Progress of immune checkpoint LAG-3 in immunotherapy (Review)

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Abstract. Immune checkpoint inhibition has been shown to successfully reactivate T cell responses directed against tumor-associated antigens, resulting in significantly prolonged overall survival in patients with various types of solid tumors. Among them, cytotoxic T-lymphocyte protein 4 (CTLA-4) and programmed cell death protein 1 (PD-1) play key roles in tumor immune escape and are well-established targets of cancer immunotherapy. However, the low response rate PD-1 and CTLA-4 is a limitation and a challenge. Hence, studies have focused on investigating the tumor microenvironment for alternative therapeutic targets. Lymphocyte activation gene 3 protein (LAG-3) negatively regulates T lymphocytes by binding to the extracellular domain of the ligand, thus avoiding autoimmunity caused by T cell overactivation. LAG-3 is an important immune checkpoint *in vivo* and plays a balanced regulatory role in the human immune system. LAG-3 is now regarded as a new generation of immunotherapy targets. The present review describes the research progress of LAG-3 to provide reference for further investigation of LAG-3. The immune checkpoint of LAG-3 plays a crucial role in cancer development and may be used in future clinical practice of cancer therapy.

Contents

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1. Introduction

In the past few decades, there has been a rapid development in tumor immunotherapy, which has been recognized as a key strategy to control the progression of malignant tumors (1). Among these immunotherapies, immune checkpoint inhibitors (ICIs) (2), chimeric antigen receptor T cells (3) and bispecific antibodies (4) are the most promising immunotherapeutic strategies. ICI therapy is one of the most well-studied immunotherapies (2,5). Inhibitors block the transmission of inhibitory signals, stimulate the activation of cytotoxic T lymphocytes (CTL) and induce the antitumor effects of T lymphocytes. Two ICIs have been investigated, including cytotoxic T lymphocyte antigen 4 (CTLA-4) (6) and programmed cell death 1/cell death 1 ligand, (PD-1/PD-L1) (7). However, the response rate of anti-PD-1/PD-L1 monoclonal antibodies (mAbs) and anti-CTLA-4mAb in patients with cancer overall is poor (8,9). Most patients show primary or acquired resistance to these ICIs (10,11). As a member of the ICI family, LAG-3 is an inhibitory molecule with multiple biological effects on the function of T cells (12). LAG-3 is highly expressed in various types of tumor infiltrating lymphocytes (TILs) and participates in the immune escape mechanism of tumors (12). Therefore, LAG-3 could be used as an indicator of tumor prognosis and a target of tumor therapy. The present review primarily describes the research progress of LAG-3 in immunotherapy.

2. Protein structure and function of LAG-3

LAG-3 is a member of the immunoglobulin superfamily, which was identified in 1990 (13). LAG-3, also known as CD223, has a molecular weight of 70kDa and is located on human chromosome 12 (12p13) (14). The LAG-3 gene is located in the same region as the CD4 gene, and there is a certain homology between the two (15); however, they share<20% homology at the protein level (13). Similar to CD4, LAG-3 binds to major histocompatibility complex-II (MHC-II) on antigen presenting cells (APCs), but with a much stronger affinity (13). The LAG-3 gene contains 8 exons, and the corresponding cDNA encodes a membrane protein, with 498 amino acids (16). LAG-3 is

