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Recent advances and challenges in cancer immunotherapies for patients with autoimmune diseases

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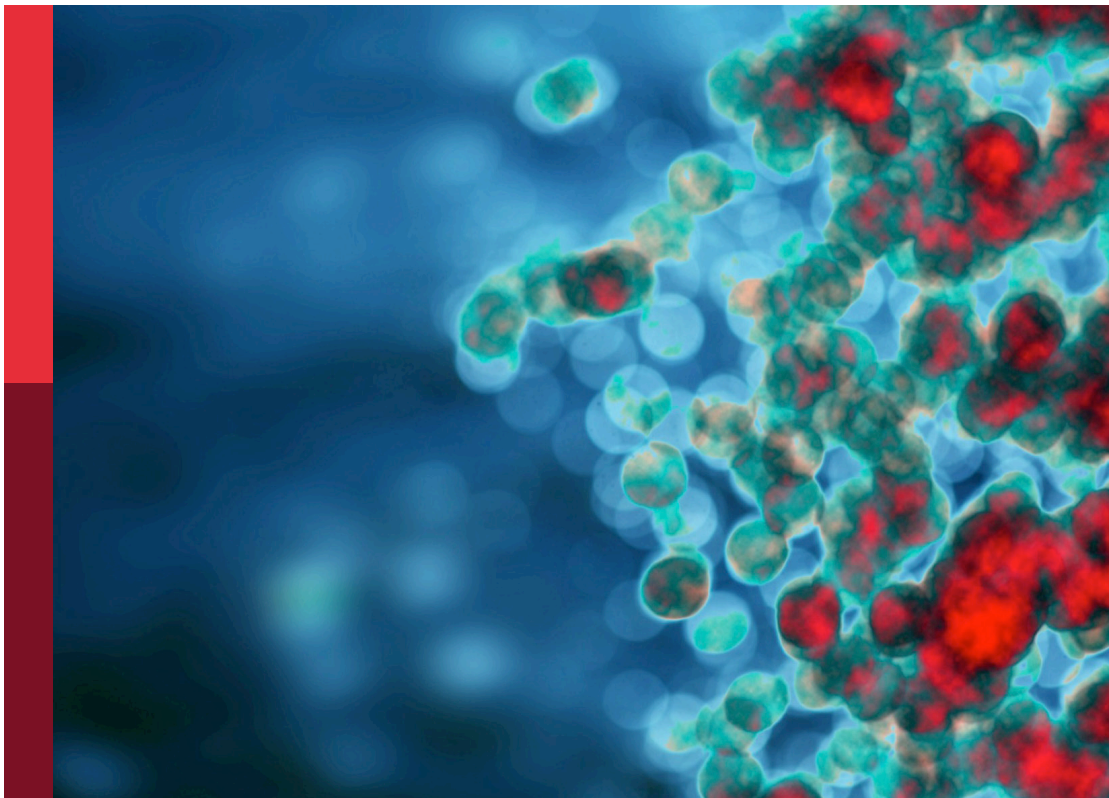

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Recent advances and challenges in cancer immunotherapies for patients with autoimmune diseases

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Editorial: Recent advances and challenges in cancer immunotherapies for patients with autoimmune diseases

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KEYWORDS

cancer, immune checkpoints, case reports, predictive biomarkers, personalized medicine, autoimmune disease

Editorial on the Research Topic

Recent advances and challenges in cancer immunotherapies for patients with autoimmune diseases

This Research Topic gathers different contributions (case reports, research articles and review articles) describing immune related adverse events in cancer patients with preexisting autoimmune diseases or exposed to immunotherapy. Furthermore, the topic discusses new approaches to discover new biomarkers that predict the development of adverse events post immunotherapy.

The first article of this Topic (Zhou et al.), introduces a case report describing autoimmune encephalitis (AIE), for the first time, as a neurological immune-related adverse event (n-irAE) of toripalimab (a new immune checkpoint inhibitor targeting PD-1) in metastatic melanoma patient. This has revealed the safety profile of this new immune checkpoint inhibitor and further studies are required to validate this finding in other cancer types.

The second article by Tang et al. describes a retrospective study in cancer patients with pre-existing autoantibodies treated with anti-PD-1 therapy. Authors reported the association between the presence of autoantibodies and overall survival in patients. Authors concluded that PD-1/PD-L1 inhibitors could be administered safely and effectively in patients with preexisting autoantibodies but without active autoimmune disease. However, patients positive for antithyroid antibody should monitor closely thyroid dysfunction during anti-PD-1/PD-L1 treatment.

Computational approaches to optimize patient selection for immunotherapy in breast cancer patients were described in Jiao et al.. This would particularly help in improving personalized medicine and avoid unnecessary irAEs.

Comprehensive review articles by Tang et al. and Zhou et al. shed the light on anti-PD-1/PD-1 therapy and novel immune checkpoint inhibitor, anti-VSIG and the associated risk of developing irAEs in cancer patients following treatment. These

articles emphasized the importance of finding new biomarkers that predict the risk of developing irAEs to optimize future application in cancer patients.

The connection between the development of autoimmune reactions and immune checkpoint inhibitors has been investigated in Mari et al.. Authors describe a case report of a refractory bullous pemphigoid (BP) which occurred four years after nivolumab introduction and lasted despite nivolumab discontinuation in a patient whose metastatic renal carcinoma is still controlled after more than two years without any anticancer treatment. This may highlight the potential association between irAE and response to immune checkpoint inhibitors. This underlines the existence of late-onset and long-lasting irAEs even after discontinuation of treatment, which should encourage clinicians to remain vigilant over the long term.

Haas et al. proposed a new immune checkpoint receptor, Siglec-7, which its expression could improve CD8+ T cell anti-tumour immune response and potentially improve approaches for personalized medicine and for T cell-driven autoimmune diseases and cancer.

The efficacy of anti-PD-L/anti-CTLA-4 immunotherapy in lung cancer patients and the potential of developing irAEs were assessed in Dey et al.. Authors found that the efficacy of immunotherapy was improved in patients who developed on-treatment irAEs. This was independent of severity of iAEs or the need for steroid treatment, which is important in allowing patients to remain on treatment and derive optimal clinical benefit. Further research is warranted to establish the correlation between incidence of irAEs and efficacy in this patient population.

Alternatively, a computational approach by Glehr et al. concluded that finding predictive biomarkers for irAEs in cancer patients treated with immunotherapy is challenging and requires further studies.

We hope that the reader will find in this Research Topic a useful reference for the connection between autoimmune diseases and the application of immunotherapy in cancer patients, and recent approaches to find new predictive biomarkers to reduce the risk of irAE development and help in optimization therapy selection for patients.

Author contributions

The author confirms being the sole contributor of this work and has approved it for publication.

Conflict of interest

The author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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CASE REPORT
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Anti-GAD65 Antibody-Associated Autoimmune Encephalitis With Predominant Cerebellar Involvement Following Toripalimab Treatment: A Case Report of a Novel irAE of Toripalimab

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Toripalimab (Junshi Bioscience Co., Ltd) is a new immune checkpoint inhibitor (ICI) that targets programmed cell death protein 1 (PD-1) in various cancers, including metastatic melanoma. No neurological immune-related adverse events (n-irAEs) of toripalimab have been reported, except for neuromuscular involvement. We report a case of a 63-year-old woman who presented with severe vertigo, vomiting, nystagmus, cerebellar ataxia, and cognitive impairment after toripalimab treatment for metastatic melanoma. Compared with the concomitant cognitive dysfunction and a pathological reflex involving the cerebral cortex, the signs and symptoms of cerebellar involvement were much more prominent. Anti-glutamic acid decarboxylase 65 (anti-GAD65) antibody was positive in both serum and cerebrospinal fluid (CSF). After intravenous immunoglobulin (IVIG) and methylprednisolone (IVMP) administration, the symptoms of vertigo and vomiting resolved, with cognitive impairment and cerebellar ataxia remaining. This is the first report of autoimmune encephalitis (AIE) as an n-irAE of toripalimab.

Keywords: autoimmune, cerebellum, encephalitis, toripalimab, irAE, anti-GAD65

INTRODUCTION

In the last decade, immune checkpoint inhibitors (ICIs) targeting programmed cell death protein 1 (PD-1) and its ligand PD-L1, as well as cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4), have been a landmark development in cancer treatment, enhancing survival in various cancers by reactivating antitumor immunity. However, ICIs also trigger immune responses against self-antigens, leading to various irAEs, including neurological events. Several n-irAEs have been described in recent years, including encephalitis, myelopathy, aseptic meningitis, meningoradiculitis, Guillain–Barré-like syndrome, peripheral neuropathy, and myasthenic syndrome (1). Most n-irAEs were observed with nivolumab and pembrolizumab, inhibitors of

PD-1, and ipilimumab, an inhibitor of CTLA-4. As novel ICIs, n-irAEs related to toripalimab have rarely been reported. Luo et al. first described a neuromuscular triad of myositis, myocarditis, and myasthenia gravis overlap following toripalimab treatment in a patient with metastatic thymoma (2). To date, there have been no other reports on the n-irAEs associated with toripalimab.

Antibodies against glutamic acid decarboxylase (GAD), the rate-limiting enzyme in the synthesis of inhibitory neurotransmitter GABA, are associated with type 1 diabetes mellitus (T1DM) and some neurological disorders, including stiff-person syndrome (SPS), cerebellar ataxia, epilepsy, and limbic encephalitis (3). GAD65-positive neurological irAEs have been observed in several cases, including SPS, cerebellar ataxia, epilepsy, and limbic encephalitis, following ipilimumab and nivolumab treatment (4–6). Herein, we report a case of toripalimab-induced anti-GAD65-associated encephalitis that may expand the irAE profile of toripalimab and provide further experience for clinical oncologists and neurologists.

CASE PRESENTATION

We report a case of a 63-year-old woman with a history of metastatic melanoma who developed severe vertigo and weakness on the day after her first toripalimab injection (3 mg/kg). She was diagnosed with cutaneous left foot melanoma and treated with wide local excision in 2018. Groin and iliac lymph node metastases were detected 20 and 26 months later, respectively, and they were managed with lymph node dissection. The patient received interferon alfa-2b from 2018 to the first dose of toripalimab. The most recent positron emission tomography–CT (PET-CT) before toripalimab administration showed no tumor progression. On the day after toripalimab administration, she developed vertigo triggered by a change in head position, bilateral upper limb tremors, dysarthria, and transient nausea and vomiting for several minutes. The symptoms gradually worsened, and she was confined to the bed. She developed psychiatric disturbances the following week, characterized by confused soliloquy, disorganized thinking, and agitation. The nausea and vomiting worsened after admission.

At admission, neurological examination revealed cognitive impairment (spatial disorientation, memory disorientation, and count disorientation), apparent horizontal nystagmus, ataxia syndrome with bilateral upper limb intentional tremor in the finger–nose test, and right dysmetria in the heel–knee–tibia test. The Babinski sign was positive on the right.

Laboratory studies revealed normal hepatic and renal function, elevated counts of white blood cells and neutral lobulated granulocytes, and a decreased serum potassium level (3.2 mmol/L). Antibodies against Epstein–Barr virus, herpes simplex virus, rubella virus, and cytomegalovirus were negative in the serum. The patient had a history of type 2 diabetes, and her serum glucose level was significantly elevated, ranging from 15 to 25 mmol/L.

Cerebrospinal fluid (CSF) analysis revealed elevated CSF glucose of 6.1 mmol/L (normal 2.5–4.4 mmol/L), elevated protein of 0.49 g/L (normal 0.15–0.45 g/L), normal chloride of 125 mmol/L (normal 120–130 mmol/L), and a cell count of 0 (normal $0–10\times10^6$/L). Anti-GAD65 antibody was detected with a titer of 1:30 in CSF and 1:100 in serum, both based on a cell-based assay (CBA). Other autoimmune antibodies against IgLON5, DPPX, GlyR1, DRD2, mGluR5, NMDA, AMPA1, AMPA2, LGI 1, CASPR2, GABAB, mGluR1, amphiphysin, CV2, Hu, Ma1, Ma2, Ri, SOX1, Titin, Tr (DNER), Yo, Zic4, Recoverin, and PKCγ were negative. CSF Gram staining and bacterial and fungal cultures were negative too.

Electroencephalography (EEG) mainly showed a 14- to 20-Hz β-wave. Head magnetic resonance imaging (MRI) with a contrast agent showed no remarkable abnormalities. Chest computed tomography (CT) showed bilateral infiltrates in the lower lobes of the bilateral lungs.

The patient was diagnosed with anti-GAD65-associated autoimmune encephalitis secondary to immune checkpoint inhibitor therapy. Considering the lung infection and hyperglycemia, high-dose glucocorticoid therapy was not considered the first choice. Intravenous immunoglobulin (IVIG) (0.4 mg/kg/day) treatment was initiated in the third week after symptom onset and it lasted for 5 days. After IVIG treatment, the symptoms of vertigo and the psychiatric disturbances resolved. One week after the last IVIG dose, intravenous methylprednisolone (IVMP) (500 mg/day for 5 days) was initiated after the infection, and hyperglycemia was effectively controlled. However, vomiting was not relieved, prompting plasma exchange (PE) 5 days after the last dose of IVMP. The first attempt of PE failed because of a sharp decline in blood pressure, pallor, and tachycardia. We suspected allergic shock related to PE and stopped the PE thereafter. Vomiting symptoms showed gradual alleviation. At discharge, the symptoms of vertigo, vomiting, and weakness had significantly improved, and the psychiatric disturbances had resolved; however, the patient did not achieve complete remission of ataxia and memory disorientation.

Toripalimab and interferon alfa-2b therapy were discontinued since admission. Eight months later, the patient could stand independently and walk slowly with the support of others, presenting with an ataxic gait with significantly short step length, low step height, and wide support base during walking. Memory dysfunction neither improved nor worsened in the eight months. The word immediate recall test and the word delayed recall test in the Mini-Mental State Examination (MMSE) showed obvious impaired memory function. Repeat head MRI did not reveal any significant changes. Recent computed tomography of the chest and abdomen, as well as lymph node ultrasonography, showed no tumor progression. The clinical manifestations, important results of examinations and related diagnosis and treatments have been organized as a timeline (**Figure 1**).

DISCUSSION

Compared with myositis and peripheral neuropathies, autoimmune encephalitis (AIE) is not a common n-irAE, but

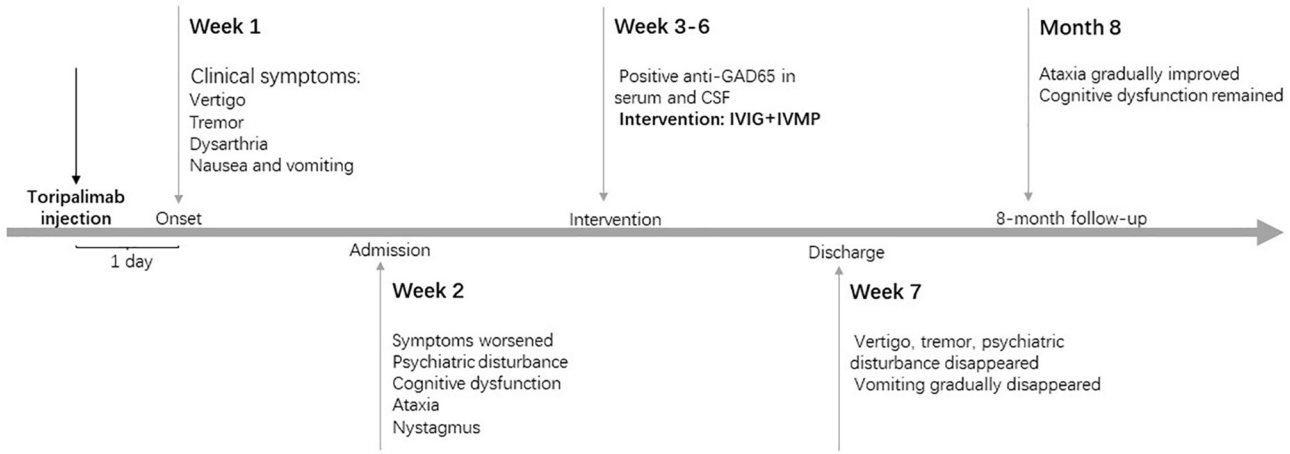

FIGURE 1 | Timeline of patient with relevant data of the episodes and interventions. IVIG, intravenous immunoglobulin; IVMP, intravenous methylprednisolone.

it accounts for more than half of the irAEs in the central nervous system (CNS) (7). The clinical symptoms of autoimmune encephalitis related to ICIs include altered mental status, focal CNS deficits, psychiatric symptoms, seizures, autonomic dysfunction, working memory deficits, ataxia, and dyskinesia (8). According to current studies, most n-irAEs occur early in ICI treatment, usually within 6 months of treatment initiation (9, 10). For most of the reported AIE cases, the first symptoms develop within a mean of 58 days from the first dose of ICIs (nearly 3 cycles of ICI treatment), with a minimum of 3 days (8). The case in our study appears to be the most acute-onset AIE. It is worth noting that cerebellar involvement was prominent, both at onset and at six-month follow-up. Cerebellar ataxia, nystagmus, vertigo, and dysarthria were observed. Immune-induced encephalitis with cerebellar involvement following ICI treatment has been rarely reported. Reina et al. reported a patient presenting with nystagmus and cerebellar ataxia 2 weeks after the initiation of nivolumab therapy for lung adenocarcinoma (11). Acute cerebellitis and corresponding imaging findings of cerebellar edema, early tonsillar herniation, and early hydrocephalus were described in a patient with primary refractory Hodgkin lymphoma being treated with the immune checkpoint inhibitor nivolumab (12). A case of acute cerebellar ataxia due to Epstein–Barr virus (EBV) infection following ICI administration was interpreted as activation of the virus under the affected immune system (13). Autoimmune antibodies, including anti-Zic4, anti-TRIM9, and GAD65, have been detected in some cases (4, 14, 15). Additionally, obvious cognitive impairment supports parenchymal involvement, which is not limited to the cerebellum.

Regarding autoimmune antibodies of AIE related to ICIs, antibodies against intracellular antigens (anti-Ma2, anti-Hu, anti-GAD, an unspecified Purkinje cell antibody, anti-Ri, anti-GFAP) were more frequent than those against cell-surface antigens (anti-NMDA receptor and anti-CASPR2) (4, 6, 8, 16–22). As an autoimmune antibody against intracellular antigens, the anti-GAD65 antibody is associated with several neurological syndromes, including stiff-person syndrome, cerebellar ataxia, and epilepsy (3, 23–25). It is recommended that high serum GAD antibody levels (positive radioimmunoassay or ELISA, positive brain immunostaining, positive cell-based assay, positive line blot) are an important indicator of an immune-mediated cause of the syndrome, and intrathecal synthesis of GAD antibodies provides the strongest evidence that a neurological syndrome is associated with GAD autoimmunity (3, 26). In our case, although we lack evidence of intrathecal synthesis due to a lack of quantitative detection, a positive cell-based assay for anti-GAD65 antibody in both serum and CSF and cerebellar ataxia indicates that the clinical neurological syndrome with anti-GAD antibody in our patient has a probable or definite autoimmune cause, according to the suggested algorithm (3).

Differential diagnoses include paraneoplastic syndrome (PNS) and acute viral encephalitis. First, cerebellar ataxia can occur as a classical paraneoplastic syndrome in patients with or without ICI treatment, always predicting a potential tumor or tumor progression. PNS is not caused by metastatic and/or local effects of cancer on the nervous system; it is instead usually related to cancer-induced immune responses against neuronal proteins (27). For example, paraneoplastic cerebellar degeneration (FCD) can occur several days or weeks after the underlying tumor has been removed and is associated with Yo autoimmune antibodies. Neuroimaging examinations of FCD show normal MRI initially, yet cerebellar atrophy several months later (28). Cerebellar ataxia is associated with anti-Hu, Yo, Tr, SOX1, and VGCC antibodies in some typical tumors such as lung cancer, breast cancer, and Hodgkin lymphoma (28–33). Consistent with other classical phenotypes caused by antitumor immunity (such as limbic encephalitis, peripheral neuropathy, and encephalomyelitis), cerebellar ataxia could also be an irAE of ICIs, in which the anticancer immune response against onconeural antigens is likely to be augmented or even altered under ICI administration because tumors (e.g., melanoma) encounter these phenotypes

and positive antibodies are not those tumors (e.g., small cell lung cancer) typically accompanied by PNS (34). We made a diagnosis of n-irAE rather than PNS due to the lack of neurological manifestations before ICI treatment, the acute onset following ICI administration, and the lack of cancer progression in the examinations before ICI treatment (35). In addition, the psychiatric disturbances in the course of the disease and the residual memory impairment may result from inflammation involving the cerebral cortex, which is not reflected on MRI. Approximately 44% of AIEs related to ICIs have negative MRI findings (8). Second, viral encephalitis primarily involving the cerebellum may also present with acute cerebellar ataxia and psychiatric disturbances, usually caused by rotavirus, varicella-zoster virus, Epstein–Barr virus, herpes simplex virus, respiratory syncytial virus, mumps virus, parvovirus B19, and other rare virus types. Autoimmune antibody-mediated encephalitis may also result from postinfectious autoimmunity. However, viral CNS infections always have distinctive CSF changes and elevated cell counts, and viral encephalitis always has precursor viral infectious symptoms. Despite the lack of nucleic acid detection of the virus in CSF, negative results of antibodies against Epstein–Barr virus and herpes simplex virus could also help in differentiation.

Encephalitis has the second-highest mortality rate after myasthenia gravis and a relatively lower improvement rate in n-irAEs (7, 10). According to the guidelines, pulse corticosteroids, methylprednisolone, and IVIG are recommended (36). If autoimmune antibodies are positive and there is limited or no improvement, rituximab or plasmapheresis is considered (36). Patients without focal syndromes and those with negative antibody focal syndromes have a good prognosis, and compared with other autoantibodies, those with anti-GAD antibody or anti-cell-surface antibodies also have a favorable prognosis (37). Additionally, abnormal MRI findings are associated with poor outcomes, and prolonged cognitive deficits were observed in 79% of patients with AIE (8, 37). In our case, although cognitive impairment and ataxia gait did not achieve complete remission after first-line immunotherapy, the patient's symptoms significantly improved.

CONCLUSION

In this report, we describe a patient with metastatic melanoma who developed anti-GAD65-associated autoimmune encephalitis with predominant cerebellar involvement following toripalimab treatment and achieved some improvements after immunoglobulin and methylprednisolone therapy. As toripalimab is a new ICI targeting PD-1, its related irAEs have scarcely been reported, let alone its n-irAEs. We have provided a novel experience regarding the n-irAEs of toripalimab.

PATIENT PERSPECTIVE

Although the patient was still staggering when walking and could not think quickly, she thought the treatment was effective. It took the excruciating vertigo and vomiting away, which freed her from being completely dependent on others for help.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material. Further inquiries can be directed to the corresponding authors.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Ethics Committee on Biomedical Research, West China Hospital of Sichuan University. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

HZ and TY initiated the study and wrote the manuscript. XX, TZ, and MY collected clinical data. DZ and TY reviewed the manuscript. All authors contributed to the article and approved the submitted version.

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Safety and Efficacy of PD-1/PD-L1 Inhibitors in Cancer Patients With Preexisting Autoantibodies

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Background: Programmed cell death protein-1/programmed cell death ligand-1 (PD-1/PD-L1) inhibitors therapy is now a routine scheme in cancers. However, the effect of preexisting autoantibodies on the safety and efficacy of PD-1/PD-L1 inhibitors in cancer patients is not well understood.

Methods: The present retrospective cohort study evaluated the safety and efficacy of PD-1/PD-L1 inhibitors in patients with preexisting autoantibodies. Patients who received PD-1/PD-L1 inhibitors in the Department of Medical Oncology, Peking Union Medical College Hospital between November 2017 and August 2021 were reviewed.

Results: 67 (37.9%) of the 177 patients, 27 (20.3%) of the 133 patients, and 16 (11.0%) of 146 patients who received PD-1/PD-L1 inhibitors were positive for ANA, anti-Ro52, and antithyroid antibodies, respectively. Preexisting ANA and anti-Ro52 antibody were not associated with the increased risk of immune-related adverse events (irAEs), while thyroid dysfunction was more frequent in patients with positive antithyroid antibody (75.0% versus 13.8%, $p < 0.001$). The median progression-free survival (PFS, 13.1 versus 7.0 months, $p = 0.015$) was significantly longer in the ANA-positive patients, while the median overall survival (OS, 14.5 versus 21.8 months, $p = 0.67$) did not differ significantly between the ANA-positive and ANA-negative groups. Moreover, the preexisting anti-Ro52 and antithyroid antibodies were not significantly associated with PFS and OS.

Conclusions: The presence of ANA and anti-Ro52 antibody were not associated with a higher risk of irAEs, whereas patients positive for antithyroid antibody should monitor closely immune-related thyroid dysfunction. Preexisting ANA might be a predictor of longer PFS, while anti-Ro52 and antithyroid antibodies had no significant effect on survival outcomes in patients receiving PD-1/PD-L1 inhibitors therapy.

Keywords: programmed cell death-1, antinuclear antibody, anti-Ro52 antibody, antithyroid antibody, immune-related adverse events

INTRODUCTION

Immune checkpoint inhibitors (ICIs), especially monoclonal antibodies targeting the PD-1 (programmed cell death-1)–PD-L1 (programmed cell death-ligand 1) axis, have improved outcomes for a variety of malignancies (1). ICIs work *via* breaking the state of immune tolerance in the tumor microenvironment, resulting in robust activation of the immune system and subsequent antitumor immune response (1). However, enhanced T cell activation may cause immune-related adverse events (irAEs), which occur approximately in 40%–50% of patients treated by ICIs (2). Therefore, it is important to identify patients who are more likely to develop irAEs or respond to ICIs.

Antinuclear antibody (ANA) profile is a spectrum of heterogeneous autoantibodies against various nuclear and cytoplasmic components (3). Given that ANA positivity may indicate a predisposition to immune activation, it is understandable that some clinicians are concerned that patients positive for ANA may be at a higher risk of irAEs (4). However, the effect of ANA on the safety and efficacy of ICIs in cancer patients is still controversial (5–9). Moreover, antithyroid antibody was suggested to be associated with thyroid dysfunction after ICIs treatment (6). Anti-Ro52 (TRIM21) antibody, one member of the ANA profile, is regarded to be associated with many autoimmune diseases, especially Sjogren's syndrome, systemic lupus erythematosus, and systemic sclerosis (10). The prevalence of anti-Ro52 antibody varies in malignant diseases (11, 12). Previous studies suggested that anti-Ro52 positivity was correlated with better overall survival in patients with ovarian cancer (11). Whether the presence of anti-Ro52 antibody might affect the safety or efficacy of ICIs has remained unknown. Thus, the present retrospective cohort study aimed to evaluate the safety and efficacy of PD-1/PD-L1 inhibitors in cancer patients with preexisting autoantibodies.

MATERIALS AND METHODS

Patients

Between November 2017 and August 2021, the data of patients with cancer who received PD-1/PD-L1 inhibitors in the Department of Medical Oncology, Peking Union Medical College Hospital (PUMCH) was obtained from the hospital's medical records. Inclusion criteria were as follows: 1) patients with histopathologically confirmed cancers; 2) received at least 1 cycle of PD-1/PD-L1 inhibitor therapy; 3) ANA test completed within one month before immunotherapy initiation was available. The exclusion criteria were as follows: 1) loss of follow-up within one month after the initiation of immunotherapy; 2) survival outcomes or irAEs could not be assessed; 3) Combined with secondary primary tumors that may affect patients' survival outcomes, and confound the efficacy or irAEs evaluation. This study was approved by the Medical Ethics Committee of PUMCH (S-K1949). Patients' consents for participation and publication were waived by the Medical Ethics Committee due to the retrospective design and the deidentified data of this study.

Assessments

Testing results of ANA, ANA profile, antithyroglobulin, and antithyroid peroxidase within one month before immunotherapy initiation were screened. ANA profile was determined by line immunoassay (Euroimmun, Lubeck, Germany), which consists of autoantibodies against antigens including Ro52, SSA, SSB, dsDNA, Sm, rRNP, U1RNP, Scl-70, PM-Scl, Jo-1, CENP-B, PCNA, nucleosomes, mitochondrial M2, and Histones. ANA titer was measured by indirect immunofluorescence assay using Hep-2 cells (Euroimmun, Lubeck, Germany), while antithyroglobulin and antithyroid peroxidase were determined by the Siemens Centaur XP Chemiluminescent Immunoassay platform (Siemens, Ireland) with the antithyroglobulin antibody IgG (Siemens, Cat. No. 10492399, USA) and the thyroid peroxidase antibody IgG (Siemens, Cat. No. 10630887, USA) (13). Patients with ANA titers ≥ 1:80 were considered ANA-positive (14). Moreover, those were considered positive for antithyroid antibody if either antithyroglobulin or antithyroid peroxidase was positive. Those were considered positive for any preexisting antibody if all autoantibodies mentioned above were examined and any autoantibody mentioned above was positive. The level of LDH, IgG, IgA, IgM, hsCRP were determined by a commercial nephelometry assay using AU series clinical chemistry analyzer (Beckman Coulter, Brea, USA). ESR were measured by VACUETTE® Automated ESR Systems (Greiner Bio-One, Kremsmünster, Austria). The level of IL-6, IL-8, IL-10, TNF-α were determined by Chemiluminescent Immunoassay using the IMMULITE® 1000 system (Siemens, Erlangen, Germany). Serum free triiodothyronine (FT3), free thyroxine (FT4), thyroglobulin (Tg), and thyroid-stimulating hormone (TSH) levels were determined by chemiluminescence immunoassay (Roche Diagnostics, Germany).

PD-1/PD-L1 inhibitor therapy was provided until tumor progression or unacceptable toxicity was noted. All patients were followed up until death or loss of contact, with a follow-up deadline of January 2022. The irAEs severity was graded according to the Common Terminology Criteria for Adverse Events version 5.0. To examine thyroid dysfunction, the serum levels of FT3, FT4, Tg, and TSH were assessed at baseline and every 6 weeks during immunotherapy administration. Thyroid dysfunction or irAE was defined as described in the previously published study (15). Briefly, newly developed abnormal FT3, FT4, Tg, and TSH levels, with or without new or significant exacerbation of symptoms of hyperthyroidism/hypothyroidism, were regarded as thyroid dysfunction. All patients were evaluated by computed tomography (CT) scans or magnetic resonance imaging (MRI) every 6 to 12 weeks. Progression-free survival (PFS) and overall survival (OS) were measured as the time from immunotherapy onset to tumor progression or death due to any cause (PFS) or to the latter (OS). Tumor response was evaluated based on the Response Evaluation Criteria in Solid Tumors version 1.1 (16). The objective response rate (ORR) was defined as the proportion of patients who had a complete or partial response to therapy, whereas the disease control rate

(DCR) was defined as the proportion of patients who had a complete or partial response to therapy or stable disease.

Statistical Analysis

The Mann-Whitney U tests were used to compare continuous variables between two groups. Chi-squared and Fisher's exact tests were used to examine the correlation between two categorical variables. Survival outcome was evaluated by the Kaplan-Meier method and was compared between groups using the log-rank test. Additionally, propensity-score matching (PSM) was utilized to minimize the impact of confounding factors. The propensity scores were calculated based on age, TNM stage, cancer type, and Eastern Cooperative Oncology Group (ECOG) performance status (PS) score. Furthermore, least absolute shrinkage and selection operator (LASSO) regression analysis was conducted to prevent collinearity among the candidate indicators of survival outcomes. Furthermore, univariate and multivariate Cox proportional hazard models were performed to calculate the hazard ratios (HR) with 95% confidence intervals (CI) of variables associated with survival outcomes in patients. Only variables, which significantly associated with survival outcome in univariate analysis, will be included in multivariate analysis. All statistical analyses and visualization were performed using R software (version 3.6.1, https://www.r-project.org/). A two-tailed value of $P < 0.05$ was considered to statistically significant.

RESULT

Patient Characteristics

Of the 177 enrolled patients with available ANA testing result, the main tumor types were digestive tract cancers and non-small cell lung cancer (NSCLC). The median age was 61 (range, 22-85) years, 172 patients (97.2%) had an ECOG PS score of 0 or 1, 149 (84.2%) had stage IV disease, and 98 (55.4%) experienced no prior systemic anti-cancer therapy (**Table 1**). Of all enrolled patients, 91 (51.4%) had at least one positive autoantibody, 67 (37.9%) were positive for ANA. Among 111 patients who completed ANA, ANA profile, and

TABLE 1 | Baseline characteristics of patients with or without preexisting antibodies.

Variables	Positive ANA (n=67)	Negative ANA (n=110)	P value	Positive for any preexisting antibody (n=66)	Negative for all preexisting antibodies (n=45)	P value
Age, median (range), years	62 (32-81)	61 (22-85)	0.478	59 (32, 83)	58 (32, 85)	0.694
Sex, male	44 (65.7%)	80 (72.7%)	0.409	46 (69.7%)	35 (77.8%)	0.469
Tumor type						
NSCLC	16 (23.9%)	30 (27.3%)	**0.020**	18 (27.3%)	9 (20.0%)	**0.026**
GC	13 (19.4%)	19 (17.3%)		8 (12.1%)	7 (15.6%)	
Head and neck	11 (16.4%)	20 (18.2%)		17 (25.8%)	7 (15.6%)	
ESCC	14 (20.9%)	6 (5.5%)		11 (16.7%)	5 (11.1%)	
Others[a,b]	13 (19.4%)	35 (31.8%)		12 (18.2%)	17 (37.8%)	
Performance status						
0-1	64 (95.5%)	108 (98.2%)	0.570	64 (97.0%)	44 (97.8%)	1
2-3	3 (4.5%)	2 (1.8%)		2 (3.0%)	1 (2.2%)	
TNM stage						
III	12 (17.9%)	16 (14.5%)	0.702	12 (18.2%)	9 (20.0%)	1
IV	55 (82.1%)	94 (85.5%)		54 (81.8%)	36 (80.0%)	
Liver metastasis	16 (23.9%)	30 (27.3%)	0.747	18 (27.3%)	12 (26.7%)	1
Multiple metastases	22 (32.8%)	51 (46.4%)	0.106	23 (34.8%)	21 (46.7%)	0.293
PD-1/PD-L1 inhibitor						
Pembrolizumab	25 (37.3%)	44 (40.0%)	0.727	25 (37.9%)	18 (40.0%)	0.800
Nivolumab	14 (20.9%)	26 (23.6%)		12 (18.2%)	12 (26.7%)	
Toripalimab	7 (10.4%)	14 (12.7%)		9 (13.6%)	4 (8.9%)	
Others[c,d]	21 (31.3%)	26 (23.6%)		20 (30.3%)	11 (24.4%)	
No prior systemic therapy	41 (61.2%)	57 (51.8%)	0.289	39 (59.1%)	20 (44.4%)	0.185
Combination therapy[e,f]	51 (76.1%)	83 (75.5%)	1	53 (80.3%)	33 (73.3%)	0.528
Elevated serum LDH	16 (23.9%)	20 (18.2%)	0.29	17 (25.8%)	8 (17.8%)	0.428
Immunoglobulin, median (range)						
IgG, g/L	12.1 (8.53-19.10)	11.3 (5.17-19.40)	**0.031**	12.1 (8.53, 19.4)	11.3 (5.17, 18.0)	0.044
IgA, g/L	2.50 (1.24-4.88)	2.33 (0.64-5.71)	0.683	2.51 (0.73, 5.71)	2.27 (0.64, 4.00)	0.234
IgM, g/L	0.925 (0.43-2.20)	0.820 (0.20-2.92)	0.397	0.975 (0.26, 1.96)	0.73 (0.20, 2.92)	0.259
PD-L1 status						
Positive[g]	23 (34.3%)	28 (25.5%)	0.273	19 (28.8%)	11 (24.4%)	0.281
Negative	7 (10.4%)	8 (7.3%)		8 (12.1%)	2 (4.4%)	
Unknown	37 (55.2%)	74 (67.3%)		39 (59.1%)	32 (71.1%)	
MSI status						
MSI-H	6 (9.0%)	7 (6.4%)	0.800	5 (7.6%)	2 (4.4%)	0.763
MSS	15 (22.4%)	24 (21.8%)		14 (21.2%)	11 (24.4%)	
Unknown	46 (68.7%)	79 (71.8%)		47 (71.2%)	32 (71.1%)	

(Continued)

TABLE 1 | Continued

Variables	Positive ANA (n=67)	Negative ANA (n=110)	P value	Positive for any preexisting antibody (n=66)	Negative for all preexisting antibodies (n=45)	P value
Anti-Ro52 antibody						
Positive	16 (23.9%)	11 (10.0%)	**0.035**	25 (37.9%)	0 (0%)	**<0.001**
Negative	38 (56.7%)	68 (61.8%)		41 (62.1%)	45 (100%)	
Unknown	13 (19.4%)	31 (28.2%)		–	–	
Antithyroid antibody[h]						
Positive	9 (13.4%)	7 (6.4%)	0.065	12 (18.2%)	0 (0%)	**0.0067**
Negative	51 (76.1%)	79 (71.8%)		54 (81.8%)	45 (100%)	
Unknown	7 (10.4%)	24 (21.8%)		–	–	
Any preexisting antibody[i]						
Positive	47 (70.1%)	19 (17.3%)	**<0.001**	-	–	–
Negative	0 (0%)	45 (40.9%)		–	–	–
Unknown	20 (29.9%)	46 (41.8%)		–	–	–
Antinuclear antibody						
Positive	–	–	–	47 (71.2%)	0 (0%)	**<0.001**
Negative	–	–	–	19 (28.8%)	45 (100%)	

ANA, antinuclear antibody; ESCC, esophageal cell squamous carcinoma; GC, gastric cancer; LDH, lactate dehydrogenase; MSI, microsatellite instability; MSI-H, MSI-high; MSS, microsatellite-stable; NSCLC, non-small cell lung cancer; PD-L1, programmed death ligand-1.

[a]For ANA, 16 patients with urological cancer, 14 with colorectal cancer, 3 with cholangiocarcinoma, 3 with pancreatic cancer, 3 with peritoneal mesothelioma, 2 with cervical cancer, 2 with sarcoma, 1 with small cell lung cancer, 1 with gallbladder cancer, 1 with endometrial cancer, 1 with neuroendocrine neoplasm, and 1 with Merkel cell carcinoma.

[b]For any preexisting antibody, 10 patients with colorectal cancer, 6 with urological cancer, 3 with peritoneal mesothelioma, 2 with pancreatic cancer, 2 with cervical cancer, 1 with sarcoma, 1 with small cell lung cancer, 1 with gallbladder cancer, 1 with cholangiocarcinoma, 1 with neuroendocrine neoplasm, and 1 with Merkel cell carcinoma.

[c]For ANA, 18 patients treated with tislelizumab, 10 with sintilimab, 8 with camrelizumab, 6 with penpulimab, 3 with durvalumab, and 2 with geptanolimab.

[d]For any preexisting antibody, 10 patients treated with tislelizumab, 7 with sintilimab, 5 patients treated with camrelizumab, 5 with penpulimab, 3 with durvalumab, and 1 with geptanolimab.

[e]For ANA, 99 patients treated with combined chemotherapy, 27 with combined targeted therapy, 7 with combined chemotherapy plus targeted therapy, and 1 with combined ipilimumab.

[f]For any preexisting antibody, 63 patients treated with combined chemotherapy, 17 with combined targeted therapy, and 6 with combined chemotherapy plus targeted therapy.

[g]PD-L1 combined positive score ≥ 1 or tumor proportion score ≥ 1%.

[h]The patients were considered positive if either antithyroglobulin or antithyroid peroxidase was positive.

[i]The patients were considered positive if all autoantibodies including ANA, ANA profile, and antithyroid antibodies were examined and any autoantibody was positive.

antithyroid antibody tests before immunotherapy initiation, 66 (59.5%) had at least one positive autoantibody (we defined this as positive for any preexisting antibody). Moreover, among 146 patients who completed antithyroid antibody tests before immunotherapy initiation, 16 (11.0%) patients were positive for antithyroid antibody (either antithyroglobulin or antithyroid peroxidase was positive). Among the members of ANA profile, the most common autoantibody was anti-Ro52 antibody (27/133, 20.3%), and the positive rate of any other autoantibody was less than 6%. In particular, 3 patients were previously diagnosed with autoimmune diseases before immunotherapy, including 1 each with immune thrombocytopenia, Hashimoto's thyroiditis, and vitiligo. At the time of immunotherapy initiation, no patient had active autoimmune diseases. Moreover, no patient had newly developed autoimmune diseases during immunotherapy. At the time of analysis, the median follow-up duration was 8.2 (range, 0.4-36) months.

As shown in **Table 1** and **Supplementary Table S1**, there was no significant difference in age, sex, PS, TNM stage, treatment line, PD-L1 status, and microsatellite instability (MSI) status between patients with or without preexisting ANA, anti-Ro52, antithyroid antibody, or any antibody. However, positive ANA was associated with higher serum IgG level and was more common in patients with esophageal cell squamous carcinoma.

Safety Analysis

Eighty-two (46.3%) patients experienced irAEs of any grade, 14 (7.9%) patients developed irAEs of grade 3-5. Specifically, 42 (23.7%) developed skin reactions, 36 (20.3%) developed thyroid dysfunction, whereas 10 (5.6%) developed pneumonitis. These irAEs readily resolved with symptomatic treatments and did not lead to interruption of therapy in most cases. However, 26 (14.7%) patients required systemic immunosuppressants, and 22 (12.4%) patients discontinued immunotherapy. Notably, the timing of irAEs occurrence ranged from 1 day to 2 years following the initiation of PD-1/PD-L1 inhibitors but occurred mainly at 1 to 10 weeks (75/82, 91%).

As shown in **Table 2**, thyroid dysfunction was more frequent in patients with positive antithyroid antibody (75.0% versus 13.8%, $p < 0.001$). However, the presence of positive ANA, anti-Ro52, or any antibody had no significant association with the development of irAEs of any grade or grades 3-5, and the development of skin reactions and thyroid dysfunction. Moreover, preexisting ANA, anti-Ro52, antithyroid, or any antibody was not correlated with the early emergence of irAEs, systemic immunosuppressant treatments required for irAEs, and immunotherapy discontinuation due to irAEs.

Evaluation of Efficacy

After receiving PD-1/PD-L1 inhibitors therapy, 4 patients achieved complete response, ORR and DCR in enrolled patients were 37.9% and 81.9%, respectively. As shown in **Table 2**, preexisting ANA, anti-Ro52, antithyroid, or any antibody had no significant influence on ORR and DCR in patients treated with PD-1/PD-L1 inhibitors. However, there was a trend for a higher DCR in those positive for anti-Ro52

TABLE 2 | Development of irAEs and treatment response among patients with or without preexisting autoantibodies.

	Positive ANA (n=67)	Negative ANA (n=110)	P value	Positive anti-Ro52 (n=27)	Negative anti-Ro52 (n=106)	P value	Positive antithyroid (n=16)	Negative antithyroid (n=130)	P value	Positive for any preexisting antibody (n=66)	Negative for all preexisting antibodies (n=45)	P value
irAEs												
Any grade	32 (47.8%)	50 (45.5%)	0.886	14 (51.9%)	50 (47.2%)	0.827	14 (87.5%)	53 (40.8%)	**0.001**	34 (51.5%)	18 (40.0%)	0.317
Grades 3–5	4 (6.0%)	10 (9.1%)	0.646	5 (18.5%)	8 (7.5%)	0.177	2 (12.5%)	10 (7.7%)	0.858	6 (9.1%)	5 (11.1%)	0.979
Skin reactions	16 (23.9%)	26 (23.6%)	1	5 (18.5%)	27 (25.5%)	0.615	6 (37.5%)	30 (23.1%)	0.339	14 (21.2%)	13 (28.9%)	0.484
Thyroid dysfunction	17 (25.4%)	19 (17.3%)	0.269	5 (18.5%)	23 (21.7%)	0.922	12 (75.0%)	18 (13.8%)	**<0.001**	19 (28.8%)	6 (13.3%)	0.093
Gap between irAEs occurrence and ICI initiation (days)	39.5 (1-238)	42.5 (3-738)	0.202	40 (3-238)	34.5 (1-738)	0.828	46.5 (1- 126)	36 (2- 738)	0.523	39.5 (1-238)	25 (3-738)	0.596
Systemic immunosuppression required for irAEs	7 (10.4%)	19 (17.3%)	0.305	6 (22.2%)	16 (15.1%)	0.549	3 (18.8%)	17 (13.1%)	0.812	10 (15.2%)	7 (15.6%)	1
ICI discontinuation due to irAEs	6 (9.0%)	16 (14.5%)	0.391	5 (18.5%)	15 (14.2%)	0.791	2 (12.5%)	14 (10.8%)	1	7 (10.6%)	7 (15.6%)	0.631
ORR	31 (46.3%)	36 (32.7%)	0.101	14 (51.9%)	41 (38.7%)	0.307	5 (31.2%)	50 (38.5%)	0.773	32 (48.5%)	14 (31.1%)	0.104
DCR	58 (86.6%)	87 (79.1%)	0.293	26 (96.3%)	83 (78.3%)	0.059	13 (81.2%)	105 (80.8%)	1	57 (86.4%)	32 (71.1%)	0.082

ANA, antinuclear antibody; DCR, disease control rate; ICIs, immune checkpoint inhibitors; irAEs, immune-related adverse events; ORR, objective response rate.

antibody than those negative for anti-Ro52 (96.3% versus 78.3%, p = 0.059).

Intriguingly, the median PFS was significantly longer in the ANA-positive patients (13.1 versus 7.0 months, p = 0.015), while the median OS did not differ significantly (14.5 versus 21.8 months, p = 0.67) between the ANA-positive and ANA-negative groups (**Figures 1A, B**). Similarly, the median PFS was significantly longer in those with any preexisting antibody (10.9 versus 4.1 months, p = 0.019), while the median OS did not differ significantly (21.9 versus 15.1 months, p = 0.19) between those with and without any preexisting antibody (**Figures 2A, B**). With adjusting the impact of confounding factors using PSM analysis, the patients with preexisting ANA, or any antibody had robustly longer PFS, and the OS did not differ significantly (**Supplementary Figures S1**, **2**). However, there were no significant differences in PFS (14.5 versus 8.1 months, p = 0.31) or OS (14.5 versus 21.8 months, p = 0.80) between those with or without ≥ 1:160 ANA titers (**Supplementary Figures S3A, B**). Moreover, the preexisting anti-Ro52 and antithyroid antibodies were not associated with PFS (13.1 versus 7.4 months, p = 0.094; 8.5 versus 7.4 months, p = 0.48, respectively) and OS (not reached versus 20.1 months, p = 0.80; not reached versus 21.8 months, p = 0.46, respectively) (**Figures 3**, **4**).

Considering the sample size and collinearity between variables, we first performed LASSO regression analysis on variables that might affect patient survival outcomes. Variables including age, gender, PS, cancer type, TNM stage, liver metastasis, multiple metastases, treatment line, combination

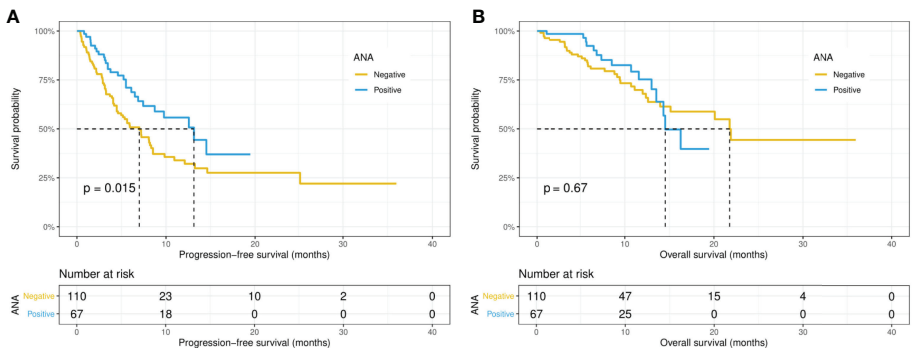

FIGURE 1 | Kaplan-Meier curves of progression-free survival **(A)** and overall survival **(B)** in the positive and negative ANA groups. ANA, antinuclear antibody.

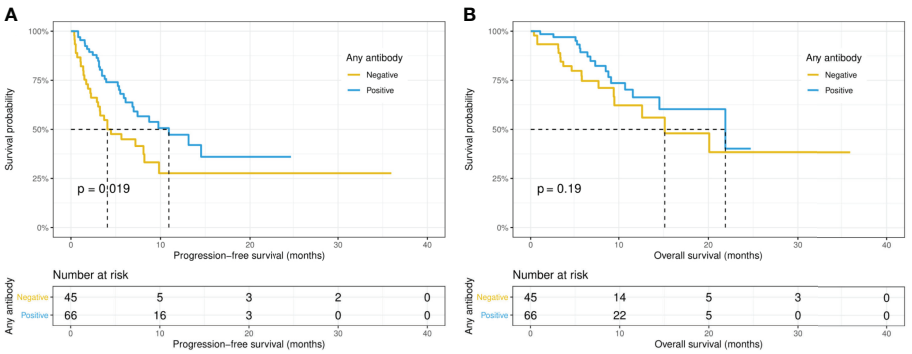

FIGURE 2 | Kaplan-Meier curves of progression-free survival **(A)** and overall survival **(B)** in patients with or without any preexisting antibody.

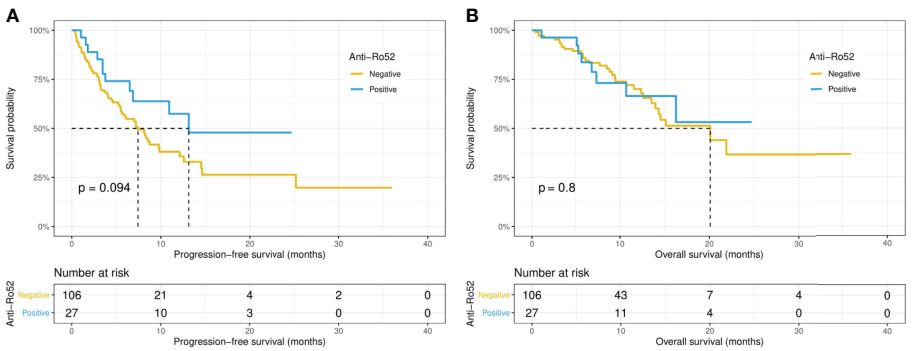

FIGURE 3 | Kaplan-Meier curves of progression-free survival **(A)** and overall survival **(B)** in the positive and negative anti-Ro52 antibody groups.

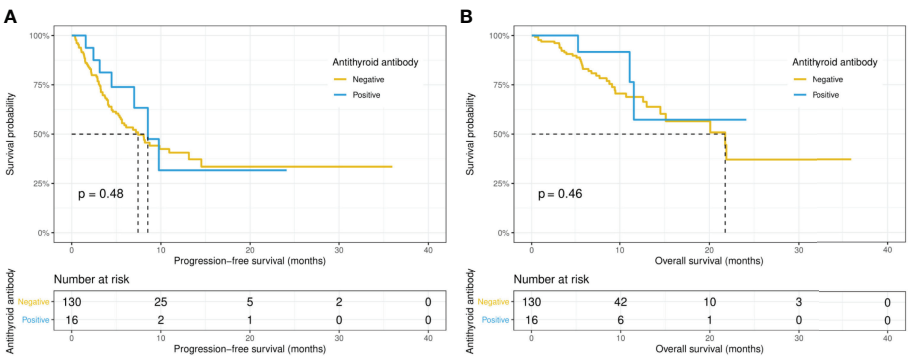

FIGURE 4 | Kaplan-Meier curves of progression-free survival **(A)** and overall survival **(B)** in the positive and negative antithyroid antibody groups.

therapy, the level of LDH, IgG, IgA, IgM, IL-6, IL-8, IL-10, TNF-α, hsCRP, ESR, PD-L1 status, MSI status, any preexisting antibody (positive or negative), ANA (positive or negative), ANA titer (≥1:160 or <160), anti-Ro52 antibody (positive or negative), and antithyroid antibody (positive or negative) were examined in LASSO regression. After performing LASSO regression, age, gender, PS, liver metastasis, multiple metastases, treatment line, the level of LDH, MSI status, ANA, and anti-Ro52 antibody were identified as the potential predictors of PFS (**Supplementary Figure S4**), while gender,

PS, TNM stage, liver metastasis, multiple metastases, treatment line, the level of LDH, MSI status, and any preexisting antibody were identified as the potential predictors of OS (**Supplementary Figure S5**). Furthermore, as shown in **Table 3**, the univariate analysis demonstrated that preexisting ANA, MSI status, treatment line, liver metastasis, and multiple metastases were significantly associated with the PFS of patients treated by PD-1/PD-L1 inhibitors. Further multivariate analysis confirmed that positive ANA (HR: 0.59, 95% CI: 0.37-0.92, p = 0.021) was an independent indicator of better PFS. However, preexisting autoantibodies were not independently associated with the OS of the patients (**Table 4**).

DISCUSSION

Although PD-1/PD-L1 inhibitors for cancer immunotherapy are currently in common use in oncology, their safety and efficacy are still unknown for patients with preexisting autoantibodies, which are recognized as biomarkers of autoimmune diseases. Naturally, clinicians would be more concerned about severe and fatal irAEs in patients with potential autoimmune diseases, which occur occasionally in the general population with the use of ICIs (17). In the present study, we evaluated the effect of preexisting autoantibodies on the safety and efficacy of PD-1/PD-L1 inhibitors.

Previous research showed that the presumptive percentage of positive ANA in the general population is 10% to 15% with the cutoff value at 1:80 (18, 19). However, the rate of ANA positivity is even as high as 17% to 51% in patients with cancer (20–22). In the present retrospective cohort, the frequency of ANA positivity was 37.9%. Our results supported that the ANA positivity rate in patients with cancer was higher than that in the general population, but the role of ANA in tumorigenesis and cancer development remains unclear. Preclinical studies suggested ANA has anti-tumor activity (3, 23, 24), ANA positivity in lung cancer patients was reported to be associated with an improved PFS and OS (25), but there were also contrary reports (26).

