

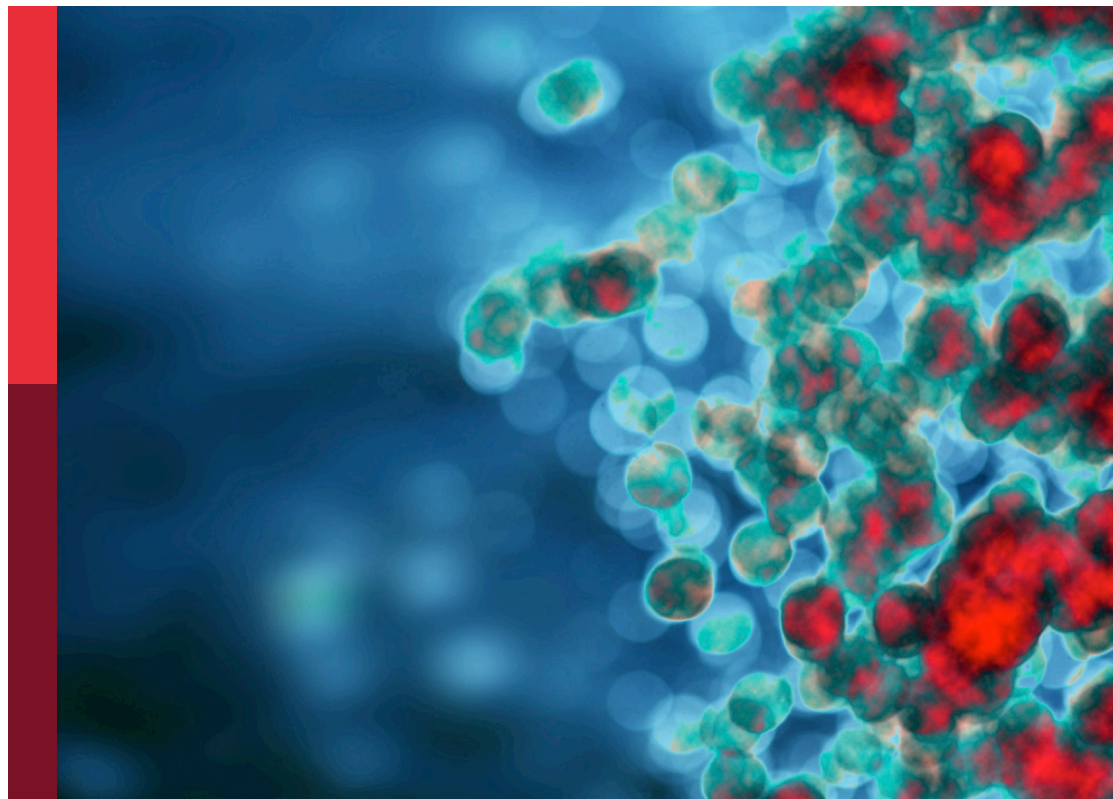
Immune tolerance dual role: advancements in cancer and autoimmune diseases

Edited by

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and Shisan Bao

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Immune tolerance dual role: advancements in cancer and autoimmune diseases

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Editorial: Immune tolerance dual role: advancements in cancer and autoimmune diseases

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KEYWORDS

immune tolerance, cancer, autoimmunity, inflammatory disorder, immune modulation

Editorial on the Research Topic

Immune tolerance dual role: advancements in cancer and autoimmune diseases

In the evolving landscape of immunology and oncology, the immune system is increasingly recognised as both a sentinel and a saboteur—capable of maintaining homeostasis or fuelling disease. Advances in immunotherapies and systems-level approaches such as multi-omics have exposed the dual nature of immune regulation: it can suppress tumours or promote their progression, preserve tolerance or incite autoimmunity. This paradox underscores the central challenge in harnessing immunity therapeutically. The studies featured in this Research Topic collectively reflect the complexity of immune modulation and highlight emerging opportunities for context-aware interventions across cancer and inflammatory diseases.

Zeng et al. investigate the PYHIN family—AIM2, IFI16, IFIX, and MNDA—cytosolic DNA sensors that traditionally mediate inflammasome activation and pyroptosis (<https://doi.org/10.3389/fimmu.2025.1576674>). While these proteins may promote anti-tumour immunity through immune-mediated cell death, they can also enhance tumour progression in certain contexts by sustaining chronic inflammation or aiding immune evasion. Their findings caution against indiscriminate therapeutic targeting and emphasise the need for tumour-specific strategies that consider immune landscape, microenvironment, and PYHIN expression dynamics.

Torniai et al. present a rare case of a patient with sarcomatoid urothelial carcinoma and pre-existing dermatomyositis (DM) who responded to pembrolizumab without autoimmune exacerbation, supported by intravenous immunoglobulin (IVIG) (<https://doi.org/10.3389/fimmu.2025.1558964>). Although the case suggests a possible role for immunomodulatory co-therapies in managing immune-related adverse events (irAEs), it remains anecdotal. Broader evidence is needed to establish standardised protocols and assess long-term safety of immune checkpoint inhibitors (ICIs) in autoimmune populations.

The review by Huang et al. explores the immunomodulatory potential of mixed-dose radiotherapy (RT) in non-small cell lung cancer (<https://doi.org/10.3389/fimmu.2024.1508007>). By combining high- and low-dose RT, the authors observed enhanced chemokine expression (e.g. CCL17), increased CD8⁺ T cell infiltration, and

improved synergy with ICIs. These findings reinforce RT's emerging role as an immune-modulating tool, not merely a cytotoxic one. However, optimal fractionation schedules, timing relative to immunotherapies, and tumour-specific responses require further investigation before broad clinical adoption.

In the realm of microbiota and cancer, the review on *Akkermansia muciniphila* highlights its depletion in hepatocellular carcinoma patients with poor ICI response (Yang and Shi). Although mechanistic details remain incomplete, this commensal bacterium appears to influence anti-tumour immunity through gut–liver axis modulation. Translating this into clinical practice will require well-designed trials that carefully standardise interventions and identify optimal microbial strains or metabolites.

Wu et al. report on the traditional herbal formula Yinchenhao decoction (YCHD) in an autoimmune hepatitis mouse model (<https://doi.org/10.3389/fimmu.2024.1488125>). Their work suggests that YCHD restores Th1/Treg balance and promotes gut-derived short-chain fatty acids, thereby enhancing immune tolerance. While mechanistically insightful, the translation of herbal medicine into clinical immunology remains hindered by challenges in formulation consistency, pharmacokinetics, and reproducibility across batches and populations.

A promising therapeutic innovation is described by Song et al., who evaluate IAH0968—a first-in-class, afucosylated anti-HER2 monoclonal antibody designed to enhance antibody-dependent cellular cytotoxicity (<https://doi.org/10.3389/fimmu.2024.1481326>). In a heavily pretreated cohort, IAH0968 achieved a disease control rate above 50%, albeit with modest objective responses. This study reinforces the relevance of Fc-engineering in strengthening immune effector functions, though further trials in earlier treatment settings or in combination regimens are needed to realise its full potential.

Chen et al. identify SPAG4 as a novel glioblastoma target associated with disulfidoptosis, an emerging form of regulated cell death (<https://doi.org/10.3389/fimmu.2024.1462064>). Elevated SPAG4 correlated with poor prognosis and increased CD47 expression, a known immune evasion marker. While these findings introduce an intriguing biomarker, future studies must address SPAG4's tissue specificity, targetability, and functional role within the tumour–immune microenvironment to support its translational utility.

Addressing a diagnostic challenge, Lyu et al. identify serum CCL4 as a candidate biomarker to distinguish gliomas from inflammatory CNS diseases (<https://doi.org/10.3389/fimmu.2024.1461450>). Their findings suggest that peripheral immune profiling could complement conventional neuroimaging. However, external validation in larger, diverse cohorts is essential before clinical integration.

Finally, Jiao et al. present a sobering case of immune-related haemophagocytic lymphohistiocytosis following tislelizumab treatment in a patient with microsatellite instability-high colorectal cancer and systemic lupus erythematosus (<https://doi.org/10.3389/fonc.2025.1585133>). Despite rapid intervention, the patient died within 16 days of diagnosis. This rare but fatal irAE illustrates the precarious immunological balance in patients with pre-existing autoimmunity and underscores the urgent need

for risk stratification tools and mechanistic insights to guide immunotherapy in high-risk populations.

Taken together, the studies in this Research Topic illuminate the complexity—and, at times, unpredictability—of immune modulation in cancer and inflammatory disorders. They converge on a common message: effective immunotherapy requires nuanced understanding of immune contexture, patient history, and the evolving crosstalk between tumour, host, and environment. Bridging mechanistic discovery with translational insight will be key to transforming immune complexity into therapeutic precision.

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CCL4 as a potential serum factor in differential diagnosis of central nervous system inflammatory diseases and gliomas

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Computed tomography (CT) scans and magnetic resonance imaging (MRI) are commonly utilized to detect brain gliomas and central nervous system inflammation diseases. However, there are instances where depending solely on medical imaging for a precise diagnosis may result in unsuitable medications or treatments. Pathological analysis is regarded as the definitive method for diagnosing brain gliomas or central nervous system inflammation diseases. To achieve this, a craniotomy or stereotaxic biopsy is necessary to collect brain tissue, which can lead to complications such as cerebral hemorrhage, neurological deficits, cerebrospinal fluid leaks, and cerebral edema. Consequently, the advancement of non-invasive or minimally invasive diagnostic techniques is currently a high priority. This study included samples from four glioma patients and five patients with central nervous system inflammatory diseases, comprising both serum and paired cerebrospinal fluid (CSF). A total of 40 human cytokines were identified in these samples. We utilized a receiver operating characteristic (ROC) analysis to assess the sensitivity and specificity for distinguishing central nervous system inflammation diseases and gliomas. Additionally, we examined the correlation of these factors between serum and CSF in the patients. Ultimately, the identified factors were validated using serum from patients with clinically confirmed gliomas and central nervous system inflammation diseases followed by detection and statistical analysis through ELISA. The levels of serum factors IL-4, IFN- α , IFN- γ , IL-6, TNF- α , CCL4, CCL11, and VEGF were found to be significantly higher in gliomas compared with inflammatory diseases of the central nervous system ($p < 0.05$). Furthermore, a strong correlation was observed between the levels of CCL4 in serum and CSF, with a correlation coefficient of $r = 0.92$ (95% CI = 0.20–0.99, $p = 0.027$). We gathered more clinical samples to provide further validation of the abundance of CCL4 expression. A clinical study analyzing serum samples from 19

glioma patients and 22 patients with central nervous system inflammation diseases revealed that CCL4 levels were notably elevated in the inflammatory group compared with the glioma group ($p < 0.001$). These results suggest that assessing serum CCL4 levels may be useful in distinguishing those patients for clinical diagnostic purposes.