composed of an extracellular region, a transmembrane region and an intracellular region (17). The membrane protein contains four extracellular immunoglobulin superfamily domains: One V region and three C2 regions (16,17). The V region is special, as there is an extra ring in the middle of the domain and also contains an abnormal in-chain disulfide bridge (18). The cytoplasmic region of LAG-3 is composed of three parts: A serine phosphorylation site, a 'KIEELE' motif and a glutamate-proline dipeptide repeat sequence (EP sequence), among which the 'KIEELE' motif is a highly conserved sequence that has not been found in other proteins and is involved in intracellular signal transduction of the LAG-3 molecule (17,19) (Fig. 1). It was initially hypothesized that the EP sequence was the key basis for the downstream signal of LAG-3. However, the result of mutation analysis found that this was not the case. The 'KIEELE' motif in the LAG-3 molecule is conserved (17), and molecules that lack this domain are unable to exert an inhibitory effect on T cells, suggesting that this motif may be associated with the downstream inhibitory signal of LAG-3 (17). However, the mechanism of downstream signal transduction is still unclear. Under the action of the membrane-penetrating metalloproteinases disintegrin and metalloproteinase domain-containing protein (ADAM) 10 and ADAM17, the LAG-3 molecule on the cell membrane splits into two parts at the junction peptide: Soluble LAG-3(sLAG-3) and a transmembrane-cytoplasm molecule (20). sLAG-3 has been found to induce maturation of dendritic cells and target tumor cells (21). Previous studies have discovered five primary ligands of LAG-3. In a quantitative cell adhesion experiment, COS-7 cells transfected with LAG-3 and DaudiB lymphocytes labeled with ^{51}Chromium were co-cultured together, and it was found, for the first time, that a garland was formed between the two cells, which was specifically dependent on LAG-3 and MHC-II molecules (14,22). Anti-LAG-3 or HLA-DR Ab have been found to destroy the formation of this garland, confirming that the ligand of LAG-3 is the MHC II protein and combines with a conservative extension ring in the LAG-3 D1 domain (14,22). LAG-3, once combined with MHC-II, transmits inhibitory signals via the cytoplasmic domain; however, the signal transduction mechanism is still unclear (17). Galectin-3 (Gal-3), a 31-kDa galactose-binding lectin that regulates T cell activation and immunoprecipitation, was found to interact with LAG-3 and inhibits the secretion of interferon-γ by CD8$^+$T cells *in vitro*, indicating that Gal-3 is also an LAG-3 ligand (23). Gal-3 can be expressed on the surface of different cancers, such as lung cancer (24), prostate cancer (25), colorectal cancer (26), breast cancer (27) and so on. Thus it exerting its regulatory function on CD8$^+$T cells via multiple mechanisms (28). LSECtin has also been proposed to be a LAG-3 ligand. LSECtin binds to the four glycosylated sites on LAG-3 and is a member of the DC-SIGN family of molecules (28). LSECtin is expressed in the liver and in melanoma tumor cells, suggesting a mechanism by which LAG-3 can regulate CD8$^+$T cells and natural killer (NK) cell function in these environments (29). α-synuclein (α-syn) fibrils is a protein aggregation, which exists in the substantia nigra of the brain in patients with paralysis tremor and is a member of the Syn fibrils family (28-30). α-syn was found to bind to LAG-3, which leads to the intercellular delivery of pathological α-syn fibrils, while blocking their combination with a LAG-3 Ab could significantly reduce the toxicity and the intercellular delivery of pathological α-syn fibrils, suggesting that α-syn fibrils were also a LAG-3 ligand (31). Fibrinogen-like protein 1(FGL1) was recently identified as the primary ligand of LAG-3, and a genome-scale reporter array identified that the FGL1 protein was tightly bound to the LAG-3 receptor (32). Functional detection of the FGL1 protein revealed that upon binding to the LAG-3 receptor on the surface of T cells, T cell proliferation was inhibited and immune activity was also affected (33). Blocking the interaction between FGL1 and LAG-3 could enhance the antitumor effect of T lymphocytes, which has important significance in the research of tumor immunotherapy (32). LAG-3 is expressed on activated CD4$^+$ (34) and CD8$^+$T cells (35), regulatory T cells (Tregs) (36), a subpopulation of NK cells (37), B cells (38) and plasmacytoid dendritic cells (39). As an inhibitory molecule expressed on the surface of lymphocytes, LAG-3 is involved in regulating the proliferation and activation of T lymphocytes and effector T lymphocytes, and plays a specific role in maintaining environmental stability *in vivo* (36). The process of T cell inactivation and death are present both in cancer and chronic infection (40). As a co-inhibitory receptor of PD-1, LAG-3 is highly expressed in chronic virus infection and various tumors (41). The high expression of LAG-3 is also associated with autoimmune diseases, tumors and chronic toxic infectious diseases (18,42-44).

