD-1/PD-L1 inhibitors and they are still at early discovery stage without much information available at the moment. Shenzhen Chipscreen Biosciences' leading candidate CS-17938 and Jubilant Biosys' candidate compound JBI-426 are currently under preclinical studies.

More recently, it was reported that a new class of partially or fully symmetric compounds that can induce the formation of more symmetrically arranged PD-L1 homodimer and thus demonstrate better PD-1/PD-L1 inhibition potency. Tad A. Holak, Japan Tobacco Inc., Arising International LLC, Polaris Pharmaceutical Inc., Incyte Corporation, and Gilead Sciences Inc. are the front runners with this strategy and their representative structures are illustrated in Fig. 22.5 (BMS patents; Incyte patents; Basu et al. 2019; Kawashita et al. 2019; Arising International LLC patent; Polaris Pharmaceutical Inc.; Gilead patents).

PD-1/PD-L1 antibodies have revolutionized the conventional approaches cancers were treated, but the limited response rate, immune-related adverse events, and high cost made small-molecule PD-1/PD-L1 inhibition as an appealing alternative

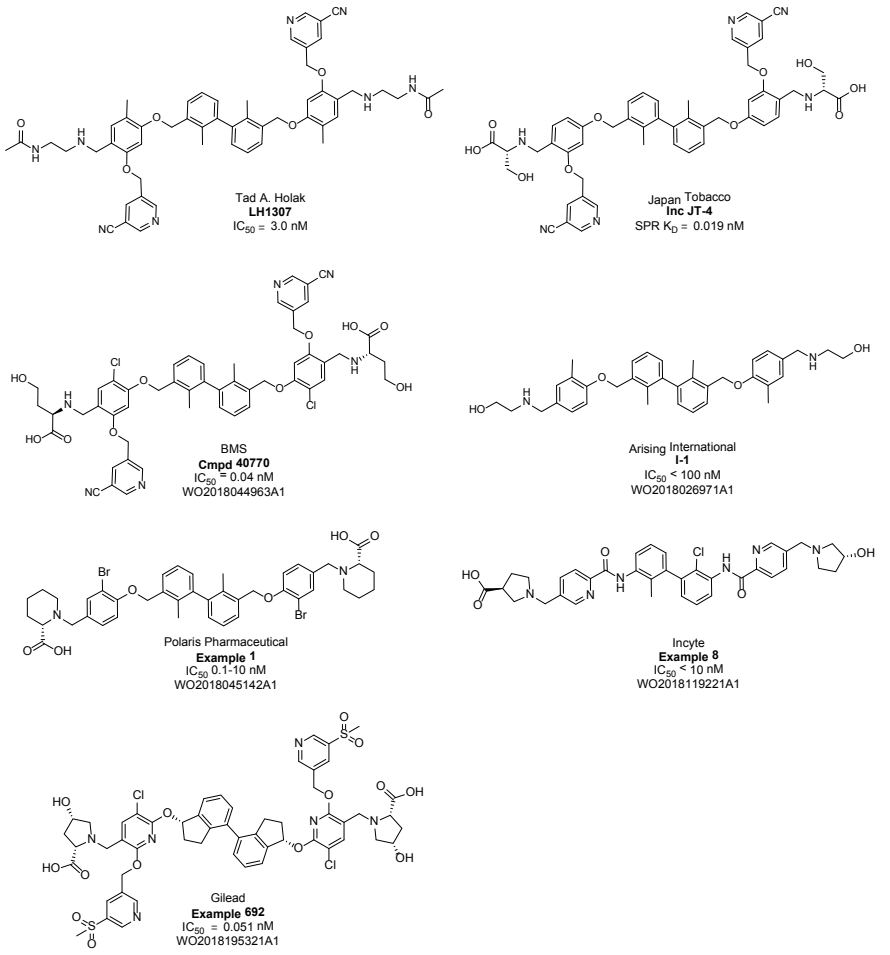


Fig. 22.5 Selected symmetric small-molecule PD-1/PD-L1 inhibitors

for cancer immunotherapy. Even though the development of small-molecule PD-L1 inhibitors is still at early stage, it is believed that its cell and tissue penetration capability, amenable dose adjustment, and low cost could bring additional clinical benefit, not just as an option to PD-1/PD-L1 antibodies.

22.2.2 Adenosine Pathway (A2A, A2B, CD39, CD73)

ATP in tumor microenvironment is interpreted as a danger signal by the immune system since it can activate the NLRP3 inflammasome in dendritic cells (DCs), which leads to secretion of IL1-β and further promotes the inflammatory response

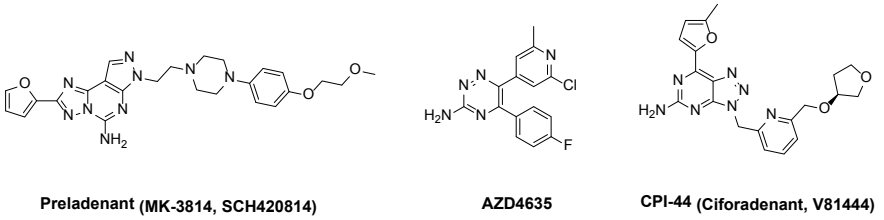


Fig. 22.6 Selected A2A receptor antagonists

in infection and cancer. However, in tumor microenvironment, Treg cells express extracellular ectonucleotidases (CD73, CD39) which dephosphorylate ATP to produce adenosine. The resulted adenosine binds to adenosine receptors (A2A and A2B) on lymphocytes in tumor, which subsequently suppresses their ability to mediate antitumor effect, such as cytolysis (Deaglio et al. 2007). By binding to A2A receptor on lymphocyte surface, adenosine amplifies the immunosuppressive effects of Treg cells (Ohta et al. 2012). Hypoxia-adenosinergic axis is proposed in the tumor microenvironment, where the hypoxia-inducible transcription factor (HIF-1α) activates several of the above receptors that contribute to immune suppression in tumor microenvironment (Adams et al. 2015). Thus, by targeting either ectonucleotidases (CD39, CD73) or adenosine receptors (A2A or A2B), small molecules could serve as potential therapeutics to reduce the immunosuppressive milieu present in tumor (Kerr and Chisholm 2019).

A number of such small molecules have been discovered, including CD73 inhibitors PSB-12379 (Bhattarai et al. 2015), AMPCP (Wang et al. 2011), CD39 inhibitor ARL67176 (Bastid et al. 2013), A2B antagonists PSB1115 (Iannone et al. 2013), PSB603 (Borrmann et al. 2009), MRS1754 (Acurio et al. 2014), A2A antagonists SCH58261 (Beavis et al. 2013), ZM241365 (Iannone et al. 2014), A2A-IN-1 (Duan et al. 2018), SCH420814 (preladenant) (Chen et al. 2013), vipadenant (Gillespie et al. 2009), and CPI-444 (Fig. 22.6). So far, only A2A antagonists have been developed into clinical trials, of which the most advanced agent is CPI-444. The preliminary phase I clinical trial results of adenosine A2AR antagonist CPI-444, either as monotherapy or in combination with atezolizumab for advanced solid cancer, demonstrated 42% overall response in 42 patients (10 of 24) resistant to previous anti-PD-1/PD-L1 therapy. Grade 1 and 2 toxicities were the most common side effects, with only one case of grade 3 autoimmune hemolytic anemia (Emens et al. 2017). Several A2A antagonists that were originally developed as therapeutics for Parkinson disease have now been repurposed for immunotherapy and are being tested clinically, either as single agents or in combination with ICI therapies (Kerr and Chisholm 2019). These include preladenant (former for Parkinson disease but discontinued in phase III), PBF-509 (NCT02403193) and AZD4635 (Congreve et al. 2012; Littleson et al. 2019). AZD4635 has been studied in 4 clinical trials starting from June 2016 (Table 22.2, NCT03710434), but no clinical results have been reported yet. Recently, Aurigene announced the discovery of a dual CD73-A2AR

Table 22.2 Summary of ongoing clinical trials of A2A receptor antagonists

Compound	Sponsor	Indication(s)	Status	ClinicalTrials.gov identifier
Preladenant (MK-3814, SCH420814) (alone and combination with pembrolizumab)	Merck Sharp & Dohme	Advanced solid tumors	Phase I, discontinued	NCT03099161
CPI-444 (alone and combination with pembrolizumab)	Corvus	Advanced cancers	Phase II	NCT02655822
CPI-444 (combination with atezolizumab)	Genentech	Advanced SCCHN	Phase II	NCT03708224
CPI-444 (combination with atezolizumab)	Roche	NSCLC	Phase I/II	NCT03337698
PBF-509 (alone and combination with PD-1 Ab PDR001)	Novartis	NSCLC	Phase II	NCT02403193
AZD4635 (monotherapy)	AstraZeneca	Solid malignancies	Phase I	NCT02740985
AZD4635 (combination with durvalumab)	AstraZeneca	Solid malignancies	Phase I	NCT03980821
AZD4635 (combination with oleclumab)	MedImmune	EGFRm NSCLC	Phase I/II	NCT03381274

SCCHN Squamous cell carcinoma of the head and neck

antagonist. Due to its critical role in tumor microenvironment, A2A is considered as a highly valuable molecular target in the hypoxia–adenosinergic axis that contributes to immune suppression.

22.2.3 VISTA

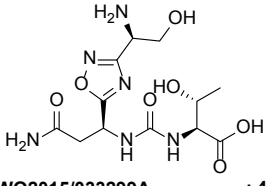
V-domain Ig suppressor of T-cell activation (VISTA), also called as programmed death-1 homolog (PD-1H), is a unique checkpoint in B7 family and regulates a broad range of immune responses. It is predominantly expressed in hematopoietic cells such as myeloid and granulocytic cells, and with low expression on T-cells. VISTA behaves as a stimulatory ligand for antigen-presenting cells (APCs) causing

immune activation and as a negative ligand for T-cell activation, proliferation, and cytokine production (Lines et al. 2014a). Le Mercier et al. reported that VISTA is mainly expressed in tumor microenvironment lymphocytes but with low expression in tumor cells. They also demonstrated that VISTA blockade improved TIL activation and enhanced tumor-specific T-cell responses in the periphery despite the presence of high PD-L1 levels or the lack of expression of VISTA in tumor cells (Le Mercier et al. 2014). Therefore, VISTA and PD-1/PD-L1 pathways are considered independent, and simultaneous blockade of PD-1 and VISTA is rationally considered as synergistic (Lines et al. 2014b).

Since VISTA inhibition-induced T-cell activation seems to be nonredundant from the PD-1/PD-L1 pathway, this implies that a combined VISTA/PD-1 blockade might be a promising combination option for cancer treatment, whose efficacy was indeed demonstrated in murine tumor models (Liu et al. 2015a; Kondo et al. 2016). Interestingly, VISTA expression levels vary among different tumors, its blockade was proved to be effective even with undetectable level of expression. This may offer the possibility and advantage for a broader clinical applicability, especially in combination with other immunotherapies. However, specific biomarkers and more translational research are still needed to predict response and improve the clinical benefit (Lines et al. 2014a).

Even though VISTA inhibition seems a very promising approach for cancer treatment, not much clinical studies either as monotherapy or in combination were reported yet. JNJ-61610588 is the first reported VISTA monoclonal antibody (mAb) in clinical trial (NCT02671955) and the clinical trial was terminated recently for not disclosed reasons. The second VISTA antagonist in clinical trial is a small-molecule VISTA inhibitor, CA-170 (Fig. 22.7, structure undisclosed). CA-170 is a first-in-class, orally available, dual VISTA/PD-L1 antagonist (WO2015033299A1 2015). CA-170 seems to be safe and efficacious in phase I studies and is currently undergoing phase II clinical investigation for treatment of lymphoma and solid tumors in India (see this chapter, Sect. 22.2.1).

Due to its dual inhibition of VISTA and PD-L1, we are eagerly waiting to find out whether this dual inhibition demonstrates improved clinical outcome as the mechanism may suggest. It is also expected that more VISTA inhibition therapies, either as small molecule or biologics, will be developed in the near future.



WO2015/033299A compound 4

Fig. 22.7 Representative structure of CA-170 class compounds

22.2.4 TIM-3

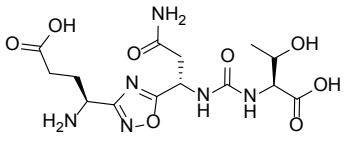
TIM-3, also known as HAVCR2, belongs to the TIM gene family. In humans, the TIM gene family is located on chromosome 5q33.2 and includes three members: TIM-1, TIM-3, and TIM-4 (Li et al. 2013).

TIM-3 is a negative regulatory immune checkpoint and generally expressed in different types of immune cells, including T-cells (Tregs), dendritic cells (DCs), B-cells, macrophages, natural killer (NK) cells, and mast cells. TIM-3 is a type I membrane protein of 281 amino acids and has an extracellular domain, a single transmembrane domain, and a *C*-terminal cytoplasmic tail (He et al. 2018).

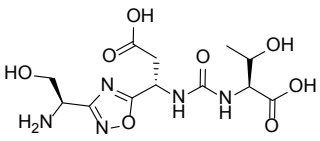
TIM3 is a potent T-cell functions suppressor and co-expressed with programmed cell death-1 (PD-1) receptors on certain T-cells. TIM3's ligands include galectin 9 (GAL9), phosphatidylserine, high mobility group protein B1 (HMGB1) and Ceacam-1. By binding to one or more of those ligands, TIM3 can modulate its immune response. Preclinical in vivo studies have shown that simultaneous inhibition of PD-L1 and TIM3 resulted in improved antitumor responses in certain tumor models, indicating their independent roles in regulating immune response to tumors (http://www.curis.com/pipeline/ca-327).

TIM-3 could induce immunological tolerance, inhibit the immune responses of T-cells, and was associated with immune exhaustion, which was induced by chronic viral infection (Lee et al. 2011).

Most of the TIM-3 antagonists were monoclonal antibodies (mAb). Recently, Curis has provided details on its small-molecule inhibitor CA-327, which selectively and potently inhibits both PD-L1 and TIM3 (Lazorchak et al. 2018). CA-327 can dose-dependently activate T-cells inhibited by exogenous PD-1 ligand or TIM3 in a similar degree of response observed using PD-1 or TIM3 antibodies. CA-327 is orally bioavailable across multiple preclinical species and inhibits tumor growth in immunocompetent mice and the structure was not disclosed (Huck et al. 2018). Representative compounds from Aurigene's PD-L1 and TIM3 antagonist patent, WO2019/087092A1 are shown in Fig. 22.8.



WO2019/087092, Compound 1



WO2019/087092, Compound 32

Fig. 22.8 Representative structures of WO2019/087092

22.3 Small Molecules Enhance Cellular Immunity

22.3.1 Kynurenine Pathway (IDO/TDO)

The kynurenine pathway of tryptophan metabolism attracted much attention in recent years since its metabolism products "kynurenines" play a key role in immune regulation and diverse physiological functions. Tryptophan dioxygenase (TDO) mainly exists in the liver, whereas indoleamine 2,3-dioxygenase (IDO) exists elsewhere. The kynurenines are produced in many different tissues, mostly in liver by TDO (Kanai et al. 2009), while in immune cells and brain, tryptophan (TRP) is converted to kynurenine (KYN) by IDO. Under normal physiological conditions, TDO controls > 95% of TRP degradation in the liver and makes TRP availability for other tissues and organs. However, under conditions of immune activation, IDO's activity can be induced in manyfold by some cytokines such as interferon-gamma (IFNγ) and assumes a major role in the control of TRP degradation.

IDO plays an important role in enabling tumor cells to evade the immune system and has recently become as an attractive onco-immunology target (Mandi and Vecsei 2012; Vecsei et al. 2013; Dounay et al. 2015). Van den Eynde and co-workers reported that indoleamine 2,3-dioxygenase 1 (IDO1) is overexpressed in cancer cells and demonstrated how this enzyme contributes to immune evasion of tumor cells (Uyttenhove et al. 2003). It was reported that many human tumor cells express IDO1, and expression of IDO1 in immunogenic mouse tumor cells prevented tumor rejection by pre-immunized mice.

IDO1, the most broadly expressed catalytic enzyme, belongs to three heme-dependent dioxygenases that catalyze the first step of tryptophan metabolism in the kynurenine pathway (Fig. 22.9) (Weinmann 2016). Even though tryptophan 2,3-dioxygenase (TDO), IDO1, and indoleamine 2,3-dioxygenase 2 (IDO2) all catalyze the same biochemical transformation, they share only limited structural homology.

In tumor immune microenvironment, degradation of L-tryptophan by IDO1 activates general control nonderepressible 2 kinase (GCN2K) and aryl-hydrocarbon receptor (AhR). Activated GCN2K upregulates the transcription factor p53 and downregulates the transcription factor c-Myc, and in turn reduces the consumption of glucose and glutamine by decreasing the expression of glucose transporter 1 (GLUT1) and glutaminase. The reduced utilization of these pivotal sources of

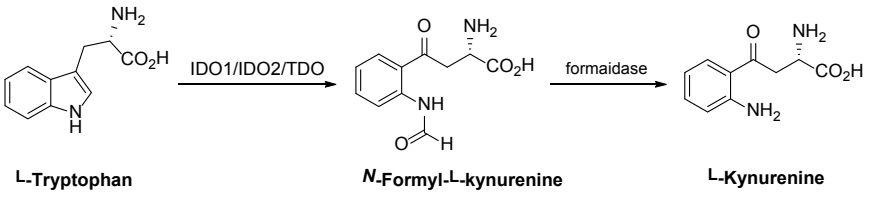


Fig. 22.9 Metabolism of L-tryptophan in the kynurenine pathway

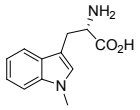
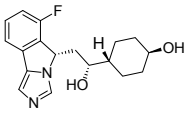
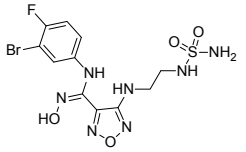
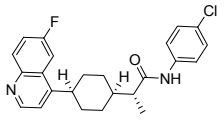
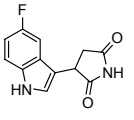
energy by activated T-cells results in reduced ATP production. In parallel, activation of AhR increases the expression of CPT1 isoenzymes which control free fatty acid oxidation. These IDO-induced alterations promote free fatty acid oxidation as an alternative fuel for ATP production, supplying the required energy for $CD4^+$ T-cell survival and proliferation (Eleftheriadis 2018).

IDO1-mediated tryptophan metabolism enhances T-regulatory cell activity. Preclinical data indicate that its role in tumorigenesis is context-dependent on host and tumor interaction, which might imply the challenges in understanding the molecular oncology of this target. Results from phase I/II trials of IDO-1 inhibitors as monotherapy have been disappointing, and the emphasis of the current clinical trials has been on the combination strategy using IDO-1 inhibitors with other immunotherapy agents. Combinations with anti-PD-1/PD-L1 antibodies showed early promise, and related strategies are still under evaluation (Zhu et al. 2019). A number of small-molecule inhibitors of tryptophan metabolism have been reported (Platten et al. 2019) as summarized in Table 22.3.

With the exciting development and advance of checkpoint inhibitors, enormous attention has been focused on the IDO1 enzyme as a metabolic mediator of immune escape in cancer to improve the efficacy and response scope of PD-1/PD-L1 inhibitors. The preliminary data of multiple phase I/II trials resulted in much excitement that small-molecule inhibitors of IDO1 may improve patient responses to anti-PD1 immune checkpoints. However, recent results from ECHO-301, the first large phase III trial to evaluate combined IDO1 inhibitor epacadostat to pembrolizumab (anti-PD1 antibody) in advanced melanoma, failed to show the increased benefit. Even though one trial is by no means conclusive for the question, there is clearly a need for the more in-depth understanding of the impact and limitation of IDO1 inhibition to immune regulation and cancer treatment. While biomarker information yet to be gleaned from ECHO-301 may reveal more useful information regarding IDO1 inhibition, better rationalized compounds and trial designs will be important to accurately gauge its clinical impact in the future. In addition, administration of IDO1 inhibitors may be beneficial to certain but not all cancers. Beyond tumor IDO1 expression, assessment of other factors such as the status of p53 and free fatty acids in the tumor microenvironment may be necessary to stratify the right patient population.

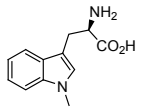
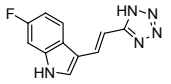
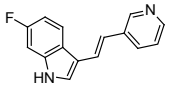
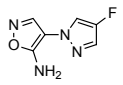
While IDO1 can suppress T-cell proliferation, induce T-cell apoptosis, and promotes the T-cell differentiation toward a regulatory phenotype, the presence of free fatty acids in the tumor microenvironment may temper the antiproliferative and proapoptotic properties of IDO1. In addition, inhibition of IDO1 may cause some other unknown compensation mechanisms. More translational research is needed to unearth the epigenetic changes after IDO inhibition, and a multiple mechanism drug combination may be needed to achieve the desired efficacy for cancer treatment.

Table 22.3 Selected small-molecule inhibitors of tryptophan metabolism

Compound	Comments	Indications/development stage
IDO1 inhibitors		
 1-Methyl-L-tryptophan (L-1-MT)	• Trp-competitive inhibitor • Moderate IDO1 inhibition, low specificity • Substrate analogue	Experimental, diverse fields/preclinical
 Navoximod/NLG-919; NewLink, Genetics	• Based on 4-phenylimidazole scaffold • Directly binds to ferric haem iron	Cancer/Phase I
 Epacadostat/INCB024360; Incyte	• Trp-competitive inhibitor • Directly binds to ferric haem iron	Cancer/Phase III
 BMS-986205/F001287; Bristol-Myers Squibb (originator: Flexus)	• Selective IDO1 inhibitor • Binds to haem-free apo IDO1	Cancer/Phase III
 EOS200271/PF-06840003; iTeos Therapeutics	• Noncompetitive kinetics with respect to Trp • Does not bind to haem iron • Central nervous system penetration	Cancer/Phase I
KHK2455; Kyowa Kirin	Binds to haem-free apo IDO1	Cancer/Phase I

(continued)

Table 22.3 (continued)

Compound	Comments	Indications/development stage
LY3381916; Eli Lilly	Binds to newly synthesized apo-IDO1 but does not inhibit mature haem-bound IDO1	Cancer/Phase I
MK-7162; Merck	No information available	Cancer/Phase I
IDO pathway inhibitors		
 Indoximod/NLG8189/1-methyl-D-tryptophan (D-1-MT); NewLink Genetics	Does not inhibit IDO1 in vitro	Cancer/Phase II/III
NLG802; NewLink Genetics	Prodrug of indoximod	Cancer/Phase I
TDO inhibitor		
 LM10	• Less potent but better solubility and bioavailability than 680C91 • Investigated in mouse cancer models	Experimental, cancer/preclinical
 680C91; GlaxoSmithKline	• Nanomolar activity in vitro • Low aqueous solubility • Poor oral bioavailability	Experimental, depression, cancer/preclinical
 4-(4-fluoropyrazol-1-yl)-1,2-oxazol-5-amine; Genentech	• Nanomolar cellular activity • Sixfold selectivity over IDO1 • Whole blood stability	Cancer/preclinical
Fused imidazo-indoles; Redx Pharma	Potent and TDO selective	Experimental/preclinical
Indazoles; IOmet Pharma	Potent and TDO selective	Experimental/preclinical
Dual IDO1-TDO inhibitors		
HTI-1090/SHR9146; Atridia, Hengrui, Therapeutics	Potent, orally bioavailable dual IDO1/TDO inhibitor	Cancer/Phase I

(continued)

Table 22.3 (continued)

Compound	Comments	Indications/development stage
DN1406131; Jiangxi Qingfeng Pharmaceutical	No information available	Cancer/Phase I
RG70099; Roche (originator: Curadev)	Significantly reduces Kyn levels in preclinical tumor models	Cancer/preclinical
EPL-1410; Emcure Pharmaceuticals	• Good oral bioavailability in rodents • Reduces tumor volume and Kyn:Trp ratio in cancer models	Cancer/preclinical

22.3.2 STING Agonists

Stimulator of interferon genes (STING) plays an important role in the production of type I interferons in response to cytosolic nucleic acid. Recent studies indicate that STING is involved in the induction of antitumor immune responses. Therefore, STING has been extensively explored as a promising immune-oncology therapeutic target (Chen et al. 2019).

