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Review

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Shedding light on the role of LAG-3 in hepatocellular carcinoma: unraveling immunomodulatory pathways

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Abstract

Hepatocellular carcinoma (HCC) stands as a primary malignant liver tumor characterized by chronic inflammation and complex alterations within the tumor microenvironment (TME). Lymphocyte activation gene 3 (LAG-3), also known as CD223, has gained prominence as a potential next-generation immune checkpoint, maintaining continuous expression in response to persistent antigen exposure within the TME, warranting our attention. In patients with HCC, LAG-3 expression on T cells, regulatory T cells (Tregs), and natural killer (NK) cells contributes to immune evasion, while high expression of LAG-3 leads to increased angiogenesis and poor prognosis. By interacting with major histocompatibility complex class II molecules, LAG-3 promotes T cell exhaustion and suppresses antitumor responses, often in collaboration with other immune checkpoints like programmed cell death protein 1 (PD-1), while on Tregs and NK cells, LAG-3 modulates their suppressive functions, indirectly facilitating tumor immune escape. LAG-3 expression may offer prognostic insights, correlating with disease progression and outcomes in HCC patients, while various preclinical studies highlight the potential of LAG-3-targeted therapies in reinvigorating immune responses against HCC, with a few combination approaches targeting LAG-3 alongside other checkpoints demonstrating synergistic effects in restoring T cell function. Therefore, harnessing LAG-3 as a

therapeutic target holds promise for enhancing antitumor immunity and potentially improving HCC treatment outcomes. Our narrative review aims to delve into the full spectrum of LAG-3 signaling in HCC, with the goal of a better understanding of the pathophysiological and immunological basis of its use to arrest HCC growth and development.

Keywords: Lymphocyte activation gene-3, hepatocellular carcinoma, tumor microenvironment, innate immunity, immunotherapy

INTRODUCTION

Hepatocellular carcinoma (HCC) stands out as the predominant form of primary liver cancer[1]. On a global scale, liver cancer is the fourth leading cause of cancer-related mortality, accounting for 782,000 deaths, holding the sixth position in terms of newly diagnosed cases, and affecting 841,000 patients annually[2]. Additionally, estimations regarding liver cancer-related death predict a significant surge in its impact on cancer-related mortality by 2030, demonstrating a tendency to become the third primary cause of such fatalities[2,3]. In most countries, men experience incidence rates two to four times higher than women[4], whereas a direct correlation between the incidence rate of HCC and age has been established in most populations persisting until approximately 75 years old, although the median age of diagnosis is considered to be at a younger age. The process of hepatocellular carcinogenesis involves angiogenesis, chronic inflammation, and complex modifications within the macro- and micro-environment of the tumor[5-7]. This initiation is driven by intrinsic factors, comprising inherited or acquired genetic mutations, as well as extrinsic factors such as chronic alcohol consumption, smoking, nonalcoholic fatty liver disease (NAFLD) and hepatitis B virus (HBV), hepatitis C virus (HCV), and hepatitis D virus (HDV)[8-10]. These factors assume crucial roles in the development of HCC by triggering alterations in mature hepatocytes or stem cells, leading to processes such as apoptosis, cell proliferation, dysplasia, and eventually neoplasia[11,12].

HCC primarily emerges in a cirrhotic liver where both repeated inflammation and fibrogenesis are prevalent, while the interplay between them predisposes to liver dysplasia and, consequently, malignant transformation. Signaling pathways including Wnt/β-catenin, P53/cell cycle regulation, oxidative stress, and epigenetic modifiers undergo frequent mutations in HCC[13,14]. Immune checkpoint molecules are necessary for immune regulation and the preservation of immune balance as they become upregulated during immune cell activation[15], while their role in inhibiting immunopathological responses contributes significantly to the maintenance of self-tolerance[16]. Monoclonal antibodies (mAbs) directed at inhibitory receptors (IRs) such as cytotoxic T-lymphocyte antigen 4 (CTLA4) and programmed cell death protein-1 (PD-1) have shown substantial clinical efficacy in certain types of tumors, highlighting the potential role of targeted drug therapies in HCC[17]. Lymphocyte activation gene-3 (LAG-3) is a prominent member of the inhibitory receptor family, with extensive expression observed across various immune cell types[18]. A recently discovered primary ligand for LAG-3, fibrinogen-like protein 1 (FGL1), has been confirmed to be abundantly produced by human tumor cells, while increased levels of FGL1 have been strongly associated with an unfavorable prognosis and resistance to therapy[19]. Most importantly, however, concomitant blockade of LAG-3 and PD-1 receptors has demonstrated remarkable synergistic effects in boosting the immune response in clinical trials[20], indicating that LAG-3-related treatment options could contribute to achieving better outcomes for patients struggling with HCC. Even though LAG-3 is acknowledged as a significant inhibitory receptor, its biology and mechanism of action are yet to be clearly defined.

To date, most research regarding LAG-3 has put the emphasis on its implication in T cell dysfunction and its negative regulation of the immune response in various types of cancer and viral infections, but the function and ligands of LAG-3 in the setting of HCC have not been thoroughly defined yet. Our narrative

review aims to delve into the potential role of LAG-3 signaling in HCC, aiming for a better understanding of the pathophysiological and immunological basis of its use to arrest HCC growth and development. In spite of the presence of many clinical trials regarding LAG-3, the expression of the FGL1/LAG-3 pathway and its association with PD-1 ligand are yet to be clearly defined in HCC. Enhanced comprehension of the role of LAG-3 in HCC could confer valuable insights into its future management, potentiating the development of novel therapeutic modalities.