There were five published studies estimating the effect of ANA on ICIs toxicity and efficacy (5–9). Four of the studies included only patients with NSCLC (5–8). Giannicola et al. (8) reported metastatic NSCLC patients positive for ANA had significantly prolonged PFS and OS, which contradicts the conclusion reached by another study (7). The other two studies (5, 6) suggested that the efficacy and safety of ICIs therapy in patients with NSCLC and positive ANA were comparable to those negative for ANA. Accordingly, there was no significant difference in the incidence of irAEs, ORR, and survival outcome between the 16 NSCLC patients positive for ANA and 30 NSCLC patients negative for ANA retrieved from our cohort (data not shown). Intriguingly, PFS was found to be longer in ANA-positive patients treated by PD-1/PD-L1 inhibitors across

TABLE 3 | Univariate and multivariate analyses of factors for progression-free survival.

Variables	Univariate analysis		Multivariate analysis	
	HR (95% CI)	P value	HR (95% CI)	P value
Age (≥60 vs. <60)	0.7 (0.47,1.05)	0.082	-	-
Sex (Male vs. Female)	1.36 (0.86,2.14)	0.187	-	-
Performance status (2-3 vs. 0-1)	2.03 (0.64,6.43)	0.229	-	-
Liver metastasis (Yes vs. No)	2.98 (1.93,4.58)	**<0.001**	2.69 (1.64,4.43)	**<0.001**
Multiple metastases (Yes vs. No)	2.15 (1.43,3.22)	**<0.001**	1.32 (0.84,2.07)	0.237
No prior systemic therapy (Yes vs. No)	0.54 (0.36,0.81)	**0.003**	0.55 (0.36,0.83)	**0.005**
Elevated LDH (Yes vs. No)	1.37 (0.84,2.24)	0.211	-	-
MSI status (MSI-H vs. MSS)	0.18 (0.05,0.6)	**0.005**	3.34 (0.97,11.43)	0.055
ANA (Positive vs. Negative)	0.58 (0.37,0.91)	**0.017**	0.59 (0.37,0.92)	**0.021**
Anti-Ro52 antibody (Positive vs. Negative)	0.58 (0.3,1.1)	0.097	-	-

ANA, antinuclear antibody; LDH, lactate dehydrogenase; MSI, microsatellite instability; MSI-H, MSI-high; MSS, microsatellite-stable.

TABLE 4 | Univariate and multivariate analyses of factors for overall survival.

Variables	Univariate analysis		Multivariate analysis	
	HR (95% CI)	P value	HR (95% CI)	P value
Sex (Male vs. Female)	1.38 (0.73,2.59)	0.324	-	-
Performance status (2-3 vs. 0-1)	6.55 (1.97,21.7)	**0.002**	3.33 (0.91,12.23)	0.07
TNM stage (IV vs. III)	8.36 (1.15,60.73)	**0.036**	5.74 (0.73,44.8)	0.096
Liver metastasis (Yes vs. No)	3.9 (2.22,6.86)	**<0.001**	2.1 (1.02,4.32)	**0.043**
Multiple metastases (Yes vs. No)	2.58 (1.46,4.57)	**0.001**	1.05 (0.54,2.05)	0.881
No prior systemic therapy (Yes vs. No)	0.43 (0.24,0.78)	**0.005**	0.53 (0.28,0.98)	**0.042**
Elevated LDH (Yes vs. No)	2.38 (1.3,4.34)	**0.005**	1.88 (0.97,3.63)	0.06
MSI status (MSI-H vs. MSS)	0.1 (0.01,0.77)	**0.027**	0.13 (0.02,1.09)	0.06
Any preexisting antibody (Positive vs. Negative)	0.64 (0.32,1.27)	0.201	-	-

ANA, antinuclear antibody; LDH, lactate dehydrogenase; MSI, microsatellite instability; MSI-H, MSI-high; MSS, microsatellite-stable.

tumor types (13.1 versus 7.0 months, p = 0.015), even when the confounding factors (including TNM stage and cancer type) were adjusted. Furthermore, Yoneshima et al. (7) observed the ANA titer increased in 3 patients who were initially positive for ANA, consequently, all of them developed irAEs. However, there was only one patient who developed grade 2 thyroid dysfunction among 7 patients with the increased ANA titer during PD-1 inhibitor treatment. As for the effect of ANA titer, the study of Mouri et al. (5) suggested the incidence of irAEs was not significantly different between the ANA-positive and ANA-negative groups, regardless of the cutoff of ANA titers (1:40 or 1:80). Our results also reached the similar conclusion that patients with preexisting ANA had no increased risk of irAEs, regardless of the cutoff of ANA titers (1:80 or 1:160) (data not shown). Regrettably, there were only 9 patients who had an ANA titer of 1:320 in our cohort, thus, we were unable to analyze the influence of ANA antibody titer ≥ 1:320 on the safety and efficacy of immunotherapy. On the one hand, ANA does not necessarily indicate autoimmune disease, and the general population can also carry ANA, hence, ANA positivity may not represent an increased risk of irAEs. On the other hand, autoantibodies probably are associated with the release of tumor neoantigens (3), ANA positivity is related to immune cells (including NK, T and B cells) activation (3, 27), so ANA-positive patients have a theoretical possibility to achieve better immunotherapy efficacy. These may be the underlying mechanisms for successful immunotherapy in ANA-positive patients in our cohort. However, the association of ANA with anti-tumor immunity needs to be verified by further research.

The rate of anti-Ro52 antibody positivity is about 12% in the general population, and ranges from 5.9% to 30% in cancers (11, 28). However, the presence of anti-Ro52 antibody may not indicate an increased risk of cancer (29). Our data showed the frequency of anti-Ro52 antibody positivity was 20.3% across malignancies, no significant association between cancer type and anti-Ro52 antibody positivity was observed. Furthermore, there was no significant difference in the efficacy and safety of PD-1/PD-L1 inhibitors between patients in the positive anti-Ro52 group and those in the negative group. Notably, there was a trend for better PFS (p = 0.094) in patients positive for anti-Ro52 antibody. Studies with a larger sample size will better clarify the effect of anti-Ro52 antibody on ICIs therapy.

Toi et al. (6) reported NSCLC patients with any preexisting antibody (including ANA, rheumatoid factor, and antithyroid antibody) had significantly better PFS and OS, while no significant differences in PFS and OS were observed between patients with or without preexisting ANA. Nevertheless, in the present study, Kaplan-Meier curves and log-rank tests showed patients with ANA or any preexisting antibody had better PFS, while LASSO and Cox regression analysis demonstrated that only ANA was an independent indicator of better PFS. In addition, our result suggested positive antithyroid antibody was associated with thyroid dysfunction during anti-PD-1/PD-L1 treatment. This is consistent with the conclusion reached by Toi et al. (6).

Our study adds to the growing evidence supporting the use of immunotherapy in patients with preexisting autoantibodies. Moreover, to the best of our knowledge, this is the first study to evaluate the effect of anti-Ro52 antibody on ICIs administration. However, our study has several limitations. First, potential inherent selection bias cannot be excluded using an observational retrospective design. Patients with severe or active autoimmune diseases were underrepresented. Besides, the incidence of diverse irAEs observed in our study might be influenced by monitoring bias. Second, the single-center approach and the relatively small size of a variety of cancer types may limit the generalization of our results to other settings. Third, the titer change of autoantibodies may reflect the change in the immune activation state of the body, but we failed to analyze the effect of the titer change of autoantibodies on efficacy and irAEs induced by ICIs.

CONCLUSION

In summary, our data suggested that PD-1/PD-L1 inhibitors could be administered safely and effectively in patients with preexisting autoantibodies but without active autoimmune disease. However, patients positive for antithyroid antibody should monitor closely thyroid dysfunction during anti-PD-1/PD-L1 treatment.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by The Medical Ethics Committee of Peking Union Medical College Hospital. Written informed consent was not provided because Patients' consents for participation and publication were waived due to the retrospective design and the deidentified data of this study.

AUTHOR CONTRIBUTIONS

LZ, HT, and RG were involved in conceptualization. HT, RG, XX, YW, JZ, SZ, and CB had contributed to data acquisition. HT and RG carried out data analysis and wrote the original draft. LZ and MG critically revised the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2022.893179/full#supplementary-material

Supplementary Table 1 | Baseline characteristics of patients with or without preexisting anti-Ro52 or antithyroid antibodies. ANA, antinuclear antibody; ESCC, esophageal cell squamous carcinoma; GC, gastric cancer; LDH, lactate dehydrogenase; MSI, microsatellite instability; MSI-H, MSI-high; MSS, microsatellite-stable; NSCLC, non-small cell lung cancer; PD-L1, programmed death ligand-1. [a] For anti-Ro52 antibody, 12 patients with colorectal cancer, 6 with urological cancer, 3 with peritoneal mesothelioma, 2 with pancreatic cancer, 2 with cervical cancer, 2 with sarcoma, 1 with small cell lung cancer, 1 with cholangiocarcinoma, 1 with gallbladder cancer, 1 with neuroendocrine neoplasm, and 1 with Merkel cell carcinoma. [b] For antithyroid antibody, 12 patients with urological cancer, 11 with colorectal cancer, 3 with cholangiocarcinoma, 3 with pancreatic cancer, 3 with peritoneal mesothelioma, 2 with cervical cancer, 1 with sarcoma, 1 with small cell lung cancer, 1 with gallbladder cancer, 1 with neuroendocrine neoplasm, and 1 with Merkel cell carcinoma. [c] For anti-Ro52 antibody, 12 patients treated with tislelizumab, 8 with sintilimab, 5 with camrelizumab, 5 with penpulimab, 3 with durvalumab, and 2 with geptanolimab. [d] For antithyroid antibody, 13 patients treated with tislelizumab, 8 with sintilimab, 8 with camrelizumab, 6 with penpulimab, 3 with durvalumab, and 1 with geptanolimab. [e] PD-L1 combined positive score ≥ 1 or tumor proportion score ≥ 1%. [f] The patients were considered positive if all autoantibodies including ANA, ANA profile, and antithyroid antibodies were examined and any autoantibody was positive. [g] The patients were considered positive if either antithyroglobulin or antithyroid peroxidase was positive.

Supplementary Figure 1 | Kaplan-Meier curves of progression-free survival **(A)** and overall survival **(B)** in the positive and negative ANA groups with the adjustment of the impact of confounding factors using propensity-score matching analysis. ANA, antinuclear antibody.

Supplementary Figure 2 | Kaplan-Meier curves of progression-free survival **(A)** and overall survival **(B)** in patients with or without any preexisting antibody with the adjustment of the impact of confounding factors using propensity-score matching analysis.

Supplementary Figure 3 | Kaplan-Meier curves of progression-free survival **(A)** and overall survival **(B)** in the patients with or without ≥ 1:160 ANA titers. ANA, antinuclear antibody.

Supplementary Figure 4 | Identification of the potential predictors of progression-free survival. **(A)** LASSO coefficient profiles of the selected variables. **(B)** Parameter selection in the LASSO model. LASSO, least absolute shrinkage and selection operator.

Supplementary Figure 5 | Identification of the potential predictors of overall survival. **(A)** LASSO coefficient profiles of the selected variables. **(B)** Parameter selection in the LASSO model. LASSO, least absolute shrinkage and selection operator.

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A genomic instability-related lncRNA model for predicting prognosis and immune checkpoint inhibitor efficacy in breast cancer

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Breast cancer has overtaken lung cancer as the most frequently diagnosed cancer type and is the leading cause of death for women worldwide. It has been demonstrated in published studies that long non-coding RNAs (lncRNAs) involved in genomic stability are closely associated with the progression of breast cancer, and remarkably, genomic stability has been shown to predict the response to immune checkpoint inhibitors (ICIs) in cancer therapy, especially colorectal cancer. Therefore, it is of interest to explore somatic mutator-derived lncRNAs in predicting the prognosis and ICI efficacy in breast cancer patients. In this study, the lncRNA expression data and somatic mutation data of breast cancer patients from The Cancer Genome Atlas (TCGA) were downloaded and analyzed thoroughly. Univariate and multivariate Cox proportional hazards analyses were used to generate the genomic instability-related lncRNAs in a training set, which was subsequently used to analyze a testing set and combination of the two sets. The qRT-PCR was conducted in both normal mammary and breast cancer cell lines. Furthermore, the Kaplan–Meier and receiver operating characteristic (ROC) curves were applied to validate the predictive effect in the three sets. Finally, the Cell-type Identification by Estimating Relative Subsets of RNA Transcripts (CIBERSORT) algorithm was used to evaluate the association between genomic instability-related lncRNAs and immune checkpoints. As a result, a six-genomic instability-related lncRNA signature (U62317.4, MAPT-AS1, AC115837.2, EGOT, SEMA3B-AS1, and HOTAIR) was identified as the independent prognostic risk model for breast cancer patients. Compared with the normal mammary cells, the qRT-PCR showed that HOTAIR was upregulated while MAPT-AS1, EGOT, and SEMA3B-AS1 were downregulated in breast cancer cells. The areas under the ROC curves at 3 and 5 years were 0.711 and 0.723, respectively. Moreover, the patients classified in the high-risk group by the

prognostic model had abundant negative immune checkpoint molecules. In summary, this study suggested that the prognostic model comprising six genomic instability-related lncRNAs may provide survival prediction. It is necessary to identify patients who are suitable for ICIs to avoid severe immune-related adverse effects, especially autoimmune diseases. This model may predict the ICI efficacy, facilitating the identification of patients who may benefit from ICIs.

KEYWORDS

genomic instability, lncRNAs, prognostic model, immune checkpoint, breast cancer, autoimmune diseases

Introduction

Breast cancer had overtaken lung cancer as the most frequently diagnosed cancer type and remained the leading cause of death for women worldwide by 2020 (1, 2). The most widely used classification of breast cancer is defined according to the expression of the progesterone receptor, estrogen receptor (ER), and human epidermal growth factor receptor 2 (HER2) (3). Moreover, breast cancer is a disease with high heterogeneity, resulting in challenges in treatment. Because of the high death rate, it is critical to identify novel prognostic biomarkers and develop suitable treatment plans for breast cancer patients.

Genomic instability (GI) refers to cells acquiring genomic alterations at an increased rate, which is divided into small structural variations and significant structural variations (4). It is reported that GI can be deemed a hallmark of cancer development due to the enhanced survival ability of cancer cells (5, 6). Moreover, the mechanism underlying increased GI involves the failure of DNA damage repair, DNA replication stress, transcription-associated stress, and chromothripsis (7). Notably, the immune checkpoint inhibitors (ICIs) have achieved unprecedented success in microsatellite instability-high (MSI-H)/deficient mismatch repair (dMMR) colorectal cancer (8). Meanwhile, programmed cell death protein 1 (PD-1) blockade has become a first-line treatment option for MSI-H/dMMR metastatic colorectal cancer as recommended in the guideline (9). Thus, it is suggested that GI may be closely associated with immune checkpoint blockade treatment. However, nearly half of the patients receiving immunotherapy are refractory to ICIs (10), and there are few biological predictive factors to stratify the patients who receive the ICI therapy, which can be a novel research direction for GI. Recently, efforts to further understand GI in breast cancer have also been focused on its roles in tumor initiation, progression, and, particularly, prognostic prediction.

Long non-coding RNAs (lncRNAs) are transcripts that include more than 200 nucleotides without the potential of coding proteins (11). During the past decades, numerous lncRNAs were identified to be aberrantly expressed in manifold cancers owing to the rapid development of next-generation sequencing technologies, and the roles of lncRNAs in the different biological processes have been realized gradually (12, 13). Emerging studies showed a noticeable link between lncRNAs and genomic stability (14, 15). The most well-known example is the non-coding RNA activated by DNA damage (NORAD, also termed as LINC00657), which can maintain genomic stability *via* sequestering pumilio RNA binding family member 1 proteins (16). Another study revealed that the interaction between NORAD and RNA binding motif protein X-linked, a component of the DNA-damage response, contributed to the maintenance of genomic stability (17). Although abundant studies have verified the connection of lncRNAs with genomic stability, the roles of GI-associated lncRNAs and their clinical value remain to be further investigated.

At present, lncRNAs are considered as an independent prognostic biomarker in cancer (18), such as HOX transcript antisense RNA (HOTAIR) in ER^+ breast cancer (19). Nonetheless, a single lncRNA as the predictive biomarker is not gratifying, due to tremendous false-positive and -negative results (20). Here, we analyzed the lncRNA expression and somatic mutation data in breast cancer from The Cancer Genome Atlas (TCGA) and developed a six-mutator-derived lncRNA signature to reflect GI and predict the survival prognosis for breast cancer patients.

Materials and methods

Data source

The RNA-seq data, somatic mutation features, and clinical information of breast cancer patients were acquired from the TCGA database (https://portal.gdc.cancer.gov/). Then, the RNA-seq data were divided into lncRNA and mRNA

expression profiles. A total of 1,109 patients with breast cancer were included in the study to identify the lncRNA-related prognostic model. Moreover, the prognostic value of these lncRNAs was validated in an interactive web, Gene Expression Profiling Interactive Analysis (GEPIA, http://gepia.cancer-pku.cn/detail.php?gene=&clicktag=survival) (21). GEPIA included data from TCGA and the Genotype-Tissue Expression (GTEx) projects. TCGA and GEPIA are open public databases, and there was no need for ethics approval in the study.

Identification of GI-related lncRNAs

To begin with, the mutation count of each patient was calculated and ranked by analyzing somatic mutation profiles from TCGA (https://portal.gdc.cancer.gov/). The top 25% of patients were assigned as the genomically unstable (GU) group, while the last 25% were defined as the genomically stable (GS) group (22). Secondly, differentially expressed lncRNAs were identified by analyzing the lncRNA expression differences between the GU and GS groups with the Wilcoxon test. GI-related lncRNAs were defined when the |log fold change| (logFC) > 1 and the false discovery rate (FDR)-adjusted $p< 0.05$.

Functional enrichment analysis

All the patients with lncRNA expression data were divided into a GU-like or a GS-like group using genome instability-related lncRNAs and conducting hierarchical cluster analyses. The somatic mutation count and the expression of some immune checkpoints, including PD-1, programmed cell death receptor ligand 1 (PD-L1), indoleamine 2,3-dioxygenase 1 (IDO1), and tryptophan 2,3-dioxygenase 2 (TDO2), between the two groups, were determined. Furthermore, the correlation test between the genome instability-related lncRNAs and mRNA expression was conducted to get the Pearson correlation coefficients. The paired top 10 mRNAs were regarded as co-expressed followers of each GI-related lncRNA. To discover the potential function of these lncRNAs, we screened related protein-coding genes and performed Gene Ontology (GO) analysis and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis (23).

Definition of the GI-related lncRNA prognostic model

All breast cancer patients were defined as the TCGA set and were also divided into two sets randomly, including a training set and a testing set. We performed the Chi-square test to evaluate the association of each set with other critical clinical characteristics. Subsequently, univariate and multivariate analysis by Cox proportional hazards regression model was used to evaluate the link between the expression of GI-related lncRNAs and prognosis in breast cancer patients in the training set. After univariate Cox regression analysis, the survival-related lncRNAs were shown as the forest plot when the p-value was<0.05, in which hazard ratio (HR) and 95% confidence interval (CI) were calculated with the survival and survminer package in R. After multivariate analysis, the prognostic risk model independent of other clinical features was built. According to the expression and coefficients of the GI-related lncRNAs and patient survival, the formula of an lncRNA-based prognostic risk score for a breast cancer patient was defined as follows:

$$\text{Risk score} = \sum_{i=1}^{n} \text{expression}(\text{lncRNA}_i) * \text{Coefficient}(\text{lncRNA}_i)$$

Firstly, the risk score of each patient in the training set was computed. Then, the median risk score of patients was regarded as the cutoff value. On the basis of the cutoff value of the training set, the patients in the training set, the testing set, and the TCGA set were categorized into high- or low-risk groups separately. Finally, the testing set along with the TCGA set was used to verify the feasibility of the prognostic risk model acquired from the data of the training set.

Validation of the GI-related lncRNA prognostic model

Survival curves were plotted in the training set, the testing set, and the TCGA set to validate the predictive ability of the risk score, in which the log-rank test was performed with a $p< 0.05$ as statistical significance. The receiver operating characteristic (ROC) curves with 3 and 5 years were used to test the performance of the lncRNA-related prognostic model, which showed the sensitivity and specificity. Furthermore, the association between the risk score and the expression of each lncRNA in the prognostic model was investigated in the three sets. Likewise, the relations between the risk score and somatic mutation level, and the expression level of IDO1 as well as TDO2 were explored. Then, the prognostic lncRNAs were validated in GEO datasets with breast cancer patients. The landscape profiling of somatic gene mutations in the high- or low-risk group from the TCGA was conducted as a waterfall plot with the Maftools package in the R software. Moreover, stratification analysis of the prognostic risk model by age, stage, and gender was estimated using the univariate Cox analysis and the log-rank test. Finally, the prognostic lncRNA signature was compared with other signatures published in existing studies.

The relation between the risk score and immune function

The Cell-type Identification by Estimating Relative Subsets of RNA Transcripts (CIBERSORT) algorithm (24) was applied to evaluate the immune-related signature of each patient with breast cancer. The expression of immune checkpoints was analyzed to identify the association of lncRNA-related risk score with cancer immunity. To determine the difference of signaling pathways between the high- and low-risk groups, multiple gene set enrichment analysis (GSEA) was performed through the GSEA software (4.1.0) and R packages (25).

Cell culture

Normal mammary epithelial cells HBL100 as well as five human breast cancer cell lines MDA-MB-231, MDA-MB-468, Sum159, H578T, and SKBR3 were obtained from the Department of Oncology (Tongji Hospital, Wuhan, China) and cultured in Dulbecco's modified Eagle medium (DMEM, Hyclone), which contained 10% fetal bovine serum (FBS). Cells were incubated in an incubator containing 5% CO_2 at 37°C.

RNA extraction and real-time PCR assay

Total RNA was extracted using Trizol reagent (Invitrogen, Carlsbad, USA) and the manufacturer's manual was followed. Complementary DNA for reverse transcription was synthesized by the Prime Script RT kit (Takara, Tokyo, Japan). Real-time PCR analysis was then performed. The $2^{-\Delta\Delta}$Ct method was applied to determine differences between multiple samples. The primer sequences are as follows. HOTAIR primer sequences: forward strand, 5′-ACTCTGACTCGCCTGTGCTCTG-3′; reverse strand, 5′-AGTGCCTGGTGCTCTCTTACCC-3′; SEMA3B-AS1 primer sequences: forward strand, 5′-GTCCTGAAGCTGAGTCTGGTGAAC-3′; reverse strand, 5′-CTCCACTCTGCCACTGTCAACATAC-3′; EGOT primer sequences: forward strand, 5′-TAACGCACTAGAGGAGACAGAGACG-3′; reverse strand, 5′-GTTGCTAGTTGGACAGTCGGTATGG-3′; MAPT-AS1 primer sequences: forward strand, 5′-CGGAACCAGAAGGGAGGGATTTG-3′; reverse strand, 5′-CACAGAGACACACAGGGAGAATGC-3′.

Statistical analysis

Statistical analysis was conducted with Perl version 5.18.4 (https://www.perl.org/) and R version 4.0.3 (Package: limma, pheatmap, sparcl, ggpubr, clusterProfiler, org.Hs.eg.db, enrichplot, ggplots, survival, caret, glmnet, survminer, timeROC, e1071, parallel, preprocessCore, plyr, grid, gridExtra, and maftools). GSEA was performed for functional annotation. The real-time PCR data were analyzed with the GraphPad Prism 8.0 software and the two-sample *t*-test. Two-tailed $p< 0.05$ was considered as statistically significant ($^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$).

Results

Identification of GI-related lncRNAs

After calculating the somatic mutation count of each breast cancer patient, the top 25% (n = 252) and the last 25% (n = 259) of the patients were grouped as GU and GS, respectively. Subsequently, 1,833 differentially expressed lncRNAs between GU and GS were identified by analyzing the lncRNA expression data with the Wilcoxon–Mann–Whitney test. Based on the criteria of $|\log FC| > 1$ and FDR< 0.05, 128 differentially expressed lncRNAs were identified as GI-related lncRNAs in breast cancer, in which 63 were upregulated and 65 were downregulated. Then, a volcano plot was produced to show the 128 GI-related lncRNAs (Figure 1A), and a heatmap was used to demonstrate the differential expression of the top 20 upregulated and 20 downregulated GI-related lncRNAs (Figure 1B).

Analysis of GI-related lncRNAs between the GS-like and GU-like groups

LncRNA expression profiles with 1,109 breast cancer patients were analyzed by unsupervised hierarchical clustering using the 128 GI-related lncRNAs. Then, the 1,109 samples were clustered into the GS-like group (n = 700) and GU-like group (n = 409) (Figure 2A). The GU-like group had a higher somatic mutation count than the GS-like group ($p< 0.001$, Figure 2B).

Moreover, given the potential relation between GI and immune checkpoints, the mRNA levels of PD-L1 (Figure 2C), PD-1 (Figure 2D), cytotoxic T-lymphocyte-associated protein 4 (CTLA4) (Figure 2E), IDO1 (Figure 2F), and TDO2 (Figure 2G) between the GS-like and GU-like groups were compared. The result indicated that the GU-like group had a significantly higher expression level of the five immune checkpoints mentioned above.

Through Spearman's correlation analysis, the top 10 protein-coding genes were chosen for each GI-related lncRNA, which produced 1,280 genes in all (Figure 3A). The enriched GO terms and KEGG pathways were analyzed for the 1,280 genes. In the biological process terms of GO, our analysis indicated that most protein-coding genes were enriched in "hormone transport" (GO:0009914), "hormone secretion" (GO:0046879), and "stem cell differentiation"

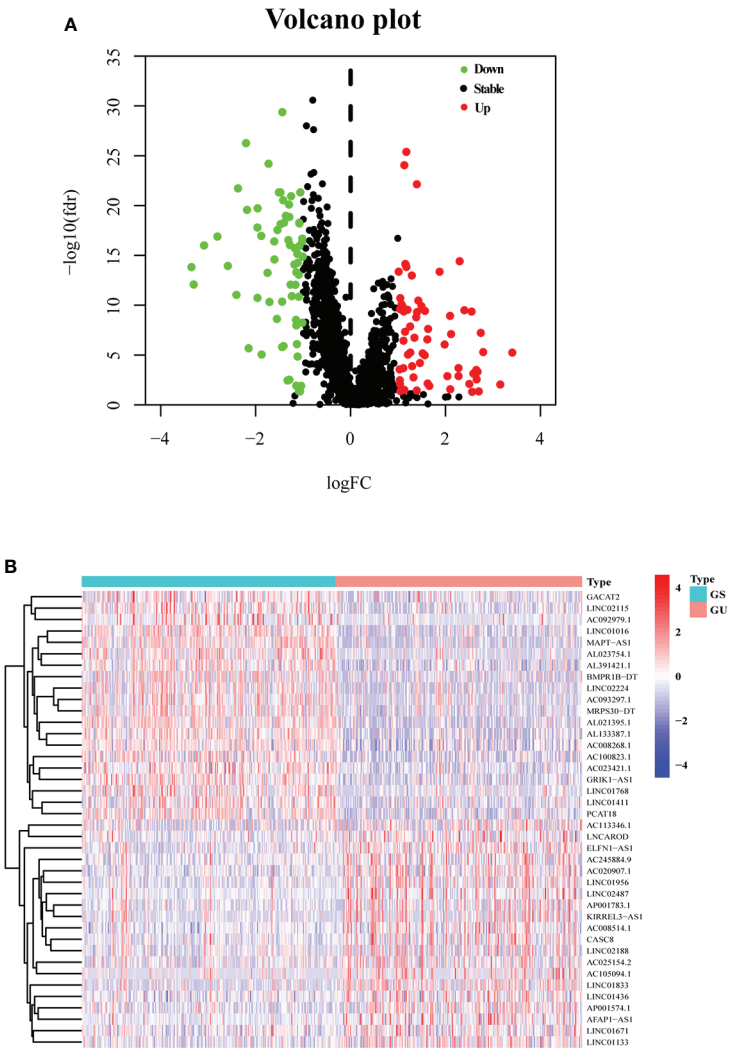

FIGURE 1
One hundred twenty-eight GI-related lncRNAs in breast cancer from TCGA. **(A)** A total of 128 GI-related lncRNAs are shown in a volcano plot. Sixty-three were upregulated and shown in red. Sixty-five were downregulated and shown in green. **(B)** Heatmap of the top 20 upregulated and top 20 downregulated GI-related lncRNAs. The top 25% (*n* = 252) and the last 25% (*n* = 259) mutated patients were selected as GU and GS. The green and red bars represent GU and GS, respectively. Red represents upregulated lncRNA, and blue denotes downregulated lncRNA.

(GO:000048863) (Figure 3B). In the KEGG analysis, our result showed that these genes were enriched in the "MAPK signaling pathway" (hsa04010) and "PI3K–Akt signaling pathway" (hsa04151) (Figure 3C).

Identification of 6-GI-related lncRNA prognostic signature for breast cancer

All patients with survival information were divided into a training set with 520 patients and a testing set with 519 patients at random. As shown in Table 1, there was no correlation between the two groups in the demographic, clinical, or pathological features as shown in the χ^2 test. Using the 128 lncRNA expression levels in 1,039 breast cancer patients with survival information, we further investigated survival-related lncRNAs with univariate Cox proportional hazard regression analysis in the training set. We found that nine lncRNAs were markedly correlated with the prognosis of breast cancer patients (Figure 4). Then, multivariate proportional hazards (Cox) regression analysis was conducted to identify the independent prognostic model using the nine lncRNA expression levels and demographic and clinical features, including age, gender, and stage. Finally, six of the nine

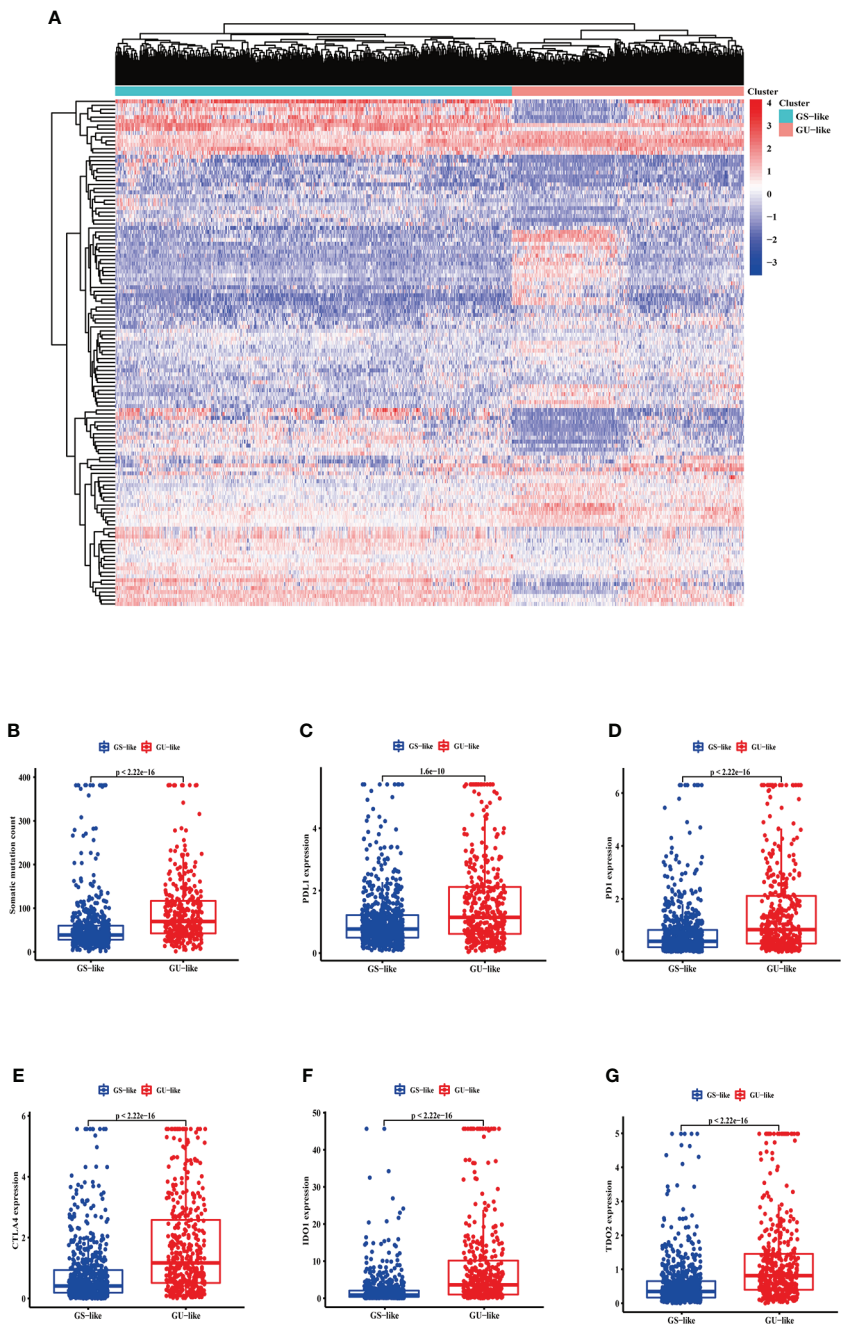

FIGURE 2
The somatic mutations and the expression level of some pivotal immune checkpoints in the GS-like and GU-like group. **(A)** Unsupervised clustering of 1,109 breast cancer patients according to the expression levels of 128 genomic instability-related lncRNAs. The right red cluster is the GU-like group, and the left blue cluster is the GS-like group. **(B)** Boxplots of somatic cumulative mutations in the GU-like and GS-like groups. The somatic mutation counts in the GU-like group are higher than in the GS-like group. The expression level of PDL1 **(C)**, PD1 **(D)**, CTLA4 **(E)**, IDO1 **(F)**, and TDO2 **(G)** in the GU-like group are higher than the GS-like group. Horizontal lines are median values. GS, genomically stable group; GU, genomically unstable group.

candidate lncRNAs [U62317.4, MAPT antisense RNA 1 (MAPT-AS1), AC115837.2, glutathione reductase and glutamic oxaloacetic transaminase (EGOT), Semaphorin 3B antisense RNA 1 (SEMA3B-AS1), and HOTAIR] were identified as the independent prognostic signature for breast cancer patients (Table 2). According to the coefficients and the expression of the six lncRNAs, the mutator-related lncRNA prognostic signature was defined as follows: risk score =

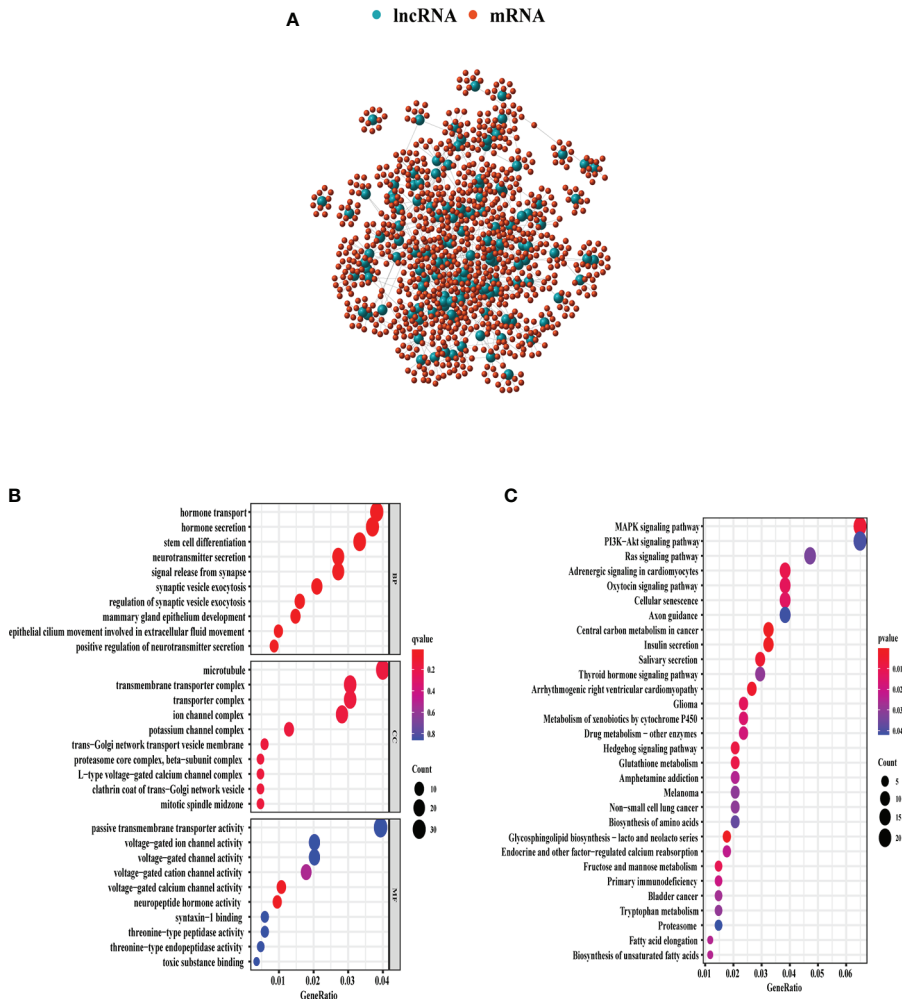

FIGURE 3
GO and KEGG enrichment analyses of 1,280 genes related with 128 lncRNAs demonstrated in the bubble plot. **(A)** The 1,280 genes related to 128 lncRNAs. **(B)** The top 10 enriched terms of BP, CC, and MF in GO analysis. **(C)** The top 30 enriched terms in KEGG analysis. The bubble size shows the count of related genes enriched under each term. GO, gene ontology; BP, biological process; CC, cellular component; MF, molecular function; KEGG, Kyoto Encyclopedia of Genes and Genomes.

(−0.6608 × expression level of U62317.4) + (−0.5443 × expression level of MAPT-AS1) + (0.0295 × expression level of AC115837.2) + (−0.2304 × expression level of EGOT) + (−0.1102 × expression level of SEMA3B-AS1) + (0.0529 × expression level of HOTAIR). The formula could evaluate the risk score and prognosis of breast cancer patients. In these lncRNAs, the coefficients of AC115837.2 and HOTAIR were positive, which showed that they were implicated in poor survival. However, the U62317.4, MAPT-AS1, EGOT, and SEMA3B-AS1 had negative coefficients associated with a good prognosis. Based on the prognostic signature consisting of the six mutator-related lncRNAs, the risk score of each patient in the training set, testing set, and the TCGA set was computed. The median risk score of the training set (1.557) was used as the cutoff value to divide the breast cancer patients in every set into high or low risk.

Validation of the 6-GI-related lncRNA prognostic model

The survival analysis in the training set (Figure 5A), testing set (Figure 5B), and TCGA set (Figure 5C) suggested that the patients with a high risk had poorer survival rates than those with a low risk (log-rank test, $p< 0.05$). The time-dependent ROC curve analysis was conducted in the training set, and the result showed that the area under the ROC curve (AUC) for 3-year and 5-year overall survival (OS) was 0.765 and 0.772,

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TABLE 1 The correlation between the two groups in demographic and clinical characteristics for breast cancer patients.

Covariates	Type	Total (n = 1,039)	Test (n = 519)	Train (n = 520)	p-value
Age	≤65	746 (71.8%)	366 (70.52%)	380 (73.08%)	0.3971
Age	>65	293 (28.2%)	153 (29.48%)	140 (26.92%)	
Gender	Female	1027 (98.85%)	515 (99.23%)	512 (98.46%)	0.3855
Gender	Male	12 (1.15%)	4 (0.77%)	8 (1.54%)	
Stage	Stage I–II	767 (73.82%)	374 (72.06%)	393 (75.58%)	0.2091
Stage	Stage III–IV	250 (24.06%)	134 (25.82%)	116 (22.31%)	
Stage	Unknown	22 (2.12%)	11 (2.12%)	11 (2.12%)	
T	T1–2	871 (83.83%)	425 (81.89%)	446 (85.77%)	0.0895
T	T3–4	165 (15.88%)	93 (17.92%)	72 (13.85%)	
T	Unknown	3 (0.29%)	1 (0.19%)	2 (0.38%)	
M	M0	862 (82.96%)	430 (82.85%)	432 (83.08%)	0.3972
M	M1	21 (2.02%)	8 (1.54%)	13 (2.5%)	
M	Unknown	156 (15.01%)	81 (15.61%)	75 (14.42%)	
N	N0	485 (46.68%)	237 (45.66%)	248 (47.69%)	0.6118
N	N1–3	537 (51.68%)	272 (52.41%)	265 (50.96%)	
N	Unknown	17 (1.64%)	10 (1.93%)	7 (1.35%)	

Chi-square test was used.

respectively (Figure 5D), showing a high sensitivity and specificity of the GI-related lncRNA prognostic signature. Furthermore, the AUC for 3-year OS in the testing set was 0.653 and that for 5-year OS was 0.674 (Figure 5E), while the AUC for 3-year OS in the TCGA set was 0.711 and was 0.723 for 5-year OS (Figure 5F). On the basis of the risk score, we stratified the patients in the training set (Figure 6A), testing set (Figure 6B), and TCGA set (Figure 6C), and demonstrated the expression levels of the six GI-related lncRNAs and the somatic mutation counts. With the risk score increasing, the expression levels of AC115837.2 and HOTAIR were upregulated, while U62317.4, MAPT-AS1, EGOT, and SEMA3B-AS1 were downregulated.

We found that MAPT-AS1, EGOT, SEMA3B-AS1, and HOTAIR in the six GI-related lncRNAs were covered by GEPIA. The survival prediction of these lncRNAs was performed separately. The results indicated that the high MAPT-AS1 expression was significantly associated with a longer OS (log-rank test, $p< 0.001$, Figure 7A), and so were EGOT (log-rank test, $p< 0.001$, Figure 7B) and SEMA3B-AS1

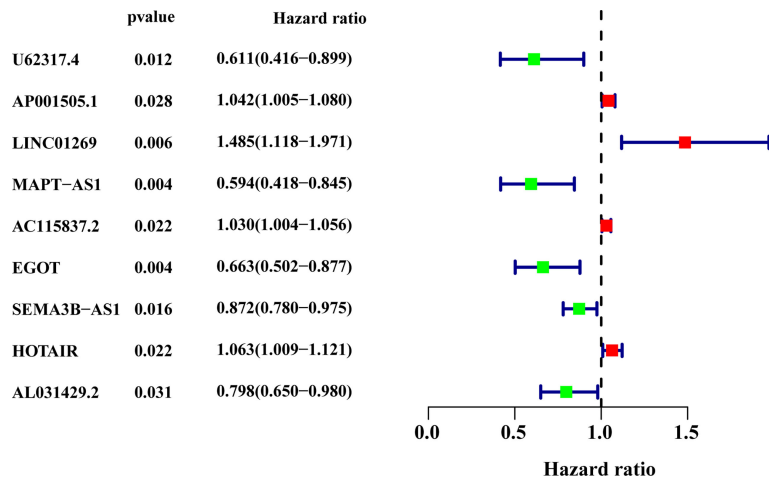

FIGURE 4
The forest plot of the nine lncRNAs generated from the univariate Cox regression analysis (criteria: p-value < 0.05). HR, hazard ratio; CI, confidence interval.

TABLE 2 The expression of the six lncRNAs generated from the multivariate Cox regression analysis.

Ensemble ID	Gene symbol	Strand	Genomic location	Coefficient	HR	95% CI	*p*-value
ENSG00000273272	U62317.4	+	chr22: 50,541,414-50,543,013	−0.660753711	0.516	0.335–0.796	0.003
ENSG00000264589	MAPT-AS1	–	chr17: 45,799,390-45,895,630	−0.544291478	0.580	0.395–0.852	0.005
ENSG00000235947	EGOT	–	chr3: 4,749,192-4,751,590	−0.230389874	0.794	0.618–1.021	0.073
ENSG00000232352	SEMA3B-AS1	–	chr3: 50,266,641-50,267,371	−0.110195358	0.896	0.801–1.002	0.054
ENSG00000228630	HOTAIR	–	chr12: 53,962,308-53,974,956	0.052897129	1.054	1.001–1.111	0.048
ENSG00000254080	AC115837.2	–	chr8: 74,609,698-74,633,320	0.029465848	1.030	1.006–1.054	0.013

CI, confidence interval; HR, hazard ratio; lncRNAs, long non-coding RNAs.

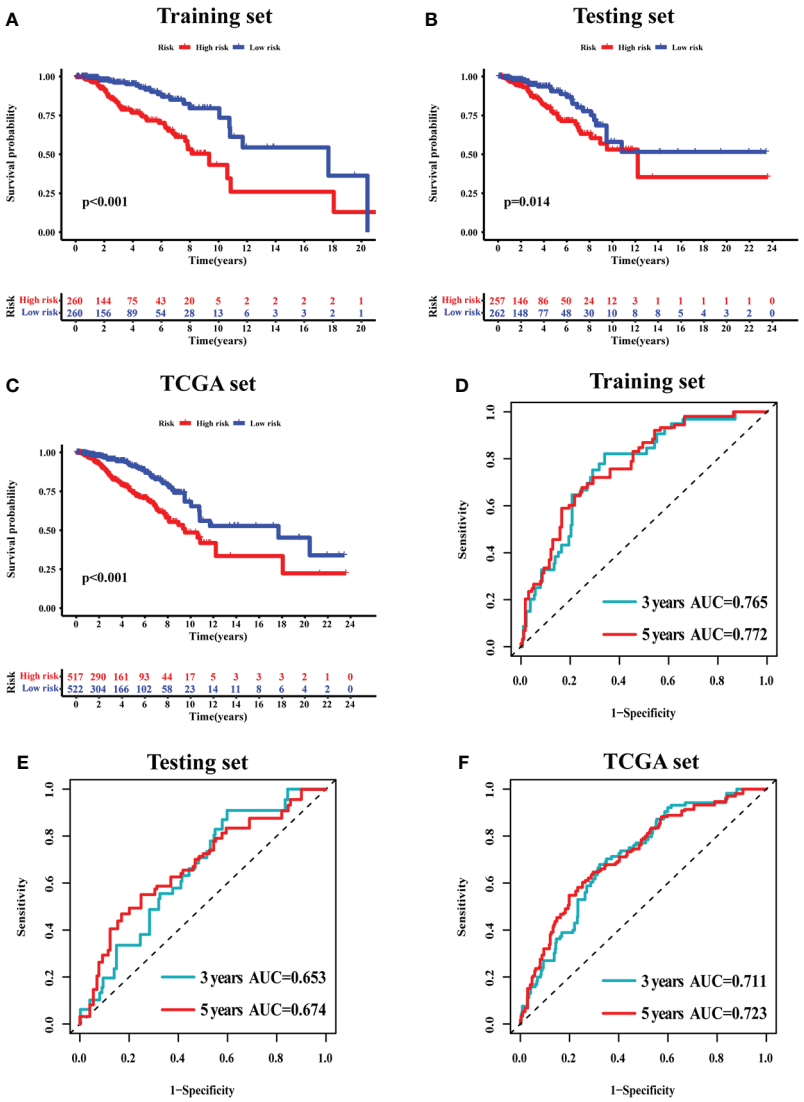

FIGURE 5
The prognostic value of the 6-GI-related lncRNA prognostic model in breast cancer patients. Overall survival was estimated by Kaplan–Meier for patients with a low or high risk predicted by the lncRNA-related model in the training set **(A)**, testing set **(B)**, and TCGA set **(C)**. Time-dependent ROC curves analysis of the lncRNA-related model was performed for 3-year and 5-year overall survival in the training set **(D)**, testing set **(E)**, and TCGA set **(F)**. AUC, area under ROC curve; ROC, receiver operating characteristic; TCGA, The Cancer Genome Atlas.

(log-rank test, $p < 0.001$, Figure 7C). However, high HOTAIR expression showed no significant association with a poorer OS (log-rank test, $p = 0.09$, Figure 7D).

In addition, MAPT-AS1, EGOT, SEMA3B-AS1, and HOTAIR were further verified in normal mammary epithelial cells (HBL100) as well as five human breast cancer cell lines by real-time PCR. The results indicated that MAPT-AS1, EGOT, and SEMA3B-AS1 were downregulated while the expression of HOTAIR was increased in breast cancer cell lines, which were consistent with the predicted results (Figure 8).

Landscape profile of somatic gene mutations

We obtained the somatic mutation profiles of 467 patients in the high-risk group and 459 patients in the low-risk group in the TCGA database. Most of the breast cancer patients had somatic mutations, with 85.87% (401/467) and 83.01% (381/459) in the high-risk group and low-risk group, respectively. The waterfall plot demonstrated the top 20 mutated genes in the patients in the high-risk group (Supplementary Figure 1A) and low-risk group (Supplementary Figure 1B). We found that the most frequently mutated gene in the high-risk group was TP53 (50%), while that in the low-risk group was PIK3CA (37%). In most cases, there was more mutability for each gene in the high-risk group. Furthermore, the most frequent gene alteration type was missense mutation.

Independent prognostic value of the 6-GI-related-lncRNA signature

The independence of the 6-GI-related-lncRNA signature from other clinical characteristics, including age, gender, and stage, was investigated by adopting univariate and multivariable Cox proportional hazards regression analysis. The risk score was significantly associated with OS and could be regarded as an independent prognostic predictor in each patient set ($p < 0.01$, Table 3). Besides risk score, both age and stage were independent factors and significantly associated with OS. To further investigate whether the prognostic model had a broad sphere of application, we sorted the patients according to their clinical features and observed the survival difference between the patients in the high- and low-risk groups. All the patients were divided into older patients with age > 65 and younger patients with age ≤ 65, female and male, and patients with stage I–II and patients with stage III–IV. In each group, patients were further stratified into high or low risk according to the median risk score. Our results suggested that patients with age > 65 and a high-risk score tended to have a poorer OS (log-rank test $p = 0.012$; Supplementary Figure 2A), and so were patients with age ≤ 65 and a high-risk score (log-rank test, $p < 0.01$; Supplementary Figure 2B). There was a significant association between the low-risk score and better OS in female patients (log-rank test $p < 0.001$; Supplementary Figure 2C), which was not observed in male patients (log-rank test $p = 0.102$; Supplementary Figure 2D). In patients with stage I–II (log-rank test $p < 0.001$; Supplementary Figure 2E) and stage III–IV (log-rank test $p = 0.042$; Supplementary Figure 2F), the higher-risk score predicted

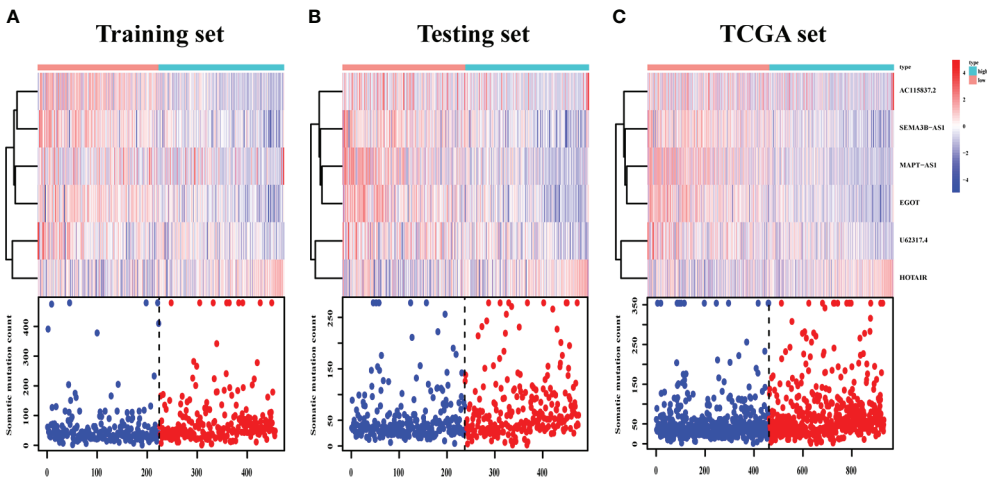

FIGURE 6
LncRNA expression and IDO1/TDO2 expression difference between patients with a high and low risk. LncRNA expression patterns and the distribution of somatic mutation counts in the training set **(A)**, testing set **(B)**, and TCGA set **(C)** with increasing risk score. TCGA, The Cancer Genome Atlas.