STING (also known as MITA, MPYS, ERIS, and TMEM173) is a ubiquitously expressed adaptor protein localized predominantly on the endoplasmic reticulum (ER) membrane, where it is anchored through transmembrane domains (Ishikawa and Barber 2008). STING is expressed in numerous cell types such as macrophage, dendritic cells, haematopoietic cells, T-cells, endothelial cells, and epithelial cells (Barber 2015).

STING exists as a monomer and becomes a dimer by stimulation (Sun et al. 2009). It can be activated in cytosol by different DNA sensors such as DNA-dependent activator of interferon regulatory factors (DAI) (Wu and Chen 2014). The most critical receptor for this pathway is cyclic GMP-AMP (cGAMP) synthase (cGAS). Activated STING translocates from the ER through the Golgi apparatus to the perinuclear microsomal compartments (Ishikawa et al. 2009). The modification of STING results in the recruitment of the interferon regulatory factor 3 (IRF3) to the complex and its phosphorylation by tank-binding kinase 1 (Liu et al. 2015b). Then, IRF3 translocates to the nucleus and triggers transcription of IFNB1 and several other genes, which promote expression of proinflammatory cytokines, such as interleukin 6 (IL6) and tumor necrosis factor TNF (Woo et al. 2014). Activation of STING leads to maturation of innate dendritic cells and production of interferon-β (IFN-β), which activates $CD8^+$ T-cells infiltrated in tumors (Li et al. 2017).

Several non-CDN small molecules have been developed and tested in clinics as immune-boosting therapies. The earlier reported STING agonists were the anticancer flavonoids FAA, DMXAA, and CMA (Kim et al. 2013; Cavlar et al. 2013), non-CDN small molecules (Fig. 22.10). FAA was discovered as an STING agonist through

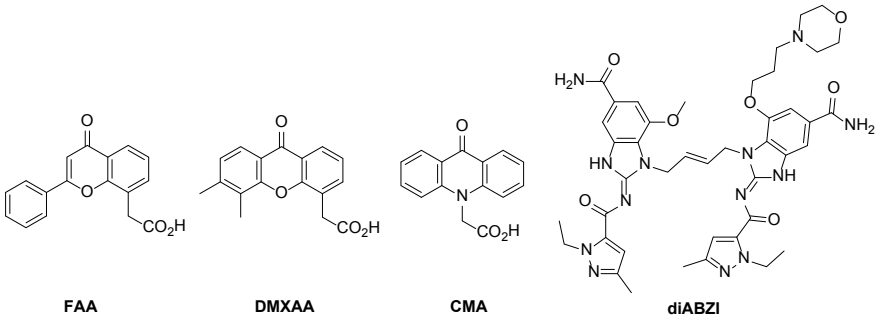


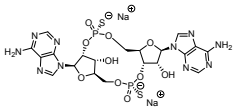
Fig. 22.10 Structures of non-CDN STING agonists FAA, DMXAA, CMA, and diABZI

screening natural flavonoids on mouse solid tumor models (Bibby et al. 1991). Unfortunately, this agent failed in the phase I clinical trial, which did not demonstrate desired activity in solid tumors (Cummings and Smyth 1989). DMXAA was developed as a more potent analogue of FAA and unfortunately failed in a phase III clinical trial in advanced non-small cell lung cancer (McKeage et al. 2009; Kerr and Kaye 1989; Bibby and Double 1993). DMXAA does not bind to human STING, which may explain its lack of efficacy or mechanism-related toxicity in human (Kim et al. 2013). Recently, a dimeric amidobenzimidazole analogue (*diABZI*) was reported as a potent non-CDN agonist that can be administered intravenously (Ramanjulu et al. 2018). This compound is currently in phase I clinical trial.

CDN analogues such as ADU-S100 (Aduro/Novartis) and MK-1454 (Merck) have been recently advanced to the clinical development to mimic the natural agonistic ligand cGAMP to activate the cGAS-STING pathway (Kerr and Chisholm 2019; Huck et al. 2018; Li et al. 2017; Toogood 2018; Cheng et al. 2018). The synthetic CDN analogue ADU-S100 is an orthosteric agonist and highly efficacious in various syngeneic mouse tumor models when administered intratumorally. In preclinical studies, it exhibited significant distal effect, indicating that even local administration of ADU-S100 could have abscopal effects on tumors that are not directly treated. Since 2015, this compound has been under phase I/II trials to treat various cutaneously accessible tumors both as a single agent and in combination with other types of anticancer drugs including cytotoxic chemotherapies, targeted therapies, and checkpoint inhibitors (Table 22.4). MK-1454 (structure undisclosed) is also being evaluated in phase I clinical trial as monotherapy and in combination with pembrolizumab.

Despite the recent success in the development of STING agonists in antitumor therapy, an intratumoral injection may be necessary for avoiding the potential autoimmune response and for the effective activation of STING receptor. This administration approach may limit clinical uses of this class of molecules. It is desirable to identify safe and systemically available STING agonists in order to treat tumors that are inaccessible through direct injection (Kerr and Chisholm 2019). Despite vadimezan's failure, it is encouraging to see that drug-like, non-nucleotide molecules

Table 22.4 STING agonists currently in clinical trials

Compound	Structure	Development status	Combination agent	ClinicalTrials.gov identifier
ADU-S100		Phase II	Anti-PD1	NCT03937141
		Phase I	PDR001	NCT03172936
		Phase I	Ipilimumab	NCT02675439
MK-1454	Undisclosed	Phase I	Pembrolizumab	NCT03010176

like vadimezan work well in mice and demonstrate the potential for oral use with full agonist properties (Huck et al. 2018).

22.3.3 Toll-Like Receptor (TLR) Agonists

Toll-like receptors (TLRs) are a family of pattern recognition receptors (PRR) that function as primary sensors of the innate immune system to recognize microbial pathogens (Gnjatic et al. 2010). TLRs recognize conserved pathogen-associated molecular patterns (PAMPs) expressed on a wide array of microbes, as well as endogenous DAMPs released from stressed or dying cells (Rakoff-Nahoum et al. 2004; Adams 2009). TLR1, –2, –4, –5, –6, and –10 are expressed on the cell surface, whereas TLR3, –7, –8, and –9 are mainly expressed on endosomal membranes within the cell (Rakoff-Nahoum and Medzhitov 2009; So and Ouchi 2010). In humans, various families of TLRs have been identified and emerging evidence have shown that many TLRs are potential drug targets due to their important role in defense against pathogens, autoimmune disorders, and cancers (Achek et al. 2016). Studies have demonstrated that TLRs could not only activate DC and other members of the immune system to eliminate cancer cells but also induce autophagy and apoptosis of cancer cells (Shi et al. 2016).

A number of small-molecule TLR agonists have been reported and evaluated in the preclinical and clinical studies. TLR3, TLR4, TLR7/8 and TLR9 agonists represent promising cancer immunotherapeutics and have been included in the ranked National Cancer Institute's list of immunotherapeutic agents with the highest potential to treat cancer as monotherapy or in combination with other antitumor therapies (Li et al. 2017; Adams 2009; Cheever 2008). TLR-mediated DC activation leads to enhanced phagocytosis, maturation with upregulation of MHC and is critical for the initiation of T-cell immunity, antigen presentation, and secretion of cytokines (Iwasaki and Medzhitov 2004). The most important TLR-induced cytokines for cancer treatment are IL-6 and IL-12 (Pasare and Medzhitov 2003; Reis e Sousa et al. 1997). IL-6 enhances antigen-specific T-cell activation through suppression of Tregs while IL-12 enhances the host responses to intracellular pathogens by inducing IFN-γ production and Th-1 responses (Trinchieri 2003).

The research activity on the development of TLR7/8 agonist is particularly active and a number of them are currently in clinical studies (Table 22.5). Imiquimod is so far the only US FDA approved small-molecule TLR7 agonist for clinical use to treat genital warts, superficial basal cell carcinoma, and actinic keratosis. It targets the TLR7–MYD88-dependent pathway and induces the secretion of the pro-inflammatory cytokine, tumor necrosis factor (TNF) and interleukin-6 (IL-6) (Salazar et al. 2017).

Imiquimod's anticancer effects may attribute to its induction of innate and adaptive immunity and alteration of the tumor microenvironment suitable for antigen cross-presentation and infiltration by effector T-cells and DCs (Wolf et al. 2007; Broomfield et al. 2009; Hemmi et al. 2002; Clark et al. 2008; Stary et al. 2007). It was also found that imiquimod can inhibit angiogenesis, cause direct apoptosis of tumor cells, and induce NK-mediated cytotoxicity (Dumitru et al. 2009; Majewski et al. 2005; Schon et al. 2003).

In phase III studies, treatment with 5% imiquimod cream resulted in histologic clearance rates of 79–82% of sBCC (Geisse et al. 2004). Objective responses have also been observed in selected melanoma patients when imiquimod was directly applied onto cutaneous metastases (Wolf et al. 2003; Bong et al. 2002). A phase II study of imiquimod in breast cancer patients with chest wall recurrences or skin metastases is ongoing.

Topical use of imiquimod cream has shown an excellent safety profile with reversible local reactions and minimal systemic exposure (Harrison et al. 2004). Orally administered imiquimod induced systemic IFN-α in patients with advanced cancers, but tumor responses were not observed due to the dose-limiting immune-related side effects (Witt et al. 1993).

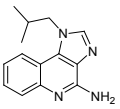
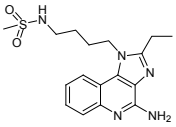
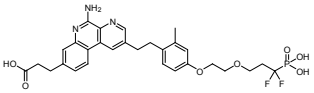
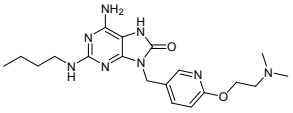
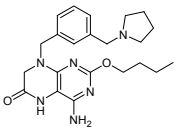
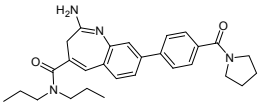
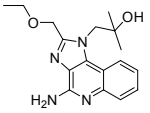
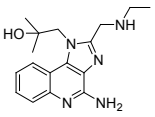
PF-4878691 (3M-852A) is an orally bioavailable TLR7 agonist and has shown modest clinical efficacy with disease stabilization observed in some patients in phase I and II studies of advanced cancer. The systemic side effects prevent the further evaluation of this agent at greater doses (Dudek et al. 2007; Dummer et al. 2008).

LHC165 is a selective TLR7 agonist and currently in phase I study to evaluate the safety and efficacy either as a single agent or in combination with PDR001 in patients with advanced malignancies using intratumoral injection administration (NCT03301896) (Bourquin et al. 2019).

DSR-6434, a potent TLR7 agonist ($EC_{50} = 7.2$ nM), can activate several immune effector cells in vitro, suppress metastasis in vivo, and enhance the efficacy of radiation therapy in a colorectal carcinoma model using intravenous delivery of the drug. DSR-6434 leads to the induction of type 1 interferon and activation of T- and B-lymphocytes, NK- and NKT-cells. Systemic administration of DSR-6434 enhanced the efficacy of ionizing radiation (IR) and led to improved survival in mice bearing either CT26 or KHT tumors with 55% complete tumor elimination and the distal antitumor effect was also observed (Adlard et al. 2014).

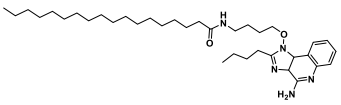
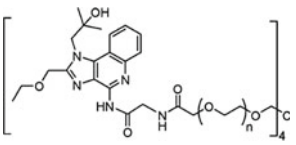
Vesatolimod (GS-96200), an orally available TLR7 agonist, is currently in a phase II study to evaluate the safety and efficacy in combination with tenofovir disoproxil fumarate (TDF) for the treatment of subjects with chronic hepatitis B. Preliminary

Table 22.5 TLR7/8 agonists in clinical development

Compound	Structure	Target	Development status
Imiquimod		TLR7 agonist	Approved for genital warts, superficial basal cell carcinoma, and actinic keratosis; Phase II: breast cancer cutaneous metastases
PF-4878691 (3M-852A)		TLR7 agonist	Phase II: advanced cancers
LHC-165		TLR7 agonist	Phase I: solid tumors
DSR-6434		TLR7 agonist	Preclinical: cancers
Vesatolimod (GS9620)		TLR7 agonist	Phase II: HBV, in combination with tenofovir disoproxil fumarate
Motolimod (VTX-2337)		TLR8 agonist	Phase Ib in cancers
Resiquimod (R848)		TLR7/8 agonist	Phase II: warts; actinic keratosis; melanoma; bladder cancer; glioblastoma
Gardiquimod		TLR7/8 agonist	Interventional trial: systemic lupus musculoskeletal pain

(continued)

Table 22.5 (continued)

Compound	Structure	Target	Development status
Telratolimod		TLR7/8 agonist	Phase I: terminated intratumoral injection; solid tumors or CTCL
NKTR262		TLR7/8 agonist	Phase Ib; intratumoral injection; locally advanced or metastatic solid tumors; in combination with bempegaldesleukin (NKTR-214)

data showed that oral administration of GS-9620 enhanced the antiviral activity without systemic IFN-α relating adverse effect (NCT02579382). No studies on cancer treatment were reported for this agent (Fosdick et al. 2014; Lanford et al. 2013; Bam et al. 2017).

Motolimod (VTX-2337), a selective TLR8 agonist activating monocytes, DCs, and natural killer cells, was shown to increase IFN**γ** production, cytolytic activity, and to enhance rituximab-mediated therapeutic effects (Lu et al. 2012). VTX-2337 is currently in clinical studies for treatment of cancers. The phase Ib clinical trial of VTX-2337 plus cyclophosphamide in treating patients with a solid tumor was terminated (NCT02650635). Additional three phase Ib studies are still undergoing: one is evaluating the combination of anti-PD-1 inhibitor tislelizumab with motolimod delivered either subcutaneously or by intratumoral injection in subjects with squamous cell carcinoma of the head and neck (NCT03906526); another is an open-label, non-randomized, multicenter phase I/II study of MEDI4736 in subjects with recurrent, platinum-resistant ovarian cancer, scheduled to receive pegylated liposomal doxorubicin (PLD) (NCT02431559); the third trial is a phase Ib study of neoadjuvant of cetuximab plus motolimod and cetuximab plus motolimod plus nivolumab (NCT02124850).

Another TLR8 agonist, VTX-1463, is currently in development for the treatment of allergic rhinitis (Horak 2011). The basis for its use relies on the induction of T-helper 1 (Th1)-associated inflammatory mediators after TLR8 activation, and the cytokine response may activate antigen-presenting cells, thereby inducing a Th1 type response and shifting the balance of the Th1/Th2 ratio in favor of a reduction in the allergic reaction (Kanzler et al. 2007).

In addition to TLR7 activation-induced IFN-α expression by pDCs, TLR8 activation of monocytes and mDCs can promote the secretion of pro-inflammatory

cytokines and chemokines such as IL-12 and macrophage inflammatory protein-1(MIP-1) (Gorden et al. 2005; Tomai et al. 2007). The induction of IL-12, enhancement of NK cytotoxicity, and TLR8 activation-mediated Treg suppression are essential for cancer immunotherapies. Resiquimod, a dual TLR7/8 agonist, has demonstrated great potential in preclinical and early clinical studies as a single agent or in combination therapy. 25 clinical studies under the name of resiquimod and 7 under R-848 are undergoing at phase I and phase II for the treatment of warts, actinic keratosis, cutaneous melanoma, bladder cancer, and glioblastoma (Schon and Schon 2008). In actinic keratosis patients, treatment with resiquimod gel resulted in 77–90% complete clearance of the lesions (Szeimies et al. 2008). In a phase I study with twelve cutaneous T-cell lymphoma patients, 75% of the patients had improved lesions, 30% of the patients has all the lesions cleared, 90% of the patients had decreased malignant T-cells and 30% of the patients has the malignant T-cells completely eliminated (Rook et al. 2015).

Gardiquimod is another dual TLR7/8 agonist and currently in an interventional clinical trial to treat systemic lupus musculoskeletal pain (NCT0282298). It has been reported that gardiquimod promoted murine splenocyte proliferation; activated splenic T, NK, and natural killer T (NKT) cells; and enhanced the expression of IL-12 by macrophage and DCs. In a murine model, gardiquimod demonstrated superior antitumor activity compared with imiquimod (Ma et al. 2010).

Telratolimod (also called MED-9197 or 3 M-052), a dual TLR7/8 agonist bearing a C18 lipid moiety, is designed for slow dissemination from the site of application and reducing the systemic cytokine such as circulating TNFα or Th1 cytokines (Smirnov et al. 2011). The phase I study using intratumoral injection for treatment of solid tumors or CTCL in combination with durvalumab and/or palliative radiation was terminated (NCT02556463). Preclinical studies have demonstrated that intratumoral injection of telratolimod induced systemic antitumor activity and inhibited both local and distal tumor growth in B16.F10 bearing mice. Addition of checkpoint inhibitor further enhanced the antitumor efficacy (Singh et al. 2014).

NKTR-262 is a novel dual TLR7/8 agonist and designed to retain locally for longer period of time and temper the systemic side effects. NKTR-262 is currently in phase Ib clinical trial in combination with bempegaldesleukin (NKTR-214, CD122-biased agonist) in patients with locally advanced or metastatic solid tumors (NCT03435640). The preliminary results showed that the treatment is generally well tolerated with mild adverse events and 45.5% of disease control rate (Diab et al. 2019).

There are a few TLR9 agonists that are currently under clinical studies with most of them as CpG ODN derivatives. CMP-001(QbG10, CYT003), a leading small-molecule TLR9 agonist (see Fig. 22.11 for structure), is currently in several phase Ib, phase II clinical trials in combination with different cancer therapies for treatment of patients with melanoma, lymph node cancer, colorectal neoplasms, liver metastases, and NSCLC. The combination of CMP-001 and pembrolizumab showed a manageable safety profile and meaningful clinical activity (NCT03618641). The preliminary findings revealed that 15 out of the 69 assessable patients showed significant tumor reduction and 2 of them showed complete response (CR). It is worth

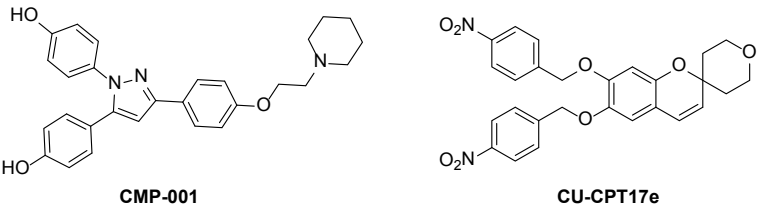


Fig. 22.11 Chemical structures of CMP-001 and CU-CPT17e

mentioning that in some patients, metastatic tumors of lymph nodes, liver, and spleen also significantly shrank (Milhem et al. 2018).

CU-CPT17e is a novel multi-toll-like receptor agonist, targeting TLR3, 8, and 9 (Fig. 22.11). Pro-inflammatory studies showed that CU-CPT17e induced a robust immune response via the expression of various cytokines in human monocytic THP-1 cells. It was reported that this compound can also induce cell apoptosis and cell cycle arrest in human cervical cancer cells (Zhang et al. 2017).

While a number of TLR agonists have demonstrated promising clinical efficacy for cancer treatment, intratumoral injection or local delivery might be necessary to avoid the systemic autoimmune complications. It is anticipated that TLR agonists will provide more clinical benefit when used in optimal combinations with other therapeutics such as tumor vaccines and immune modulators, especially checkpoint inhibitors.

22.3.4 OX40

OX40 is an immune stimulator and plays a crucial role in the regulation of immune cell activation and sustained inflammatory responses (Croft et al. 2009). OX40 receptor (CD134) is a co-stimulatory protein that is expressed on the surfaces of activated T-cells and NK-cells (Redmond et al. 2009), whereas its ligand OX40L is a member of the TNF superfamily and mostly found on APCs such as dendritic cells (DCs), macrophages, and activated B-cells as well as endothelial cells and T-cells (Croft et al. 2009; Redmond et al. 2009).

Preclinical studies have demonstrated that agonistic anti-OX40 monoclonal antibodies (mAbs) and OX40L-Fc fusion proteins can increase antitumor immunity and improve tumor-free survival while OX40L-Fc fusion proteins are more efficacious (Linch et al. 2015). It has been shown that anti-OX40 mAbs and OX40L-Fc fusion proteins can cause OX40 dependent CD8 and CD4 T-cell expansions, and a proportion of mice showed evidence of a strong memory sufficient to provide resistance upon tumor re-challenge (Jensen et al. 2010).

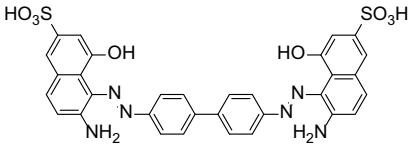


Fig. 22.12 Structure of OX40 agonist CVN

A number of agonistic anti-OX40 antibodies and OX40L-Fc fusion proteins are currently in early-stage clinical trials either as monotherapy or in combination to evaluate the safety and primary antitumoral efficacy. These compounds include 9B12 (IgG1, anti-OX40, NCT018629000), MEDI0562 (anti-OX40, NCT02318394), MEDI6469 (anti-OX40, NCT02205333), MOXRO0916 (anti-OX40, NCT02219724), and MEDI6383 (OX40L fusion protein, NCT02221960 (Aspeslagh et al. 2016). The early clinical results indicated that anti-OX40 antibodies showed strong bioactivity and certain antitumor efficacy but failed to achieve dramatic antitumor efficacy compared with anti-PD1/PDL1 mAbs (Curti et al. 2013).

Even though the researches and development of anti-OX40 antibodies and OX40L-Fc fusion proteins are very active, related studies using small molecules are still limited. Since the OX40–OX40L interaction is a typical protein–protein interaction (PPI), the design and discovery of the appropriate OX40/OX40L agonists is very challenging. Buchwald P. et al. first reported the discovery of several small molecules such as DB36, DB71, DB15, and CVN as partial agonists. These small molecules can directly interrupt the OX40-OX40L interaction and activate OX40 downstream signaling in vitro (Song et al. 2014). Notably, CVN was found to have the best effect of inhibiting Treg generation as well as stimulating helper T-cell (Th9) generation, which is ideal for maintaining an antitumor immune response (Fig. 22.12). These molecules have low micromolar potency as partial agonists and are found to have comparable efficacious to the OX40 human mAb, which is a promising sign for their clinical application. To date, these compounds remain as the only example that has produced promising results. Further development and clinical testing remain to be done (Smith et al. 2019).

While targeting OX40/OX40L is an attractive strategy with great potential for cancer treatment, especially in combination with other immunotherapies such as PD-1/PD-L1 inhibitors and chemotherapies, better understanding of the biology of this pathway for rational combination is still needed. With the advantage of better tumor tissue penetration and more amenable for fine-tuning of the dosing regime, it is expected that there will be more research activities in the discovery of small molecules targeting OX40/OX40L pathway in the near future.

22.3.5 GSK-3 Inhibitors

Glycogen synthase kinase-3 (GSK-3) is a serine/threonine-protein kinase with two highly homologous isoforms, GSK-3α and GSK-3β which have different tissue-specific functions and substrates (Kaidanovich-Beilin and Woodgett 2011). GSK-3 was discovered in the context of glycogen metabolism and emerged as a ubiquitous regulator of multiple signaling pathways (Woodgett 1990).

The role of GSK-3 signaling pathways was initially described in the Wnt/β-catenin pathway (Walz et al. 2017). GSK-3-mediated β-catenin phosphorylation has a critical role in Wnt/β-catenin signaling. Wnt signaling inhibits GSK-3β, thus preventing β-catenin degradation and stabilizing β-catenin, which in turn increases transcriptional activity of c-Myc and cyclin-D (Yost et al. 1996). GSK-3β can also phosphorylate several upstream and downstream components of PI3K/AKT/mTOR pathway including AKT, RICTOR, and PTEN (Hermida et al. 2017). Moreover, the regulatory roles of GSK-3β in cell cycle (Wang et al. 2008), apoptosis (Beurel and Jope 2006), tumor invasion, and metastasis have been well described (Kroon et al. 2014; Matsuo et al. 2018).