3. Biological characteristics of LAG-3

Immunosuppressive receptor molecules play an important role in the maintenance of immune homeostasis. When T lymphocytes are activated to a certain extent, immunosuppressive molecules, such as LAG-3, CTLA-4 and PD-1 are expressed to maintain the immune response in a stable state (45). The molecule LAG-3 blocks the signal transduction pathway of T cell activation; however, the intracellular segment of the LAG-3 molecule produces immunosuppressive signals, which have been found to regulate CD4$^+$T cell activity (46). LAG-3 regulates the immune response of T cells in three ways: Firstly, it directly inhibits the proliferation and activation of T cells via negative regulation of T cells. Secondly, it can promote the inhibitory function of Tregs, and the T cell response can then be indirectly inhibited. Thirdly, it can prevent T cell activation by regulating the function of APCs (47). A LAG-3 knockout mouse model showed increased numbers of CD4$^+$T and CD8$^+$T cells in the compared with wild-type mice; however, there were no significant changes in the ratio of CD4$^+$T and CD8$^+$T cells (48). Additionally, studies also found that LAG-3mAbs or LAG-3 knockout cells did not significantly alter the apoptosis of T cells. However, the proportion of S phase cells increased significantly, suggesting that the inhibitory effect of LAG-3 on T cell proliferation may be by controlling the cell cycle rather than through apoptosis (49). Therefore, LAG-3 plays a key role in regulating immune homeostasis. Under normal physiological conditions, naive CD8$^+$T cells are expressed at low levels (48). LAG-3 expression was significantly increased when tumor antigen cells were stimulated (50). LAG-3 has also been shown to bind to tumor infiltrating lymphocytes (TILs) in multiple solid tumors, and TILs are highly expressed in human solid tumors (51-53). LAG-3 negatively regulates TILs

Table I. LAG-3 immunotherapy clinical trials.

Intervention (trial number)	Company	Drug form	Date of first approved testing	Clinical trial disease
IMP321 (NCT00351949, NCT00349934, NCT00324523 NCT03252938, NCT02614833, NCT02676869, NVT03625323)	Prima BioMed/Immutep	Soluble LAG-3 Ig	September 2015	Phase I: Stage IV renal cell carcinoma; stage III/IV phase melanoma; pancreatic cancer; solid tumor peritoneal carcinoma Phase II: Non-small cell lung cancer; squamous cell carcinoma of head and neck; Phase IV: Breast cancer
BMS 986016 (NCT01968109, NCT02966548, NCT03470922, NCT03743766, NCT02519322, NCT03623854, NCT02488759, NCT02060188)	Bristol-Myers Squibb	Relatlimab, anti-LAG-3 mAb	January 2013	Phase I: Glioblastoma; gliosarcoma; gastric cancer; esophageal cancer; gastroesophageal cancer; recurrent brain tumors Phase II: Blood tumor; melanoma; gastroesophageal cancer; chordoma
GSK2831781 (NCT02195349)	GlaxoSmithKline	Humanized IgG1	July 2014	Phase I: Psoriasis
LAG525 (NCT03365791, NCT02460224, NCT03499899, NCT03484923)	Novartis	Anti-LAG-3 mAb	June 2015	Phase I: Advanced solid tumor Phase II: Small cell lung cancer; gastric adenocarcinoma; esophageal adenocarcinoma; antiprostatic adenocarcinoma; soft tissue sarcoma; ovarian adenocarcinoma; neuroendocrine tumor; diffuse large B cell lymphoma
RENG3767 (NCT03005782)	Regeneron Pharmaceuticals	Anti-LAG-3 mAb	November 2016	Phase I: Malignant tumor
BI-754111 (NCT03156114)	Boehringer Ingelheim	Anti-LAG-3 mAb	January 2017	Phase I: Non-small cell lung cancer; head and neck tumors Phase II: Metastatic tumors
TSR-033 (NCT03250832)	Tesaro, Inc.	Anti-LAG-3 mAb	August 2017	Advanced solid tumor
MGD013 (NCT03219268)	MacroGenics	PD-1/LAG-3 bispecific DART protein	January 2017	Phase I: Advanced metastatic solid tumor; blood tumor
FS118 (NCT03440437)	F-starDelta Limited	LAG-3/PD-1 bispecific antibody	April 2018	Phase I: Advanced metastatic tumor

Table I. Continued.

Intervention (trial number)	Company	Drug form	Date of first approved testing	Clinical trial disease IMP321
Sym022 (NCT03489369)	Symphogen A/S	Anti-LAG-3 mAb	May 2018	Phase I: Metastatic tumor; Solid tumors; lymphoma
INCAGN02385 (NCT03538028)	Incyte Biosciences International Sàrl	Anti-LAG-3 mAb	June 2018	Phase I: Cervical cancer; gastric cancer; esophageal cancer; hepatocellular carcinoma; melanoma (excluding uveal melanoma); Merkel cell carcinoma; Mesothelioma; MSI colorectal cancer; non-small cell lung cancer; ovarian cancer; squamous cell carcinoma of the head and neck; renal cell carcinoma; triple negative breast cancer; urothelial carcinoma; diffuse large B cell lymphoma
EOC202	EddingPharm	Human recombinant fusion protein	September 2018	Phase I: Adult solid tumor
(NCT03600090)	OncologyCo., Ltd.			