UNDERSTANDING LAG-3 SIGNALING

A deeper understanding of the molecular structure, interactions, and signaling of LAG-3 will provide valuable insight into targeting LAG-3. In more detail, LAG-3, also known as CD223, is a cell surface molecule with significant involvement in the functions of various immune cells. It was first discovered by Triebel *et al.* in 1990 as a new 498-amino acid type I transmembrane protein present on activated human natural killer (NK) cells and T cell lines[18]. The LAG-3 locus is situated on chromosome 12 (12p13.32) in humans and chromosome 6 in mice, demonstrating high structural homology with CD4. The protein structure of LAG-3 can be further subdivided into extracellular, transmembrane, and intracellular components. Initially, the structural insight of LAG-3 provided important information regarding the molecular structure of the LAG-3 ectodomain (D1-D4) and demonstrated that LAG-3 forms a homodimer, with a central axis *via* a hydrophobic D2 domain with the rest of the domains forming a V-shaped architecture[21]. Moreover, LAG-3 ectodomains were recognized as dimers *via* the D2 domain, while the binding sites for FGL1 and major histocompatibility complex class II (MHCII) occurred via the flexible loop 2 region in the LAG-3 D1 domain. Afterwards, the main pathway of LAG-3 inhibitory signaling was found, demonstrating the presence of a relationship between LAG-3 and T cell receptor (TCR)-CD3 at the immunological synapse, inhibiting TCR signaling *via* the co-receptor-Lck signaling pathway[22]. In more detail, LAG-3 was demonstrated to function independently of MHCII and that it alternatively utilized the TCR-CD3 complex as a ligand inside the synapse, leading to disconnection of Lck from CD4 and CD8 co-receptors, leading to suppressed TCR signaling, providing important information for the development of LAG-3-focused immunotherapies.

Despite existing gaps in our knowledge and comprehension of LAG-3 biology, immunotherapeutics against LAG-3 have demonstrated their utility in restoring T cell function. The extracellular segment of LAG-3 is comprised of four immunoglobulin (Ig)-G domains (D1-D4) with folding patterns closely resembling those of CD4, while LAG-3 includes an individual short amino acid sequence termed "extra loop", in the D1 domain exhibiting a higher affinity to MHCII molecules compared to CD4[23]. Furthermore, the intracellular segment of LAG-3 is relatively short, featuring a distinctive motif (KIEELE) required for modulating T cell function[24]. Notably, transmembrane and cytoplasmic regions do not exhibit many similarities to CD4, suggesting that even though both genes have probably evolved from a pre-existing common evolutionary ancestor, they diverged early in evolution, explaining the differences between those molecules, indicating the unique signaling and inhibitory function of LAG-3. LAG-3 is present in most activated CD4 differentiation subsets including T helper cell type 1 (Th1), but is absent in T helper cell type 2 (Th2)[25]. It is also widely expressed in CD8+ T cells, regulatory T cells (Tregs), B cells, and NK cells[26]. Secretion of specific interleukins is associated with higher expression of LAG-3 molecules in T cell populations. Cytokines like interleukin (IL)-2 and IL-12 enhance LAG-3 expression on activated T cells[27], with its presence being also linked to upregulated IL-10 production[28], Moreover, the surface expression of LAG-3 protein and its shedding by activated CD4 T cells greatly aligns with interferon (IFN)-γ production, whereas LAG-3 expression shows an inverse correlation with IL-4 secretion[25]. Besides various blood cell types, LAG-3 messenger RNA (mRNA) was also identified in the medulla of the thymus gland and at the cerebellum, indicating a potential association of LAG-3 cellular signaling in conditions like Parkinson's

disease[29].

While LAG-3 evidently assumes a negative regulatory function in managing T cell activation and function, the precise mechanisms of LAG-3 signal transduction are not yet fully understood. Initially, it was demonstrated that the high affinity between LAG-3 and MHCII elucidated the role of LAG-3 in T cell activation. MHCII molecules are recognized as the primary ligands for LAG-3, forming a stable interaction predominantly through the D1 domain of the extracellular segment. This binding is considered to exert a negative regulatory effect on T cell activation and cytokine production[30]. According to this hypothesis, LAG-3-Ig fusion proteins function as rivals in CD4/MHCII-dependent cellular adhesion assays[31], modulating the immune system response by intervening in the TCR signaling pathway. On the other hand, the possible role of the tailless LAG-3 molecules as competitors to CD4/MHCII ligands binding is highly disputed, and counterarguments propose that LAG-3's cytoplasmic domain plays a key role in CD4 T cell activation through a single lysine residue (K468) within the KIEELE motif of the intracellular segment of the LAG-3 molecule[19]. The LAG-3 signaling pathway also plays a crucial role in dendritic cells (DCs). When LAG-3 engages with MHCII, it triggers an ITAM-mediated inhibitory signaling pathway. This pathway involves the Fc region of IgG (FcγR) and extracellular signal-regulated kinase (ERK)-mediated recruitment of the tyrosine phosphatase SHP-1, leading to the suppression of DC maturation and immune-stimulatory capacity. Notably, the cytoplasmic tailless form of LAG-3 alone was sufficient for inhibition, indicating that MHCII signaling in DCs, rather than LAG-3 signaling within T cells, was essential for LAG-3-mediated suppression of DC maturation[32]. Despite discrepancies in the proposed signaling mechanisms, there is substantial evidence supporting the idea that the binding of LAG-3 to MHCII leads to cancer cell evasion from apoptosis by facilitating the aggregation of tumor-specific CD4 T cells[33], indicating its unique role in the physiological processes underlying carcinogenesis [Figure 1].

The distinct role of LAG-3 in reducing CD8+ T cell response has been evidenced in various models[34]. Research reveals that LAG-3 is briefly expressed on CD8+ T cells following acute stimulation, while it persists at elevated levels under tolerizing conditions[35]. As CD8+ T cells do not engage with MHCII, immunologists have posited that LAG-3 potentially modulates the function of these cells via alternative ligands. The initial molecule suggested as a potential ligand is Galectin-3, an extensively distributed lectin across various tissues and cell types, playing a crucial role in regulating immune responses and inflammation[36]. The association between the two molecules has been supported through *in vitro* experiments, wherein the expression of LAG-3 was found to be necessary for Galectin-3-mediated inhibition of CD8+ T cells[37]. Another possible ligand that could interact with LAG-3 in a comparable fashion is considered to be liver sinusoidal endothelial cell lectin (LSECtin), a cell surface lectin expressed in the liver that belongs to the DC-SIGN family[38]. Recent data have demonstrated that interactions between LAG-3 and LSEC contribute to increased tumor growth in melanomas by inhibiting the IFN-γ secretion of effector T cells, supporting its possible role in mechanisms concerning neoplasia[39]. Engagements with these two potential alternative ligands might also extend the influence of LAG-3 on further T cell function, especially concerning its inherent role on CD8+ T cells within the tumor microenvironment (TME). Finally, it is worth mentioning that there might be additional ligands for LAG-3 that are yet to be identified.