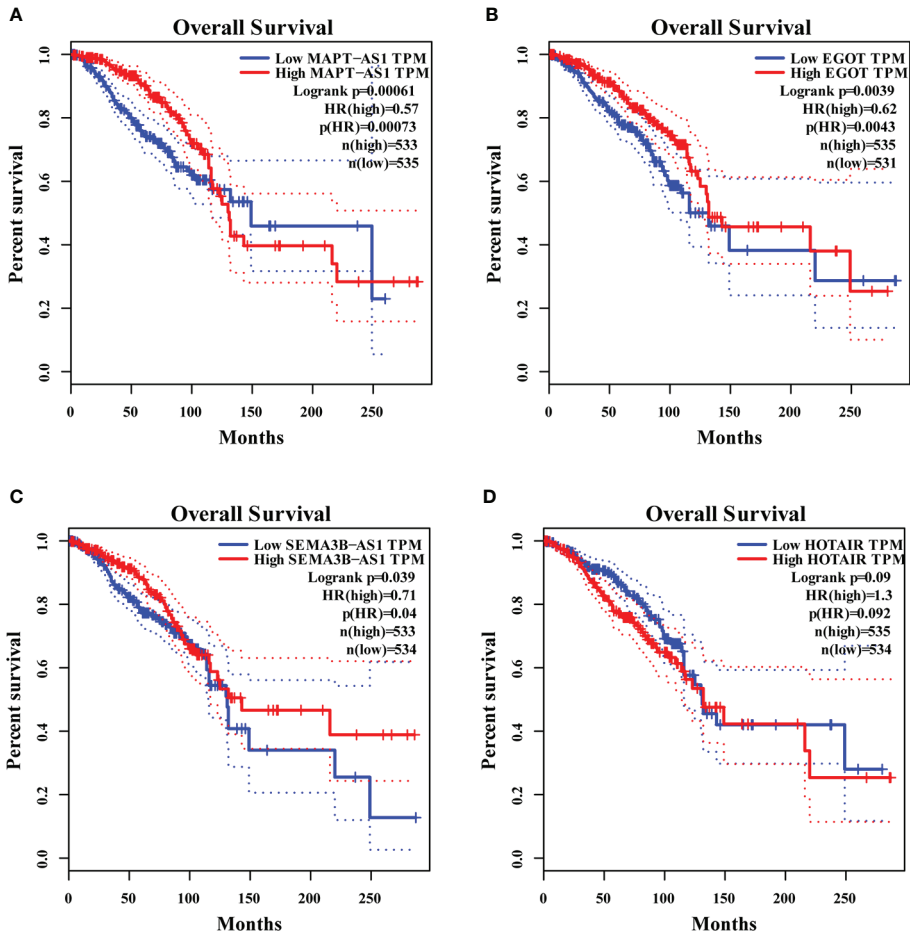

FIGURE 7
The overall survival analyses of MAPT-AS1, EGOT, SEMA3B-AS1, and HOTAIR on the online web, GEPIA. The high expression of MAPT-AS1 **(A)**, EGOT **(B)**, and SEMA3B-AS1 **(C)** predicts favorable survival, while the low HOTAIR **(D)** indicates favorable survival. GEPIA, gene expression profiling interactive analysis; TPM, transcripts per million.

poorer OS. Moreover, our results showed that the prognostic model could be adapted in patients with T, N, and M stages (Supplementary Figure 3).

Analysis and comparison of the 6-GI-related lncRNA signature with other prognostic models in breast cancer

The effect of survival prediction was compared between our six-lncRNA signature (from now on referred to as JiaolncSig) and two other prognostic lncRNA models, the immune-related (referred to as LiulncSig) (26) and stemness-related signature (referred to as LilncSig) (27) in the same TCGA database with breast cancer patients. The AUC of JiaolncSig for 3-year OS was 0.711, while the AUC of LilncSig was 0.708 and 0.608 for LiulncSig (Supplementary Figure 4A). As for the AUC for 5-year OS, JiaolncSig (0.723) was also superior to the other two models (Supplementary Figure 4B).

Moreover, the JiaolncSig only included 6 lncRNAs, which was fewer than LilncSig (12 lncRNAs) and LiulncSig (7 lncRNAs). These results suggested our signature to be a better lncRNA-related prognostic model than the other two existing lncRNA signatures in breast cancer with more potential in clinical applications.

Association between the 6-GI-related lncRNA signature and the immune checkpoints in breast cancer

The GU-like and GS-like groups had distinct immune checkpoint expression levels, including CTLA4, IDO1, and TDO2. We next analyzed the association of the risk score with the expression of some immune checkpoint molecules in breast

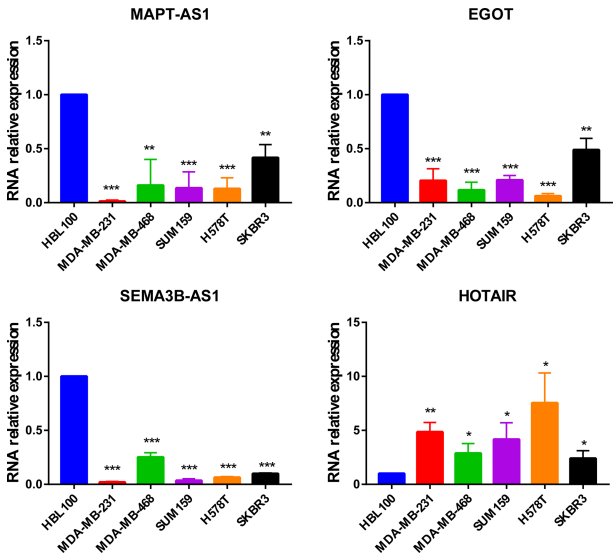

FIGURE 8
The expression of MAPT-AS1, EGOT, SEMA3B-AS1, and HOTAIR in human normal mammary epithelial cells and breast cancer cell lines. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

cancer. As shown in Figure 9A, the higher risk score was significantly associated with higher expression of some negative immune checkpoint molecules in breast cancer patients, including CTLA4, CD276, TIGIT, PVR, HMGB1, TDO2, IDO1, CXCL9, and CXCL10. However, there was no link between the risk score and PDCD1 (PD-1) or CD274 (PD-L1) expression. As shown in Figure 9B, the lower-risk score was positively associated with the expression of positive immune checkpoint molecules, including tumor necrosis factor receptor superfamily (TNFRSF) 9, TNFRSF14, and TNFRSF18. Multiple GSEAs indicated that the group with a high-risk score was enriched in DNA replication (NES = 1.893, $p< 0.01$), cell cycle (NES = 2.077, $p< 0.001$), pathways in cancer (NES = 1.631, $p< 0.05$), and tryptophan metabolism (NES = 1.560, $p< 0.05$) (Figure 9C).

Discussion

Breast cancer pathogenesis partly originated from GI. Anti-HER2 therapy has improved the survival rate for patients with HER2 amplification (4, 28). Although the improvement of early detection and treatment has decreased the death rate for breast

TABLE 3 Univariate and multivariate Cox regression analyses in the training, test, and TCGA sets.

Group	Variables	Univariable analysis			Multivariable analysis		
		HR	95% CI of HR	*p*-value	HR	95% CI of HR	*p*-value
Training set (n = 520)	Age	1.035	1.016–1.055	<0.001	1.042	1.021–1.064	<0.001
	Gender	1.173	0.162–8.479	0.874			
	Stage	2.068	1.539–2.779	<0.001	2.025	1.505–2.724	<0.001
	Risk Score	1.262	1.178–1.352	<0.001	1.206	1.124–1.293	<0.001
Testing set (n = 519)	Age	1.033	1.014–1.054	0.001	1.030	1.010–1.050	<0.001
	Gender	0.000	0–inf	0.996			
	Stage	2.255	1.581–3.217	<0.001	2.122	1.500–3.003	<0.001
	Risk Score	1.092	1.024–1.164	0.007	1.086	1.014–1.163	0.018
All patient set (n = 1,039)	Age	1.035	1.021–1.049	<0.001	1.036	1.022–1.050	<0.001
	Gender	0.852	0.119–6.104	<0.001			
	Stage	2.189	1.742–2.751	<0.001	2.142	1.717–2.673	<0.001
	Risk Score	1.128	1.086–1.173	<0.001	1.118	1.072–1.165	<0.001

CI, confidence interval; HR, hazard ratio.

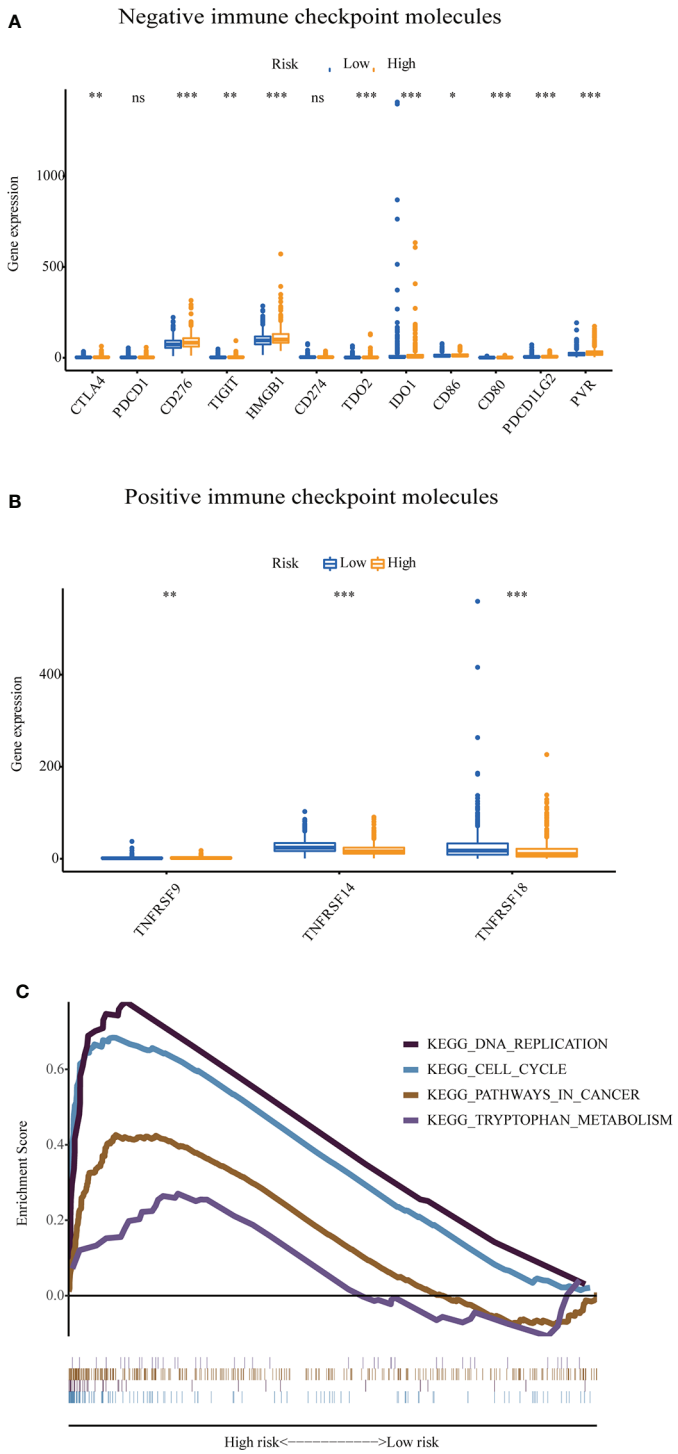

FIGURE 9
The expression level of some immune checkpoint molecules and representative transcriptome traits of biological function between the patients in the high-risk group and low-risk group. **(A)** The negative immune checkpoint molecules. **(B)** The positive immune checkpoint molecules. **(C)** Representative transcriptome traits of biological function in multiple GSEAs of patients in the high-risk group and low-risk group.GSEA, gene set enrichment analysis. *p < 0.05, **p < 0.01, ***p < 0.001, ns, no significance.

cancer patients during the past decades, almost all metastatic patients eventually succumb to death (29). Currently, single-cell approaches and high-throughput multicellular sequencing technologies can detect genetic alteration for cancer patients (30), but the degree of GI still needs to be explored. NORAD has been proven to be indispensable for keeping GI (14, 15), indicating a close association of lncRNA and GI.

This study determined 128 GI-related lncRNAs using somatic mutation and lncRNA expression data of breast cancer patients. After analyzing the co-expressed genes, we found that the expression levels of some negative immune checkpoints, including CTLA4, IDO1, and TDO2, were closely associated with these lncRNAs. Furthermore, the functional analysis suggested that the 1,280 co-expressed genes were mainly enriched in the MAPK signaling pathway. It has been reported that the MAPK pathway participates in regulating cell differentiation, proliferation, survival, and death and is considered as the most frequently mutated pathway in cancer patients (31). Using univariate and multivariable Cox proportional hazards regression analysis, we generated the prognostic model of six lncRNAs, including U62317.4, MAPT-AS1, AC115837.2, EGOT, SEMA3B-AS1, and HOTAIR. The model was proved to predict survival independently from other clinical features, including gender, age, and stage.

According to the coefficient of each lncRNA, we found that HOTAIR and AC115837.2 increased the risk score of a breast cancer patient, while U62317.4, EGOT, MAPT-AS1, and SEMA3B-AS1 tended to decrease the score. The survival analysis in GEPIA demonstrated that high expression of EGOT, MAPT-AS1, and SEMA3B-AS1 predicted favorable OS, which was consistent with their coefficients. Xu et al. reported that low level of EGOT expression was associated with poor OS (32). A previous study showed that HORAIR was overexpressed in primary and metastatic breast cancer patients, which could predict the possibility of metastasis and death (31). In contrast to the lncRNAs above, the function of AC115837.2 remains not clear yet. For U62317.4, it was suggested to be an autophagy-related lncRNA and was included in prognosis-related risk models in breast cancer and bladder cancer (33, 34). Qiu et al. demonstrated that EGOT was lowly expressed in cell lines and breast cancer tissues and may suppress cell migration and viability (35). MAPT-AS1 exists at the anti-sense strand of the MAPT promoter region. Pan et al. indicated that reducing the expression of MAPT-AS1 restrained the migration and proliferation of ER^- breast cancer cells (36). It has been reported that SEMA3B-AS1 was deemed as a novel cancer suppressor in gastric cardia adenocarcinoma, esophageal squamous cell carcinoma, and hepatocellular carcinoma (37–39). Moreover, Li et al. suggested that SEMA3B-AS1could be used as part of the stemness-associated lncRNA prognostic signature in breast cancer (27).

According to the prognostic model in this study, the breast cancer patients in the training set could be divided into two groups with high or low risk, indicating an utterly different OS and somatic mutation level. Moreover, the result has been validated on the independent testing set and the TCGA set. Most importantly, the prognostic model could be applied on breast cancer patients with any age and pathologic stages. However, there was a significant association between the low-risk score and favorable OS in female patients rather than male patients, probably due to the insufficient number of male patients. The ROC area of OS showed that our prognostic model was superior to the other existing two models in breast cancer. Four lncRNAs in our model were covered in GEPIA and survival analysis showed that they were closely related with OS. These validation results demonstrated that our prognostic model may predict prognosis of breast cancer. Additionally, drugs that target certain aberrantly expressed genes or non-coding RNAs show a more potent anticancer efficiency and lower toxicity than conventional chemotherapies (40). Thus, the six lncRNAs may serve as potential therapeutic targets.

Nowadays, cancer immunology and immunotherapy provide a novel perspective for cancer therapeutics (41). Cancer immune escape mechanism is considered a potential target in cancer immunotherapy (42, 43). Therefore, we analyzed some immune checkpoint molecules between the high- and low-risk groups. The result implied that the patients with a high risk had higher expression of negative immune checkpoints, such as CTLA4, CD80, CD86, IDO1, and TDO2. CTLA-4 on T cells binds to B7 molecules (CD80 and CD86) on the antigen-presenting cells, blocking co-stimulation and then terminating T-cell activation (44–46). Tryptophan catabolism has a pivotal role in forming immune evasion and immune tolerance (47). Both IDO1 and TDO2 are rate-limiting enzymes in tryptophan degradation. With ICIs becoming a powerful new strategy for cancer therapy, it is necessary to identify patients who are suitable for ICIs to avoid severe immune-related adverse effects (irAEs), especially autoimmune diseases. A systematic review reported that irAEs could occur in any organ and impact 89% of patients treated with CTLA-4 inhibitors and 74% of those receiving PD-1/PD-L1 inhibitors (48). Thereinto, ICI-induced endocrinopathies are the most common irAEs, which are presumed to result in permanent, irreversible endocrine dysfunction (49). Though transient inflammation affecting most systems resolves with steroid therapy and is followed by restoration of normal organ function, administering ICIs to patients should still be deliberately considered. This model may predict ICI efficacy, facilitating the identification of patients who are responsive to ICIs precisely. Therefore, patients potentially benefiting from ICIs can be screened out and needless irAEs are avoided as the unresponsive population has been excluded.

Furthermore, the multiple GSEA results suggested that the breast cancer patients with a high risk were mainly associated with genes involved in DNA replication, cell cycle, pathways in cancer, and tryptophan metabolism. This suggested that

there tends to be a rapid progression in breast cancer patients with a high risk. Meanwhile, the patients with a low risk had more positive immune checkpoints, such as TNFRSF9, TNFRSF14, and TNFRSF18. The proliferation of antigen-primed $CD8^+$ T cells could be stimulated by the interaction between the tumor necrosis factor ligand and cognate TNFRSF, which is beneficial for protective immunity and cancer immunotherapy (50).

Conclusion

To sum up, we constructed a GI-related prognostic risk model comprising six lncRNAs (U62317.4, MAPT-AS1, AC115837.2, EGOT, SEMA3B-AS1, and HOTAIR) in breast cancer. This model may have improved predictive value compared to other existing models and provide novel therapeutic opportunities for breast cancer patients.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding author.

Author contributions

YJ collected data, prepared the figures, and drafted the manuscript. SL prepared the figures, organized the structure, and checked the manuscript. XW conducted the real-time PCR assay. LZ, MY, HW, SR, and KZ participated in the discussion. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2022.929846/full#supplementary-material

SUPPLEMENTARY FIGURE 1
Mutation landscape of breast cancer patients with a high **(A)** or low **(B)** risk. Del, deletion; Ins, insertion; OS, overall survival.

SUPPLEMENTARY FIGURE 2
The prognostic value of 6-GI-related lncRNA prognostic model in breast cancer patients with distinct clinical features. **(A)** age > 65; **(B)** age ≤ 65; **(C)** female; **(D)** male; **(E)** stage I-II; **(F)** stage III-IV.

SUPPLEMENTARY FIGURE 3
The prognostic value of the lncRNA-related model in breast cancer patients with different T, N, or M stages. **(A)** T1-2, **(B)** T3-4, **(C)** N0, **(D)** N1-3, **(E)** M0, and **(F)** M1.

SUPPLEMENTARY FIGURE 4
The ROC analyses for 3-year **(A)** and 5-year **(B)** overall survival for the JiaolncSig, LilncSig, and LiulncRNA.

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Glossary

AUC	area under ROC curve
CI	confidence interval
CIBERSORT	Cell-type Identification by Estimating Relative Subsets of RNA Transcripts
CTLA-4	cytotoxic T-lymphocyte-associated protein 4
EGOT	glutathione reductase and glutamic oxaloacetic transaminase
ER	estrogen receptor
dMMR	deficient mismatch repair
FDR	false discovery rate
GEPIA	gene expression profiling interactive analysis
GI	genomic instability
GO	gene ontology
GS	genomically stable group
GSEA	gene set enrichment analysis
GU	genomically unstable
HER2	human epidermal growth factor receptor 2
HR	hazard ratio
HOTAIR	HOX transcript antisense RNA
ICI	immune checkpoint inhibitor
IDO1	indoleamine 2, 3-dioxygenase 1
KEGG	Kyoto Encyclopedia of Genes and Genomes
lncRNA	long non-coding RNA
MAPT-AS1	MAPT antisense RNA 1
MSI-H	microsatellite instability-high
NORAD	non-coding RNA activated by DNA damage
OS	overall survival
PD-1 (PDCD1)	programmed cell death 1
PD-L1	programmed cell death receptor ligand 1
ROC	receiver operating characteristic
SEMA3B-AS1	Semaphorin 3B antisense RNA 1
TCGA	the cancer genome atlas
TDO2	tryptophan 2,3-dioxygenase 2
TNFRSF	tumor necrosis factor receptor superfamily.

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The role of PD-1/PD-L1 and application of immune-checkpoint inhibitors in human cancers

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Programmed cell death protein-1 (PD-1) is a checkpoint receptor expressed on the surface of various immune cells. PD-L1, the natural receptor for PD-1, is mainly expressed in tumor cells. Studies have indicated that PD-1 and PD-L1 are closely associated with the progression of human cancers and are promising biomarkers for cancer therapy. Moreover, the interaction of PD-1 and PD-L1 is one of the important mechanism by which human tumors generate immune escape. This article provides a review on the role of PD-L1/PD-1, mechanisms of immune response and resistance, as well as immune-related adverse events in the treatment of anti-PD-1/PD-L1 immunotherapy in human cancers. Moreover, we summarized a large number of clinical trials to successfully reveal that PD-1/PD-L1 Immune-checkpoint inhibitors have manifested promising therapeutic effects, which have been evaluated from different perspectives, including overall survival, objective effective rate and medium progression-free survival. Finally, we pointed out the current problems faced by PD-1/PD-L1 Immune-checkpoint inhibitors and its future prospects. Although PD-1/PD-L1 immune checkpoint inhibitors have been widely used in the treatment of human cancers, tough challenges still remain. Combination therapy and predictive models based on integrated biomarker determination theory may be the future directions for the application of PD-1/PD-L1 Immune-checkpoint inhibitors in treating human cancers.

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KEYWORDS

PD-1/PD-L1, immunecheckpoint inhibitor, clinical application, biomarker, human cancers

Introduction

PD-1 is a representative immunosuppressive checkpoint and mainly expressed in macrophages, B lymphocytes, dendritic cells (DCs), monocytes, tumor-specific activated T cells, myeloid cells and natural killer (NK) cells under conditions of chronic antigen exposure (1–3). PD-L1 is one of the PD-1 ligands. PD-L1 expression has been shown to be a valuable biomarker for the prognosis and prediction of the sensitivity of PD-1/PD-L1 inhibitors. The expression of PD-L1 is mainly expressed in tumor cells, tumor-infiltrating cells and antigen-presenting cells (APCs) in many cancers (1, 4). In recent years, a number of studies have confirmed the clinical significance of PD-1/PD-L1 antibodies and their prognostic impact on human cancers (5, 6). However, the relationship between this biomarker and its clinical significance is imperfect and varies in different types of human cancers (7).

In general, PD-1/PD-L1 inhibitory checkpoints suppress T cell receptor-mediated cytotoxicity and $CD8^+$ T cell proliferation by interacting with the ligand PD-L1, thus avoiding the killing effect of the autoimmune system on tumor cells and immune surveillance (8–10). Immune checkpoint antibodies as promising cancer therapeutic strategies are based on their natural role acting as T cell-activated co-inhibitory receptors. Undoubtedly, the co-stimulatory and co-inhibitory receptors of T cells play an important role in the treatment of PD-1/PD-L1 immune checkpoint inhibitors (11). Expression of PD-L1 in tumor cells or tumor-associated stromal cells is a potential predictive marker for response and outcome of anti-PD-1/PD-L1 immunotherapy (1, 12).

Despite the remarkable efficacy of PD-1/PD-L1 immunocheckpoint inhibitors in the treatment of tumors, some problems also remain, such as drug resistance and adverse events. The presence of drug resistance significantly reduces the efficacy of anti-PD-1/PD-L1 immunotherapy. Exploring the mechanisms of PD-1/PD-L1 immunocheckpoint inhibitors resistance will contribute to the discovery of new immunotherapeutic strategies to control disease progression and provide a more sustainable survival benefit for patients (13). In addition, PD-1/PD-L1 immunocheckpoint inhibitors acting as immunomodulatory drugs can significantly enhance the natural defense of the immune system against cancers, while inevitably leading to some immune-related adverse events, the erroneous stimulation of the immune system leads to immune injuries to the normal tissues of the body (14). Therefore, in order to further improve the treatment outcome and reduce the risk of patients, it is necessary to learn more about the immune-related adverse events of PD-1/PD-L1 immunocheckpoint inhibitors in the treatment of human cancers.

In any case, we believe that tumor immunotherapy based on PD-1/PD-L1 inhibitors will become a promising strategy for human cancers. This article will focus on the role of PD-1/PD-L1 and the application of PD-1/PD-L1 inhibitors in human cancers.

Mechanisms of tumor immunotherapy based on PD-1/PD-L1

At present, the immunotherapy mechanisms of anti-PD-1/PD-L1 antibody have been relatively clear. The activation of T cells relies mainly on dual signals. The first signal consists of the binding of MHC-presenting antigen to T cell receptor (TCR). The second signal constitute by co-stimulatory and co-inhibitory signals (15). The interaction between PD-1 on T cells and PD-L1 on tumor cells or APCs can effectively inhibit T cell activation and even cause T cell apoptosis, decreased cytokine production, t-cell lysis and induction of tolerance to antigen, thus making the tumor escape from the immune surveillance (16). PD-1/PD-L1 inhibitors respectively bind to PD-1 or PD-L1 to prevent the interaction between PD-1 and PD-L1, then the recognition and killing effect of immune cells is restored and the immune escape of tumor cells is avoided (Figure 1).

PD-1 activation significantly inhibits TCR signaling, CD28 co-stimulatory signaling and inducible T cell co-stimulator(ICOS) signaling (17–19). Recent studies have suggested that after activation by its ligand PD-L1, PD-1 is phosphorylated by protein tyrosine kinase Lck to recruit tyrosine phosphatase Shp2 (Src homologous phosphatase 2), followed by dephosphorylation of TCR and CD28, and subsequent inhibition of T cell-associated signaling (20–23)(Figure 1). When PD-1/PD-L1 immune checkpoint inhibitors intervene, the intramembrane motif of PD-1 cannot be phosphorylated by lymphocyte-specific protein tyrosine kinase (LCK), resulting in the failure of cell recruitment to SHP-2. TCR and CD28 dephosphorylation is blocked, which leads to efficient delivery of activation signals to downstream proteins and signaling pathways, ultimately stimulating T cell proliferation and differentiation. Eventually, the immune function of T cells is

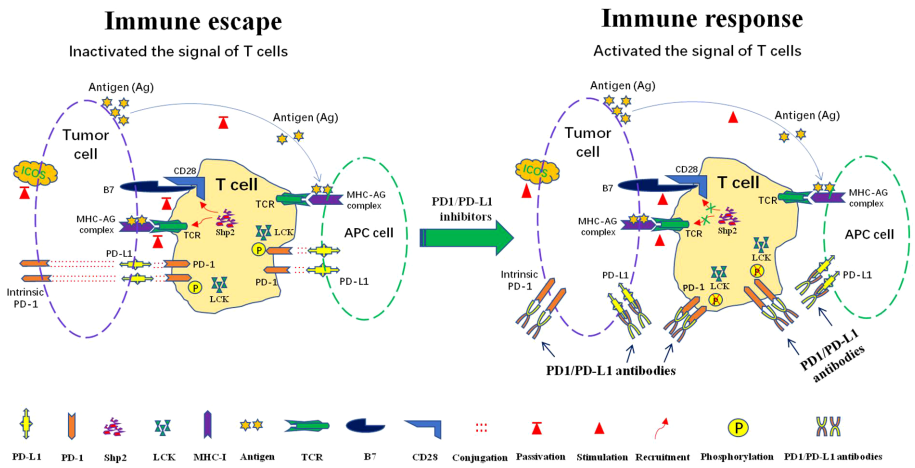

FIGURE 1
Mechanisms of the response to anti-PD1/PD-L1 immunotherapy: PD-L1 is expressed in tumor cells and antigen presenting cells (APCs). PD-1 is mainly expressed in T cells, some tumor cells also express intrinsic PD-1. Immune escape occur after interaction of PD-1 and PD-L1. PD-1 can be phosphorylated by LCK to recruit tyrosine phosphatase Shp2, consequently inactivating CD28 and T cell receptor (TCR) function and signaling pathway, thus attenuating the activation signal of T cells and causing immune escape. Lck kinase activity is required to maintain PD-1/Shp2-mediated inhibitory signaling. The intervene of PD-1/PD-L1 immunocheckpoint inhibitors can effectively block the interaction between PD-L1 and PD-1, which in turn blocks the recruitment of SHP-2 and reactivates T cells signal for immune function.

effectively performed (24) (Figure 1). Interestingly, some tumor cells also express intrinsic PD-1 to promote the occurrence and development of tumors independent of adaptive immunity. PD-1 checkpoint inhibitors can also block the binding of intrinsic PD-1 and PD-L1 to inhibit tumor growth (25, 26) (Figure 1).

Tumor cells evolve themselves to lose the ability to present tumor antigens in order to avoid recognition by cytotoxic T cells and APCs (27). Recent studies showed that major histocompatibility complex class-I and -II (MHC-I and MHC-II) were required for tumor antigen presentation and immunosurveillance (28–30). In many malignancies, downregulation of MHC-I/II is associated with immunosuppression, metastatic progression and poor prognosis, as well as predict anti-PD-1/PD-L1 therapy response (31). Researchers have attempted to find ways to upregulate MCH-II expression in tumor cells with a view to improve the response rate to PD-1/PD-L1 immunotherapy. They found that epigenetic and ERK signaling cascades were effective in suppressing the expression of intrinsic MHC II in non-small cell lung cancer (32). Therefore, the combined blocking strategy for these pathways may generate a novel positive response to PD-1/PD-L1 immune checkpoint therapy in human cancers. In addition, lung epithelial MHC-II was needed for surface expression of PD-L1 (33). The results of a clinical study showed that recurrent or metastatic nasopharyngeal carcinoma with high expression of both MHC-II and PD-L1 responded better to treatment with camrelizumab (anti-PD-1) (34). In conclusion, the above results suggest that MHC-II and PD-L1 influence each other not only in expression but also in function for the treatment of PD-1/PD-L1 Immune-checkpoint inhibitors in human cancer.

Mechanisms of PD-1/PD-L1 inhibitors resistance

In recent years, immune checkpoints blockade therapy targeting the PD-1/PD-L1 axis has pushed tumor immunotherapy to a new revolutionary-like milestone and achieved surprising therapeutic effects in a variety of malignancies. However, most patients have developed resistance to PD-1/PD-L1 inhibitors, which severely limits its application and becomes a serious clinical problem that cannot be ignored in this field. Therefore, it is urgent to deeply reveal the molecular mechanism of immune checkpoint inhibitor resistance and improve the response rate of patients to PD-1/PD-L1 immunotherapy. Herein, we have summarized the molecular mechanisms of resistance to common PD-1/PD-L1 immune checkpoint inhibitors (Figure 2).

The resistance to PD-1/PD-L1 inhibitors includes primary resistance and acquired resistance. Primary resistance is defined as patients who have never shown clinical response or stable disease when using PD-1/PD-L1 blockade. The mechanism of primary resistance includes lack of tumor immunogenicity (35), T cell exclusion (36), lack of interferon responsiveness (37), epidermal growth factor receptor (EGFR) mutations and anaplastic lymphoma kinase(ALK) rearrangements (38), local immunosuppressive factors in tumor microenvironment (39)

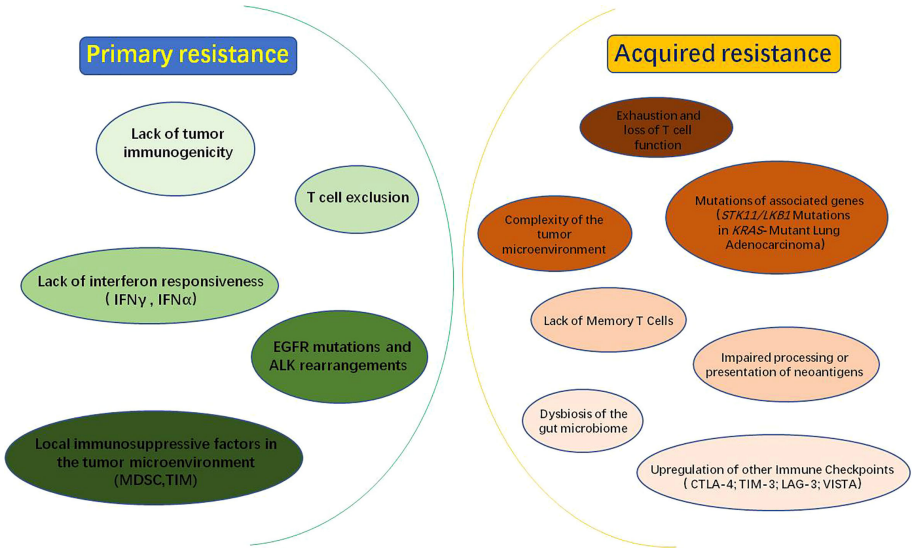

FIGURE 2
Mechanisms of PD-1/PD-L1 inhibitors resistance: PD-1/PD-L1 inhibitor resistance is divided into primary resistance and acquired resistance. Mechanisms of primary resistance include lack of tumor immunogenicity; T-cell rejection; lack of interferon responsiveness, such as IFNγ (interferon Gamma) and IFNα (interferon alpha); EGFR (epidermal growth factor receptor) mutations and ALK (anaplastic lymphoma kinase) rearrangements; local immunosuppressive factors within the tumor microenvironment, such as MDSC (myeloid-derived suppressor cell) and TIM (tumor-infiltrating myeloid cell). While, the mechanisms of acquired resistance may be related to the following factors: exhaustion and loss of T cell function; impaired processing or presentation of neoantigens; complexity of the tumor microenvironment; mutations in associated genes, such as STK11/LKB1; dysbiosis of the gut microbiome; lack of Memory T Cells and upregulation of other Immune Checkpoints, such as CTLA-4(cytotoxic T-lymphocyte antigen-4), TIM-3(T cell immunoglobulin and mucin domain-containing molecule-3), LAG-3(lymphocyte activation gene-3) and VISTA(V-domain Ig suppressor of T cell activation).

and other factors (Figure 2). While acquired drug resistance means that PD-1/PD-L1 inhibitors show a durable and effective response at the beginning of treatment, but therapeutic effect of inhibitors is significantly diminished or non-responsive after a period of treatment for some patients (40). The mechanisms may be closely associated with exhaustion and loss of T cell function (41–43), impaired processing or presentation of neoantigens (44), complexity of the tumor microenvironment (45), mutations in associated genes (46), dysbiosis of the gut microbiome (47), lack of Memory T Cells (48) and upregulation of others immune checkpoints (49) (Figure 2).

Tumor cells may interact closely with stromal cells, immune cells, other suppressive immune checkpoints and cytokines in the surrounding environment, thus protecting them from detection and elimination by immune surveillance (45). In general, T lymphocytes accomplish immune clearance of tumors by recognizing specific antigens on the surface of tumor cell membranes, thereby killing tumor cells. Therefore, effective tumor-specific antigen is an important factor for the efficacy of immune response. If the structure of the specific antigen is similar to the immune tolerance antigen or autoantigen, resulting in the inability of APCs to recognize and initiate T-cell activation, thus acquired resistance may developed (50, 51). In addition, some tumors cause a decrease of normal mature dendritic cells (DCs) and an increase in the number of immature DCs by secreting certain suppressors, such as IL-10 and VEGF. When tumors recruit these immature DCs, effector T cells are not effectively activated during antigen presentation (24, 52). These patients will fail to generate an effective immune response with PD-1/PD-L1 blockers, resulting in drug resistance and immune escape.

Main clinical trials and outcomes for PD-1/PD-L1 inhibitors in the treatment of human cancers

In recent years, a large number of clinical trials have been conducted with PD-1/PDL1 Immune-checkpoint inhibitors, and their therapeutic effects have been evaluated from different perspectives, including overall survival (OS), objective response rate (ORR) and medium progression-free survival (PFS), respectively (53–55). Herein, we reviewed some clinical trials of PD-1/PDL1 immunocheckpoint inhibitors (Table 1). We found that the same inhibitor has completely different therapeutic effects and responsiveness against different cancers (Table 1). This will provide better reference for the selection of PD1/PD-L1 inhibitors for different cancers in future clinical practice.

TABLE 1 Clinical trials of PD-1/PD-L1 inhibitors in human cancers.

Inhibitors	Style	Cancers	Trial number	N	OS	ORR	PFS	References
Pembrolizumab	PD-1	Gastric cancer	NCT02589496	61	N/A	85.7% in microsatellite instability-high mGC 100% in Epstein-Barr virus-positive mGC	N/A	(56)
		Pancreatic cancer	NCT02054806	475	N/A	0.0% to 14.2%	1.7 months 1.5 to 2.9 months)	(57)
		Small-cell lung cancer			N/A	33% (15.6% to 55.3%)	N/A	
		Thyroid cancer			N/A	N/A	6.8 months (1.9 to 14.1 months)	
		Non-Small-Cell Lung Cancer	NCT02142738	305	N/A	N/A	10.3 months	(58)
		Breast cancer	NCT02447003	84	18 months		2.1 months	(59)
		Non-small-cell lung cancer	NCT02775435	559	15.9 VS 11.3 months (pembrolizumab-combination group VS placebo-combination group)	N/A	(6.4 VS 4.8 months pembrolizumab-combination group VS placebo-combination group)	(60)
		Gastric cancer	NCT01848834	39	N/A	22%	N/A	(61)
		Non-small-cell lung cancer	NCT01295827	495	12 months	19.40%	3.7 months	(62)
		Melanoma	NCT01295827	655	N/A	8%, 12%, 22%, 43%, 57%, and 53% for MEL scale of 0, 1. 2, 3, 4 and 5	N/A	(1)
		Hepatocellular carcinoma	NCT02702414	104	N/A	17%	N/A	(63)
		Malignant pleural mesothelioma	NCT02054806	25	N/A	20%	N/A	(64)
Nivolumab	PD-1	Advanced hepatocellular carcinoma	NCT01658878	262	N/A	20%	N/A	(63)
		Hodgkin Lymphoma	NCT02181738	80	N/A	66.30%	N/A	(65)
		ovarian cancer	NCT02873962	38	N/A	40% in platinum-sensitive and 16.7% in platinum-resistant participants	8.1 months	(66)
		Follicular lymphoma	NCT01592370	10		40%	N/A	(67)
		Diffuse large B- cell lymphoma		11	N/A	36%	N/A	
		Peripheral T-cell lymphoma		5	N/A	40%	N/A	
		Melanoma	NCT01844505	945	N/A	N/A	11.5 VS 2.9 months (nivolumab plus ipilimumab group VS ipilimumab alone group)	(68)
Atezolizumab PD-L1	PD-1	Triple-Negative Breast Cancer	NCT02425891	451	21.3 VS 17.6 months (atezolizumab plus nab-paclitaxel group VS placebo plus nab- paclitaxel group)	N/A	7.2 VS 5.5 months (atezolizumab plus nab-paclitaxel group VS placebo plus nab-paclitaxel group)	(69)
			NCT02008227	1225		N/A	N/A	(70)

(Continued)

TABLE 1 Continued

Inhibitors	Style	Cancers	Trial number	N	OS	ORR	PFS	References
		Non-small-cell lung cancer			15.7VS 10.3 months (atezolizumab group VS docetaxel group)			
Toripalimab	PD-1	Alveolar soft part sarcoma	NCT02836834	12	34.7 months	22.70%	5.7 months	(71)
		Lymphoma		11	N/A	90.90%	8.3 months	
		Non-Small Cell Lung Cancer	NCT03301688	41	13. 8 months among 28 patients included in the response and survival analysis	N/A	2.8 months among 28 patients included in the response and survival analysis	(72)
Durvalumab PD-L1		Head and neck squamous cell carcinoma	NCT02207530	112	7.1 months	N/A	2. 1 months	(12)
		Non-Small Cell Lung Cancer		406	13.- months; 3.4 VS16.2 months (HPD VS Non-HPD)	18.90%	2.1 months	(73)
Avelumab	PD-L1	Metastatic breast cancer	NCT01772004	168	N/A	3.0% overall 5.2% in patients with TNBC	N/A	(74)
Tislelizumab	PD-1	Hodgkin lymphoma	NCT03209973	70	N/A	87.10%	74.5% (9-month progression-free survival rate)	(75)
Camrelizumab	PD-1	Hodgkin lymphoma	NCT03155425	75	N/A	76.00%	N/A	(76)
GLS-010	PD-1	Hodgkin lymphoma	NCT03713905	24	N/A	87.50%	N/A	(77)
		Peripheral NK T lymphoma		14	N/A	21.4%	N/A	

N/A, Not Applicable.

Immune-related adverse events caused by PD-1/PD-L1 inhibitors in the treatment of human cancers

Over the past few decades, cancer immunotherapies represented by PD-1/PD-L1 immune checkpoint inhibitors have changed the landscape of cancer treatment. However, this has also inevitably led to some immune-related adverse events (irAEs), and these irAEs are usually characterized by long duration and delayed onset (58, 78–86). In this study, we have reviewed some of the common immune-related adverse events associated with PD-1/PD-L1 immune checkpoint inhibitors for cancer treatment (Table 2).

PD-1/PD-L1 and inhibitors in human solid cancers

Lung cancer

Lung cancer is the most common cancer and the leading cause of cancer death worldwide. Fortunately, the advent of immune checkpoint inhibitors has improved the outlook for patients with advanced lung cancers. Tumor immunotherapy targeting PD-1/PD-L1 have revolutionized the treatment of lung cancer (70, 72, 90).

Elevated PD-L1 expression correlates with higher efficacy of immunotherapy, implying that PD-L1 has high predictive value as a cancer biomarker (62, 91). The anticancer efficacy of PD-L1 inhibitors is significantly better than that of chemotherapy in advanced non-small cell lung cancer (NSCLC) patients with high PD-L1 expression (58),as well as in patients with previously untreated metastatic squamous NSCLC (60). Remarkably, PD-L1 expression may also be induced by chemotherapy or targeted therapies (92). Therefore, if PD-L1 protein expression is to be used as a biomarker to guide immunotherapy, fresh specimens may need to be collected after other treatments and before the start of immunotherapy to assess PD-L1 expression. Moreover, tumor microenvironment plays an important role in the anticancer effect of PD-1/PD-L1 immunocheckpoint inhibitors. Recent studies have shown that low-dose apatinib (VEGFR2-TKI) significantly improves the therapeutic effect of PD-1/PD-L1 inhibitors by modulating the tumor microenvironment, delaying tumor growth, reducing the number of metastases and prolonging survival in mouse models (93).

TABLE 2 Immune-related adverse events caused by PD-1/PD-L1 inhibitors in human cancers.

Inhibitors	Styles	Cancers	Total number of patients (N)	Immune-related adverse events (Number of events/total number of patients, n/N)					References
				Rash	Hypothyroidism	Elevated AST	Colitis	Pneumonitis	
Nivolumab	PD-1	Squamous-CellCarcinoma	236	18/236	9/236	2/236	N/A	5/236	(80)
		Hepatocellularcarcinoma	48	11/48	N/A	10/48	N/A	N/A	(63)
		Ovarian Cancer	38	4/38	N/A	10/38	N/A	4/38	(66)
		Diffuse Large B Cell lymphoma	121	6/121	N/A	N/A	N/A	N/A	(87)
		Hodgkin lymphoma	80	1/80	N/A	2/80	N/A	1/80	(65)
Pembrolizumab	PD-1	NSCLC	1034	29/1034	28/1034	10/1034	N/ A	16/1034	(81)
		NSCLC	154	6/154	14/154	N/A	3/154	9/154	(58)
		Gastric cancer	39	N/A	4/39	N/A	N/A	1/39	(61)
		Hepatocellularcarcinoma	104	10/104	6/104	7/104	N/A	1/104	(88)
		Advanced urothelial cancer	266	23/266	19/266	N/A	N/A	N/A	(82)
Atezolizumab	PD-L1	NSCLC	144	N/A	N/A	6/144	2/144	4/144	(83)
		Hepatocellularcarcinoma	58	6/58	N/A	6/58	N/A	N/A	(84)
		NSCLC	609	N/A	N/A	N/A	2/609	6/609	(70)
		Urothelial carcinoma	119	6/119	8/119	4/119	N/A	N/A	(85)
Tislelizumab	PD-1	Solid tumors	451	61/451	N/A	23/451	6/451	13/451	(86)
Toripalimab	PD-1	NSCLC	41	6/41	3/41	5/41	N/A	1/41	(72)
		Gastric Cancer	58	5/58	7/58	7/58	N/A	N/A	(89)

N/A, Not Applicable.

In patients with lung cancer, PD-1 and PD-L1 can be detected not only in tissues but also in the serum and plasma of patients (94–96). The clinical diagnosis and prevention of soluble PD-1 and PD-L1 lung cancer in blood is of great importance because blood samples are easily available and easily detectable (96). By detecting the expression levels of PD-1 and PD-L1 in the blood of lung cancer patients, the drug treatment regimen of PD-1 and PD-L1 can be formulated based on the test results, and the patients' response to immunotherapy can be further assessed. The purpose of individualized treatment of lung cancer is absolutely achieved.

Unfortunately, although many patients have achieved long-term survival benefits with PD-1/PD-L1 inhibitors, some patients have experienced rapid tumor progression after immunotherapy, known as hyperprogressive disease (HPD) (97, 98). In pretreated NSCLC patients, HPD is more common with PD-1/PD-L1 inhibitors compared to chemotherapy, and patients treated with PD-1/PD-L1 inhibitors are also associated with a high metastatic burden and poor prognosis (73). Currently, the combination of PD-1/PD-L1 immune checkpoint inhibitors with other antitumor agents has become an important treatment strategy. For example, the combination of pembrolizumab antibody (anti-PD-1) with carboplatin plus paclitaxel reduced the risk of death in advanced NSCLC. Atezolizumab antibody(anti-PD-L1) combined with carboplatin plus paclitaxel also improved the treatment outcome in advanced NSCLC (99).

The rapid development of anti-PD-1/PD-L1 inhibitors for advanced NSCLC has greatly improved patient prognosis. However, the vast majority of NSCLC patients are ineffective to PD-(L)1 blockade. Therefore, more clinical trials are required to explore immunomodulatory pathways in an effort to enhance non-responders or hyposensitive individuals to achieve desired therapeutic outcomes. In addition, understanding the mechanisms of resistance to immune checkpoint inhibitors will help determine combination therapy strategies for advanced lung cancer.

Breast cancer

In the past few years, anti-PD-1/PD-L1 antibodies have shown promising therapeutic effects, and great anti-tumor effects have been observed when used alone or in combination with conventional treatment. However, immunotherapy is still rarely used in the treatment of breast cancer. In fact, breast

cancer is generally thought to have a weaker immunogenicity than other types of tumors (100).

Encouragingly, specific PD-1/PD-L1 antibodies can effectively block PD-1 or PD-L1 in breast cancer. Especially, metastatic triple negative breast cancer (mTNBC) shows a potential response to PD-1/PD-L1inhibitors. For example, pembrolizumab antibody(anti-PD-1) has significant antitumor activity and safety in patients with PD-L1-positive mTNBC (16, 59). Moreover, atezolizumab antibody (anti-PD-L1) effectively prolong progression-free survival in patients with mTNBC, and paclitaxel enhanced the therapeutic effect of atezolizumab (69). In addition, patients with metastatic breast cancer (MBC) were treated with avelumab (anti-PD-L1) for 2-50 weeks and followed up for 6-15 months. The results showed that the objective response rate (ORR) was significantly increased in patients with PD-L1 positive tumor-associated immune cells, which suggested that PD-L1 is associated with higher response rates to avelumab in patients with MBC (74).

However, it has also been suggested that PD-1 inhibitors are less effective in mTNBC and that better strategies should be adopted to make the tumor microenvironment more sensitive to PD-L1 inhibitors. For example, short-term adriamycin and cisplatin may induce a more favorable tumor microenvironment in mTNBC and increase the anticancer effect of PD-1 blockers (101). In the past, chemotherapy was the standard first-line treatment for mTNBC, but the efficacy was not satisfactory. Recent study have found that the combination of PD-1/PD-L1 Immune-checkpoint inhibitors and chemotherapy may be a new promising clinical paradigm for the treatment of triple-negative breast cancer (102).

Moreover, PD-L1 expression is associated with high-risk clinicopathological parameters and poor prognosis in patients with primary breast cancer (PBC). A meta-analysis, including 47 studies with a total of 14,367 PBC patients, suggested that PD-L1 high expression associates with large tumor size, histologic grade, Ki-67 high level, ER and PR negative, TNBC subtype and shorter survival time (103). In addition, the expression of PD-L1 in breast cancer stem cells has attracted interest in recent years, and studies have found a significant increase in PD-L1 protein in breast cancer stem cells; therefore, targeting PD-L1 in stem cells may become a new promising therapeutic strategy for breast cancer (104). In any case, PD-1 and PD-L1 have been increasingly studied in breast cancer in recent years, and PD-1/PD-L1 immunocheckpoint inhibitors have shown promising applications in the treatment of breast cancer.

Gastric cancer

Gastric cancer (GC) is a common malignancy and the third leading cause of cancer death worldwide. The 5-year survival rate of patients with advanced gastric cancer was only 5% and 20%, and the median overall survival rate was 10 months. Advanced gastric cancer has a poor prognosis and limited therapeutic options (89, 105, 106). Therefore, it is urgent to explore some new molecular targets and treatments.

Clinical studies have confirmed the efficacy of programmed cell death 1 (PD-1)-targeted therapy for patients with metastatic GC. For example, the anti-PD-1 antibody pembrolizumab has promising anti-tumor activity and manageable toxicity in the treatment of patients with recurrent or metastatic GC (61). Moreover, anticancer effect and responsiveness of pembrolizumab are closely related to PD-L1 expression. Pembrolizumab has a significantly higher ORR in PD-L1-positive GC than in PD-L1-negative GC (56).

It is well known that autophagy is a highly conserved homeostasis process that plays a key role in tumor formation, cell survival, cell metabolism, immune response and tumorigenesis (107–109). Recent studies have shown that autophagy is highly associated with the expression level of PD-L1. Autophagy inhibition increases the expression of PD-L1 in GC, autophagy-related protein LC3 expression is also positively correlated with PD-L1 in primary GC (110). Therefore, autophagy may be closely related to anti-PD-1/PD-L1 immunotherapy in human GC.

Although anti-PD-1/PD-L1 immunotherapy is widely recognized in the clinical practice of gastric cancer, some studies have questioned that single PD-1/PD-L1 inhibitor do not result in relative improvements in OS and PFS compared with chemotherapy in patients with advanced GC or gastroesophageal junction cancer. However, they also determined that PD-1/PD-L1 inhibitors appear to enhance antitumor activity in patients with advanced gastric junction cancers (111). Therefore, further randomized clinical trials are needed to confirm those findings.

Angiosarcoma

Angiosarcoma (AS) is rare malignant endothelial-cell tumors of vascular or lymphatic origin, and is among the most aggressive subtypes of soft-tissue sarcomas (112, 113). In recent years, immunotherapy targeting PD-1/PD-L1 has become a hotspot in the treatment of AS (114, 115). A recent study analyzed PD-L1 expression levels in angiosarcomas at different sites in humans and showed that PD-L1 was abnormally expressed in about 66% of the samples (116). In addition, a 63-year-old male patient with nasal AS that received pembrolizumab 2 mg/kg every 21 days for 13 cycles had no new tumor progression during the 8 months after therapy, which suggested that PD-1/PD-L1 immune checkpoint inhibitors have significant efficacy against angiosarcomas (117).

Cutaneous angiosarcoma (CAS) is the most common form of AS. Positive PD-L1 expression predicts worse outcome in CAS (118). Malignant progression and prognosis of CAS are closely associated not only with high expression of PD-L1, but

also with the presence of tumor-infiltrating lymphocytes (TILs) (114). Since high PD-L1 expression is closely related to the progression of CAS, it is also critical to explore upstream regulators that can increase PD-L1 expression. Recent study revealed that PD-L1 expression was closely associated with atypical protein kinase C lambda/iota (aPKCλ). Inhibition of aPKCλ expression in HUVECs significantly reduced the expression of PD-L1 (119). Therefore, the combination of immune checkpoint inhibitors and aPKC inhibitors may be a potential therapeutic strategy for patients with CAS.

With the development of genomic sequencing technology, treatment strategies for advanced diseases have advanced significantly. Whole genome sequencing (WGS) can provide valuable information for treatment of PD-1/PD-L1 Immune-checkpoint inhibitors in the treatment of AS. For example, patients with metastatic AS who underwent WGS analysis were found to have hypermutated tumor characteristics associated with a positive response to PD-1 Immune-checkpoint inhibitors (120). Subsequently, corresponding scheme was established for the patient to receive the anti-PD-1 antibody pembrolizumab, and the metastases almost completely disappeared after 4 weeks therapy (120). Taken together, PD-1/PD-L1 expression is related to AS progression, and growing evidence suggests that the treatment with PD-1/PD-L1 immunocheckpoint inhibitors may be a promising strategy for AS patients.

Prostate cancer

Prostate cancer (PC) remains the most commonly diagnosed malignant disease in men worldwide (121). At present, PD-1/PD-L1 immunocheckpoint inhibitors have brought significant clinical benefits to some patients with PC. Further study will help guide the development of immunotherapy for advanced PC (122, 123). Patients with high density of PD-1+ lymphocytes were at significantly higher risk of clinical failure, and it was positive association between a high density of PD-1+ lymphocytes and worse clinical failure-free survival (124). Pembrolizumab(anti-PD-1) achieve durable objective responses in a group of severely pretreated patients with advanced PD-L1 positive PC (125). Moreover, recent study suggested that the combination of PD-1/PD-L1 checkpoint inhibitors and radiotherapy was a promising strategy for treating PC (126). Interestingly, PD-1/PD-L1 inhibitors significantly enhanced the efficacy of SA-GM-CSF surface-modified tumor vaccines against PC, which may be a new application for PD-1/PD-L1 inhibitors in the treatment of PC (127). In addition, PD-1/PD-L1 inhibitors had potential of lasting response to microsatellite instability-high (MSI-H) or defective mismatch repair (dMMR) molecular phenotype of prostate cancer (128). Nevertheless, more relevant studies may be needed to confirm this issue.

However, it was reported that PD-L1/PD-1 blockade had a poor effect in PC, due to the low immunogenicity of PC. Early clinical trials confirmed that patients with metastatic castration-resistant prostate cancer (mCRPC) did not significantly respond to PD-1 inhibitors, and they believed that loss of PTEN is responsible for upregulation of PD-L1, followed by constituting innate immune resistance (129). However, subsequent studies revealed that high PD-L1 expression is not significantly related to the loss of PTEN, but rather to the regulation of inflammatory cytokines (130).

All in all, although PD-1/PD-L1 Immune-checkpoint inhibitors have made revolutionary breakthroughs in the treatment of a wide range of human cancers, only a small percentage of prostate cancer patients have achieved significant clinical benefit. However, many experts support that we should still encourage clinical trials with PD-1/PD-L1 inhibitors in PC patients and exploring the mechanisms of PD-1/PD-L1 inhibitors resistance will optimize treatment options and guide the next steps in immunotherapy for PC.