Several GSK-3β inhibitors (Fig. 22.13), including tideglusib, LY2090314, solasodine, AR-A014418, 9-ING-4, SB-216763, SB-415286, TDZD-8, TWS119, AZD2858, AZD1080, and IM-12, have been discovered and advanced to early-stage clinical trials for multiple cancer types (Palomo and Martinez 2017). The majority

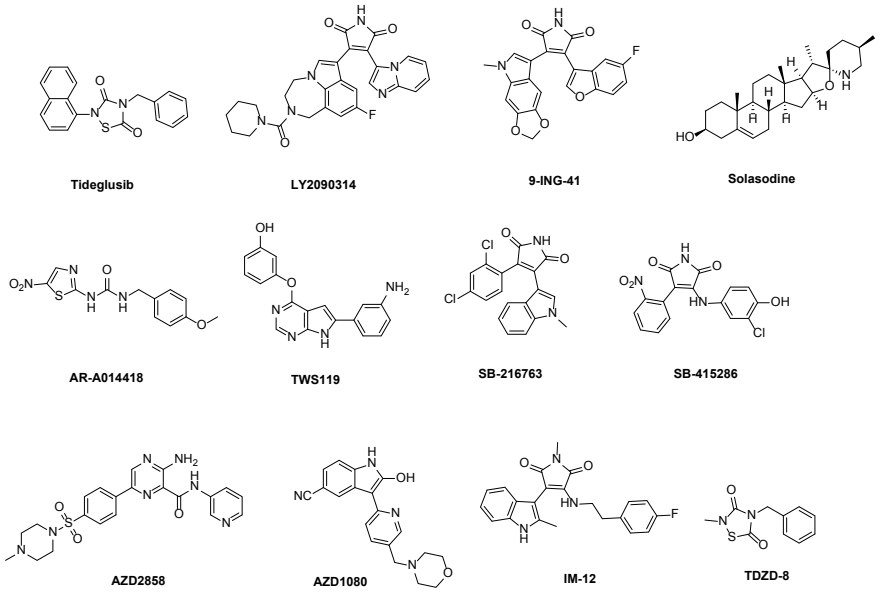


Fig. 22.13 Structures of representative GSK-3β inhibitors

Table 22.6 GSK-3 inhibitors in clinical trial

Compound	Target	Development status	Indication(s)	ClinicalTrials.gov identifier
Tideglusib (NP031112)	GSK-3α; GSK-3β	Phase II	Myotonic dystrophy	NCT02858908
		Phase II	Autism spectrum disorders	NCT02586935
		Phase II/III	Congenital myotonic dystrophy	NCT03692312
		Phase II	Alzheimer's disease	NCT01350362
		Not applicable	Progressive supranuclear palsy	NCT01049399
		Phase I/II	Alzheimer's disease	NCT00948259
LY2090314	GSK-3α; GSK-3β	Phase I	Advanced cancer	NCT01287520
		Phase I/II	Pancreatic cancer	NCT01632306
		Phase II	Acute leukemia	NCT01214603
9-ING-41	GSK-3α; GSK-3β	Phase I/II	Advanced cancers	NCT03678883

of the GSK-3 inhibitors are ATP competitive with low selectivity. No selective GSK-3β inhibitor has been approved by FDA for treatment of cancer or other diseases (Sahin et al. 2019). However, emerging data support GSK-3β as a promising cancer therapeutic target, and a number of GSK-3β inhibitors are currently in clinical development (Table 22.6).

Tideglusib is an ATP noncompetitive GSK-3β inhibitor and was originally developed for treatment of Alzheimer disease through decreasing tau phosphorylation and increasing proapoptotic proteins, which was validated in murine neuroblastoma models (Mathuram et al. 2016). Tideglusib was also found being able to decrease colony formation and increased G0/G1 population in tumor cells. The intracranial glioblastoma xenograft study showed that tideglusib can sensitize temozolomide with improved survival in mice (Zhou et al. 2016). It was also found that tideglusib increased NK cell cytotoxic activity in human AML mouse models. Tideglusib is well tolerated and currently being tested in phase II clinical studies for Alzheimer's disease and progressive supranuclear palsy (Lovestone et al. 2015; Tolosa et al. 2014). To our best knowledge, there is no active cancer clinical trial going on with tideglusib.

LY2090314 is an ATP-competitive GSK-3 inhibitor with obvious antiproliferative effects in preclinical melanoma and neuroblastoma models (Atkinson et al. 2015; Le Page et al. 2018). A phase I study found that LY2090314 was safe and well

tolerated, and primary antitumor activities were observed when in combination with pemetrexed and carboplatin in mesothelioma, non-small cell lung cancer (NSCLC), and breast cancer patients (Gray et al. 2015). The most reported adverse effects are minor, including nausea, decreased appetite patients, anemia, thrombocytopenia, and neutropenia in 11 out of 20 of patients (Rizzieri et al. 2016). However, none of the patients in the study had either complete remission or partial response. This implies that it may be necessary to combine LY2090314 together with other therapies, such as chemotherapy or immunotherapy (such as PD-1/PD-L1 antibodies) for future studies to improve the clinical efficacy.

Solasodine, a naturally occurring aglycone of glycoalkaloid, was reported with antitumor activities in different cancers by inhibiting GSK-3β pathway (Zhuang et al. 2017; Hameed et al. 2017).

AR-A014418 is a highly specific GSK-3β inhibitor and was reported to be able to enhance the cytotoxic effects of temozolomide and gemcitabine in GBM and pancreatic cancer models (Domoto et al. 2016). A preclinical study using AR-A014418 demonstrated that inhibition of GSK-3 decreased gastric cancer cell survival and proliferation through downregulating telomerase reverse transcriptase expression and telomerase activity (Mai et al. 2009). AR-A014418 is currently in clinical studies to evaluate the safety and efficacy.

9-ING-41 is an ATP-competitive, dual GSK-3α and GSK-3β inhibitor with better selectivity for GSK-3β over 320 other related kinases. In renal cancer, GSK-3β inhibition by 9-ING-41 decreased proliferation via G0–G1 and G2-M phase arrest and induced autophagy by modulating glucose metabolism (Pal et al. 2014). The study also showed the antitumor activity of 9-ING-41 in two RCC xenograft models. A phase I study designed to evaluate the safety and primary clinical efficacy of 9-ING-41 as monotherapy and in combination with other cytotoxic agents in advanced cancer patients is underway (NCT03678883).

Recent studies have demonstrated the important role of GSK-3β in regulating the immune response. Gattinoni et al. elaborated how effector T $CD8^+$ cells are able to differentiate into stem cells by inhibition of GSK-3β (Gattinoni et al. 2009). By blocking T-cell differentiation via GSK-3β inhibition, Wnt signaling was able to generate multipotent $CD8^+$ memory stem cells with enhanced antitumor capacities both in vitro and in vivo in a gastric cancer study (Zhang et al. 2018).

In a GBM-specific CAR-T-cells model, inhibition of GSK-3β increased survival and memory phenotype generation with enhanced tumor-killing ability in GSK-3β-inhibited IL13 CAR-T-cells (Sengupta et al. 2015, 2018). These results suggest that GSK-3 inhibitors could enhance antitumor response of T-cells and combination with CAR-T immunotherapy could further enhance the antitumoral capacity of CAR-T immunotherapy.

GSK-3 is linked to increased programmed cell death-1 (PD-1) expression and its inhibition enhanced T-cell response (Taylor et al. 2016, 2018). Taylor et al. investigated further how GSK-3 inhibition decreased tumor growth and metastasis by downregulating PD-1 on $CD8^+$ T-cells in a melanoma model, while having a minimal effect on NK-cells and no obvious effect on $CD4^+$ T-cells (Taylor et al. 2016). Despite these findings, a recent study suggested a role of GSK-3β in destabilizing PD-L1 (Li

et al. 2016), thus GSK-3β inhibitors may potentiate PD-L1-dependent immunosuppression. In this regard, the effects of GSK-3β inhibitors should be interpreted with enough caution in both basic and translational studies.

The emerging promising preclinical results have demonstrated the important role of GSK-3β in regulating anticancer immune response. Further characterization on the molecular and cellular events following GSK-3β inhibition would help understand the therapeutic effects and determine the strategies for patient selection and combination therapies.

22.4 Tumor Microenvironment Modulators

22.4.1 CSF-1R Inhibitors

It is well acknowledged that tumor-associated macrophages (TAMs) are involved in carcinogenesis and immunosuppression, correlated with poor prognosis and survival (Tamimi et al. 2008; Zhang et al. 2012). CSF-1R, also known as macrophage colony-stimulating factor receptor (M-CSFR), is a cell-surface protein encoded in humans by CSF-1R proto-oncogene (also known as c-FMS) (Sherr et al. 1985) and acts as a cell-surface receptor for cytokine CSF-1 and IL-34 (Lin et al. 2008). The CSF-1R/CSF-1 pathway is involved in the regulation of the survival, proliferation, differentiation, recruitment, and function of the tumor-associated macrophages (TAMs) in tumor microenvironment (Hume and MacDonald 2012; Hamilton 2008; Chitu and Stanley 2006).

CSF-1R activation requires CSF-1 binding and the subsequent receptor dimerization. Blockage of CSF-1R using either monoclonal antibodies (mAbs) or small-molecule CSF-1R inhibitors can prevent CSF-1 and IL-34 binding, which decreases TAM proliferation, differentiation, survival, and relieves the effect of TAMs in tumors. Moreover, blocking CSF-1R removes the immunosuppressive influence of TAMs, which enhances the activity of tumor-reactive T-cells and can potentially lead to antitumor activity (Ries et al. 2014).

A number of small molecules (Fig. 22.14), including pexidartinib (PLX3397), ARRY-382, PLX-7486, BLZ945, ABT-869, JNJ-40346527, directed at CSF-1R are in clinical development both as monotherapy or in combination with standard treatment therapies such as chemotherapy as well as other cancer immunotherapy (Tables 22.7 and 22.8) (Cannarile et al. 2017).

PLX-3397 (pexidartinib) is a multi-target receptor tyrosine kinase inhibitor and is the most advanced selective CSF-1R inhibitor under clinical development. A phase II study in 38 patients with recurrent GBM treated with pexidartinib did not show significant improvement in 6-month progression-free survival (PFS) compared to historical control data. Of 38 patients, seven (18%) experienced stable disease; no partial or complete responses were observed (Butowski et al. 2016). A clinical trial for the treatment of 126 patients with tenosynovial giant cell tumor (TGCT) using

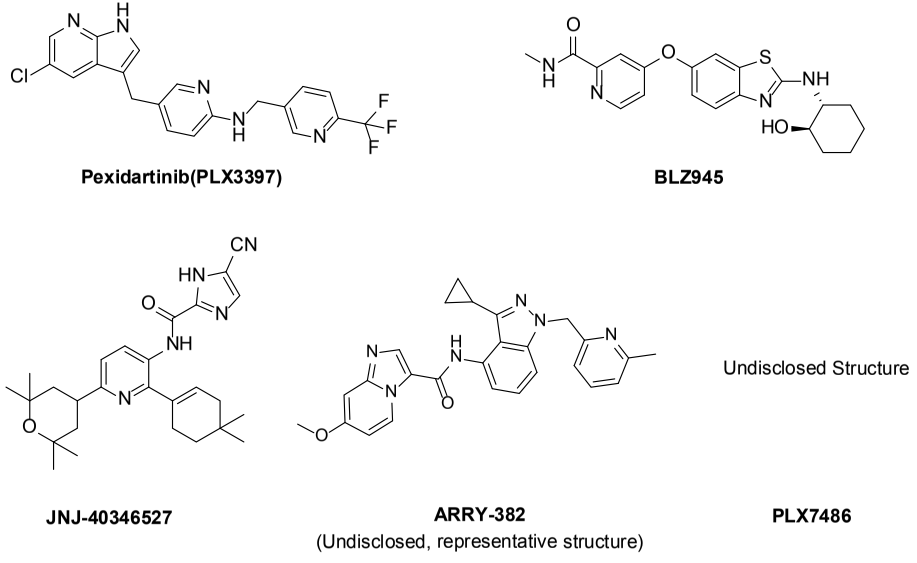


Fig. 22.14 Representative CSF1R inhibitors

Table 22.7 CSF1/CSF1R inhibitors as monotherapy in clinical trial

Compound	Target(s)	ClinicalTrials.gov identifier	Phase	Indication(s)	Status/results
Pexidartinib (PLX3397)	CSF-1R (and c-Kit, Flt3)	NCT02975700	1/2	Melanoma	Ongoing
		NCT02071940	2	Malignant melanoma	Ongoing
		NCT02390752	1/2	Refractory leukemia and refractory solid tumor	Ongoing
		NCT01499043	2	Advanced CRPC	Not yet reported
		NCT01349036	2	Recurrent glioblastoma	ORR: 0% CBR: 18%
		NCT01090570	1	Rheumatoid arthritis	Withdrawn
		NCT01217229	2	Hodgkin lymphoma	ORR: 5%
		NCT01349049	1/2	Acute myeloid leukemia	Ongoing
		NCT01004861	1	Solid tumor	Ongoing
		NCT02371369	Marketed	PVNS, GCT-TS	Approved in 2019 by FDA
ARRY-382	CSF-1R	NCT01316822	1	Metastatic cancer	ORR: 0% CBR: 25%
PLX7486	CSF-1R (and Trk)	NCT01804530	1	Solid tumor	On hold
BLZ945	CSF-1R	NCT02829723	1/2	Advanced solid tumors	Ongoing
JNJ-40346527 (PRV-6527)	CSF-1R	NCT03557970	2	Refractory AML Recurrent adult AML	Ongoing
		NCT03177460	1	Prostate adenocarcinoma	Ongoing
		NCT01054014	1	Health	Not yet reported
		NCT01572519	1	Relapsed or refractory Hodgkin lymphoma	ORR: 5% CBR: 52%
		NCT01597739	2	Arthritis, rheumatoid	Not yet reported
		NCT03854305	2	Crohn's disease	Ongoing

Table 22.8 Clinical trials with CSF1/CSF1R inhibitors in combination with cancer immunotherapy agents

Compound	Target	Combination	Phase	Indication(s)	ClinicalTrials.gov identifier
Pexidartinib (PLX3397)	CSF-1R (and c-Kit, Flt3)	Pembrolizumab	1/2	Melanoma, NSCLC, GIST, HCCHN, ovarian carcinoma	NCT02452424
		Durvalumab	1	Colorectal cancer, pancreatic cancer, metastatic cancer, advanced cancer	NCT02777710
ARRY-382	CSF-1R	Pembrolizumab	1/2	Advanced solid tumors	NCT02880371
BLZ945	CSF-1R	PDR001	1/2	Advanced solid tumors	NCT02829723
JNJ-40346527	CSF-1R	Daratumumab	1	Prostate adenocarcinoma	NCT03177460

pexidartinib met its primary end point, and US FDA approved its TGCT treatment on August 2, 2019. In a study of 20 heavily pre-treated patients with cHL, an objective response rate (ORR) of 5% was reported with single-agent PLX3397 treatment (Moskowitz et al. 2012; Butowski et al. 2014). Comparable efficacy in relapsed or refractory cHL was demonstrated with JNJ-40346527 in a phase I/II clinical study. Out of 21 patients enrolled, one showed a complete response (ORR 5%) and 11 (52%) experienced stable disease (von Tresckow et al. 2013).

ARRY-382 (structure undisclosed) is a potent and highly selective oral small-molecule CSF-1R inhibitor. Results from a phase I study investigating ARRY-382 in advanced solid tumors were recently reported by Bendell et al. (2013). Out of the 26 patients, four (15%) had stable disease, and no objective responses were observed.

Gabrilovich et al. reported that the lack of antitumor effect in mice and several failed clinical trials of CSF-1R inhibitors may be caused by the migration and accumulation of tumor-promoting polymorphonuclear myeloid-derived suppressor cells (PMN-MDSCs) in tumor microenvironment after CSF-1R blockage (Kumar et al. 2017). The study showed that CSF-1 produced by tumor cells repressed the production of granulocytic chemokines by carcinoma-associated fibroblasts (CAF) and limited granulocyte recruitment. CSF-1R inhibition altered the chemokine expression by CAF and caused substantial accumulation of PMN-MDSCs, and thus resulted in poor efficacy. The combination of CSF-1R and CXCR2 inhibitors significantly blocked MDSC recruitment and reduced tumor growth. This tumor inhibition effect was further improved by the addition of anti-PD-1 to this combination.

In summary, emerging data from clinical trials demonstrated favorable safety profile and primary clinical efficacy for CSF1/CSF-1R inhibition. Due to the plasticity of TAM, the antitumor effect of CSF-1R inhibitors could be tempered by other compensatory pathways, and the combined use of CSF-1R inhibitors and other agents such as CXCR2 inhibitors may provide more meaningful clinical benefit for cancer patients.

22.4.2 TGF-β and ALK5

TGF-β signaling pathway has pleiotropic functions in regulating cell growth, differentiation, apoptosis, motility and invasion, extracellular matrix production, angiogenesis, and immune response (Neuzillet et al. 2015). Elevated TGF-β expression by NSCLC, CRC, gastric, and prostate cancer has correlated with tumor progression and poor prognosis (Massague 2008). Although a functional switch known as the "TGF-β paradox" exists in the TGF-β signaling pathways due to its differential effects at the early and late stages of carcinogenesis (Wendt et al. 2012), it is undisputed that the TGF-β pathway plays a critical role in generating a favorable microenvironment for tumor growth and metastasis throughout all the steps of carcinogenesis (Neuzillet et al. 2015). Targeting TGF-β pathway in cancer may be considered primarily as a microenvironment-targeted strategy. This also allows for potential combination with other therapies such as immune checkpoint inhibitors and chemotherapies (Marin-Acevedo et al. 2018).

Preclinical and clinical studies demonstrated that targeting TGF-β signaling could be useful in treatment of fibrosis and tumors, particularly in augmentation of existing cancer therapies, such as radiation and chemotherapy, as well as in tumor vaccines (Akhurst and Hata 2012). There are three methods for blocking the TGF-β pathway: blocking the ligand, disrupting the ligand–receptor interaction, or inhibiting the receptor tyrosine kinase activity.

TGF-β-receptor 1 (TGFBR1, ALK5), a member of the TGF-β receptor subfamily, is a Ser/Thr protein kinase that forms a heteromeric complex with type II TGF-β receptors when binding to TGF-β. This heterodimer complex phosphorylates the intracellular proteins SMAD2 and SMAD3, activating a signaling cascade to induce several nuclear transduction proteins (Herbertz et al. 2015).

Among the TGF-β inhibitors, small-molecule inhibitors (SMIs) represent a large and diverse group of chemical entities including galunisertib (Sawyer et al. 2004) and TEW-7197 (Jin et al. 2014). Galunisertib (LY2157299 monohydrate) is an orally bioavailable small-molecule inhibitor against TGFBR1 kinase that specifically downregulates the phosphorylation of SMAD2, abrogating activation of the canonical pathway. In 2013, galunisertib received orphan drug designation in HCC by the EMA (March) (EMA/COMP/95768/2013) and the FDA (April) (Herbertz et al. 2015). It is currently being evaluated in a phase II/III clinical study for myelodysplastic syndromes (MDS) (NCT02008318). The primary result of NCT02008318 in patients with very low, low, or intermediate risk MDS showed that galunisertib induced a

hematological improvement (HI) rate of 26%, HI rate of the subgroup of patients who required ≥4 units of packed red blood cells/8 weeks was 38% (Valcarcel et al. 2015). However, the final efficacy data are not yet available. Another phase II clinical study in recurrent glioblastoma failed to demonstrate the improved OS compared to placebo (Brandes et al. 2016). This molecule is also being studied in NSCLC, hepatocellular carcinoma, pancreatic cancer, rectal adenocarcinoma, metastatic breast cancer, glioblastoma, and metastatic colorectal cancer (ClinicalTrials.gov). Vactosertib (TEW-7197) is another TGFBR1 small-molecule inhibitor in clinical investigation. In mid-2014, a phase I study of TEW-7197 was initiated in patients with breast cancer, melanoma, hepatocellular carcinoma, and glioblastoma (MedPacto 2019). Currently, no clinical data is reported yet.

As of today, there are only two TGFBR1 small-molecule inhibitors in clinical investigation, perhaps due to the complicated biological function of TGF-β signaling pathway and the observed severe cardiac toxicities in animal studies (Garber 2009). Undoubtedly, there exist challenges with the development of TGFBR1 inhibitors. First, the activity of TGF-β inhibition is dependent on a subtle modulation of the EMT, stem cell function, and immune function. Hence, for the drug development, it will be important to investigate new biomarkers that are related to EMT, stem cell function, and immune responses. Second, the patient selection tools defining the population most likely benefitting from TGF-β inhibition remain one of the most challenging questions to date. Alternatively, combining TGF-β inhibitors with other agents may pose other unpredictable challenges (Herbertz et al. 2015).

22.4.3 CXCR Antagonists

CXCR4, a G-protein-coupled receptor, is mainly expressed in the hematopoietic and immune cells under normal physiological conditions (Duda et al. 2011), and frequently overexpressed in a number of cancers including breast, brain, ovary, and prostate cancer, melanoma, and cancer stem cells. Increased CXCR4 expression is associated with the recurrence, distant metastasis, and poor survival rates of cancers (Porcile et al. 2004; Furusato et al. 2010). Several researches have shown that cancers expressing CXCR4 tend to metastasize to the bones through the bloodstream in CXCL12 (also called SDF-1)-dependent manner through the CXCR4/CXCL12-mediated trafficking/homing mechanism (Woodard and Nimmagadda 2011; Highfill et al. 2014; Holm et al. 2007; Muller et al. 2001). High levels of CXCL12 in organs and tissues such as the lymph nodes, lungs, liver, and bones are believed to attract the migration of CXCR4-expressing cancer cells (Geminder et al. 2001). Therefore, inhibition of CXCL12/CXCR4 interaction is an attractive approach for potential cancer treatment (Varmavuo et al. 2012). Plerixafor, a first-in-class CXCR4 antagonist, was approved by the FDA in 2008 for the mobilization of hematopoietic stem cells in patients with cancer (Duda et al. 2011; Ha et al. 2017). Several other small-molecule CXCR4 inhibitors including MSX–122 and USL311 are currently being evaluated

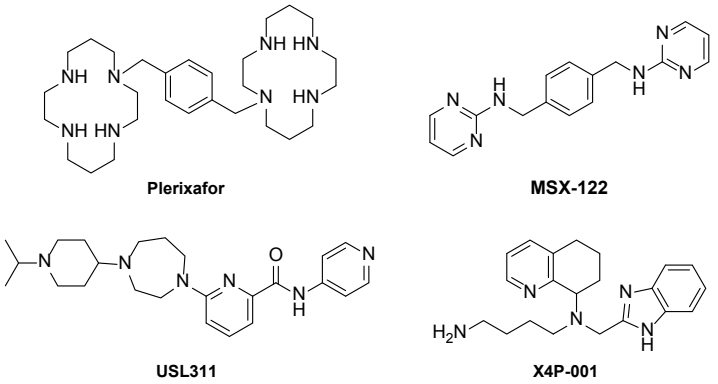


Fig. 22.15 Structures of CXCR4 antagonists

in clinical trials in participants with advanced solid tumors or relapsed/recurrent glioblastoma multiforme (GBM) (see Fig. 22.15 for structures).

X4P-001 (X4Pharma), an orally bioavailable CXCR4 inhibitor, has been shown to be able to reduce the tumor growth and increase the survival of mice in several preclinical cancer models. The clinical trials of combining X4P-001 with nivolumab for the treatment of renal cell carcinoma (NCT02923531) and combining with pembrolizumab in patients with advanced melanoma (NCT02823405) are currently undergoing.