THE IMMUNOLOGIC BASIS OF ANTI-LAG-3 THERAPIES

Cancer growth and development involves evading immune checkpoint mechanisms and molecules such as PD-1, CTLA4, and LAG-3, which regulate immune responses against infectious agents and cancer cells[40]. The expression of LAG-3 extends beyond T cells, as it has also been demonstrated on various other immune cells. Notably, LAG-3 is expressed on activated B cells in a T cell-dependent manner, as well as on 20% of γδ T cells[41]. While the functional role of LAG-3 on the aforementioned cells remains to be identified,

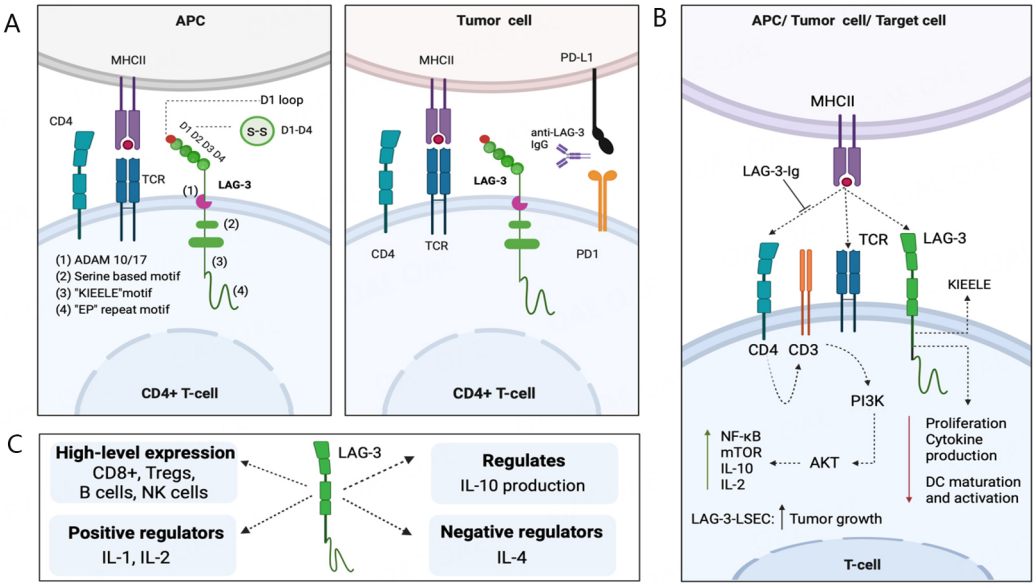

Figure 1. (A) MHCII is one of the main ligands of LAG-3 and the inhibition of LAG-3 in tumor cells is a potential therapeutic option for patients resistant to PD-L1 immunotherapy. The LAG-3 protein structure consists of an extracellular, a transmembrane, and a cytoplasmic part. The extracellular part is composed of four IgG domains: D1, which contains a loop domain rich in proline and an in-chain species-specific disulfide bond, and D2-D4. In the transmembrane part, the metalloproteinases ADAM10/17 regulate LAG-3's breakaway from the cell membrane. The cytoplasmic part consists of the serine phosphorylation site, the KIEELE motif, and the EP repeat motif. (B) The principal ligand of LAG-3 is MHCII, to which it binds with greater affinity than CD4. LAG-3 is involved in DC maturation and activation; it is involved in decreased T cell proliferation and cytokine production, upregulating the production of interleukins and NF-κB, via PI3K/Akt signaling. LAG-LSEC interaction is positively correlated with tumor growth, while LAG-3-Ig fusion proteins inhibit CD4/MHCII-dependent cellular adhesion assays, modulating MHCII-TCR signaling. (C) Cells with increased LAG-3 expression, as well as basic regulators of LAG-3 expression and interleukins regulated by LAG-3. LAG-3 is highly expressed in various types of immune cells (CD8+, Tregs, B cells, and NK cells), and is positively (IL-1, IL-2) or negatively (IL-4) regulated by interleukins. Additionally, it acts as a regulator of IL-10. ADAM: a disintegrin and metalloproteinase; APC: antigen-presenting cell; CD3: cluster of differentiation 3; CD4: cluster of differentiation 4; CD8: cluster of differentiation 8; IL-1: interleukin 1; IL-2: interleukin 2; IL-4: interleukin 4; IL-10: interleukin 10; LSECs: liver sinusoidal endothelial cells; MHCII: major complex histocompatibility complex class II; mTOR: mammalian target of rapamycin; NFκB: nuclear factor kappa-light-chain-enhancer of activated B cells; NK cells: natural killer cells; PD1: programmed cell death protein 1; PD-L1: programmed death-ligand 1; PI3K/Akt: phosphatidylinositol 3-kinase/protein kinase B pathway; S-S: disulfate bond; TCR: T-cell receptor; Tregs: T regulatory cells.