Colorectal carcinoma

Colorectal cancer (CRC) is one of the most common neoplasms accompanied by a high rate of morbidity and mortality, immune checkpoint molecules have been identified as a novel treatment for CRC, such as PD-1 and PD-L1 (131, 132).

Currently, several clinical studies gave the clinical conclusions for PD-1 inhibitors in CRC with dMMR and MSI-H (133, 134). In a non-randomized phase II clinical trial enrolling 74 metastatic CRC(mCRC) patients with dMMR/MSI-H, patients were treated with nivolumab antibody (anti-PD-1) with relatively satisfactory clinical results. The results showed that ORR is 31.1% was achieved, disease control longer than 12 weeks was achieved in 69% of patients, twelve months PFS was 50.4% and 12 months OS was 73.4% (135). Immune checkpoint inhibitors have achieved clearer therapeutic effect in mCRC with dMMR or high microsatellite instability (MSI-H) (133, 136). However, patients with proficient mismatch repair (pMMR) or microsatellite stable (MSS) tumors have not gained enough benefit from immunotherapy (137). This may be related to the higher expression of PD-1 and PD-L1 in dMMR tumors compared to pMMR tumors (138). For pMMR CRC patients with poor responsiveness to immunotherapy, recent study has demonstrated a significant synergistic inhibitory effect of pembrolizumab(anti-PD-1) combined with ibrutinib (139). The combination of pembrolizumab and azacitidine for chemotherapy-refractory mCRC has also achieved a safe, tolerable and positive clinical efficacy (140). Moreover, the chemotherapy agent FOLFOXIRI plus bevacizumab is known to increase the immunogenicity of pMMR or MSS tumors. Importantly, the addition of atezolizumab(anti-PD-L1) to the

first-line FOLFOXIRI plus bevacizumab significantly improve progression-free survival in patients with previously untreated metastatic colorectal cancer (141). Taken together, the application of PD-1/PD-L1 inhibitors in combination with other antitumor agents will bring light to the treatment of refractory or metastatic CRC.

It is generally believed that PD-1/PD-L1 cause immune escape from tumors by inhibiting tumor immune processes. Reduction of T cell cytotoxicity may be one of the key mechanisms of tumor PD-L1-induced inhibition of antitumor immunity. A recent study suggested that PD-L1 expressed in CRC significantly inhibited the cytotoxicity of $CD8^+$ T cells, which led to tumor immune escape (142). In addition, tumor immune escape may be caused by upregulation of tumor cell infiltrating immune cells (TIICs) or their ligands at suppressive immune checkpoint in CRC, such as PD-1, PD-L1 and CTLA-4. Changes in DNA methylation patterns and enrichment of methylated histone markers in promoter regions may be the main reasons for upregulation of immune checkpoint (ICs) in CRC (143). Furthermore, a study aimed at analyzing the prognostic value of PD-L1 in CRC cells and tumor cell infiltrates (TILs) revealed that high expression of PD-1 and PD-L1 was associated with a better prognosis in colorectal cancer patients and TILs-PD-1 may be an independent prognostic factor for OS and disease-free survival (DFS) in CRC patients (144).

Anyway, PD-L1 inhibitor-based immunotherapy is considered a promising approach for targeting colorectal cancer. The binding of PD-L1 and PD-1 in tumor cells or tumor microenvironment induces immunosuppressive signals that reduce T cell proliferation and lead to tumor immune escape.

Hepatocelullar carcinoma

Hepatocelullar carcinoma (HCC) is the third leading cause of cancer death and the sixth most common malignancy worldwide (145). Recent study showed that PD-1/PD-L1 expression played an important role and interacted with $CD8^+$ T-cell immune responses to regulate the immune homeostasis and prognosis of HCC patients (146). Recent studies revealed that amplification or high expression of PD-L1 was significantly and independently associated with poor survival in HCC patients, which confirmed that the PD-1/PD-L1 axis is a promising potential target for HCC immunotherapy (147).

Recent studies have shown that immunocheckpoint inhibitor therapy significantly improves the overall survival of HCC patients (148, 149). For example, some PD-1 inhibitors, such as pembrolizumab and nivolumab, were effective and well tolerated in patients with advanced HCC (63, 88). PD-L1 mediated the growth inhibition of herbal medicines baicalin and flavonol on HCC by decreasing STAT3 activity, thereby restoring the anti-cancer sensitivity of T cells (150). More importantly, anti-PD-1/PD-L1 antibodies combined with other therapies was considered an effective strategy for the treatment of HCC (151). In addition to binding to cell membranes, PD-1/PD-L1 could also dissociate in the blood of patients and is called as soluble PD-1/PD-L1 (94, 152). Recent studies have shown that high expression of soluble PD-L1 was significantly associated with an increased risk of death (153, 154). Furthermore, soluble PD-1 and PD-L1 were independent prognostic factors with opposite roles in predicting disease-free survival (DFS) and overall survival (OS) in HCC patients (155).

Currently, clinical trials about the application of PD-1/PD-L1 checkpoint inhibitors in HCC are underway or completed, some of which have shown promising therapeutic expectations. Nonetheless, the clinical benefits for other patients are not satisfactory. Comprehensive predictive biomarkers are necessary to identify HCC patients who are more likely to respond to immunosuppression and thus guide clinical therapy strategies.

Bladder cancer

Bladder cancer (BC) is the most common cancer of the human urinary system, with poor prognosis and high recurrence rate (156, 157). Immunotherapy based on PD-1/PD-L1 immunecheckpoints has been approved and successfully performed in the treatment of BC (158–160). Recent studies have identified significant differences in the expression levels of PD-1 and PD-L1 between higher-grade BC and lower-grade BC. The expressions of PD-1 and PD-L1 in higher-grade BC are higher than those in lower-grade BC. Therefore, PD-1 and PD-L1 may be important biomarkers related to the pathological grading of BC and play a mediating role in the progression of BC (161). Moreover, PD-L1 may be novel combined biomarkers for predicting tumor invasitivity and immune checkpoint response in BC (162). Taken together, PD-1/PD-L1 are closely associated with tumorigenesis, treatment and prognosis of human BC.

PD-L1 positive BC patients with heavily pretreated have shown a manageable safety profile and meaningful clinical outcomes after treatment of durvalumab antibody (Anti-PD-L1), ORR was 46.4% and responses were ongoing in 12 of 13 responding patients (163). Another study evaluated the safety and antitumor activity of avelumab (Anti-PD-L1) in patients with metastatic urothelial BC. Results suggested that patients achieved a median progression-free survival of 11.6 weeks, median OS of 13.7 months and 12-months OS rate of 54.3% (164). There were many other similar clinical studies that demonstrate that PD-1/PD-L1 Immune-checkpoint inhibitors are well associated with durable responses and prolonged survival in metastatic urothelial BC. Recent studies revealed that the expression and function of PD-L1 in BC were closely related to autophagy. PD-L1 could be upregulated by autophagy-

related proteins (e.g ATG7), ultimately enhancing the stem cell-like properties and invasive capacity of BC cells (165). Therefore, PD-1/PD-L1 Immune-checkpoint inhibitors combined with autophagy inhibitors may be a promising therapeutic approach for human BC. In addition, anti-PD-1/PD-L1 immunetherapy combined with radiotherapy has significant local and distal synergistic anticancer effects (166).

Overall, PD-1/PD-L1 Immune-checkpoint inhibitors have shown promising results in terms of clinical efficacy in patients with advanced and metastatic BC. However, more additional data is urgently needed for the evaluation of reliability and safety of anti-PD-1/PD-L1 treatment. These exciting advances will bring more benefit and hope for BC patients.

Ovarian cancer

Ovarian cancer(OC) is the seventh most common cancer and the eighth leading cause of cancer death in women worldwide (167). Recent studies have shown that the application of PD-1/PD-L1 inhibitors in the treatment of OC has attracted extensive attention of researchers (168–170). PD-1 and its ligand were significantly expressed in tumor cells and immune system cells of OC patients (171).

Recent studies claimed that PD-1/PD-L1 immune checkpoint inhibitors do not perform well in the treatment of recurrent epithelial OC (172, 173). However, well efficacy was achieved by the combination of PD-1/PD-L1 inhibitor (Nivolumab) and anti-angiogenic drug (Bevacizumab). Their interaction may exert synergistic effects by modulating the microenvironment (66). In addition, combination therapy with PARP and immune checkpoint inhibition has yielded encouraging results in ovarian cancer (174, 175). Taken together, combination therapy may be an effective strategy for the treatment of OC, offering a potential therapeutic opportunity for OC.

Although combined immunotherapy have evolved rapidly in the treatment of OC and have been successfully applied, some emerging issues need to be addressed in clinical practice, such as the dose and sequence of optimal synergy, differences in immunotherapy response across OC subtypes and possible side effects of the interaction of two medicines. Therefore, we appeal that more clinical trials of combination immunotherapy should be performed to obtain relevant clinical data, including efficacy, stability and immune-related adverse effects. so that the combined immunotherapy will be more widely applied in the treatment of OC in the near future.

Pancreatic cancer

Pancreatic cancer (PC) is one of the leading causes of cancer death worldwide. In the last two decades, the number of pancreatic cancer patients diagnosed each year has doubled worldwide (176). PD-L1 was identified as a novel maker of prognosis in patients with PC, and the up-regulation of PD-L1 was found in human PC tissues (177). PD-L1 is involved in the regulation of PC stemness, epigenetic mechanisms and metastasis. Blocking PD-1 significantly inhibited the PC growth by enhancing INF-γ production and decreasing IL-10 production in a mouse model (178). Therefore, PD-1/PD-L1 expression and related signaling may play an important role in the progression of PC.

In recent years, although PD-1/PD-L1 immunecheckpoint inhibitors have been rapidly developed in the treatment of various cancers. However, the outcomes of PD-1/PD-L1 immunecheckpoint inhibitors monotherapy are not satisfactory in PC (179). Currently, two main reasons are believed to be responsible for such failures. First, pancreatic cancer is inherently non-immunogenic. Second, immunosuppression due to high tumor burden is another reason for which PC cannot be treated by PD-1/PD-L1 blockade alone, immune escape of pancreatic tumors is closely related to the excessive development of immunosuppressive T cells (177). Therefore, the combination therapeutic strategies may bring new hope for the treatment of PC with PD-1/PD-L1 inhibitors. For example, the combination of anti-PD-1 inhibitory antibodies and anti-ox40 agonist antibodies decreased the proportion of T regulatory, and increased the number of memory CD4+ and CD8+ T cells, thereby attenuating the immune escape response and enhancing the anticancer effects of anti-PD-1 in PC (180). In addition, a study suggested that Anti-TNFR2 and anti-PD-L1 combination therapy significantly inhibited the growth of PC through relieving tumor immunosuppression and generating robust memory recall (181). Moreover, Anti-PD-1 antibody immunotherapy combined with gemcitabine significantly inhibited PC and liver metastasis by enhancing the immune response mediated by Th1 lymphocytes and M1 macrophages (182).

All in all, although anti-PD-1/PD-L1 inhibitors have rapidly developed as a priority of immunotherapy strategy for various cancers. However, the poor therapeutic outcomes were observed in the treatment of PC because of the particularity of pancreatic cancer, such as a high tumor burden, non-immunogenicity and immunosuppressive tumor microenvironment. The combination of anti-PD-1/PD-L1 immunotherapy with other anti-tumor agents that overcome these specific properties will significantly improve the therapeutic effect of anti-PD-1/PD-L1 immunotherapy in PC.

PD-1/PD-L1 and inhibitors in human hematological malignancies

Leukemia

Leukemia is the common name for several malignant diseases with an increasing number of white blood cells in the

blood and/or bone marrow. Leukemia includes acute myeloid leukemia, chronic myeloid leukemia, acute lymphoblastic leukemia, chronic lymphocytic leukemia, and so on (183). Acute myeloid leukemia (AML) is the most common form of leukemia and hematological malignancy with a poor clinical prognosis and characterized by uncontrolled proliferation of hematopoietic stem cells in the bone marrow (184, 185).

Recent results has shown that high expression of PD-1 and PD-L1 was associated with poorer overall survival (OS) and clinical outcome in AML patients (186, 187). In addition, a clinical trial has shown encouraging response and overall survival rates for patients with relapsed/refractory (R/R) acute myeloid leukemia (AML) treated with nivolumab(anti-PD-1) and azacitidine, which suggested that nivolumab in combination with azacitidine appears to be a safe and effective treatment for AML (188).

However, the clinical response to PD-1/PD-L1 blockade varied in different AML patients (189). A recent study revealed that the majority of immune-checkpoint receptor genes were downregulated in bone marrow (BM)-infiltrating CD8+ T cells and partially in CD4+ T cells due to pathological chromatin remodeling via histone deacetylation. Therefore, the dysfunction of CD8+ T cells in AML was mainly due to pathological epigenetic silencing of activated IC receptors rather than due to signaling by immune inhibitory IC receptors (190). This may explain the limited role of PD-1/PD-L1 antibodies in AML patients. In conclusion, anti-PD-1/PD-L1 therapy may be a new immunotherapeutic strategy for AML. However, further studies are still necessary.

Multiple myeloma

Multiple myeloma (MM) is a genetically heterogeneous clonal plasma cell disorder, which is the second most common malignancy in the hematological system (191, 192). The immune dysfunction is critical for the genesis of MM. The interaction of PD-L1 and PD-1 inhibited the body's immune function and promoted immune escape by preventing tumor-reactive T cells from being activated and functioning (193). PD-L1 and PD-1 were higher on their tumor cells and T-cells in MM patients, respectively. MM cells with high PD-L1 expression effectively protected themselves against MM-specific t-cell killing, which could be reversed by anti-pd-1 or PD-L1 antibodies (194). In addition, PD-L1 expression on malignant myeloma plasma cells was related to an increased risk of MM (195).

However, the role of PD-L1/PD-1 axis in MM is still debated, the clinical outcomes of PD-1/PD-L1 inhibitors alone for MM are not very encouraging, the combination of PD-1/PD-L1 inhibitors with other drugs for multiple myeloma appears to be promising (196, 197). Recent study showed that pembrolizumab (anti-PD-1) in combination with belapectin (Galectin-3 Inhibitor) significantly enhanced the activation of effector memory T cells and the percentage of effector memory T cell proliferation in MM patients. Moreover, pembrolizumab in combination with belapectin was associated with fewer immune-related adverse events compared to pembrolizumab monotherapy (198)). Moreover,PD-1inhibitor in combination with CD38 monoclonal antibody was also a promising strategy for the treatment of CD38-positive MM (194). In fact, *in vitro* experiments have also demonstrated that PD-1/PD-L1 inhibitors directly enhance NK cell- and T cell-mediated immune responses against MM, and lenalidomide (immunomodulator) significantly enhanced such immune responses (199). Overall, PD-1/PD-L1 expressions in MM have shown an important clinical significance and its inhibitors have a certain potential in the treatment of MM, but the conclusions of their effectiveness are inconsistent and more rigorous clinical and basic studies are required to confirm that.

Lymphoma

Lymphoma is a kind of heterogeneous lymph-like malignancy (200).The World Health Organization classifies lymphomas into more than 80 subtypes in 2017 year based on their morphology, immunophenotype, genetic lesion, molecular profile, clinical features and cell type of origin, such as B cell lymphoma, T cell lymphoma, Hodgkin's lymphoma and so on (200, 201). Preliminary clinical data suggested that checkpoint inhibitors were a promising therapeutic strategy for certain lymphoid malignancies. However, the expression level and role of PD-1/PD-L1 in lymphoma cells and tumor microenvironment varied depending on the subtype (202). For example, increased infiltration of PD-1+ tumor-infiltrating lymphocytes (TILs) was a positive prognostic predictor in diffuse large B-cell lymphoma (DLBCL) but not in Hodgkin's lymphoma (HL) (202). The NK cell-associated and monocyte/macrophage-associated immune escape due to the PD-1/PD-L1 pathway was more prominent in HL than DLBCL (203).

Anyway, PD-1/PD-L1 inhibitors have still made some promising achievements in the research and treatment of lymphoma. For instance, a clinical trial suggested that PD-L1 may be the most promising soluble biomarker for classical Hodgkin lymphoma (CHL) (204). The objective response rate (ORR) to nivolumab was 66.3% (53/80) in a multicenter, multicohort, single-arm phase 2 trial for classical Hodgkin's lymphoma after failure of autologous stem cell transplantation and brentuximab vedotin (65). Moreover, GLS-010, a recombinant human anti-programmed death-1 monoclonal antibody, has demonstrated favorable response and safety in clinical trials for the treatment of advanced solid tumors or lymphomas (77). In a recent phase 2 study, pembrolumab significantly improved the PFS and OS in patients with

relapsed/refractory (R/R) CHL after autologous stem cell transplantation (ASCT) and achieved 82% PFS at 18 months and 100% OS at 18 months, which suggested that pembrolumab is a promising approach for post-ASCT consolidation in patients with R/R CHL (205). Furthermore, the results of a small phase 1b study showed that the ORR was 36% in patients with R/R diffuse large B-cell lymphoma (DLBCL) treated with nivolumab (67). However, in a subsequent larger phase 2 study, the ORR to nivolumab treatment was only 10% and 3% respectively, median response time was 11 and 8 months respectively in patients with R/R DLBCL who are ineligible for autologous hematopoietic cell transplantation (AHCT) or experienced failure with AHCT (87). Taken together, considering the diversity and complexity of lymphomas, more precise and individual clinical trials are necessary to elucidate the role of PD-1/PD-L1 inhibitors for the treatment of lymphomas in the future.

Problems and prospects

Currently, there is an increasing number of studies targeting PD-1/PD-L1 immune checkpoint inhibitors in human cancers including solid tumors and hematological malignancies. PD-1 and PD-L1 are expressed in tumor-infiltrating immune cells and most solid tumors, and they are closely associated with tumor development and prognosis (206–209). PD-L1-positive patients have a significantly lower 5-year survival rate than patients with non-PD-L1-positive tumors, and PD-L1 expression is an independent prognostic indicator (210). PD-L1 expression is associated with many factors, such as age, tumor size, depth of infiltration, lymph node metastasis, lymphovascular infiltration, venous infiltration and disease stage. Recent studies have shown that PD-L1 and PD-1 expressions are often closely linked. In patients with high PD-L1 expression, PD-1 levels in T cells are also high, which may be an intrinsic factor for the immune escape of tumors (210). Activated T cells play a key role in tumor suppression. PD-1 is mainly expressed in activated T cells and inhibits T cell function through binding to PD-L1, thereby promoting immune escape (22).Therefore, blockade of interaction between PD-1 and PD-L1 can significantly enhance immune function and inhibit tumor growth. A multicenter phase 1 trial showed that intravenous anti-PD-L1 antibody significantly inhibited tumor progression (objective remission rate of 6-17%) and prolonged disease stability (12-41% at 24 weeks) (211).

Although PD-1/PD-L1 expression is closely associated with tumor progression and treatment, using PD-1/PD-L1 as the only predictive biomarker for cancer immunotherapy still remains problematic. For example, the low accuracy of PD-L1 detection brings unnecessary obstacles for examination and anti-PD-L1 treatment in patients. The main reasons are considered as follows: Firstly, different studies may use antibodies of varying sensitivity. Secondly, the criteria for positive PD-L1 staining were inconsistent across studies. Thirdly, the expression level of PD-L1 in different sites of tumor tissues is variable, and even if the same sites are sampled at different times, the results of PD-L1 detection can be affected (212). Fortunately, some studies have found that some other factors can work together with PD-L1 as biomarkers to better predict the responsiveness of anti-PD-1/PD-L1 therapy. For example, high expression of TMB (tumor mutational burden), T-cell-inflamed gene-expression profile (GEP) and PD-L1 together reflect the potential for higher response of pembrolizumab in various types of cancers (57).

Taken together, PD-1/PD-L1 immune checkpoint inhibitors have been widely used in the treatment of a variety of cancers (64, 65, 68, 71, 75, 76). However, serious challenges still remain, such as the small number of beneficiary populations, primary and acquired drug resistance, lack of predictive and prognostic biomarkers, and treatment-related adverse effects (22). In addition, there are few predictive biomarkers that can identify the type of patients who will benefit from treatment (213, 214). Moreover, PD-L1 expression is heterogeneous and dynamic in tests with different antibodies and different scoring criteria complicate the interpretation of the test results (215–217). Therefore, considering the multifactorial characteristics of tumor immune crosstalk, prediction models based on comprehensive biomarker determination theory may be more feasible for future applications. Finally, in response to the treatment resistance of PD-1/PD-L1 blockers, some investigators believe that combined therapy, nanoimmunotherapy and intestinal microbial therapy may be promising therapeutic strategy (218). In any case, PD-1/PD-L1 Immune-checkpoint inhibitors definitely have wildly application prospects and clinical value in the treatment of human cancers.

Author contributions

QT, YC, and XL drafted the manuscript and were involved in data analysis in the whole manuscript. SL, YS, and YY were involved in technical support and revised the manuscript. WW and LH contributed to funding and writing suggestions. SW revised the manuscript and contributed to the conception and design of the work. All authors approve it for publication and agree to be accountable for all aspects of the work.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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V-Set and immunoglobulin domain containing (VSIG) proteins as emerging immune checkpoint targets for cancer immunotherapy

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The development of immune checkpoint inhibitors is becoming a promising approach to fight cancers. Antibodies targeting immune checkpoint proteins such as CTLA-4 and PD-1 can reinvigorate endogenous antitumor T-cell responses and bring durable advantages to several malignancies. However, only a small subset of patients benefit from these checkpoint inhibitors. Identification of new immune checkpoints with the aim of combination blockade of multiple immune inhibitory pathways is becoming necessary to improve efficiency. Recently, several B7 family-related proteins, TIGIT, VSIG4, and VSIG3, which belong to the VSIG family, have attracted substantial attention as coinhibitory receptors during T-cell activation. By interacting with their corresponding ligands, these VSIG proteins inhibit T-cell responses and maintain an immune suppressive microenvironment in tumors. These results indicated that VSIG family members are becoming putative immune checkpoints in cancer immunotherapy. In this review, we summarized the function of each VSIG protein in regulating immune responses and in tumor progression, thus providing an overview of our current understanding of VSIG family members.

KEYWORDS

immune checkpoint, VSIG4/CRIg, VSIG, TIGIT, cancer immunotherapy, antitumor T-cell response, coinhibitory receptor

Abbreviations: ICB, immune checkpoint blockade; CTLA-4, cytotoxic T-lymphocyte-associated protein 4; AML, acute myeloid leukemia; TCGA, The Cancer Genome Atlas; GEO, Gene Expression Omnibus; COAD, colon adenocarcinoma; VISTA, V-domain immunoglobulin suppressor of T-cell activation; Co-IP, coimmunoprecipitation; MM, multiple myeloma; TIGIT, T-cell immunoreceptor with immunoglobulin and ITIM domain; PVR, poliovirus receptor; TFH, follicular T helper; FDC, follicular DC; ITT, immunoglobulin tyrosine tail; ITIM, immunoreceptor tyrosine-based inhibitory motif; LTA, recognizing lipoteichoic acid; ELISA, Enzyme-linked Immuno Sorbent Assay; MST, Microscale Thermophoresis; ECD, extracellular domain; TTF1, subcellular localization of thyroid transcription factor 1; SLE, systemic lupus erythematosus; NSCLC, non-small cell lung carcinoma; FECD, fuchs endothelial corneal dystrophy; JAM, junctional adhesion molecule; SNP, single nucleotide polymorphisms; EMT, epithelial-mesenchymal transition; IBS-D, intestinal biopsy of irritable bowel syndrome; HCC, hepatocellular carcinoma.

Introduction

Immune checkpoint receptors are membrane molecules that can modulate lymphocyte activation upon encoding their cognate ligands on antigen-presenting cells or target cells. They play an essential role in controlling excessive immune responses by transmitting a stop signal to attenuate T-cell activation and maintain immune homeostasis. However, tumors always take advantage of these inhibitory pathways to escape attack from antitumor immune cells (1, 2). Various malignancies are found to confer an overall immunosuppressive tumor microenvironment by upregulating the expression of immune checkpoint receptors and their ligands. To unleash effector T-cell responses and enhance endogenous antitumor activity, therapies targeting these immunoregulatory proteins are becoming an encouraging approach. The most successful immune checkpoint blockade (ICB) therapy is anti-PD-1/PD-L1, which has been shown to confer therapeutic advantages for a variety of cancers, such as non-small cell lung carcinoma (NSCLC), malignant melanoma, kidney cancer, and liver cancer (3–8). Another well-studied immune checkpoint is cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) (1). The first immune checkpoint inhibitor (ipilimumab) targeting CTLA-4 was approved in 2011 by the Food and Drug Administration (FDA) and has been demonstrated to control tumor growth and prolong survival in melanoma (9–11). Since then, the application of ICBs has brought a groundbreaking paradigm shift in cancer treatment, particularly for advanced-stage cancers (9, 12–14). With the growing research interest in cancer immunotherapy, many new checkpoints have been identified and extensively studied in recent years, such as TIM3, TIGIT, VISTA, LAG-3, BTLA, B7-H3, B7-H4, and B7-H5 (15–19). Most of them belong to the B7 family (VISTA, B7-H3, B7-H4, B7-H5), which is characterized by typical extracellular IgV-like and IgC-like domains and is categorized as the immunoglobulin superfamily (IgSF) (20). These proteins can function as coinhibitory receptors to deliver negative signaling towards T cells upon TCR engagement, therefore inhibiting T-cell activation, expansion, and functional polarization. Recently, the V-Set and immunoglobulin (Ig) domain-containing (VSIG) family, which also belongs to IgSF and exhibits structural similarity with the B7 family proteins, has been increasingly recognized as a potential immune checkpoint contributing to tumor evasion. This family is currently comprised of eight members, including VSIG1, VSIG2, VSIG3, VSIG4, VSIG8, VSIG9, VSIG10, and VSIG10 L, which are all type I transmembrane proteins that are expressed by a variety of both immune and nonimmune cells, and many possess immunosuppressive properties. For example, VSIG3, a ligand of VISTA, is essential for its role in T-cell suppression (21, 22). Another member, VSIG4, is also well known for its potent ability to suppress T-cell responses (23). A star member of the VSIG family is VSIG9, also known as TIGIT, which has emerged as a promising cancer therapeutic target due to its apparent function in limiting antitumor T-cell and NK-cell responses (24–26). These studies suggested that the members of the VSIG family could be potent candidates for developing novel ICB therapies. However, there is still a range of related proteins in this family that have yet to be studied extensively, and the mechanism whereby VSIG family proteins inhibit immune responses is not fully understood. To attract more attention to this family, this review aims to introduce VSIG family members and their role in regulating the T-cell response in cancers.

Overview of the VSIG family

The discovery of the VSIG protein family dates back to the 1990s. The first identified member of the VSIG family is VSIG2, initially called CTX. This gene was first cloned by Chrétien and colleagues from the cortical thymocyte of Xenopus in 1996 (27). The homolog gene of *VSIG2* in chickens, mice, and humans was cloned two years later (28). It is located on chromosome 11, 11q24.2. The second member, *VSIG4*, was cloned and found to be localized in the pericentromeric region of the human X chromosome (29). Since then, other members showing sequence similarities with these identified VSIG proteins have been discovered, including VSIG1, VSIG3, VSIG8, VSIG9 (widely known as TIGIT), VSIG10, and VSIG10 L. The corresponding genes of those VSIG family members are present on different chromosomes in humans, with the exception of *VSIG1*, which is also present on the X chromosome at a different position from *VSIG4* (Xq22.3 and Xq12, respectively). Of these eight members, only the structures of VSIG4 and TIGIT (RCSB-PDB ID: 5IMK and 3Q0H, respectively) were experimentally resolved through X-ray crystallization, while the rest had computationally predicted structures available. From these data, VSIG family members have been shown to have either an IgV domain, an IgC2 domain, or both (23, 26, 28–33). The IgV domain is shared by all members except for VSIG10 and VSIG10 L (UniProt Accession # Q8N0Z9 and Q86VR7), while IgC2 is a common feature of all members except for VSIG8 (UniProt Accession # P0DPA2) and TIGIT. Moreover, they are all type I transmembrane proteins with a wide range of expression. The structural composition of the extracellular domain (ECD) is highly conserved between hVSIG and mVSIG members.

Although the tissue distribution of VSIG proteins is not fully described due to antibody unavailability for some of the family members, genetic analysis and sequencing data reveal a diverse range of expression of VSIG proteins from testicular germ cells (VSIG1 and VSIG3) (32, 34) to the hair shaft (VSIG8) (35, 36) and immune cells (VSIG4 and TIGIT) (23–26, 33, 37–39), hence serving a wide array of functions in both humans and mice. A brief overview of VSIG family members and their functions,

expression patterns, and roles in immunotherapy are summarized in Table 1 and Table 2. In the following context, we will discuss the role of each member of the VSIG family in detail, provide a comprehensive summary of our current understanding of these proteins, and highlight their potential as new targets for ICB therapy.

VSIG1

IgSF is a large protein superfamily of cell surface and soluble proteins involved in adhesion processes, binding, and cell recognition. Members of this superfamily are defined by structural similarities to immunoglobulins and the presence of an Ig domain (40). VSIG1 is a typical IgSF with two extracellular Ig-like domains and a short cytoplasmic domain. It is also known as the radioiodinated cell surface A33 antigen or glycoprotein A34, which was first characterized to be a tissue-restricted cell surface protein predominantly expressed in the gastric mucosa (30, 41). It was subcellularly localized in the adherens junctions of glandular epithelia and was critical for ensuring proper differentiation of glandular gastric epithelium (41). Three alternatively spliced isoforms of VSIG1, including VSIG1A, B and C, were identified in mice, with the latter being specifically expressed in the testis (41). In this tissue, VSIG1 was found to be a ZO1 (zonula occludens-1)-binding junctional adhesion molecule (JAM) localized on the surface of sperm cells to facilitate their interactions with Sertoli cells, suggesting that VSIG1 may be involved in supporting spermatogenesis (34). However, this has been challenged by a recent report showing that *VSIG1* knockout mice had normal development and function of sperm cells, and whether the absence of phenotype upon *VSIG1* deletion was caused by unknown compensatory mechanisms or genetic redundancy remains to be investigated (42).

Due to its abundant expression in the stomach, VSIG1 has been extensively studied in gastric cancers. In a cohort of 232 gastric adenocarcinoma samples, Chen et al. reported that VSIG1 was significantly reduced at both the mRNA and protein levels in gastric tumor tissues compared to paired noncancerous gastric mucosal tissues (43). Inoue et al. also reported a dramatic decrease in VSIG1 expression in 219 of 362 gastric cancer specimens (44). Furthermore, downregulation of VSIG1 was significantly correlated with poor overall survival and worse clinical outcome in gastric cancer patients (43–45), suggesting that *VSIG1* may function as a tumor suppressor gene. In support of this, overexpression of VSIG1 diminished the proliferation and migration of multiple gastric cancer cell lines *in vitro* (44).

In contrast, VSIG1 seemed to be upregulated in a variety of nongastric carcinomas (30, 44, 46, 47). It was identified as a signature gene for gastric-type differentiation of serrated pathway-associated colon carcinoma (47–49) and lung adenocarcinoma (50). The coexpression of VSIG1 in the cytoplasm of hepatocytes with thyroid transcription factor 1 (TTF1) was also considered to be a potential lineage shift indicator of conventional to gastric-type hepatocellular carcinoma (HCC) (51). In the same study, Gurzu et al. further revealed that VSIG1 was strongly correlated with epithelial-mesenchymal transition (EMT) genes, such as E-cadherin and N-cadherin and VIM (51). Since VSIG1 is known to function as a JAM involved in tight junction assembly, these data implicated a potential role of VSIG1 in modulating EMT during tumor metastasis. In support of this, Bernal et al. showed that VSIG1

TABLE 1 Brief overview of VSIG family members.

	VSIG1	VSIG2	VSIG3	VSIG4	VSIG8	VSIG9	VSIG10	VSIG10 L
Aliases	GPA34	CTH, CTXL	BT-IgSF, IgSF11	VSIG4, Z39IG	C1orf204	TIGIT, VSTM3, WUCAM	–	–
Cytogenic location	Xq22.3	11q24.2	3q13.222	Xq12	1q23.2	3q13.31	12q24.23	19q13.41
Exons	10	7	12	8	7	6	11	10
Discovery/First Report	2006(30)	1996(19)	2002(32)	2000(29)	2006(36)	2009(26)	2015(140)	2016(144)
Binding Partners	–	–	VISTA	C3 (C3b),LTA, MS4A6D	VISTA	CD155, CD112, CD113	–	–
Structure Of ECD	IgV-IgC2	IgV-IgC2	IgV-IgC2	IgV-IgC	IgV-IgV	IgV	IgC2-IgC2-IgC2- IgC2	IgC2(type 1)-IgC2 (type 2)
Length of amino acids (ECD domain) for Human & Mouse	H:387 aa (211aa)	H:227 aa (220aa)	H:431 aa (219aa)	H:399 aa (264aa)	H:414 aa (242aa)	H:244 aa (120aa)	H:540 aa (383aa)	H:867 aa (749aa)
	M:407 aa (212aa)	M:328 aa (220aa)	M:428 aa (218aa)	M:280 aa (unreviewed)	M:417 aa (241aa)	M:249 aa (120aa)	M:558 aa (406aa)	M:868 aa (736aa)
Uniprot ID	Q86XK7	Q96IQ7	Q5DX21	Q9Y279	P0DPA2	Q495A1	Q8N0Z9	Q86VR7
Similarity with mVSIG	81% *	85% *	95% *	80% **	88% *	65% *, 77% ***	63% *	75% *

*Based on ECD, ** Based on IgV domain, *** Based on cytoplasmic region.

TABLE 2 Diverse expression and function of VSIG family members.

VSIG Members	Expression in Immune cells	Expression In Tissue	Expression in Cancer	Role in Immunotherapy
VSIG1	–	Stomach, testis, ovary, liver	Esophageal carcinomas, gastric cancer, Ovarian cancers(30,44,47) HCC (51) Colon cancer(47,52) Lung cancer(46)	–
VSIG2	Macrophage B cell	Colon, stomach, prostate, trachea, thyroid glands	AML (58); Colon adenocarcinoma(62) Pancreatic cancer(60) Lung adenocarcinoma(61)	Positively correlated with B cell and M1 macrophage infiltration (62)
VSIG3	–	Brain, testis	Colorectal cancer, hepatocellular carcinomas, Gastric cancer (63,69) ; gliomas (70)	Negative regulation of T cell activation(21,22,67)
VSIG4	Tissue resident macrophage	Liver, peritoneum, Pancreas, colon	Lung cancer (93); Breast cancer (94) Ovarian cancer (95) Multiple myeloma (MM) (96) High-grade glioma (97)	Negative regulation of Tcell activation (23,73,74,92)
VSIG8	–	Oral epithelium, hair shaft & follicle, nail matrix	Head and neck cancer(#) Thyroid cancer(#) Colorectal cancers(#) …	Negative regulation of T cell activation(99,101);
TIGIT	T cell, NK cell, Treg	Lymphoid tissue	Melanoma, NSCLC, Colorectal cancer,HCC, AML, Glioblastoma(124-128,137) …	Negative regulator of immune cells (26,107,131,135…)
VSIG10	DC	Intestinal epithelium	Adenocarcinoma (141)	Negative regulation of CD4+ T cell activation(*)
VSIG10L	–	Saliva gland, oesophagus	Lung squamous cell carcinoma (145)	–

(*Reference from US patent (Application #20200270343).
(#)Reference from THE HUMAN PROTEIN ATLAS.
Website: https://www.proteinatlas.org/ENSG00000243284-VSIG8/pathology.

knockdown increased while gain of VSIG1 inhibited the migration of colon cancer cells (52). Overall, although the function of VSIG1 in modulating antitumor immune responses has not been explored thus far, given its importance as a cell surface tumor suppressor in contraining tumor growth and metastasis, targeting VSIG1 would be of great value for the treatment of different types of cancer (30).

VSIG2

VSIG2 is composed of an ECD of 220 aa containing IgC2 and IgV domains and a cytoplasmic tail of 63 aa (28). VSIG2 is also known as CTXL (cortical thymocyte-like protein). It was initially identified as a marker predominantly expressed on cortical thymocytes in *Xenopus* and was designated CTX (cortical thymocyte of Xenopus). Due to the abundant expression of VSIG2 on double-positive thymocytes of Xenopus and on recent T-cell immigrants in chickens, it was considered to be involved in T-cell development in these species (27). However, by cloning its mouse and human homologues, namely, CTM (cortical thymocyte of mouse) and CTH (crotical thymocyte of human), respectively, VSIG2 was found to be abundantly expressed in the thyroid glands, trachea, prostate, colon, and stomach but weakly expressed in the lung and bladder but not in the thymus (28). These initial data suggest that VSIG2 may be an ancestral lymphocyte receptor before the introduction of somatic rearrangement in mammals.

Although the physiopathological function of VSIG2 remains to be explored, a close association of VSIG2 with the progression of various human diseases has been demonstrated in recent years using multiomics approaches, highlighting the potential of VSIG2 as a biomarker for the diagnosis of many diseases. VSIG2 was found to be significantly upregulated in the corneal samples of Fuchs endothelial corneal dystrophy (FECD) patients (53), in the intestinal biopsy of irritable bowel syndrome (IBS-D) patients (54), in the plasma of acute tubular injury and interstitial fibrosis/tubular atrophy patients (55), and in the plasma of incident heart failure patients (56). Moreover, the single nucleotide polymorphisms (SNPs) of VSIG2 are strongly

associated with serologic profile and cytokine phenotype in systemic lupus erythematosus (SLE) (57). Aberrant VSIG2 expression was also found in tumors. Heimeng et al. reported that VSIG2 expression in acute myeloid leukemia (AML) patients correlated with poor prognosis according to The Cancer Genome Atlas (TCGA) and Gene Expression Omnibus (GEO) databases (58). VSIG2 expression has been found to be significantly upregulated in patients with nonmuscle invasive bladder cancer but downregulated in patients with muscle invasive bladder cancer; thus, it could serve as a biomarker of invasiveness in bladder cancers (59). In primary lung adenocarcinoma and pancreatic cancer, VSIG2 was characterized as a member of the DNA methylation-related prognostic signature (60, 61). High expression and methylation of VSIG2 correlated with poor survival in these cancer patients (58, 60, 61).

The potential effect of VSIG2 in modulating antitumor immunity was also implicated in colorectal cancers. In a recent study, Cui Z et al. reported lower expression of VSIG2 in colon adenocarcinoma (COAD) samples, and its downregulation was associated with a poor overall survival rate in COAD patients (62). However, it appears that *VSIG2* functions as a tumor suppressor gene to ensure tumor immune surveillance rather than an immune checkpoint molecule in this particular cancer type. Interestingly, VSIG2 expression positively correlated with B-cell and M1 macrophage infiltration (62). Since these cells are normally the most abundant antigen-presenting cells in tumor tissues, dissecting the role of VSIG2 in these cells may have implications for understanding the biology of T-cell activation in the tumor microenvironment.

VSIG3

VSIG3 contains a V-type and C2-type immunoglobulin domain, a C-terminal PDZ domain, and a transmembrane domain (63). It was first cloned in 2002 and was then indicated to be a cell adhesion molecule that mediates homophilic cellular interactions (32). *VSIG3* is also known as IgSF gene 11 (IgSF11) or brain- and testis-specific IgSF (BT-IgSF) because of its abundant expression in these two organs in mammals (32, 64). hVSIG3 contains 12 exons encoding two isoforms that share 97% amino acid identity and are thus considered to be identical in function. VSIG3-mediated cell adhesion can regulate the development of neurons and excitatory synaptic transmission and the differentiation of osteoclasts, and its mutation in zebrafish was associated with impaired migration and survival of melanophores (65, 66).

In addition to its role in cell adhesion, one of the most notable functions of VSIG3 relies on its capacity to regulate immune responses. VSIG3 is reported to be a ligand for the novel B7 family immune checkpoint V-domain immunoglobulin suppressor of T-cell activation (VISTA) (21, 22, 67). VISTA is mainly expressed on naïve T cells and functions as an important regulator in maintaining T-cell tolerance through the induction of peripheral T-cell deletion (68). Truncated VSIG3 ECD containing either the IgV- or IgC2-type domain bound to human VISTA protein in a similar manner as the full-length ECD. Most importantly, the crosslinking of VSIG3 during TCR stimulation significantly inhibited T-cell activation by reducing the production of cytokines and chemokines. Blockade of VISTA significantly attenuated VSIG3-mediated T-cell inhibition, suggesting that this process is dependent on its recognition with VISTA (67). The VSIG3 and VISTA interaction was further demonstrated by Xie et al. using a coimmunoprecipitation (Co-IP) assay (21). They also revealed the crystal structure of the human VSIG3 ECD and designed a small molecule inhibitor, K284-3046, based on protein–protein docking analysis. This chemical inhibitor showed potent effects in diminishing VSIG3-mediated T-cell suppression (21).

The identification of VSIG3 as a binding partner for VISTA has important implications for tumor immunotherapy. As a well-known coinhibitory molecule for T cells, VISTA is highly expressed in myeloid cells and T cells that infiltrate into tumors and help create an immunosuppressive tumor microenvironment by enhancing Treg differentiation and inhibiting T-cell activation. In contrast, VSIG3 was highly expressed in a number of cancers, such as colorectal cancers, gastric cancer, hepatocellular carcinomas, and gliomas, but not on immune cells (63, 69). Suppression of VSIG3 by small interfering RNA (siRNA) attenuated the proliferation of gastric cancer cells *in vitro*, suggesting that the expression level of VSIG3 is essential for the fate of cancer cells (63). Ghouzlani et al. also found that high VSIG3 expression was related to a strong immunosuppressive microenvironment and functionally compromised T cells in glioma (70). Therefore, it is speculated that highly upregulated VSIG3 in tumor cells could reinforce immune inhibitory signals to VISTA-expressing T cells in the tumor microenvironment, generating antibodies or chemical inhibitors that specifically block the VSIG3-VISTA interaction and could increase the efficiency of VISTA-based ICB therapy (22).

VSIG4

VSIG4, also known as Z39Ig or CRIg, was first described in 2000 as an X chromosome-located gene (29). VSIG4 contains two Ig-like domains, one complete IgV domain and a truncated IgC domain, and shares all the conserved amino acids with known B7 family members; thus, it is also considered a B7 family-related protein (23). There exist two alternatively spliced forms of human VSIG4 protein: the long isoform of VSIG4(L) encodes both IgV and IgC2 domains, while the short isoform of VSIG4(S) encodes only a single IgV domain (37). In comparison, murine VSIG4 only shows the short isoform of a

single IgV domain. Human and mouse VSIG4 share 83% sequence homology within the IgV domain (71). The IgV domain is responsible for binding to the β chain of C3b to promote phagocytosis (72). The intracellular portion of VSIG4 contains a cAMP/cGMP-dependent protein kinase phosphorylation site and a protein kinase C phosphorylation site, yet the function of these sites remains unclear (29). Notably, VSIG4 expression is restricted to tissue resident macrophages, including liver Kupffer cells (37), peritoneal macrophages (23), pancreatic macrophages (73, 74), synovial lining macrophages in the joint, and interstitial macrophages in the heart (38, 75, 76); however, the extent of expression on these macrophages can be downregulated by inflammatory cytokines (77, 78). This expression pattern suggests a role for VSIG4 in maintaining tissue homeostasis.

VSIG4 is well known as the complement receptor of the Ig superfamily (CRIg) with a high binding affinity to complement components C3b and iC3b. Upon activation, C3—the central component of the complement system—is cleaved to a small C3a fragment and a large C3b fragment by C3 convertase, and iC3b is subsequently produced by complement factor I-mediated cleavage of C3b (79, 80). Both C3b and iC3b are potent opsonins that can coat the surface of invading pathogens, apoptotic cells, or immune complexes to facilitate their clearance by the mononuclear phagocytic system. By recognizing C3b- and iC3b-tagged pathogens, accumulating evidence suggests a critical role of VSIG4 in host defense against bloodstream infections by promoting Kupffer cells to take up complement-tagged bacteria, fungi, and viruses (37, 81, 82). This function is vital to prevent the dissemination of pathogens to some vulnerable organs, such as the heart and kidney (37). However, it was also reported that VSIG4 facilitated a relatively slow clearance of circulating bacteria when compared to scavenger receptor-mediated fast clearance (83). This slow clearance of circulating bacteria may be essential to enable a timely induction of adaptive immune responses. Moreover, VSIG4 was suggested to be a pattern recognition receptor that directly binds and captures blood-borne gram-positive bacteria by recognizing lipoteichoic acid (LTA) during *Staphylococcus aureus* infection (84).

Structural analysis revealed that VSIG4 can also dramatically inhibit the activity of C3 and C5 convertase upon binding to C3b, thereby preventing the alternative pathway of complement activation (72). Given that inappropriate activation of complement is usually associated with unwanted and exacerbated inflammation, recombinant VSIG4-Fc protein has been exploited as a decoy receptor to efficiently alleviate a variety of inflammatory diseases by preventing excessive complement activation, such as experimental autoimmune uveoretinitis (EAU) (85), intestinal ischemia/reperfusion (IR) injury (86), type 1 diabetes (73, 74), arthritis (71, 72), and SLE (87). This complement inhibitory function could be further improved by fusing VSIG4 with the alternative pathway inhibitory domain of factor H (FH) (88). In addition, recent studies reported that VSIG4 can directly inhibit macrophage-mediated inflammation independent of complement. Huang et al. found that macrophages lacking VSIG4 showed increased activation of the NLRP3 inflammasome upon stimulation (89). VSIG4 was found to interact with the transmembrane protein MS4A6D to form a surface inhibitory signaling complex, leading to attenuated NLRP3 inflammasome activation *via* a JAK2-STAT3-A20 signaling cascade (89, 90). In addition, VSIG4 was also able to intervene in mitochondrial pyruvate metabolism in macrophages by activating the PI3K-Akt-STAT3 pathway, thereby resulting in reduced oxidative phosphorylation and diminished M1 polarization of macrophages (91). These data suggest the possible applications of targeting VSIG4 in treating inflammatory diseases.

As a macrophage-specific immune regulator, VSIG4 is a potent coinhibitory ligand that strongly suppresses T-cell proliferation and cytokine production. T-cell stimulation in the presence of recombinant VSIG4 caused T-cell anergy, cell cycle arrest at the G0/G1 phase (23, 73, 74, 92), and skewed differentiation of $CD4^+$ T cells towards $Foxp3^+$ Treg cells. Importantly, this T-cell inhibitory effect was only found with plate-bound but not soluble VSIG4 protein, suggesting that the crosslink of VSIG4 with a putative binding partner on the surface of T cells is required to deliver inhibitory signals. Indeed, VSIG4 can directly bind activated T cells without the need for serum, demonstrating the existence of a complement-independent ligand of VSIG4 on T cells, which remains to be determined. Although VSIG4 expression is restricted in tissue-resident macrophages at a steady state, several studies have reported an upregulation of VSIG4 expression in lung cancer (93), breast cancer (94), ovarian cancer (95), and multiple myeloma (MM) (96). By examining VSIG4 expression in tumor tissues, it was found to be highly enriched in tumor-associated macrophages but not in tumor cells or other immune cells (93). High expression of VSIG4 is correlated with poor prognosis of high-grade glioma (97), and its deficiency led to significantly inhibited growth of Lewis lung cancer cells (LLC) in mice (93). Based on these findings, VSIG4 is becoming an attractive macrophage-specific immune checkpoint molecule in cancer immunotherapy. Identifying the ligand of VSIG4 on T cells would be pivotal for understanding the mechanisms whereby VSIG4 modulates antitumor T-cell responses and is fundamentally important for developing high-efficacy inhibitors that aim to block VSIG4-mediated T-cell suppression in cancer Figure 1.

VSIG8

VSIG8 is a relatively less explored member of the VSIG family, approximately 45 kDa in size. Mature hVSIG8 contains two Ig-V domains, which are a part of the ECD spanning 242 aa.

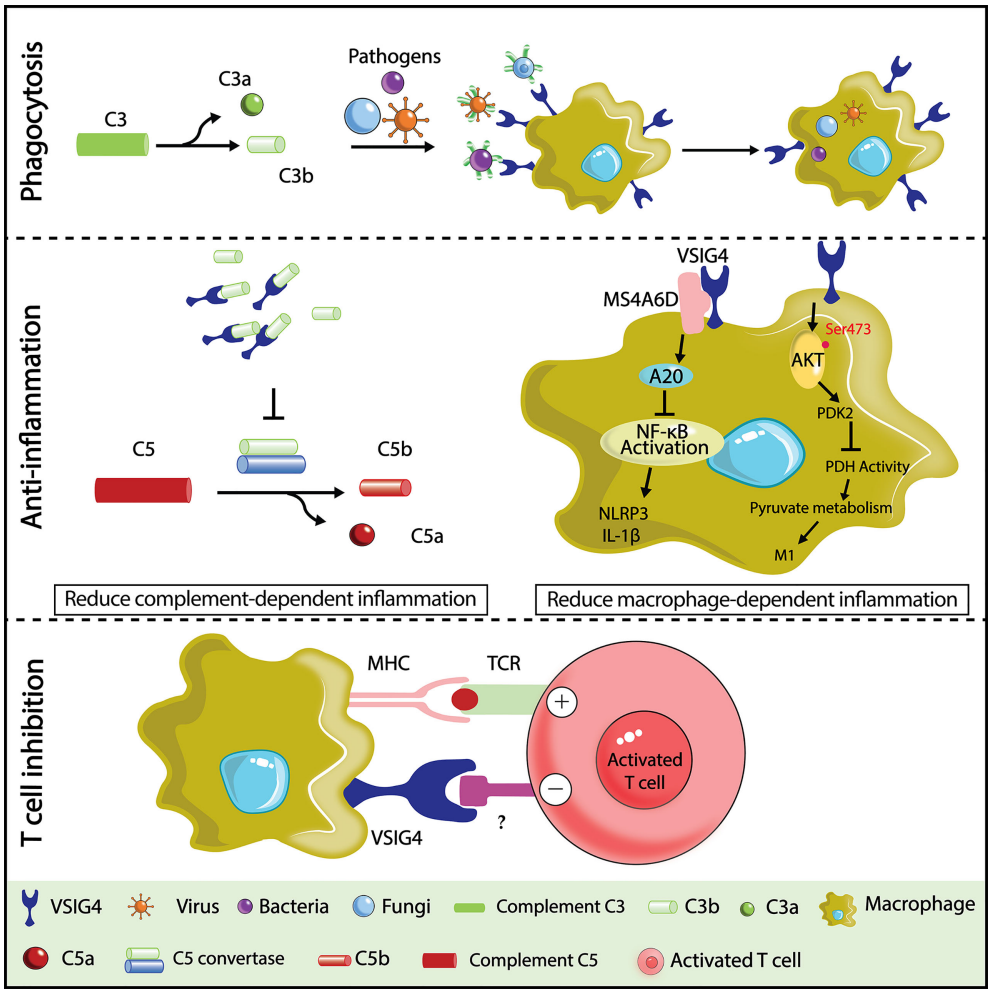

FIGURE 1
Illustration of VSIG4 functions. In host defense against bloodstream infections,VSIG4 recognizes C3b and helps macrophages phagocytose C3b- or iC3b-tagged pathogens (bacteria, viruses, fungi, etc.). In a variety of complement activation-dependent inflammatory diseases, VSIG4 delivers anti-inflammatory signals by binding C3b to prevent the alternative pathway of complement activation. VSIG4 also inhibits macrophage M1 activation by regulating inflammasome activation and pyruvate metabolism. VSIG4 also inhibits T-cell activation, proliferation, and IL-2 production upon binding to an unknown ligand on T cells.

hVSIG8 shares 88% and 89% identity with the VSIG8 of mouse and rat, respectively. It was identified through proteomic analysis of the human hair shaft (35, 36) and found to also be expressed in the oral epithelium, superficial layers of the nail matrix, and hair follicles (98). Interestingly, Wang et al. reported that immobilized recombinant VSIG8 could suppress human T-cell proliferation and cytokine production and decrease the differentiation of naïve $CD4^+$ T cells towards Th1 cells, confirming its role as a negative regulator of T-cell responses (99). VSIG8 was later reported to be a putative binding partner of VISTA (100), and a US patent (WO2016090347 A1) also reported the interaction of VSIG8 and VISTA, demonstrating the suppressive effects of this interaction on T cells. In addition, Chen et al. demonstrated the VSIG8-VISTA interaction by ELISA, MST and Co-IP assays and confirmed its function in inhibiting human T-cell activation (101). However, a recent study using a functional ELISA suggested no interaction between human VSIG8 and VISTA (67). Similarly, George et al. generated a two-sided fusion protein that contained the ECD of both VSIG8 and OX40 L and reported no binding between this fusion protein and recombinant VISTA, although it was able to bind VISTA-expressing macrophages or tumor cells. Nevertheless, this VSIG8-Fc-OX40 L fusion protein stimulated T-cell activation and antitumor activity, possibly by blocking VSIG8-mediated inhibitory signaling (102). Future studies generating VSIG8-deficient animals and blocking antibodies will further enhance our understanding of this potential immune checkpoint molecule in cancer immunotherapy.

VSIG9

VSIG9, well known as TIGIT, with a full name of T-cell immunoglobulin and ITIM domain (also known as WUCAM or Vstm3), is currently one of the most attractive and promising immune checkpoint targets. TIGIT also belongs to the poliovirus receptor (PVR)/nectin family and is widely expressed on activated NK cells, $CD8^+$ T cells, $CD4^+$ T cells, and Treg cells (24–26, 33, 39). TIGIT was discovered in 2009 by three independent groups (25, 26, 33). One reported that TIGIT was an adhesion molecule mediating TFH (follicular T helper) and FDC (follicular DC) interactions (33), whereas the other two identified TIGIT as a coinhibitory receptor on T and NK cells (25, 26). The structure of TIGIT comprises a short intracellular domain with one immunoglobulin tyrosine tail (ITT)-like motif and one immunoreceptor tyrosine-based inhibitory motif (ITIM), a type I transmembrane domain, and an extracellular IgV domain (26, 33, 39). While there is a 58% overall sequence identity between human and mouse TIGIT (25, 26), their ITIM-containing sequences that confer immune inhibitory functions are identical. TIGIT has three ligands, including CD155, CD112, and CD113, which all belong to the PVR/nectin family receptors (25, 103, 104). Their expression features and binding affinity with TIGIT are listed in Table 3.