KEYWORDS

gliomas, central nervous system inflammatory diseases, non-invasive testing, non-invasive testing of biological structures, potential biomarker, clinical diagnosis

1 Introductions

Brain tumors are among the deadliest types of tumors and contribute significantly to illness and death globally (1). It is estimated that brain tumors occur at a rate of 10 cases per 100,000 individuals annually (2). Gliomas are the most prevalent form of malignant brain tumor, making up 81% of all brain tumors, with an incidence rate of seven cases per 100,000 people (3). Adult brain tumors have a high mortality rate, particularly gliomas (4). In contrast, intracranial demyelinating inflammation has a lower mortality rate, but their imaging characteristics can be easily mistaken for one another, making it challenging to rely solely on medical imaging for accurate identification. Gliomas and central nervous system inflammatory diseases can be differentiated based on their underlying difference mechanisms, treatment approaches, and prognoses (5). Gliomas are typically managed through a combination of methods, including surgical removal, followed by chemotherapy and radiotherapy, as well as biological therapies like tumor treating fields (TTF) (6–9). Most central nervous system inflammatory diseases are multiple sclerosis, neuromyelitis optica, concentric sclerosis, disseminated encephalomyelitis, and others, which can lead to the formation of tumor-like demyelinating lesions (10–12). Immune therapy, hormone therapy, CD20 therapy to eliminate antibodies, or plasma exchange are necessary treatments (13–15). Brain tumors can develop at any age, but they are more frequently seen in young adults, middle-aged individuals, and older adults. They are present in 2% of standard autopsies. Therefore, there is an urgent need to discover novel biomarker that are sensitive, specific, and easy to practice (16).

Given the challenging circumstances surrounding imaging diagnosis and treatment, surgical biopsy is primarily used to collect tissue samples from lesions, allowing for pathological diagnosis to guide treatment. However, this procedure may lead to bleeding or nerve damage. Therefore, there is an urgent need to identify less invasive or non-invasive detection methods that can accurately differentiate between brain inflammations and gliomas.

In our study, we examined 40 abundance factors in CSF and serum related to gliomas and central nervous system inflammatory

diseases. We assessed the correlation of these immune factors and compared them between the glioma group and the central nervous system inflammatory diseases group in both CSF and serum. We found that CCL4 levels were notably elevated in cases of encephalitis compared with glioma, suggesting that it could serve as a potential biological marker.

2 Materials and methods

2.1 Patients' information

In our study, we analyzed samples from nine patients, comprising four with gliomas and five with central nervous system inflammatory diseases, to assess 40 human cytokines. In addition, we included CSF and serum from eight patients with cranial hypertension as a control group to validate the previous results. The diagnoses of the four glioma patients were confirmed through imaging and pathology. The five patients with central nervous system inflammatory diseases were diagnosed with myelitis (two patients), inflammatory demyelinating disease of the central nervous system (two patients), and tumor-like demyelinating lesions. Serum tests and C-reactive protein levels in these five patients indicated abnormal findings, but they showed improvement in clinical symptoms and imaging lesions following anti-inflammatory treatment.

2.2 Analysis flowchart of 40 human cytokines

Human cytokine analysis was conducted using the ABplex Human Cytokine 40-Plex Assay Kit (Cat. No: RK04368), supplied by Beijing Qiangxin Biorepublic Co., Ltd., in Beijing, China. The ABplex Human Cytokine 40-Plex Assay Kit is designed for the simultaneous quantification of 40 different human cytokines, chemokines, and growth factors in various sample types, including serum, plasma, and cell culture supernatants.

It allows simultaneous measurement of 40 analytes in a single well, increasing throughput and conserving sample volume. This kit is commonly used in immunology, oncology, and infectious disease research to analyze cytokine responses and to identify and validate biomarkers in various disease states (17).

After collecting samples from patients, including serum and CSF from various groups, the standards and reagents were prepared. Then, 50 μ L of standards or test samples was added to each well, followed by 50 μ L of coded microspheres and 50 μ L of the working detection antibody. The mixture was incubated for 30 min at 37°C and washed once, and then 50 μ L of SA-PE was added. This was incubated for 15 min at 37°C in the dark, followed by the addition of 70 μ L of wash buffer. Detection was performed using the ABplex-100.

2.3 Patients' inclusion criteria for ELISA

Patients with gliomas were diagnosed using a combination of pathological and imaging techniques, with evaluations conducted by two different neuropathologists and two independent imaging experts. Serum samples were obtained from 19 glioma patients prior to surgery at Tiantan Hospital. Furthermore, 22 patients with inflammation were identified through imaging and serum C-reactive protein tests, and their serum was also collected at Tiantan Hospital.

2.4 ELISA flowchart

At 4°C overnight, 100 ng of recombinant CCL4 in carbonate buffer (pH 9.6) was added to 96-well ELISA plates (MM-61999H1, No. 2024022699H). After a 2-h blocking period at room temperature with 300 μ L of blocking buffer (10 mM pH 7.4 PBS with 15% goat serum), serum samples were added to the wells and incubated for 1 h at 37°C, followed by five washing steps. Then, 100 μ L of diluted HRP-labeled goat anti-human IgG antibody (Jiangsu Meimian Industrial Co., Ltd., China) was added and incubated for another hour at 37°C. After three washes with the washing buffer, the plates were treated with 50 μ L of 2 mol/L H₂SO₄ and 100 μ L of TBM per well for 15 min at 37°C to stop the reaction. A microplate reader set to 450 nm was used to measure the optical density of each well over a period of 5 min.

2.5 Specimen collection

The matched serum and CSF were enrolled for analysis. Those samples were collected from Beijing Tiantan Hospital and Beijing Children's Hospital. All patients were diagnosed with brain tumors or different kinds of brain inflammation. These glioma patients were diagnosed by two independent neuropathologists according to the 2021 WHO classification. The brain inflammation was diagnosed by two independent clinical neurologists and ancillary examination. In addition, weighted kappa analysis was used to evaluate the consistency of the gliomas and brain inflammation.

2.6 Statistical analysis

Data analysis and graphing were conducted using GraphPad Prism version 9.0.0 (San Diego, CA, USA). To assess concentration differences between brain inflammation and brain tumors, we utilized two-way ANOVA along with Sidak's *post-hoc* test to adjust for multiple comparisons of serum and CSF from the same patient. We also carried out ROC curve analysis to evaluate whether any of the measured immunological molecules could distinguish between brain inflammation and brain tumors. This analysis yielded information on cutoff values, area under the curve (AUC) values, and specificities, sensitivities, and p values. Cutoff values were determined through ROC analyses by optimizing the Youden index (sensitivity + specificity – 1) and selecting the most favorable likelihood ratio. Lastly, we applied the Pearson correlation coefficient to assess the relationships between the concentrations of immunological molecules in serum and CSF. Statistically significant differences were considered those with a p value of less than 0.05.

3 Results

A total of four gliomas and five inflammatory diseases of the central nervous system were examined. An analysis of 40 human cytokines was performed using the ABplex Human Cytokine 40-Plex Assay Kit on serum or CSF samples via a non-invasive method (Figure 1). The weighted kappa values for the glioma patients as determined by two neuropathologists according to the 2021 WHO classification was 0.81, and the weighted kappa values for the brain inflammation as determined by two independent clinical neurologists was 0.73. There are cases where it is challenging to distinguish between intracranial inflammation and glioma using imaging methods like T1, T2, FLAIR, and T1 enhancement (Figure 2). Our study found that 30 factors exhibited no notable differences in serum and cerebrospinal fluid (Figure 3). Pathological analysis is essential for identifying the specific type of disease. However, procedures like craniotomy or biopsy may risk normal neurological function. Thus, there is a pressing need for non-invasive or minimally invasive methods to improve diagnostic accuracy for these conditions. Our research revealed that over 30 factors showed no significant differences in serum and CSF (Figure 4). On the basis of these results, we used CSF and serum from patients with intracranial hypertension for control and showed that CCL4 was significantly higher in both the inflammatory and tumor groups than in the control group (Supplementary Figures 1, 2).

In contrast, levels of IL-4, IFN- α , IFN- γ , IL-6, TNF- α , CCL4, P-cadherin, TRAIL, CCL11, and VEGF were significantly higher in serum than in CSF for both glioma and brain inflammation groups. However, there were no differences in CSF levels between the glioma and brain inflammation groups. The specific p-values for these factors were as follows: IL-4 p = 0.023, IFN- α p = 0.027, IFN- γ p = 0.049, IL-6 p = 0.028, TNF- α p = 0.024, CCL4 p = 0.038, P-cadherin p = 0.035, TRAIL p = 0.022, CCL11 p = 0.013, and VEGF p = 0.012 (Figure 4). ROC analysis of immunological