observations in aged LAG-3-deficient mice revealed noteworthy findings[42]. Specifically, apart from a greater number of T cells, these mice exhibited a proportional rise in B cells, macrophages, granulocytes, and DCs compared to wild-type mice, suggesting a potential role for LAG-3 in cellular homeostasis beyond its other well-documented functions. While soluble LAG-3 (sLAG-3) does not seem to be the primary focus of clinical research employing LAG-3-specific monoclonal antibodies, it is pertinent to consider the effect of sLAG-3 in T cell immunity[43]. Analogous to LAG-3, the soluble form of LAG-3 also interacts with MHCII molecules, but sLAG-3 is believed to selectively bind to MHCII molecules located within lipid raft microdomains on a small subset of antigen-presenting cells (APCs). Moreover, the immunoactivity of the fusion of sLAG-3 and immunoglobulin (sLAG-3-Ig) has been validated, and it has been demonstrated that it effectively enhances the functionality of antitumor T cells in response to a vaccine containing irradiated tumor cells[44]. *In vitro* experiments have further supported these findings, revealing that the presence of sLAG-3-Ig upon the activation of human CD8+ T cells, individualized for tumor-associated antigens, leads to an augmented clonal expansion of T cells[45]. The clinical relevance of sLAG-3 is evident *in vivo*, as demonstrated by improved overall survival in patients diagnosed with breast cancer who exhibit increased levels of sLAG-3 during diagnosis as compared to those with decreased levels[46]. The aforementioned findings underscore the potential clinical significance of sLAG-3 in modulating immune responses and

suggest its utility as an immunotherapeutic adjuvant.

PD-1 curbs T-cell receptor signaling, inhibiting T-cell proliferation and cytotoxic mediator secretion, potentially leading to T-cell exhaustion[47]. Immune checkpoint inhibitors targeting PD-1 and its ligand, programmed death-ligand 1 (PD-L1), including nivolumab, pembrolizumab, camrelizumab, tislelizumab, durvalumab, and atezolizumab, induce objective tumor responses in about 15% of advanced HCC patients in phase II and III trials, correlating with prolonged survival[48]. The randomized phase 2/3 trial RELATIVITY-047 revealed that relatlimab, a monoclonal antibody against LAG-3, when combined with nivolumab (anti-PD-1), demonstrated a 12-month progression-free survival (PFS) of 47.7% in melanoma patients, surpassing the 36% PFS observed with nivolumab monotherapy[49]. These compelling results marked a significant milestone in 2022, as the US Food and Drug Administration (FDA) granted approval for the relatlimab/nivolumab combination drug Opdualag for the therapy of unresectable or metastatic melanoma[50]. This decision positioned LAG-3 as the third checkpoint inhibitor that demonstrates efficacy in clinical settings when specifically targeted[51]. As mentioned above, monotherapy with PD-1/PD-L1 inhibitors showed limited improvement in overall survival compared to standard treatment[52]. To address this, combination strategies were explored, aiming either to stimulate T-cell activation via checkpoint inhibition or modify the tumor immune microenvironment to favor immune responses[53]. CTLA4 blockade enhances immune responses by targeting T-cell co-stimulation and Tregs[54]. Combining CTLA4 and PD-1/PD-L1 blockade leads to varied immune-stimulating effects, including modulation of effector CD8+ T cells[48]. Nonetheless, around 30% of HCC tumors exhibit intrinsic resistance to PD-1/PD-L1 inhibitors, and on rare occasions, treatment may accelerate tumor growth[54]. In murine HCC models, the combined treatment produced heightened CD4+ and CD8+ T-cell infiltration, decreased Treg infiltration, and enhanced efficacy[55]. The immunosuppressive effects of angiogenesis hinder immune infiltration, and bevacizumab inhibits them, stimulating dendritic cell and cytotoxic T-cell activation while reversing vascular endothelial growth factor (VEGF)-induced T-cell exhaustion[56]. However, VEGF inhibition can lead to a more hypoxic TME, activating immunosuppressive mechanisms[57,58]. The immunological impact of tyrosine kinase inhibitors (TKIs) is still being explored, with varying effects on macrophage polarization, T-cell function, and immunosuppressive cell populations observed in preclinical models[59]. The potential effect of TKI dosing is crucial, as low doses have shown differential impacts on immune responses[60]. Different TKIs can exert distinct immune-modulating effects, influencing immune-related parameters such as PD-L1 expression[61]. Finally, it is important to note that the effects observed in animal models should be interpreted cautiously, considering the challenges of replicating the human TME [Figure 2].

THE CLINICAL LANDSCAPE OF LAG-3-TARGETED HEPATOCELLULAR CARCINOMA THERAPY

Unfortunately, the therapeutic options currently available for HCC, comprising of surgical resection, liver transplantation, and radiofrequency ablation, exhibit an effect only at the early stages of the disease. The majority of patients with HCC, however, are diagnosed at later stages and therefore have poor prognosis, disqualifying them from such treatment options[62]. HCC exhibits resistance to chemotherapy, but due to the presence of an inflammatory TME that has been associated with improved patient survival[63], immunotherapy is nowadays considered an important therapeutic modality for HCC[64]. It has been established that intratumoral Treg accumulation is a leading mechanism contributing to the immunoevasion of HCC, while inhibitory receptor-ligand pathways can also lead to the inhibition of antitumor immunity in the TME[65]. It has been demonstrated that tumor-infiltrating T cells, under the effect of chronic tumor antigens, are deprived of their effector and tumoricidal capacity, followed by a gradually greater variety and quantity of inhibitory receptors which are expressed on them, such as PD-1 and LAG-3[47]. When contacting their ligands on tumor cells and tumor-infiltrating APCs, they inhibit T-