CXCR1 and CXCR2 are highly related receptors. CXCR1/2 and their ligands are essential for the activation and trafficking of inflammatory mediators as well as for tumor progression and metastasis. The CXCR2-dependent accumulation of MDSCs in the tumor microenvironment has recently been demonstrated in both colitis-associated and rhabdomyosarcoma tumor models (Debnath et al. 2013; Hall and Korach 2003). Currently, several CXCR1/2 antagonists are in clinical trials with advanced cancer patients (see Fig. 22.16 for structures). Navarixin (MK-7123), a dual CXCR1/2 allosteric antagonist, is tested in a phase II clinical study to assess the efficacy and safety in combination with pembrolizumab (MK-3475) in adults with NSCLC, prostate cancer, or colorectal cancer (NCT03473925). SX-682, another CXCR1/2 dual antagonist, is currently in a phase I study with advanced melanoma to evaluate the safety and whether SX-682 can block cancers from attracting MDSCs

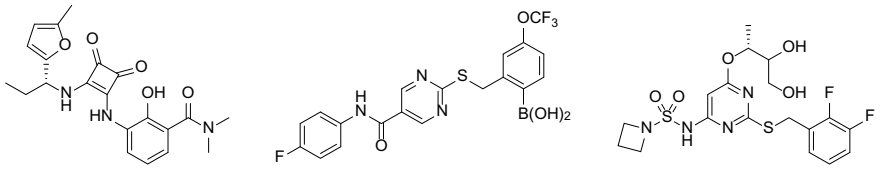


Fig. 22.16 Structures of CXCR2/(CXCR1) antagonists in clinical development

(NCT03161431). AZD5069, a specific CXCR2 inhibitor, is in phase II clinical study with advanced solid tumors.

22.4.4 CCR Antagonists

CCL2 and CCL5 are abundant in tumor tissue and attract tumor-associated macrophages (TAM) to tumor tissue to form the immune-suppressive environment for tumor growth and immune evasion. Various cells in the tumor microenvironment produce these cytokines, whose total abundance will define the ability of the tissue to attract inflammatory cells (Svensson et al. 2015). Inhibition of these axes has been actively explored as a novel approach to limit macrophage infiltration and pro-tumorigenic activities (Stewart and Smyth 2011).

Many CCL2/CCR2 pathway inhibitors have been developed and evaluated in clinical trials, including PF–4136309 (NCT02732938, Fig. 22.17), BMS–813160 (a dual CCR2/5 antagonist, NCT03767582 and NCT03496662, Fig. 22.17), and CCX872-B (structure not disclosed, NCT02345408, NCT03778879). In a human pancreatic cancer model, treatment with CCR2 inhibitor PF4136309 depleted inflammatory monocytes and macrophages from the primary tumor and premetastatic liver, which resulted in enhanced antitumor immunity, decreased tumor growth, and reduced metastases (Sanford et al. 2013).

CCL5 can be expressed by leukocytes, fibroblasts, endothelial cells, and MSCs at the tumor site as well as tumor cells; CCR5 is mainly expressed on TH1 cells, $CD8^+$ T-cells, monocytes, and macrophages. CCL5 in tumor attracts the infiltration of Treg

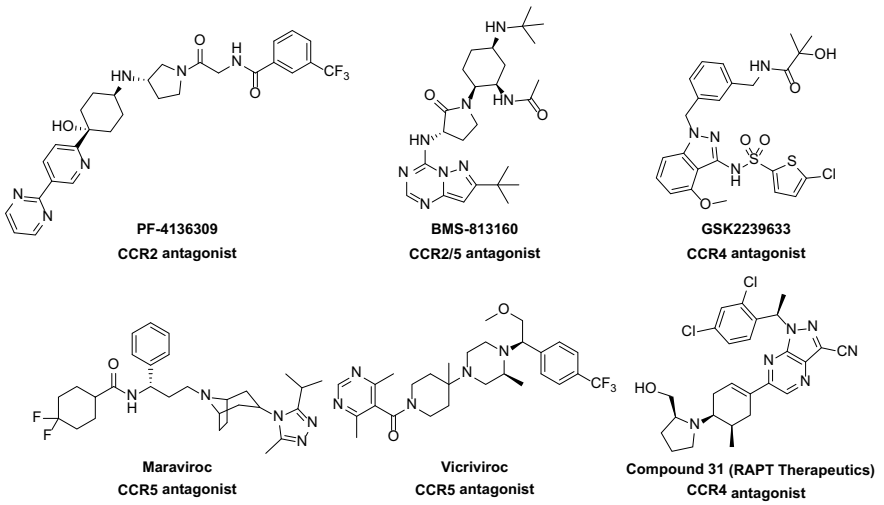


Fig. 22.17 Structures of representative CCR antagonists

cells as well as progenitor cells to generate the immune-suppressive tumor microenvironment filled with TAMs and MDSCs (Weitzenfeld and Ben-Baruch 2014). Maraviroc (Fig. 22.17) is a small-molecule CCR5 antagonist and was approved in 2007 for the treatment of HIV. The phase I study of Maraviroc for treatment of colorectal cancer was completed in 2014 and no results were reported (NCT01736813). Another CCR5 inhibitor, Vicriviroc (MK-7690, Fig. 22.17) is currently in phase I studies for patients with advanced/metastatic microsatellite stable (MSS) colorectal cancer (CRC) (NCT03631407).

CCR4 is highly expressed on Treg and plays a dominant role in the recruitment of highly immunosuppressive $CD4^+$, $CD25^+$, and $FOXP3^+$ Treg to the TME. Therefore, targeting CCR4 to inhibit the Treg trafficking pathway becomes an attractive immuno-oncology approach (Jackson et al. 2019). In addition, CCR4 plays an important role in the recruitment of T-helper type 2 cells (Th2) in autoimmune disorders such as asthma, allergic rhinitis, and atopic dermatitis. Several CCR4 antagonists have been discovered for treatment of autoimmune diseases, such as GSK2239633 (Fig. 22.17), which was originally developed for asthma treatment. The clinical development of GSK2239633 was terminated due to low target engagement and low plasma exposure (Jackson et al. 2019). FLX475, a small-molecule CCR4 antagonist developed by RAPT Therapeutics (formerly known as FLX Bio Inc.), is well tolerated without immune-related adverse events or significant clinical adverse events in the phase I studies (https://rapt.com/pipeline/flx475/). A phase I/II study (NCT03674567) of evaluating FLX475 as a monotherapy or in combination with pembrolizumab in patients with charged and virally associated tumors is undergoing. Although the exact structure of FLX475 has not yet been disclosed, RAPT therapeutics discloses the structure of analogues (Fig. 22.17) recently (Jackson et al. 2019).

Due to the role of CCR2/CCL2, CCR5/CCL5, and CCR4/CCL22 axes in TME, it is expected that the combination of CCR2, CCR5, and CCR4 antagonists with other immune therapies such as checkpoint inhibitors may significantly improve the cancer treatment outcome.

22.5 Epigenetic Regulation of Immune Response

22.5.1 HDAC Inhibitors

Tumor occurrence and development involve a substantial change in functions of both oncogenes and tumor suppressor genes. It has been well established that epigenetic regulation of genes is a major mechanism in carcinogenesis, apart from the genetic abnormality of the cancer-related genes. The mechanisms of epigenetic control of genes involve changes of gene expression patterns through modifications of histones and/or DNA. Of these modifications, histone acetylation/deacetylation plays a central role in epigenetic regulation of genes. Histone deacetylases (HDACs) are critical

regulators of gene expression that enzymatically remove the acetyl group from histones. The activity of HDACs on nonhistone proteins is also a key aspect of HDAC function (Spange et al. 2009).

HDACs remove the acetyl group from lysine residue in the presence of a Zn^{2+} ion, producing an unacetylated lysine and acetate (Li et al. 2010). Depending on their structure and intracellular localization, HDACs are classified as three classes (Class I, II, and IV) containing 11 HDAC isoforms. Class III HDACs are generally called sirtuins, which function as lysine deacetylases in the presence of NAD^+ and will not be discussed here.

Class I HDACs (including HDAC1, 2, 3 and 8) are usually located in the nucleus and catalyze a set of nonhistone substrates including transcription factors besides histones. Class II HDACs (including HDAC4, 5, 6, 7, 9, 10) show different sequence homology and domain organizations compared with class I HDACs, and conducting different downstream functions (Yang and Gregoire 2005). Class II HDACs are further divided into two subgroups, IIa and IIb. Class IIa members include HDAC4, HDAC5, HDAC7, and HDAC9, and are localized in both nucleus and cytoplasm. Some cytoplasmic proteins such as structural proteins are regulated by Class IIa HDACs. HDAC6 and 10 are mostly confined to cytoplasm (Guardiola and Yao 2002; Tong et al. 2002; Hubbert et al. 2002; Grozinger et al. 1999; Kao et al. 2002). Class IV HDAC (HDAC11) contains nine deacetylase motifs shared by both class I and II HDACs (Gao et al. 2002).

A number of HDAC inhibitors with different selectivity have been developed for clinical testing with vorinostat and romidepsin approved by US FDA for refractory CTCL, belinostat recently approved by US FDA and chidamide approved by Chinese NMPA for peripheral T-cell lymphoma (PTCL), and panobinostat for multiple myeloma. Several others are currently being assessed at different stages of clinical trials.

According to their chemical structures, HDAC inhibitors can be categorized as hydroxamic acids, cyclic tetrapeptides, benzamides, aliphatic acids, and electrophilic ketones (Manzotti et al. 2019).

Recent studies have shown that HDACs are involved in various immunomodulatory activities, implying the rationale for the combinational use with immune checkpoint inhibitors (Shen et al. 2016). Much effort has demonstrated that HDAC inhibition can promote tumor cell-specific apoptosis and its anticancer efficacy has been validated clinically. The presence of dead cancer cells is essential for antigen-presenting cells (APC)-mediated activation of cytotoxic T-cells, and enhances the response of immune-stimulatory therapies (Christiansen et al. 2011). In addition, HDAC inhibition can inhibit T-cell apoptosis, prevent activation-induced cell death, and enhance $CD4^+$ T-cells infiltration (Cao et al. 2015). Woods and Booth reported that HDACi treatment enhanced the effect of anti-PD1 immunotherapy in melanoma through different molecular mechanisms. Woods and colleagues discovered that inhibition of class I HDAC increased the expression of PD-L1 and PD-L2 (Woods et al. 2015), while Booth and colleagues observed the upregulation of class I MHC protein expression, following the treatment with pan-HDAC inhibitors (Booth et al. 2017). It has also been shown that HDAC inhibition can impair myeloid-derived suppressor

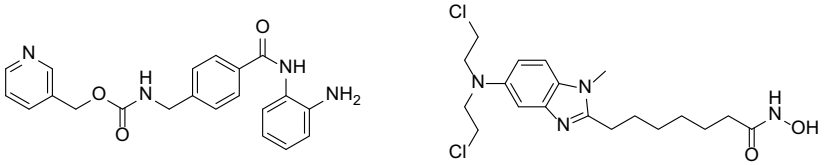


Fig. 22.18 The structures of entinostat and EDO-S101

cells (MDSCs), a population of immunosuppressive cells. Through inhibiting the function of MDSCs, the combination use of HDAC inhibitors improved the efficacy of both anti-PD1 and anti-CTLA4 anticancer therapies (Kim et al. 2014; Orillion et al. 2017).

There are several ongoing clinical trials for combinations of HDAC inhibitors with immunotherapeutic agents. A phase I/II study (NCT01038778) result for combination of entinostat (Fig. 22.18) with interleukin-2 (IL2) in metastatic renal carcinoma has been reported and demonstrated promising clinical efficacy (Pili et al. 2017). Professor M. Joerger recently started the first-in-human clinical trial (NCT03903458) of combing anti-PD-1 monoclonal antibody nivolumab with tinostamustine (EDO-S101), a first-in-class alkylating histone deacetylase inhibitor (Fig. 22.18) in patients with refractory, locally advanced, or metastatic melanoma (Tinostamustine and Nivolumab in advanced melanoma (ENIgMA) 2019). With more translational research and further understanding the biological function of different isoforms of HDACs in tumorigenesis and immune regulation, it is expected that more selective HDAC inhibitors will be developed, especially for the combination use with immunotherapy agents in the future.

22.5.2 BET

The bromodomain and extra-terminal domain (BET) family comprises four members, BRD2, BRD3, BRD4, and BRDT. These four members all share two *N*-terminal tandem bromodomains and a *C*-terminal extra-terminal motif. These proteins bind and "read" acetylated histones, and in turn recruit other proteins to form complexes that stimulate transcription initiation and elongation. The basic functions and the pathological functions of the four paralogous BET proteins are summarized by Taniguchi (2016).

BRD4 is the most characterized member of this family and heavily implicated in transcriptional regulation and tumorigenesis (Donati et al. 2018). BRD4 localizes on both gene promoters and enhancers, and has been shown to accumulate specifically on regulatory regions termed "super-enhancers" (Loven et al. 2013). Aberrant BRD4 expression contributes to carcinogenesis by mediating hyperacetylation of the chromatin containing the cell proliferation-promoting genes. Many oncogenes are

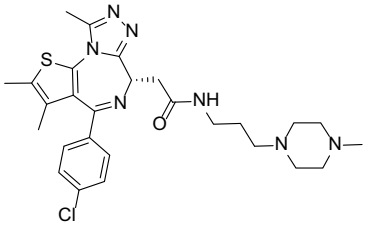
under the control of BRD4 to regulate cancer cell proliferation, apoptosis resistance, and tumor aggressiveness. c-MYC is the first oncogene that has been described to be regulated by BRD4, both in solid tumors and hematological malignancies (Delmore et al. 2011; McCleland et al. 2016), providing the rationale for the development of pharmacological inhibitors of BET proteins. Besides c-MYC, it is reported that several other oncogenes are controlled by BRD4, including FOSL1 (FRA-1), BCL-2, RUNX2, and c-KIT (Lockwood et al. 2012; Wyce et al. 2013; Sancisi et al. 2017; Zhao et al. 2016). In addition, BRD4 has been shown to regulate molecular mechanisms related to the repair of damaged DNA and to be implicated in aberrant telomere regulation in cancer, highlighting the diversity of functions of this protein in carcinogenesis (Li et al. 2018; Wang et al. 2017). A translocation between NUTM1 gene and BRD3 or BRD4 has been reported in 75% of NUT-midline carcinomas, leading to a formation of a fusion protein that is believed to promote transcription of oncogenes (French et al. 2003).

A number of clinical trials evaluating different BETi in different cancer types are currently undergoing, however without certain conclusions yet. Small-molecule BET inhibitors including I-BET762, I-BET151 (GSK2820151), JQ1, OTX-015, TEN-010, CPI-0610, FT-1101, INCB054329, INCB057643, BMS-986158, ABBV-075, ABBV-744, GS-5829, PLX51107, and BAY1238097 are in clinical development both as monotherapy or in combination with standard treatment therapies such as chemotherapies and other cancer immunotherapies.

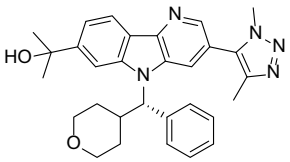
Lai et al. developed a mathematical model for cancer treatment by combination of BET inhibitors and CTLA-4 inhibitor (Lai et al. 2018). In ovarian cancer xenograft models, BET inhibition resulted in decreased PD-L1 expression in immune and cancer cells, suggesting a possible synergism between BET and immune checkpoint inhibitors (Zhu et al. 2016). Hogg's group also reported that BET bromodomain inhibitors promote antitumor immune responses through transcriptional repression of immune checkpoint ligand PD-L1 in genetically diverse tumor models and in response to inflammatory stimuli (Hogg et al. 2017).

BET inhibitors have been successfully used in preclinical combination with immune checkpoint inhibitors (PD-L1) (Hogg et al. 2017). Two recent papers showed that BETi can increase the efficacy of both anti-PD1 and anti-CTLA4 monoclonal antibodies. In particular, in a KRAS-mutated NSCLC model, it has been shown that BETi remodeled the cancer-immune microenvironment, by reducing tumor-infiltrating Treg and inducing T-helper type1 lymphocytes (Lai et al. 2018; Adeegbe et al. 2018). The clinical trial of BMS-986158 (NCT02419417, Recruiting, Fig. 22.19) combining with nivolumab (PD-1) for the treatment of solid malignancies is still undergoing and the trial of TEN-010 (NCT03292172, Fig. 22.19) combining with atezolizumab (PD-1) is put on hold for undisclosed reasons (Manzotti et al. 2019).

While the potential of using BET inhibitors for treatment of cancer is promising, more clinical trials are still needed to verify the clinical efficacy of BET inhibitors either as monotherapy or as combination therapy with immune checkpoint



TEN-010



BMS-986158

Fig. 22.19 TEN-010 and BMS-986158 structures

inhibitors. With further mechanistic elucidation of BET in regulating the transcription of immune-modulating genes, BET inhibitors would find their special place in cancer treatment, most likely in combination with other immunotherapies.

22.5.3 EZH2 Inhibitors

Enhancer of zeste homolog 2 (EZH2) is a histone methyltransferase that functions as the catalytic subunit of the polycomb repressive complex 2 (PRC2) (Gulati et al. 2018). The other core subunits in this complex include embryonic ectoderm development (EED), suppressor of zeste 12 (SUZ12), and retinoblastoma (Rb) associated protein 46. EZH2 is responsible for the methylation of lysine 27 of histone 3 (H3K27) to generate H3K27me3, a histone mark associated with a more condensed chromatin and transcriptional gene repression.

EZH2 has been shown to be essential for the proliferation of cancer cells. EZH2 overexpression and/or mutation have been found correlated with the development, progression, and aggressiveness of a variety of cancers, including prostate cancer, breast cancer, bladder cancer, endometrial cancer, and melanoma (Varambally et al. 2002; Bracken et al. 2003; Bachmann et al. 2006; Victora and Nussenzweig 2012).

Due to the evidence for EZH2 enzymatic gain of function being a cancer driver, the development of EZH2 inhibitors as cancer treatment therapy has attracted extensive interest. A number of EZH2 inhibitors have been developed for clinical testing with early results suggesting potential clinical activity (see Fig. 22.20 and Table 22.9).

Tazemetostat is the leading EZH2 inhibitor in clinical development. A phase II study showed that tazemetostat resulted in clinically meaningful and durable responses with well-tolerated safety profile. The phase II study data on sarcoma treatment were presented at the 2019 American Society of Clinical Oncology (ASCO) Annual Meeting. Based on the ongoing phase II study results, US FDA granted a priority review to a new drug application for the accelerated approval of tazemetostat to treat metastatic or locally advanced epithelioid sarcoma not eligible for curative surgery.

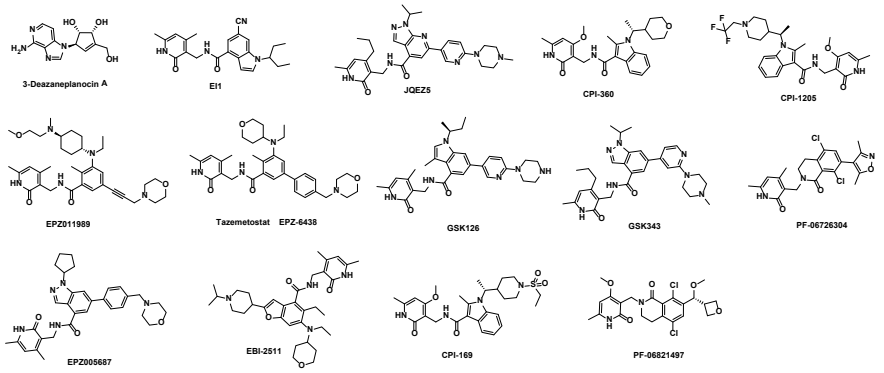


Fig. 22.20 Structures of EZH2 inhibitors

Table 22.9 The summary of EZH2 inhibitors in clinical trials

Compound	NCT ID	Development stage	Tumor type
Tazemetostat	NCT01897571	Phase I/II	Phase I: B-cell NHL and solid tumors; Phase II: DLBCL, FL
	NCT03213665 Phase II subprotocol: NCT03155620	Phase II	NHL and solid tumors
	NCT02889523	Phase Ib-II	DLBCL
	NCT02220842	Phase I	DLBCL, FL
	NCT03217253	Phase I	B-cell NHL and solid tumors
CPI-1205	NCT02395601	Phase I	B-cell NHL
GSK126	NCT02082977	Phase I	NHL, MM expansion cohort: DLBCL

DLBCL Diffuse large B-cell lymphoma, *FL* follicular lymphoma, *NHL* non-Hodgkin lymphomas, *MM* multiple myeloma

The phase I clinical trial of EZH2 inhibitor GSK126 was less encouraging and recently terminated as the maximal dose and schedule showed insufficient evidence of clinical activity. Haixia Long and Bo Zhu et al. reported that treatment with EZH2 inhibitor GSK126 inhibited tumor growth in immune-deficient, but not in immune-competent hosts. In the immune-competent hosts (C57BL/6 mice), GSK126 promoted myeloid-derived suppressor cells (MDSCs) generation which suppressed anti-tumor T-cell immunity (Huang et al. 2019). Besides the increase of MDSC numbers, $CD4^+$ and IFN-γ^+CD8^+ T-cells decreased. Addition of a neutralizing antibody against the myeloid differentiation antigen GR-1 or MDSC depletion agents gemcitabine/5-fluorouracil (5Fu) alleviated MDSC-mediated immunosuppression and increased $CD4^+$ and $CD8^+$ T-cell tumor infiltration and therapeutic efficacy. This may explain

the less desirable clinical efficacy of GSK126 in clinical trial and imply the need for combination use with other MDSC suppression agents.

Lukas Sommer and Onur Boyman et al. reported that EZH2 serves as a molecular switch controlling melanoma immunosurveillance escape (Zingg et al. 2017). In the melanoma models, treatment with anti-CTLA-4 and IL-2 immunotherapies lead to increased EZH2 activity that was dependent on T-cells and TNF-α, resulted in loss of immunogenicity, antigen presentation, and upregulation of the PD-1/PD-L1 axis. The inhibition of EZH2 resulted in the restored presentation of several dominant melanoma antigens while downregulating PD-L1 on melanoma cells. In parallel, PD-1 expression on tumor-antigen-specific and polyclonal melanoma-infiltrating $CD8^+$ T-cells also decreased significantly, which increased IFN-γ production and cytotoxicity. The combined treatment using EZH2 inhibitor and anti-CTLA-4 and/or IL-2 showed clear synergistic effect in tumor inhibition.

The research on the combination of EZH2 inhibitors with immunotherapy agents is still limited. It can be predicted that more research of using EZH2 inhibitors in immunotherapy application will occur in the future due to the role of EZH2 in epigenetic modulation of immune response.

22.6 Other Anticancer Targets That Involve in Tumor Immune Modulation

22.6.1 VEGFR Inhibitors

Vascular endothelial growth factor (VEGF) is a signaling protein that stimulates angiogenesis—the formation of new blood vessels. VEGF family members in mammals consist of VEGF-A, -B, -C, -D, -E, and placenta growth factor (PLGF). There are three main isoforms of VEGFR: VEGFR-1, VEGFR-2, and VEGFR-3. VEGF/VEGFR2 signaling is one of the most important molecular signaling pathways that control tumor angiogenesis (Zhao and Adjei 2015). VEGFR-3 is mainly expressed on lymphatic vessel cells, but the other VEGFR and the Tie receptor family primarily exist in endothelial cells. VEGFR2 is phosphorylated after stimulation by VEGF-A binding and subsequently activates various downstream signaling pathways (Kowanetz and Ferrara 2006). With the understanding of function of VEGF/VEGFR in cancer cell growth and metastasis, not surprisingly, VEGFR has become a highly sought drug target with great success (Hato et al. 2016; Carmeliet and Jain 2011). A number of small-molecule VEGFR inhibitors have been approved for the treatment of various cancers as summarized in Table 22.10.