LAG-3, lymphocyte activation gene3; mAb, monoclonal antibody; PD-1, programmed cell death protein 1; DART, dual-affinity re-targeting antibody.

and anti-LAG-3 Ab can enhance antitumor immunity (54). Woo *et al* (55) found that LAG-3 and PD-1 were expressed in $CD4^+$ and $CD8^+$TIL in mouse cancer models and can enhance the cellular immune response of $CD8^+$T cells by co-inhibiting LAG-3 and PD-1. Surprisingly, despite the high affinity, only a handful of residues located in the D1 loop of LAG-3 are involved in MHC-II and LAG-3 binding, in contrast to the extensive molecular interaction between MHC-II and CD4 (22). In 2006, Casati *et al* (56) found that sLAG-3 binds to MHC-II and mediates APC activation, thereby activating and promoting the production and proliferation of $CD8^+$T lymphocytes. This indicated that sLAG-3 could compete with LAG-3 molecules to bind MHC-II and thus inhibiting LAG-3 from exerting a negative regulatory role on the proliferation and activation of T cells. Blocking with an inhibitor or knock-down of the LAG-3 gene has been found to accelerate the progression of autoimmune diseases in several animal models of autoimmune disease (17). The progression of diabetes was found to accelerate in mice lacking the LAG-3 gene in an obese diabetic mouse model (57). The infiltration of $CD4^+$T and $CD8^+$T cells around the islet cells in the mice increased significantly, accelerated the destruction of islet cells and all of the mice eventually developed diabetes (57). LAG-3 was also found to be involved in immune homeostasis and autoimmune diseases with other immunosuppressive molecules, such as Tim-3, TIGIT (58).

Typically, LAG-3 is expressed on the surface of activated T and NK cells to maintain immune response homeostasis and prevent the occurrence of immune overreaction or autoimmune diseases (22). However, in the tumor microenvironment (TME) or chronic infectious diseases, the continuous activation of T cells leads to co-expression of immunosuppressive molecules, such as LAG-3, PD-1, T-cell immunoglobulin-and mucin domain-containing (Tim)-3, TIGIT and CD160, resulting in the incapacitant and even apoptosis of T cells, which is the 'deceptive mechanism' by which tumors and chronic infectious diseases escape from being killed by the immune system (59). On the surface of activated $CD4^+$and $CD8^+$T cells, LAG-3 is physically integrated with CD3 (60) and then specifically binds to the CD3-TCR complex after TCR participation acting as a co-receptor for negative signals (61).

4. Role of LAG-3 in tumor development

The immune system plays an important role in removing malignant cells (62). ICs are inhibitory signals that exist in the immune system which continuously regulates the immune response intensity of peripheral tissues in the normal healthy body and maintains the tolerance of autoantigens to prevent damage to tissues (63). However, immune or tumor cells in the TME highly express inhibitory IC co-stimulatory molecules, leading to the depletion and disability of antitumor T cells, which play an important role in tumor immune escape (63). LAG-3 expression is increased in TILs and in numerous types of cancer (64). In NSCLC, LAG-3 expression has been observed in TILs and studies have shown that its expression is associated with poor prognosis (65). LAG-3 negatively regulates TILs and anti-LAG-3 Ab was found to enhance antitumor immune function (54). LAG-3 and PD-1 are co-expressed on $CD4^+$ and $CD8^+$TILs, and when LAG-3 and PD-1 are jointly