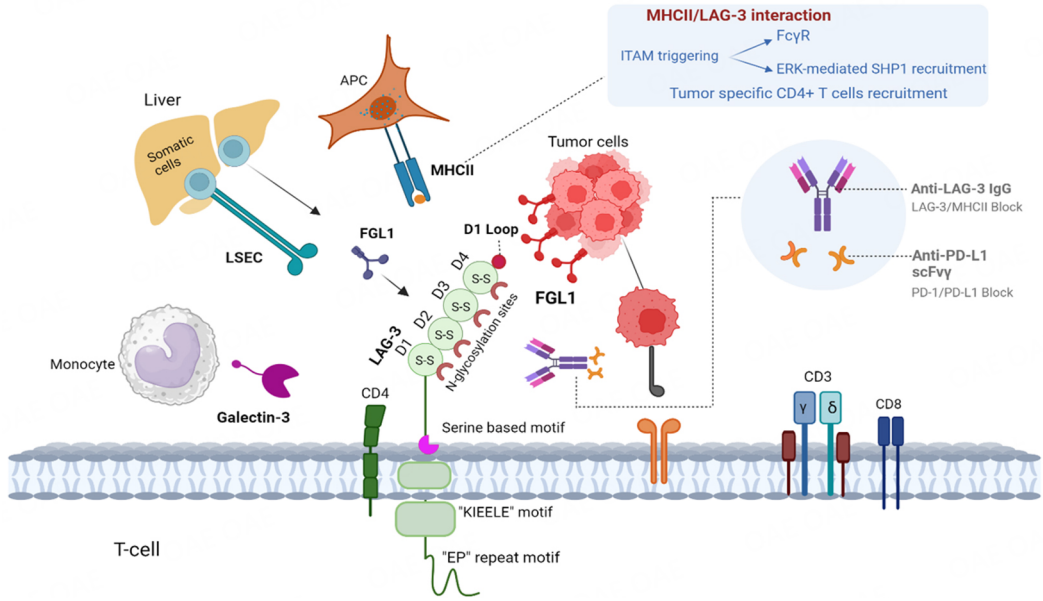

Figure 2. Multiple interactions of LAG-3 within the TME of HCC have made LAG-3 a potential therapeutic target with the aid of bispecific antibodies targeting both LAG-3 and PD-L1. The confirmed ligands of LAG-3 are MHCII, Galectin-3, LSECtin, and FGL1. MHCII is the principal ligand of LAG-3, showing higher binding affinity than CD4, while LAG-3 is highly glycosylated and can interact with Galectin-3, regulating T cell responses. LAG-3/LSECtin interaction is considered to promote tumor growth through the suppression of the antitumor T cell response, while the interaction of FGL1/LAG-3 is considered to promote tumor immune escape through the inhibition of the antigen-specific T cell. Galectin-3: galactose lectin-3; FGL1: fibrinogen-like protein 1; LSECtin: liver sinusoidal endothelial cell lectin; MHCII: major histocompatibility complex II; APC: antigen-presenting cell; ITAM: immuno-receptor tyrosine-based activation motif; FCγR: receptors for the Fc region of IgG; ERK: extracellular signal-regulated kinase.

cell responses of tumor antigens, thus providing potentially unprecedented clinical benefits for patients[66]. In addition, Yang *et al.* demonstrated that there is an elevated LAG-3 expression in CD8+ tissue-resident memory T cells (T_{rm}) in advanced HCC, while FGL1 levels are negatively associated with CD103 expression and were correlated with worse patient survival in HCC[67]. Patients with higher levels of CD8+ T_{rm} had better outcomes, and FGL1/LAG-3 binding decreased the levels of CD8+ T_{rm} cells in HCC, demonstrating its capabilities as an immunotherapeutic target for HCC.

Although LAG-3 mechanism of action has not been investigated as extensively as PD1, the pleiotropic roles of LAG-3 can potentiate the utilization of other pathways for LAG-3 blockade. LAG-3 is upregulated on tumor-infiltrating lymphocytes (TILs) and inhibition of LAG-3 can potentiate antitumor T cell responses, while combined blockade of the PD1 pathway and LAG-3 has been demonstrated to have a greater effect on antitumor immunity, as compared with targeting each molecule separately, representing a promising combinatorial treatment strategy for HCC[68]. Interestingly, Zhou *et al.* demonstrated on human HCC biopsies that inhibitory receptors PD-1, T cell immunoglobulin and mucin domain-containing protein 3 (TIM3), LAG-3, and CTLA4 were upregulated on tumor-infiltrating T cells, while PD-1, TIM3, and LAG-3 were elevated on tumor-associated antigen-specific CD8+ TIL[69]. Moreover, the blockade of PD-L1, TIM3, and LAG-3 increased the *ex vivo* CD4+, CD8+ TIL, and effector cytokine production, while antibodies against PD-L1, TIM3, or LAG-3 restored HCC-derived T cell response to tumor antigens alongside antibodies demonstrating synergistic effects, suggesting that inhibiting antibodies against TIM3, LAG-3, or CTLA4, alongside their respective combinations with PD-L1 blockade, may hold promise for the immunotherapy of individuals with HCC. The FGL1/LAG-3 pathway is also a potential target for immunotherapy and has demonstrated an additive effect with PD-1/PD-L1, while elevated levels of LAG-3+ cells and CD8+ T cells constitute adverse and advantageous prognostic biomarkers for HCC,

respectively[70]. Individuals expressing PD-L1 have greater chances to respond to anti-PD-1/PD-L1 therapy, while individuals with positive expression of LAG-3 (≥ 1%) show better response rates to immunotherapy[71]. Hence, LAG-3+/PD-L1 tumor cell-negative individuals with worse prognosis may adequately respond to the anti-LAG-3 mAb or the combination of anti-LAG-3 and anti-PD-L1 mAb. Along the same line, Oxysophocarpine, a bioactive alkaloid with neuroprotective, antiviral, anticonvulsant, and antinociceptive functions, suppressed HCC growth and sensitized the LAG3 immuno-effect of CD8+ T cells in HCC both *in vivo* and *in vitro*, by decreasing the expression of FGL1 via inhibiting IL-6-mediated Janus kinase 2 (JAK2)/signal transducer and activator of transcription 3 (STAT3) signaling, providing preclinical evidence that oxysophocarpine may be effectively applied in HCC treatment, as a combination therapy with anti-LAG-3 immunotherapy[72]. It is also worth noting that in a study of the clinical significance of LAG-3 on microvessel density in primary human HCC, increased expression of LAG-3 was associated with increased preoperative alpha-fetoprotein level, tumor diameter, N stage, and the presence of HBV infection, microvessel density, angiogenesis, and poor prognosis in HCC patients[73].