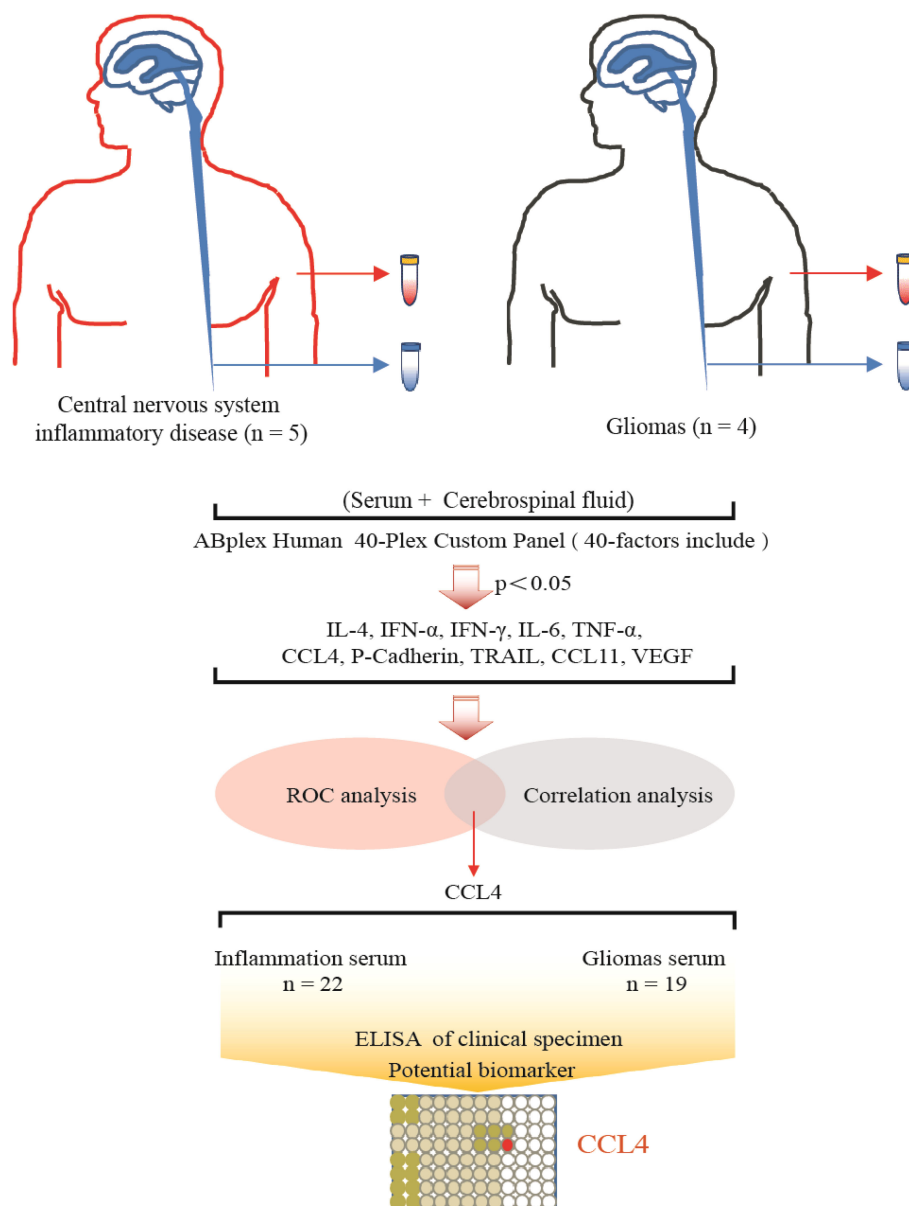


FIGURE 1

Summary graphical chart for this research. Brain inflammation groups (n=5) and brain glioma groups (n=4) samples were included in the research. Totally 40 immunological factors were analyzed. Two-way ANOVA, ROC analysis, and Pearson correlation coefficient analysis were performed, with the p value <0.05. ELISA of patients' serum was analyzed.

molecules in serum and CSF showed that the expression levels of IL-4, IFN- α , IFN- γ , IL-6, TNF- α , CCL4, CCL11, and VEGF exhibited high sensitivity and specificity in distinguishing between brain inflammation and brain tumor groups ($p < 0.05$) (Table 1). In our correlation analysis in serum and CSF, we found that CCL4 was significantly correlated between serum and CSF in the brain inflammation group ($r = 0.92$, 95% CI = 0.20–0.99, $p = 0.027$) (Figure 5, Table 2), whereas no other factors showed significant differences between groups. To further assess the effectiveness of CCL4, the researchers gathered more data from the serum of 19 patients diagnosed with glioma and 22 patients suffering from brain inflammation. The results showed a notable rise in CCL4 levels, indicating that elevated serum CCL4 expression could be a marker

for intracranial inflammatory diseases (Figure 6). ROC analysis showed a cutoff value of 969.6 and an AUC of 0.8182.

4 Discussion

To detect gliomas or intracranial inflammatory diseases, gadolinium-enhanced T1-weighted MRI or contrast-enhanced CT scans are utilized. Imaging tests can occasionally complicate the distinction between the two disease types, necessitating a biopsy for a clearer understanding of the disease's pathology (18, 19). There is an urgent need for non-invasive techniques to distinguish between intracranial tumors and inflammation (20, 21).

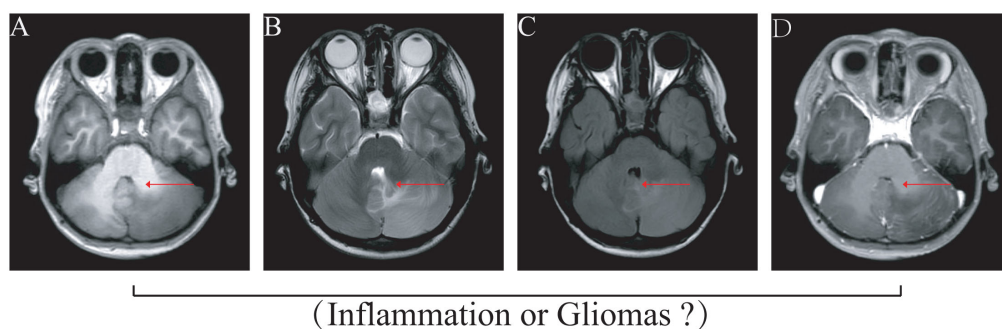


FIGURE 2

Imaging illustrates that differentiating glioma from inflammation disease is difficult. The images of MRI difficulty to gliomas or intracranial demyelinating inflammation disease. (A) T1 image, (B) T2 image, (C) FLAIR (fluid-attenuated inversion recovery), (D) Enhanced nuclear magnetism.

In our study, we gathered samples from four brain glioma diagnoses along with corresponding pathological diagnoses, serum, and CSF. We also collected serum samples from five cases of brain inflammation, matched with CSF. Using these serum samples, we aimed to identify potential factors that could differentiate between brain gliomas and brain inflammation. Our findings revealed

that the serum levels of IL-4, IFN- α , IFN- γ , IL-6, TNF- α , CCL4, P-cadherin, TRAIL, CCL11, and VEGF were significantly higher in cases of brain inflammation compared with brain gliomas. However, we did not find any significant factors in the CSF samples. Additionally, we examined the relationships between serum and CSF in both brain glioma and brain inflammation

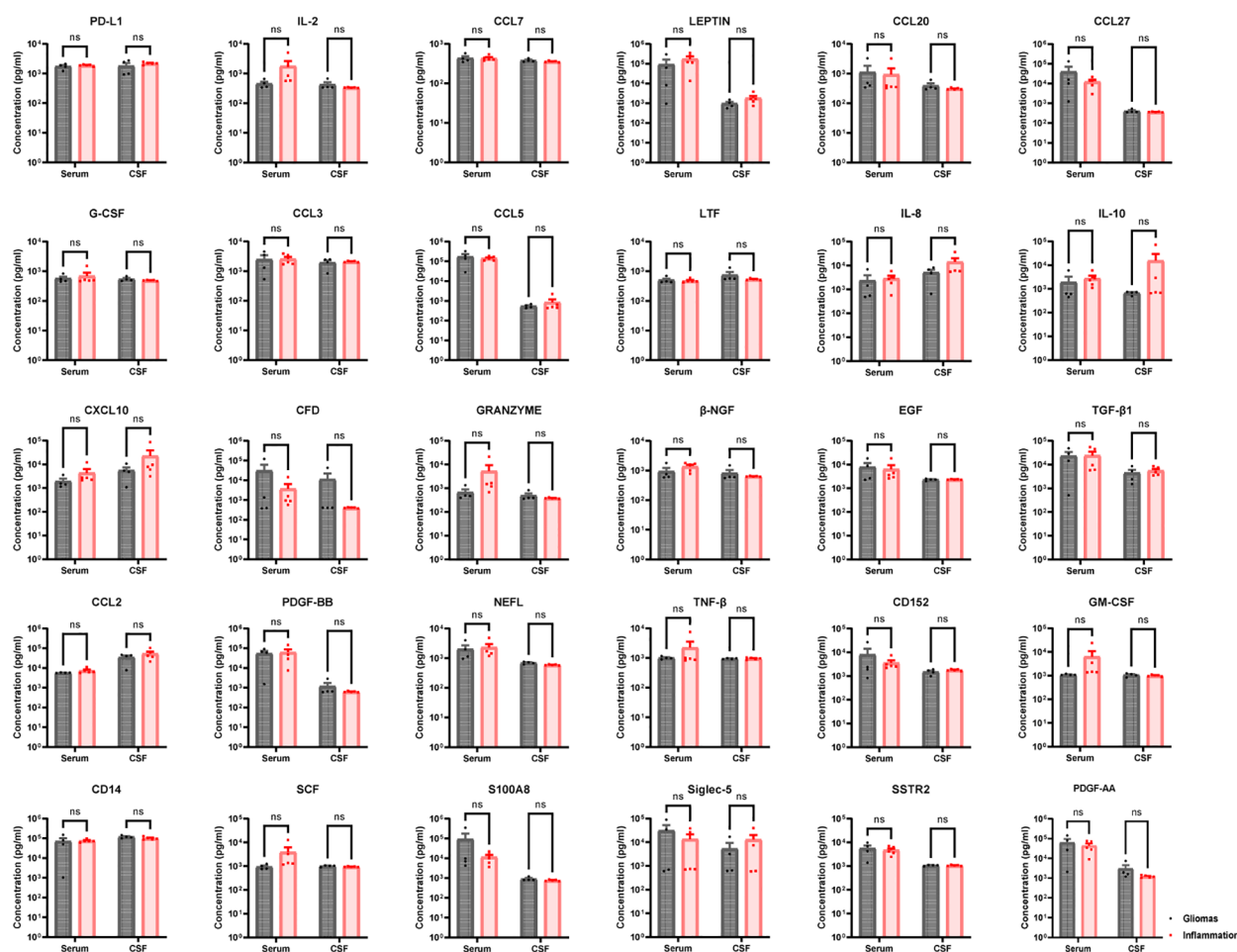


FIGURE 3

Thirty no-sense factors compared in gliomas and inflammation group. PD-L1, IL-2, CCL7, LEPTIN, CCL20, CCL27, G-CSF, CCL3, CCL5, LTF, IL-8, IL-10, CCL10, CFD, GRANZYME, β -NGF, EGF, TGF- β 1, CCL2, PDGF-BB, NEFL, TNF- β , CD152, GM-CSF, CD14, SCF, S100A8, Siglec-5, SSTR2, and S100A8—30 factors in serum had non-sense in brain inflammation groups than brain gliomas groups ($p > 0.05$). ns, not significant.