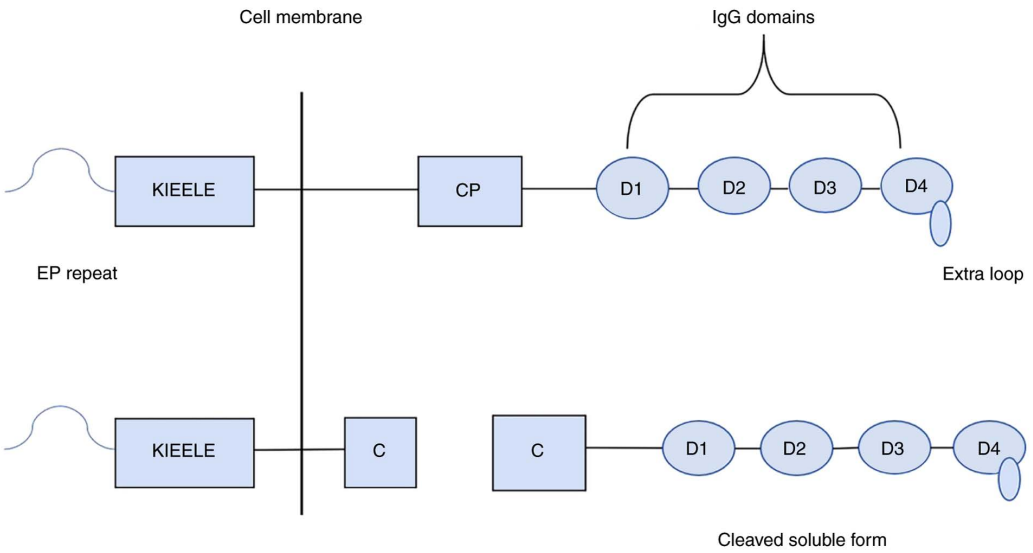

Figure 1. LAG-3 structure. LAG-3 is composed of four Ig-like domains and contains three highly conserved regions in the cytoplasmic tail. LAG-3, lymphocyte activation gene-3; D, domain.

inhibited, the immune response of tumor $CD8^+$T cells is enhanced. Blocking the LAG-3 receptor with an inhibitor can be used in combination with antitumor vaccines to improve the activation of tumor-specific $CD8^+$T cells. Notably, this effect does not involve $CD4^+$T cells, but rather by LAG-3 directly regulating $CD8^+$T cells (48). The co-expression of LAG-3 and PD-1 in inactivated and dying T cells has been observed in human ovarian cancer tissues (66). In a mouse model of ovarian cancer, blocking of LAG-3 and PD-1 was found to promote tumor antigen specific $CD8^+$T cells to produce cytokines, and joint blocking of LAG-3 and PD-1 in the treatment of ovarian cancer was more effective compared with immune inhibitors and blocking of a single molecule (66). LAG-3 expression in $CD4^+$ and $CD25^-$T cells in non-small cell lung cancer was found to be upregulated and significantly associated with poor prognosis (67,68). LAG-3 expression on the surface of $CD8^+$T cells in TIL was significantly increased in patients with hepatocellular carcinoma compared with on peripheral blood lymphocytes (64). The mRNA expression levels of LAG-3 molecules was increased on the $CD8^+$T cell surface of TILs and led to the functional defects of $CD8^+$T cells (69). LAG-3 is also highly expressed in Treg cells. LAG-3^+FoxP3^+Treg cells have been shown to be highly expressed in the tumor tissues of peripheral blood mononuclear cells (including lymphocyte and dendritic cells), TIL and in patients with melanoma and with colorectal cancer (70). These LAG-3^+Treg cells show an activation phenotype, producing interleukin (IL)-10 and transforming growth factor (TGF)-β1, and LAG-$3^+$$CD49b^+$cells are associated with poor prognosis in colorectal cancer (70). LAG-3 expression on Tregs induced IL-10 and TGF-β1 production (71). This indicated that LAG-3^+Treg cells have an immunosuppressive effect and are associated with tumor immune escape (72). A recent study found that co-culture of DLBCL cell lines with primed T cells in the presence of anti-LAG-3 and anti-TIM-3Ab induced potent dose-dependent increases in *in vitro* cell death using AcellaTox and IL-2 ELISA assays, suggesting potent antitumor activity of these compounds (73). Woo *et al* (55) established B16 melanoma, MC38 colorectal adenoma and Sa1N fibrosarcoma mouse models and found that most tumors in groups with double-blocking of LAG-3/PD-1 disappeared. The results of flow cytometry analysis showed that the number of $CD4^+$ and $CD8^+$T cells in the group of mice with the combined treatment increased significantly. Wierz *et al* found that (74) in a mouse model of chronic lymphocytic leukemia, double blocking of PD-1 and LAG-3 significantly decreased the number and percentage of chronic lymphocytic leukemia cells in the blood and spleens of mice. Additionally, the serum levels of IL-2, IL-23 and TNF-α in mice with double blocking of PD-1 and LAG-3 was also increased, along with the immune response, proving that the dual target of PD-1 and LAG-3 could control the increase in the yield of chronic leukemia cells in mice. Huang *et al* (75) randomly assigned ovarian cancer-bearing C57BL/6 mice into different groups and treated them with anti-PD-1, anti-LAG-3 or anti-PD-1/LAG-3 Ab treatments. The results showed that the double blocking of LAG-3 and PD-1 inhibited tumor growth, significantly increased the number of $CD4^+$ and $CD8^+$TILs and enhanced the antitumor immune response. LAG-3 is often co-expressed with PD-1 on exhausted T cells, and targeting of both using anti-LAG-3 and PD-1Ab was effective at reinvigorating T cells and eliminating mouse tumors (74,76). Accumulating evidence suggested that multiple ICIs could optimize antitumor immunity (63,77,78). However, the mechanism by which PD-1 and LAG-3 pathways operate together to inhibit T-cell functions is not known. The Yale Cancer Center found that the protein FGL1 plays an important role in LAG-3 immunosuppression (32). Inhibition or knockdown of FGL1/LAG-3 promoted the effectiveness of T cells against tumors in the body (32). Blocking this pathway could be used in conjunction with anti-PD-1/PD-L1 therapy to achieve antitumor efficacy (32).