Anti-LAG-3-based therapies can be subdivided into three categories: anti-LAG-3 monoclonal antibodies, bispecific LAG-3 antibodies, and LAG-3 immunoglobulin fusion proteins. However, there is growing evidence that relying solely on LAG-3 blockade might not constitute the ideal therapeutic approach, mainly due to the capacity of tumor cells to evade antitumor immune responses through various molecular mechanisms. Both PD-1 and LAG-3 constitute emerging mechanisms of HCC immunoescape and hold promise as therapeutic targets, with current evidence indicating that combined blockade of both pathways may be a better alternative than targeting either of the pathways alone in order to overcome the resistance to anti-PD-1/anti-PD-L1 or anti-CTLA4, while combining single or dual immune checkpoint blockade (ICB) therapy with agents that promote immunogenic cell death, is postulated to enhance tumor immunogenicity and potentially expand the pool of patients responsive to immunotherapy. In more detail, a recent study of 191 HCC-patient samples comprising resection specimens from ICB-naïve and before-treatment samples, from individuals with advanced HCC having received immune checkpoint blockade therapy, demonstrated a correlation between before-treatment LAG-3, CD8, STAT1, and LAG-3 CD8+ tissue expression and response rate to mAb immunotherapy in individuals with advanced HCC, while LAG-3 CD8+ cells were the most favorable biomarker. They demonstrated that immunohistochemical analysis of LAG-3 and CD8, as individual markers and the LAG-3+ CD8+ phenotype, was beneficial for predicting the response to ICB in before-treatment individuals with advanced HCC[74]. Interestingly enough, only the LAG-3+ CD8+ cell proportion was statistically correlated with response to ICB, irrespective of viral-related and non-viral HCC sample status, concluding that immunohistochemical staging of before-treatment LAG-3 and CD8 levels in the TME might aid in predicting ICB response in individuals with HCC. Similarly, another study by Guo *et al.* provided evidence that serum levels of LAG-3 and PD-L1 were highly increased in individuals with HCC compared to healthy individuals. In more detail, individuals with decreased pre-transarterial chemoembolization (TACE) and post-TACE levels of LAG-3 and not PD-L1 had an increased probability of objective response (OR) after TACE[75]. Moreover, greater pre-TACE LAG-3 levels were associated with poorer disease outcomes, and individuals with both high LAG-3 and PD-L1 serum levels had shorter OS, as compared with individuals who had high PD-L1 or LAG-3 or low PD-L1 and LAG-3, while in post-TACE patients, LAG-3 level in the serum was significantly decreased at day 3 after TACE. The aforementioned findings may indicate that the LAG-3-related pathway holds promise for individuals with HCC undergoing TACE, while LAG-3 inhibition can potentiate the tumoricidal effect of TACE [Table 1].

A recent study by Yang *et al.* implemented neoantigen peptide vaccination alongside anti-PD-1 and investigated the tumoricidal effects, concluding that the combination immunotherapy had better antitumor outcomes than monotherapy and resulted in more significant HCC regression, providing concrete evidence

Table 1. Summary of studies evaluating the role of LAG-3 in hepatocellular carcinoma

Study (Year)	Study characteristics and treatment	Study findings
Yang *et al.* (2023)[67]	Prospective, early- and advanced-stage human HCC FGL1 knockdown of Hepa1-6 murine HCC (*n* = 35)	Higher LAG-3 expression in CD8+ TRM in end-stage HCC FGL1/LAG-3 binding leads to greater CD8+ TRM exhaustion in HCC
Zhou *et al.* (2017)[69]	Prospective, early- and advanced-stage human HCC Anti-PD-L1/anti-TIM3/anti-LAG-3 as monotherapy, or combined anti-PD-L1 with anti-TIM3/anti-LAG-3/anti-CTLA-4 (*n* = 59)	LAG-3 blockade increased CD8+ and CD4 proliferation in HCC LAG-3 expression was higher on CD8+ and CD4 T cells in HCC
Guo *et al.* (2020)[70]	Retrospective, early- and advanced-stage human HCC Anti-FGL1, anti-LAG-3, anti-PD-L1 or anti-CD8 in human HCC (*n* = 143)	Elevated density of LAG-3 positive cells correlated with poor disease outcome in patients with HCC Elevated PD-L1 combined with high LAG-3 positive cells had a poorer prognosis
Wang *et al.* (2020)[72]	Prospective, early- and advanced-stage murine HCC Oxysophocarpine, anti-LAG-3 therapy in Hep-G2 and Hepa1-6 murine HCC	Oxysophocarpine hindered HCC growth Oxysophocarpine increased LAG-3 blockade effect and decreased FGL1 expression
Tian *et al.* (2022)[73]	Prospective, early- and advanced-stage human HCC (*n* = 127)	Elevated LAG-3 levels demonstrated shorter overall survival in HCC High LAG-3 correlated with increased MVD, preoperative AFP level, tumor diameter, and N stage
Cheung *et al.* (2023)[74]	Retrospective, early- and advanced-stage human HCC (*n* = 191)	High LAG-3 was associated with shorter median progression-free survival and overall survival in HCC LAG-3 positive CD8+ proportion had a better predictive value of overall survival in long-likelihood models
Guo *et al.* (2021)[75]	Prospective, advanced-stage human HCC TACE in individuals with HCC (*n* = 100)	High LAG-3 and PD-L1 levels presented with shorter overall survival High pre-TACE LAG-3 was correlated with poor disease outcome, cirrhosis, elevated AST/ALT and overall survival
Yang *et al.* (2023)[76]	Prospective, advanced-stage murine HCC Neoantigen vaccination and anti-PD-1 in Hep-55.1C, Dt-81 and Hepa1-6 murine HCC	Combined neo-antigen vaccination and PD-1 blockade led to increased tumor regression in HCC Combined treatment reduced exhaustion-related markers PD-1 and LAG-3 on CD8+ T cells
Jia *et al.* (2022)[77]	Retrospective, early- and advanced-stage human HCC (*n* = 1,884)	Anti-SPINK1 antibodies impeded HCC growth There was a positive correlation between SPINK1 and LAG-3
Li *et al.* (2022)[78]	Prospective, early- and advanced-stage human and murine HCC STAT3 inhibitor in Huh7 and Hepa1-6 cells (*n* = 371)	STAT3 inhibition prevented HCC growth STAT3 inhibition led to lower LAG-3 and PD-1 in HCC
Macek Jilkova *et al.* (2019)[79]	Prospective, advanced-stage human HCC Sorafenib or Anti-PD-1/PD-L1 in human HCC (*n* = 21)	Patients irresponsive to Sorafenib had elevated LAG-3 levels Low baseline CD4/CD8+ T cells ratio was associated with better overall survival