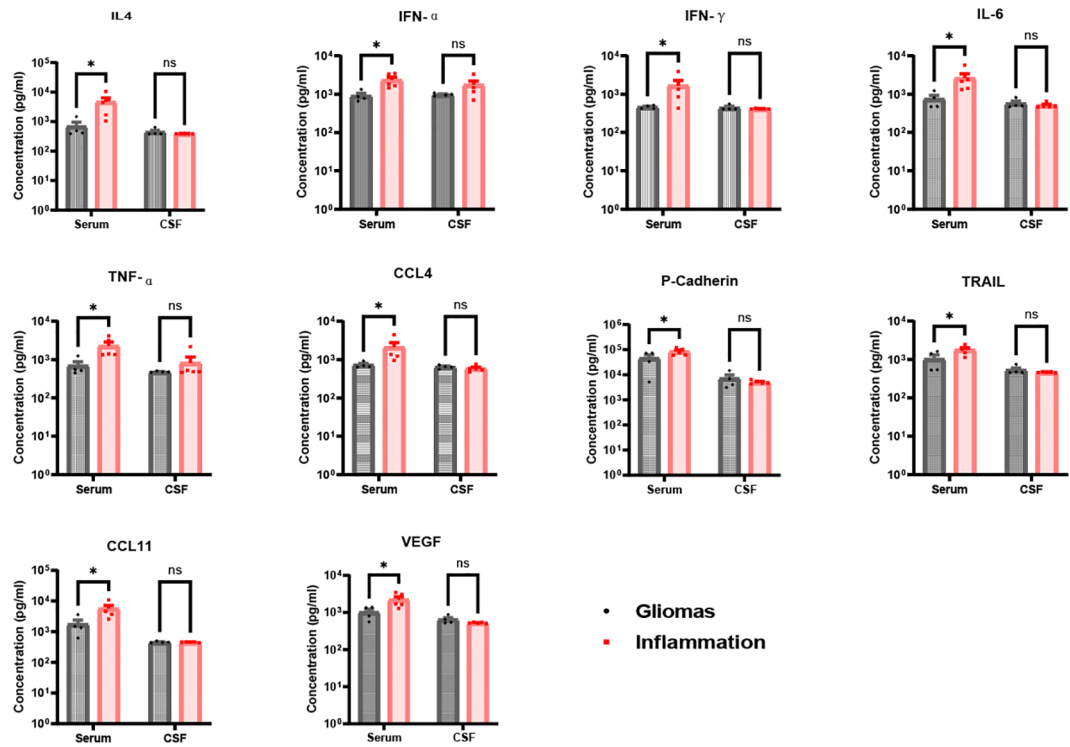


FIGURE 4
Ten significance factors compared in brain gliomas and inflammation group. IL-4, IFN-α, IFN-γ, IL-6, TNF-α, CCL4, P-Cadherin, TRAIL, CCL11, and VEGF factors in serum were significantly in brain inflammation groups than brain gliomas groups (IL-4 p = 0.023, IFN-α p = 0.027, IFN-γ p = 0.049, IL-6 p = 0.028, TNF-α p = 0.024, CCL4 p = 0.038, P-Cadherin p = 0.035, TRAIL p = 0.022, CCL11 p = 0.013, VEGF p = 0.012), but less significance in CSF between brain inflammation than brain tumor (p > 0.05). * p < 0.05; ns, not significant.

TABLE 1 ROC analysis of immunological molecules in CSF and serum.

Cytokines	Sample	Cutoff (pg/ml)	Sensitivity (95% CI) [%]	Specificity (95% CI) [%]	AUCs (95% CI)	P-value
IL-4	Serum	> 1593	80.00 (37.55-98.97)	100.0 (51.01-100)	0.95 (0.81-1.00)	* 0.0275
	CSF	> 385.5	100.0 (56.55-100)	50.0 (8.89-91.12)	0.50 (0.01-0.99)	>0.9999
IFN-α	Serum	> 1402	100.0 (56.55-100)	100.0 (51.01-100)	1.00 (1.00-1.00)	* 0.0143
	CSF	> 1119	80.00 (37.55-98.97)	100.0 (51.01-100)	0.80 (0.45-1.00)	0.1416
IFN-γ	Serum	> 681.0	80.00 (37.55-98.97)	100.0 (51.01-100)	0.90 (0.68-1.00)	* 0.0500
	CSF	< 412.0	80.00 (37.55-98.97)	50.00 (8.89-81.12)	0.70 (0.34-1.00)	0.3272
IL-6	Serum	> 1275	100.0 (56.55-100)	100.0 (51.01-100)	1.00 (1.00-1.00)	* 0.0143
	CSF	< 491.5	80.00 (37.55-98.97)	75 (30.06-98.72)	0.75 (0.41-1.00)	0.2207
TNF-α	Serum	> 1288	100.0 (56.55-100)	100.0 (51.01-100)	1.00 (1.00-1.00)	* 0.0143
	CSF	> 476.0	80.00 (37.55-98.97)	75 (30.06-98.72)	0.75 (0.41-1.00)	0.2207
CCL4	Serum	> 936.0	100.0 (56.55-100)	100.0 (51.01-100)	1.00 (1.00-1.00)	* 0.0143
	CSF	< 611.0	80.00 (37.55-98.97)	75 (30.06-98.72)	0.70 (0.32-1.00)	0.3272
P-Cadherin	Serum	> 75108	60 (23.07-92.89)	100.0 (51.01-100)	0.80 (0.49-1.00)	0.1416
	CSF	> 4176	100.0 (51.01-100)	50.0 (8.89-91.12)	0.50 (0.01-0.99)	>0.9999
TRAIL	Serum	> 1748	60 (23.07-92.89)	100.0 (51.01-100)	0.85 (0.59-1.00)	0.0864

(Continued)

TABLE 1 Continued

Cytokines	Sample	Cutoff (pg/ml)	Sensitivity (95% CI) [%]	Specificity (95% CI) [%]	AUCs (95% CI)	P-value
	CSF	> 459.5	80.00 (37.55-98.97)	50.0 (8.89-91.12)	0.50 (0.08-0.92)	>0.9999
CCL11	Serum	> 3645	80.00 (37.55-98.97)	100.0 (51.01-100)	0.95 (0.81-1.00)	* 0.0275
	CSF	> 452.5	80.00 (37.55-98.97)	75 (30.06-98.72)	0.65 (0.23-1.00)	0.4624
VEGF	Serum	> 1511	80.00 (37.55-98.97)	100.0 (51.01-100)	0.90 (0.68-1.00)	* 0.0500
	CSF	< 522.0	60 (23.07-92.89)	100.0 (51.01-100)	0.80 (0.49- 1.00)	0.1416

* p <0.05.
Bold values indicates p-values < 0.05.

groups. Notably, we discovered a strong correlation between the serum and CSF levels of CCL4 in the brain inflammation group. To confirm our findings, we collected serum again from clinical patients diagnosed with brain inflammation disease and glioma and measured the CCL4 levels present. Our results indicated that the expression of CCL4 in cases of brain inflammation disease was notably higher compared with that in glioma, and this difference was statistically significant ($p < 0.001$, AUC = 0.8182). CCL4 may serve as a potential biomarker to distinguish between brain gliomas and brain inflammation in serum.

CCL4, or macrophage inflammatory protein-1 beta (MIP-1 β), is a chemokine that plays a significant role in the immune response. It serves as a crucial chemokine that attracts and activates immune cells, influences the immune response, and encourages inflammation. When it binds to the CCR5 receptor, it activates several signaling pathways, such as NF- κ B, MAPK, and JAK/STAT, all of which play a role in disease development. In the context of gliomas, CCL4 aids in tumor progression by altering the tumor microenvironment, thereby facilitating tumor growth, invasion, and the formation of new blood vessels. In low-grade gliomas (LGG), CCL4 is a key element for the survival of LGG stem cells (22). Studies have shown that CCL4 can enhance tumor growth by attracting factors that promote tumorigenesis, and it also interacts with fibroblasts and endothelial cells to create a tumor microenvironment that supports tumor development (23, 24). Additionally, CCL4 facilitates the migration

of osteosarcoma cells by activating signaling pathways such as focal adhesion kinase (FAK), protein kinase B (AKT), and hypoxia-inducible factor 1 subunit alpha (HIF-1 α). Our goal is to discover a minimally invasive or non-invasive approach to identify brain gliomas and brain inflammation. To achieve this, we collected serum and CSF samples from four brain glioma patients and five with brain inflammation for immune factor analysis and statistical evaluation. We also measured CCL4 levels in confirmed clinical serum samples using ELISA. Our findings indicate that serum CCL4 could serve as a potential marker for identifying between intracranial inflammation disease and gliomas.

This study offers important insights into the potential of serum CCL4 levels as a biomarker for differentiating between brain gliomas and central nervous system inflammatory diseases, but it also has several limitations. Firstly, the sample size was relatively small, especially in the initial analysis, which may restrict the applicability of the results. To validate the diagnostic value of CCL4 in various populations, larger and more diverse groups are needed. Secondly, the research concentrated on a specific group of cytokines, potentially overlooking other relevant biomarkers that could be significant. Future investigations should include a wider range of biomarkers to create a more thorough diagnostic tool. Additionally, it is clinically difficult to obtain cerebrospinal fluid and serum from completely normal individuals; therefore, cerebrospinal fluid and serum from patients with intracranial