LAG-3 expression in the TME has also been associated with increased tumor mutational burden. For example, cancers with high microsatellite instability (MSI^{hi}), which includes a subset of patients with colorectal cancer, exhibited higher somatic mutations and higher levels of immunogenic neoantigens. The

TME of these MSI^{hi} tumors are characterized by increased expression of multiple IRs including LAG-3, PD-1, PD-L1, CTLA4 and indoleamine 2,3-dioxygenase (IDO) compared with in microsatellite stable tumors (79). These data may suggest an association between tumor mutational burden, antitumor immune response and the co-expression of LAG-3 and other IRs. In fact, the presence of LAG-3 and other co-expressed IRs in the TME may explain why MSI^{hi} tumors are not naturally eliminated, despite a hostile immune microenvironment (79).

5. Conclusion

ICIs have innovated the treatment of cancer, and the combination of LAG-3 and PD-1 drugs has been effective (54). The corresponding clinical trials of LAG-3 and its effects on tumors have been performed in the USA, Australia, etc. (54,80). Until 2019, one LAG-3 fusion protein and 11 LAG-3 inhibitors were in clinical trials or are being used as anticancer therapies (Table I). A phase I clinical trial using LAG-3 as an immunotarget for cancer is under way, including a single center trial for hematological malignancies (clinical trial no. NCT02061761). In addition, LAG-3 specific Abs have been used in the combination of anti-PD-L1 and anti-LAG-3 therapy for solid tumors (clinical trial no. NCT01968109). However, there are still numerous problems to be solved in the investigation of LAG-3. Firstly, the biological function of LAG-3 binding to ligands is still unclear, and the specific mechanism of its negative regulation of T cell function is also still unknown. Therefore, further investigation is required. Secondly, there might be other potential ligands of LAG-3 that could be used as biological markers for the diagnosis and evaluation of prognosis. Thirdly, whether LAG-3 can play an antitumor role by regulating NK cells or B cells remains to be further investigated. Fourthly, how the molecular mechanism of different ICIs plays a co-role is not clear, and it is still important to identify the optimal IC combination. Lastly, whether modern advanced biological technology can be used to optimize the molecular structure of LAG-3 inhibitors and reduce production costs and to ensure improvements in a clinical setting remains to be determined. Taken together, LAG-3 has the potential to target molecules involved in biological functions and play a therapeutic role. There are numerous unknown links in the specific mechanism, and more depth understanding is required, such as ensuring LAG-3 inhibitors can be applied to clinical patients earlier for more beneficial effects, such as improved survival. The immune checkpoints of LAG-3 play crucial roles in cancer development and may be used in future clinical practice for cancer therapy. Along with the progress of additional and further observations, a broad variety of scientific questions will emerge and require to be addressed by scientists and physicians.

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CS and JZ conceived and designed the review. CS drafted the manuscript. XL and JZ critically revised the article for intellectual content. All authors read and approved the final manuscript.

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Competing interests

The authors declare that they have no competing interests.

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