n: number of subjects; HCC: hepatocellular carcinoma; LAG-3: lymphocyte activating gene 3; TRM: tissue-resident memory T cells; FGL1: fibrinogen-like protein 1; MVD: micro-vessel density; AFP: alpha-fetoprotein; HBV: hepatitis B virus; TACE: transarterial chemoembolization; ALT: alanine aminotransferase; AST: aspartate aminotransferase; PD-1: programmed cell death protein 1; STAT3: signal transducer and activator of transcription 3; SPINK1: serine protease inhibitor Kazal type 1; TIM3: T cell immunoglobulin and mucin-domain containing-3.

for further clinical research implementing neoantigen vaccination alongside anti-PD-1 therapy for the treatment of individuals with HCC[76]. Neoantigen vaccination enhanced the antitumor potency of anti-PD-1 on HCC, as results demonstrated decreased Tregs and monocytic myeloid-derived suppressor cells, elevated CD8+ T cells, increased granzyme B expression, and decreased PD-1 and LAG-3 on CD8+ T cells. Moreover, Jia *et al.* provided evidence that serine protease inhibitor Kazal type 1 (SPINK1) could be a potential biomarker for earlier detection, targeted therapy, and prediction of ICB response to therapy in the management of HCC, as SPINK1 enhanced the proliferation, anti-apoptosis, tumorigenic and metastatic potential of HCC cells, while the anti-SPINK1 antibody hindered the proliferation of HCC, indicating that SPINK1 can serve both as a tumor marker and as a target for immunotherapy for HCC management[77]. In more detail, in a transcriptomic analysis of 368 HCC specimens, a positive association between SPINK1 and immune checkpoints was demonstrated, including PD-1, LAG-3, TIM-3, TIGIT, HAVCR2, and CTLA4.

Along the same line, the inhibition of STAT3-mediated direct regulation of CD47 and glycolysis via GLUT1 in HCC cells led to anti-HCC immune memory and the accumulation of important antitumor effector CD8+ T cells, expressing lower levels of LAG-3 and PD-1, facilitating deeper knowledge of targeted inhibition of immune-checkpoints in HCC, while also shedding some light on new therapeutic options for HCC[78]. It is also worth noting that in a study of immunologic features of advanced HCC patients before and during sorafenib or anti-PD-1/PD-L1 therapy, a trend of enhanced activity of LAG-3 and TIM3 inhibitory immune checkpoints on circulating T cells was observed in individuals who were unresponsive to PD-1/PD-L1 pathway blockade, highlighting the clinical necessity of detailed immunomonitoring during therapies, in order to predict factors of response to therapy and to identify the pathway of resistance to immunotherapy, so as to broaden the pool of responders to HCC treatment[79]. Finally, similar to HCC, high expression of LAG-3 was associated with poor survival in head and neck squamous cell carcinoma, melanoma, breast cancer, and soft tissue sarcoma[80-82], whereas other studies evaluating the role of LAG-3 in gastric cancer, esophageal squamous cell carcinoma, and non-small cell lung carcinoma (NSCLC) demonstrated the opposite results[83-85]. In more detail, LAG-3 expression in gastric cancer was positively associated with a better prognosis, as sLAG-3 hindered tumor growth, enhancing the secretion of CD8+ T cells, IL-12, and IFN-γ, while the clinicopathological associations and prognostic value of LAG-3 in NSCLC are histotype-dependent, as a result of discrepancies in the TME of adenocarcinomas and squamous cell carcinomas[86]. Differences among patient cohorts, tumor cell types, and cut-off margins may have also contributed to the aforementioned differences, highlighting the need for more research and clinical trials in order to consolidate or disute these findings.

DISCUSSION AND FUTURE PERSPECTIVES

LAG-3 holds promise as a target for immunotherapy, marked by the approval of the anti-LAG-3 antibody relatlimab, a therapeutic agent designed to target LAG-3. This approval has opened up avenues for advancing clinical development in this field, positioning LAG-3 as the third checkpoint inhibitor to demonstrate efficacy in clinical targeting. Furthermore, RELATIVITY-073, an ongoing, randomized, phase 2 study in immune-oncology-naive patients with advanced HCC who progressed on prior TKI therapy in the advanced/metastatic setting, will evaluate the efficacy and safety of the combination of relatlimab and nivolumab, aiming to leverage potentially synergistic pathways to enable T-cell activation and improve immune response in patients with HCC[87]. However, numerous unresolved questions persist, and gaining further insights is crucial for optimizing the utilization of current LAG-3-targeting therapeutics for HCC. Apart from therapeutics specifically targeting LAG-3, numerous bispecific antagonists concurrently addressing LAG-3 and PD-1 or PD-L1 are currently undergoing clinical evaluation, while it remains intriguing to ascertain whether these therapeutic agents exhibit efficacy comparable to the existing combination of relatlimab and nivolumab. Moreover, the exploration of the predictive value of RNA and protein biomarkers within the TME has emerged as a central focus of scientific inquiry, utilizing methodologies such as RNA-sequencing (RNA-seq) and immunohistochemistry (IHC), while the principal benefit of IHC methodologies over RNA sequencing lies in their greater translatability and adoptability in clinical practice. Notably, a particularly promising predictive marker for HCC is the four-gene inflammatory signature, encompassing CD8, PD-L1, LAG-3, and STAT1 genes, as demonstrated by the HCC CheckMate 040 clinical trial, which provided evidence that the expression of the four-gene inflammatory signature identified *via* RNA sequencing was correlated with enhanced responsiveness to nivolumab and improved overall survival[88]. An additional aspect to contemplate is whether the optimal blockade of all ligand interactions, including TCR-CD3 interactions, necessitates the use of antagonists. This consideration underscores the need for a comprehensive understanding of the therapeutic landscape, probing potential benefits and challenges associated with the simultaneous targeting of multiple pathways in immunomodulation.