In addition to promoting angiogenesis and vascular permeability, VEGF/VEGFR axis also plays an important role in the regulation of multiple biological functions in tumor microenvironment, such as immunosuppression (Hato et al. 2016). VEGF acts as an immunosuppressive molecule via multiple mechanisms. By binding to VEGFR receptors, VEGF can inhibit the maturation of dendritic cells (CDs), increase the

Table 22.10 Clinically approved VEGFR inhibitors and their kinase inhibition profile

TKI	VEGFR1	VEGFR2	VEGFR3	Other targets
Sorafenib		+		Raf-1, B-Raf, B-Raf(V599E)
Sunitinib		+		c-Kit, FLT3, PDGFRβ
Lenvatinib	+	+	+	PDGFRα, PDGFRβ, FGFR1
Cabozantinib		+		c-MET, AXL, RET, c-Kit, FLT3, TRKB, Tie-2
Axitinib	+	+	+	PDGFRα, PDGFRβ, Kit, BCR-ABL1
Vandetanib		+	+	EGFR
Dovitinib	+	+	+	c-Kit, FLT3, FGFR1
Pazopanib	+	+	+	PDGFR, FGFR, c-Kit
Foretinib	+	+	+	MET, Tie2
Apatinib		+		RET

number of MDSC cells, and subsequently inhibit/suppress immune response. VEGF can also promote the infiltration of regulatory T-cell (Treg) and myeloid-derived suppressor cell (MDSC), while MDSCs inhibit both antigen presentation and $CD8^+$ cytotoxic T-cell (CTL) activity. In addition, VEGF can also enhance the expression of immune checkpoint molecule PD-1 on CTLs, which suppresses the biological activity of CTLs. Due to the function of increasing the number of intratumoral effector T-cells, reducing the accumulation of immunosuppressive regulatory T-cells and MDSCs, VEGFR inhibitors may find utility in combination with immuno-oncology agents besides the ability to normalize aberrant tumor vasculature (Desar et al. 2011).

A number of clinical studies are undergoing to evaluate the combination of VEGFR inhibitors with either anti-PD-1 or anti-PD-L1 agents for treatment of various cancers (Fig. 22.21). Encouraging clinical benefits have been observed with several combination therapies in advanced clinical studies (Table 22.11) (Amin et al. 2014).

Antiangiogenesis, especially VEGF/VEGFR targeted therapy, emerged as the standard of care for mRCC. The mccRCC treatment landscape is rapidly changing with the exploration of the combinations of immune checkpoint inhibitors (ICI) and anti-VEGF therapies. The results of IMmotion151, a randomized phase III study of atezolizumab plus bevacizumab (anti-VEGF antibody) versus sunitinib in untreated metastatic renal cell carcinoma (mRCC), were recently reported and demonstrated the superiority of the combination of atezolizumab plus bevacizumab to sunitinib treatment in terms of progression-free survival (PFS) (11.2 vs. 7.7 months, hazard ratio [HR] 0.74, p Z 0.02) and ORR (43% vs. 35%) in PD-L1 positive patients, per investigator assessment (Motzer et al. 2018).

The combination of Keytruda and axitinib was approved by FDA as the first-line therapy for the treatment of late-stage renal cell carcinoma (RCC) in April 2019. This is the second immune checkpoint inhibitor approved as 1st line treatment for RCC following the approval of the combination of nivolumab and ipilimumab in 2018.

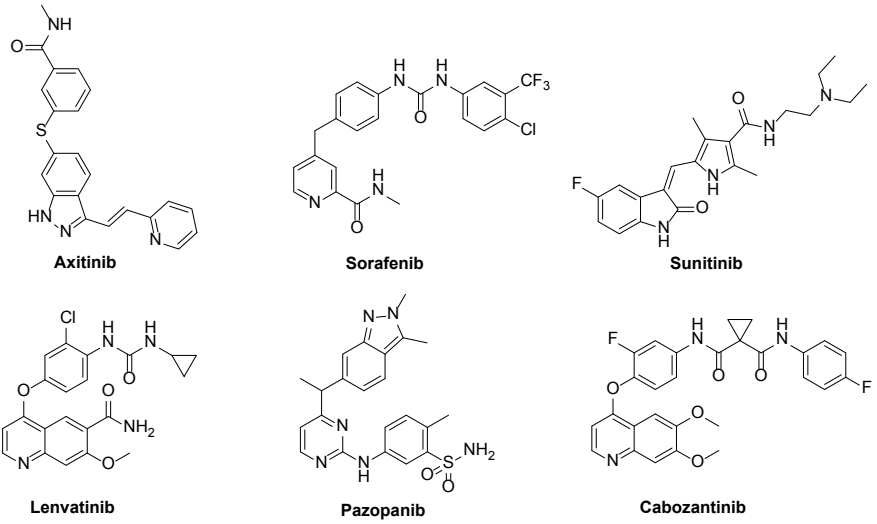


Fig. 22.21 The structures of clinically approved VEGF inhibitors

Table 22.11 VEGFR small molecules currently in immuno-oncology combination clinical trials (Astudy of SHR-1210 in combinationwith apatinib in advanced non-small cell lung cancer (NSCLC) 2017)

VEGFR inhibitor	Anti-PD-1 or anti-PD-L1 agent	Clinical trial ID	Status
Axitinib	Pembrolizumab	NCT02133742	Active, not recruiting
	Avelumab	NCT02684006	Active, not recruiting
		NCT02493751	Active, not recruiting
Lenvatinib	Pembrolizumab	NCT02501096	Recruiting
Sorafenib	PDR001	NCT02988440	Active, not recruiting
Sunitinib	Nivolumab	NCT01472081	Unknown
	Avelumab	NCT03035630	Withdrawn
Pazopanib	Nivolumab	NCT01472081	Unknown
	Pembrolizumab	NCT03260894	Active, not recruiting
Cabozantinib	Nivolumab or nivolumab and ipilimumab	NCT02496208	Recruiting

Several VEGFR tyrosine kinase inhibitors (TKI) have been approved for the first-line treatment of RCC, including sunitinib, cabozantinib, pazopanib, and lenvatinib. A number of clinical trials of combining different VEGFR inhibitors with PD-1/PD-L1 inhibitors are currently undergoing (Table 22.11) and some have shown encouraging clinical outcome.

The clinical study for late-stage renal cancer using the combination of pembrolizumab plus multiple kinase inhibitor Lenvatinib showed 83% ORR and 100%

DCR (30 enrolled patients). Based on this result, pembrolizumab plus lenvatinib was granted "Breakthrough Therapy" designation for late-stage renal cancer treatment. The combination of avelumab and axitinib was also awarded "Breakthrough Therapy" designation as first-line treatment of renal cancer.

Stephanie Du Four et al. studied the effect of combination of VEGFR inhibitor axitinib with CTLA-4-blockade on immune cells using subcutaneous and intracranial melanoma mouse models (Du Four et al. 2016). The study showed an increased number of $CD4^+$ and $CD8^+$ T-cells after combination treatment. Moreover, combination treatment increased intratumoral dendritic cells (DCs) and deceased monocytic myeloid-derived suppressor cells (moMDSCs). These results suggest that the combination of antiangiogenesis and checkpoint inhibition may lead to an enhanced antitumor effect and survival benefit due to the increased antigen-presenting function of intratumoral DCs and reduced intratumoral moMDSCs.

Even though preclinical and clinical results of combination of VEGF/VEGFR inhibition with immune checkpoint blockage (IBC) are encouraging, there are more challenges ahead for this combination use. It has been reported that the effect of anti-VEGF therapies was transient with a window of short duration (days to weeks) of vascular normalization depending on the tumor and dose of anti-VEGF agent used (Winkler et al. 2004; Huang et al. 2012). Winkler et al. discovered that ANG1/ANG2–TIE2 signaling mediates the recruitment of pericytes to the tumor vessels and played as a compensation mechanism by elevated expression of ANG2 to abrogate the benefit of anti-VEGF therapy (Winkler et al. 2004; Chae et al. 2010). The concomitant blockade of ANG2 and VEGF extends both the window of normalization and the survival benefit compared with single inhibition, in part, by reprogramming the immune-suppressive TME (Kloepper et al. 2016; Peterson et al. 2016). In glioblastoma patients, circulating ANG2 levels was elevated following treatment with VEGF pathway inhibitors (Batchelor et al. 2010). For melanoma patients with unfavorable response to checkpoint inhibitors, ANG2 level has also been observed to be elevated or increased. Therefore, ANG2 level could be used as a predictive marker of treatment response to ICB (Wu et al. 2017).

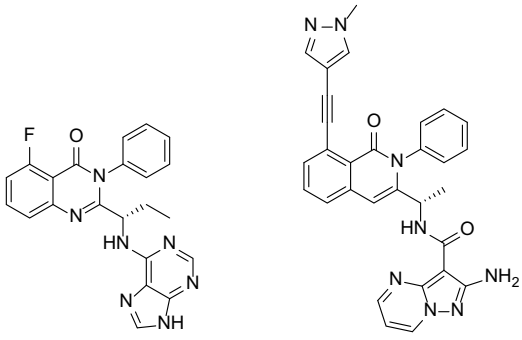
Besides the compensation pathways, the doses of anti-VEGF agents seem important to the treatment outcome. It has been noted that high doses of antiangiogenic agents resulted in a short normalization window by causing hypoxia and acidosis in the TME (Jain 2014). In addition, high doses of anti-VEGF agents also led to increased deposition of extracellular matrix that, together with hypoxia, promote the infiltration of immunosuppressive and/or pro-tumor immune cells, such as monocytic and granulocytic MDSCs (Rahbari et al. 2016; Jung et al. 2017a, b). In a mouse model of advanced-stage hepatocellular carcinoma (HCC), it was discovered that blockade of VEGF signaling using high doses of sorafenib, a multikinase inhibitor, increased the stromal cell-derived factor 1 (SDF1)-mediated recruitment of immunosuppressive cells, such as Treg cells and M2 macrophages, in addition to increased hypoxia. The shift toward an immunosuppressive TME was inhibited by blockade of SDF1 receptor C-X-C-chemokine receptor 4 (CXCR4) and resulted in tumor growth and metastasis inhibition and improved survival (Chen et al. 2015).

With the understanding of the compensation mechanisms after VEGF/VEGFR inhibition treatment and the impact of different dosing regime (dosage amount and dose schedule) to tumor microenvironment (TME), it is predicted that improved antitumor immune response could be obtained through proper combination of antiangiogenesis therapies and immunotherapies.

22.6.2 PI3K Inhibitors

Constitutive activation of the PI3K-AKT signal transduction pathway stimulates cell growth and proliferation. PI3Kα and PI3Kβ are found in most cell types, but PI3Kδ and PI3Kγ are primarily expressed in leukocytes (B- and T-cells and myeloid lineage cells). With their role in leukocytes, selective inhibition of either PI3Kδ or PI3Kγ is particularly attractive to regulate innate immunity while minimizing the effects on normal cells (Adams et al. 2015).

Significant progress has been made in the development of selective PI3Kδ or PI3Kγ inhibitors in the past decade. PI3Kδ plays a major role in B-cell signaling and is frequently overexpressed in B-cell lymphomas. This led to the discovery and clinical development of idelalisib (Fig. 22.22), a selective PI3Kδ inhibitor, which was approved by US FDA for the treatment of various B-cell malignancies, including CLL, follicular lymphoma, and small lymphocytic lymphoma (Kerr and Chisholm 2019; Huck et al. 2018; Yang et al. 2015). PI3Kδ plays a key role in the regulation of Treg. Inhibition of PI3Kδ disrupts the function of Treg and possibly MDSC too results in the shift of the balance from immune tolerance toward effective anticancer immunity, which has been validated in preclinical studies (Patton et al. 2006; Ali et al. 2014). The combination of idelalisib with the checkpoint inhibitor pembrolizumab is currently in phase II clinical studies in patients with CLL or B-cell lymphoma (NCT02332980).



Idelalisib
PI3Kδ inhibitor

IPI-549
PI3Kγ inhibitor

Fig. 22.22 The structures of PI3K inhibitors

PI3Kγ serves as a critical regulator of immune suppression by regulation of pro-inflammatory immune responses in macrophages, supporting immunosuppressive myeloid cells within TME (Kaneda et al. 2016). Selective inactivation of macrophage PI3Kγ stimulates and prolongs NFκB activation and inhibits C/EBPβ activation, thus promoting an immunostimulatory transcriptional program that restores $CD8^+$ cell activation and cytotoxicity. IPI-549 (Fig. 22.22) is an oral selective PI3Kγ inhibitor. In preclinical melanoma models, inhibition of PI3Kγ with IPI-549 resulted in the reprogramming of immunosuppressive macrophages (M2) into a pro-inflammatory (M1) state, which overcome the intrinsic resistance to checkpoint inhibitors. This enhanced the activation and recruitment of cytotoxic T-cells into tumor tissues. In melanoma mice models rich with macrophages, combination treatment using IPI-549 and immune checkpoint inhibitors significantly improved the survival: treatment with anti-CTLA-4 or anti-PD-1 therapy alone resulted in 20% remission and addition of IPI-549 increased the remission rate to 80%. It should be noted that the effect upon using IPI-549 is only demonstrated in the tumors with high myeloid cell content, the appropriate patient stratification could be essential for the best possible outcome of this combination therapy (De Henau et al. 2016).

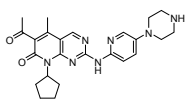
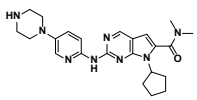
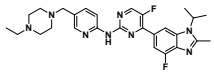
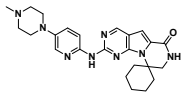
The clinical testing of IPI-549 with nivoluminab or atezolizumab against different cancer types is currently undergoing (NCT02637531, NCT03980041, NCT03961698). Preliminary results (NCT02637531) demonstrated that only mild adverse events including nausea and fatigue were reported, and no dose-limiting toxicities were observed. It is encouraging that 12 out of 15 patients have demonstrated durable clinical benefit, and 50% of patients remained on the treatment for more than 16 weeks (Tolcher et al. 2017).

22.6.3 CDK4/6 Inhibitors

The cyclin-dependent kinases, CDK4 and CDK6 (CDK4/6), control the progression of cell cycle process through the early G1 phases. CDK4/6 function is positively regulated by the association with cyclins D1/D2/D3 (Sherr 1996) and negatively regulated by tumor suppressors, such as p16INK4A through the interaction with D-type cyclins. Cyclin D-CDK4/6 complexes hyper-phosphorylate retinoblastoma protein (RB) in response to stimulatory mitogens, then uncouple RB from E2F transcription factors and regulate E2F-driven transcription of genes for cell cycle progression. Both p16INK4A and RB play an important role in cell proliferation regulation, the inactivating mutations and deletions in their encoding genes are frequent found in many tumor types (Young et al. 2014; Schutte et al. 1997; Classon and Harlow 2002). Besides RB, CDK4/6 also phosphorylates FOXM1, NFAT4, and SMAD3 (Anders et al. 2011; Matsuura et al. 2004; Deng et al. 2018).

CDK4/6 inhibitors proved to be beneficial in both preclinical and clinical trials for ER^+ breast cancer, especially in combination with antiestrogen therapies. Three of them have been approved for clinical use and one is in phase II studies for TNBC (Table 22.12). Palbociclib and ribociclib received FDA approval in Febru-

Table 22.12 FDA approved and clinical stage CDK4/6 inhibitors

Drug name	Structure	Targets (IC_{50}, nM)	Status and dose regime	Major toxicities
Palbociclib		**CDK4**: 11 **CDK6**: 15	Approved 125 mg PO; 21 days out of a 28-day cycle	Neutropenia Thrombocytopenia
Ribociclib		**CDK4**: 10 **CDK6**: 39	Approved 600 mg PO; 21 days out of a 28-day cycle	Neutropenia Thrombocytopenia QT prolongation
Abemaciclib		**CDK4**: 0.4–2 **CDK6**: 2.4–5 **CDK9**: 57	Approved 300–400 mg PO continuously	Diarrhea Fatigue
Trilaciclib		**CDK4**: 1 **CDK6**: 4 **CDK9**: 50	Phase II 200–240 mg/m^2 IV; 1–5 days out of 21 days	Thrombocytopenia

ary 2015 and March 2017, respectively, for the first-line treatment of ER+/HER2- advanced breast cancer for postmenopausal women in combination with antiestrogen therapy. Abemaciclib received its first FDA approval in September 2017 as a single agent for women with $ER^+/HER2^-$ advanced BC after disease progression following endocrine therapy and prior chemotherapy. Following the positive results of the MONARCH-2 trial, abemaciclib also received approval in combination with fulvestrant for women with $ER^+/HER2^-$ advanced BC after disease progression following endocrine therapy. These three approved CDK4/6 inhibitors have different toxicities, PK, and efficacy profiles, which should be carefully considered when in combinations with other therapies (Sobhani et al. 2019).

Although CDK4/6 inhibitors have rendered greater clinical benefits, patients tend to develop resistance to these drugs. The combination with different anticancer agents is under clinical studies to overcome the resistance (Vasan and Dickler 2017). Recent studies have shown that CDK4/6 inhibition can enhance antigen presentation, stimulate effector T-lymphocyte activation, and repress the proliferation of immunosuppressive Treg cells, which provide the rationale for combination of CDK4/6 inhibitors with immunotherapies (Deng et al. 2018; Goel et al. 2017).

Mechanistically, CDK4/6 inhibition can cause cell cycle arrest and induce cellular phenotypes consistent with senescence rather than inducing breast cancer cell apoptosis. Goel et al. reported that CDK4/6 inhibitors induced breast cancer cell cytostasis without directly causing their apoptosis, and their anticancer effect may

attribute to the capability in enhancing antigen presentation and stimulating cytotoxic T-cells (Goel et al. 2017). In a MMTV-rtTA/tetO-HER2 transgenic mouse model of mammary carcinoma, treatment with CDK4/6 inhibitors (abemaciclib or palbociclib) increased cell-surface expression of β2 M and MHC class I proteins. The increased expression of antigen processing and presentation genes was also observed in a patient-derived breast cancer xenograft of a treatment-refractory breast cancer (PDX 14-07). In addition, CDK4/6 inhibitor treatment caused significant increase of $CD3^+$ T-cells and reductions of $CD4^+FOXP3^+$ regulatory T-cells in tumor tissue. The total circulating Treg numbers were also significantly lower. In tumor-free mice, both abemaciclib and palbociclib significantly reduced Treg numbers and the Treg/$CD8^+$ T ratio in the spleen and lymph nodes, demonstrating tumor-independent effects of these agents. This study also discovered that intratumoral $CD8^+$ T-cells in abemaciclib-treated mice displayed significantly reduced expression of PD-1, Tim-3, CTLA-4, and LAG3. By using the combination treatment of abemaciclib and anti-PDL-1 antibody in the MMTV-rtTA/tetO-HER2 tumor-bearing mice, tumor volume shrank by ~70% by day 13 and did not resume growth by day 35. However, abemaciclib-treated tumors initially decreased and ultimately resumed growth by day 21. Similar results were observed in the CT-26 colorectal carcinoma model. The combination treatment induced complete tumor regression in all cases and the treated mice rejected the re-challenged CT-26 tumor cells 5 weeks after stopping the therapy.

The decreased expression of PD-1 and CTLA4 in both $CD4^+$ and $CD8^+$ T-cells after palbociclib or trilaciclib treatment was also reported by Jiehui Deng and colleagues (Deng et al. 2018). In the same study, it was found that CDK4/6 inhibition reduced the abundance of $CD11c^+$ myeloid cells, reduced the expression of IL6, IL10, and IL23, and increased the secretion of IFNγ. In an immunocompetent genetically engineered mouse model of human non-small cell lung cancer (NSCLC) ($Kras^{LSL\text{-}G12D}Trp53^{fl/fl}$ (KP)), transient treatment with either palbociclib or trilaciclib increased the infiltration of $CD4^+$ and $CD8^+$ T-cells into lung tumors. In the CT26 colon carcinoma cell mice model, treatment with PD-1 inhibitor alone was less effective but the combination of palbociclib with PD-1 inhibitor nearly eliminated all the tumors. Profiling of TILs from tumors revealed that anti-PD-1 alone increased $CD8^+$ IFNγ production but not $CD4^+$ IL2 production. Addition of CDK4/6 inhibitor to PD-1 blockade resulted in approximately twofold increase of $CD4^+$ IL2 production in addition to tenfold increase of IFNγ in $CD8^+$ TILs.

Teo et al. (2017) showed in a syngeneic TNBC mouse model that combined PI3Kα and CDK4/6 inhibition resulted in increased activation and cytotoxicity of both adaptive and innate immune cells as well as decreased numbers of immune-suppressive MDSCs within the tumor environment. It was further demonstrated that addition of immune checkpoint inhibitors (anti-PD-1 or anti-CTLA-4) to the combination of PI3Kα and CDK4/6 inhibitors resulted in complete and durable regressions of established TNBC tumors for more than 1 year.

Several clinical trials using combination of CDK4/6 inhibitors with PD-1/PD-L1 inhibitors are currently undergoing, including the combination of abemaciclib plus pembrolizumab in participants with non-small cell lung cancer or breast cancer

(phase II, NCT02779751) and adding pembrolizumab to palbociclib plus letrozole in patients with stable disease on palbociclib plus letrozole (phase II, NCT02778685). With further understanding of the effect of CDK4/6 inhibition to immune regulation and tumor microenvironment, the proper combination of immune checkpoint inhibitor with CDK4/6 inhibitor may improve the treatment outcome and potentially overcome the resistance to CDK4/6 inhibitors.

22.6.4 MEK and BRAF Inhibitors Combination with Immunotherapy

The successful development of BRAF inhibitor, MEK inhibitors, and immune checkpoint inhibition (ICI) for clinical use in the last decade has dramatically improved the survival and quality of the life of the patients with advanced melanoma (Rozeman and Blank 2019). Current standard-of-care therapies for advanced melanoma include anti-PD-1/PD-L1 checkpoint inhibitors (nivolumab or pembrolizumab), either as monotherapy or in combination with anti-CTLA-4 therapy (nivolumab plus ipilimumab), BRAF and MEK inhibitor combination (dabrafenib plus trametinib, vemurafenib plus cobimetinib, or encorafenib plus binimetinib) for patients with $BRAF^{V600}$-mutated advanced melanoma (see Fig. 22.23 for approved BRAF and MEK inhibitors) (Luke et al. 2017).

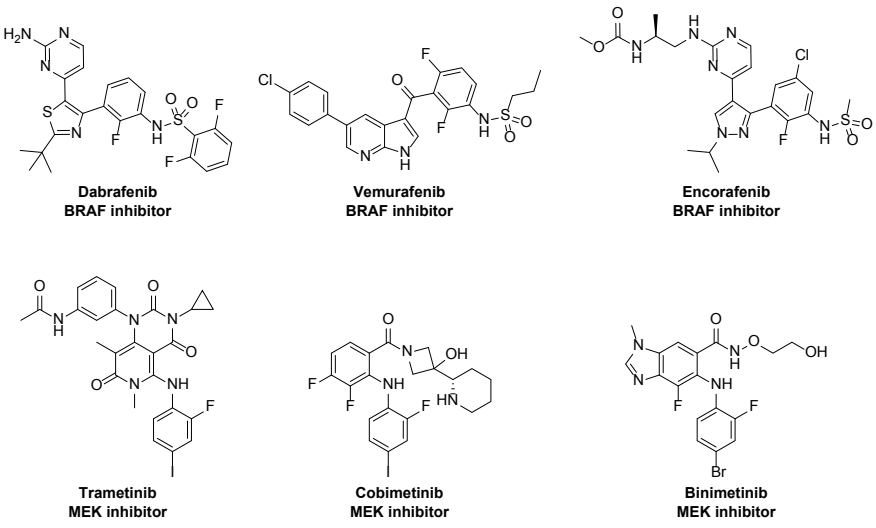


Fig. 22.23 Structures of approved BRAF and MEK inhibitors

Inhibition of MAPK pathway enhances host antitumor immunity through multiple mechanisms, including elevation of melanoma antigen expression and improving T-cell infiltration and function. These changes may serve to prime the tumor microenvironment for response to immunotherapy (Hughes et al. 2016).