Among these ligands, CD155 exhibited the highest affinity with TIGIT (104, 105). CD155 is mainly expressed on dendritic cells (DCs), T cells, B cells and macrophages. Engaging of TIGIT with CD155 has been shown to prevent excessive immune cell activation and sustain immune homeostasis (33, 106–108). Notably, there are two other PVR family receptors, CD226 and CD96, which share sequence homology with TIGIT and compete with TIGIT for CD155 binding (109–111). However, as opposed to TIGIT and CD96, CD226 acts as an activating receptor that promotes T-cell and NK-cell activation opon CD155 ligation (112–114). TIGIT binds CD112 and CD113 with lower affinity than CD155, and the functional consequences of their binding have been less characterized. CD112 is also known as the ligand for the coinhibitory receptor CD112R, which was recently discovered as an immune checkpoint receptor expressed on T cells and NK cells (109, 115). Similar to CD155, CD112, another common ligand of TIGIT, can also bind CD226 (109). The competition and balance among TIGIT, CD226, CD96, and CD112R for the same ligands is quite complex and has been extensively reviewed elsewhere (112, 116, 117). The interactions between TIGIT and its ligands with other VSIG members are summarized in Figure 2.

Because of the binding affinity advantage, the TIGIT-CD155 interaction prevails in TIGIT-mediated immune inhibition (26). The TIGIT-CD155 axis exerts its inhibitory effects on T and NK cells through three distinct mechanisms of action, including 1) a cell intrinsic manner by transmitting inhibitory signals to the effector cells (26); 2) a cell extrinsic manner by modulating the cytokine profile of CD155-expressing cells, such as DCs and macrophages (26, 107); and 3) by competing with CD226-mediated costimulatory signals (118). The cell intrinsic effect of TIGIT was well characterized by Inozume et al. by expressing truncated CD155 without a cytoplasmic domain, which was sufficient to suppress T-cell production of IFN-γ in a TIGIT-dependent manner (119). Similarly, agonistic anti-TIGIT mAbs were capable of dampening mouse and human T-cell proliferation and cytokine production (39, 118, 120). Following TIGIT-CD155 binding, the ITT-like motif is phosphorylated and subsequently bound to growth factor receptor-bound protein 2 (Grb2) or β-arrestin 2, which leads to the recruitment of SHIP-1 and SHP2 and abolishment of PI3K and MAPK and NF-κB signaling (121). TIGIT can also work in a cell-extrinsic manner by modulating the cytokine profile of CD155-expressing cells, such as DCs and macrophages. The altered cytokine milieu, e.g., increased IL-10 levels and decreased IL-12 levels, in turn, can lead to attenuated activation of NK and T cells (26).

The tumor microenvironment has taken advantage of the TIGIT-CD155 inhibitory pathway as an important strategy to evade immune surveillance and thus result in uncontrolled tumor growth (122, 123). TIGIT is highly expressed on $CD8^+$ tumor-infiltrating lymphocytes (TILs) in various tumors, such as gastric cancer, colon cancer, breast cancer, melanoma, and NSCLC (124–127). $TIGIT^+$ $CD8^+$ TILs are dysfunctional with reduced effector cytokine production and impaired degranulation, exhibiting a typical feature of exhaustion. Blocking the TIGIT pathway can drastically reverse T-cell exhaustion. In AML, $TIGIT^+$ $CD8^+$ exhausted T cells were reinvigorated by knockdown of TIGIT expression (128). The prominent advantage of TIGIT over other immune checkpoints lies in its potent ability to restrain NK-cell responses. Upon binding to its ligand CD155 expressed by tumor cells, TIGIT-expressing NK cells dramatically diminish their cell cytotoxicity (129, 130). Zhang Q et al. showed that antibody blockade of TIGIT prevented NK exhaustion and unleashed antitumor

TABLE 3 Expression patterns of TIGIT ligands and their relative binding affinity and function.

	CD155	CD112	CD113
Expression cell types	DCs, T cells, B cells, macrophages, tumor cells	DCs, T cells and B cells, CD14+ cells, monocyte, tumor cells	T cells, tumor cells
Receptors and Binding Affinity(+)	TIGIT (++) CD226(+) CD96(+)	TIGIT (+) CD226 (+) CD112R (++)	TIGIT (+)
Function	Inhibitory ligand	Inhibitory ligand	Inhibitory ligand

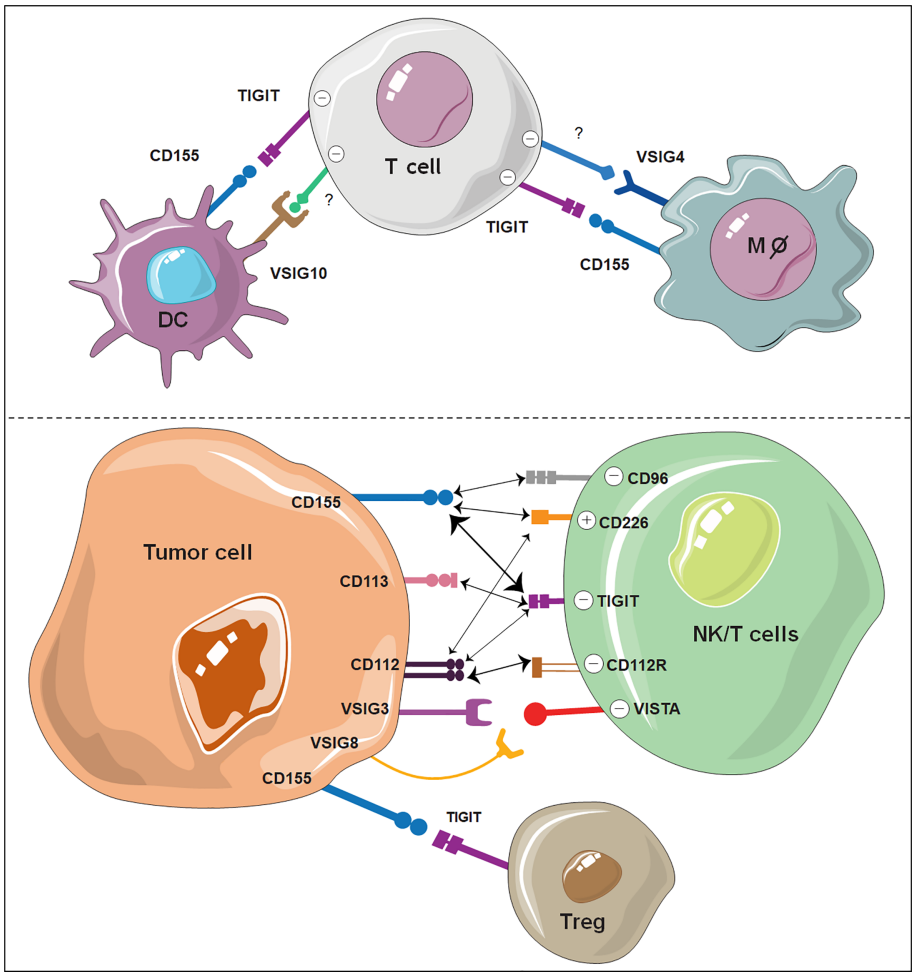

FIGURE 2
Illustration of VSIG family members as potential ICBs on immune cells. The interaction of TIGIT, VSIG3, VSIG8 and VSIG4 with other ligand-expressing cells shows great potential as novel immune checkpoints. VSIG10 also shows potential as a coinhibitory receptor on DCs. The width of the arrows is proportional to the relative binding affinities. The strongest interactions are between TIGIT/CD155 and CD112R/CD112. Negative (-) represents an inhibitory signal, and positive (+) represents an activating signal.

immunity in an NK-cell-dependent manner, collectively leading to tumor regression (131). Moreover, TIGIT is highly expressed on Treg cells and is essential to maintain the suppressive capabilities of Tregs, which potentially inhibit a variety of immune cells by suppressing Th1 and Th17 cells (132–135). A study demonstrated that TIGIT suppresses antitumor immunity primarily *via* Tregs but not $CD8^+$ T cells (135). In addition, TIGIT also functions as a ligand to skew the maturation or polarization of intratumoral myeloid cells, including DCs and macrophages, towards a state with increased IL-10 but decreased IL-12 secretion (26). This results in DC tolerance and alternative activation of macrophages, both contributing to tumor immune tolerance.

Owing to the importance of TIGIT-CD155 engagement in NK and T-cell dysfunction in tumors, developing therapeutic agents to block this pathway holds great promise for cancer immunotherapy. There is strong evidence that TIGIT blockade has a direct effect in reversing T-cell dysfunction in cancer patients. Anti-TIGIT mAb treatment has been shown to escalate the proliferation, cytokine production, and degranulation of bone marrow $CD8^+$ T cells from MM patients and peripheral blood $CD8^+$ T cells from melanoma patients (125, 136). Recent advances have also proposed a dual blockade of PD-1 and TIGIT as a more inspiring method for cancer immunotherapy than a single TIGIT blockade. Whereas blocking each of the PD-1/PD-L1 or TIGIT pathways does not remarkably impede the growth of CT26 tumors, a dual blockade synergizes to increase the proliferation and function of antitumor $CD8^+$ T cells, which results in protective memory T cells, complete tumor rejection, and overall prolonged survival

(126, 131). These effects have been abrogated upon $CD8^+$ T-cell deficiency. The translational potential of dual PD-1/TIGIT inhibition has already been demonstrated; it increases the proliferation and function of intratumoral antigen-specific $CD8^+$ T cells in melanoma patients to an extent that is much more dramatic than a single blockade (119, 125). A recent phase II study also indicated that dual PD-L1/TIGIT blockade (atezolizumab/tiragolumab) has superior clinical benefits compared to PD-L1 blockade alone in NSCLC patients, despite similar profiles of toxicity and tolerability (137). Apart from PD-1, TIGIT blockade could also synergize with other ICBs in cancer immunotherapy. For instance, TIGIT and TIM-3 inhibition in mice cooperated to promote an antitumor immune response (135); dual blockade of TIGIT and LAG3 improved the treatment efficacy in a mouse model of anti-PD-1-resistant lung cancer (138). In conclusion, TIGIT, as a new immune checkpoint, possesses great potential for cancer immunotherapy. Effective tumor control for certain types of cancer can be expected by combining anti-TIGIT with other ICB inhibitors.

VSIG10 and VSIG10 L

VSIG10 contains four Ig-like C2-type domains in its ECD with 63% identity between hVSIG10 and mVSIG10 (139, 140). VSIG10 was highly expressed on both normal and cancer epithelial cells based on transcriptional data. Moreover, Papasotiriou et al. reported the overexpression of VSIG10 in adenocarcinoma; however, no expression was observed in melanoma, prostate, breast, or pancreatic cancer (141). Until recently, there was no report about its biological function. According to a US patent (Application #20200270343), recombinant VSIG10-Fc fusion protein was able to suppress $CD4^+$ T-cell activation and cytokine production, pinpointing its potential as an immune checkpoint inhibitor. Interestingly, VSIG10 was also predicted to be abundantly expressed by DC subsets both in humans and mice. Growing data points toward the importance of immune checkpoints expressed on DCs in dampening the antitumor response. For instance, DCs highly express PD-L1 and have been demonstrated to be an important target of PD-L1 blocking antibodies (142). These PD-L1-expressing DCs are identified to attenuate T-cell activation and regulate its response to ICBs (143).

Hence, the generation of anti-VSIG10 antibodies, as reported in the patent, may present a promising DC-targeting ICB cancer immunotherapy. Similar to VSIG10, VSIG10 L also contains IgC2 in its ECD. VSIG10 L is normally expressed in the healthy esophagus and squamous mucosa; however, it is downregulated in esophageal adenocarcinoma and Barrett's esophagus (144). High expression is found in lung squamous cell carcinoma (145), pointing to the dual nature of VSIG10 L in cancers. Further exploration is needed, as they could be potential biomarkers or immune checkpoints.

Conclusion

ICB treatment has brought a revolutionary advance for cancer therapy in the past decade. Antibodies targeting PD-1 and CTLA-4 were approved by the FDA and were proven to be effective against several cancer types. Despite tremendous success, over half of the patients remain poorly responsive to these regimens, possibly due to the involvement of multiple immune inhibitory pathways in the cancer microenvironment. Therefore, seeking new immune checkpoint molecules is becoming increasingly important for the optimization of ICB-based cancer immunotherapy. Here, we show that many VSIG family members show potent effects of T-cell inhibition in cancer, and antitumor immunity can dramatically benefit from the blockade of these molecules. The most attractive and promising member of the VSIG family is TIGIT, and its blockade has achieved great success in reinvigorating antitumor NK and T-cell responses (126, 131, 135). There are currently over 50 clinical trials underway to study the therapeutic effect, safety, and tolerability of TIGIT blockade in cancer, either using it alone or in combination with other cancer therapeutics. Apart from TIGIT, VSIG3, VSIG8, and VSIG4 also show great potential as novel immune checkpoints. As putative binding partners for the well-known coinhibitory molecule VISTA, both VSIG3 and VSIG8 were able to negatively regulate T-cell responses and can be targeted in certain cancer types in which antitumor immunity is predominantly affected by the VISTA pathway. VSIG4 is of particular interest because it is specifically expressed by tissue resident macrophages, which are becoming increasingly appreciated as critical contributors to tumor progression and metastasis. Blockade of VSIG4 to functionally reprogram macrophages thus stands out as an important complement to the current T-cell-based immunotherapy regimens. In addition, although not fully validated, VSIG10 shows potential as a coinhibitory receptor expressed by another important type of myeloid immune cell, namely, DCs, which are also largely overlooked in the field of immune checkpoint therapy. Overall, VSIG family proteins represent an important group of transmembrane receptors that emerge as immune checkpoints controlling the fates of multiple types of immune cells in tumors, spanning from myeloid cells to lymphoid cells. Therapeutically targeting these proteins could be beneficial to the current regimen of ICB treatment in cancer.

Author Contributions

XZ and SK wrote the manuscript, LL and DH supervised the study and revised the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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Durable disease control and refractory bullous pemphigoid after immune checkpoint inhibitor discontinuation in metastatic renal cell carcinoma: A case report

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Background: Immune checkpoint inhibitors deeply modified metastatic renal cell carcinoma's management, and confront us to adverse events that we were not used to with conventional anti-cancer therapies. We report the case of a patient who received nivolumab as second-line treatment of a metastatic clear cell renal cell carcinoma and who developed bullous pemphigoid four years after nivolumab introduction, with persistent exacerbations even after its discontinuation.

Case presentation: A 66-year-old man was diagnosed with lung metastasis eight years after radical nephrectomy for a clear cell renal cell carcinoma. He firstly received an anti-angiogenic agent combination, and then received anti-programmed death 1 (PD1) nivolumab as second-line treatment. Nivolumab led to prolonged disease control, but after four years of exposure the patient developed skin lesions consistent with bullous pemphigoid. After seven years of nivolumab administration and perfect disease stability, nivolumab was discontinued and surveillance was proposed. Despite nivolumab discontinuation, the patient continued to develop bullous pemphigoid exacerbations. Metastatic renal cell carcinoma was still perfectly stable more than two years after immune checkpoint discontinuation with no further anti-cancer therapy.

Discussion: We report the case of a refractory bullous pemphigoid which occurred four years after nivolumab introduction and lasted despite nivolumab discontinuation, in a patient whose metastatic renal cell carcinoma is still controlled after more than two years without any anticancer treatment. This highlights the potential association between immune-related adverse events and response to immune checkpoint inhibitors, and underlines the occurrence of late-onset and long-lasting immune-related adverse events even after

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discontinuation of treatment, which must encourage us to remain vigilant in the long term.

KEYWORDS

renal cell carcinoma, immunotherapy, bullous pemphigoid, durable response, late adverse event

Background

Kidney cancer, among which renal cell carcinoma (RCC) is the most common form, represents the 7th most common cancer in men, and the 10th most common cancer in women (1). Metastatic renal cell carcinoma's management has been deeply modified by the approvement of immune checkpoint inhibitors (ICI). Nivolumab, an anti-programmed death 1 (PD1) monoclonal antibody, was firstly used as monotherapy for advanced clear cell renal cell carcinoma (ccRCC) who experienced progression after antiangiogenic therapy (2). It was secondly approved as first-line treatment for ccRCC in association with ipilimumab – another ICI that targets the Cytotoxic-T-Lymphocyte-Antigen 4 protein (CTLA4) – for intermediate and poor risk patients (3), and then in association with the tyrosine kinase inhibitor (TKI) cabozantinib, regardless of the patient risk (4). Pembrolizumab, another PD1 monoclonal antibody, has also been approved as first-line treatment in this setting, in association with the TKI axitinib or lenvatinib (5, 6). These new agents, whose mechanism of action is based on anti-tumor immunity enhancing, present a very specific safety profile, resulting in several immune-related adverse events (irAE) whose management is very different from those we were used to (7). Since the first approval of ipilimumab in advanced melanoma in 2011 (8), the knowledge of ICI's safety profile is evolving rapidly over time consequently to the duration of utilization and the increasing number of tumors in which they are used.

We report the case of a refractory bullous pemphigoid (BP) following nivolumab discontinuation in metastatic ccRCC with durable response.

Case presentation

In 2001, a 58-year-old Caucasian man had radical nephrectomy for pT3N0 ccRCC, Fuhrman grade III. In 2009, lung metastasis was histologically proven. The patient had a favorable risk metastatic ccRCC, according to the IMDC (International Metastatic RCC Database Consortium). He was enrolled in a clinical trial and received a combination of sunitinib and trebananib (AMG 386) – a recombinant fusion protein which neutralizes interaction between angiopoietin-1/2 and its receptor (9). Trebananib was stopped in 2012 for toxicity, and sunitinb was maintained until progression in 2013. The patient was then included in the Checkmate 025 trial (NCT01668784) which compared second line treatment by nivolumab versus everolimus, and received nivolumab.

In April 2014, he presented a single lung injury treated by stereotactic radiation and nivolumab continuation. In 2017, after four years of well-tolerated nivolumab administration, the patient developed pruriginous skin lesions. The histologic analysis of the cutaneous biopsy was compatible with BP. This hypothesis was reinforced by the presence of anti-basal membrane antibodies in the patient's serum. Oral corticosteroid therapy with prednisone was introduced and then progressively decreased. In May 2020, after seven years of nivolumab administration and perfect disease stability, nivolumab was discontinued and surveillance was proposed. In October 2020, as systemic corticosteroids had been decreased to 10 mg per day, the patient presented new pruriginous skin lesions associated with cutaneous blisters, which biopsy and direct immunofluorescence revealed a junctional bullous auto-immune dermatitis, concordant with the BP diagnosis that had previously been established (Figure 1). Serum analysis revealed anti basal membrane (>80 UR/mL), antiBP180 (27 UR/mL, N <20) and anti BP230 (50 UR/mL, N < 20) antibodies, consistent with this diagnosis. Oral corticosteroid therapy was thus increased, and then very progressively decreased. In October 2021, as anti PD1 had been stopped for more than one year, the patient presented a new BP exacerbation, and oral corticosteroid therapy was reintroduced (60 mg per day), and then replaced by Methotrexate in December 2021. In the meantime, regular follow up of the ccRCC was continued, and computed tomography scan continued to show perfect stability of the disease at the last follow-up in August 2022. BP is relatively controlled by Methotrexate, even if he still presents pruritis than requires prolonged symptomatic treatment (Figure 2).

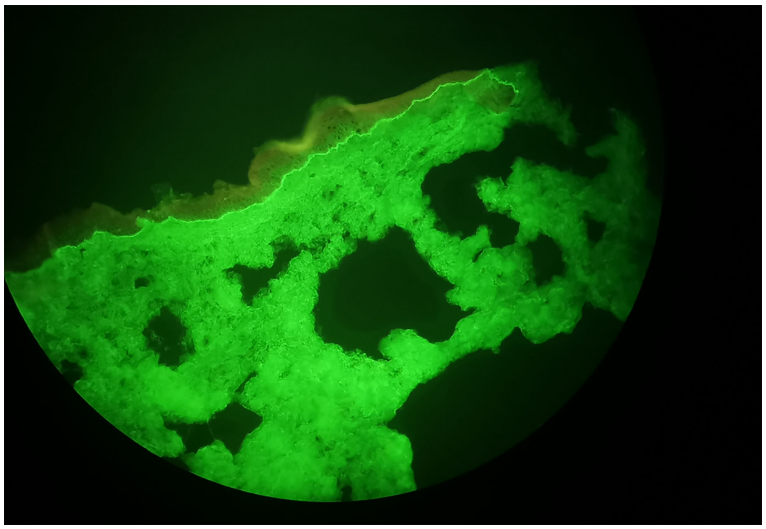

FIGURE 1
Direct Immunofluorescence consistent with Bullous Pemphigoid Diagnosis.

Discussion

We report the case of a durable disease control even after ICI discontinuation, in a patient treated for a lung metastatic ccRCC for more than twelve years, and who presented a junctional bullous auto-immune dermatitis four years after nivolumab introduction. He had no history of auto-immune disease, and had not taken any other treatment likely to trigger this BP, which suggest that it could be an irAE. He continued to present BP exacerbations despite oral corticosteroid therapy and nivolumab discontinuation for 18 months, and was offered methotrexate treatment, which finally seems to control the BP. To date, his metastatic ccRCC is still perfectly controlled more than two years after nivolumab discontinuation.

The arrival of immunotherapy has profoundly changed our way to manage cancer therapy toxicities, and irAE are now well-known side effects of ICI. In the phase III CheckMate 025 trial, irAE were described in approximately 79% of patients receiving nivolumab for TKI refractory metastatic RCC, with 19% being grade 3 or 4 (2). Pruritus was the second most observed treatment adverse-event (14% of patients), and cutaneous rash was the sixth (10% of patients).

Regardless of the type of cancer, cutaneous toxicities are one of the most common irAE, observed in one third of the treated patients, mainly in the form of pruriginous rash (10). Vitiligo is another well-known cutaneous adverse event, most frequently observed in melanoma but also reported in other cancer types, with an estimated overall incidence of 7,5% for nivolumab (11). The occurrence of vitiligo has been shown to be associated with tumor response to ICI in some cases, especially in melanoma but also in cases of non-small cell lung cancer (NSCLC) or RCC (12–14).

Autoimmune bullous cutaneous disorder, such as BP, are less common cutaneous irAE, but several cases of immune induced BP have been described, especially in melanoma and NSCLC (15).

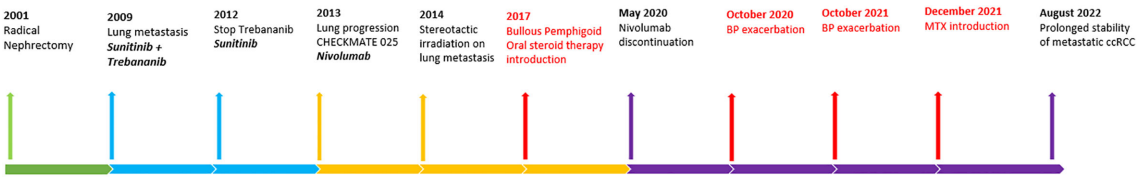

FIGURE 2
Diagnosis and Treatments Timeline.

BP is an acquired skin disorder in which auto antibodies led to subepidermal detachment of the epidermis from the underlying dermis. This results in cutaneous blisters, often preceded by an initial non-bullous phase of pruritus and non-specific maculopapular eruption. Direct immunofluorescence classically shows linear deposits of IgG and C3 at the dermoepidermal junction. Circulating antibodies directed against BP180 and BP230 – two hemidesmosomal structural proteins – may be found in serous samples.

Whereas classic BP is idiopathic, some BP are triggered by drugs such as antibiotics, non-steroidal anti-inflammatory drugs, diuretic, hypoglycemic agents. These drug-induced BP usually resolve after withdrawal of the causative agent (16). Immune-related BP mechanism is unclear, but PD1 pathway's blockade may increase antibodies production against the hemidesmosomal protein BP180, through a process that may be T-cell and B-cell induced. Some cases of immune related BP associated with tumor response have been described, especially in melanoma and NSCLC (15, 17), but to our knowledge this is the first case of durable disease control associated with BP in RCC. It has been suggested that BP180 may be a common antigen, found both in the dermo-epidermal junction and on the surface of malignant melanocytic tumor cells and NSCLC cells, and that this cross-reactivity between tumor neoantigens and normal tissue antigens may explain the association between immune related BP and tumor response (18, 19). However, we did not find any data concerning BP180 expression by renal cancer cells.

More generally, the association between irAE and efficacy of ICI has already been reported, especially in melanoma, in which a pooled analysis of 576 patients treated with nivolumab for advanced melanoma showed a significant overall response rate improvement in patients who experienced irAE (20). It has also been reported in NSCLC, with improved overall and progression-free survivals in patients who had experienced irAE (21, 22), and this association was particularly reported for immune related thyroid dysfunction, which is consistent with other studies (23, 24). Furthermore, late adverse events might be associated with better response rate and overall survival, as it has been shown in a recent study which compared outcomes in patients with NSCLC and other types of cancer, and showed better outcomes in patients who experienced irAE occurring more than 3 months after ICI initiation, compared to patients whose irAE occurred earlier (25). Concerning RCC, some studies also reported an association between the occurrence of irAE and efficacy of ICI, resulting in improved overall and progression-free survival (26, 27). However, to our knowledge, specific association between cutaneous irAE, and more specifically immune induced bullous disease, has not been reported yet.

For our patient, this cutaneous irAE occurred four years after nivolumab introduction, and lasted even after ICI discontinuation, underlining the fact that irAEs can occur tardily, and that long-lasting irAEs can persist despite ICI discontinuation. Some cases are reported concerning late irAEs that had occurred several months after the introduction of ICI. For example, a case was described concerning a patient treated for a metastatic ccRCC who experienced immune-related renal toxicity after 19 months of nivolumab, and who maintained clinical response even after ICI discontinuation (28). Another patient, treated by nivolumab for platinum refractory laryngeal carcinoma, firstly presented pseudo-progression followed by progressively complete response achieved after 16 courses of nivolumab. A grade 1 interstitial pneumonitis was simultaneously identified, that lead to ICI discontinuation after 18 courses. He then experienced grade 2 immune-related colitis 5 months after ICI discontinuation. Complete response was still maintained 18 months after nivolumab discontinuation (29). Another case was reported concerning a patient who presented reappearance of immune-related colitis one year after ICI discontinuation, underlying the potential of delayed and prolonged alteration of gastro-intestinal immune response (30). These cases underline the fact that irAE can occur after prolonged exposure to ICI, and can last even after ICI discontinuation, suggesting that the PD1 occupancy on T cells could remain for months after ICI exposure (31).

Conclusion

We herein report the case of a refractory BP which occurred four years after nivolumab introduction and lasted despite nivolumab discontinuation in a patient whose metastatic ccRCC is still controlled after more than two years without any anticancer treatment. As previously reported, this could highlight the potential association between irAE and response to ICI. It also underlines the existence of late-onset and long-lasting irAEs even after discontinuation of treatment, which should encourage clinicians to remain vigilant over the long term.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding author.

Ethics statement

Written informed consent was obtained from the individual(s) for the publication of any potentially identifiable images or data included in this article.

Author contributions

GG and RM: conception and manuscript writing. RM, MG, CV, JW, NB, GP and GG: final approval. GG, MG and CV: Patient's management.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Siglec-7 represents a glyco-immune checkpoint for non-exhausted effector memory CD8+ T cells with high functional and metabolic capacities

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While inhibitory Siglec receptors are known to regulate myeloid cells, less is known about their expression and function in lymphocytes subsets. Here we identified Siglec-7 as a glyco-immune checkpoint expressed on non-exhausted effector memory CD8+ T cells that exhibit high functional and metabolic capacities. Seahorse analysis revealed higher basal respiration and glycolysis levels of Siglec-7$^+$ CD8+ T cells in steady state, and particularly upon activation. Siglec-7 polarization into the T cell immune synapse was dependent on sialoglycan interactions *in trans* and prevented actin polarization and effective T cell responses. Siglec-7 ligands were found to be expressed on both leukemic stem cells and acute myeloid leukemia (AML) cells suggesting the occurrence of glyco-immune checkpoints for Siglec-7$^+$ CD8+ T cells, which were found in patients' peripheral blood and bone marrow. Our findings project Siglec-7 as a glyco-immune checkpoint and therapeutic target for T cell-driven disorders and cancer.

KEYWORDS

Siglec-7, CD8+ T cells, acute myeloid leukemia, immune checkpoint, tumor immunity and immunotherapy, sialoglycans, hypersialylation

Introduction

Siglecs are surface receptors that are differentially and broadly expressed on immune cells, and have recently emerged as critical immune checkpoints in health, inflammatory disease and cancer (1–4). These lectin receptors are thought to protect from autoreactivity by recognition of sialic-acid containg carbohydrates (sialoglycans) as so-called self-associated molecular patterns (SAMPs) (5). However, such sialoglycan ligands are overexpressed in a variety of different types of malignancies (6), eventually leading to tumor immune evasion by engagment of inhibitory Siglec receptors (7). Whereas Siglec-mediated suppression of immune responses could be therapeutically exploited in autoimmune and inflammatory disease, targeting Siglecs might restore anti-tumor responses as a form of normalization cancer immunotherapy (8). Indeed, a broad range of anti-Siglec therapies are currently explored in pre-clinical studies and first candidate drugs have been forwarded to evaluation in clinical trials (1, 9, 10).

The expression of Siglecs has been reported to be low or absent on human T cells (11–13). However, we and others recently reported enhanced Siglec expression on peripheral blood and tumor infiltrating T cells of cancer patients (14, 15). Notably, in melanoma tissues the majority of tumor-infiltrating lymphocytes consisted of Siglec-9$^+$ CD8$^+$ T cells (15). Mechanistic studies revealed that Siglec-7 and -9 can directly suppress TCR signaling (12, 15), which results in synergistic effects yet involves disparate signaling pathways compared to the immune checkpoint receptor PD1 (15). Together, these observations invigorate the interest in the role of Siglecs for T cell biology (3).

In the present study, we identified Siglec-7 as a glyco-immune checkpoint receptor on non-exhausted effector memory CD8$^+$ T cells with high functional and metabolic capacities, and a history of previous clonal expansion. Notably, Siglec-7 was found to congregate within the T cell immune synapse which was associated with reduced CD8$^+$ T cell effector functions including cytotoxicity and cytokine production. Primary leukemic cells and stem cells from patients with acute myeloid leukemia (AML), a malignancy so far ineffectively treated with immune checkpoint therapy, expressed high levels of Siglec-7 ligands. This might adversily affect effector responses of Siglec-7$^+$ CD8$^+$ T cells, which were found in the blood and bone marrow of AML patients. The understanding of Siglec-7 as a clonality-associated glyco-immune checkpoint might inspire novel therapeutic approaches to T cell-associated disorders and cancer.

Materials and methods

Cells and tissues

Blood from healthy donors was collected upon informed consent or buffy coats were purchased from the Blood Transfusion Center of Bern, Switzerland. Mononuclear cells were obtained by density centrifugation using Pancoll solution (PAN-Biotech, Aidenbach, Germany). For functional experiments, CD8+ T-cells were isolated using the EasySep™ Human CD8$^+$ T Cell Isolation Kit (StemCell Technologies, Vancouver, Canada), according to the manufacturer's instructions. The purity of isolated cells was >95%. For experiments with CD8$^+$ T cells subsets, cells were isolated using fluorescence-activated cell sorting (FACS Aria, BD Biosciences, Franklin Lakes, USA). Informed consent was obtained from all patients prior to tissue sample collection. Peripheral blood and BM aspiration samples were obtained from untreated AML patients at the University Hospital of Bern (Switzerland) after informed consent. Samples were stored in liquid nitrogen. AML bone marrow samples were thawed using citrate-dextrose solution in a concentration 1:10 in order to avoid cell clumping. Samples were washed and resuspended in FCS-containing medium prior to staining. All studies using human material were in accordance with the Helsinki Declaration and approved by the cantonal ethics committee of Bern, Switzerland. Written informed consent was received from participants prior to inclusion in the study. For redirected cytotoxic assay, the mouse mastocytoma cell line P815 (American Type Culture Collection ATCC, Manassas, VA, USA) was used exclusively between passage 5 and 8. No mycoplasma testing was performed.

Cell culture

Isolated CD8$^+$ T cells were cultured in RPMI medium (Sigma-Aldrich, Missouri, USA) containing 10% fetal calf serum (FCS) (Life Technologies, Waltham, USA) and 1% penicillin/streptomycin (Life Technologies) supplemented or not with 100 U/mL rhIL-2 (Peprotech, USA). When required, cells were activated with plate-bound αCD3 (1 μg/mL; OKT-3, BioXcell, Lebanon, USA) and soluble αCD28 (1 μg/mL, BioLegend, San Diego, USA) antibodies for 1 h at 37°C in supplemented medium. The mouse mastocytoma cell line P815 was cultured in DMEM medium (Sigma-Aldrich) containing 10% fetal calf serum (FCS) (Life Technologies) and 1% penicillin/streptomycin (Life Technologies).

Monoclonal antibodies and cell labeling

PBMCs, lymphocytes isolated from tissues or purified CD8$^+$ T cells, were labeled using fluorescent mAbs directed against surface molecules (20 min at 4°C), washed in PBS with 0.2% BSA (Sigma-Aldrich), and acquired using FACSVerse or FACSLyric (BD Biosciences, Franklin Lakes, NJ, USA). When required, cells were blocked using FC-block (human Trustain FcX, BioLegend, San Diego, CA, USA), and viability was analyzed using the Zombie NIR or Violet viability kit (BioLegend). Cells were labeled either directly *ex vivo* or, where indicated, after 30 min

of treatment with 25 mU neuraminidase (Roche Diagnostics, Rotkreuz, Switzerland) at 37°C. All mAbs were purchased from BioLegend, with the exception of fluorochrome-conjugated antibodies against CD3, CD8, CLA, CD45RA, LAG3, CXCR3, CCR4, and CCR7 (BD Bioscience); Siglec-9, CCR1 and CCR7 (R&D Systems, Minneapolis, USA); Siglec-7 (Beckman Coulter, Brea, CA, USA), and TNF-α (eBioscience, Waltham, MA, USA). Each mAb was titrated on PBMCs before use.

RNA analysis on TCGA database

TCGA RNA-seq data were retrieved from the GDC portal (https://portal.gdc.cancer.gov/) using the following filtering rules: Disease Type IS myeloid leukemias AND Workflow Type IS HTSeq - Counts AND Experimental Strategy IS RNA-Seq. The dataset consisted of 818 samples derived from 740 patients (274 primary cancer/bone marrow; 265 primary cancer/peripheral blood; 140 recurrent cancer/bone marrow; 139 recurrent cancer/peripheral blood). We defined the top/bottom 25% (n = 205) samples based on *SIGLEC7* (ENSG00000168995.12) and *SIGLEC9* (ENSG00000129450.7) expression values (transcript per million), respectively. Differential gene expression analysis was done between the "top" and "bottom" groups of samples (separately for Siglec-7 and Siglec-9) using DESeq2 and raw gene counts. Gene set enrichment analysis was done with GSEA 4.0.3 and the MSigDB v7.0 (16), using the "hallmark gene sets", and the following options: -nperm 1000 -scoring_scheme weighted -plot_top_x 200 -rnd_seed timestamp -set_max 500 -set_min 15. Input genes were all the differentially expressed genes with a padj value below 0.05. Plots were made in R 3.6.3 using ggplot2.

Telomere length measurement by automated multicolor flow-FISH

For telomere length analysis, human $CD8^+$ T cells Siglec-9^+, Siglec-7^+ and Siglec-$9^-/7^-$ subsets were isolated from the peripheral blood of 3 healthy donors by fluorescence-activated cell sorting as described above. Telomere length measurement by *in situ* hybridization and flow cytometry (automated multicolor flow-FISH) was performed as previously done (6): Briefly, 2.5×10^3 to 2×10^6 cells were used for *in situ* hybridization. Cells were incubated with 170 µL hybridization mixture containing 75% deionized formamide (Sigma-Aldrich), 20 mM Tris (pH 7.1; Sigma-Aldrich), and 1% BSA (Sigma-Aldrich) with no probe (unstained) or 0.3 µg/mL telomere-specific FITC conjugated $(C_3TA_2)_3$ peptide nucleic acid (PNA) (Applied Biosystems, Foster City, USA). Denaturation was done at 87°C for 15 min, and hybridization was performed in the dark and at room temperature (RT) for 90 min. Excess and nonspecifically bound telomere PNA probes were removed by 4 washing steps at RT using 1 mL washing solution containing 75% formamide, 10 mM Tris, 0.1% BSA, and 0.1% Tween 20 (Sigma-Aldrich), followed by 1 × 1 mL wash with a solution containing PBS, 0.1% BSA, and 0.1% Tween 20 at RT. DNA counterstaining was performed using a solution containing Sheath Fluid (BD Bioscience), 0.1% BSA, and a subsaturating amount of LDS 751 (0.01 µg/mL; Invitrogen, Waltham, MA, USA) overnight. Acquisition of telomere fluorescence was performed using FACSCalibur (BD Biosciences). For each sample, unstained and telomere-stained samples were tested. FlowJo version 10 (Tree Star Inc.) was used for analysis of telomere length in the specific cell subsets. Specific telomere fluorescence was determined as the difference between the fluorescence of the stained samples minus the (auto-) fluorescence of the corresponding unstained sample. Using calibration beads and an internal standard of cow thymocytes, the telomere fluorescence was calculated into kilobases of telomere length.

Intracellular cytokine measurements

Isolated $CD8^+$ T cells were stimulated for 1 h at 37°C in 5% CO_2 with αCD3 (1 µg/mL, plate bound) and αCD28 (1 µg/mL, soluble) antibodies or with αCD3 mAb-coated P815 cells (see below). Thereafter, GolgiPlug and GolgiStop (BD Biosciences) were added to the cultures followed by incubation for 5 h. Cells were spun down and incubated with fluorochrome-conjugated mAbs for multiparametric flow cytometric analysis, when required. Cells were then washed, fixed with 2% paraformaldehyde in PBS, permeabilized, and stained intracellularly with fluorochrome-conjugated mAbs against cytokines. Finally, cells were washed and analyzed on a BD FACSVerse (BD Biosciences). Data were analyzed with FlowJo 10.0.6 software (Tree Star Inc., Ashland OR, USA).

Redirected cytotoxicity assay

Cytolytic $CD8^+$ T cell activity was evaluated in a redirected cytotoxicity assay against P815 cells. To this end, the P815 cells were coated with 20 µg/mL of αCD3 (OKT-3) for 1 h. When indicated, P815 cells were treated with neuraminidase (25 mU, Roche Diagnostics) for 30 min at 37°C. CD3-coated P815 cells were co-cultured (3:1 E/T ratio) with $CD8^+$ T cells at 37°C. After 4 h of incubation, the specific lysis of P815 cells was assessed by measuring the LDH activity in the supernatant using the Cytotoxicity Detection Kit^{PLUS} LDH (Roche), according to the manufacturer's instructions. Specific lysis was calculated as (experimental – spontaneous release)/(total – spontaneous release)*100 and expressed as a fold change between treated and untreated groups (specific lysis fold change).

Transwell cell migration assay

2 x 10^5 Siglec-7^+ and Siglec-7^- $CD8^+$ T cells were resuspended in 200 µL RPMI medium and preactivated using αCD3 and αCD28 co-stimulation for 1 h as described above. Cells were loaded on Transwell inserts with 5 µm pores (Stemcell). Transwell inserts were placed over 24 wells plate well containing 600 µL serum-free RPMI, supplemented with or without 100 ng/mL of rhRANTES (CCL5, BioLegend) or rhCXCL9 (BioLegend). Cells were incubated for 4 h at 37°C and then analyzed using flow cytometry (see above).

TCRVβ sequencing

$CD8^+$ T cells were isolated from peripheral blood of healthy donors and separated by fluorescence-activated cell sorting into Siglec-9^+, Siglec-7^+ and Siglec-$9^-/7^-$ populations as previously described. Genomic DNA from $CD8^+$ T cells subsets was extracted using NucleoSpin® Tissue kit from Macherey-Nagel according to the manufacturer instructions. Genomic DNA quantity and purity were assessed through spectrophotometric analysis. 1.47 to 29.1 ng/µL of genomic DNA were analyzed by high-throughput sequencing of the TCRVβ using the ImmunoSEQ immune profiling platform at the survey level (Adaptive Biotechnologies Corp, Seattle, WA), which represents a detection capacity of 1 cell in 40'000. Raw data can be retrieved from the immuneACCESS repository (DOI:10.21417/haas-2022-fi URL: https://clients.adaptivebiotech.com/pub/haas-2022-fi).

Flow cytometric quantification of Siglec ligands on AML cells

Detection of Siglec-7 ligands by flow cytometry was performed as previously described (6). AML-derived samples were analyzed as previously described (17). In brief, non-specific antibody binding by Fc receptors was blocked using 100 µL of Fc Receptor Blocker (Innovex Biosciences, Richmond, CA, USA) and dead cells were excluded from analysis by staining with a Fixable Viability Dye (ThermoFisher). For the Siglec ligands staining, recombinant human Siglec-7-hFc (R&D Systems, Minneapolis, MN, USA) was pre-incubated with PE-conjugated goat anti-human Ig (Jackson ImmunoResearch Laboratories, West Grove, USA) for 1 h at 4°C and then applied to the samples for 1 h at RT together with lineage-associated antibodies. Lineage-positive cells were stained with biotinylated αCD2, αCD14, αCD16, αCD19, αCD56, and αCD235, together with fluorophore-conjugated αCD8, αCD4, αCD45, αCD34, αCD38 (all from BioLegend), followed by a second step with streptavidin-FITC conjugate (BD Biosciences). Cells were washed and analyzed on a BD FACSVerse or BD FACSlyric (BD Biosciences). Data were analyzed using the FlowJo 10.0.6 software (Tree Star Inc.).

Immune synapse analysis

Isolated $CD8^+$ T cells subgroups were obtained by cell sorting as previously described. P815 cells were incubated with 20 µg/mL of αCD3 (OKT-3) for 1 h. When indicated, P815 cells were treated with neuraminidase (25 mU, Roche Diagnostics). CD3-coated P815 cells were co-cultured (3:1 E/T ratio) with $CD8^+$ T cells subgroups for 30 min. After incubation, the cells were centrifuged on poly-lysine (Sigma)-treated coverslips, fixed using 3% paraformaldehyde for 20 min at 4°C and permeabilized with Triton X-100 for 1 min. After extensive washing with fish skin gelatin buffer (PSG), αSiglec-7 antibody and BODIPY 488 Phalloidin (actin dye, Life Technologies) were applied for 1 h at RT. After another round of washing, the secondary antibody (goat anti-mouse, Alexa-Fluor 555, Invitrogen) was applied for 1 h at RT. Coverslips were mounted on slides (Fisherbrand, Thermo Fisher) using ProLong Gold anti-fade reagent (Invitrogen). The mounted slides were cured for 2 d in the dark at RT. Long-time storage at 4°C. Conjugates were analyzed by confocal laser scanning microscopy (LSM510, Carl Zeiss, Jena, Germany). Acquired images were analyzed using the ImageJ software version 1.51 (NIH, Bethesda, MD, USA).

Analysis of bioenergetic profiles

$CD8^+$ T cells subsets were isolated from healthy donors as previously described. T cells were either analyzed directly following sorting or were activated for 7 d using αCD3 and αCD28 (each at 1 µg/mL) as described above. Bioenergetic profiles were assesed using a seahorse approach (18). Briefly, to overcome machine detection limitations, sorted $CD8^+$ T cells from 2 to 4 patients were pooled together for each condition. XF96 cell culture microplate (Agilent, Santa Clara, USA) was treated with 30 µL poly-D-lysine (Sigma-Aldrich) for 1–2 h, before washing with ddH_2O. Sorted $CD8^+$ T cells subsets were harvested, washed in non-buffered DMEM containing 25 mM glucose, 2 mM L-glutamine and 1 mM sodium pyruvate (Seahorse XF DMEM medium, Agilent). Cells were then resuspended in medium at a concentration of at least 5×10^6 cells/mL. 40 µL of cells were plated into the bottom of the analysis wells (=$2x10^5$ cells/well). Cells were centrifuged 5 min at 400xg to adhere and form a monolayer at the bottom of the plate. 140 µL of medium was added to each well and cells were incubated at 37°C in a non-CO_2 incubator for 60 min. 10x stocks of compounds (oligomycin, fluoro-carbonyl cyanide phenylhydrazone, rotenone, antimycin A and 2-deoxy-D-glucose; all purchased from Sigma-Aldrich) in medium were prepared and loaded into delivery ports of XFe96 sensor

cartridges. Oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) were measured under basal conditions and in response to sequentially injected compounds at a final concentration of 1 µM oligomycin, 1.5 µM fluoro-carbonyl cyanide phenylhydrazone and 100 nM rotenone + 1 µM antimycin A and 50 mM of 2-deoxy-D-glucose using the XF-96 Extracellular Flux Analyzer (Agilent).

Statistics

Statistical analysis was performed using Prism 7.0 (GraphPad Software, San Diego, CA, USA). For quantitative comparisons between two groups the paired Student's *t* test and between multiple groups one-way ANOVA tests with Bonferroni or Dunn posttest were used. All statistical tests were two-sided and $P < 0.05$ was considered significant. Unless otherwise indicated, data represent mean ± standard deviation (SD).

Results

Siglec-7 defines a distinct subset of effector memory CD8$^+$ T cells

In line with earlier reports (11, 12), we observed a subset of Siglec-7$^+$ CD8$^+$ T cells in the peripheral blood of healthy individuals (Figures 1A, B and Supplementary Figure 1A). Our flow cytometric analysis revealed that these cells represented a distinct and more frequent subtype compared to Siglec-9$^+$ CD8$^+$ T cells, and were predominantly negative for NK cell (CD56) and natural killer T cell (NKT, TCR Vα24-Jα18) markers (Supplementary Figures 1B, C). Unmasking of potential sialoglycan ligands bound *in cis* by neuraminidase had no further effect on the flow cytometric assessment of Siglec-7 on CD8$^+$ T cells (Supplementary Figure 2). Further phenotypic analysis based on CCR7 and CD45RA cell surface expression (19), indicated that Siglec-7$^+$ CD8$^+$ T cells predominantly constitute effector memory (EM), and to a lesser extent effector memory cells re-expressing CD45RA (EMRA), T cell subsets (Figure 1C).

Deep sequencing of TCRVβ chains on genomic DNA performed by multiplex polymerase chain reaction (PCR) assays (20), was performed to decipher the TCR repertoires of Siglec-7$^+$, Siglec-9$^+$ and Siglec-7/9$^{-/-}$ CD8$^+$ T cell subsets from three donors (TS-01, TS-02, and TS-03). The low clonality scores of Siglec-7/9$^{-/-}$ CD8$^+$ T was consistent with the highly diverse repertoire of circulating T cells (Figure 1D). In contrast, both Siglec-7$^+$ and Siglec-9$^+$ CD8$^+$ T cells exhibited high normalized productive clonality scores based on diversity and sample entropy, indicative of limited TCR rearrangements and enriched clones within these subsets. Corroborating results were obtained when maximum productive frequency was assessed (Supplementary Figure 3). Furthermore, a frequency distribution analysis of clonotypes based on the 10 most prevalent nucleotide TCRvβ chains confirmed the clonal expansion pattern of Siglec-7$^+$ and Siglec-9$^+$ CD8$^+$ T cells (Figure 1E).

We went on to compare the clonotype repertoires of Siglec-7$^+$, Siglec-9$^+$ and Siglec-7/9$^{-/-}$ subsets of peripheral blood T cells (Figure 1F and Supplementary Figure 4). The Siglec-7$^+$ T cell subset enclosed a higher proportion (2.7-10.6%) of the total of all clonotypes, as compared to the Siglec-9$^+$ subset (0.9-2.8%) (Figure 1F and Supplementary Figure 4), eventually in line with their higher occurrence in the circulation. The analysis of the TCRVβ nucleotide chain distribution and clonotypes frequency in Siglec-7$^+$ CD8$^+$ T cells (x-axis) and Siglec-9$^+$ CD8$^+$ T cells (y-axis) from donor TS-02 confirmed the distinct clonotype profile of the Siglec-7$^+$ subset (Figure 1G). Taken together, these data suggest that Siglec-7$^+$ CD8$^+$ T cells, similarily to their Siglec-9 positive counterparts, are expanded oligoclonal effector memory T cells, but represent a distinct subset in light of the TCR repertoire.

High metabolic capacities of Siglec-7$^+$ CD8$^+$ T cells in steady state and upon activation

Metabolism plays a key role in immune cell functionality and activated effector T cells elevate aerobic glycolysis and oxidative phosphorylation (OXPHOS) (21, 22). Using Seahorse technology, we investigated the oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) in presence of oligomycin (complex V blocker), FCCP (mitochondrial uncoupler), 2-deoxy-D-glucose (2-DG, glycolysis inhibitor), or combined antimycin A (complex III blocker) and rotenone (complex I blocker) treatment (Figures 2A, B). OCR analysis of sorted CD8$^+$ T cell subsets revealed significantly higher basal respiration (Figures 2C, D) and ATP-linked respiration (Figure 2E) rates of Siglec-7$^+$ CD8$^+$ T cells at steady state. Compared to their Siglec-7 negative counterparts, Siglec-7$^+$ CD8$^+$ T cells also exhibited an overall amplified ECAR profile (Figure 2F), and a trend towards higher basal ECAR (Figure 2G) and glycolysis (Figure 2H). The increased OXPHOS capacity of Siglec-7$^+$ CD8$^+$ T cells at steady state matched the metabolic profile previously described for effector T cells (22–24), corresponding with the surface marker analysis.