However, a plethora of additional significant questions pertain to the way that LAG-3 influences or is influenced by various immune and non-immune pathways, while understanding the mechanisms driving the additive combination of treatment options targeting LAG-3 and PD-1 is essential for making informed decisions regarding the selection of new combinatorial treatments. In more detail, LAG-3 exhibits notable interactions with other immune checkpoints and, particularly, PD-1, collectively inhibiting T cell activation[33]. The interaction between LAG-3 and MHCII prevents its binding to both a TCR and CD4, thereby inhibiting TCR signaling, while LAG-3 emits an inhibitory signal through the KIEELE motif in its cytoplasmic tail. Additionally, LAG-3 interacts with two newly proposed ligands, Galectin-3 and LSECtin, which are found on melanoma cells regulating the function of CD8+ T cells within the TME, and when crosslinked with the CD3/TCR complex, it can hinder T cell proliferation and cytokine secretion[39,89]. Moreover, both LAG-3 and CTLA4 have been demonstrated to suppress the TCR signaling pathway, leading to the arrest of cell cycle progression and thereby negatively impacting T-cell homeostasis, promoting the immunosuppressive function of Tregs and exerting significant effects on DCs[90]. Antagonistic antibodies targeting these inhibitory immune checkpoints have demonstrated the potential to restore T cell function and have shown promise in ongoing clinical trials. The interaction of LAG-3 with its major ligand FGL1 is associated with HCC cell infiltration, proliferation, and secretion, while LAG-3 abundance is associated with increased microvessel density in primary HCC, a hallmark tumor angiogenesis[73]. The expression of the FGL1/LAG-3 pathway accelerates tumor proliferation due to the activation of Akt and mTOR, leading to accelerated cell growth, heightened cell migration, tumor angiogenesis, and epithelial-mesenchymal transition[91,92].

Due to their effect on restricting TCR signaling, LAG-3 therapeutics may synergize effectively with other treatment modalities targeting inhibitory molecules, such as TIM3 and TIGIT, co-stimulatory agonists, cytokines, or chemotherapy, while identifying optimal combinations will significantly contribute to advancing therapeutic strategies in the complex landscape of immune-related treatments. A puzzling inquiry arises as to why LAG-3 exhibits lower potency compared to PD-1, despite its focus on targeting TCR signaling, while PD-1 predominantly aims at the subordinate co-stimulatory signaling through CD28. The aforementioned discrepancy may be attributed in part to the broader expression of PD-1 as compared to LAG-3, but further evidence should be provided. Finally, a study of the PI3K/AKT/mTOR pathway-associated genes (PAGs) revealed a prognostic signature associated with immune infiltration in HCC, providing neoteric insights into the immunotherapeutic targets and the personalized treatment in HCC[93]. In more detail, the risk score was correlated with the levels of CD8 T cells, CD4 memory T cells, and Th cells, and the expression of immune checkpoints (PD-1, TIGIT, TIM3, LAG-3, and CTLA4) was positively associated with the risk score, concluding that the signature might be indicative of the status of the TME, and even pave the way for individualized immunotherapy in HCC. In summary, the clinical approval of a therapeutic agent targeting LAG-3 serves as a foundational milestone for advancing HCC immunotherapy, but further investigations are imperative to optimize the efficacy of this therapeutic approach.

CONCLUSION

In conclusion, HCC is, to this day, a significant global health challenge, being the fourth leading cause of cancer-related mortality worldwide, and with its incidence predicted to rise significantly by 2030, there is an urgent need for innovative therapeutic approaches. This narrative review has delved into the emerging importance of the LAG-3 signaling pathway in HCC, shedding light on its diverse roles in modulating T cell function and its potential significance in the context of HCC. Through a detailed examination of its structure, expression, and signaling mechanisms, it becomes evident that LAG-3 operates as a negative regulator of T cell activation, presenting a promising target for therapeutic intervention. We have also emphasized the potential role of LAG-3 signaling in HCC growth and development, aiming for a better

understanding of the pathophysiological and immunological basis of the disease, highlighting the prognostic value of LAG-3 expression in HCC, with increased levels being associated with unfavorable outcomes. Recent studies have provided compelling evidence supporting the efficacy of LAG-3-targeted immunotherapies, both as monotherapy and in combination with existing treatments, while the association between pre-treatment LAG-3 levels and responsiveness to immune checkpoint blockade therapy offers a promising avenue for predicting treatment outcomes in advanced HCC patients and the exploration of LAG-3 in conjunction with other immune checkpoint molecules such as PD-1, TIM3, and CTLA4 presents a multifaceted approach to improving therapeutic responses. As we navigate the complexities of HCC treatment, it is crucial to acknowledge the limitations and ongoing challenges in this field, with the heterogeneity of HCC, intricate immune responses, and the dynamic nature of the TME posing significant ongoing challenges. Nevertheless, the promising results from preclinical and clinical studies underscore the potential of LAG-3-targeted therapies in reshaping the landscape of HCC treatment, while the insights gained from such studies lay the foundation for further research, offering hope for the development of novel and effective treatment modalities that can significantly impact the prognosis and outcomes for patients grappling with HCC.

DECLARATIONS

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