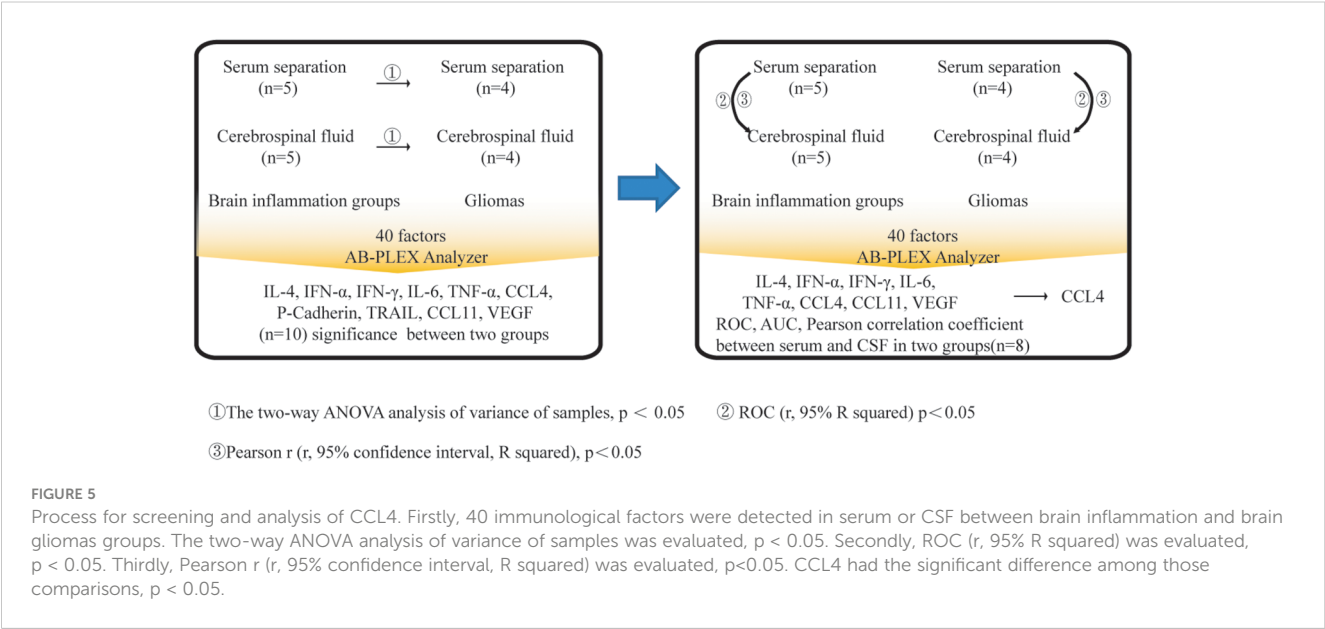
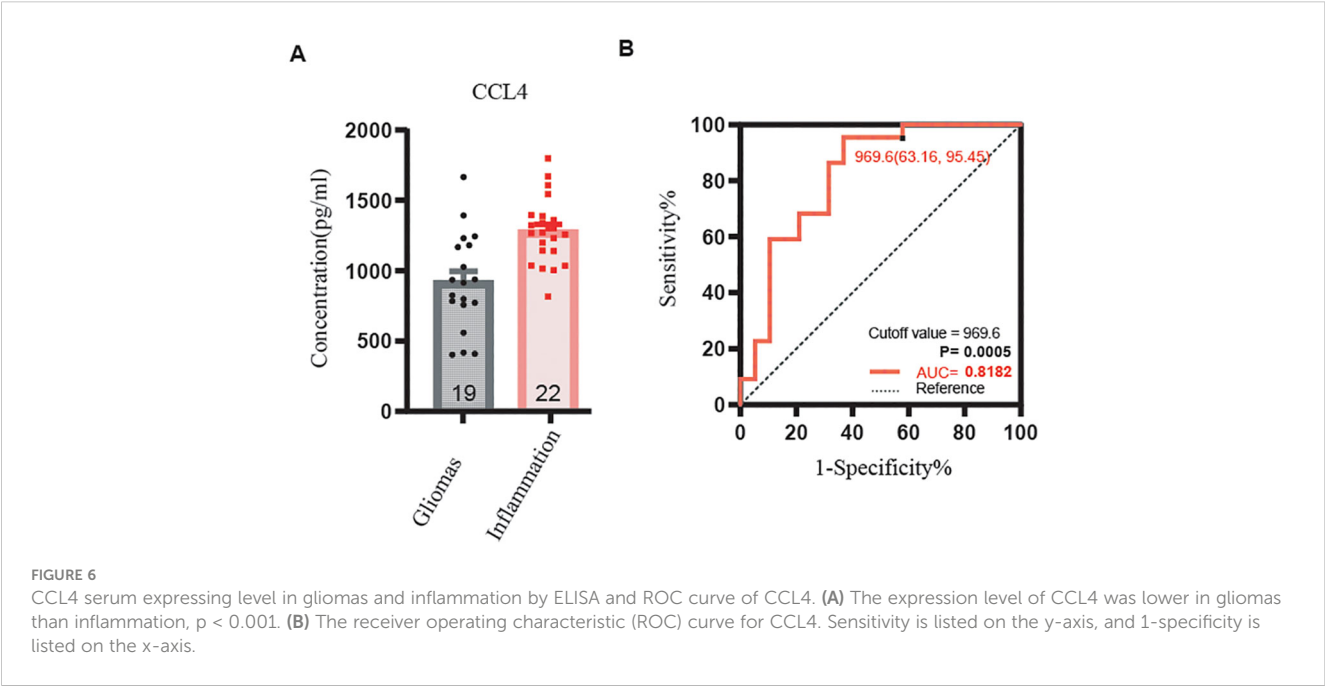


TABLE 2 Correlation analysis of immunological molecules in CSF and serum.

Cytokines	Patient group	Pearson r (95% CI)	R squared	P value (two-tailed)
IL-4	Tumor	−0.20 (−0.97 - 0.94)	0.0403	0.7992
	Inflammation	−0.55 (−0.96 - 0.64)	0.3047	0.3347
IFN-α	Tumor	0.76 (−0.75 - 0.99)	0.5719	0.2438
	Inflammation	0.15 (−0.84 - 0.91)	0.0234	0.8062
IFN-γ	Tumor	0.43 (−0.91 - 0.98)	0.1808	0.5747
	Inflammation	−0.39 (−0.95 - 0.75)	0.1535	0.5143
IL-6	Tumor	0.17 (−0.95 - 0.97)	0.0275	0.8342
	Inflammation	−0.29 (−0.93 - 0.80)	0.0844	0.6354
TNF-α	Tumor	−0.11 (−0.97 - 0.95)	0.0118	0.8915
	Inflammation	−0.39 (−0.95 - 0.75)	0.1554	0.5114
CCL4	Tumor	−0.69 (−0.99 - 0.81)	0.4730	0.3122
	Inflammation	0.92 (0.20 - 0.99)	0.8456	* 0.0270
P-Cadherin	Tumor	0.71 (−0.79 - 0.99)	0.5050	0.2894
	Inflammation	0.34 (−0.92 - 0.98)	0.1139	0.6626
TRAIL	Tumor	0.43 (−0.90 - 0.98)	0.1888	0.5655
	Inflammation	0.08 (−0.86 - 0.90)	0.0063	0.8992
CCL11	Tumor	−0.45 (−0.98 - 0.90)	0.1996	0.5533
	Inflammation	0.45 (−0.72 - 0.95)	0.1987	0.4518
VEGF	Tumor	−0.58 (−0.99 - 0.86)	0.3323	0.4236
	Inflammation	0.23 (−0.82 - 0.92)	0.0517	0.7131

Bold values indicates p-values < 0.05.



hypertension were included in this study as a control group. Moreover, although a strong correlation between serum and cerebrospinal fluid CCL4 levels was found, the study did not investigate the mechanisms behind this relationship, which is essential for understanding the pathophysiology of these diseases. Lastly, the research did not consider possible confounding factors such as patient comorbidities, medication use, or other neurological conditions that might affect cytokine levels and the accuracy of the biomarker. Future studies should aim to confirm its clinical relevance, explore its mechanistic functions, and develop targeted therapies. These initiatives will contribute to personalized medicine strategies, ultimately enhancing patient care and outcomes.

5 Conclusion

This research reveals a significant relationship between CCL4 concentrations in serum and cerebrospinal fluid, indicating its possible role as a biomarker. Additional validation indicated that serum CCL4 levels were notably higher in patients with central nervous system inflammation than in those with gliomas. These results imply that serum CCL4 levels may act as a non-invasive biomarker to differentiate between gliomas and inflammatory conditions, providing a valuable resource for enhancing diagnostic precision and potentially minimizing the necessity for invasive diagnostic methods in clinical settings.

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 humans were approved by Tiantan hospital ethics committee. The studies were conducted in accordance with the local legislation and institutional requirements. Written informed consent for participation in this study was provided by the participants' legal guardians/next of kin.

Author contributions

T-JL: Writing – original draft, Writing – review & editing. JW: Conceptualization, Investigation, Software, Writing – original draft. FZ: Data curation, Methodology, Supervision, Writing – original draft. KS: Formal analysis, Project administration, Validation, Writing – original draft. ZZ: Data curation, Supervision, Writing – original draft. RT: Software, Writing – review & editing. ZG: Investigation, Writing – review & editing. HW: Validation, Writing – review & editing. XZ: Conceptualization, Formal analysis, Project administration, Writing – review & editing. WM: Data curation, Methodology, Supervision, Writing – original draft. MZ: Formal analysis, Project administration,

Validation, Writing – review & editing. WX: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Software, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing.

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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.

The reviewer QC declared a shared affiliation, with no collaboration, with several of the authors, T-JL, ZZ, RT, HW, MZ, WX, to the handling editor at the time of the review.

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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.2024.1461450/full#supplementary-material>

SUPPLEMENTARY FIGURE 1

Comparison of CCL4 expression abundance between intracranial hypertension control group and glioma group. ($p < 0.05$).

SUPPLEMENTARY FIGURE 2

Comparison of CCL4 expression abundance between intracranial hypertension control group and inflammation group. ($p < 0.05$).

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Integrated multi-level omics profiling of disulfidptosis identifis SPAG4 as an innovative immunotherapeutic target in glioblastoma

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Objective: To investigate the association between disulfidptosis-related genes (DFRGs) and patient prognosis, while concurrently identifying potential therapeutic targets in glioblastoma (GBM).

Methods: We retrieved RNA sequencing data and clinical characteristics of GBM patients from the TCGA database. We found there was a total of 6 distinct clusters in GBM, which was identified by the t-SNE and UMAP dimension reduction analysis. Prognostically significant genes in GBM were identified using the limma package, coupled with univariate Cox regression analysis. Machine learning algorithms were then applied to identify central genes. The CIBERSORT algorithm was utilized to assess the immunological landscape across different GBM subtypes. *In vitro* and *in vivo* experiments were conducted to investigate the role of SPAG4 in regulating the proliferation, invasion of GBM, and its effects within the immune microenvironment.

Results: 23 genes, termed DFRGs, were successfully identified, demonstrating substantial potential for establishing a prognostic model for GBM. Single cell analysis revealed a significant correlation between DFRGs and the progression of GBM. Utilizing individual risk scores derived from this model enabled the stratification of patients into two distinct risk groups, revealing significant variations in immune infiltration patterns and responses to immunotherapy. Utilizing the random survival forests algorithm, SPAG4 was identified as the gene with the highest prognostic significance within our model. *In vitro* studies have elucidated SPAG4's significant role in GBM pathogenesis, potentially through the regulation of fatty acid metabolism pathways. Our *in vivo* investigations using a subcutaneous xenograft model have confirmed SPAG4's influence on tumor growth and its capacity to modulate the immune microenvironment. Advanced research hints that SPAG4 might achieve immune evasion by increasing CD47 expression, consequently reducing phagocytosis.

Conclusions: These findings highlight SPAG4 as a potential GBM therapeutic target and emphasize the complexity of the immune microenvironment in GBM progression.

KEYWORDS

glioblastoma, disulfidptosis, tumor immune microenvironment, prognostic model, SPAG4

1 Introduction

Glioma stands as the predominant primary malignant neoplasm within the central nervous system (CNS), comprising 47.7% of the total malignancies occurring in the CNS (1). Glioma is categorized into lower-grade gliomas (LGG, WHO grade I and II) and glioblastoma (GBM, WHO grade III and IV). Patients with glioblastoma generally experience a less favorable prognosis compared to those with lower-grade gliomas (2). The median survival period for glioblastoma (GBM) patients typically stands at 12 months post-surgery and radiotherapy. In contrast, the survival duration for lower-grade glioma (LGG) patients can vary significantly, spanning from 1 to 15 years (3). Therefore, it is of paramount importance to elucidate the molecular mechanisms that underlie the progression of GBM.