Next, we compared the metabolic behavior of Siglec-7$^+$ and Siglec-7$^-$ CD8$^+$ T cells upon activation by αCD3 and αCD28

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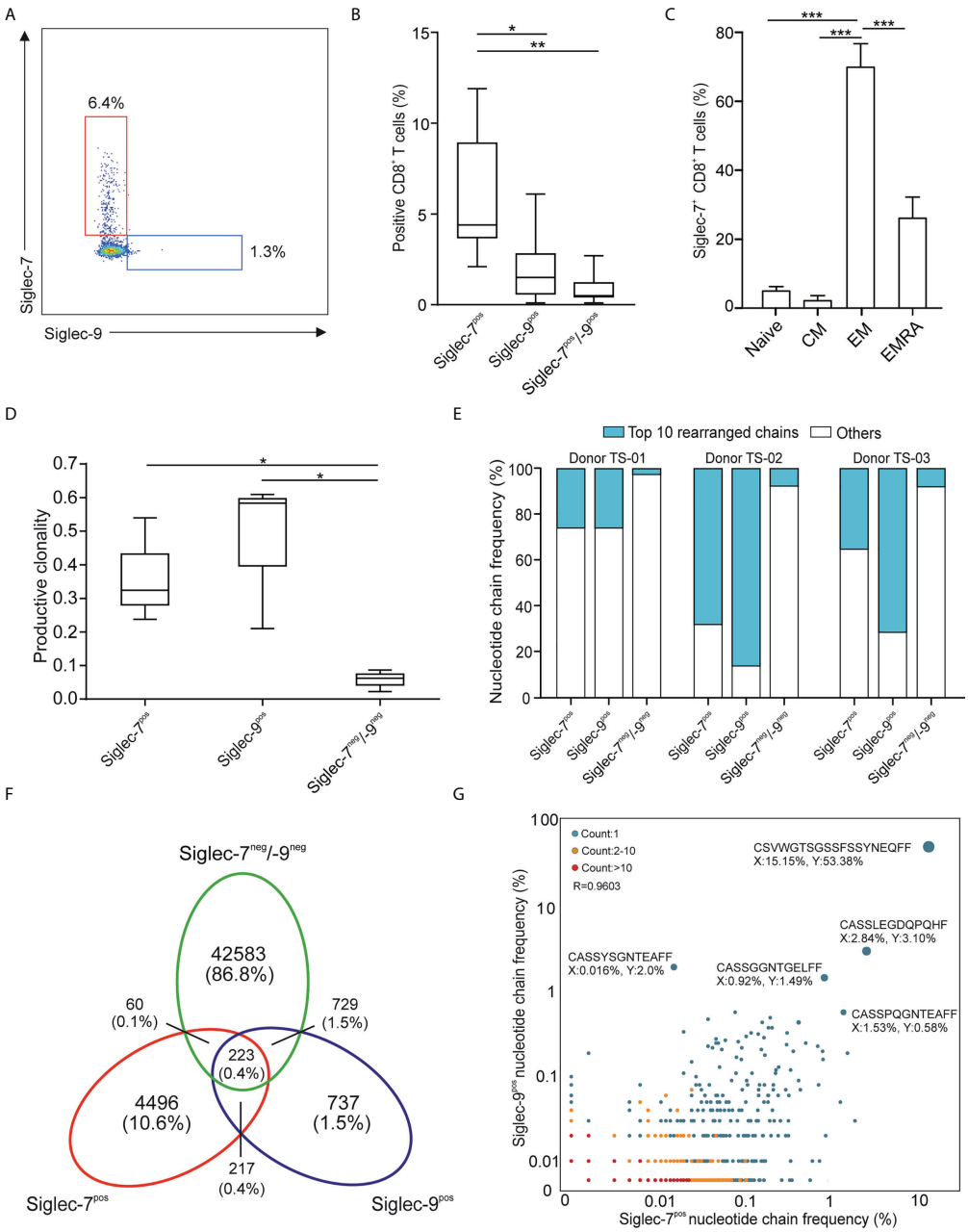

FIGURE 1
Siglec-7 defines a unique subset of effector memory CD8$^+$ T cells in healthy donor peripheral blood. **(A, B)** Representative flow cytometry plot **(A)** and quantitative analysis (n=23) **(B)** for comparison of Siglec-7 and Siglec-9 surface expression on healthy donor peripheral blood CD8$^+$ T cells. **(C)** Composition of Siglec-7$^+$ CD8$^+$ T cell subsets, including naive, central memory (CM), effector memory (EM), and CD45RA$^+$ effector memory (EMRA) cells (n=8). **(D–G)** TCRvβ chain analysis of peripheral blood CD8$^+$ T cell subsets. Productive clonality **(D)** and clonotype frequency distribution **(E)** for three individual donors. Venn diagram **(F)** and scatter plot **(G)** representation displaying clonotypes distribution among CD8$^+$ T cell subsets for donor TS-02. Statistical analysis was performed by one-way ANOVA followed by **(B, C)** Bonferroni or **(D)** Dunn posttest. *P < 0.05; **P < 0.01; ***P < 0.001. Error bars, SD.

mAbs co-stimulation for a duration of 7 days (25). In analogy to steady state conditions, upon activation Siglec-7$^+$ CD8$^+$ T cells displayed elevated OCR and ECAR profiles (Figures 3A, B), significantly increased basal respiration (Figure 3C), and augmented ATP-linked respiration (Figure 3D). Furthermore, upon activation the basal ECAR (Figure 3E) and glycolytic capacity (Figure 3F) of Siglec-7$^+$ CD8$^+$ T cells raised dramatically compared to Siglec-7$^-$ CD8$^+$ T cells at steady-

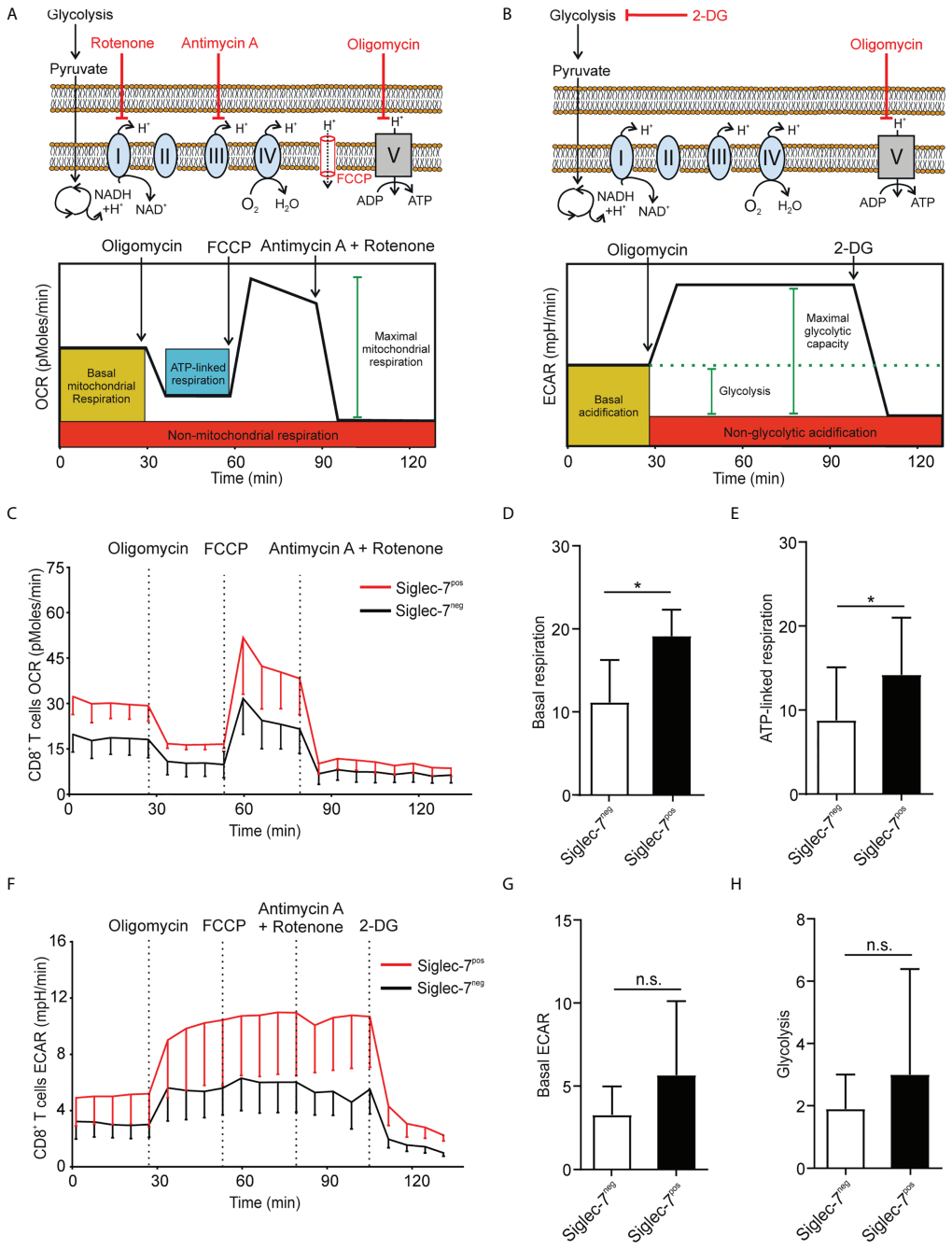

FIGURE 2
Siglec-7+ CD8+ T cells display a higher oxidative phosphorylation capacity at steady state. **(A, B)** Schematic illustrations of expected effects of oligomycin (ATPase inhibitor), FCCP (mobile ion carrier), 2-deoxy-D-glucose (2-DG, glucoprivic agent), and antimycin A (cytochrome C reductase blocker) and rotenone (complex I blocker) on oxygen consumption rate (OCR) **(A)** and extracellular acidification rate (ECAR) **(B)**. **(C–H)** Comparative OCR or ECAR analysis of isolated Siglec-7$^+$ and Siglec-7$^-$ CD8$^+$ T cells from healthy donors by Seahorse (three independent experiments with 2-4 donors each, 11 donors in total). OCR profile **(C)**, basal respiration (steady state mitochondrial respiration) **(D)**, and ATP-linked respiration (ΔOCR after oligomycin injection) **(E)**. ECAR profile **(F)**, basal acidification (ECAR at steady state) **(G)**, and glycolysis (ΔECAR at steady state minus non-glycolytic acidification in response to 2-DG injection) **(H)**. Statistical analysis was performed by paired *t* test **(D, E, G, H)**. *P < 0.05; n.s., not significant. Error bars, SD **(D, E, G, H)** or SEM **(C, F)**.

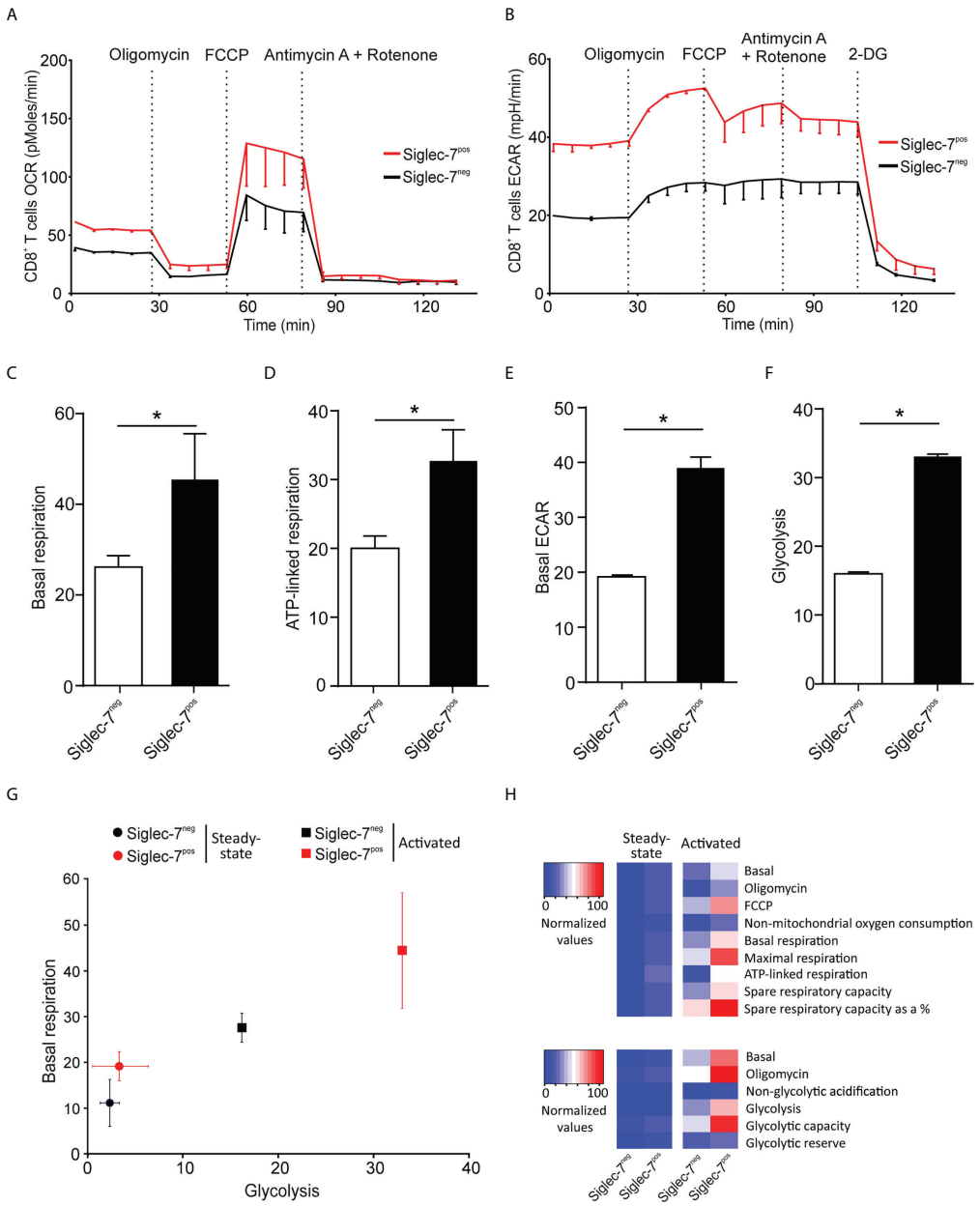

FIGURE 3
Upon activation Siglec-7$^+$ CD8$^+$ T cells exhibit a higher increase in oxidative phosphorylation and glycolysis compared to Siglec-7$^-$ CD8$^+$ T cells. Seahorse analysis of Siglec-7$^+$ or Siglec-7$^-$ CD8$^+$ T cells after 7 days of co-stimulation by αCD3 and αCD28 (each at 1 µg/mL) (three independent experiments with 2-4 donors each, 11 donors in total). Oxygen consumption rate (OCR) **(A)** and extracellular acidification rate (ECAR) **(B)** profiles, basal respiration (steady state mitochondrial respiration) **(C)**, and ATP-linked respiration (ΔOCR after oligomycin injection) **(D)**, basal acidification (ECAR at steady state) **(E)**, and glycolysis (ΔECAR at steady state minus non-glycolytic acidification in response to 2-DG injection) **(F)**. **(G, H)** Comparison of the metabolic profiles of αCD3 and αCD28 co-stimulated and unstimulated Siglec-7$^+$ and Siglec-7$^-$ CD8$^+$ T cells. Plot representation of basal respiration and glycolysis rates **(G)** and heatmap representation **(H)**. Statistical analyses were performed by paired *t* test **(C–F)**. *P < 0.05. Error bars, SEM **(A, B)** or SD **(C–G)**.

state. Figure 3G highlights the higher basal respiration and glycolysis levels of Siglec-7$^+$ CD8$^+$ T cells compared to Siglec-7$^-$ CD8$^+$ T cells in steady state, and particularly upon activation. The heatmap in Figure 3H summarizes the key metabolic characteristics, illustrating the bioenergetic advantage of activated Siglec-7$^+$ CD8$^+$ T cells over Siglec-7$^-$ cells, particularly in regard of ATP-linked respiration and spare respiratory capacity.

Siglec-7$^+$ CD8$^+$ T cells represent a non-exhausted and effective cell subset

We went on to further explore the phenotypic and functional characteristics of Siglec-7$^+$ CD8$^+$ T cells. Previously, we reported that Siglec-9$^+$ T cells (15), but not Siglec-9$^+$ NK cells (6), exhibit a shorter telomere length than their Siglec-9 negative counterparts. In the present study, telomere length analysis of sorted T cell subsets by automated multicolor flow-FISH (15, 26), revealed that the telomere length of Siglec-7$^+$ CD8$^+$ T cells is around two kilobases shorter than in Siglec-7/9$^{-/-}$ T cells (Figure 4A), but similar to the Siglec-9$^+$ T cell subset (15).

When redirected against αCD3-coated P815 cancer cells, Siglec-7$^+$ CD8$^+$ T cells demonstrated a higher cytotoxic capacity than their Siglec-7 negative counterparts (Figure 4B), and co-stimulation with αCD3 and αCD28 mAbs resulted in stronger IFN-γ and TNF-α production in Siglec-7$^+$ CD8$^+$ T cells (Figures 4C, D). Flow cytometric analysis revealed broad surface expression of chemokine receptors on Siglec-7$^+$ CD8$^+$ T cells (Figure 4E). Given the significant expression of CCR5 and CXCR3 on this subset, migration towards the chemokines RANTES (CCR5 ligand) and CXCL9 (CXCR3 ligand), was assessed. Indeed, Siglec-7$^+$ CD8$^+$ T cells demonstrated a higher migration capacity towards these chemokines (Figures 4F, G). Together, these data confirm a history of previous clonal expansion for Siglec-7$^+$ CD8$^+$ T cells and indicate that this subset of effector memory T cells is not exhausted but exhibits high functional and chemotactic capabilities.

Inhibition of Siglec-7$^+$ CD8$^+$ T cells by sialoglycans on tumor cells

The formation of an immune synapse (IS) involves the spatio-temporal organization of molecular events at the interface between effector and target cells. For IS formation analysis by confocal microscopy, sorted CD8$^+$ T cell subsets were redirected to αCD3-coated P815 target cells, which express Siglec-7 surface ligands that can be removed by enzymatic digestion with neuraminidase (Supplementary Figure 5). We observed polarization of the Siglec-7 receptor into the IS, which was diminished upon neuraminidase treatment (Figures 5A-E), indicating the requirement of sialic acid-dependent receptor-ligand interactions *in trans* for effective Siglec-7 polarization. Moreover, neuraminidase treatment of target cells further enhanced actin polarization, required for the establishment of a stable and functional IS (27), in synapses formed with Siglec-7$^+$ CD8$^+$ T cells but not with Siglec-7$^-$ CD8$^+$ T cells (Figure 5F). Functional experiments revealed that the digestion of Siglec-7 ligands by neuraminidase treatment on αCD3-coated P815 target cells increased effector functions of Siglec-7$^+$, but not Siglec-7$^-$ CD8$^+$ T cells, including redirected cytotoxicity (Figure 5G), as well as IFN-γ and TNF-α (Figures 5H, I) production. Together, these mechanistic studies provide functional evidence for the sialic acid-Siglec axis as an immune checkpoint that directly regulates effector functions of Siglec-7$^+$ CD8$^+$ T effector memory cells.

Siglec-7$^+$ T cell glyco-immune checkpoints on AML and leukemic stem cells

Using flow cytometry, we went on to analyze the expression of Siglec-7 ligands on leukemic cells (Lin$^-$CD90$^-$CD34$^+$CD38$^+$) and leukemic stem cells (Lin$^-$CD90$^-$CD34$^+$CD38$^-$) in AML patient-derived peripheral blood (PB) and bone marrow (BM) (17). High surface expression of Siglec-7 ligands was detected on both AML cells and leukemic stem cells in peripheral blood and bone marrow (Figures 6A–C). An analysis of RNA-seq data from the AML data set (n=818) from The Cancer Genome Atlas (TCGA) project using a clustering algorithm revealed heterogenous expression of the twenty human sialyltransferases (Figure 6D), which are involved in the biosynthesis of sialoglycans. Consistently high expression levels were found for sialyltransferases (ST) predicted to be involved in the biosynthesis of Siglec-7 ligands, including *ST3GAL1*, *ST3GAL4*, *ST6GAL1*, and *ST6GALNAC6* (27–31). A gene set enrichment analysis of the AML TCGA data revealed a strong correlation between Siglec-7 expression and hallmark gene sets linked to effector CD8$^+$ T cell activity, such as IFN-γ response, inflammatory response, glycolysis or IL-2-STAT5 signaling (Figure 6E).

Next, we used multi-parametric flow cytometry to explore the expression of Siglec-7 on CD8$^+$ T cells in peripheral blood (n=8) and bone marrow (n=5) from AML patients. We observed that the majority of CD8$^+$ T cells in the bone marrow of AML patients expressed Siglec-7, while circulating Siglec-7$^+$ CD8$^+$ T cells in AML patients were found at similar levels as compared to healthy donors (Figure 6F). Further analysis of Siglec-7$^+$ CD8$^+$ T cells isolated from the bone marrow of four AML patients revealed co-expression with CTLA4, and, to a lesser extent with other check-point receptors, such as PD1, LAG3, BTLA, TIM3 (Figure 6G). These data suggest a potential role of the sialic acid-Siglec axis as an immune checkpoint in AML.

Discussion

Increased expression of multiple inhibitory receptors is commonly considered a hallmark of exhausted CD8$^+$ T cells (32). However, in this study we observed that in humans Siglec-7 defines a non-exhausted effector memory CD8$^+$ T cell subset characterized by high functional and metabolic capacities. For effective functionality, effector memory CD8$^+$ T cells need to reprogram their metabolism, which also allows to overcome

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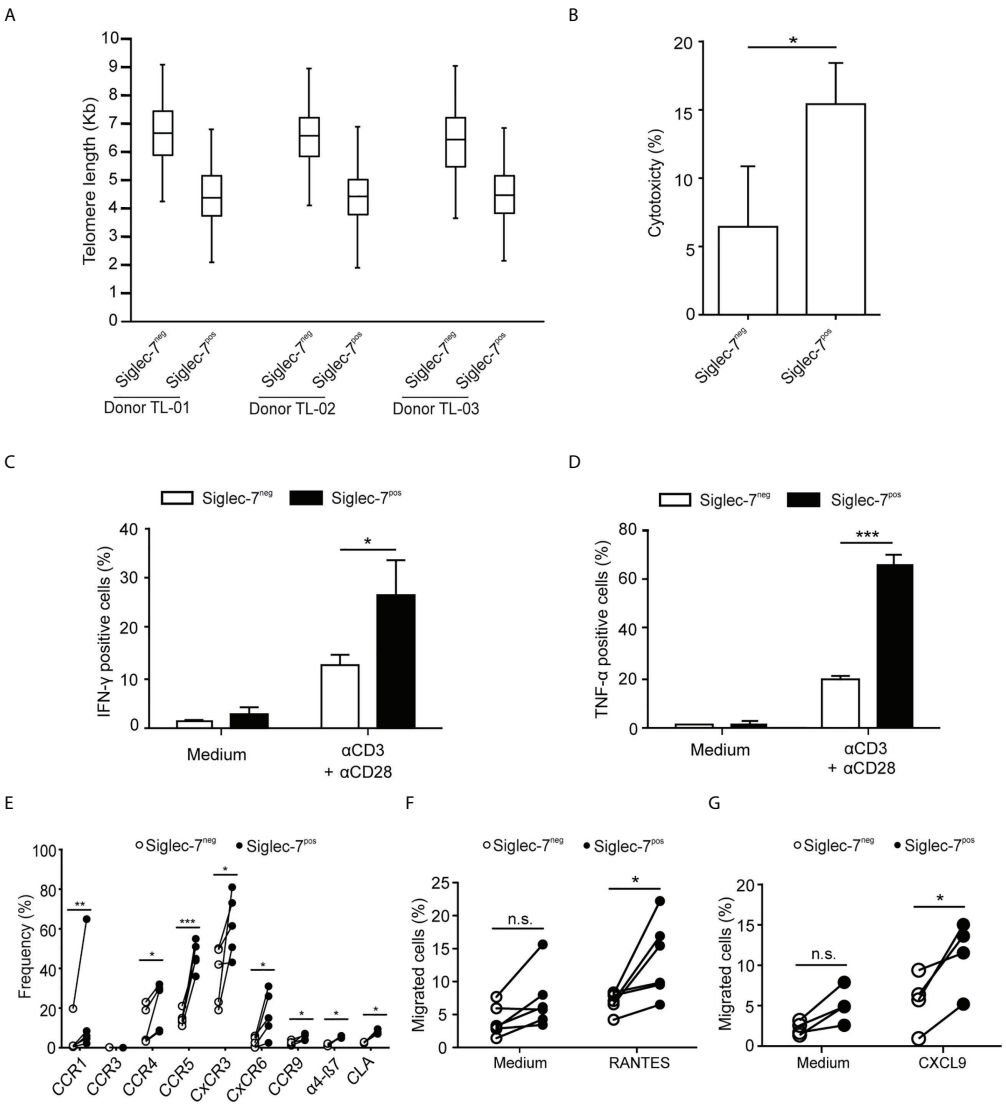

FIGURE 4
Siglec-7+ CD8+ T cells display a highly proliferative and functional phenotype. **(A)** Telomere length analysis of CD8$^+$ T cell subsets from three healthy donors. Box plot representation indicating 25th to 75th percentiles with median; error bars, 1st to 99th percentiles. **(B)** Redirected cytotoxicity of CD8$^+$ T cell subtypes upon co-culture with αCD3–loaded P815 tumor cells for 4 h (n=4). **(C, D)** Flow cytometric quantitative analysis of intracellular IFN-γ or TNF-α production by CD8$^+$ T cell subsets following 5 h of culture upon costimulation by αCD3 and αCD28 (each at 1 µg/mL; n=5). **(E)** Flow cytometric quantitative analysis of chemokine receptors on the surface of CD8$^+$ T cell subsets (n=5). **(F, G)** Migration of CD8$^+$ T cell subsets towards 100 ng/mL of RANTES (E, n=6) or CXCL9 (F, n=5) through transwell inserts with 5 µm pores after 4 h incubation. Statistical analyses performed by paired *t* test **(C–F)** or one-way ANOVA followed by Bonferroni posttest **(A)**. *P < 0.05; **P < 0.01; ***P < 0.001. n.s., not significant. Error bars, SD.

barriers imposed by challenging environments such as the tumor microenvironment (TME) (33). While exhausted T cells are known to progressively undergo metabolic dysfunction (32), Siglec-7$^+$ CD8$^+$ T cells were found to exhibit a high potential for concomitant glycolysis and oxidative phosphorylation upon activation, as assessed by ECAR and OCR measurements, respectively. Indeed, Siglec-7$^+$ CD8$^+$ T cells demonstrated superior functionality in terms of cytotoxicity, cytokine production, and migratory potential compared to Siglec-7 negative cells.

Rather than exhaustion, Siglec-7 expression on CD8$^+$ T cells was linked to clonality. Indeed, Siglec-7$^+$ CD8$^+$ T cells exhibit short telomere length and high TCRvβ chain clonality, which together is indicative of a history extensive of previous clonal expansion. The clonotype repertoire of Siglec-7$^+$ CD8$^+$ T cells was distinct and showed higher clonality compared to other

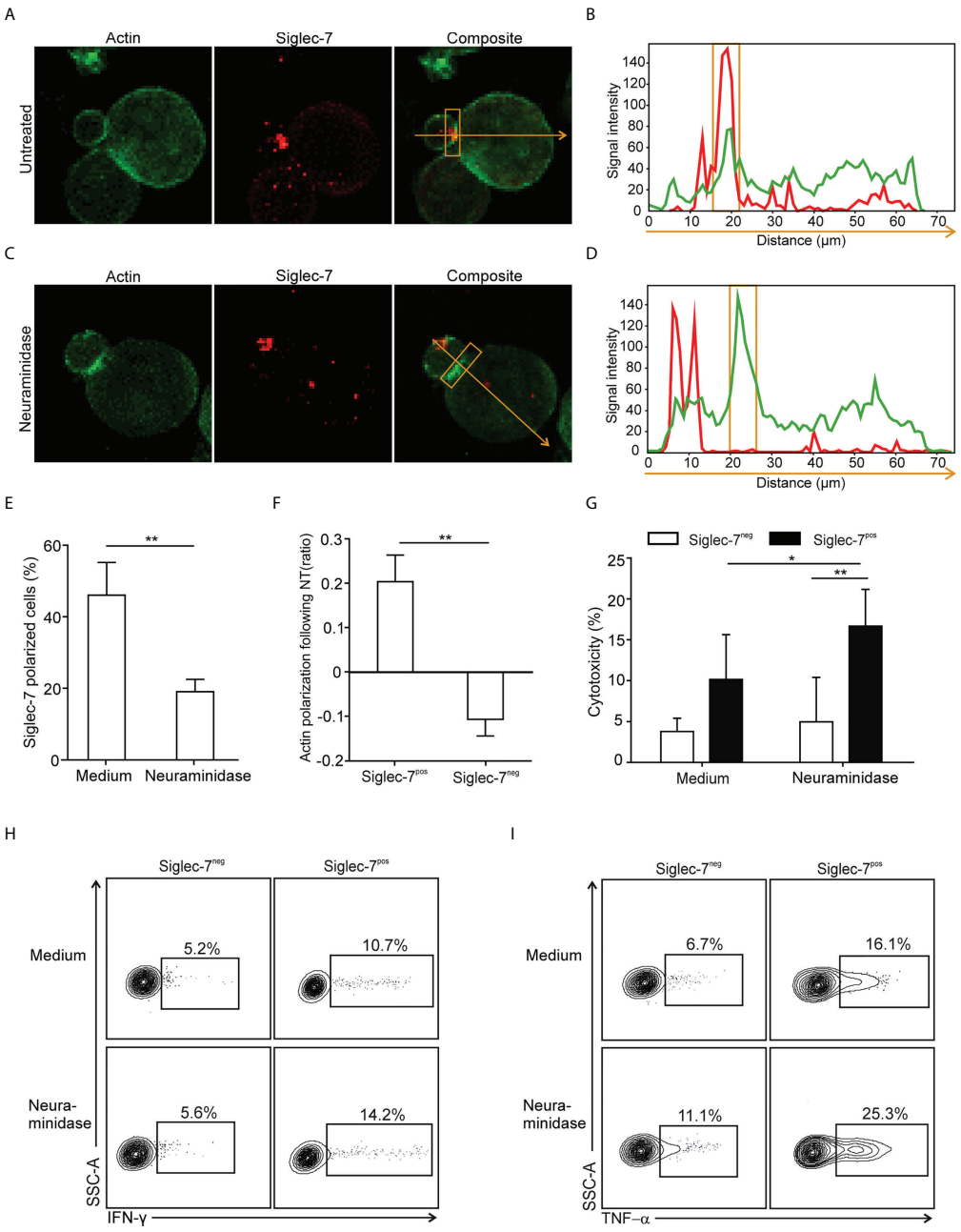

FIGURE 5
Polarization of Siglec-7 and inhibition of Siglec-7$^+$ CD8+ T cells induced by sialoglycans on tumor cells. **(A–F)** Confocal immunofluorescence microscopy analysis of immunological synapse (IS) formation between redirected CD8+ T cell subsets and αCD3 mAb-loaded P815 tumor cells in absence or presence of target cell neuraminidase treatment (NT). Representative images **(A, C)** and corresponding fluorescence profiles plotted along the indicated trajectory **(B, D)**. Quantification of Siglec-7 polarization on T cells towards the IS **(E)**, or actin staining intensity at the synapse **(F)**, compared with the opposite side of the same T cell. **(G–I)** Effects of neuraminidase pre-treatment of P815 target cells on redirected cytotoxicity **(G)** and intracellular IFN-γ **(H)** or TNF–α **(I)** production 4 h after target cell loading with αCD3 mAb (n=5). **(A–F)** analysis of at least 40 conjugates from three independent experiments with cells from at least three donors. Statistical analyses were performed by paired *t* test **(E–G)** or one-way ANOVA followed by Bonferroni posttest **(H, I)**. *P < 0.05; **P < 0.01. Error bars, SD.

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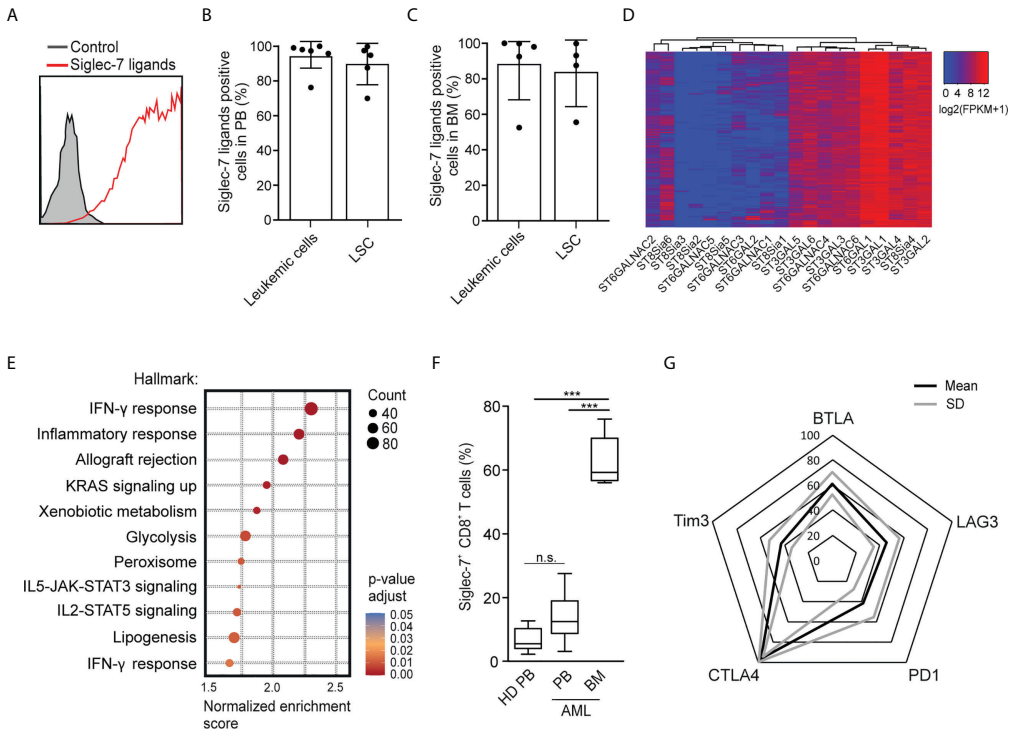

FIGURE 6
Occurrence of Siglec-7$^+$ CD8+ T cells glyco-immune checkpoints in AML. **(A)** Representative flow cytometric histogram demonstrating Siglec-7 ligands surface expression on AML cells isolated from bone marrow (BM). **(B–C)** Siglec-7 ligands expression on AML patient peripheral blood leukemic cells (n=6) and leukemic stem cells (LSC; n=5) **(B)**, or bone marrow leukemic cells (n=5) and leukemic stem cell (LSC; n=4) **(C)**. **(D)** RNA expression of sialyltransferases in AML patients based on TCGA Network data computed by a dendrogram clustering algorithm and **(E)** gene set enrichment analysis performed using hallmark gene sets (n=818). **(F)** Quantitative analysis of Siglec-7 expression by CD8+ T cells from the PB (PB, n=8) and from the bone marrow (BM, n=5) of patients with acute myeloid leukemia (AML). **(G)** Radar chart of flow-cytometric data demonstrating surface coexpression of Siglec-7 with PD1, CTLA4, BTLA, LAG3, or TIM3 on CD8+ T cells from AML bone marrow (n=4). Statistical analyses were performed by one-way ANOVA followed by Bonferroni posttest **(F)** ***P < 0.001; n.s., not significant. Error bars, SD.

CD8$^+$ T cell subsets, including Siglec-9 positive oligoclonal counterparts found at lower frequency in peripheral blood. Notably, it has been reported that pre-treatment clonality is predictive of response to anti-PD1 treatment, while TCR diversity in tumor-infiltrating lymphocytes (TILs) is prognostic for overall survival in absence of immune checkpoint therapy (34). In line with this observation, results from our study support the conceptual perspective that the expression of at least certain inhibitory receptors is not a defining phenotype of exhausted T cells but is associated with clonality, as a consequence of previous T cell expansion. Furthermore, our data suggest that in combination with T cell repertoire analysis (35), the exploration of clonality-associated immune checkpoints may provide novel avenues for personalized immunotherapy.

Recent advances in immune checkpoint therapy highlight the importance of CD8$^+$ T cells in anti-tumor responses and the need for “immune normalization” to restore tumor-induced immune deficiency (34). Current immune checkpoint therapy successfully used in patient subsets of select solid tumors and lymphomas, but ineffective in other malignancies such as AML in which epigenetic silencing of activating immune checkpoint receptors rather than inhibitory signaling might lead to T cell dysfunction (36). However, in this study we observed high expression of Siglec-7 ligands on leukemic cells and leukemic stem cells in peripheral blood and bone marrow from AML patients. Intriguingly, the majority of CD8$^+$ T cells in the bone marrow of AML patients expressed Siglec-7, with high co-expression of CTLA4, but lower expression of other inhibitory receptors (PD1, LAG3, BTLA, TIM3). These findings suggest that the sialic acid-Siglec axis provides a glyco-immune checkpoint in AML that restrains effective anti-tumor responses of CD8+ T cells in the bone marrow environment. Indeed, in mechanistic experiments, we observed Siglec-7 repolarization on CD8+ T cells into the immune synapse with target cells expressing the cognate ligand, as well as sialic-acid dependent suppression of cytotoxicity and cytokine production selectively of Siglec-7$^+$ CD8+ T cells.

There is increasing awareness that reverse translational research from bedside to bench is needed for more personalized pharmacotherapy and to explore the complexity of processes at play in human diseases (37). In this study, we identified Siglec-7 as an immune checkpoint receptor on non-exhausted effector memory CD8+ T cells that is acquired upon clonal expansion. Strategies considering the combined analysis of T cell repertoires and clonality-associated immune checkpoint receptors, such as Siglec-7, may lead to novel and more personalized treatment approaches for T cell-driven autoimmune disorders and for cancer immunotherapy.

Data availability statement

The data presented in the study are deposited in the immuneACCESS repository, accession number haas-2022-fi (DOI:10.21417; URL: https://clients.adaptivebiotech.com/pub/haas-2022-fi).

Ethics statement

The studies involving human participants were reviewed and approved by the ethics committee of the canton of Bern, Switzerland. The patients/participants provided their written informed consent to participate in this study.

Author contributions

SG designed the study. QH and SG wrote the manuscript. TCGA data were analyzed by AB, CN and QH. Experimental work was performed by QH, NM, LM, MH, and VR under supervision by SG, H-US, GB, CR and CM. Patient material was provided by CR and AO. All authors had full access to the data, helped draft the report or critically revised the draft, contributed to data interpretation, reviewed and approved the final version of the report.

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Conflict of interest

SG receives remuneration for serving on the scientific advisory board of Palleon Pharmaceuticals.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2022.996746/full#supplementary-material

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External validation of biomarkers for immune-related adverse events after immune checkpoint inhibition

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Immune checkpoint inhibitors have revolutionized treatment of advanced melanoma, but commonly cause serious immune-mediated complications. The clinical ambition of reserving more aggressive therapies for patients least likely to experience immune-related adverse events (irAE) has driven an extensive search for predictive biomarkers. Here, we externally validate the performance of 59 previously reported markers of irAE risk in a new cohort of 110 patients receiving Nivolumab (anti-PD1) and Ipilimumab (anti-CTLA-4) therapy. Alone or combined, the discriminatory value of these routine clinical parameters and flow cytometry biomarkers was poor. Unsupervised clustering of flow cytometry data returned four T cell subsets with higher discriminatory capacity for colitis than previously reported populations, but they cannot be considered as reliable classifiers. Although mechanisms predisposing some patients to particular irAEs have been described, we are presently unable to capture adequate information from pre-therapy flow cytometry and clinical data to reliably predict risk of irAE in most cases.

KEYWORDS

biomarker, checkpoint inhibition, irAEs, immune-related adverse events, validation, prediction

Abbreviations: AUC, Area-under-the-curve; BMI, body mass index; CMV, cytomegalovirus; EBV, Epstein-Barr virus; FDR, false discovery rate; γ-GT, gamma-glutamyltransferase; GOT, glutamic oxaloacetic transaminase; GPT, glutamic pyruvic transaminase; HHV, human herpesvirus; ICI, Immune Checkpoint Inhibitor; irAE, immune-related adverse event; KSHV, Kaposi's sarcoma-associated virus; LDH, lactate dehydrogenase; LOOCV, leave-one-out cross-validation; MLR, monocyte-to-lymphocyte ratio; ROC, receiver operating characteristic.

Introduction

Combined checkpoint blockade with anti-PD-1 (Nivolumab) and anti-CTLA-4 (Ipilimumab) antibodies is now a standard treatment for inoperable metastatic melanoma. The clinical efficacy of dual therapy is evident from the excellent clinical response rate, progression-free survival and overall survival (1–3). However, immune-related adverse events (irAE) complicate immune checkpoint inhibitor (ICI) treatment in a high proportion of patients, which significantly impacts their quality of life (4). Although life-threatening irAEs are infrequent, even moderate reactions lead to interruption of immunotherapy, multidisciplinary management and treatment with immunosuppressive medication (5). Disruption of ICI treatment, and costs associated with monitoring or treatment of irAEs, are burdensome; therefore, reliable prognostic methods to assess an individual's risk of irAE prior to therapy would greatly impact patient care.

Factors predisposing individual patients to irAE are incompletely understood. ICI therapy can worsen autoimmune conditions and patients with pre-existing autoimmune diseases stand a greater risk of developing other immune-mediated adverse reactions after treatment (6–8). Immunogenetics also play a role in irAE susceptibility (9–11). Prior exposure to viruses has recently surfaced as a significant predisposing factor in some patients (12). Infection with human herpesviruses (HHV) may play a particularly important role in the context of malignant melanoma. Our own studies revealed that chronic or recurrent cytomegalovirus (CMV; HHV-5) reactivation drives proliferation of virus-specific $CD4^+$ effector memory T cells (T_{EM}) in patients with metastatic melanoma before starting immunotherapy. These expanded T_{EM} cells are responsible for hepatitis after combined Nivolumab and Ipilimumab treatment (13–15). Similarly, others have implicated Epstein-Barr virus (EBV; HHV-4)-specific memory T cells in a case of fatal encephalitis after anti-PD-1 therapy (16). An unexpectedly high rate of seropositivity against Kaposi's sarcoma-associated virus (KSHV; HHV-8) has been reported in Stage IV melanoma patients, again hinting at a peculiar susceptibility to HHV infections (17). Beyond ICI therapy, autoimmunity may be triggered by persistent T cell immunity against various herpesgroup viruses; for example, Hashimoto's thyroiditis has been associated with seroconversion to roseolovirus (HHV-6) (18, 19).

Recently, many groups have reported biomarkers associated with irAE risk, which include leucocyte subsets measured in peripheral blood by flow cytometry. We systematically searched for these reports to independently assess the discriminatory value of biomarkers they identified. We found 20 relevant articles published between 2006 and 2022 that examined a range of tumor entities, treatment strategies and analytical methods (20–39). Here, we tested the general validity of these biomarkers by asking whether they predicted irAEs in a related, but non-identical clinical setting. Specifically, we asked whether any reported biomarkers measured prior to starting combined Ipilimumab and Nivolumab therapy predicted the incidence of hepatitis, colitis or thyroiditis in patients with advanced melanoma.

Materials and methods

Patients

This single-center, non-interventional clinical study was conducted in accordance with the Declaration of Helsinki and all applicable German and European laws and ethical standards. Blood samples were obtained from patients with Stage III/IV melanoma enrolled in an observational trial authorized by the Ethics Committee of the University of Regensburg (approval 16-101-0125) and registered with clinicaltrials.gov (NCT04158544). All participants gave full, informed written consent. The first reported case was recruited in OCT-2016 and the last reported case was recruited in JUN-2021. Patients received standard-of-care treatment according to local guidelines. Patients with unresectable metastatic disease who received first- or second-line checkpoint inhibitor therapy were initially treated with Nivolumab (αPD-1; 1 mg/kg; Bristol-Myers Squibb) plus Ipilimumab (αCTLA-4; 3 mg/kg; Bristol-Myers Squibb) for four cycles at 3 week intervals. Thereafter, patients received 3 mg/kg Nivolumab monotherapy at 3 week intervals.

irAE grading

All irAE were assessed by an expert Dermatological Oncologist (Supplemental Figure 1A). ICI-related hepatitis was diagnosed when: (i) GOT, GPT, γ-GT or total bilirubin substantially deviated from pretreatment values; (ii) this change was not attributable to other causes, such as co-medication or viral disease; and (iii) liver injury was sufficiently severe that ICI therapy was suspended or stopped, or immunosuppression was given. Colitis was diagnosed according to stool frequency and consistency, abdominal discomfort, suspension or cessation of ICI therapy, and introduction of immunosuppressive treatment. Thyroiditis was diagnosed based on decreased T3/T4 and elevated TSH levels measured at routine clinic visits.

Routine investigations

Hematological, Biochemical and Microbiological investigations were performed by accredited diagnostic laboratories at University Hospital Regensburg.

Literature search

We searched Medline at the National Library of Medicine through the NCBI website on 11-JUN-2022. Our search terms were 'immunotherapy', 'immune checkpoint inhibitor', 'irAEs', 'biomarkers', 'prediction' and synonyms. We followed-up on relevant citations from articles returned in our original search. We identified 20 articles (Supplemental Table I) describing 59 unique biomarkers (Supplemental Table II).

Flow cytometry

Step-by-step protocols for preparing and analyzing clinical samples by flow cytometry can be accessed through Nature Protocol Exchange (40). Briefly, blood was collected into EDTA-vacutainers by peripheral venepuncture before delivery to the immune monitoring lab at ambient temperature. Samples were stored at 4°C for up to 4 h until processing. Whole blood samples were stained using DURAClone reagents (DURAClone IM Phenotyping Basic Tube, B53309; DURAClone IM T Cell Subsets Tube, B53328; DURAClone IM TCRs Tube, B53340; DURAClone IM Treg Tube, B53346; DURAClone IM B Cell Tube, B53318; DURAClone IM Dendritic Cell Tube, B53351; DURAClone IM Granulocytes Tube, B88651; all from Beckman Coulter, Krefeld, Germany). For the flow cytometry anaylsis of exhausted T cells the following liquid antibodies were used (EXH_CD8 panel): CD49b FITC, 359306, BioLegend, Amsterdam, Netherlands; CD160 PE, IM3657; CD27 ECD, B26603; CD244 PC5.5, B21171; CD279 (PD-1) PC7, A78885; CD127 APC, B42026; CD8 AA700, B49181; CD3 AA750, A94680; CD4 PB, B49197 and CD45 KrO, B36294; all from Beckman Coulter, Krefeld, Germany. Data were collected with a Navios™ cytometer running Cytometry List Mode Data Acquisition and Analysis Software version 1.3 (Beckman Coulter). An experienced operator performed blinded analyses of all datasets in Kaluza version 2.1, as far as possible replicating gating strategies described in the original reports.

Statistics

Our main dataset comprised 110 samples and 59 features extracted from publications and 9 routine clinical parameters; one missing value for GOT was imputed with the median "25" from all other 109 samples. In the extended analysis of DURAClone IM Tube panels, we extracted 80 cell population frequencies by manual gating. Additionally we included 8 clinical parameters, 9 clinical biochemistry values and 18 cell counter values in this extended feature set. One missing value of the presence of liver metastases was imputed with the median "no presence" from all other samples. Univariate analysis was performed for each condition per feature. P-values were calculated using a two-sample Wilcoxon test using a significance level of 0.05 (41). For false discovery rate (FDR) correction (42) of the p-values we used a significance level of 0.1. Discriminatory capability of the features was additionally assessed using ROC-curves and the corresponding area under the curves (AUCs). We report features with AUC > 0.65 as discriminatory. All calculations and plots were made with R 4.2.0 (43).

Models were built in leave-one-out cross-validation and the predictions for each left-out sample were gathered to report the final performance of each model. The penalized logistic regression models were built with glmnet (44) using the elastic-net (45) with an alpha=0.9, and 250 lambda steps in inner cross-validation. The random forest model was built using mlr3 (46) for each binary classification problem with alpha=0.5, num.trees=500, replace=True and splitrule=gini. We also assessed ROC-curves and the AUCs here. AUC $\simeq$ 0 were obtained when penalization of the linear model excluded all features in multiple cross-validation steps, leading to a null-model of only the intercept.

Clustering was performed using FlowSOM (47) in CytoBank on $CD45^+$ $CD3^+$ T cells (DURAClone IM T Cell Subsets Tube) and $CD45^+$ $CD19^+$ B cells (DURAClone IM B Cell Tube). All channels were used as clustering markers except for CD3 or CD19, CD45, FSC, SSC and time. We used hierarchical consensus clustering with 10 metaclusters and 100 (T Cell Tube) or 49 (B Cell Tube) clusters. Feature standardization was applied.

Results

Reported biomarkers are weak predictors of irAE

Our first objective was to test the predictive performance of reported biomarkers of irAE risk in our cohort of 110 metastatic melanoma patients treated with dual checkpoint blockade. Patient characteristics are shown in Table 1. Reviewing the literature, we catalogued 20 publications that reported associations between irAE risk and the frequencies of 55 unique cell populations in peripheral blood or 4 routine clinical parameters (Supplemental Table I) (20–39). In addition, we selected another 9 routine clinical parameters with possible prognostic relevance – namely, sex, CMV seropositivity, GOT, GPT, γ-GT, total bilirubin, LDH, Protein-S100 and presence of liver metastases. Although many of these biomarkers were identified in different clinical contexts, such as anti-PD-1 monotherapy or other malignancies, we reasoned that any robust, mechanistically relevant biomarker could be reasonably expected to have some predictive capacity in

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TABLE 1 Characteristics of study cohort.

Patient cohort characteristics	
Total number of cases	110
Female	37 (33.6%)
Male	73 (66.4%)
Baseline characteristics	
Age (years)	62 (22-84)
BMI	26.6 (15.4-54.6)
Stage III	8 (7.3%)
Stage IV	102 (92.7%)
Liver metastases present	30 (27.3%)
CMV seropositive	52 (47.3%)
ANA positive	65 (59.1%)
Pretreatment	
None	3 (2.7%)
Surgical excision	102 (92.7%)
Radiosurgery	3 (2.7%)
Radiation	42 (38.2%)
Monotherapy	17 (15.5%)
IFNa therapy	9 (8.2%)
Braf/Mek inhibitor therapy	21 (19.1%)
T-VEC therapy	7 (6.4%)
Chemotherapy	6 (5.5%)
Rounds of Ipi/Nivo	
1 round	13 (11.8%)
2 rounds	24 (21.8%)
3 rounds	20 (18.2%)
4 rounds	53 (48.2%)
Complications	
Hepatitis	48 (43.6%)
Colitis	40 (36.4%)
Thyroiditis	41 (37.3%)
No complication	23 (20.9%)
1 complication	50 (45.5%)
2 complications	32 (29.1%)
3 complications	5 (4.5%)

110 patients with Stage III/IV melanoma were enrolled into the study cohort. For Age and BMI, median values were calculated. Minimum and maximum values are given in brackets. Baseline characteristics were obtained before start of Ipi/Nivo therapy.

closely related situations. Hence, our aim was not to directly confirm or refute any previous findings through replication, but to test whether they could be generalized.

To externally validate these 55 flow cytometry and 13 clinical features as predictors of irAEs, we performed uni- and multivariate analyses. We particularly focused on 3 common irAE – hepatitis (44%), colitis (36%) and thyroiditis (37%). Each complication was treated as an separate outcome, but we also considered the occurrence of (i) hepatitis and/or colitis, and (ii) hepatitis and/or colitis and/or thyroiditis (henceforth, "any irAE"). Hence, we tested the value of 68 features in predicting 5 clinical outcomes in our dataset of 110 cases (Supplemental Table II).

Considering all five clinical outcomes, significant associations were discovered for 16 features using the Wilcoxon test without correcting for multiple comparison (Supplemental Table IIIA). However, after adjustment for multiple testing using the false discovery rate (FDR) (42), no associations with hepatitis, colitis, thyroiditis, or "hepatitis and/or colitis" were significant. Four features remained significantly associated with "any irAE" – notably, these were all B cell subsets. Next, we assessed the discriminatory capacity of all 68 features using the area under Receiver-Operating-Characteristics (ROC) curves (Figure 1A). An area under the curve (AUC) of 0.5 implies no discrimination, whereas a maximum AUC of 1 implies perfect discrimination. We found 7 features with AUC > 0.65 (Supplemental Table IIIA). Next, we asked whether these discriminatory features were capturing similar information by grouping them into immunologically relevant classes (Figure 1B). The most discriminatory marker for hepatitis was $CD4^+$ T cell frequency (AUC=0.630) (Supplemental Table IV). Discriminatory markers of colitis risk related primarily to T cells, especially the frequency of $CD4^+$ T cells (AUC = 0.652). The most discriminatory feature for thyroiditis risk was the platelet count (AUC = 0.659). The five most discriminatory features of "any irAE" were B cell markers (best AUC = 0.727). Unfortunately, no single biomarker was powerful enough to reliably identify predisposed individuals.

Combining features does not improve discriminatory power

We next asked whether combining previously reported features predicted irAEs better than single features alone. Therefore, we generated simple penalized logistic regression models (44) and random forest (48) analyses in leave-one-out cross-validation (LOOCV). Neither approach found reliable predictive models (Figure 2). AUC $\simeq$ 0 were obtained when penalization of the linear model excluded all features in multiple cross-validation steps, leading to a null-model of only the intercept. The prediction of each left-out sample is then the mean prediction of all other samples, which always leads to incorrect class prediction.

Our inability to find a reliable predictive model combining different discriminatory biomarkers could be explained in several ways: (i) There may be no immunological predisposition to particular irAEs; (ii) immunological factors might be only partly responsible for any such predisposition; (iii) hepatitis or colitis may be the end-result of more than one immune aetiology; (iv) although we may be capturing predictive information, we might be unable to extract the signal from background noise; or (v) our selection of biomarkers might not capture all phenotypic information necessary for a reliable prediction. Importantly, the hope of finding predictive

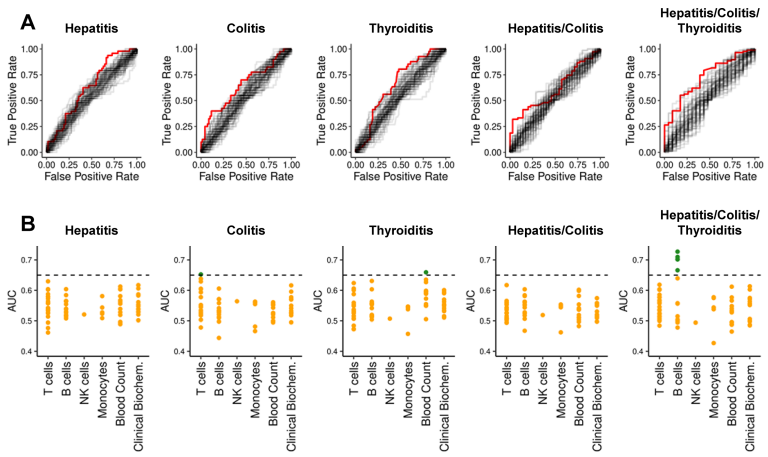

FIGURE 1
ROC-curves and AUCs for previously reported biomarkers and clinical parameters per condition. **(A)** ROC-curves for all 68 features regarding each dependent variable are shown. For each dependent variable, the features with highest AUC is highlighted in red. **(B)** AUCs from ROC-curves in subfigure **(A)** grouped according to immunological classes. The y-axis represents the AUC. Orange dots denote AUC ≤ 0.65; green dots denote AUC > 0.65.

biomarkers of irAE risk that could drive personalized treatment decisions rests upon there being measurable predisposing factors of irAE. There is now good mechanistic evidence for immunological predisposition to irAEs in some cases. For instance, our group and others reported that irAE risk is predicted by oligoclonal expansion of T cells prior to immunotherapy, likely as a consequence of chronic or recurrent viral exposure (13, 20).

To investigate whether our selection of features or analytical methods were limiting the performance of our predictive models, we extended our set of biomarkers by making a finer manual re-gating of our flow cytometry data before repeating our uni- and multivariate analyses. After correction for multiple testing, no extended-set features were significantly associated with hepatitis, colitis, thyroiditis or "hepatitis and/or colitis" risk. However, three B cell subpopulations were significant markers of "any irAE" (Supplemental Table IIIB). The extended-set feature that returned the highest AUCs in prediction of hepatitis was $CD4^+$ T_{EM} (AUC = 0.677), whereas colitis was weakly predicted by immature neutrophils (AUC = 0.670) (Supplemental Table IV). Unfortunately, combining the extended-set features did not return a more stable predictive model for any of the outcomes (Supplemental Figure 1B).