The benefit of BRAF inhibitors is typically limited due to the quick development of resistance. BRAF inhibition leads to MAPK activation and increased expression of PD-L1 in melanoma cells via activation of c-Jun and STAT3. Combination of MEK inhibitor with BRAF inhibitor in the BRAF inhibitor-resistant melanoma cells downregulated MAPK activation and significantly decreased PD-L1 expression. This study suggests that BRAF and MEK are involved in and may play an important role in the onco-immune regulation in tumors. Even though the combination of BRAF inhibitor and MEK inhibitor significantly improved the progression-free survival (PFS), the median duration of response is only about 1 year due the developed resistance (Larkin et al. 2014; Long et al. 2018b). PD-1/PD-L1 antibodies have provided durable response for advance melanoma patients but the response rate is still low (Long et al. 2018a; Hodi et al. 2018). Due to the role of BRAF and MEK involved in onco-immune regulation, mechanistically, the combination of BRAF and MEK inhibitors with PD1/PD-L1 inhibitors may overcome the resistance developed against BRAFi/MEKi and improved the response rate of PD-1/PD-L1 inhibitors. The synergistic effect of combining immunotherapy with BRAF and/or MEK inhibitors were indeed validated in a number of murine melanoma models (Ebert et al. 2016; Cooper et al. 2014; Hu-Lieskovan et al. 2015). The recent clinical trials in patients with previously untreated metastatic $BRAF^{V600E}$-mutant melanoma showed encouraging preliminary results (Sullivan et al. 2019; Ribas et al. 2019; Ascierto et al. 2019). A phase Ib study with 39 patients receiving the combination of vemurafenib (BRAF inhibitor), cobimetinib (MEK inhibitor) and atezolizumab demonstrated that the combination treatment is generally tolerable with 72% ORR, 21%CR and 12.9 months of mean progress free survival (PFS) (Sullivan et al. 2019). In another randomized phase II trial (KEYNOTE-022), 120 patients were enrolled: 60 patients received dabrafenib and trametinib plus pembrolizumab (60 patients, triplet group) and 60 patients received dabrafenib and trametinib (60 patients, control group). Comparing the triplet group with control group, the progression-free survival was 16.0 months versus 10.3 months; median duration of response was 18.7 months versus 12.5 months, the estimated response rate lasted for more than 18 months was 59.8% versus 27.8%, respectively. Grade 3–5 treatment-related adverse events occurred in 58.3% of patients for the triplet group compared with 26.7%with the control group (Ascierto et al. 2019).

These studies clearly demonstrate the clinical benefit of combining two oncogene-targeted therapies (BRAF+MEK) with an ICI in the treatment of melanoma (Killock 2019). However, for other solid tumors such as colorectal cancer, the combination of atezolizumab plus cobimetinib did not meet its primary endpoint of overall survival (OS) compared to regorafenib (Eng et al. 2019). More studies will be needed to explore the potential of combining BRAFi and/or MEKi with immune checkpoint inhibitors.

22.6.5 PARP Inhibitors and Tumor Microenvironment

DNA damage and its repair are essential to the induction of mutations and the development of cancers. Normal cells defend themselves against DNA damage through the DNA damage response (DDR), which detects DNA damage and mediates the DNA repair process to maintain the genome integrity. Poly(ADP-ribose) polymerase 1 and 2 (PARP1 and PARP2) are the key enzymes to sense DNA damage and signal the DNA repairing process (Smulson et al. 1994; Eustermann et al. 2015; Dawicki-McKenna et al. 2015).

At early stage of study of PARP functions, it was believed that inhibition of PARP could sensitize tumor cells to conventional DNA damage treatments such as chemotherapy or radiotherapy (Helleday 2011). The synthetic lethal (SL) interaction between PARP inhibition and BRCA1 or BRCA2 mutation was demonstrated that BRCA-mutant tumor cells were 1000 times more sensitive to PARP inhibition than wild type BRCA cells (Farmer et al. 2005), which suggests that the inhibition of PARP can serve as a novel treatment strategy for patients with BRCA-mutant tumors (Bryant et al. 2005; Ashworth et al. 2011). Four PARP inhibitors (see Fig. 22.24 for structures) have been approved for clinical use to treat cancer patients with BRCA mutations, and a number of new PARP inhibitors are still at different stages of clinical development (Table 22.13).

It has been observed that acquired resistance occurred in most patients with advanced cancer after PARP inhibitor treatment. The secondary mutations in BRCA1 or BRCA2 have been validated as one of the mechanisms leading to PARP inhibition resistance (Edwards et al. 2008; Barber et al. 2013). Recent research in DDR pathway has demonstrated its involvement in the antitumor immune response and the combination with checkpoint inhibitors may overcome the resistance and improve the clinical outcome (Chatzinikolaou et al. 2014). In a study using BR5-AKT ovarian cancer syngeneic mouse model, the combination of PARP inhibitors and CTLA4

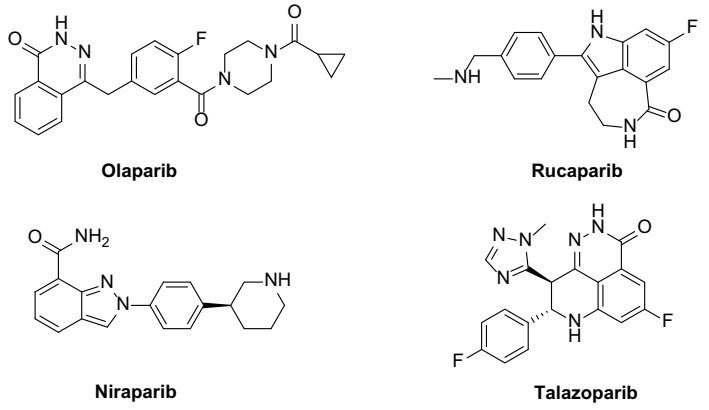


Fig. 22.24 The structures of FDA approved PARP inhibitors

Table 22.13 PARP inhibitors in clinical use and clinical development

Drug	Developer	Development status	Therapeutic indications
Olaparib	AstraZeneca	Marketed	Ovarian cancer, fallopian tube cancer, or primary peritoneal cancer with abnormal BRCA gene
Rubraca	Clovis	Marketed	Ovarian cancer, fallopian tube, primary peritoneal cancers
Zejula	Tesaro/Merck	Marketed	Recurrent ovarian, fallopian tube, or peritoneal cancer
Talzenna	Medivation/Pfizer	Marketed	Breast cancer (HER2-negative with BRCA mutations)
Veliparib	Abbvie	Phase III	Breast cancer, NSCLC
CEP-8983	Teva	Phase II	Solid tumors
BGP-15	N-Gene	Phase II	Type II diabetes
JPI-289	Jell	Phase II	Stroke
Fluzoparib	Hengrui	Phase I	Solid tumors
Fuzuopali	Haosen	Phase I	Solid tumors
BGB-290	BeiGene	Phase I	Breast cancer, ovarian cancer, other solid tumors
ABT-767	Abbvie	Phase I	Breast cancer, ovarian cancer, other solid tumors
MP-124	Kateiki	Phase I	Acute ischemic stroke
Simmiparib	SIMM	Phase I	Solid tumors, lymphoma
Meifupairui	SIMM	Phase I	Solid tumors
IMP-4297	Impact therapeutics	Phase I	Breast cancer, ovarian cancer, other solid tumors
SC-10914	Qingfeng pharmaceutical	Phase I	Ovarian cancer, peritoneal carcinoma
WXFL-10040340	Humanwell healthcare (Group) Co.	Phase I	Breast cancer, ovarian cancer, NSCLC

antibody demonstrated a synergistic therapeutic effect. However, such synergistic effect using the anti-PD-1 and PARPi combination in the same animal model was not observed (Higuchi et al. 2015). This could be explained by the fact that BR5-AKT tumors display high AKT activities and PARP inhibition might not be able to inhibit GSK-3β enough in the presence of high level of AKT to induce PD-L1 expression. In a different study using MDA-MB-231 and BT549 cells, it was also discovered that PARP inhibition caused the increase of PD-L1 level in a dose-dependent manner no matter which PARP inhibitor was used. In vivo studies using BT549, SUM149, and MDA-MB-231 cells also demonstrated the increased expression of PD-L1 in tumor tissues. It was further elucidated that PARP inhibition upregulates PD-L1 expression primarily through GSK-3β inactivation (Jiao et al. 2017). The increased expression of PD-L1 in small cell lung cancer (SCLC) cells was also reported after inhibition of DNA damage response (DDR) proteins, PARP, and checkpoint kinase 1 (CHK1) (Sen et al. 2019). In several immunocompetent SCLC models, inhibition of PARP or CHK1 augmented cytotoxic T-cell infiltration and significantly improved the anti-tumor effect of PD-L1 blockade. In addition, it was found that DDR inhibition also activated the STING/TBK1/IRF3 innate immune pathway, leading to increased levels of chemokines such as CXCL10 and CCL5, and in turn induced the activation of cytotoxic T-lymphocytes.

These preclinical studies have provided the scientific basis for the combination of PARP inhibitors with immunotherapies, and three early-stage clinical studies of such combination are currently undergoing. These trials include the combination of olaparib with PD-L1 inhibitor MEDI4736 for advanced solid tumors and advanced or recurrent ovarian, triple-negative breast (TNBC), lung, prostate, and colorectal cancers (NCT02484404), the combination of niraparib with pembrolizumab for TNBC or ovarian cancer (NCT02657889), the combination of PARPi BGB-290 with PD-1 inhibitor BGB-A317 for advanced solid tumors (NCT02660034).

22.7 Conclusions and Future Perspectives

In summary, recent studies have demonstrated that small-molecule therapies can modulate the immune response to cancer by restoring the antitumor immunity, promoting more effective cytotoxic lymphocyte responses, and regulating tumor microenvironment, either directly or epigenetically. Small-molecule approaches offer inherent advantages over biologic immunotherapies since they can cross cell membranes and penetrate into tumor tissue and tumor microenvironment more easily, which make it possible to access a wider range of molecular targets, such as intracellular targets. The clinical use of small molecules is more amenable to be finely controlled than biological agents, which may help reduce immune-related adverse events seen with biologic therapies and provide more flexibility for the combination use with other therapies and more clinical benefit. In addition, the relatively low cost of small-molecule drugs should provide greater access to advanced immunotherapy for patients. Compared with the success and development activities of cancer

immunobiologic agents, the development success and impact of small molecular targeting immune modulation are still limited. The effort around several small molecular targets, such as IDO, OX40, and STING, has encountered great challenges in clinical studies as combination therapy. Future research may need to focus on the translational medicine research to develop more predictive biomarkers for patient stratification, and the optimization of the right therapy combination and treatment regime. Given the significant clinical success with checkpoint inhibitory biological agents and CAR-T therapies, the combination of small-molecule drugs with these agents is particularly attractive for expanding their treatment scope and efficacy. In 2017 alone, 469 new combination trials were started with a combined target enrollment of 52,539 patients. A number of small-molecule agents such as VEGFR, MEK and HDAC inhibitors, etc., have been explored in clinical studies with early encouraging clinical results for combination with immunological checkpoint monoclonal antibodies. With more understanding of system cancer biology and comprehensive immune system biology, it can be expected that the combination of right small molecular drugs with the appropriate immunological agents shall generate more efficacious and more durable treatment options for cancer patients.

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Chapter 23
Therapeutic Development of Immune Checkpoint Inhibitors

Jilin Wang, Teddy Yang and Jie Xu

Abstract Immune checkpoint blockade (ICB) has been proven to be an effective strategy for enhancing the effector activity of anti-tumor T cells, and checkpoint blockers targeting CTLA-4, PD-1, and PD-L1 have displayed strong and durable clinical responses in certain cancer patients. The new hope brought by ICB therapy has led to the boost in therapeutic development of ICBs in recent years. Nonetheless, the therapeutic efficacy of ICBs varies substantially among cancer types and patients, and only a proportion of cancer patients could benefit from ICBs. The emerging targets and molecules for enhancing anticancer immunity may bring additional therapeutic opportunities for cancer patients. The current challenges in the ICB therapy have been discussed, aimed to provide further strategies for maximizing the efficacy of ICB therapy.

Keywords Immune checkpoint blocker · CTLA-4 · PD-1 · PD-L1

23.1 Introduction

Immunotherapy has emerged as an attractive treatment option for many kinds of cancer patients, in particular, immune checkpoint blockade (ICB) therapies that enhance the function of anti-tumor T lymphocytes have been especially promising in the clinic. Compared with other immunotherapies, ICB therapies often show higher response

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rates and long-lasting responses, even in patients with advanced cancer (Busato et al. 2019). Ipilimumab, a cytotoxic T-lymphocyte-associated antigen-4 (CTLA-4) blocker, was the first ICB approved by the FDA in 2010 (Lipson and Drake 2011). It provided a new treatment option for the metastatic melanoma patients who previously lacked any effective treatments (Graziani et al. 2012). Until now, many kinds of ICB agents that targeting CTLA-4, programmed cell death-1 (PD-1), and PD-L1 have been approved for many kinds of cancers, such as metastatic melanoma (Postow et al. 2015; Deeks 2016; Rosenberg et al. 2016), non-small-cell lung cancer (NSCLC) (Wolchok et al. 2010; Rizvi et al. 2015; Dang et al. 2016), Hodgkin lymphoma (Kasamon et al. 2017), urothelial cancer (Kim et al. 2019; Burgess et al. 2019), hepatocellular carcinoma(HCC) (Hage et al. 2019; Kudo 2019), gastric cancer (Chen et al. 2019; Park et al. 2018), head and neck squamous cell carcinoma(HNSCC) (Yu et al. 2018; Sim et al. 2019), microsatellite instability-high colorectal cancer (CRC), and other MSI^{high} cancers (Middha et al. 2019; Overman et al. 2017; Marginean and Melosky 2018). The common mechanism of these ICBs is through the activation of anti-tumor T-lymphocyte responses and overcoming tumor immune supervision.

However, the ICB therapy usually has many shortcomings, such as the effectiveness of ICBs varies in different kinds of cancers, and even if in melanoma, most of the patients cannot benefit from the ICB therapy (Puglisi et al. 2010; Khalil et al. 2016). Other more common cancers, such as breast cancer and MSI^{low} CRC patients rarely could benefit from ICB therapies (Reck et al. 2016; Kindler et al. 2012; Alexandrov et al. 2013; Polk et al. 2018; Hermel and Sigal 2019). The reason for this phenomenon is that the effectiveness of ICBs in different tumors and patients is strongly affected by the tumor's mutation load and the local tumor microenvironment (TME) (Tumeh et al. 2014; Hamada et al. 2018).

In this chapter, we will first summarize the clinical development of the ICBs related to CTLA-4, PD-1, and PD-L1, then we will describe the emerging new ICB agents besides the CTLA-4, PD-1, and PD-L1, and finally we will explore the current challenges of immune checkpoint blockade therapy in cancer.

23.2 Therapeutic Development of CTLA4 Blockade

23.2.1 The CTLA-4 Immune Checkpoint

CTLA-4 was the first identified negative regulator of T-cell activation. It belongs to the immunoglobulin superfamily and has similar structures as T-cell surface molecule CD28 with similar functional properties (Linsley et al. 1994). CTLA-4 shares the same B7 ligands as CD28, including B7-1 (CD80) and B7-2 (CD86), but the affinity of CTLA-4 for both ligands is about 100-fold higher than that of CD28 (Sansom 2000). CD28 ligation by the B7 family ligands results in a positive co-stimulatory signal needed by the T lymphocytes for optimal cytokine secretion and proliferation (Chen et al. 2019). However, after T-cell receptor (TCR) activation, CTLA-4

is upregulated and binds CD80/CD86, resulting in reduced T-lymphocyte proliferation and lessened cytokine secretion (Engelhardt et al. 2006). More and more studies supported the idea that CTLA-4 functioned as an important negative regulator of T-lymphocyte activation. In the early stage of carcinogenesis, CTLA-4 could decrease the T lymphocyte activation by producing inhibitory signals to weaken the immune response against tumor cells (Rowshanravan et al. 2018); CTLA-4 could trigger reverse signaling through B7 ligands to induce indoleamine-2, 3-dioxygenase (IDO) and results in inhibition of T-cell proliferation (Boasso et al. 2005); recent studies also revealed that CTLA-4 could induce inhibition of PI3K/Akt pathways, cyclin-dependent kinases, and nuclear transcription factor (NF-κB) (Parry et al. 2005; Ghorpade et al. 2011); in addition, CTLA-4 inhibition may also involve in regulatory CD4+ T-cell (Treg) activation which then suppresses CTL functions by "stripping" CD80/CD86 from APCs (Qureshi et al. 2011).

However, in spite of extensive researches on CTLA-4, the mechanism of CTLA-4 interacting with its ligands or its downstream targets and the action of CTLA-4 blockade still need to be further investigated.

23.2.2 Immune Checkpoint Inhibitors Targeting CTLA-4

Based on CTLA-4's role in the negative regulation of T-cell activation, antibodies that block CTLA-4 and B7 ligands interaction have become attractive targets for cancer therapies. Antibodies that block CTLA-4 have demonstrated anti-tumor effect first in mouse models, then in cancer patients (Peggs et al. 2009). Based on the encouraging outcome of a pivotal clinical trial in 2010, the anti-CTLA-4 monoclonal antibody ipilimumab became the first immune checkpoint inhibitor approved for cancer therapy by the U.S. FDA in 2011. This study found that patients with unresectable stage III/IV melanoma exhibited improved survival following treatment by ipilimumab compared with glycoprotein 100 (gp100) peptide vaccine (median overall survival (OS) of 10 vs. 6.4 months, respectively) (Hodi et al. 2010). In the meanwhile and after that, more clinical trials were conducted to explore the treatment of melanoma by ipilimumab, and most of the studies support the idea that ipilimumab is effective for the treatment of unresectable melanoma (Zimmer et al. 2015; Chiarion Sileni et al. 2014; Alexander et al. 2014). A pooled analysis from 10 prospective and 2 retrospective studies including 1861 advanced melanoma patients found that the 3-year survival rate could reach 22% for patients receiving ipilimumab (Schadendorf et al. 2015). Meanwhile, the 3-year survival rate was only 12.2% for the metastatic melanoma patients treated with the FDA approved chemotherapeutic agent dacarbazine (Robert et al. 2011). Therefore, ipilimumab was recommended for the treatment of metastatic or unresectable melanoma patients by the National Comprehensive Cancer Network (NCCN). However, ipilimumab is not effective for a large amount of melanoma patients, and a recent clinical trial found that ipilimumab had no clinical activity in patients with metastatic uveal melanoma (Zimmer et al. 2015).

In the meanwhile, many clinical trials are exploring the potential clinical use of ipilimumab in many other cancers, but most of the results are not encouraging. Ipilimumab has been found to have a partial response in stage IIIB/IV non-small-cell lung cancer (Lynch et al. 2012), and a high dose of ipilimumab could result in a durable response in some subtypes of hematologic cancers after allogeneic hematopoietic stem-cell transplantation (HSCT) (Davids et al. 2016). The clinical activity of ipilimumab in prostate cancer was controversial (Slovin et al. 2013; Kwon et al. 2014; Beer et al. 2017). Furthermore, more clinical trials have suggested that ipilimumab monotherapy is not effective in some other solid tumors, such as colorectal cancer (O'Mahony et al. 2007), extensive-small-cell lung cancer (Reck et al. 2013), unresectable locally advanced or metastatic gastric or gastroesophageal junction cancer (Bang et al. 2017), gastrointestinal stromal tumor (GIST) (D'Angelo et al. 2017), metastatic sarcoma (D'Angelo et al. 2018), and pancreas cancer (Royal et al. 2010).

Although ipilimumab did not show complete response in many kinds of cancers, it did show some clinical activity in most kinds of cancers, therefore, some efforts have been made to assess the efficacy of combination therapy, and some twilight has been seen. A phase I clinical trial of a combination of ipilimumab and imatinib in patients with advanced GIST and melanoma have shown partial response and long-time disease stable property (Reilley et al. 2017), another RCT has shown that ipilimumab plus paclitaxel and carboplatin could improve progression-free survival (PFS) of stage IIIB/IV non-small-cell lung cancer patients (Lynch et al. 2012), Sakamuri's study also revealed combination of ipilimumab and lenalidomide demonstrated preliminary signals of activity in patients with refractory Hodgkin lymphoma and other advanced cancers (Sakamuri et al. 2018), Formenti's study also found that radiotherapy enhances responses of lung cancer to CTLA-4 blockade (Formenti et al. 2018). Some preclinical studies also found combination of CTLA-4 inhibitor and other chemotherapy agents could result in better response, Charlotte's study has found local chemotherapy combined with CTLA-4 inhibitor results in a durable response to cancer therapy in melanoma and prostate cancer (Ariyan et al. 2018), Liu's study revealed combination immunotherapy of the vaccine and anti-CTLA-4 monoclonal antibody could significantly enhance anti-tumor immune response for triple-negative breast cancer (TNBC) (Liu et al. 2018). More clinical trials are warranted to clarify the efficacy of combination therapy in these kinds of cancer patients.

While ipilimumab has received FDA approval for the treatment of advanced melanoma, there is another CTLA-4 inhibitor, tremelimumab, a fully human IgG2 monoclonal antibody marketed by AstraZeneca that is also being investigated in many clinical trials. Unfortunately, it has not improved patient survival as monotherapy in most of the trials although tremelimumab has a comparable affinity and longer serum half-life (22 days versus 12 days) than ipilimumab. For example, Forty-four patients with non-small-cell lung cancer (NSCLC) were treated with 15 mg/kg of tremelimumab, only two out of 44 patients (4.5%) showed a partial response (PR) (Corrales et al. 2018); Seventeen patients diagnosed with hepatocellular carcinoma received 15 mg/kg of tremelimumab, only three of those patients experienced a confirmed PR (Sangro et al. 2013); Tremelimumab was also investigated as a second-line treatment for patients with gastric and esophageal adenocarcinomas, only one out of

18 patients achieved a PR > 32 months (Ralph et al. 2010); Tremelimumab did not significantly prolong overall survival compared with placebo in patients with previously treated malignant mesothelioma (Maio et al. 2017). According to melanoma, a phase II clinical trial gave a promising data, in this study, tremelimumab (15 mg/kg) was administered to 32 patients with metastatic melanoma, four patients benefitted with an overall response(OR), where the OS fluctuated between 2 months and 41 months, and seven patients survived > 2 years (Ribas 2010). This promising data led to the development of a two arm Phase III clinical trial, although the patients treated with tremelimumab had an objective response of 10.7 months, with a median OS of 12.6 months, there were no clinical differences between the tremelimumab and the temozolomide or dacarbazine arms (Ribas et al. 2013). Therefore, tremelimumab was not approved as cancer monotherapy to date. Tremelimumab is currently being investigated in combination with other regimens to assess whether it will have greater efficacy as part of combinatorial regimens (Jiang et al. 2019; Xie et al. 2019). The difference observed in clinical outcome between ipilimumab and tremelimumab may be attributed to their antibody isotypes. Ilipilumab in human IgG1 depletes immune-suppressive CTLA-4 high expressing regulatory T cells through antibody dependent cell-mediated cytotoxicity (ADCC) while tremelimumab in IgG2 isotype does not engage ADCC pathway (Borrie and Maleki 2018).

23.3 Therapeutic Development of PD-1/PD-L1 Blockade

23.3.1 The PD-1/PD-L1 Immune Checkpoint

Programmed cell death 1(PD-1) is a transmembrane protein, mainly expressed on the surface of activated T cells, B cells, and macrophages cells (Chemnitz et al. 2004). PD-L1(CD724) and PD-L2(CD723) were discovered as dual ligands for PD-1, and both were shown to inhibit T-cell effector activity following PD-1 engagement (Panjwani et al. 2018). Although the engagement between PD-1 and PD-L2 in cancer tissues could contribute to PD-1-mediated inhibition of cytotoxic T-lymphocyte(CTL) responses, there is no evidence that antibodies against PD-1 show higher clinical activity than antibodies against PD-L1, suggesting that PD-L1 is the dominant inhibitory ligand of PD-1 on T cells (Yearley et al. 2017). Binding of PD-L1 by PD-1 has been proposed to deliver survival signals to cancer cells, enhancing their resistance to proapoptotic effects of Fas, interferons, and CTLs (Gato-Canas et al. 2017; Kythreotou et al. 2018). In fact, tumor cells could escape the immune attack by abnormally expressing a series of negative co-stimulatory molecules such as PD-L1, which binds to PD-1 on the surface of immune cells, forming a unique immune escape microenvironment and inhibiting anti-tumor immunity (Topalian et al. 2015; Choueiri et al. 2014). This is the main mechanism of tumor immune escape. In view of this, the PD-1/PD-L1 immune checkpoint pathway has become an ideal target for immunotherapy that aim to restore the effector function of anti-tumor-specific T cells.