In 2023, Liu et al. (4) proposed a novel form of cell death termed dual sulfur death. In their investigation, they observed that under conditions of glucose starvation, cells with elevated expression of solute carrier family 7 member A11 (SLC7A11) led to the continuous accumulation of intracellular disulfides. This subsequently induced a stress response resulting in increased disulfide bond content in the actin cytoskeleton, a component of the cell structure. The heightened disulfide bonds prompted contraction of actin filaments, ultimately disrupting the cytoskeletal architecture and leading to cell death (disulfidptosis). This new form of cell death differs from well-known mechanisms such as apoptosis, autophagy, and necroptosis. An increasing body of research suggests that disulfidptosis plays a crucial role in the initiation and progression of tumors and may serve as a novel molecular target (5, 6). However, as of now, the specific role of disulfidptosis in GBM remains unclear.

In this study, our objective was to comprehensively investigate the role of disulfidptosis-related genes (DFRG) in the immune microenvironment and prognosis of GBM. We stratified GBM patients into distinct risk groups based on the expression levels of disulfidptosis-related genes (DFRG) and their association with clinical outcomes. We developed and validated a prognostic model associated with DFRG, which demonstrated high accuracy in predicting prognosis and immune therapy response across various independent cohorts. SPAG4 emerged as the most crucial gene in the model. Mechanistically, SPAG4 was found to facilitate the transformation of macrophages into an M2

phenotype, thereby promoting the proliferation and invasion of GBM. These findings provide potential therapeutic targets for the treatment of GBM.

2 Materials and methods

2.1 Ethics statement

This study has been approved by the Ethics Committee of The First Affiliated Hospital of Hainan Medical University. The acquisition of tumor and adjacent normal tissues for GBM patients has been agreed by the patients themselves, and the informed consent has been signed. The specimen tissues of these clinical patients were used for subsequent Western blot and immunohistochemical analysis.

2.2 GBM data acquisition

In this study, the TCGA-GBM cohort comprising 374 glioblastoma (GBM) samples and 50 normal tissue samples was utilized. Gene expression profiles and clinical data, including TNM classification, age, gender, and overall survival, were obtained from the TCGA data portal (<https://portal.gdc.cancer.gov/>).

In addition, we acquired the GSE14520 dataset, consisting of 221 GBM samples, from the GEO database (<https://www.ncbi.nlm.nih.gov/geo/>), and the ICGC dataset (<https://icgc.org/>), which included 240 GBM samples. Only datasets with comprehensive clinical information were included in the analysis.

Furthermore, single-cell data was sourced from the Tumor Immune Single-Cell Hub (TISCH2; <http://tisch.comp-genomics.org>), an extensive online repository of single-cell RNA-seq data specifically focusing on the tumor microenvironment (TME) (1). Leveraging this resource, we systematically explored TME heterogeneity across diverse datasets and cell types.

2.3 DFRGs resource

A comprehensive set of 25 disulfidptosis-related genes (DFRGs) was acquired from the GeneCards repository.

2.4 Consensus clustering

For cluster analysis, the “ConsensusClusterPlus” package was utilized, employing the k-means algorithm. To identify genes exhibiting significant alterations across distinct clusters of disulfidptosis-related genes (DFRGs), differential expression analysis was performed using the “limma” software. Criteria for determining the significance of changes included a False Discovery Rate (FDR) < 0.05 and an absolute log2 fold change ($|\log_2\text{FC}|$) > 1.

2.5 Cell culture and cell lines

HEB cells, U87 cells, and U251 cells were cultured separately in DMEM high-glucose medium containing 10% FBS and 1% penicillin-streptomycin solution (dual antibiotics, P/S). MCF-10A cells were cultured in DMEM/F12, supplemented with 5% horse serum, 20 ng/mL epidermal growth factor (EGF), 0.5 µg/mL hydrocortisone, 10 µg/mL insulin, 1% non-essential amino acid solution (NEAA), and 1% penicillin-streptomycin solution (dual antibiotics, P/S). All cells were maintained in a 37°C, 5% CO₂ incubator, with passaging conducted every 2-3 days. The culture medium was replaced twice a week.

2.6 Measurement of cellular reactive oxygen species levels

Following various treatments, cells were cultured for 3 days in a 37°C, 5% CO₂ incubator. Staining was conducted according to the instructions provided by the cell proliferation assay kit. Images were captured and observed under a laser confocal microscope, and the proportion of DCFH-DA-stained positive cells was analyzed.

2.7 Western blot experiment

Total proteins were extracted using a lysis buffer. Subsequently, total proteins (40 µg/sample) were separated through 10% SDS-PAGE, running at 300 mA for 120 minutes. Following electrophoresis, proteins were transferred to a PVDF membrane, and the membrane was blocked with 5% milk at room temperature for 1 hour. Next, the membrane was incubated overnight at 4°C with the primary antibody, followed by a 2-hour incubation with the corresponding secondary antibody at room temperature. Finally, the expression levels of the target protein bands were analyzed. The following primary antibodies were utilized: anti-SPAG4 (1: 1000, CSB-PA989865), and anti-GAPDH (1: 5000, CSB-MA000071M1m) were purchased from Cusabio Biotech Co. Ltd, Hubei, China. Anti-CD47 (1: 2000, AF6423) was purchased from Beyotime Institute of Biotechnology, Nantong, China.

2.8 Cell scratch assay

Cell scratch assay was performed to assess cell migration. U87 and U251 cells from siNC group, siRNA1 group, and siRNA2 group

were trypsinized, counted using a cell counting plate, and seeded into 6-well plates at a density of 2×10^5 cells/well in serum-free medium. After cell fusion, scratches were made using a 200 µL pipette tip. Subsequently, the cells were washed twice with PBS, and the cell-free areas were photographed at 0 and 24 hours, respectively.

2.9 Transwell invasion assay

This experiment was employed to assess the invasive capabilities of U87 and U251 cells. Cells from siNC group, siRNA1 group, and siRNA2 group were starved for 24 hours and then seeded at a density of 3×10^4 cells/well in the upper chamber of Transwell, which was pre-coated with Matrigel. The upper chamber was devoid of FBS, while the lower chamber contained DMEM with 10% FBS. After 24 hours, cells were fixed with 4% paraformaldehyde for 30 minutes, followed by staining with 0.1% crystal violet. Non-invasive cells in the upper chamber were gently removed with a cotton swab, and images were captured using a high-power inverted microscope.

2.10 Immunohistochemical staining

Tissue cores on the microarray were deparaffinized in xylene, followed by graded ethanol hydration. Antigen retrieval was achieved by microwave treatment in a citrate buffer solution. Subsequent immunohistochemical staining was performed according to the kit instructions, with the primary antibody of SPAG4 (CSB-PA989865, Cusabio Biotech) diluted to a working concentration of 1:400 and incubated overnight at 4°C.

2.11 Immunofluorescence staining

Tissue sections were deparaffinized in xylene, underwent graded ethanol hydration, and antigen retrieval was performed using microwave treatment in a citrate buffer solution. After room temperature blocking, the sections were incubated with the primary antibody overnight at 4°C. The next day, the sections were incubated with a mixture of goat anti-rabbit red fluorescence secondary antibody (ab150080, Abcam USA, working concentration 1:1000) and goat anti-mouse green fluorescence secondary antibody (ab150117, Abcam USA, working concentration 1:1000). After washing, the sections were counterstained with DAPI-containing anti-fluorescence quenching mounting medium, cover-slipped, stored in the dark at 4°C, and examined and imaged using an upright fluorescence microscope. The following primary antibodies were utilized: anti-induction of brown adipocytes 1 (Iba-1, 1: 500, GB15105-100), anti-inducible nitric oxide synthase (1: 500, iNOS GB115703-100), and anti-CD206 (1: 500, GB113497-100) were purchased from Servicebio, Wuhan, China.

2.12 Subcutaneous tumor xenograft in nude mice

U251 cells in logarithmic growth phase were centrifuged, counted, and based on the cell count, a portion of the cells was

resuspended in DMEM to achieve a concentration of 2×10^6 cells/100 μ L. Nude mice were prepared by disinfecting the left shoulder with iodophor using a cotton ball. Using a syringe, 100 μ L of cell suspension was injected into the left shoulder subcutaneously. After injection, gentle pressure was applied to stop bleeding. After approximately one week of normal housing, the tumors on the nude mice were measured using calipers. Once the tumors reached an approximate volume of 70 mm³, mice were randomly divided into groups of 5, housed separately as control and intervention groups. The mice's weight was recorded during each administration to monitor any changes after drug administration.

2.13 Statistical analysis

R version 4.2.3 software was employed for data processing, statistical analysis, and visualization. The optimal cut-off value was determined using the “survminer” R package, and Kaplan-Meier analysis was conducted using the survival program. Comparisons between two groups regarding continuous variables were performed using the Wilcoxon rank-sum test, while Spearman correlation analysis was employed to assess the interrelationships among continuous variables. Statistical significance was defined as $P < 0.05$ for all conducted statistical analyses.

3 Results

3.1 The mutation profile of DFRG in GBM

In order to elucidate the molecular underpinnings of glioblastoma (GBM) and identify potential therapeutic targets, we initiated our investigation by examining the expression patterns of DFRGs within GBM, utilizing the comprehensive GTEx database. Utilizing the criteria of $|\log FC| > 1$ and adjusted P -Value < 0.05 , we identified a total of 217 upregulated and 149 downregulated differentially expressed genes, encompassing 23 DFRGs (Supplementary Figure S1). As depicted in Figure 1A, our comparative analysis of 23 DFRGs. Building upon these findings, we further explored the genetic stability of the 23 DFRGs. Mutational profiling was conducted to assess whether these genes harbored any oncogenic mutations. It showed that 19 of the DFRGs exhibited mutations, while 4 genes remained mutationally unaltered (Figure 1B). To gain insight into the cooperative or mutually exclusive relationships among these genes, we performed a gene alteration co-occurrence and mutual exclusivity analysis (Figure 1C). Gene copy number variations, including gains and losses, are known to significantly impact gene expression and contribute to cancer phenotypes, which is shown in Figure 1D. In order to contextualize the chromosomal localization of DFRGs and infer potential mechanisms of genomic instability, we generated a graphical representation of the chromosome regions harboring these genes. Figure 1E presents a genomic landscape of DFRGs, suggesting that their spatial distribution may have implications for GBM genetics and therapeutic targeting.