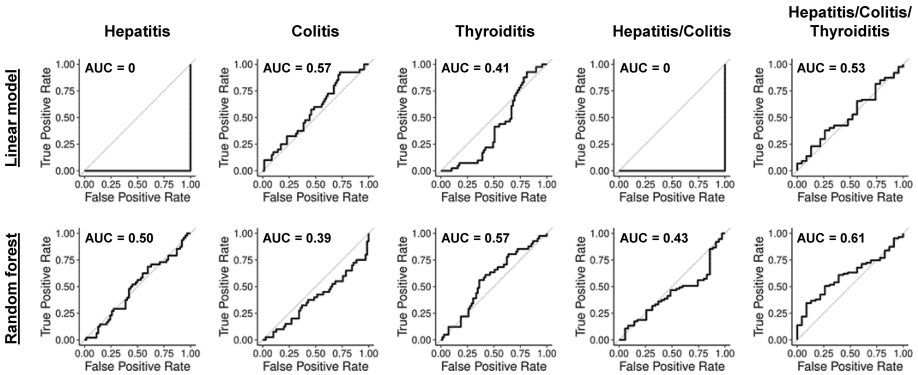

FIGURE 2
ROC-curves for linear models and random forests with previously reported biomarkers and clinical parameters. ROC-curves in LOOCV for penalized logistic regression and random forest models predicting hepatitis (AUC 0 and 0.50), colitis (AUC 0.57 and 0.39), thyroiditis (AUC 0.41 and 0.57), hepatitis and/or colitis (AUC 0 and 0.43) and hepatitis and/or colitis and/or thyroiditis (AUC 0.53 and 0.61).

ICI-related hepatitis may have more than one cause

We previously reported that $CD4^+$ T_{EM} expansion in CMV-seropositive patients before therapy is a strong predictor of hepatitis risk after combined Nivolumab and Ipilimumab treatment (13). We were able to rederive this result in a subset of patients comprising the validation set from our original publication (n=45) plus an additional 30 patients added in this study: The AUC for $CD4^+$ T_{EM} (%) was 0.729. In addition, we used the full dataset to discover another 12 markers of CMV IgG^+ hepatitis with AUC > 0.65, which were mainly T cell subsets (Supplemental Table IIIC). Interestingly, for the CMV IgG^- samples, monocyte frequency (AUC = 0.705) and absolute numbers (AUC = 0.657) predicted hepatitis risk, suggesting there may be more than one aetiological route to ICI-related liver inflammation (Supplemental Table IIID & Supplemental Table IV).

Unsupervised clustering returns new predictive features

It is conceivable our flow cytometry dataset captured predictive information about irAE risk, but that our manual gating strategy failed to identify the most informative cell subsets. Therefore, we applied an unsupervised clustering algorithm (FlowSOM) to samples stained with B cell or T cell markers, then used clusterwise cell abundances as predictive features (47). Univariate analyses after clustering of B cell markers identified no new features with greater discriminatory value than previously considered features (Supplemental Tables IIIE, F). Furthermore, the top-performing models after combining B cell (meta-)clusters in LOOCV were not superior to single features alone (Supplemental Table IV, Supplemental Figures 2A, B).

Likewise, clustering T cells revealed no better discriminatory features for hepatitis, thyroiditis, "hepatitis or colitis" or "any irAE" (Supplemental Tables IIIG, H, Supplemental Table IV & Supplemental Figure 2C). Surprisingly, 4 clusters (C45, C50, C56 and C63) were significantly associated with colitis after FDR correction. These clusters returned AUCs of 0.690, 0.709, 0.711 and 0.713, respectively – hence, they showed greater discriminatory value than previously considered features (Supplemental Table IIIH). Unfortunately, combining C45, C50, C56 and C63 in LOOCV did not improve their predictive performance (Supplemental Figure 2D).

We next asked why these particular T cell clusters might encode more information about colitis risk than other T cell subsets. C63, C56 and C45 were $CD4^+$ memory T cells with a $CD45RA^-$ $CCR7^{int/-}$ $CD27^+$ $CD28^+$ $CD57^-$ phenotype, possibly representing transitional states between recently activated central memory (T_{CM}) and T_{EM} cells (Figure 3). Apart from CCR7 expression, these T cell clusters differed only in PD-1 expression. C50 was a minor population of $CD8^+$ $CD45RA^+$ $CCR7^-$ $CD27^+$ $CD28^-$ PD-1^- $CD57^+$ T_{EMRA} cells. We speculate that C50 overlaps with a non-exhausted, recirculating subset of $CD8^+$ T_{EMRA} cells that others have reported as important for

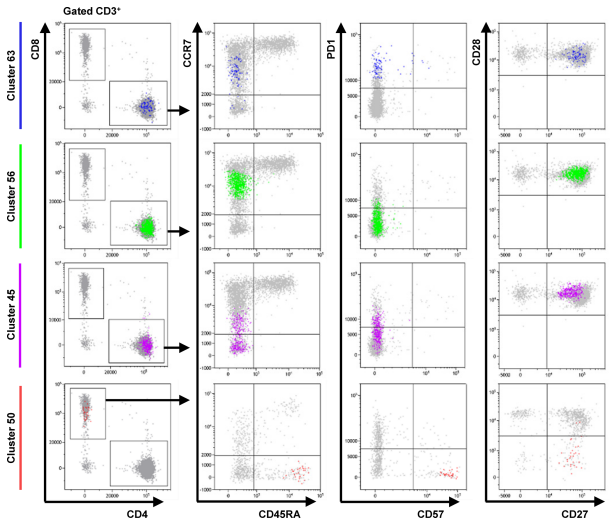

FIGURE 3
Phenotype of cells in FlowSOM clusters associated with colitis. Dot plots show the phenotype of the cells in each cluster (color) and all gated cells for reference (grey). Clusters 63 and 56 are $CD4^+$ $CD45RA^-$ $CCR7^{int}$ $CD27^+$ $CD28^+$ $CD57^-$ T cells that differ only in expression of PD-1. Cluster 45 is $CD4^+$ $CD45RA^-$ $CCR7^{low/-}$ PD-1^{int} $CD27^+$ $CD28^+$ $CD57^-$ T cell population. Cluster 50 represents a $CD8^+$ $CD45RA^+$ $CCR7^-$ $CD27^+$ $CD28^-$ PD-1^- $CD57^+$ T_{EMRA} subpopulation. Data from one representative patient.

maintaining anti-viral immunity (49). Our results suggest that patients at risk of ICI-related colitis might have on-going immune responses – possibly against subclinical viral infections – and that our predictive features actually capture information about the rate of T_{CM} to T_{EM} differentiation.

Discussion

Reproducibility studies using external data are an important validation step in clinical biomarker development. Here, we showed that previously reported flow cytometry-based biomarkers of irAE are not generally reliable enough to predict hepatitis, colitis or thyroiditis as a basis for clinical decision-making. Promisingly, however, unsupervised clustering revealed four T cell subpopulations associated with risk of colitis that returned AUC > 0.65, which we take as a sign that better predictions of irAE risk might be possible with a refined marker selection and more sophisticated computational methods. We conclude that deeper phenotyping of monocytes and $CD4^+$ memory T cells transitioning between T_{EM} and T_{CM} might lead to more informative biomarkers in future.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding author.

Ethics statement

The studies involving human participants were reviewed and approved by Ethics Committee of the University of Regensburg. The patients/participants provided their written informed consent to participate in this study.

Author contributions

GG performed statistical evaluation and wrote the manuscript. JY performed literature search. PR and LC analyzed data and revised the manuscript. H-LS revised the manuscript. MK provided expert flow cytometry advice. HS, EG and RB provided infrastructural support and critical feedback. BS provided expert virological opinion. SH provided clinical samples and expert dermatological opinion. JH designed study and wrote the manuscript. KK performed experiments, analyzed data and wrote the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

MK is a Beckman Coulter Life Sciences associate. SH has received consulting fees and speaker's honoraria from BMS and Merck Sharp & Dohme (MSD).

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2022.1011040/full#supplementary-material

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Knowledge mapping of targeted immunotherapy for myasthenia gravis from 1998 to 2022: A bibliometric analysis

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Background: The treatment of myasthenia gravis (MG) has advanced from steroids and traditional immunosuppressants to targeted immunotherapy. Targeted immunotherapy has been successfully employed in clinical practice in recent years. This study aimed to explore the emerging trend of targeted immunotherapy in MG and summarize the knowledge structure through bibliometric methods.

Methods: The Web of Science Core Collection database (WoSCC) was chosen to retrieve the literature on targeted immunotherapy for MG. Two bibliometric analysis software, VOSviewer and CiteSpace, and bibliometric online platform were mainly used to evaluate the contributions from countries/regions, institutions, journals, and authors through the construction and visualization of bibliometric networks. By systematically reviewing a knowledge domain, future research developments were determined. The R version 4.1.2 and Microsoft Excel 365 were used for statistical analysis.

Results: A total of 562 original articles and 262 reviews relevant to MG targeted immunotherapy were included. The number of publications on targeted immunotherapy for MG exhibited a two-phase advancement. The first stage showed a steady growth trend from 1998 to 2016, with an annual number of no more than 35 publications. The second stage revealed an explosive growth trend from 2017, reaching a peak number of publications in 2020. The United States ranked first in the number of publications, citations, and h-index. The author with the highest citation and h-index was Vincent A. And 28.03% of the articles were published in the top 10 journals. In addition to "myasthenia gravis", the keyword with the highest consideration was "rituximab", followed by "double-blind", which indicate research hotspots gradually from basic research to clinical research over time, especially in the field of targeted immunotherapy. The MG treatment has entered a personalized precision treatment phase. Exploration into new target molecules and conducting high-quality randomized controlled trials on existing biological agents are the further research direction.

Conclusion: The current study summarized the global research trends concerning targeted immunotherapy for MG. Research interests gradually advanced from basic research to clinical research. MG treatment has entered a personalized precision treatment phase. Further investigations into new target molecules and high-quality randomized controlled trials on existing biological agents are required urgently to direct future immunotherapy research.

KEYWORDS

myasthenia gravis, bibliometric, VOSviewer, Citespace, targeted immunotherapy

Introduction

Myasthenia gravis (MG) is an acquired autoimmune disease, manifested by disruption of neuromuscular junction (NMJ) transmission caused by autoantibody, cellular immune dependence, and complement (1). Anti-acetylcholine receptor antibodies (AChR-Ab) are the frequent cause of pathogenesis, and the main clinical manifestations are fluctuating skeletal muscle weakness and fatigue (2). The global annual incidence of MG is 0.4-1 per 100,000 people and the worldwide prevalence was 15-25 per 100,000 people (3–5). Presently, the treatment of MG is based on steroidal and other traditional immunosuppressants, such as tacrolimus, azathioprine, and mycophenolate mofetil (6). Nevertheless, long-term use of steroids can cause serious side effects such as abdominal obesity, elevated blood pressure, elevated plasma glucose level, osteoporosis, and necrosis of the femoral head (7). The traditional immunosuppressive agents not only have a slower onset of effect but are associated with the risk of tumorigenesis, myelosuppression, and infection. Therefore, these side effects will further burden the disease. Furthermore, the selection of MG treatment remains very challenging due to the heterogeneity in pathogenesis, clinical manifestations, and drug reactions (1). Targeted biological agents are a class of small molecule inhibitors that specifically target inflammatory cytokines, immune cells, and intracellular kinases (8). The clinical use of these drugs has changed the treatment landscape for autoimmune diseases. A variety of targeted biological agents targeting immune cells, complements, neonatal Fc receptors, and cytokines have entered phase II and III clinical trials (9–12). These targeted biological agents can alleviate the clinical symptoms quickly, significantly, and continuously with favorable tolerability and safety (13). These targeted biological agents can reduce the dosage of steroids and accelerate precision medicine. Therefore, the targeted biological agents possess significant research value and promising clinical applications.

Bibliometric analysis and data visualization, a well-established bioinformatics tool, are used to analyze a field of research quantitatively and qualitatively, provide evidence for the impact of an area of research, find the emerging area of research, and identify potential research collaborators (14, 15). Different from meta-analysis and systematic review, bibliometric analysis integrates information visualization techniques with mathematical and statistical analyses to assess institutions performing research, contributing authors, journals publishing a specific area of research, and countries/regions with a research area of interest (16). It primarily evaluates the characteristics of the literature, such as the number of publications, citations, and research or clinical collaborations (17, 18). These analyses provide guidelines for assessing research trends and developing research areas (19). This analytical method of the literature is used in all areas of basic and clinical research.

A team published high-impact articles on targeted immunotherapy for MG. Who are the core authors of these studies? Who are their collaborators? What research topics were they interested in? Which topic received the most attention? What journals were they published in? How did the specific research area develop and evolve? No individual can read all the high-impact articles on a specific area due to limited time and energy (20). Therefore, bibliometrics provides a new method for literature analysis, so that readers can rapidly understand the emerging subjects in their research areas of interest and read the selected literature (21, 22). Researchers can use these data to quickly identify potential new collaborators in their respective research fields.

No bibliometric studies have been published on targeted immunotherapy for MG thus far. This paper aimed to systematically summarize and visually analyze the literature in the field of targeted immunotherapy for MG based on the Web of Science and using CiteSpace and VOSviewer software to understand the frontiers and emerging trends of research. The outcome can provide more references, novel insights, and directions for future clinical research and guidelines establishments. This bibliometric analysis is the first attempt relevant to this area of research.

Methods

Data source

There were only a few articles on MG targeted immunotherapy before 1998, while after rituximab was used for treatment MG in 2000 firstly (23), the articles on MG targeted immunotherapy gradually increase over time. Therefore, the Science Citation Index Expanded (SCI-Expanded, 1998-present) of the Web of Science Core Collection (WoSCC) database was selected after considering the limitations and strengths of diverse databases (24). The Web of Science (WoS) was created by Thomson Scientific to make citation indices (that E. Garfield assessed since the early 1960s) accessible *via* the internet, which is the oldest citation database and is currently owned by Clarivate Analytics Company (Philadelphia, United States of America) (24). The selection to use the WoSCC database was justified for the following reasons. First, the WoSCC is the most comprehensive and authoritative database when compared with other databases, such as PubMed, Scopus, and Embase (25, 26). Second, it is a classic citation database, including literature abstracts and other relevant data, such as citations and research collaboration information, which is useful for bibliometric analysis (17). Finally, it can directly provide reference information that is required for the construction and visualization of bibliometric networks by VOSviewer and CiteSpace. Otherwise, an additional operation is required to change the file format if the information is retrieved from another database. Therefore, the WoSCC is considered the most suitable online database for bibliometric analysis (27–29).

Retrieval strategies

The advanced retrieval function was used to improve the quality of information. The specific retrieval rules were as follows: #1: TS= (myasthenia gravis); #2: TS=(eculizumab) OR TS=(rituximab) OR TS=(RTX) OR TS=(tocilizumab) OR TS= (belimumab) OR TS=(rozanolixizumab) OR TS=(efgartigimod) OR TS=(Zilucoplan) OR TS= (monoclonal antibody) OR TS= (biologic drugs) OR TS= (targeted immunotherapy) OR TS= (targeted immunotherap*) OR TS= (novel therap*) OR TS= (novel treatment strategies); the ultimate dataset: #1 AND #2. Literature in English only were included. The search was limited to systematic reviews and original articles. A truncation symbol "*" was used and the use of truncation searches improved recall and prevented missing inspection. All contents including the titles, authors, abstracts, keywords, and cited references were recorded. A total of 993 records on targeted immunotherapy for MG were searched from 1998 to 2022 (retrieved on April 25, 2022). The exclusion materials were 169 records including meeting abstract, editorial material, revision, letters, journalism, and non-English works of literature. Consequentially, 824 valid literatures (562 articles and 262 reviews) were retrieved as the final dataset and exported in the form of "full record and cited references" for further analysis. Subsequently, the text files were renamed as "download∗.txt", which were recognized by CiteSpace software. The detailed literature screening process is shown in Figure 1.

Data extraction

These data were imported into Microsoft Excel 365 (Microsoft Corporation, Redmond, Washington, WA, United States) for further processing. Two researchers (YS and ZR) performed data extraction and literature selection and analysis to ensure the reliability of the results independently. Any discrepancies between the two researchers were discussed to reach a consensus. The disagreements were resolved through discussion or a third-party consultation (RW and SH). The indicators such as the annual number of publications and citations, countries/regions, journals, institutions, authors, co-cited references, and keywords were primarily focused on. The citation reporting of the WoSCC was used to assess the h-index and citation frequency. The h-index was calculated considering a scientist/country has published h articles, each of which has been cited at least h times (30). This index was typically used to assess the scientific impact and productivity of a researcher/country (31). The journal impact factor (IF) and category (Q1, Q2, Q3, or Q4) were retrieved from the Journal Citation Reports (JCR) 2021, which is the most widely used reference standard for evaluating the journal performance within its field.

Data visualization and analysis

Three bibliometric tools, including two software (CiteSpace (5. 8. R3) and VOSviewer (1. 6. 18)) and an online platform were used in this study for a more comprehensive analysis.

VOSviewer

VOSviewer (version 1.6.18, the Netherlands, downloaded from http://vosviewer.com) is a literature knowledge visualization software that uses the Visualization of Similarities (VOS) technology, which was developed by Professors Eck and Waltman from Leiden University using the Java language. The VOSviewer assesses and visualizes research characteristics from different perspectives, such as co-authors, research institutions, countries/regions, keywords, and co-cited references (32). In the network visualization map, each node corresponds to parameters, such as countries/regions, institutions, journals,

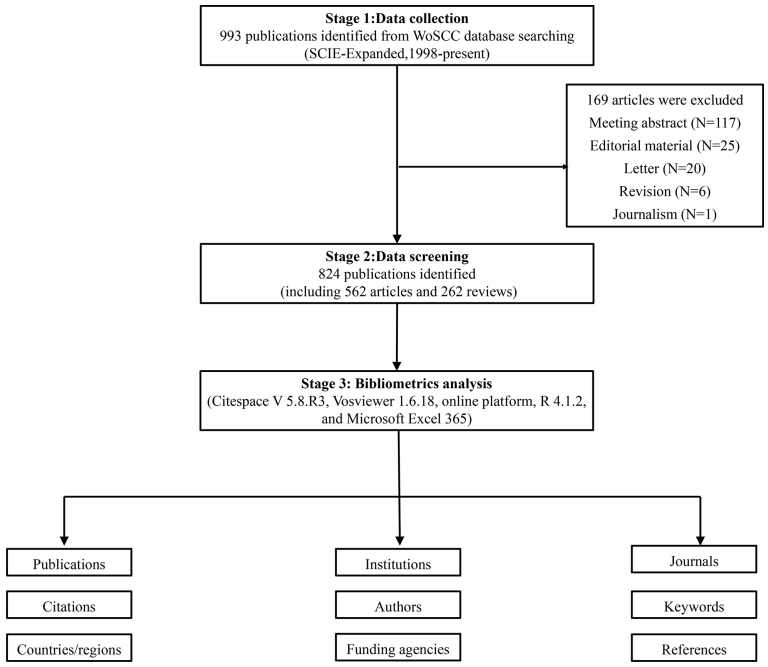

FIGURE 1
Flowchart of the literature search and selection process.

authors, or keywords. The diameter of its size is roughly proportional to the number of publications, citations, or occurrences. Closer terms in the same publications are automatically assigned to a cluster with the same color. Otherwise, the nodes are set apart with different color coding. The link between nodes represents the network connection and the strength of the link. The total link index is used to quantitatively evaluate the strength (TLS), which is the sum of the link strength of all other terms (26, 27). Furthermore, the VOSviewer can provide three types of network maps, including the network visualization map, the overlay visualization map, and the density visualization map (33).

CiteSpace

CiteSpace (Version 5.8. R3, downloaded from https://sourceforge.net/projects/citespace/) is a Java-based computer program designed by professor Chen from Drexel University (34), and it is an influential visualization software to obtain quantitative information and discover the related development trends and dynamics in a particular scientific research field (34). The network maps generated by the CiteSpace were also composed of links and nodes. The nodes normally represent the authors, country/regions, or institutions, whereas links represent co-authorship between these nodes. The centrality is an important indicator that unveils the importance of a node in the network, and the higher the centrality the node has, the larger the impact the node has on the map (35). The burst detection of references and keywords recognizes the sharp increases in scientific activities over a limited period and captures the increasing interest in a specific research field (36).

Bibliometric analysis using an online platform

In addition to the above methods, an online platform for bibliometric analysis and visualization, https://bibliometric.com/ (accessed on 28 April 2022), plotted the distribution and international collaboration of countries/regions.

Materials and methods ethics statement

This study did not involve human or animal subjects and all data used in this manuscript were obtained from public databases. Therefore, ethical approval was not required.

Statistical analysis

Statistical analysis was performed using R version 4.1.2 (The R Foundation for Statistical Computing, Vienna, Austria) and

Microsoft Excel 365 (Microsoft Corporation). The VOSviewer (version 1.6.18, Leiden University, Leiden, the Netherlands) and CiteSpace (version 5.8.R3, Drexel University, Philadelphia, PA, USA) were used for the analysis of basic metrics.

Result

The growth trend of publication outputs and citations

Quantitative analysis of the published papers can reflect the productivity of a given scientific research field over the years and exhibit the trend in development in a specific area. Utilizing the aforementioned search strategies, a total of 993 articles were retrieved. After excluding invalid articles, 824 publications, including 562 original research articles and 262 reviews were included in the final analysis (Figure 1). The annual distribution of publications and total citations of annual publications from 1998 to 2022 are shown in Figure 2. During the past 24 years, with the exception of decrease in number at some time points, the annual number of articles on targeted immunotherapy for MG has shown a steady growth trend and reached its peak in 2020.There were two growth phases according to the curve: an early stationary growth phase from 1998 to 2016 and a rapid-growing phase from 2017 to 2022. Based on the WoSCC database analysis, 34.95% of them (824) were published in the last four years, all publications related to targeted immunotherapy for MG have been cited 22184 times (18346 times after the removal of self-citations) with 26.92 citations per paper and the H-index of 70.

Analysis of published articles by countries/regions

Table 1 summarizes these publications from the top 10 countries/regions. The retrieved articles were from 59 countries/regions. The USA ranked first in research productivity [320 (38.83%)], followed by China [89 (10.80%)], England [81 (9.83%)], Germany [71 (8.62%)], and Italy [70 (8.50%)]. After removing self-citations, the USA had 9696 citations and an h-index of 54. Both parameters ranked first among all countries/regions analyzed, followed by England, Germany, Italy, and France. The overall citations and h-index were 3097, 2665, 1979, and 1524 and 32, 28, 23, and 24, respectively (Figure 3A). The geographical distribution map based on the total number of publications from the distinct country is shown in Figure 3B. On the map, the lighter colors represent the low density of publications, and the darker colors represent the high density. Annual trends in the number of articles are displayed in Figure 3C, and the USA was the leading country in the annual number of publications from 1998 to 2022. A collaboration analysis was conducted to examine the international collaboration observed from 1998 to 2022. Figure 4 demonstrates that the USA had the greatest international collaboration in this area followed by China. The United Kingdom has the strongest connection with the USA. Links represent international collaboration pathways between countries. Only countries/regions with a minimum number of 3 publications were included in the network. Only 34 countries/regions that met the threshold were analyzed using the VOSviewer (Figure 3D). There were 34 nodes, 8 clusters, and 176 links on the network map. The top three countries with the largest TLS were the USA (TLS =200), England (TLS = 129), and Germany (TLS = 99).

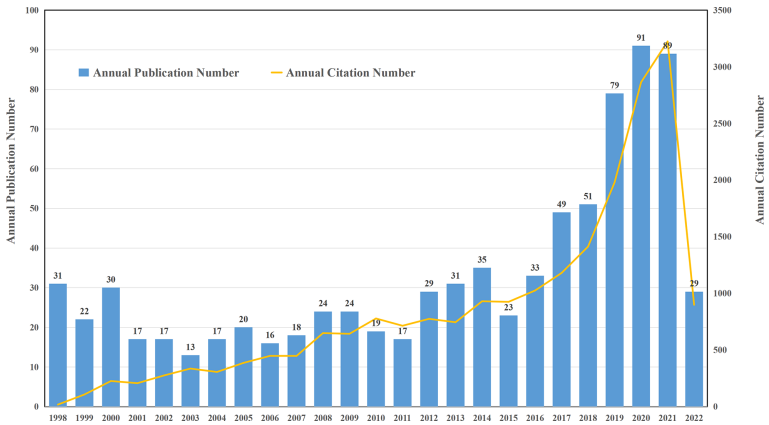

FIGURE 2
Global trend of annual publications and citations related to targeted immunotherapy for MG from 1998 to 2022.

TABLE 1 The top 10 countries/regions contributing to targeted immunotherapy for MG.

Rank	Country/Region	Number of publications	Number of citations	Citations per article	H-Index
1	USA	320	9696	32.89	54
2	CHINA	89	1146	12.88	21
3	ENGLAND	81	3097	39.56	32
4	GERMANY	71	2665	38.2	28
5	ITALY	70	1979	29.53	23
6	GREECE	67	1193	20.12	22
7	FRANCE	52	1524	30.06	24
8	JAPAN	52	1112	22.1	17
9	NETHERLANDS	52	1451	29.08	21
10	CANADA	37	937	27	19

Analysis of the institutions with the most productivity

A total of 1084 institutions published scientific articles on targeted immunotherapy for MG during the defined study period. As shown in Table 2, the top 10 institutions accounted for 323 (39.20%) of literatures in this field, and the League of European Research Universities was the largest contributor in terms of numbers of publications with 81 (9.83%) articles, followed by the University of Oxford with 40 (4.85%) articles and the Hellenic Pasteur Institution with 35 articles (4.25%). The institution citation analysis is presented in Figure 5A. The publications originating from 81 institutions were selected, with a minimum number of documents of more than 5 from each country. The data were analyzed by using the VOSviewer and there were 81 nodes, 4 clusters, and 1862 links on the network map, the hellenic pasteur institution at the center of node.

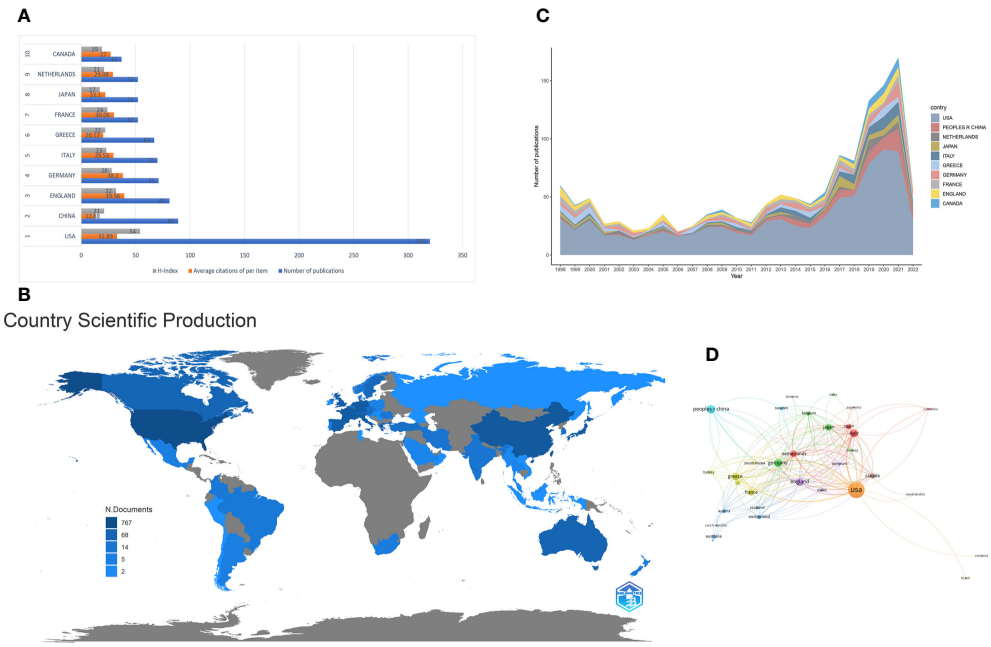

FIGURE 3
(A) The number of publications, average citations of per items and H-index of the top 10 countries/regions. **(B)** Geographic distribution map displaying the global distribution of targeted immunotherapy for MG. Different countries/regions were denoted with different colors based on the number of articles published. **(C)** The annual number of publications from the top 10 countries/regions between 1998 and 2022. **(D)** Citation map of countries/regions on targeted immunotherapy for MG generated by the VOSviewer. Each node represents a country/region, and node size indicates the number of publications. The connection between the nodes represents a citation relationship, and the thickness of the lines indicates citation strength (weights on the TLS).

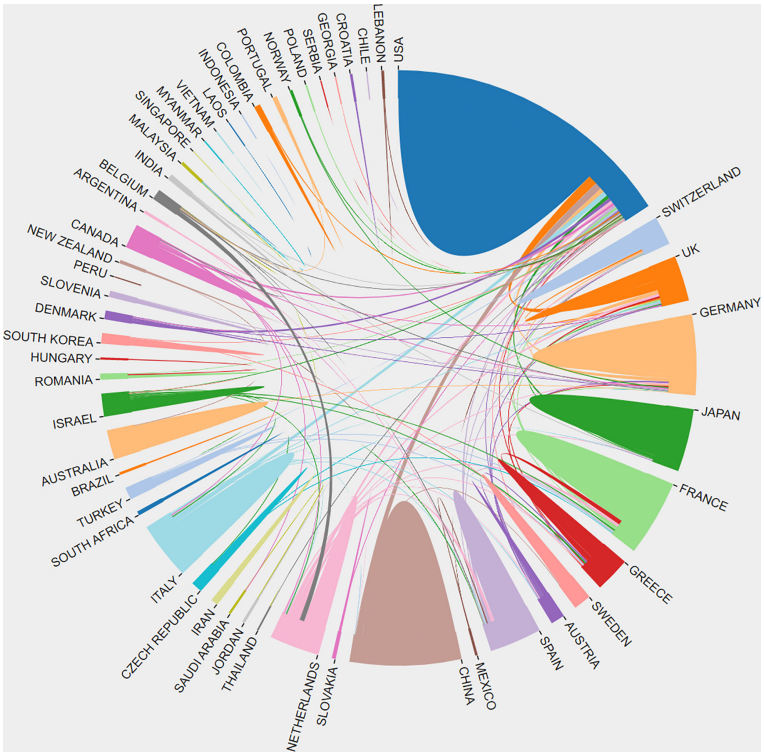

FIGURE 4
Distribution and international cooperation of countries/regions that are involved in targeted immunotherapy for MG. The thickness of the line reflects the frequency of the cooperation. The thicker the line, the stronger the cooperation.

Analysis of funding agencies

As noted above, the economic foundation contributed the most to scientific development. A summary of the top 10 most active funding agencies in this area is provided in Table 3 and Figure 5B. The funding organizations from the USA including the National Institutes of Health (NIH), United States Department of Health and Human Services (HHS), and NIH National Institute of Neurological Disorders Stroke (NIDS) occupied the top three positions in contributing to this field, and funded 105, 105, and 56 studies, respectively. The remaining funding agencies were from China, Belgium, France, and Japan. In addition to having well-established institutions, the USA maintained its leading position in the domain of targeted immunotherapy for MG, which was not separated from the support of adequate funding. Adequate funding can attract a wider variety of researchers and institutions to devote more work to this area, which is a mutually reinforcing process.

TABLE 2 The top 10 institutions with most publications in the field of targeted immunotherapy for MG.

Rank	Institution	Number of publications	Number of citations	Citations of per article	H-Index
1	League of European Research Universities Leru	81	3467	44.05	32
2	University of Oxford	40	1631	41.9	20
3	Hellenic Pasteurinst	35	595	19.46	15
4	Udice French Research Universities	26	853	33.15	16
5	University of California System	26	677	26.5	12
6	Yale University	26	849	34.46	14
7	Maastricht University	20	495	25.95	11
8	University of Texas System	25	518	21.56	13
9	University of North Carolina	23	795	36.48	13
10	University of North Carolina Chapel Hill	21	763	38.38	13

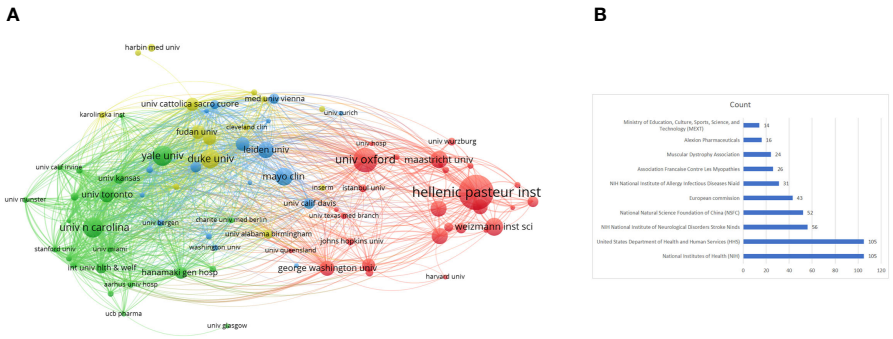

FIGURE 5
(A) Network visualization of the institution co-citation analysis on targeted immunotherapy for MG based on the VOSviewer. **(B)** The top 10 funding agencies for the output of targeted immunotherapy for MG.

Analysis of journals and co-cited journals

In total, the retrieval article was published in 323 journals in this research field. The top 10 active journals that published 231 papers on targeted immunotherapy for MG, accounted for 28.03% of all 824 publications. Table 4 summarizes the basic information on the top 10 journals. The highest number of relevant articles were published in the *Journal of Neuroimmunology* [44 (5.34%)], and *Muscle Nerve* [41 (4.98%)] ranked second, followed by *Annals of the New York Academy of Sciences* [27 (3.28%)]. According to the 2020 JCR standards, the IF of the top 10 journals ranged from 3.217 (*Muscle Nerve*) to 9.91 (*Neurology*) and was classified as Q1 to Q2 categories. In addition to the number of publications, the impact factor of journals also depends on how often they are co-cited in a particular field of research. As shown in Figures 6A, B, co-citation analysis was performed by the CiteSpace software to determine the connection between journals that were cited in other journals, and there were 300 nodes and 448 links in the co-cited network map. The *FASEB J* had the highest centrality, with a central value of 0.4, followed by the *Brain* (0.29) and *Nat Immunol* and *J Neurol Neurosur Ps* (0.2). Additionally, a dual map overlay of the journals on targeted immunotherapy for MG was constructed (Figure 7). The dual map overlay of journals described the topic distribution of academic journals, and the map of the citing journals was on the left and the map of the cited journals was on the right. Collectively, there were three main citation paths on the current map. The published studies mainly targeted the journals in three fields: i) molecular biology and immunology; ii) medical and clinical areas; and iii) neurology, sports, and ophthalmology whereas the most cited publications originated from the journals of molecular biology and genetics.

Analysis of authors and co-cited authors

The number of research papers published by an author may translate the contribution to the research in the field. A total of

TABLE 3 The top 10 funding agencies contributed to targeted immunotherapy for MG.

Rank	Funding agencies	Countries	Count	Percentage(N=824)	H-index
1	National Institutes of Health (NIH)	USA	105	12.74	37
2	United States Department of Health and Human Services (HHS)	USA	105	12.74	37
3	NIH National Institute of Neurological Disorders Stroke (NINDS)	USA	56	6.80	25
4	National Natural Science Foundation of China (NSFC)	China	52	6.31	12
5	European commission	Belgium	43	5.22	21
6	NIH National Institute of Allergy Infectious Diseases (NIAID)	USA	31	3.76	20
7	Association Francaise Contre Les Myopathies	France	26	3.16	15
8	Muscular Dystrophy Association	USA	24	2.91	17
9	Alexion Pharmaceuticals	USA	16	1.94	9
10	Ministry of Education, Culture, Sports, Science, and Technology (MEXT)	Japan	14	1.70	8

TABLE 4 Top 10 journals with most publications in the field of targeted immunotherapy for MG.

Rank	Journal	Number of publications	Number of citations	Citations of per article	H-Index
1	Journal of Neuroimmunology	44	636	15.05	15
2	Muscle Nerve	41	1246	31.61	19
3	Annals of the New York Academy of sciences	27	556	20.85	14
4	Frontiers in Immunology	26	310	12.38	11
5	Frontiers in Neurology	19	99	5.42	6
6	Neurology	19	981	52.37	13
7	Current Opinion in Neurology	15	688	46.2	13
8	Clinical and Experimental Immunology	14	471	33.71	11
9	Journal of Immunology	13	600	46.31	11
10	Journal of Neurology	13	240	18.85	7

200 researchers authored 824 articles. The top 10 most productive authors in the field are presented in Table 5. Tzartos SJ [47 (5.70%)] had the highest number of publications, followed by Vincent A [21 (2.55%)], Howard JF [21 (2.55%)], Evoli A [19 (2.31%)], and Nowak RJ [19 (2.31%)]. Additionally, the CiteSpace software analyzed the author's co-citation. Nevertheless, the centrality of the top 10 authors was not high and was <0.1 for each author, and a small number of links were observed on the network map, which indicated that there was little collaboration between different researchers in this research field.

Analysis of references with citation burst

The top 10 original articles relevant to targeted immunotherapy for MG with the most citations are summarized in Table 6. These selected articles span from 2000 to 2017. The most highly cited paper was published in 2006 and was written by Pescovitz, MD with 392 citations (37). The second co-cited paper was written by Vincent, A with 359 citations (38). The third co-cited paper was published by Zimmer, L with 354 citations (39). Burst detection, an algorithm developed by Kleinberg, was considered a tool to identify research frontiers or emerging trends in research over time (36). In our study, the burst detection algorithm was used to determine key references and keywords for targeted immunotherapy for MG. The blue line represented the period, and the red line indicated the duration of the reference burst occurrence. Among these references, REGAIN study had the strongest burst reference during the period from 1998 to 2022. Howard JF published the article, and its strength value was 22.47 (40). This study further assessed the efficacy and safety of eculizumab, a terminal complement inhibitor, in anti-acetylcholine receptor antibody-positive refractory patients using a phase 3 trial. This finding provided a novel perspective for the further development of targeted immunotherapy for MG. Citation bursts determined the frequency of citations for a

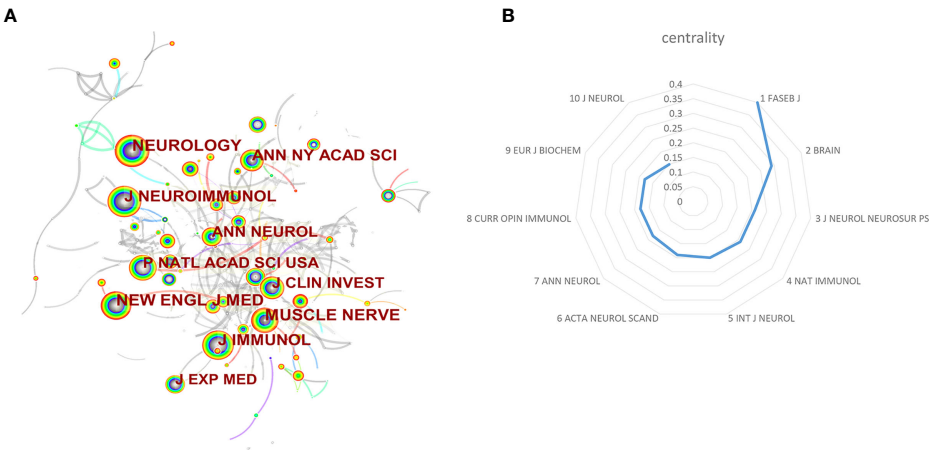

FIGURE 6
(A) Visualization map of journal co-citation analysis by using CiteSpace. **(B)** The top 10 centrality of journals for the targeted immunotherapy for MG.

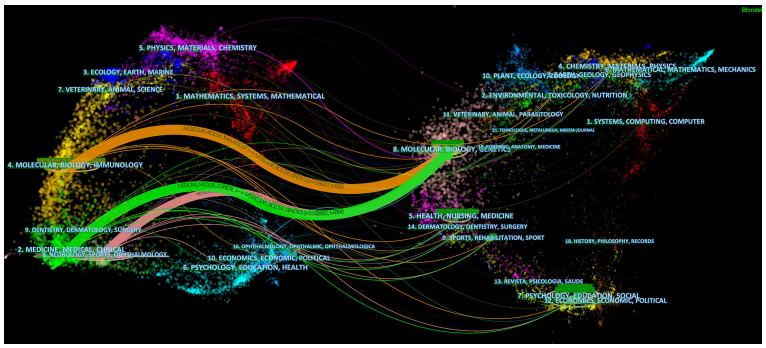

FIGURE 7
The dual-map overlay of academic journals in the field of targeted immunotherapy for MG based on the CiteSpace software. The labels represent different research subjects covered by the journals. The citing journals are on the left side, while the other side of the map represents the cited journals. Different colored lines correspond to the different paths of references, beginning with the citing map and ending at the cited map. The path widths are scaled proportionally to the frequency of z-score-scale citation.

reference over a period and the establishment of findings in this field. The CiteSpace was used (Selection Criteria: Top 25; The Number of States: 2; Minimum Duration: 2) to obtain 165 references with the most robust citation bursts for the targeted immunotherapy for MG. Figure 8 shows the top 25 among them. The first burst of co-cited reference began in retrieval time (1998), which was a review on MG. Currently, 8 of the 25 references were still in the burst. Therefore, targeted immunotherapy for MG-related research fields may advance in the future. We also performed the reference co-citation analysis (Supplementary Figure S1) and the cluster view map (Supplementary Figure S2) by CiteSpace, Supplementary Figure S1 displays the first author, and the year of the co-citations of references. Each circle represents a reference. The link between the two circles represents two references cited in the same article among the 824 articles (citing articles) retrieved in this study. A cluster view map is conducted if the two articles have many similar references and are often homogeneous. The largest eight clusters extracted from the references of the 824 citing articles are shown in Supplementary Figure S2, including #1 myasthenia gravis treatment, #2 muscle, #3 musk antibodies, #4 orphan drugs, #5 versus-host disease, #6 pembrolizumab, #7 complementary peptide, #8 complement activation. The total Modularity Q (0.7908) and Mean Silhouette (0.907) values were both greater than 0.5, suggesting that the cluster quality was reasonable.

Analysis of keywords

In addition to references, keywords can offer readers information about the research topics and methodologies of the publications, and analysis of keywords co-occurrence is often employed to detect the research hotspots and directions in the research field. The network visualization map was generated for keywords with the value of co-occurrence greater than 20 times. As shown in Figure 9A, there were 50 nodes, 839 links, and a total link strength of 4293 on the visualization map, the "myasthenia gravis" at the center of node, followed by "rituximab". The density visualization map of the keywords is

TABLE 5 The top 10 most productive authors contributed to targeted immunotherapy for MG.

Rank	Author	Number of publications	Number of citations	Citations of per article	H-Index
1	Tzartos SJ	47	896	20.61	12.5
2	Vincent A	21	1100	49.13	16
3	Howard JF	21	832	41.85	13
4	Evoli A	19	880	47.74	14
5	Nowak RJ	19	651	36	12
6	Kaminski HJ	18	435	26.28	11
7	Mantegazza R	17	617	38	12
8	Berrih aknin S	15	356	24.67	11
9	De Baets MH	15	270	19	8
10	Martinez Martinez, P	14	245	19.07	9

TABLE 6 The top 10 co-cited references of targeted immunotherapy for MG.

Rank	Title	Journal	Country	Author	Years	Number of citations
1	Rituximab, an anti-CD20 monoclonal antibody: History and mechanism of action	American Journal of Transplantation	USA	Pescovitz, MD	2006	392
2	Myasthenia gravis	Lancet	England	Vincent, A	2001	359
3	Neurological, respiratory, musculoskeletal, cardiac and ocular side-effects of anti-PD-1 therapy	European Journal of Cancer	Germany	Zimmer, L	2016	354
4	Imbalance of regulatory T cells in human autoimmune diseases	Immunology	Austria	Dejaco, C	2006	254
5	Safety and efficacy of eculizumab in anti-acetylcholine receptor antibody-positive refractory generalised myasthenia gravis (REGAIN): a phase 3, randomised, double-blind, placebo-controlled, multicentre study	Lancet Neurology	USA	Howard, JF	2017	226
6	Acetylcholine receptors and myasthenia	Muscle & Nerve	USA	Lindstrom, JM	2000	202
7	Long-lasting treatment effect of rituximab in MuSK myasthenia	Neurology	Spain	Diaz-Manera, J	2012	194
8	Myasthenia gravis: An emerging toxicity of immune checkpoint inhibitors	European Journal of Cancer	Australia	Makarious, D	2017	146
9	A randomized, double-blind, placebo-controlled phase II study of eculizumab in patients with refractory generalized myasthenia gravis	Muscle & Nerve	USA	Howard, JF	2013	131
10	Rituximab treatment of myasthenia gravis: a systematic review	Muscle & Nerve	USA	Tandan, R	2017	121

illustrated in Figure 9B, the top three keywords with the greatest number of occurrences are “myasthenia gravis” which appears 309 times; followed by “rituximab” and “monoclonal-antibodies” which appears 171 times and 111 times, respectively. The overlay visualization map is shown in Figure 9C, summarizing the keyword occurrences from a time zone perspective.

Burst keywords

The CiteSpace was used to detect burst keywords to determine the hotspots and research frontiers over time. The burst keywords are terms cited frequently over a period. The top 25 keywords with the strongest citation bursts are presented in Figure 9D. The blue line represents the time from 1998 to 2022,

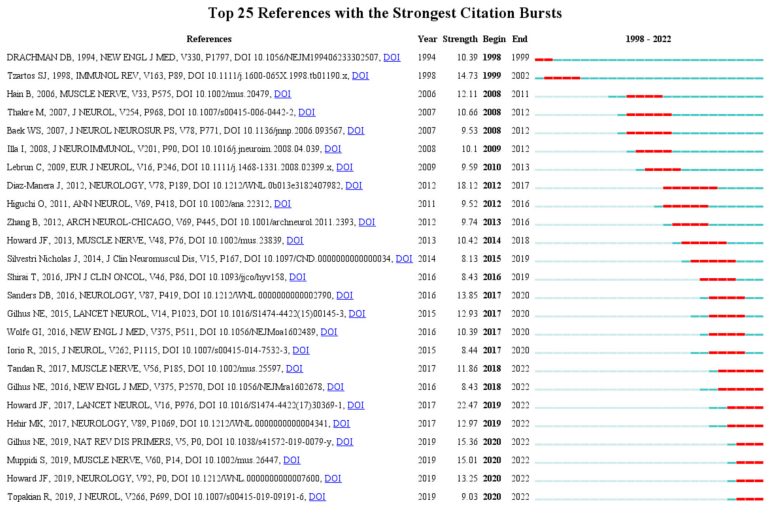

FIGURE 8
The top 25 references with the strongest citation bursts. The red segment represents the begin and end year of the burst duration.

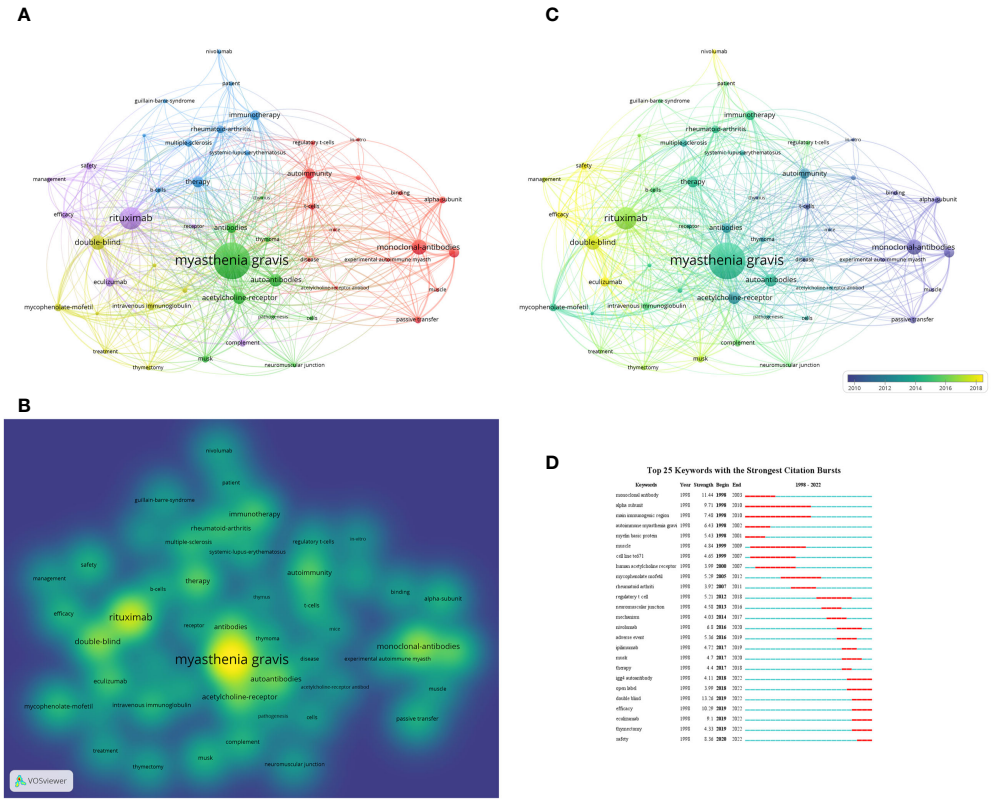

FIGURE 9
(A) Network visualization of keywords based on VOSviewer. In this network map, keywords with close relationship are assigned to one cluster with the same color. All the keywords could be divided into five clusters: cluster 1 (red nodes), cluster 2 (green nodes), cluster 3 (blue nodes), cluster 4 (yellow nodes) and cluster 5 (purple nodes). **(B)** Density visualization of keywords based on VOSviewer. **(C)** Overlay visualization of keywords based on VOSviewer. The nodes marked with purple or blue color represent the keywords that appeared relatively earlier, whereas keywords coded with yellow color represents the current research focuses. **(D)** The top 25 keywords with the strong citation bursts in articles related to targeted immunotherapy for MG.

while the periods of each burst keyword are plotted by the red line. The keywords that had citation bursts after 2018 were "igg4 autoantibody" (2018-2022, strength of 4.11), "open-label" (2018-2022, strength of 3.99), "double-blind" (2019-2022, strength of 13.26), "efficacy" (2019-2022, strength of 10.29), "eculizumab" (2019-2022, strength of 9.1), "thymectomy" (2019-2022, strength of 4.33), and "safety" (2019-2022, strength of 8.36). In particular, the burst of these six keywords including "open-label", "double-blind", "efficacy", "eculizumab", "thymectomy" and "safety" is still in progress.

Discussion

The current study was the first to use bibliometric methods to measure research trends on targeted immunotherapy for MG from 1998 to 2022. Unlike systematic reviews and scoping reviews, bibliometric analysis has become a powerful tool for summarizing the current status of knowledge and predicting future trends (41, 42). Based on information science, computer science, scientometrics, and applied mathematics, the visualization map exhibited specific knowledge domain and structural relationships, which were generated by the VOSviewer or CiteSpace (32, 34, 43). After excluding 169 studies that did not meet the inclusion criteria, 824 publications in 323 journals with 18346 co-cited references from 1084 institutions in 59 countries/regions were eligible for the analysis. Subsequently, bibliometric and visualization analysis tools were used to identify the main publications and citations, contributing countries, institutions, authors, funding agencies, knowledge base, research hotspots, and emerging topics.

From 1998 to 2022, the total number of publications on MG was 7672, and the total number of publications on targeted immunotherapy for MG was 824, which accounting for 10.74%. Supplementary Figure S3 shows that the number of publications on MG has maintained a steady growth before 2020. After 2020, the annual number of publications on MG exceeded 500 for the

first time. Supplementary Figure S4 shows the annual publication output on targeted immunotherapy for MG dramatically increased after 2017. The reason for this phenomenon is the REGAIN study was published (40) and the US Food and Drug Administration (FDA) approved eculizumab for use in the refractory AChR-GMG in 2017. Although the growth time point are different, it can be seen that the number of publications has shown a continuous growth trend over time, whether the field of MG or targeted immunotherapy for MG. However, the pathogenesis, diagnostic methods, and biomarkers of MG are also the research hotspots in the MG field. Therefore, the publication output trend of MG targeted immunotherapy can partially represent the trend of MG.

In recent years, targeted immunotherapy has gradually entered the view of neuroimmunology specialists. The research on targeted immunotherapy for autoimmune diseases has also increased over time, including NMOSD (neuromyelitis optica spectrum disorders), MS (multiple sclerosis), MG, etc. We searched the publications on targeted immunotherapy for NMOSD and MS. Supplementary Figure S5 shows that the NMOSD targeted immunotherapy have been first published since 2004, the cause of this phenomenon is the discovery of AQP4 (aquaporin 4) antibody in 2005 (44), NMOSD was independent from MS, and the public had a new understanding of the pathogenesis of NMOSD. Since then, biological agents for various targets on its pathogenesis have been developed. Supplementary Figure S6 shows that the publication outputs of targeted immunotherapy for MS is highly than NMOSD and MG and shows a continuous growth trend. This is due to the high incidence rate, heavy disease burden, high disability rate of MS, the R & D cost of DMTs (disease-modifying therapies) is high corresponding. For example, the approval and listing of ocrelizumab, natalizumab and ofatumumab have played a role in promoting the DMTs of MS.

Comparing the number of publications on targeted immunotherapy for NMOSD, MS and MG, they all show an increasing trend, but each disease has its own unique increasing trend. This is because the pathogenesis, the time of significant breakthrough was achieved and investment in different diseases are different. For example, MS is mediated by cellular immunity, while NMOSD and MG are caused by humoral immunity and the production of AQP4 antibody and AChR antibody respectively. In conclusion, the MG has a special growth trend compared with other diseases.