23.3.2 Immune Checkpoint Inhibitors Targeting PD-1/PD-L1

In recent years, PD-1 and PD-L1 antibodies have attracted more and more attention due to their promising efficacy compared with other immune therapy or chemotherapy agents. People have witnessed several different anti-PD-1 and anti-PD-L1 antibodies in simultaneous development in numerous cancer types. Among the agents, FDA has approved two PD-1 antibodies (Pembrolizumab and Nivolumab) and three PD-L1 antibodies (Atezolizumab, Avelumab, and Durvalumab) for cancer therapy. Currently, more research focused on Pembrolizumab, Nivolumab, and Atezolizumab in solid tumors.

Pembrolizumab

Pembrolizumab was the first PD-1 antibody approved by U.S. FDA for patients with metastatic melanoma in September 2014 based on two randomized clinical trials, PN002 and PN006. In trial PN002, 540 patients with ipilimumab-refractory metastatic melanoma were randomized (1:1:1) to pembrolizumab 2 or 10 mg/kg every 3 weeks or to chemotherapy (Weber et al. 2013). In trial PN006, 834 patients with ipilimumab-naive metastatic melanoma were randomized (1:1:1) to pembrolizumab 10 mg/kg every 2 or 3 weeks until disease progression or ipilimumab 3 mg/kg every 3 weeks for up to four doses (Barone et al. 2017). In both trials, patients receiving pembrolizumab demonstrated statistically significant improvements in PFS. In trial PN006, patients treated with pembrolizumab demonstrated a statistically significant improvement in overall survival compared with ipilimumab. In recent years, other more studies are conducted to assess the efficacy of pembrolizumab in different melanoma population and its long term efficacy. There was a phase 3 double-blind trial to evaluate pembrolizumab as adjuvant therapy in patients with resected, high-risk stage III melanoma (Eggermont et al. 2018). In this trial, 514 patients received 200 mg of pembrolizumab intravenously every 3 weeks for a total of 18 doses, other 505 patients received a placebo. After a median follow-up of 15 months, pembrolizumab was associated with significantly longer recurrence-free survival than a placebo. A retrospective analysis found that melanoma patients with pretreated brain metastasis could have durable systemic responses to pembrolizumab (Dagogo-Jack et al. 2017). The efficacy of pembrolizumab in melanoma was confirmed in different countries, including Spanish, Japan, and China. The long-term effect of pembrolizumab was confirmed by re-analyzing the PN006 and PN001 trials. In PN006 trial, 24-month overall survival rate was 55% in the 2-week pembrolizumab group, 55% in the 3-week pembrolizumab group, and 43% in the ipilimumab group, suggesting pembrolizumab continued to provide superior overall survival versus ipilimumab (Schachter et al. 2017). In PN001 trial (Hamid et al. 2019), 655 patients with previously treated or treatment-naive advanced/metastatic melanoma received pembrolizumab 2 mg/kg every 3 weeks, 10 mg/kg every 3 weeks, or 10 mg/kg every 2 weeks, median follow-up was 55 months. The estimated 5-year OS was 34% in all patients and 41% in treatment-naive patients; median OS was 23.8 months and 38.6 months, respectively. Estimated 5-year PFS rates were 21% in all patients and 29% in treatment-naive patients; median PFS was 8.3 months and 16.9 months,

respectively. This trial confirmed the durable anti-tumor activity and tolerability of pembrolizumab in advanced melanoma.

In addition to melanoma, pembrolizumab was found to have good anti-tumor activity in other solid tumors, especially in non-small-cell lung cancer (NSCLC). The phase 1 KEYNOTE-001 trial initially revealed the efficacy of pembrolizumab in NSCLC (Leighl et al. 2019). After that, a large international multi-center phase 2/3 randomized trial found that pembrolizumab prolonged overall survival and had a favorable benefit-to-risk profile in patients with previously treated, PD-L1-positive, advanced NSCLC (Herbst et al. 2016). Based on this trial, FDA approved pembrolizumab for second-line and above treatment of NSCLC with PD-L1 positive ($\geq$1%). Another KEYNOTE-024 trial compared the efficacy and safety of pembrolizumab and platinum-based chemotherapy in advanced NSCLC and found that in patients with advanced NSCLC and PD-L1 expression on at least 50% of tumor cells, pembrolizumab was associated with significantly longer progression-free and overall survival and with fewer adverse events than was platinum-based chemotherapy (Brahmer et al. 2017). This trial led to the approval of pembrolizumab for the first-line treatment of advanced NSCLC with PD-L1 high expression ($\geq$50%). Another KEYNOTE-21 trial assessed whether the addition of pembrolizumab to platinum-doublet chemotherapy improves efficacy in patients with advanced nonsquamous NSCLC (Langer et al. 2016). Result showed that 55% patients in the pembrolizumab plus chemotherapy group achieved an objective response compared with 29% in the chemotherapy alone group, the median PFS was significantly longer in the pembrolizumab plus chemotherapy group than in the chemotherapy group (13.0 months vs 8.9 months), with a 6-month progression-free survival rate of 77%. Based on this data, the FDA approved pembrolizumab in combination with pemetrexed/carboplatin chemotherapy for the first-line treatment of metastatic nonsquamous NSCLC. Therefore, these results have changed the first-line management of advanced NSCLC. There are other clinical trials ongoing to assess the clinical use of pembrolizumab in lung cancer. A recent KEYNOTE-042 trial suggested that pembrolizumab monotherapy can be extended as first-line therapy to patients with locally advanced or metastatic non-small-cell lung cancer without sensitizing EGFR or ALK alterations and with low PD-L1 tumor proportion score (Mok et al. 2019).

In addition to melanoma and NSCLC, based on a series of clinical trials, pembrolizumab has also got approval for other cancers, including classical Hodgkin lymphoma (Chen et al. 2017), HNSCC (Larkins et al. 2017), urothelial carcinoma (Bellmunt et al. 2017), gastric cancer (Muro et al. 2016), and colorectal cancer (Wang et al. 2019). It is noteworthy that there is another milestone clinical trial of pembrolizumab in anti-tumor therapy. It is the first time that US FDA has granted a therapeutic treatment for any cancer types with a specific genetic biomarker. This NCT01876511 clinical trial included 11 dMMR (mismatch repair deficient) CRC patients, 9 dMMR other cancer patients, and 21 pMMR (mismatch repair proficient) CRC patients, all of the patient received pembrolizumab intravenously at a dose of 10 mg per kg of body weight every two weeks. The immune-related objective response rate and immune-related progression-free survival rate were 40% and 78%, respectively, for dMMR CRC and 0 and 11% for pMMR CRC patients. Based on this trial, FDA

approved pembrolizumab for the treatment of microsatellite instability-high(MSI-H) or dMMR solid tumors (Marcus et al. 2019).

Nivolumab

Nivolumab is another PD-1 antibody that has been approved by FDA for the treatment of various types of cancer. Nivolumab was also firstly approved for the treatment of unresectable or metastatic melanoma based on CheckMate-037 trial and CheckMate066 trial. The CheckMate-037 trial revealed improved objective response rates to nivolumab versus chemotherapy in patients with unresectable or metastatic melanoma whose cancers had progressed following treatment with ipilimumab ± a BRAF inhibitor (Weber et al. 2015). CheckMate 066 trial compared the nivolumab and dacarbazine based chemotherapy in 418 previously untreated metastatic melanoma patients without BRAF mutation (Robert et al. 2015). The overall survival rate was 72.9% in the nivolumab group, as compared with 42.1% in the dacarbazine group. The median progression-free survival was 5.1 months in the nivolumab group versus 2.2 months in the dacarbazine group. The objective response rate was 40.0% in the nivolumab group versus 13.9% in the dacarbazine group. CheckMate 067 was a subsequent Phase III study that enrolled 945 untreated unresectable stage III or metastatic melanoma patients, aimed to assess the combination therapy of nivolumab and ipilimumab (Hodi et al. 2018). This trial showed that nivolumab combined with ipilimumab resulted in significantly longer progression-free survival than ipilimumab alone. This trial led to the approval of dual therapy with nivolumab and nivolumab for the first-line therapy of metastasis melanoma.

Nivolumab is also a hot topic for the treatment of NSCLC. Nivolumab was the first checkpoint inhibitor approved by FDA in 2015 for the treatment of squamous cell NSCLC based on the phase 2 CheckMate 063 trial (Rizvi et al. 2015). In this trial, 117 patients with advanced, refractory squamous NSCLC received nivolumab 3 mg/kg Q2W until progression or unacceptable toxic effects. The 6 months and 1 year PFS were 25.9 and 20.0%. Median OS was 8.2 months (95% CI, 6.1–10.9) and 1 year OS was 40.8% (31.6–49.7). Nivolumab was then approved by the FDA as a second-line therapy for patients with previously treated advanced NSCLC based on CheckMate017 (Yoo et al. 2018) and CheckMate057 trials (Horn et al. 2017). The CheckMate 017 trial evaluated the efficacy and safety of nivolumab versus docetaxel in advanced squamous cell NSCLC. The results showed that the median OS was 9.2 months with nivolumab versus 6.0 months with docetaxel, the 1 year OS rate was 42% with nivolumab versus 24% with docetaxel, the ORR was 20% with nivolumab and 9% with docetaxel. Meanwhile, the CheckMate 057 trial compared nivolumab to docetaxel in previously treated advanced non-squamous NSCLC. Median OS was 12.2 months for nivolumab and 9.4 months for docetaxel; 1-year and 18-month OS rates were 51 and 39% with nivolumab versus 39 and 23% with docetaxel; ORR was 19% for nivolumab and 12% for docetaxel; 1-year PFS was 19% for nivolumab and 8% for docetaxel. Nivolumab further improved efficacy across all endpoints compared with docetaxel.

In addition, the anti-tumor potential of nivolumab has also gained a lot of support in other tumors. The CheckMate025 trial compared nivolumab with everolimus in 821 patients with renal cell carcinoma who had received previous treatment, and

found that the median OS was 25.0 months with nivolumab and 19.6 months with everolimus, and the ORR was greater with nivolumab than with everolimus (25 vs. 5%) (Motzer et al. 2015). The CheckMate 275 trial has found that nivolumab monotherapy provided meaningful clinical benefit (ORR 28.4%) irrespective of PD-L1 expression in previously treated patients with metastatic or surgically unresectable urothelial carcinoma (Sharma et al. 2017). Nivolumab monotherapy also resulted in longer overall survival than treatment with standard therapy among patients with platinum-refractory, recurrent squamous cell carcinoma of the head and neck (Ferris et al. 2016). A phase 3 trial also found survival benefits of nivolumab in the treatment of pretreated patients with advanced gastric or gastro-oesophageal junction cancer (12-month OS rates were 26·2% with nivolumab and 10·9% with placebo) (Kang et al. 2017). Nivolumab also showed promising efficacy in other tumors, such as dMMR/MSI-H metastatic colorectal cancer (Overman et al. 2017), unresectable metastatic anal cancer (Morris et al. 2017), and platinum-resistant ovarian cancer (Hamanishi et al. 2015).

There is another PD-1 blocker, cemiplimab, which has been approved for the treatment of metastatic cutaneous squamous cell carcinoma (CSCC) or locally advanced CSCC who are not candidates for curative surgery or curative radiation (Migden et al. 2018).

Atezolizumab

Atezolizumab is the first approved PD-L1 monoclonal antibody, and there are currently two approved indications as monotherapy for the progression of metastatic urothelial carcinoma, metastatic NSCLC after platinum-based chemotherapy and three indications in combination with chemotherapy in metastatic SCLC and metastatic triple-negative breast cancer as well as in combination with bevacizumab in metastatic non-squamous non-small-cell lung cancer.

FDA approved atezolizumab for the treatment of local advanced or metastatic urothelium cell cancer based on the IMvigor210 trial. This trial revealed that the ORR reached 23.5% in patients treated with atezolizumab, and the median CR time is 14.4 months (Powles et al. 2014). FDA approved atezolizumab for the treatment of NSCLC based on POPLAR and OAK clinical trials. The POPLAR trial assessed efficacy and safety of atezolizumab versus docetaxel in previously treated NSCLC, and found that OS was 12·6 months for atezolizumab versus 9·7 months for docetaxel, 16 (11%) patients in the atezolizumab group versus 52 (39%) patients in the docetaxel group had treatment-related grade 3–4 adverse events (Fehrenbacher et al. 2016). The OAK trial also found that atezolizumab treatment results in a clinically relevant improvement of overall survival versus docetaxel in previously treated non-small-cell lung cancer, with a favorable safety profile (Rittmeyer et al. 2017).

Atezolizumab was approved for the treatment of metastatic triple-negative breast cancer based on a recent clinical trial that found atezolizumab plus nab-paclitaxel could prolong the PFS among patients with metastatic triple-negative breast cancer (Schmid et al. 2018). Another trial assessed the efficacy of first-line atezolizumab treatment plus chemotherapy in extensive-stage SCLC, and found a significantly longer overall survival and progression-free survival than chemotherapy alone (Horn

et al. 2018). This trial led to the approval of atezolizumab in combination with chemotherapy for the treatment of SCLC.

There are another two PD-L1 blockers, avelumab and durvalumab, which have got US FDA approval for indications for some types of cancers. A phase 2 clinical trial revealed that avelumab monotherapy was associated with durable responses, most of which are still ongoing, and was well tolerated; hence, avelumab represents a new therapeutic option for advanced Merkel cell carcinoma (Kaufman et al. 2016). Another phase one clinical trial found that avelumab showed anti-tumor activity for patients with platinum-refractory metastatic urothelial carcinoma with a manageable safety profile (6% complete responses and 11% partial responses; 29% of grade 1–2 AEs, 6% of grade 3–4 AEs) (Patel et al. 2018). Based on these trials, avelumab has been approved for the treatment of advanced Merkel cell carcinoma and platinum-refractory metastatic urothelial carcinoma. In the meanwhile, durvalumab was approved for the treatment of metastatic urothelial carcinoma and unresectable stage III NSCLC. A phase 3 clinical trial has found that durvalumab monotherapy could result in a significantly longer overall survival and prolonged PFS as compared with placebo, this led to the approval of durvalumab for the unresectable stage III NSCLC (24-month overall survival rate was 66.3% in durvalumab group as compared with 55.6% in placebo group; the median PFS was 17.2 months in durvalumab group as compared with 5.6 months in the placebo group) (Antonia et al. 2018). A phase 1/2 clinical study designed to assess the efficacy and safety of durvalumab in 191 locally advanced or metastatic urothelial carcinoma patients found that durvalumab demonstrated favorable clinical activity and an encouraging and manageable safety profile (ORR was 17.8%, one-year OS rate was 55, 6.8% grade 3/4 AEs) (Powles et al. 2017). Durvalumab also showed anti-tumor activity with acceptable safety in some other cancer types such as triple-negative breast cancer (Loibl et al. 2019) and PD-L1-high patients with recurrent/metastatic head and neck squamous cell carcinoma (Zandberg et al. 2019), as monotherapy or in combination with chemotherapy. However, these results warranted further investigation in phase 3 clinical trials.

23.4 Therapeutic Development of Combined Blockade of CTLA4 and PD-1

Although the drugs targeting PD1-/PD-L1 and CTLA4 have got great success in the treatment of many kinds of cancers, only a small percentage of patients were seen to respond to monotherapy. A combination of CTLA-4 and PD-1/PD-L1 blockers was suggested to have a synergistic effect in the treatment of cancer patients and could increase the response rates. A large amount of clinical trials have been conducted to test the efficacy and safety of the combination in different cancer types, and some of the trials have suggested combination therapy which showed a remarkable increase in response rates and median survival times, resulting in approval of the combination treatment of ipilimumab and nivolumab.

A combination of ipilimumab and nivolumab has been approved for the treatment of metastatic melanoma, metastatic renal cell carcinoma, and CRC with MSI-H and MMR aberrations. This combination has been studied extensively in metastatic melanoma patients and the efficacy and safety of the combination therapy were demonstrated in multiple clinical trials. Ipilimumab plus nivolumab combination was reported to increase the ORR to 61% in a phase 1 study (Postow et al. 2015), the combination therapy increased the 2-year OS rate to 63.8% in a phase 2 study (Hodi et al. 2016), and the combination therapy had higher ORR, longer median progression-free survival and lower incidence of disease progression or death compared to ipilimumab and nivolumab monotherapy in a phase 3 study (Larkin et al. 2015). The combination of ipilimumab and nivolumab was approved for the treatment of metastatic renal cell carcinoma based on two trials. A phase 1 study found that the ORR reached to 40.4% and 2-year OS rate reached to 69.6% in the combination group (Hammers et al. 2017), a following phase 3 study reported the 18-month OS rate was 75%, ORR was 42%, and median PFS was 11.6 months in the nivolumab 3 mg/kg plus ipilimumab 1 mg/kg combination group (Motzer et al. 2018). The combination of ipilimumab and nivolumab was approved for the treatment of CRC with MSI-H and MMR aberrations based on the results of CheckMate-142 trial. This trial revealed a ORR was 55%, PFS rate was 71%, and OS was 85% in 12 months (Overman et al. 2018).

There are also multiple studies exploring the efficacy and safety of anti-PD-1/PD-L1 plus anti-CTLA-4 antibodies in other types of cancer. A phase 1 study evaluated the safety and efficacy of durvalumab (anti-PD-L1) and tremelimumab (anti-CTLA-4) combination in patients with advanced NSCLC and reported the ORR was 23% (Antonia et al. 2016). Another phase 3 study has been conducted to test the safety and activity of nivolumab and ipilimumab combination as first-line therapy for NSCLC. The study showed that in patients with high tumor mutational burden, a combination of nivolumab and ipilimumab achieved ORR of 45.3%, 1-year PFS rate of 42.6%, and median PFS of 7.2 months (Hellmann et al. 2018). Combination of nivolumab plus ipilimumab was also tested in patients with malignant pleural mesothelioma (Scherpereel et al. 2019), locally advanced or metastatic esophagogastric cancers (Janjigian et al. 2018), metastatic prostate cancer (Boudadi et al. 2018), and metastatic sarcoma (D'Angelo et al. 2018), and have showed promising activity in these patients, therefore, the combination therapy may provide new option for these patients in the future.

The FDA approved indications of ICBs targeting CTLA-4, PD-1, and PD-L1 were summarized in Table 23.1.

Table 23.1 List of approved drugs targeting CTLA-4 and PD-1/PD-L1

Agents	Brand name	FDA approved indications (year of approved)
CTLA-4 blocker		
Ipilimumab	Yervoy	Metastatic melanoma and surgically resectable "high-risk" melanoma (2014)
PD-1 blockers		
Pembrolizumab	Keytruda	1. Unresectable or metastatic melanoma (2014)
		2. Recurrent or metastatic PD-L1^{+} NSCLC (non-small-cell lung cancers) (2016)
		3. Metastatic HNSCC (squamous cell carcinoma of the head and neck) (2016)
		4. Refractory or relapsed Hodgkin lymphoma (2017)
		5. Locally advanced or metastatic urothelial carcinoma (2017)
		6. Locally advanced or metastatic gastric or gastroesophageal junction PD-L1+ adenocarcinoma (2017)
		7. Unresectable or metastatic MSI-H or dMMR solid tumors (2017)
		8. Unresectable or metastatic MSI-H or dMMR colorectal cancer (2017)
		9. Refractory PMBCL (primary mediastinal large B-cell lymphoma) (2018)
		10. Recurrent locally advanced or metastatic gastric/gastroesophageal junction PD-L1 + adenocarcinoma (2018)
		11. Hepatocellular carcinoma previously treated with sorafenib (2018)
		12. Recurrent, locally advanced or metastatic Merkel cell carcinoma(2018)
		13. Recurrent or metastatic cervical cancer with PD-L1 $\geq$ 1% (2018)
		14. Melanoma with lymph node invasion after complete resection (2019)
		15. Stage III PD-L1 + NSCLC who are not candidates for surgical resection or definitive
		chemoradiation (2019)
Nivolumab	Opdivo	1. Unresectable or metastatic melanoma (2014)
		2. Advanced renal cell carcinoma (2015)
		3. Metastatic NSCLC (non-small-cell lung cancers) (2015)
		4. Recurrent or metastatic HNSCC (squamous cell carcinoma of the head and neck) (2016)

(continued)

Table 23.1 (continued)

Agents	Brand name	FDA approved indications (year of approved)
		5. Progressed Classical Hodgkin lymphoma (2016)
		6. Locally advanced or metastatic Urothelial carcinoma (2017)
		7. Progressed Hepatocellular Carcinoma (2017)
		8. Metastatic dMMR and MSI-H colorectal cancer (2017)
		9. Melanoma with lymph node invasion or metastatic following complete resection (2017)
		10. Metastatic SCLC (small-cell lung cancer) (2018)
Cemiplimab	Libtayo	Metastatic or locally advanced CSCC (cutaneous squamous cell carcinoma) (2018)
PD-L1 blockers		
Atezolizumab	Tecentriq	1. Progressed Metastatic NSCLC (non-small-cell lung cancers) (2016)
		2. Advanced or metastatic urothelial carcinoma (2017)
		3. Metastatic SCLC (2019)
		4. Unresectable, locally advanced or metastatic breast cancer with PD-L1 + (2019)
Avelumab	Bevencio	1. Metastatic Merkel cell carcinoma (2016)
		2. Locally advanced or metastatic urothelial carcinoma (2016)
Durvalumab	Imfinzi	1. Locally advanced or metastatic urothelial carcinoma (2016)
		2. Unresectable stage III NSCLC (2018)
Combination of CTLA-4 and PD-1 blockers		
Ipilimumab plus	Yervoy plus	1. Unresectable or metastatic BRAF V600 wild-type melanoma (2015)
Nivolumab	Opdivo	2. Metastatic renal cell carcinoma (2018)
		3. Colorectal cancer with MSI-H and MMR aberrations (2018)

23.5 Therapeutic Development of Next Generation Immune Checkpoint Blockade

CTLA-4 and PD-1/PD-L1 blockade only confers clinical benefits in a limited proportion of cancer patients, therefore, therapeutic agents that target immune checkpoints other than CTLA-4 and PD-1/PD-L1 are currently under clinical investigations. Here, we summarized the therapeutic development of new targets in immune checkpoint blockade, including TIM-3, LAG-3, TIGIT, VISTA, CD39, CD73, A2AR, and NKG2A.

TIM-3

T-cell immunoglobulin mucin-3 (TIM-3), also known as HAVCR2, is a member of the TIM gene family. As a negative regulatory immune checkpoint, TIM-3 is detected in different types of immune cells, including T cells, Tregs, DCs, B cells, macrophages, NK cells, and mast cells. It has four ligands including galectin-9 (Gal-9), high-mobility group protein B1 (HMGB1), carcinoembryonic antigen cell adhesion molecule 1 (CEACAM-1), and phosphatidylserine (PS) (Anderson et al. 2016). By binding to these ligands, TIM-3 could inhibit cancer immunity by negatively regulating T-cell immunity.