3.2 Expression of DFRG in the single-cell analysis within GBM

To understand the cellular context and functional significance of DFRGs in GBM, we compared their expression patterns within single-cell resolution GBM samples from the GSE241037 dataset. Our approach involved utilizing both t-SNE and UMAP analyses to visualize the cellular distribution (Figure 2A). Further exploration of DFRG expression was achieved through a bubble diagram representation presented in Figure 2B. Figure 2C illustrated the gene expression profiles and interrelationships among distinct cell types. Figure 2D showed a significant upregulation of GCSH in microglial cells, suggesting a correlation between disulfidptosis and tumor immune modulation.

3.3 Disulfidptosis subtypes compared through analyses

To elucidate the complex interactions among DFRGs in GBM and their prognostic significance, we conducted a comprehensive analysis by integrating data from TCGA and GEO (GSE74187, and GSE83300), which is displayed in Figure 3A. Subsequently, to identify prognostic DFRGs, we performed an unsupervised clustering analysis, as depicted in Figure 3B, which resulted in a consensus matrix heatmap defining two distinct clusters ($k = 2$). The DFRG-related clusters, discerned through the aforementioned methodologies, exhibit notable prognostic relevance, as illustrated in Figure 3C with Kaplan-Meier curves depicting overall survival (OS). The comparison revealed a significant difference in survival rates between the two disulfidptosis clusters (chi-square test, $p = 3.9e-04$). To further explore the heterogeneity between the DFRG-related clusters, Figure 3D presents a principal component analysis (PCA). The result illustrates the distinct distribution patterns of patients, indicating that the identified DFRGs could be used to stratify GBM patients into subgroups with different clinical outcomes.

3.4 Cluster analysis outcomes for DFRG

To understand the clinical relevance and molecular mechanisms underlying the distinct subtypes of GBM defined by DFRGs, we compared the clinical characteristics and performed functional enrichment analyses between DFRGcluster A and DFRGcluster B. GSVA method was utilized to explore the enrichment of KEGG pathways and GO terms. As shown in Figure 4A, significant disparities were found in clinical features and expression levels of DFRGs between the two clusters. Figure 4B depicts the KEGG pathway enrichment analysis using GSVA, revealing distinct biological pathways that are overrepresented in each cluster. Similarly, Figure 4C showcases the GO enrichment analysis through GSVA, indicating the functional categories that are significantly enriched in each DFRG cluster.

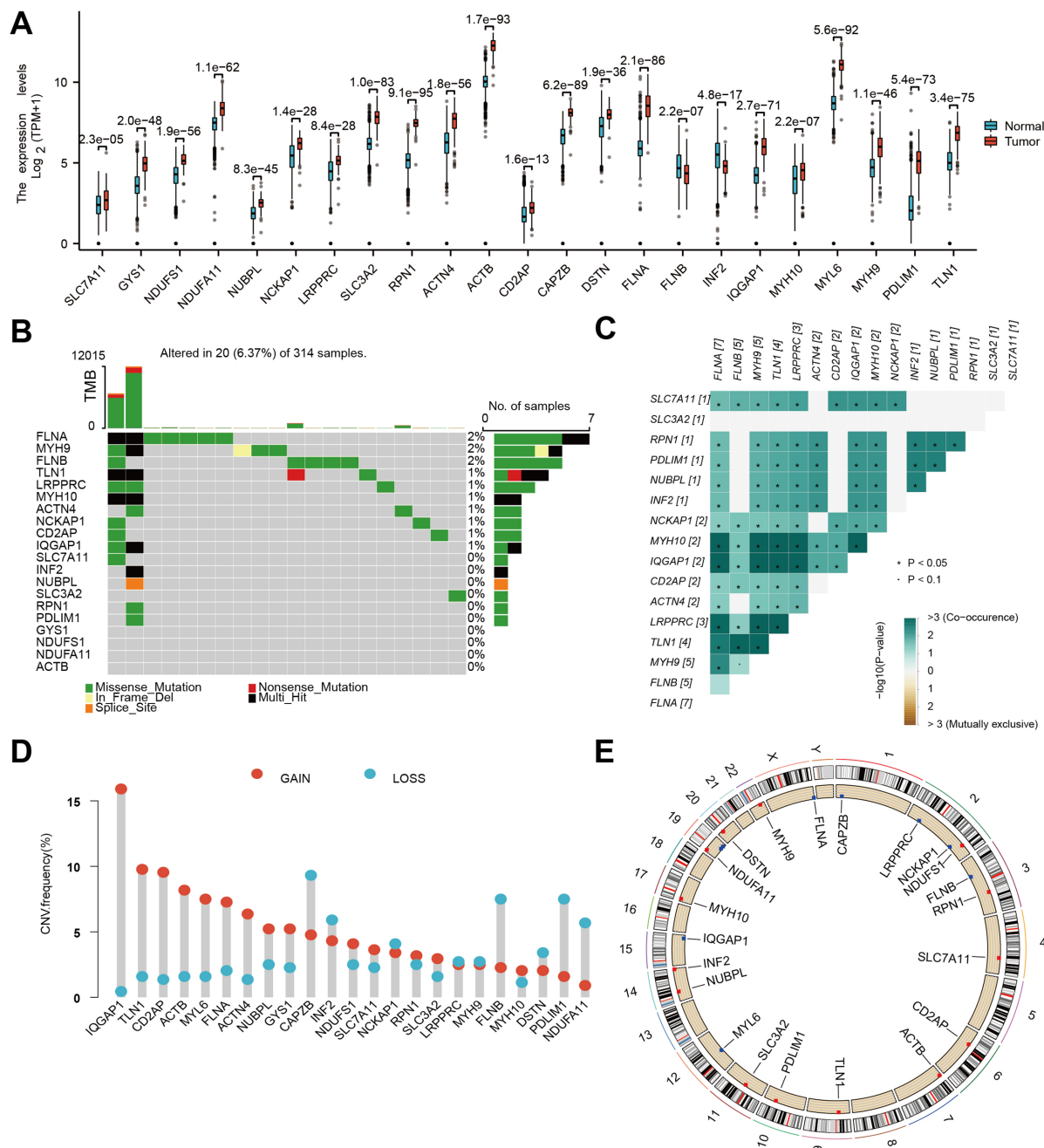


FIGURE 1

Genetic and transcriptional characteristics of DFRGs. (A) Gene expression levels from TCGA+GTEx V8 tissues. (B) Somatic mutation of DFRGs in the study population. (C) Gene alteration co-occurrence and mutual exclusivity analysis heatmap. (D) Frequency of gene copy number variations (gains and losses) in DFRGs. (E) Chromosome region of DFRGs.

3.5 Tumor-infiltrating immune cells and functional enrichment analysis results

To discern the immunological landscape and molecular pathways associated with different DFRG-related clusters in glioblastoma, we conducted a comparative analysis of tumor-infiltrating immune cell abundance and performed GO and KEGG pathway enrichment analyses. As shown in Figure 5A, certain immune cell types were found enriched in specific disulfidptosis clusters, suggesting a potential role in disease

progression or response to therapy. In Figures 5B, C, the results of the GO and KEGG enrichment analyses for DFRGs between the two clusters were provided.

3.6 Clinical and pathological characteristics among patients of distinct subtypes

To understand the clinical and molecular heterogeneity within GBM and its implications for patient outcomes, we aimed to

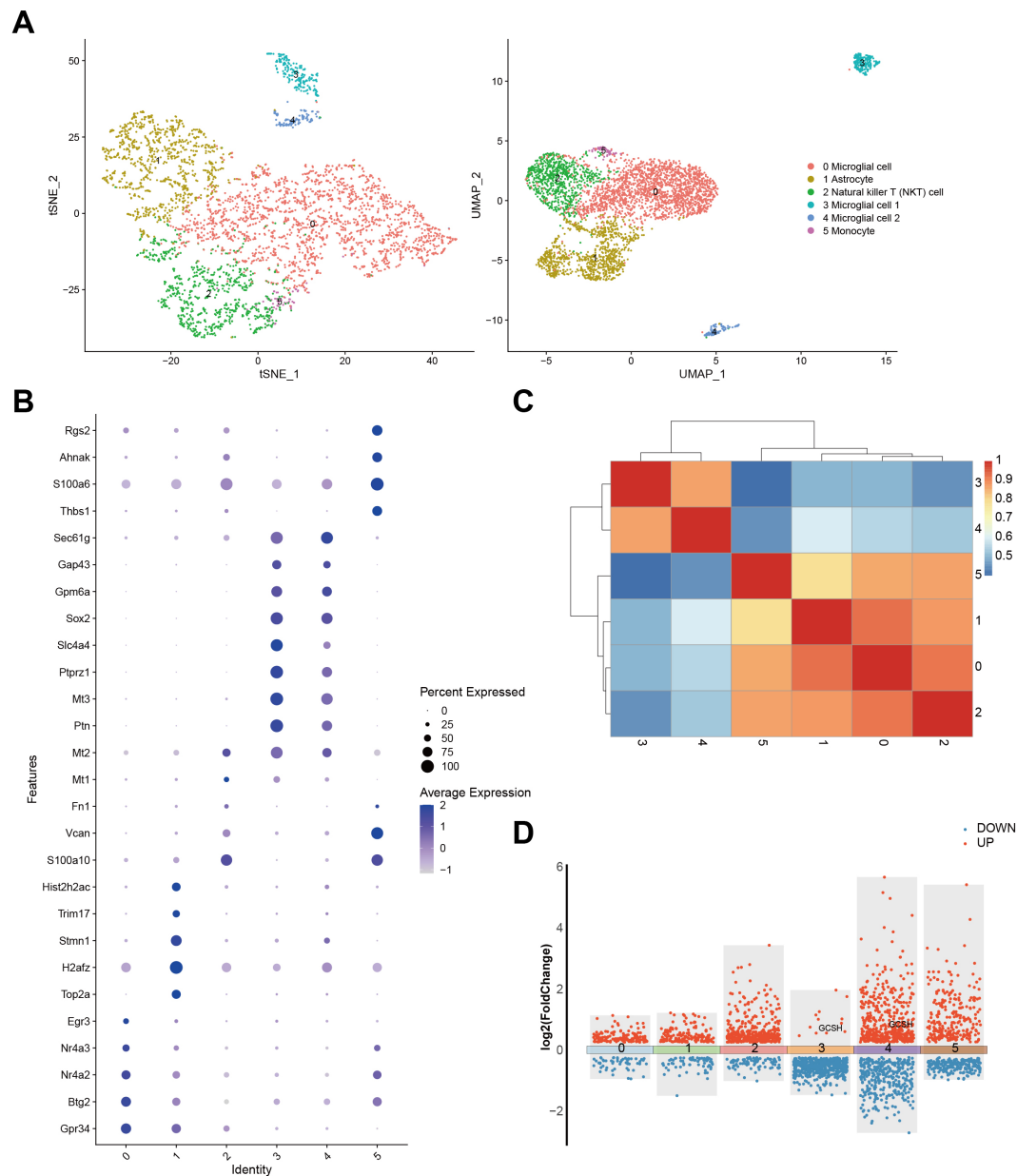


FIGURE 2

Expression of DFRG in the single-cell analysis and the data processing results of GSE241037. (A) The t-SNE analysis result of cells and the UMAP analysis plot. (B) A bubble diagram was generated to depict the expression identity of DFRG. (C) Correlation analysis was performed to examine the gene expression profiles among distinct cell types. (D) A differential expression gene map was constructed.

identify distinct gene subtypes and compare their clinical characteristics and DEG profiles. Unsupervised clustering analyses were performed to differentiate between disulfidptosis subtypes, as illustrated in Figures 6A, B. The identified gene subtypes exhibited significant differences in overall survival (OS), as demonstrated by Kaplan-Meier curves in Figure 6C. The chi-square test showed a p-value of 0.001, indicating a statistically significant difference in survival rates among the three gene subtypes. Figure 6D features a heatmap that delineates variations in clinical features and Supplementary Figure S2 shows DEGs expression levels among geneClusters A, B, and C.