A general upward trend was found in the number of targeted immunotherapies for MG-related publications, indicating that this field was actively researched in recent years. Two phases, including a slow growth period (1998-2016) and a rapid growth period (2017-2022) were noticed. The number of publications exhibited a stable increasing trend from 1998 to 2016, but the increase was not apparent because the average annual publication volume did not exceed 35 articles. After 2017, the annual publication output dramatically increased which peaked in 2020, and this stage accounted for 47% of the total publications. The growth trend of citations was consistent with the publications. This phenomenon may have been connected to the significant events in this field, Firstly, REGAIN study entitled "Safety and efficacy of eculizumab in anti-acetylcholine receptor antibody-positive refractory myasthenia gravis: a phase 3, randomized, double-blind, placebo-controlled, multicenter study" (40) was published in 2017. The safety and efficacy of targeted biological agents in generalized myasthenia gravis (GMG) were confirmed for the first time. Second, based on the above findings, the US Food and Drug Administration (FDA) approved eculizumab for use in the refractory AChR-GMG in 2017. Before that approval, targeted biological agents were widely used in the treatment of other autoimmune diseases (13, 45–47). After the FDA approval of eculizumab to treat refractory AChR-GMG in 2017, targeted immunotherapy for MG has attracted the enthusiasm and attention from pharmaceutical companies and researchers. Phase II and phase III clinical trials on targeted immunotherapy for MG have been supported by complete funding, and several new targets are also being exploited (48, 49). Therefore, the number of publications and citations on targeted immunotherapy for MG has been increasing after 2017. Although only 29 articles in the first quarter of 2022 were retrieved, the number of articles in 2022 will reach a new peak according to the current growth trend. Improved diagnosis of the disease can seemingly contribute to increased disease incidence and population. From 1998 to 2022, the detection technique of autoantibodies is diverse persistently, and the sensitivity and specificity have been improved continuously. With the improvement of diagnostic methods, the diagnosis rate of MG has been improved, which increased the prevalence greatly. The development and application of targeted immunotherapy not only reduces the side effects of hormones and traditional immunosuppressants, but also further reduces the MG relapse and MG crisis, which greatly promotes the demand of MG patients for targeted immunosuppressants. The development of these new targets and successful clinical trials in MG will provide more treatment options for MG patients.

The distribution of contributing countries/regions and institutions showed some characteristics. As shown in Table1 and Figure 3, the United States ranked first in the number of publications, citations, and h-index in this field. Furthermore, the United States had a solid foundation in the biomedical field for a long time. The United States received a large amount of financial support and showed a sufficient reserve of researchers and institutions from funding and institution analyses. The top three funding agencies were the National Institutes of Health (NIH), the United States Department of Health and Human Services (HHS), and the NIH National Institute of Neurological

Disorders and Stroke (NINDS) and were from the United States. Four of the top 10 institutions with the most publications were also from the United States, indicating that the United States is the most influential country in this research field, which is far ahead of other countries. Besides the USA, China exhibited an increasing trend in advancement in this area. Much attention has been paid to this research area in the past years in China. Following the development of the Chinese economy, the healthcare needs of the general population are on the rise, and the financial support for the medical and health fields is also gradually increasing, especially focusing on molecular biological treatment options. Nevertheless, the citations and h-index were low in China when compared with other countries. Although the economic development is rapid in China, the advancement in the biomedical field was relatively behind and the groundwork was weak. Due to the large population in China, the medical insurance is challenged because one-year treatment with eculizumab costs approximately $500,000. Further, high research and development (R&D) costs and clinical expenses limited the clinical promotion and application of targeted immunotherapy to some extent. Although the number of publications from China in international journals has significantly increased, high-quality research papers have been published infrequently in top-grade journals (50, 51). However, the matter has attracted great attention from policymakers, and they encouraged researchers to improve the research quality, not the quantity of research (52). Additionally, the United States had the largest international cooperation in this field, followed by China (Figure 4). But collaboration between China and other countries was not strong. The developing countries should encourage their institutions to participate in research, strengthen collaboration, promote the advancement of related fields, and publish high-quality articles.

An analysis of journals and co-cited journals can provide a wealth of information for researchers to choose the best journal to submit their manuscripts (29, 53). There were 28% of articles published in the top 10 journals (Figure 6 and Table 4). The most productive journals in this field were the *Journal of Neuroimmunology* (IF=3.478), followed by *Muscle Nerve* (IF=3.217) and *Annals of the New York Academy of Sciences* (IF=5.691). Due to the rare nature of MG, the option for journals is narrow. Although the impact factor of the top 3 journals was not high, *Muscle Nerve* is a professional journal in the MG field and all other journals are comprehensive in immunology or neurology. Researchers can focus on these journals to know about research trends and frontiers in targeted immunotherapy for MG. In addition, when submitting manuscripts, researchers can find the most suitable journals for timely processing, avoiding delays in time of the study. Figure 7 shows that publications in "molecular biology, genetics" are often cited in "medicine, medical and clinical", indicating that current research focuses more on clinical research and translational research.

In our analysis, Vincent A scored the highest citations and h-index. Further, Vincent A and his research team had the highest research strength and influence. They published important findings in this field when compared to others.

Knowledge base

The more frequently an article is cited, the more important it is perceived in a specific field. Therefore, the most cited publications or influential literature can be regarded as a knowledge base in a particular field (54). As shown in Table 6, among the top 10 cited articles, there were 6 reviews, 2 randomized controlled trials (RCT), and 2 clinical research. From the time of publication perspective, 4 articles were published between 2000 to 2010 (early phase), which were reviews, 2 articles were published between 2011 to 2015 (middle phase) that were clinical research, and 4 articles were published between 2016 to present (recent phase), which had 2 reviews and 2 clinical studies.

The 4 reviews published in the early phase mainly described the pathogenesis, diagnosis, and treatment of MG, and these reviews played a landmark role in elucidating the mechanism of pathogenesis and diagnosis of MG. The article titled "Rituximab, an anti-CD20 monoclonal antibody: History and mechanism of action" was published by Pescovitz MD (37), which was the most cited paper from the analysis. This article mainly reviewed the history, pharmacokinetics, and potential mechanism of action of rituximab (RTX). After Zaja F first reported that RTX could be used for the treatment of GMG patients in 2000 (23), targeted immunotherapy was first envisioned by researchers. Since then, case reports and small-sample studies on RTX treatment in refractory MG have been endlessly streaming (55–58). Although it was not supported by advanced evidence-based medical studies, it has been widely applied in the clinical field, and these studies reflected the effectiveness of RTX in patients with anti-Musk positive and some anti-AChR positive MG (59, 60). The second co-cited paper titled "myasthenia gravis" was published by Vincent A (38), and was published in *Lancet* in 2001. This article summarized the epidemiology, clinical characteristics, classification, pathophysiological parameters, treatment, diagnosis, and differential diagnosis of MG. As a high-quality review in the field of MG, it served as a very important reference value for the targeted immunotherapies for MG. The third co-cited paper "Imbalance of regulatory T cells in human autoimmune diseases" was published by Christian Dejaco (61). This article described the unique role of Treg cells in autoimmune diseases, which exhibited their inhibition function *in vitro* in a contact-dependent manner and preferentially expressed high levels of CD25, forkhead, and winged-helix family transcription factor forkhead box P3 (FOXP3) (Tregs). In autoimmune diseases, altered Tregs and

insufficient suppression of inflammation were thought to be critical factors for disease development and persistence.

During the middle phase, "A randomized, double-blind, placebo-controlled phase II study of eculizumab in patients with refractory generalized myasthenia gravis" was published by Howard JF (62). This study outcome suggested that the overall change in mean total QMG score was significantly different between eculizumab and placebo therapies (P<0.0001). Another clinical study on RTX in Musk MG patients was published in *Neurology* in 2012 (63). Previous articles were all case reports or small sample studies on RTX in the treatment of MG (64, 65). But in this article, anti-AChR positive MG patients were used as controls. The study participants were prospectively followed for up to 31 months and compared with anti-AChR-positive MG patients. All the anti-Musk-positive groups achieved remission or showed minimal manifestations status (MMS), prednisone doses were significantly reduced, and concomitant immunosuppressants were withdrawn. At the last follow-up, Musk antibodies were negative in 3 of these patients and showed a decrease of over 80% in the other three patients. This study described better treatment options for patients with anti-Musk-positive MG. These two studies developed a new perspective on targeted biological agents for the treatment of MG patients. It was translational findings between the early stage and recent stage and provided evidence for more targeted biological agents in treating MG in the future.

Four articles published during the recent stage were in the direction of targeted immunotherapy for MG, which showed that the theory and mechanism of targeted immunotherapy have been mature, which have gradually entered the clinical transformation and application stage. Especially, the REGAIN study published by Howard, JF in *Lancet Neurology* in 2017, which was the phase III clinical study on eculizumab in refractory GMG. Since the publication of this study, articles on targeted immunotherapy for MG exhibited an explosive growth trend and more and more targeted biological agents were investigated. For example, efgartigimod (a FcRn antagonist) completed phase III clinical trials and was approved by the FDA for the treatment of anti-AChR positive GMG (11). The citations of "rituximab treatment of myasthenia gravis: a systematic review" published in 2017 were ranked in the top 10 (55). Although various targeted biological agents for the treatment of MG are under development, the popularity of RTX in the treatment of MG is still advancing. Several reasons may have accounted for this phenomenon. First, the results of RTX in different studies were not consistent. Second, the RTX has been administered clinically for almost 2 decades, while other targeted biological agents only have been used to treat MG in recent years (66, 67). Compared with other targeted biological agents, the RTX therapy has been adopted for a long and had long-term safety data. Third, the affordability, accessibility, and availability of the RTX are generally good, and the cost is bearable by the patients. In 2020, Brauner et al. published an article in *JAMA neurology*, which compared the therapeutic effect of the RTX in new-onset and refractory generalized MG patients (68). Surprisingly, the RTX was more favorable in new-onset generalized MG (69, 70), and the RTX performed better than the conventional immunosuppressant therapy. These findings showed a relatively greater benefit of RTX earlier in the disease course. Therefore, a placebo-controlled randomized trial to corroborate these findings is warranted.

Although most references burst has ended, several reference bursts are still ongoing, and most of these references focused on the clinical studies of targeted immunotherapy for MG, such as RTX, eculizumab, and efgartigimod, indicating continuous advancement in recent years (Figure 8).

Research hotspot

Keywords can reflect the research hotspots and frontiers in a specific research field. In addition to "myasthenia gravis", the most representative keyword was "rituximab" (Figure 9). The keywords with a strong link with RTX were "safety", "efficiency", "therapy", and "double-blind", which was consistent with the results of references co-cited. RTX is the first targeted biological agents which was used in the clinical practice for MG. For a long time, it is also the only targeted biological agents for MG patients. Therefore, the research on RTX in the targeted immunotherapy field is most. From the keywords with the strong link with RTX, researchers are still concerned about the effectiveness and safety of RTX. Because of long-term off-label use, there urgently need for conduct high quality double-blind RCT. The publication of the REGAIN study in 2017 laid the foundation for the safety and efficacy of targeted biological agents in MG for the first time, which has evoked enthusiasm from researchers. The next focused keyword was "double-blind", which showed that a growing number of clinical studies with high-quality evidence-based medicine have been conducted recently. The safety and efficacy of many targeted biological agents have been gradually confirmed. Therefore, researchers are encouraged to develop more new target molecules in the future. Interestingly, the keywords were "mice", "experimental autoimmune MG", "regulatory T -cells", "T-cells", and "alpha subunit" between 2010 and 2012 (Figure 9C). These keywords mainly focused on the basic research of MG. The keywords were "patient", "therapy", "intravenous immunoglobulin", "mycophenolate-mofetil", "thymus", "thymoma", and "thymectomy" between 2012 and 2016,. The keywords in 2016-2018 were mainly "eculizumab", "rituximab", "nivolumab", "double-blind", "safety", "efficiency", and "management", which focused on the targeted immunotherapy for MG with high-quality clinical research. The basic research on targeted immunotherapy has been relatively mature, and multiple immune targets have been identified. With the basic research to clinical translation, the investment in new

drug research has increased, and researchers has gradually focused to clinical research.

In addition, the CiteSpace was used to analyze keywords, which were used to identify the research hotspots and frontiers of research during the period. The evolution of burst keywords over the past decade demonstrated the continued progress in the field of targeted immunotherapy for MG. The result was consistent with the VOSviewer (Figure 9D). The "double-blind" was also the strongest burst keyword. Several keywords are still in the burst presently, suggesting the safety and efficacy of targeted immunotherapy and the development of high-quality RCT is also the research hotpots. There is a need for increased research efforts in this area so that MG patients of different types will have more treatment options, which is conducive to individualized precision therapy.

Strengths and limitations

To the best of our knowledge, this was the first study using the bibliometric method to summarize the status and development of the targeted immunotherapy for MG. To comprehensively evaluate the existing literature, the data were analyzed using two bibliometric tools (CiteSpace and VOSviewer) and an online platform. Despite the above-mentioned strengths, several limitations are unavoidable, First, there are diverse factors that can affect the number of publications, both known and unknown. It is very difficult to obtain the overall funds data in different countries from the WoSCC database (the specific amount of fund support is not available from the WoSCC database). Moreover, many countries lack national epidemiological data in MG field, which may cause statistics bias. Therefore, our study adopted bibliometric analysis methodology, we only compared the number of publications in different countries, and through this important indicator to reflect the research status of different countries. This method has many limitations obviously, but it is also a feasible method at present. Second, the database selection bias was that all literatures included in this study were downloaded from the WoSCC. The relevant studies deposited in other databases might have been missed. Finally, our literature search was only dependent on the English language, hence our analysis may have excluded articles reported in non-English.

Conclusion

Taken together, the current study summarized the global research trends concerning the targeted immunotherapy for MG. The outcome of the study demonstrated that the USA is leading ahead in both the sum of publications, total citation frequency, and funding in this field. Along with improving life quality of MG patients, high efficiency, rapid onset, less side effects and good compliance have become new treatment needs. The MG treatment has entered a personalized precision treatment phase. Further exploration into new target molecules and conducting high-quality randomized controlled trials on existing biological agents are urgently needed to guide the future directions of immunotherapy research. Consequently, it is not difficult to predict that this field is likely to advance rapidly and more studies will be published in the future. Meanwhile, there are also multiple challenges for MG targeted immunotherapy in the future, such as the long-term effectiveness, safety, accessibility, and cost of biological agents. We should focus on providing precise treatment schemes for patients with different subtypes, which will help patients achieved the treatment goal rapidly, reduce the treatment burden and provide convenience for patients to the greatest extent.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Author contributions

TC conceptualized the study, secured funding, and designed uniform procedures for data collection across the study centers. TC, ZL, YS, ZR, RW and SH contributed to the study design. YS, ZR, RW and SH contributed to collect and analyzing data. TC, YS, ZR, RW and SH contributed to the drafting of the manuscript. YT, XH and TG contributed to collecting data. All authors contributed to the critical revision of the manuscript for important intellectual content and provided approval of the final manuscript for submission.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2022.998217/full#supplementary-material

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Association between immune-mediated adverse events and efficacy in metastatic non-small-cell lung cancer patients treated with durvalumab and tremelimumab

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Purpose: Immune-mediated adverse events (imAEs) may be associated with response to immune checkpoint inhibitors. We assessed the relationship between imAE development and efficacy in metastatic non-small-cell lung cancer patients treated with durvalumab (anti-programmed cell death ligand-1 [PD-L1]) alone or in combination with tremelimumab (anti-cytotoxic T-lymphocyte-associated protein 4).

Methods: The analysis used individual patient-level data from 307 and 310 patients in the monotherapy and combination arms of MYSTIC, respectively. We evaluated the association between treatment efficacy and development of imAEs using univariate and multivariate survival analyses. Using machine learning, we built a predictive model utilizing baseline clinical and laboratory features to identify patients at risk of developing imAEs and further evaluated patient survival based on a threshold index extracted from the model.

Results: Patients who developed any grade of imAE had improved overall survival versus patients without (hazard ratio [HR] 0.51; 95% confidence interval [CI]: 0.41–0.62). imAE development was associated with improved overall survival (HR 0.54; 95% CI 0.44–0.66) in a multivariate Cox proportional hazard model considering patient demographic features and baseline characteristics. Higher odds of imAE development were observed (odds ratio 3.023; 95% CI: 1.56–5.83) in responders versus non-responders in patients treated with immunotherapy. Based on baseline characteristics, the random forest classification algorithm was used to formulate a predictive model to identify patients at increased risk of developing imAEs during treatment.

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Conclusion: *Post-hoc* exploratory analysis found that the efficacy of immunotherapy was improved in patients who developed on-treatment imAEs. This was independent of severity of imAEs or the need for steroid treatment, which is important in allowing patients to remain on treatment and derive optimal clinical benefit. Further research is warranted to establish the correlation between incidence of imAEs and efficacy in this patient population.

KEYWORDS

immunology, biomarkers, clinical trials, methodology and modeling, immunotherapy, computational methods, biostatistics, lung cancer

Introduction

Immune checkpoint inhibition (ICI) has revolutionized the treatment of cancer over the last decade, with multiple checkpoint inhibitors approved in solid tumors as well as for some hematologic malignancies (1, 2). The first approved checkpoint inhibitor was ipilimumab, an anti-cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) monoclonal antibody (3). Subsequently, antibodies targeting programmed cell death-1 (PD-1; nivolumab, pembrolizumab, and cemiplimab) (4–6) or its ligand, programmed cell death ligand-1 (PD-L1; atezolizumab, avelumab, and durvalumab) (7–9) have also gained approvals and become integrated into the standard-of-care in many tumor types.

Despite significant clinical benefits, the use of immunotherapy can result in immune-mediated toxicities in up to 85% of patients, although this varies by agent and across tumor types (10). Such toxicities are commonly gastrointestinal, respiratory, endocrine, or dermatologic in nature (11). Mechanistically, this toxicity is believed to be caused by aberrant activation of autoreactive T or B cells (12), inhibition of regulatory T cells (13), and/or activation of tumor-reactive T cells that share an antigen with normal tissue (14). Frontline management includes withholding immunotherapy and starting corticosteroids (15). If response is inadequate, second-line immunosuppressive agents such as infliximab, vedolizumab, mycophenolate, and azathioprine are recommended (15). To date, there are no reliable tools to predict which patients will develop immune-mediated adverse events (imAEs) during treatment with immunotherapy. Prior studies have shown that clonal expansion of cytotoxic CD8 T cells precedes ipilimumab-related toxicity (16) and that patients with tumors characterized by a high mutational burden are at increased risk of immune-mediated toxicity (17). While these analyses investigated only single features, the role of multiple features in relation to the development of imAEs was evaluated in a proof-of concept study, which used real-world, reported adverse event (AE) data and molecular genomics to identify a bivariate regression model of lymphocyte cytosolic protein 1 (LCP1) and adenosine diphosphate dependent glucokinase (ADPGK) that could accurately predict imAEs (18). However, there remains a need for a comprehensive approach to identify patients who might experience imAEs during treatment using baseline information.

Several retrospective studies have shown an association between the development of imAEs and both treatment response and survival. This leads us to consider the fact that widespread systemic immune system activation and auto-reactivity may be simultaneously reflective of antitumor response. In a retrospective analysis combining seven trials in patients with metastatic or locally advanced urothelial carcinoma, overall survival (OS) was longer in patients with imAEs versus those without imAEs (hazard ratio [HR], 0.45; 95% confidence interval [CI], 0.39–0.52) (19). Similarly, incidence of imAEs was associated with longer relapse-free survival in patients with stage III melanoma who received pembrolizumab (HR, 0.61; 95% CI, 0.39–0.95; P = 0.03) (20).

Durvalumab is a human immunoglobulin G1 kappa monoclonal antibody that inhibits PD-L1 binding to PD-1 and CD80; tremelimumab is a monoclonal immunoglobulin G2 antibody binding to CTLA-4 which, in combination with durvalumab, has shown clinical efficacy in patients with advanced non-small-cell lung cancer (NSCLC) (7, 21). The phase III MYSTIC trial compared durvalumab alone or in combination with tremelimumab versus standard of care (SOC) chemotherapy in treatment-naïve patients with metastatic NSCLC whose tumors expressed PD-L1 in ≥25% of tumor cells (TC ≥ 25) (22). In the MYSTIC trial, durvalumab alone or in combination with tremelimumab did not meet statistical significance for OS versus SOC chemotherapy. However, a clinically meaningful improvement was observed in the durvalumab monotherapy group (HR 0.76; 97.54% CI: 0.56–1.02; $p = 0.036$) suggesting that some patients in MYSTIC did derive benefit from durvalumab monotherapy. Here, we report results from an analysis of MYSTIC patient data designed to better understand the effect of imAEs on treatment efficacy, allowing the development of a predictive model to identify

patients with a high probability of experiencing on-treatment immune-mediated toxicity.

Materials and methods

Patients

Full eligibility criteria for the MYSTIC clinical trial (NCT02453282) have been previously reported (22). In brief, adults with stage IV NSCLC were eligible provided they had not previously received systemic therapy for advanced or metastatic NSCLC, had an Eastern Cooperative Oncology Group (ECOG) performance status of 0 or 1, demonstrated measurable disease according to Response Evaluation Criteria in Solid Tumors (RECIST) version 1.1 (23), and had known tumor PD-L1 expression status prior to randomization. Patients with sensitizing epidermal growth factor receptor (EGFR) or anaplastic lymphoma kinase (ALK) genetic alterations and those with symptomatic, unstable brain metastases were excluded. With the exception of vitiligo or alopecia and hypothyroidism, patients with active or prior documented autoimmune or inflammatory disorders were also excluded from the study.

The MYSTIC study was performed in accordance with the Declaration of Helsinki and the International Conference on Harmonization Good Clinical Practice Guidelines. The protocol and all modifications were approved by the institutional review boards or ethics committees of all participating centers and the relevant regulatory authorities. All participants provided written informed consent.

Patient and public involvement statement

This study utilized patient data from the MYSTIC trial to help develop a predictive model that could identify patients at a high risk of experiencing on-treatment immune-mediated toxicity during the course of immunotherapy. Patient response and safety data were analyzed retrospectively.

Study design and treatment

Patients were randomized in a 1:1:1 ratio in a stratified manner according to PD-L1 expression (25% cutoff) and histology (squamous vs non-squamous) to receive durvalumab plus tremelimumab, durvalumab monotherapy or SOC chemotherapy (Supplemental Figure S1). Patients in the durvalumab plus tremelimumab group received durvalumab 20 mg/kg and tremelimumab 1 mg/kg *via* intravenous (IV) infusion every 4 weeks (q4w) for up to four doses/cycles and then continued with durvalumab 20 mg/kg q4w from week 16 until disease progression. Patients in the durvalumab monotherapy group received durvalumab 20 mg/kg *via* IV infusion q4w. Patients in the chemotherapy arm received 4–6 cycles of platinum-based doublet chemotherapy of the investigator's choice.

Endpoints

The primary endpoints were OS (time from randomization to death due to any cause) for both immunotherapy arms versus chemotherapy and progression-free survival (PFS; time from randomization to objective disease progression according to blinded independent central review, or death) for durvalumab plus tremelimumab versus chemotherapy, all assessed in patients with ≥25% of TCs expressing PD-L1 (PD-L1 TC ≥25%). Secondary endpoints included PFS for durvalumab versus chemotherapy, and objective response rate and duration of response (DOR) for both immunotherapy arms versus chemotherapy (all in patients with PD-L1 TC ≥25%), as assessed using RECIST version 1.1, as well as safety and tolerability (22).

Assessment

During medical review of the MYSTIC trial, an AE consistent with an immune-mediated mechanism of action with no clear alternate etiology was adjudicated as an imAE by the sponsor. Serologic, immunologic, and histologic (biopsy) data, as appropriate, were used to support characterization of an imAE. Unless otherwise indicated, all data regarding imAEs were adjudicated. The severity of imAEs was graded according to the National Cancer Institute's Common Terminology Criteria for Adverse Events (CTCAE version 4.03).

Statistical analysis

Kaplan–Meier analysis was used to compare patient survival outcomes (OS, PFS) based on development of imAEs in the immunotherapy arms, irrespective of PD-L1 expression and (separately) in subgroups based on PD-L1 expression (cut-off, 25%). Since imAE is an on-treatment phenomenon, landmark analysis based on imAE median time to onset was also used to account for immortal time bias. Treatment efficacy was compared between patients experiencing high-grade (grade ≥3) imAEs and patients experiencing low-grade (grade ≤2) imAEs using Kaplan–Meier analysis, as well as between the low-grade imAE patient cohort and the no imAE patient cohort. We also explored the effect of steroid use on treatment efficacy in patients experiencing imAEs using Kaplan–

Meier analysis. Finally, we used the Kaplan–Meier method to compare the survival benefit for patients with imAEs in the immunotherapy arms over patients in the chemotherapy arm, employing a Restricted Mean Survival Time (RMST) analysis to overcome the violation of proportional hazards assumption (as observed in early crossing over of Kaplan–Meier curves) (24).

Demographic features (age, weight, sex, and race) and potential prognostic factors or predictive biomarkers of efficacy (baseline ECOG performance status, histology, presence of liver metastasis, PD-L1 expression, and tumor mutational burden) were considered in addition to incidence of imAEs in a step-wise multivariate Cox regression model to account for any possible confounding effect. All covariates were initially considered for selection. The significance levels for entry and for stay were set at 0.25 (suggested to be set conservatively at ≥0.15) (25). The best candidate final regression model was then identified by eliminating the covariates with a *p*-value of >0.05 one at a time until all regression coefficients were significantly different from 0 at the chosen alpha level of 0.05.

The association between objective response and development of imAEs was explored using a binary outcome logistic regression model. To examine the effect of exposure duration (difference in days between the treatment stop and start dates) on the development of imAEs, responder status (Yes/No; defined as those patients who achieved either a confirmed complete response [CR] or partial response [PR]), duration of exposure, and a multiplicative interaction term were included as covariates in the model.

Machine learning classification

A predictive model was developed using the random forest algorithm (26) to identify patients at risk of experiencing immune-mediated toxicities. Baseline demographic features, potential prognostic factors and predictive markers of efficacy, and several laboratory parameters were used in model building (Supplemental Table S1). The 10 most significant features in this classification problem were noted using random forest feature selection. Both "accuracy" and "Gini" importance measures were taken into consideration for feature selection (26). A simpler predictive model was then developed using only these 10 features to reduce the complexity of the original model. A baseline threshold immune toxicity index was extracted (random forest model classification cut-off) from the model to identify patients more likely to experience imAEs during the course of immunotherapy. Patient survival was evaluated based on the model-informed baseline threshold immune toxicity index. An out-of-bag (OOB) error estimate (26) was used to measure the predictive ability of the model. In random forest, cross-validation on a separate dataset is not required to obtain an unbiased estimate of prediction error (26). OOB error is estimated internally using approximately one-third of cases excluded from the bootstrap sample. All analyses were performed using R statistical software.

Results

Data from 902 patients (307 randomized to monotherapy, 310 to combination therapy, and 285 to chemotherapy) who participated in the trial were included in the analysis. Due to restrictions on secondary usage of data, the number of patients in each treatment arm in our analysis differs from the actual number of patients in the MYSTIC trial (374 randomized to monotherapy, 372 to combination therapy, and 372 to chemotherapy) (22). Patient demographics and baseline characteristics of these 902 patients were well balanced across treatment arms (Supplemental Table S2).

imAEs were observed in 215/617 patients (35%) from the immunotherapy arms, including 84/307 patients (27%) from the durvalumab arm and 131/310 patients (42%) from the durvalumab plus tremelimumab arm. In univariate analysis of both immunotherapy arms combined and irrespective of PD-L1 status, patients with imAEs had improved OS (HR 0.51; 95% CI: 0.41–0.62) (Figure 1A) and PFS (HR 0.54; 95% CI: 0.44–0.66) (Figure 1B), compared with patients without imAEs. Median time to onset of imAES was 34 days and the landmark analysis accounting for immortal time bias, which excluded patients who died (OS) or progressed (PFS) before Day 34, also reported improved OS (HR 0.55; 95% CI: 0.44–0.67) and PFS (HR 0.60; 95% CI: 0.49–0.74) in patients who had imAEs versus those who did not (Supplemental Figure S2). We also assessed survival benefit associated with incidence of imAEs in each immunotherapy arm separately. A similar improvement in treatment efficacy was seen in each arm for patients with imAEs versus patients without immune-mediated toxicity both for OS (durvalumab arm: HR 0.44; 95% CI: 0.31–0.61 and durvalumab plus tremelimumab arm: HR 0.52; 95% CI: 0.40–0.68) and for PFS (durvalumab arm: HR 0.49; 95% CI: 0.36–0.68 and durvalumab plus tremelimumab arm: HR 0.54; 95% CI: 0.42–0.71) (Figure 2). Furthermore, when considering only patients with imAEs, there was no significant difference in OS between the two immunotherapy arms, with HR 0.70 (95% CI: 0.49–1.01) for patients in the durvalumab arm versus those in the combination arm (Supplemental Figure S3). Therefore, patients in both immunotherapy arms were grouped together for subsequent analyses.

In total, 269/617 patients (44%) treated with immunotherapy had PD-L1 TC ≥25%. The incidence of any-grade imAEs was 37% (100/269 patients) in patients with PD-L1 TC ≥25% and 33% (115/348 patients) in patients with PD-L1 <25%. The association between immune-mediated toxicity and improved OS and PFS was observed regardless of PD-L1 expression (Figure 3). For OS and PFS, respectively, the HRs for patients with imAEs versus those lacking imAEs were 0.46 (95%

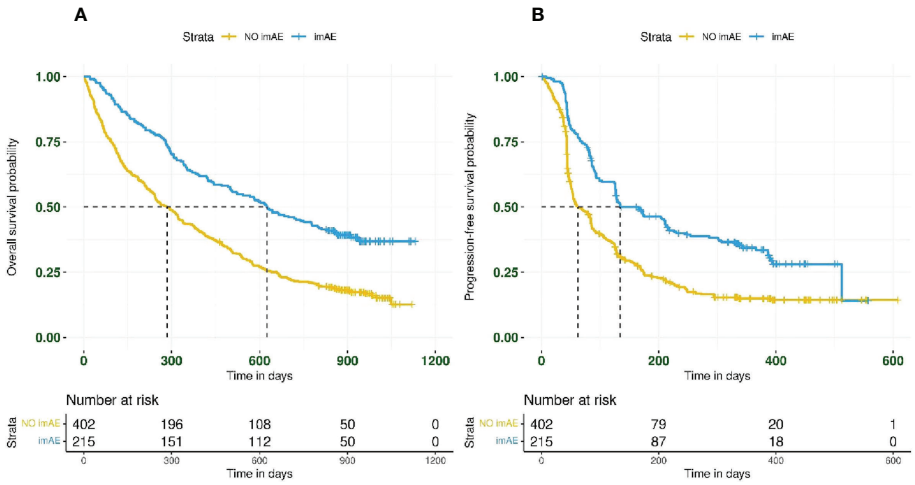

FIGURE 1
Kaplan–Meier plots for **(A)** overall survival and **(B)** progression-free survival by imAE development in patients treated with immunotherapy (*n* = 617). Data from patients randomized to durvalumab (*n* = 307) were combined with those from patients randomized to durvalumab plus tremelimumab (*n* = 310). imAE, immune-mediated adverse event.

CI: 0.33–0.64) and 0.55 (95% CI: 0.40–0.76) in the PD-L1 TC ≥25% subgroup (Figures 3A, C) and 0.55 (95% CI: 0.42–0.71) and 0.53 (95% CI: 0.41–0.68) in the PD-L1 TC <25% subgroup (Figures 3B, D).

As previously reported, neither durvalumab alone nor durvalumab plus tremelimumab improved survival outcomes compared with chemotherapy in the MYSTIC trial in patients with PD-L1 TC ≥25% (22). In the present analysis, although median OS was longer in patients with imAEs treated with immunotherapy than in those receiving chemotherapy, the Kaplan–Meier curves for these two patient groups crossed over at an early timepoint, thus violating the proportional hazards assumption (Supplemental Figure S4). An RMST analysis of OS also showed improved treatment efficacy in patients with imAEs from the immunotherapy arms compared with patients treated with chemotherapy (Supplemental Figure S5 and Supplemental Table S3).

The baseline covariates listed in Supplemental Table S2 were all considered in a multivariate analysis to further assess the relationship between treatment efficacy and incidence of imAEs. Development of imAEs was associated with improved OS (HR 0.54; 95% CI: 0.44–0.66) in the multivariate Cox regression model (where covariates were chosen in the final model by a stepwise variable selection procedure), with a similar outcome for PFS (HR 0.57; 95% CI: 0.47–0.70) (Table 1).

Of 215 patients experiencing imAEs in the immunotherapy arms, 173 patients (80%) had low-grade (grade ≤2) imAEs, including 68/84 patients (81%) in the durvalumab arm and 105/131 patients (80%) in the durvalumab plus tremelimumab arm. OS and PFS did not differ between patients with high-grade versus low-grade imAEs (Supplemental Figure S6). However, OS and PFS were both improved (Supplemental Figure S7) in patients with only low-grade imAEs (both immunotherapy arms grouped together) compared with patients who didn't experience any imAEs (OS: HR 0.50; 95% CI: 0.40–0.62; PFS: HR 0.56; 95% CI: 0.45–0.69).

Overall, 47/215 patients (22%) had immune-mediated toxicity requiring steroid use. Survival outcomes were unaffected by steroid use in patients with imAEs; for patients receiving steroids, the HR for OS was 1.27 (95% CI: 0.85–1.89) and the HR for PFS was 0.93 (95% CI: 0.62–1.41) compared with those patients who did not require steroid treatment (Figure 4).

Among the 617 patients treated with immunotherapy included in our analysis, 152 patients (25%) had an objective response (CR or PR). The incidence of imAEs was higher in responders (79/152 patients; 52% [95% CI: 44–59]) than in non-responders (136/465 patients; 29% [95% CI: 25–33]) (Table 2). The association between development of imAEs and objective response was evaluated through a logistic regression model adjusted for the duration of exposure. Higher odds of imAE development were observed (odds ratio 3.023; 95% CI: 1.56–5.83) in responders versus non-responders (Table 2). The interaction term between response and duration of exposure was significant in the model, indicating that responders and non-responders had a differential proclivity for development of imAEs given the same duration of exposure.

Using patient baseline characteristics (Supplemental Table S1), a random forest predictive model (R script; Supplementary File) was developed to identify patients with a higher likelihood of experiencing imAEs during treatment with immunotherapy.

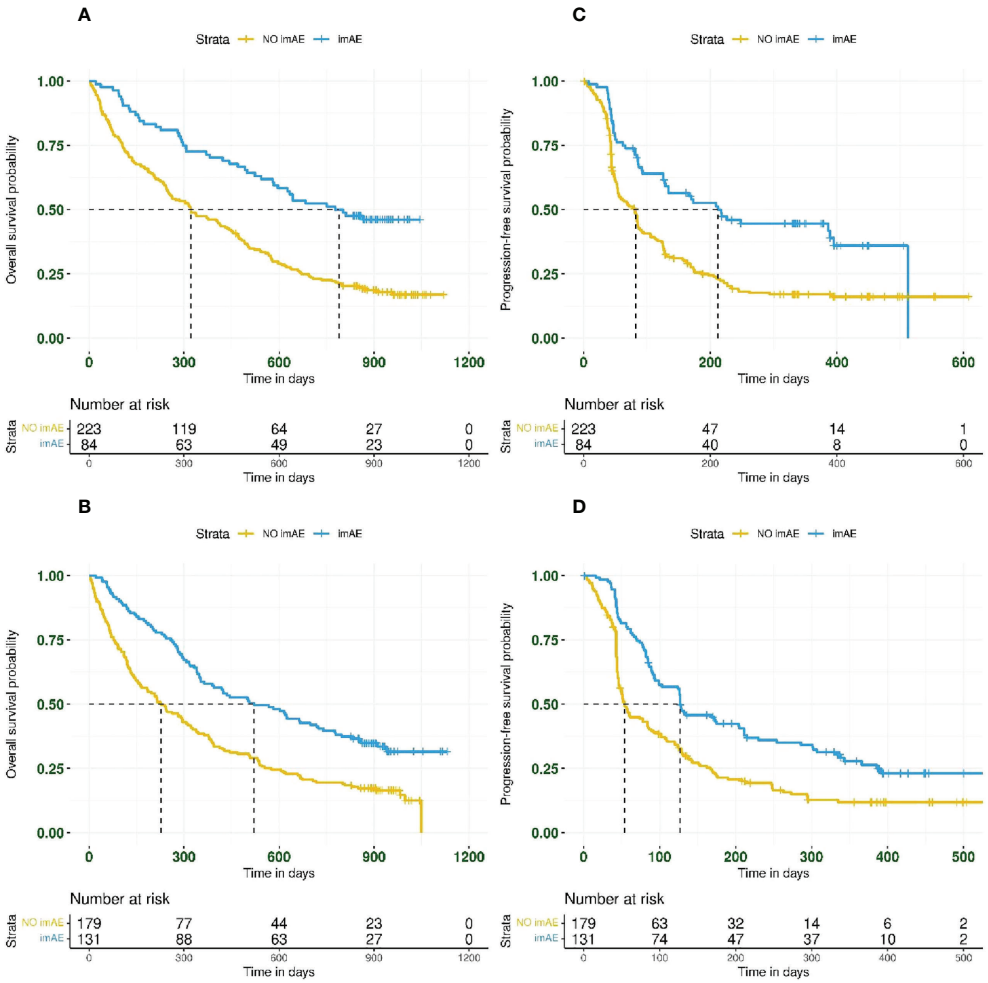

FIGURE 2
Kaplan–Meier plots for overall survival and progression-free survival based on imAE development in the durvalumab arm (panels **A**, **C** for OS and PFS, respectively) and the durvalumab plus tremelimumab arm (panels **B**, **D** for OS and PFS, respectively). imAE, immune-mediated adverse event; OS, overall survival; PFS, progression-free survival.

Patients with immune toxicity index above the reference level of 0.5 (≥0.5 vs <0.5) were classified (OOB error estimate 12.28%) as patients who would experience imAEs during the course of immunotherapy. The top 10 features associated with imAE development were identified (Supplemental Figure S8) from the entire feature-space (Supplemental Table S1) using random forest feature selection (26). The simpler model, developed using these 10 features, was able to classify patients with imAEs with an OOB error estimate of 14.3% (Supplemental Figure S9). The model-informed immune toxicity index was then assessed with other baseline covariates from Supplemental Table S2 in a multivariate Cox regression model for treatment efficacy. Based on this analysis, immune toxicity index ≥0.5 versus <0.5 was associated with improved OS (HR 0.57; 95% CI: 0.46–0.71), with a similar outcome for PFS (HR 0.60; 95% CI: 0.48–0.74) (Supplemental Table S4).

Discussion

Association between imAEs and clinical outcome

The primary analysis of the MYSTIC trial found no statistically significant difference in OS between immunotherapy (either durvalumab or durvalumab plus tremelimumab) and chemotherapy (22). The aim of this retrospective analysis was to improve our understanding of the survival benefit associated with imAE development in the immunotherapy arms of the phase III MYSTIC trial. Using both univariate and multivariate analysis, we found that both OS and PFS tended to be improved in patients who experienced imAEs on immunotherapy when compared with those who did not develop imAEs. Landmark analysis using imAE median time to onset (taking into account the time-dependent

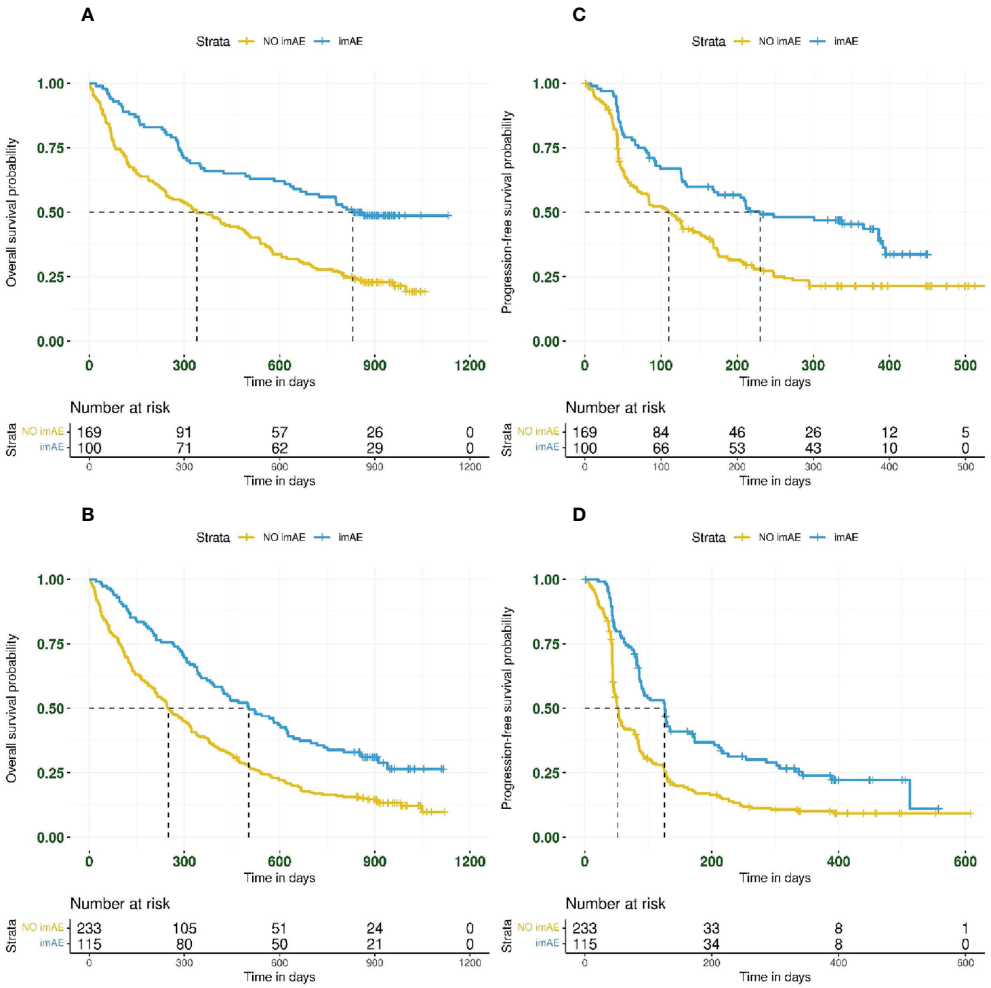

FIGURE 3
Kaplan–Meier plots for overall survival and progression-free survival based on imAE development in patients with PD-L1 TC ≥25% (panels **A**, **C** for OS and PFS, respectively) and in patients with PD-L1 TC <25% (panels **B**, **D** for OS and PFS, respectively). Data from patients randomized to durvalumab (n = 307) were combined with those from patients randomized to durvalumab plus tremelimumab (n = 310) before analyzing by PD-L1 subgroup. imAE, immune-mediated adverse event; OS, overall survival; PD-L1, programmed cell death ligand-1; PFS, progression-free survival; TC, tumor cell.

nature of imAE) further confirmed the survival benefit associated with imAEs – this is also in alignment with the results presented by Haratani et al. (27) Subgroup analysis showed that survival outcomes were independent of PD-L1 status (above or below a threshold of TC 25%), grade of imAE, and corticosteroid usage. Although overall rates of imAEs in MYSTIC were low, we also demonstrated that patients who experienced imAEs during immunotherapy had noticeably longer OS than patients randomized to SOC chemotherapy. Substantial association between development of imAEs and objective response was seen in patients treated with immunotherapy in a multivariate analysis adjusted for duration of exposure. The significant interaction term between response and duration of exposure in the logistic regression model suggests that, given the same duration of exposure, immunotherapy responders and non-responders have different likelihoods of developing imAEs. However, these analyses were limited by their retrospective and exploratory nature.

Survival benefit associated with incidence of imAEs has been previously reported in the literature across various tumor types; however, most had relatively small cohorts and significant limitations adjusting for confounders. However, recent examples of larger-scale analyses reflect the findings reported here. For example, one such analysis looked at pooled safety and efficacy data from 1783 patients with various solid tumors, including NSCLC, treated with avelumab in the JAVELIN Solid Tumor and JAVELIN Merkel 200 trials (28). Patients experiencing imAEs (overall incidence 16.5%), had a greater improvement in OS than those who did not. In exploratory

TABLE 1 Multivariate analysis for overall survival and progression-free survival in the immunotherapy arms combined.

Covariate	HR	95% CI
Overall survival		
imAE (Yes vs No[a])	0.54	(0.44–0.66)
ECOG performance status (≥1 vs 0[a])	1.55	(1.28–1.89)
PD-L1 (TC ≥25% vs TC <25%[a])	0.71	(0.59–0.86)
Histology (Squamous vs Non-squamous[a])	1.36	(1.11–1.65)
Liver metastasis (Yes vs No[a])	1.27	(1.01–1.60)
TMB (≥20 mut/Mb vs <20 mut/Mb[a])	0.66	(0.51–0.86)
Progression-free survival		
imAE (Yes vs No[a])	0.57	(0.47–0.70)
ECOG performance status (≥1 vs 0[a])	1.34	(1.10–1.62)
PD-L1 (TC ≥25% vs TC <25%[a])	0.63	(0.52–0.77)
Liver metastasis (Yes vs No[a])	1.29	(1.02–1.62)
TMB (≥20 mut/Mb vs <20 mut/Mb[a])	0.68	(0.53–0.89)

CI, confidence interval; ECOG, Eastern Cooperative Oncology Group; HR, hazard ratio; imAE, immune-mediated adverse event; mut/Mb, mutations/megabase; PD-L1, programmed cell death ligand-1; TC, tumor cell; TMB, tumor mutational burden.
imAE development was assessed along with other significant covariates chosen by the stepwise multivariate Cox regression model.
[a]Indicates reference level. For example, HR = 0.54 favors imAE = Yes.

analyses including 1747 patients with urothelial cancer who received a PD-1/PD-L1 inhibitor across seven clinical trials, patients experiencing on-treatment imAEs also demonstrated improved OS versus those with no imAEs (19). We also highlight that much of the current literature has considered only a single immunotherapy mode-of-action, thus our analysis of imAEs in patients receiving a combination of PD-(L)1 and CTLA-4 agents is of particular note.

The exact mechanism for the association between development of imAEs and ICI-triggered response is not entirely clear. It is postulated that there is a shared antigen between the tumor and otherwise normal surrounding tissue from which the tumor arises, with toxicity a byproduct of T-cell stimulation and activation at the site of the tumor. Several clinical observations support this notion, including an increase in response for patients with metastatic melanoma who develop vitiligo after treatment with immunotherapy (14, 20), the association between acute interstitial nephritis and clinical response in renal cell carcinoma (29), and the high incidence of pneumonitis in patients with NSCLC receiving immune checkpoint inhibitors (30). It has also been shown that there is an association between tumor-tissue similarity and frequency of autoimmune toxic effects on a molecular level. Using advanced sequencing techniques, researchers demonstrated that several T-cell clonotypes present in NSCLC tumors were also present at the site of skin toxicity (31), providing evidence to support the theory that shared antigenicity in two separate organ systems is one potential mechanism for the observed toxicity.

An important unanswered clinical question is the effect of glucocorticoid use on immunotherapy efficacy. While it has been shown through *in vivo* studies that corticosteroids inhibit effector T-cell proliferation and promote regulatory T-cell expansion (32), it is unclear how this may affect efficacy in the context of imAEs. This is particularly important given the need for effective management of imAEs to ensure that patients can remain on treatment and derive optimal clinical benefit. In our analysis, the improvement in PFS and OS related to imAEs was independent of steroid exposure. This is consistent with data presented in the context of urothelial cancer, which showed that use of corticosteroids did not negatively affect either the chance of developing a response to PD-1/PD-L1 inhibitors or the DOR (19). Analysis of data from patients with NSCLC is also in alignment, reporting that receipt of corticosteroids for imAEs did not affect OS (33). Our ability to make definitive conclusions based on the dataset presented is limited due to a small sample size, with only 47 patients receiving immunotherapy requiring steroid administration. Future, prospective, and biological studies are warranted to better address this question.

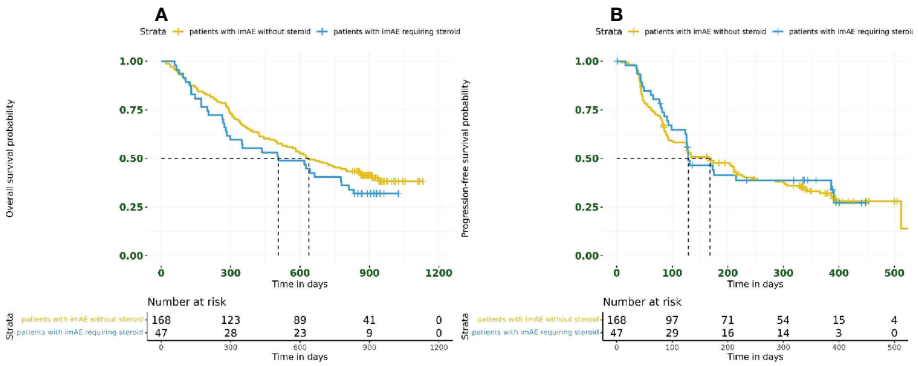

FIGURE 4
Kaplan–Meier plots for **(A)** overall survival and **(B)** progression-free survival based on steroid usage in patients with imAEs from the immunotherapy arms combined (*n* = 215). imAE, immune-mediated adverse event.

TABLE 2 Relationship between imAE development and objective response.

Odds of imAE development			
	OR	**95% CI**	***p*-value**
Response (Yes/No)[a]	3.023	1.56–5.83	0.0009
Interaction between response and imAE development			
	Coefficient	**SE**	***p*-value**
Response (Yes/No) [a]	1.10	0.33	***
Exposure duration	0.002	0.0005	***
Interaction (response x exposure duration)	−0.001	0.0007	*
Patients with imAE stratified by response			
Patients treated with immunotherapy	**Responders (*n* = 152)**		**Non-responders (*n* = 465)**
Incidence of imAEs: *n* (%)	79 (52)		136 (29)
95% CI	44–59		25–33

CI, confidence interval; imAE, immune-mediated adverse event; OR, odds ratio; SE, standard error.
[a]Responder status "No" is the reference level.
OR calculated after adjusting for duration of exposure.
Responders have higher odds of developing imAEs after adjusting for duration of exposure. A significant interaction term indicates different inclination towards imAE development among responders versus non-responders, given the same duration of exposure.
p-value significance: *, $p \leq 0.05$; ***, $p \leq 0.001$.

Developing a predictive tool for imAEs

We developed a machine learning predictive model to identify patients who might experience imAEs during treatment with immunotherapy using only baseline characteristics. A model immune toxicity index was created for this purpose. We also evaluated patient survival based on this index. In our model, in which prediction was limited by the available data, patients with a higher immune toxicity index score (≥0.5; i.e. those with a higher chance of developing imAEs on immunotherapy), had a noticeable survival benefit. However, more comprehensive predictive model development using a larger database and validation using different studies is required for patient benefit.

Various baseline patient characteristics, such as differences in peripheral cytokine levels (34, 35) and differences in B-cell abundance (36), have been correlated with immune-mediated toxicity; however, these findings are preliminary and as yet there are no validated predictive biomarkers for imAE development. As immunotherapy is incorporated into earlier stages where surgical and radiotherapy treatment alone has curative potential, the risks and benefits of additional immunotherapy treatment need to be considered with a different benefit-risk perspective to that in the metastatic context. Our hope is that a comprehensive predictive model may ultimately be available to clinicians to improve risk stratification of patients and to help guide treatment decisions.

In conclusion, our analysis demonstrated improved survival outcomes in patients experiencing imAEs during immunotherapy, regardless of the severity of imAE or the need for steroid treatment, which is important in allowing patients to remain on treatment and derive optimal clinical benefit. Further analyses across different tumor types and with different combination regimens are required to investigate this relationship in greater detail. If imAE development is confirmed to be correlated with survival benefit, a comprehensive predictive model (using only baseline characteristics) that identifies patients with a greater likelihood of experiencing immune-related toxicity during treatment would significantly aid clinicians in careful monitoring and counseling of these patients.

Data availability statement

Data underlying the findings described in this manuscript may be obtained in accordance with AstraZeneca's data sharing policy described at https://astrazenecagrouptrials.pharmacm.com/ST/Submission/Disclosure.

Ethics statement

Please refer to the primary publication for this trial: Rizvi et al. JAMA Oncol. 2020;6 (5):661-674. doi:10.1001/jamaoncol.2020.0237. The patients/participants provided their written informed consent to participate in this study.

Author contributions

AD was involved in the study concept, the collection and analysis of the data, contributed to the development of the analysis plan and modeling, and was heavily involved in the writing, reviewing, and editing of the manuscript. MA was involved in the writing, reviewing, and editing of the manuscript. HMK contributed to the development of the analysis plan and was involved in reviewing and editing the manuscript. NT contributed to the development of the analysis plan and was involved in reviewing and editing the manuscript. HM contributed to the development of the analysis plan and was involved in reviewing and editing the manuscript. NS was involved in reviewing and editing the manuscript. CM contributed to the development of the analysis plan and was involved in reviewing and editing the manuscript. DZ contributed to the development of the analysis plan and was involved in the writing, reviewing, and editing of the manuscript. GMM was involved in the study concept, contributed to the development of the analysis plan, and was involved in reviewing and editing the manuscript. AD, MA, HMK, NT, HM, NS, CM, DZ, and GMM have provided consent for the analyses detailed in this manuscript to be published.

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Conflict of interest

AD and GMM were employees of AstraZeneca at the time this work was conducted. NT, HM, NS, CM, and DZ are all employees of AstraZeneca and hold or have the option to hold stock. HMK declares receipt of institutional research grants directly to institute from Merck, Bristol-Myers Squibb, and Apexigen, personal fees outside of the work under consideration from Nektar, Immunocore, Celldex, Array Biopharma, Merck, Elevate Bio, Instil Bio, Bristol-Myers Squibb, Clinigen, Shionogi, Chemocentryx, Calithera, and Signatero, and personal fees not outside of the work under consideration from Iovance.

The remaining author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

The authors declare that this study received funding from AstraZeneca. The funder of the study participated in study design, data collection, data analysis, data interpretation, and writing of the report.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2022.1026964/full#supplementary-material

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