TIM-3 expression has several roles in cancer. Firstly, TIM-3 expression is associated with severe T-cell dysfunction in several types of cancers including NSCLC, hepatocellular carcinoma (HCC), CRC, cervical cancer, ovarian cancer, gastric cancer, RCC, head and neck cancer, and so on. TIM-3 could inhibit anti-tumor immunity by mediating T-cell exhaustion in these cancers (Zhu et al. 2015). For example, TIM-3+ CD8+ T cells could impair the functioning of CD8+ T cells in gastric cancer (Wang et al. 2015); in CRC, upregulation of TIM-3 could restrict T-cell responses and might participate in tumorigenesis (Xu et al. 2015); in RCC, TIM-3 expressed on cancer cells and in myeloid cells could inhibit cancer immunity (Komohara et al. 2015); in ovarian cancer, TIM-3 could negatively regulate various T-cell subsets (Fucikova et al. 2019). Secondly, TIM-3 expression on tumor-infiltrating T cells has been suggested to have a role in resistance to PD-1/PD-L1 blockade. It was reported that PD-1 blockade may lead to an increased expression of TIM-3 in a mouse model of lung cancer, and additional TIM-3 blockade conferred survival benefits (Koyama et al. 2016). PD-1 and TIM-3 inhibitors could enhance T cells' response to tumor antigens, and had a synergistic function, therefore, the combined use of TIM-3 blockade and PD-1 blockade could be more effective than the TIM-3 or PD-1 blockade alone. It was reported that Dual TIM-3 and PD-1 blockade synergistically restored the function of tumor-infiltrating T cells from HCC patients (Zhou et al. 2017), melanoma patients (Fourcade et al. 2014), and gastric cancer patients (Lu et al. 2017).

Currently, several clinical trials are focusing on TIM-3 alone or combined with PD-1 as a new approach for the treatment of cancer. Three anti-TIM-3 antibodies, MBG453 (Novartis Pharmaceuticals), LY3321367(Eli Lilly and Company), and TSR-022 (Tesaro, Inc.) are under clinical evaluation in combination with PD-1 blockade for patients with advanced solid tumors, and the clinical benefits are worth looking forward to (He et al. 2018).

LAG-3

Lymphocyte activation gene-3 (LAG-3) is a member of the immunoglobulin superfamily mainly expressed on activated T cells, NK cells, Tregs, B cells, and dendritic cells (DCs). LAG-3 could bind to MHC class II and LSECtin, however, recently fibrinogen-like protein 1 (FGL-1) has been identified as a major inhibitory ligand for LAG-3 (Wang et al. 2019). By binding to these ligands, LAG-3 could suppress T-cells activation and cytokines secretion, and could exert differential inhibitory impacts on various types of lymphocytes (Goldberg and Drake 2011).

Importantly, over-expression of LAG-3 is detected on various TILs and exhibits significant immune regulatory impacts. For example, expression of LAG-3 on tumor-specific CD8+ T cells was first described in ovarian cancer and co-expression of LAG-3 and PD-1 was linked to a more severe T-cell dysfunction (Matsuzaki et al. 2010); LAG-3 is also expressed at a high level on Treg cells, and LAG-3+ Treg cells have a more activated phenotype and confer higher suppressive effect (Chew et al. 2017); LAG-3 blockade can potentially affect CD4+ T-cell populations, lead to a relative skewing from a Treg phenotype, and modulate the function of CD4+ T cells to be suppressed (Durham et al. 2014); In melanoma patient samples, LAG-3 is highly expressed on tumor-infiltrating pDCs, contributing to directing an immune-suppressive environment (Camisaschi et al. 2014). Therefore, LAG-3 may be a promising therapeutic target in cancer immunotherapy.

Interestingly, LAG-3 has remarkable interactions with other immune checkpoints especially PD-1. Increasing evidence has elucidated that LAG-3 has remarkable cooperation with PD-1/PD-L1, which can conjointly mediate immune homeostasis, and enhance tumor-induced tolerance (Okazaki et al. 2011). In animal studies, the striking synergy between LAG-3 and PD-1 has been reported in melanoma, fibrosarcoma, and CRC models, the combinational blockade against LAG-3 and PD-1 could effectively eradicate most established tumors resistant to single agent treatment (Woo et al. 2012). In tumor samples from patients, co-expression of LAG-3 and PD-1 can modulate T-cells exhaustion state (Matsuzaki et al. 2010). A recent study in human NSCLC revealed that over-expression of LAG-3 on TILs significantly correlates with PD-1/PD-L1 expression (Deng et al. 2016). Overall, these preclinical data suggest an apparent synergy between LAG-3 and PD-1/PD-L1, providing the foundation for combinational treatment strategy (Dempke et al. 2017).

Currently, several anti-LAG-3 antibodies, such as BMS-986016, LAG525, MGD013, REGN3767, TSR-033, and INCAGN022385 are under clinical evaluation mostly in combination with PD-1 blockade for cancer patients (Long et al. 2018). Among these agents, BMS-986016 is actively being evaluated in various phase I or II clinical trials in hematological and solid tumors. Notably, the combination of BMS-986016 and nivolumab exhibited exciting preliminary efficacy in melanoma patients who were refractory to anti–PD-1/PD-L1 therapy (Ascierto and McArthur 2017). These promising results support the ongoing more extensive exploration of LAG-3 as an alternative immunotherapy target.

TIGIT

T-cell immunoglobulin and ITIM domain (TIGIT) is a member of the immunoglobulin superfamily that is expressed on T cells and NK cells and functions as an inhibitory checkpoint receptor (Dougall et al. 2017). TIGIT has two ligands, CD115 and CD112, and has a much higher affinity to CD115 (Zhang et al. 2014). Interaction of TIGIT with CD112 and CD155 can be happened *in trans* or *in cis*. TIGIT competes with immunoactivator receptor DNAX accessory molecule-1 (DNAM-1) for the same set of ligands CD155 (Sanchez-Correa et al. 2019). It is also reported that TIGIT could inhibit immunosurveillance through direct inhibition of DNAM-1.

TIGIT appears to have an important role in the suppression of CD8+ TILs. It is reported that TIGIT expressed at a higher level on CD8+ TILs than on other immune

checkpoint receptors, and its expression was also correlated with impaired effector function of CD8+ TILs in acute myeloid leukemia (Wang et al. 2018), multiple myeloma (Guillerey et al. 2018), and gastric cancer (He et al. 2017). TIGIT also has an important role in the suppressive activity of tumor-infiltrating Treg cells. It was proposed that TIGIT primarily suppresses anti-tumor T-cell responses via Tregs rather than CD8+ T cells in mouse models (Kurtulus et al. 2015). Zhang's study also suggested that TIGIT was highly expressed on exhausted tumor-infiltrating NK cells, and TIGIT blockade could reverse NK-cell exhaustion and restore NK cell cytotoxic activity (Zhang et al. 2018). TIGIT's role in the tumor microenvironment may also be intertwined with the microbiome. It was suggested that Fusobacterium nucleatum could directly interact with TIGIT, and cause inhibition of NK cell cytotoxicity (Gur et al. 2015). Furthermore, TIGIT and PD-1 were found to be co-expressed in multiple tumor-associated T cells, and this was seen in colon, endometroid, breast, and renal clear cell carcinoma (Chauvin et al. 2015). These findings suggested that both TIGIT and PD-1 are partners in inducing T-cell exhaustion.

Preclinical trials have revealed the anti-tumor activity of anti-TIGIT agents alone or combined with anti-PD-1 antibodies (Solomon and Garrido-Laguna 2018). Currently, several phase 1 clinical trials evaluating the therapeutic efficacy of anti-TIGIT monoclonal antibodies BMS-9862, OMP-313M32, MTIG7192A, MK-7684, AB154, CGEN-15137, and CASC-TIGIT alone or in combination with anti-PD-1 therapy are ongoing (Dixon et al. 2018).

VISTA

V-domain Ig-containing Suppressor of T-cell Activation (VISTA, also known as PD-1H) is a type I transmembrane protein of the B7 family, and shares similarities with PD-1, CD28, and CTLA-4, with the highest identity with PD-1 (Wang et al. 2011). However, analysis of the IgV domain of VISTA shows the greatest homology with PD-L1, suggesting that VISTA may act as both a ligand and receptor in regulating immune responses (Lines et al. 2014). Unlike other immune checkpoints, VISTA is primarily, if not exclusively, found in hematopoietic tissue cells, including macrophages, dendritic cells, myeloid-derived suppressor cells (MDSCs), and neutrophils. VSIG3, VSIG8, and PSGL-1 have been reported to interact with VISTA and mediate the suppressive effect of VISTA (Wang et al. 2019). In vitro binding study demonstrated that multimeric form of VISTA was bound to activated T cells at acidic pH but not at physiological pH7.0. Co-immunoprecipitation analysis has identified that PSGL-1 interacted with VISTA at acidic pH (Johnston et al. 2019).

In multiple mouse models, VISTA plays a critical role in shaping anti-tumor immunity. Wang's study initially demonstrated that over-expression of VISTA in fibrosarcoma tumor cells significantly increased tumor growth due to an impact of the ligand activity of VISTA on suppressing T-cell immunity (Wang et al. 2011). Le Mercier 's study showed that anti-VISTA monotherapy significantly reduced growth in many different solid tumor models regardless of their immunogenic status or origin (Le Mercier et al. 2014). Taking together, the preclinical studies suggested that anti-VISTA monotherapy reshapes the suppressive nature of the TME by reducing the number of MDSCs and tumor-specific Tregs, and increasing the proliferation of TIL and promoting T-cell effector function. It is also reported that a combined blockade of

VISTA and PD-1 achieved optimal synergistic anti-tumor activity in a mouse model (Liu et al. 2015). Currently, the therapeutic efficacy of CA170, a selective inhibitor of VISTA,is under evaluation (Nowak et al. 2017).

NKG2A

NKG2A is another promising inhibitory checkpoint receptor in cancer immunotherapy. NKG2A is mainly expressed on the surface of T cells and NK cells in a heterodimeric form with CD94, and the main ligand is HLA-E (Manser and Uhrberg 2016). NKG2A has important roles in tumor-infiltrating NK cells. As to know, NK cells play a major role in the anti-tumor immune response by controlling both tumor progression and metastases. However, tumor cells have the ability to escape from NK cell-mediated immune surveillance within the tumor microenvironment (Pahl and Cerwenka 2017). It is reported that cancer cells could inhibit the effector functions of tumor-infiltrating NK cells via the upregulation CD94/NKG2A heterodimer on NK cells (Schleypen et al. 2003). NK cells from AML patients also show an increased expression of NKG2A and impaired effector functions. The increased expression of NKG2A in tumor-infiltrating NK cells is also emerging as a contributor in determining the poor prognosis of cancer, such as hepatocellular carcinoma, lung carcinoma, and invasive breast cancer. Therefore, NKG2A blockade could restore the cytotoxic capacity of NK cells and targeting NKG2A represents a promising cancer immunotherapy.

Monalizumab is a humanized NKG2A blocking antibody. The impact of monalizumab had been first investigated in in vitro and in vivo studies, and the success of preliminary investigations made it possible to develop clinical trials in human cancer patients. Monalizumab was first used after haplo-HSCT because it is demonstrated that the in vitro blockade of CD94/NKG2A early after haplo-HSCT is able to promote NK cell alloreactivity (Roberto et al. 2018). The potential clinical utility of monalizumab in the Chronic Lymphocytic Leukemia (CLL) is also investigated in combination with irutinib, a tyrosine kinase inhibitor already used in the treatment of CLL (McWilliams et al. 2016). Other clinical trials are ongoing for the treatment of different solid tumors including head and neck cancer, ovarian and endometrial cancer, and metastatic colon cancer (Zandberg et al. 2019).

CD39/CD73/A2AR pathway

CD39/CD73/A2AR/adenosine pathway has recently drawn lots of attention in cancer immunotherapy field. Adenosine is involved in many pathophysiological processes particularly it supports development of immunosuppressive cells like regulatory T cells and myeloid-derived suppressor cells (MDSC) through the binding and activation of A2AR. Ectoenzyme CD39 hydrolyzes extracellular ATP to ADP and AMP, where CD73 converts AMP to adenosine. Adenosine exerts its biological functions through binding to adenosine receptors (Perrot et al. 2019). Expression of CD39 and CD73 have been shown to be upregulated in tumor microenvironment that promotes the development of immune-suppressive cells like regulatory T cells, myeloid-derived suppressor cells M2 macrophage, at the same time, inhibits T-cell functions

(through upregulation of CTLA-4, PD-L1, and LAG-3) (Zarek et al. 2008), dendritic cells activation, reduction of NK cell cytotoxic capability, neutrophils attachment. Oleclumab (<EDI9447), a fully human anti-CD73 antibody from Medimmune/AstraZeneca is currently in Ph I and II clinical studies as a single agent or in combination with anti-PDL-1 or chemotherapy across various solid tumors including advanced NSCLC (Vigano et al. 2019), metastatic TNBC, and pancreatic cancer as well as PD-1/PD-L1 resistant NSCLC. Many anti-CD39 and anti-CD73 therapeutics antibodies are in preclinical stage.

23.6 Current Challenges of Immune Checkpoint Blockade Therapy in Cancer

In order to maximize the efficacy of immune checkpoint blockade therapy for cancer patients, some major challenges in this field must be addressed.

One of the major challenges is the toxicities associated with immune checkpoint blockade therapy for cancer. The immune checkpoint inhibitors are not directed solely to tumor-specific T cells, therefore, these drugs may lead to activation of non-tumor-specific immune responses that target self antigens expressed on healthy tissue. This can result in immune-related adverse events (irAEs) due to enhanced T-cell responsiveness, and the activation of self-reactive T cells. The most common irAEs include pruritis and mucositis, vitiligo, diarrhea, and immune-mediated colitis. Less common irAEs include hepatotoxicity, endocrinopathies, and pneumonitis, and rare irAEs include renal toxicity, neurotoxicity, pancreatitis, cardiovascular toxicity, and hematological abnormalities (Kumar et al. 2017). A recent systematic review concluded that grade 3/4 treatment-related adverse events occurred in 14% of patients treated with PD-1/PD-L1 inhibitors, in contrast to 34% of patients treated with CTLA-4 blockade, increasing to 55% during PD-1/PD-L1 and CTLA-4 combination therapy (Arnaud-Coffin et al. 2019). The majority of the irAEs could be treated with corticosteroids and other immunosuppressive drugs. Such drugs might be expected to counteract the action of ICBs, although some studies have reported no obvious therapeutic disadvantage to patients treated with ICBs when corticosteroids were used to alleviate the symptoms of irAEs (Garant et al. 2017). Therefore, it is urgent to explore the methods to retain the efficacy and alleviate the side effects. Ishihara's study has shown that the safety of anti-PD-L1 antibody in mouse models can be improved by fusing it to the collagen-binding domain of von Willebrand factor, thereby allowing it to bind to the tumor stroma and exert its effects locally (Ishihara et al. 2019). Optimization of dosing regimens could also reduce irAEs in some studies (Lebbe et al. 2019). More efforts are needed to alleviate the side effects of ICBs in future studies.

Another challenge is to gain insight into factors that influence response outcomes to ICB therapy and to better understand and overcome tumor resistance to ICB therapy. It is a fact that most cancer patients do not respond or do not show long-lasting

remission after ICB treatment. Despite the clinical benefits of ICBs, the response rates to date have rarely exceeded 40% (Pitt et al. 2016). Patients that do not respond to ICB are said to have "innate resistance", while those responding transiently before disease progresses have "acquired resistance" (Park et al. 2019). There is urgent to clarify the mechanisms underpinning innate and acquired resistance, and develop accurate ways of predicting which patients will benefit from ICB therapy.

Apart from "innate resistance", another major factor that contributes to low response rate for ICB is the lack of tumor T-cell infiltration or so-called "cold tumor". The lack of T-cell infiltration includes lack of tumor-specific antigens, defect in antigen presentation by antigen presenting cells, inhibition of T-cell activation, or homing to the tumor sites. Conversion of "cold tumor" to "hot tumor" have been a focus in improving the overall response rate of ICB. Various approaches or therapeutic combinations are being tested both in patients or animal models. Combination of ICB with chemotherapy or radiation, oncolytic viruses, tumor antigen vaccination as well as DC activation agents (Toll like receptor agonist or CD40 agonist) is being explored to enhance T-cell activation or priming. Anti-TGF, anti-angiogenic agents or IL-2/IL-15 have been used to improve T-cell trafficking and infiltration into tumor microenvironment. Bispecific antibody such as T-cell engager or NK cell engager is another approach being investigated for recruiting T cells or NK cells to tumor sites. Bispecific antibodies comprising antibodies against ICI and innate immunity targets such as anti-PD-L1/anti-TGF and anti-PD-L1/anti-CD47 are under extensive investigation.

Researchers have proposed some possible mechanisms that may be responsible for this resistance. One of the mechanism is the tumor mutational burden. It is reported that high mutational burden is usually associated with a positive outcome for patients treated with ICB. This is because they contain more potential neo-Ags, therefore increasing the chance of anti-tumor T cells becoming activated (Gandara et al. 2018; Samstein et al. 2019; Hellmann et al. 2019). The second potential mechanism is T-cell priming and infiltration of the TME. Responses to ICB therapy depend on the number and diversity of previously activated tumor-specific T cells present in the tumor patient. Tumors with extensive effector T-cell infiltrates will respond best to the ICB therapy. In other words, tumors will be resistance to ICB therapy if there are insufficient tumor-specific T cells, or if these cells are unable to enter the TME to exert anti-tumor activities (Gide et al. 2019; Smith et al. 2019). Another mechanism is the accumulation of additional metabolic and immunosuppressive factors in the TME may limit the efficacy of T-cell responses elicited by ICB therapy. It is reported that tumor cells can outcompete T cells for glucose to reduce glycolytic activity and IFN-γ production by T cells, ICB therapy could restore T-cell glycolysis (Chang et al. 2015). Genetic defects in IFNγ pathway-related genes are also involved in the resistance to ICB therapy (Gao et al. 2016). It is also emerging that the microbiome could influence responses to ICB therapy. Studies on patients with melanoma or NSCLC have revealed that the certain bacterial species in the oral or gut microbiome could influence the responses to PD-1/PD-L1 blockade, and antibiotics can reduce the clinical benefit of PD-1/PD-L1 blockade therapy in cancer patients and mice (Matson et al. 2018; Routy et al. 2018). In-depth study and understanding these

mechanisms could develop effective strategies to overcome the resistance to ICB therapy.

23.7 Conclusion

Immune checkpoint inhibitor therapy, especially anti-PD-1/PD-L1 therapy has demonstrated clinical efficacy in multiple types of solid and hematologic tumors, thus FDA has approved six ICB drugs for the treatment of various tumors in recent years, and more promising clinical trials are ongoing to explore the potential anti-tumor activity in more kinds of cancer. However, the irAEs and resistance to ICB therapy are the current major challenges, and more efforts are warranted to develop more effective strategies to overcome the resistance to ICB therapy.

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Chapter 24
Concluding Remarks

Jie Xu and Mingyao Liu

Abstract The regulation of immune checkpoint is a pivotal mechanism mediating both self-tolerance physiologically and tumor immune evasion pathologically. Along with an increasing number of identified checkpoint ligand–receptor pairs, the complexity of regulation at genetic, epigenetic, transcriptional, translational, and post-translational levels makes it highly challenging to assemble a comprehensive regulatory network. Advanced animal models are required for determining the exact regulatory effects, given the differences in human and mouse immune systems. Our further understanding on checkpoint regulation may energize translational studies aimed to improve cancer immunotherapy, and collaborations between researchers with different expertise would help to tackle existing challenges in this field.

Keywords Immune checkpoint regulation · Trans-omics · Translational medicine · Precision medicine · Combinatorial therapy

In the previous chapters, we have covered different aspects of researches on immune checkpoints, with both advances and challenges highlighted. Here, we further discuss some key questions and trends in the studies in this field.

Firstly, there is an urgent need for further insights into the physiological and pathological roles of immune checkpoints. From a mechanistic perspective, the adverse effects of ICB therapies are virtually the costs of blocking certain intrinsic functions of immune checkpoint signaling, so physiological researches may help to improve the safety of ICB therapy. Tumor cells may accumulate molecular alterations during development and therapeutic stresses, so the mechanisms of acquired resistance to ICB therapies may vary between tumors with different genetic/immunological profiles. Therefore, to prolong the efficacy of ICB therapies, it is essential to gain further

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insights into the dysregulation of immune checkpoints. To increase tumor responses to ICB therapy, a comprehensive survey on the molecular and cellular events behind immune evasion is also fundamental. Recent clinical trials on PD-L1/TGFβ bispecific antibody have demonstrated higher response rate as compared to PD-L1 monoclonal antibody, and the success is based on the finding that TGFβ-mediated fibrosis may cooperate with PD-L1 signaling to suppress immunosurveillance in the tumor microenvironment (Strauss et al. 2018). Inevitably, the efficacy of bispecific antibodies and combinatorial immunotherapies currently under development would return to a fundamental question, i.e., whether these targets may jointly constitute an essential environment for tumor immune evasion. At the present stage, we are still far from fully understanding these "intercellular star wars".

The second point is actually connected to the first, regarding the methods for resolving the complex roles of immune checkpoints. As already reported, trans-omics profiling of cancer tissue samples has facilitated the characterization of tumor microenvironment at high resolution. Meanwhile, genome-wide screening approaches have facilitated the identification of new regulators for immune checkpoints (Wang et al. 2018). In this post-genomic era, it is necessary to see deeper and wider into the "intercellular star wars" using "biological hubble telescopes", i.e., trans-omics profiling techniques. Combined with the fast-evolving bioinformatic tools, cancer trans-omics data are ready to be translated into specific molecular patterns behind the dysregulated immune checkpoints, which will be useful for developing novel biomarkers and immunotherapies.

Another issue to point out here is the importance of clinical studies for confirming the findings from in vitro or animal studies. Since the interactions between cancer and immune system involve various cells, cytokines, and extracellular matrix, in vitro studies commonly cannot perfectly reflect the actual situations in vivo. The immune systems between human and mouse are of lower conservation as compared to many intracellular signaling pathways (Dominguez-Andres and Netea 2019), so the findings based on mouse models have to be interpreted with enough caution when trying to understand the mechanisms in human. Based on these, human-based studies are crucial for validating the findings on immune checkpoints, and the variance between individuals also reminds us on the importance of high-quality and large-scale clinical studies. The tight connections between basic and translational researches may be changing the mode of researches on immune checkpoints. While trying to understand how tumor cells may evade immune attack, researchers may develop antibody inhibitors for the proposed checkpoint ligands or receptors, and usually animal-based studies are used to test the efficacies of the checkpoint inhibitors. These researches naturally join basic research and drug development processes, and related patents may be filed before the research paper is published. The more complete intelligence property owned by the researchers may facilitate further translational development, which is a nice trend to see.

Perhaps immune checkpoint blockade therapies would be the best practitioners of precision medicine, given that companion diagnostics is so crucial for the success of checkpoint inhibitors (Conway et al. 2018). There is considerable difference in the response rate of tumors to PD-1 blockade with or without patient selection based

on PD-L1 expression and tumor mutation burden. It is also reasonable to expect the prevalence of companion diagnostics for using the other checkpoint inhibitors that may be approved for clinical use in the future. The heterogeneous nature of tumor immune evasion mechanisms raises the bar for the accurate use of specific checkpoint inhibitors, which make the companion diagnostics as important as the therapeutic drugs for successful treatment. Thus, the patents for companion diagnostics may provide a complementary strategy for protecting the intelligence properties associated with certain therapeutic targets and drugs.

Although antibodies represent the most successful and prevalent form of checkpoint inhibitors, there is still a need for diversifying checkpoint inhibitors. In addition to some obvious advantages such as high targeting specificity and long half-life in the body, antibody drugs share some concerns such as higher cost and production requirements, as well as the single mode of action (MOA). Along with the discoveries on the regulation of immune checkpoint ligands and receptors, more chances are provided to intervene the tumor immune evasion process, introducing small-molecular and peptidic inhibitors with various MOAs. Efforts are being made to diversify the inhibitors for an existing target, which may provide additional therapeutic opportunities for the cancer patients who have acquired resistance to antibody inhibitors.

We are entering a golden age of immune checkpoint researches, experiencing the evolvement of research methods, and witnessing the success translation of basic findings into clinical therapies. Studying the regulation of immune checkpoints represents a manner to "kill two birds with one stone", as it will help to both lower the adverse effects and increase the therapeutic benefits of immune checkpoint therapies.

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