3.7 Established and validated a risk score model

To discern the prognostic value of DEGs in GBM, LASSO regression analysis was conducted to identify candidate prognostic genes and assessed their association with survival outcomes (Figures 7A, B). We also examined the distribution of subtypes and their prognosis in GBM patients, as well as the relationship between gene expression and immune cell abundance (Figure 7C). We found that the risk scores, as depicted in Figure 7D, effectively distinguished between two DFRG subtypes, and Figure 7E showed

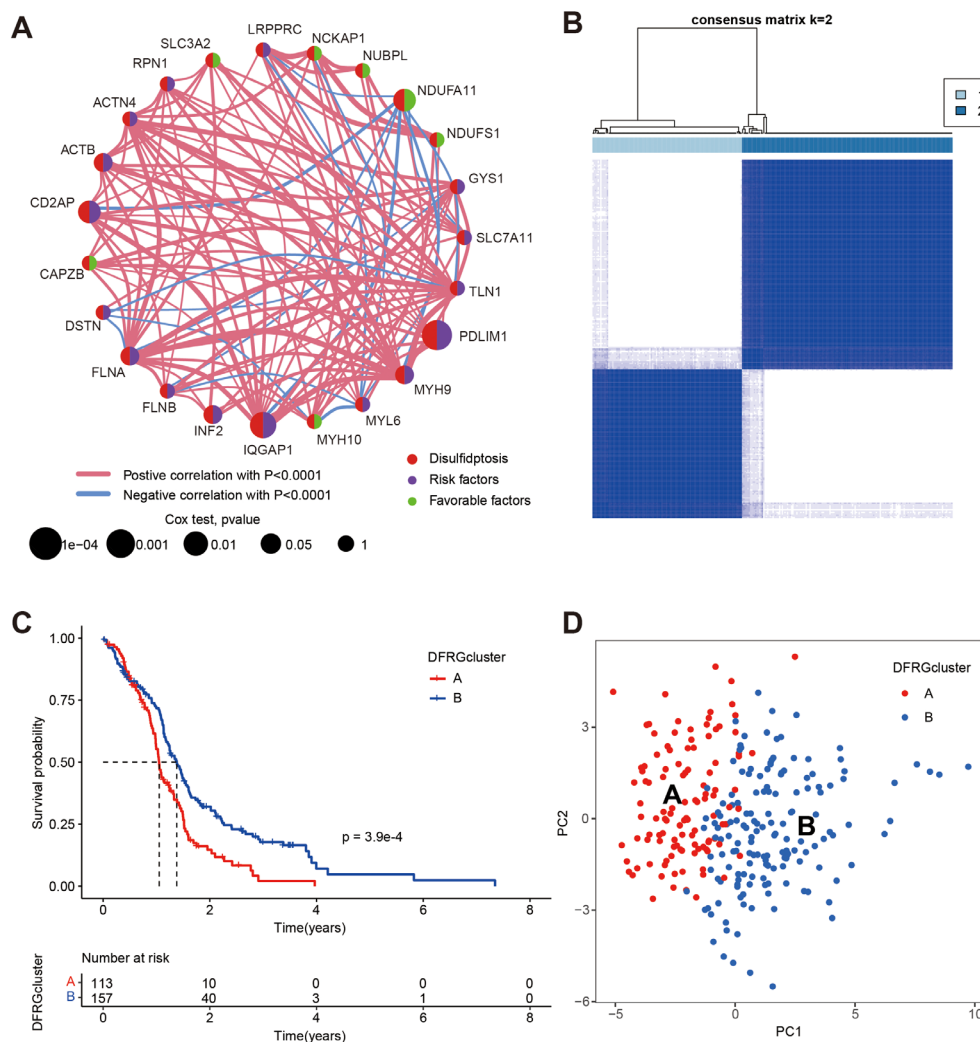


FIGURE 3

Identification of disulfidptosis subtypes and comparison of clinical outcomes between the two subtypes, data were from TCGA, GSE74187, and GSE83300. **(A)** Univariate Cox regression and correlation analysis between DFRGs. **(B)** Unsupervised clustering analysis of prognostic DFRGs. Consensus matrix heatmap defining two clusters ($k = 2$) and their correlation area. **(C)** Kaplan-Meier curves for OS of the two disulfidptosis subtypes (chi-square test, $p = 3.9e-04$). **(D)** The PCA analysis based on the prognostic DFRGs demonstrated that the patients in the different disulfidptosis subtypes were distributed in two directions.

significant distinctions among three gene subtypes. In the training set, the distribution of these risk scores was visualized in a ranked dot plot (Figure 7F). Kaplan-Meier curves in Figure 7G revealed significant differences in OS between the two risk groups (chi-square test, $p < 0.001$), underscoring the predictive power of our risk score model. Furthermore, ROC curves in Figure 7H assessed the sensitivity and specificity of predictive model in predicting 1-, 3-, and 5-year survival. The validation set was analyzed similarly, as shown in Figures 7I–K. Given the risk model's proficiency in forecasting the survival rates of GBM patients, we subsequently investigated the correlation between genes associated with the risk model (SOD3, SPAG4, FREM3, and SPP1) and the immune microenvironment. The Spearman correlation analysis, as depicted in the correlation heatmap, indicates a negative correlation between the expression of SPAG4 and the riskScore

with the expression of Macrophage M1, suggesting an association with poor prognosis in GBM patients (Figure 8A). Figure 8B displayed the three types of Tumor Microenvironment (TME) scores in both high-risk and low-risk groups using a violin plot, revealing the immune contexture associated with different risk profiles.

3.8 *In vitro* validation of the alleviating effect of SPAG4 on GBM

Firstly, an evaluation of the relative importance of five model genes is presented by random survival forests (RSF) algorithm in Figure 9A. Subsequently, Spearman correlations between SPAG4 and SOD3, SPP1, and FREM3 are depicted in Figures 9B–D, illustrating

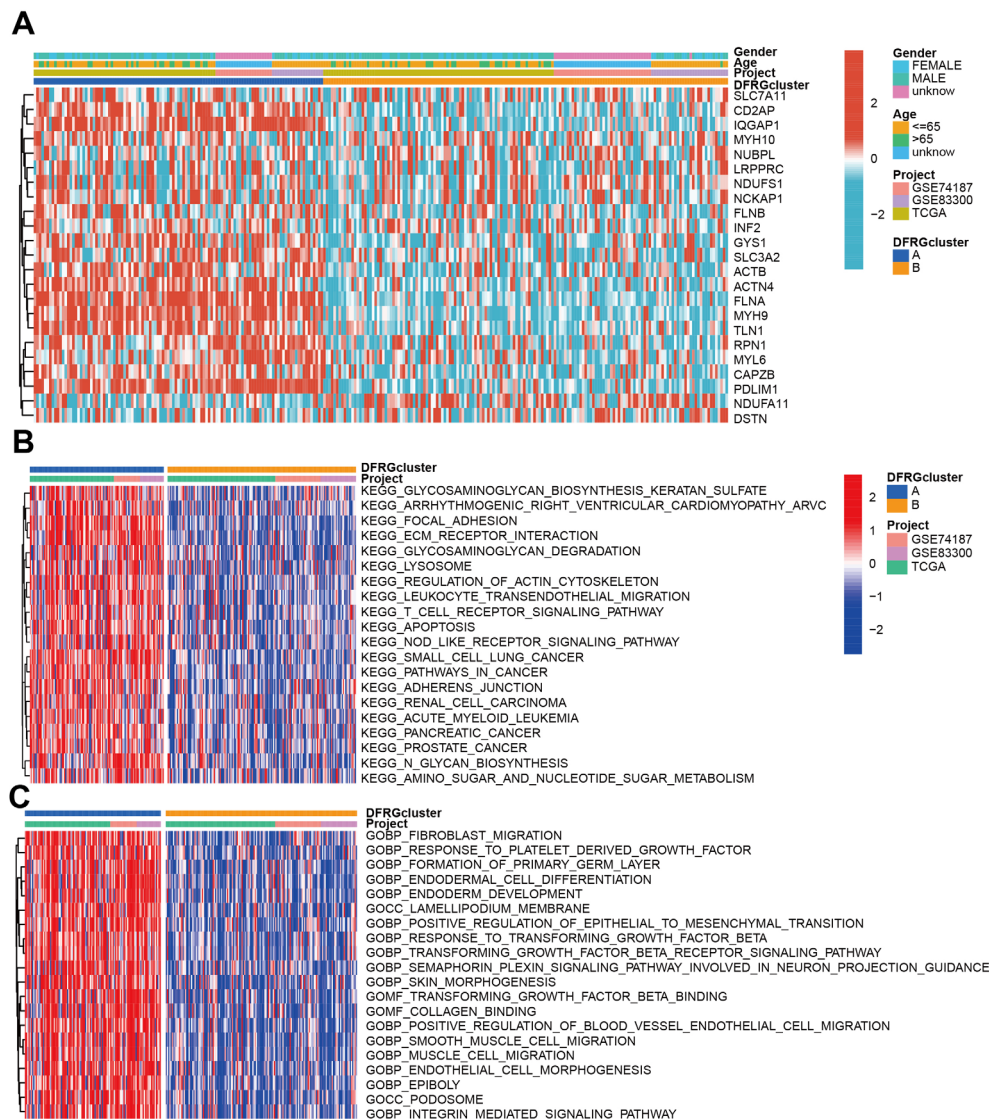
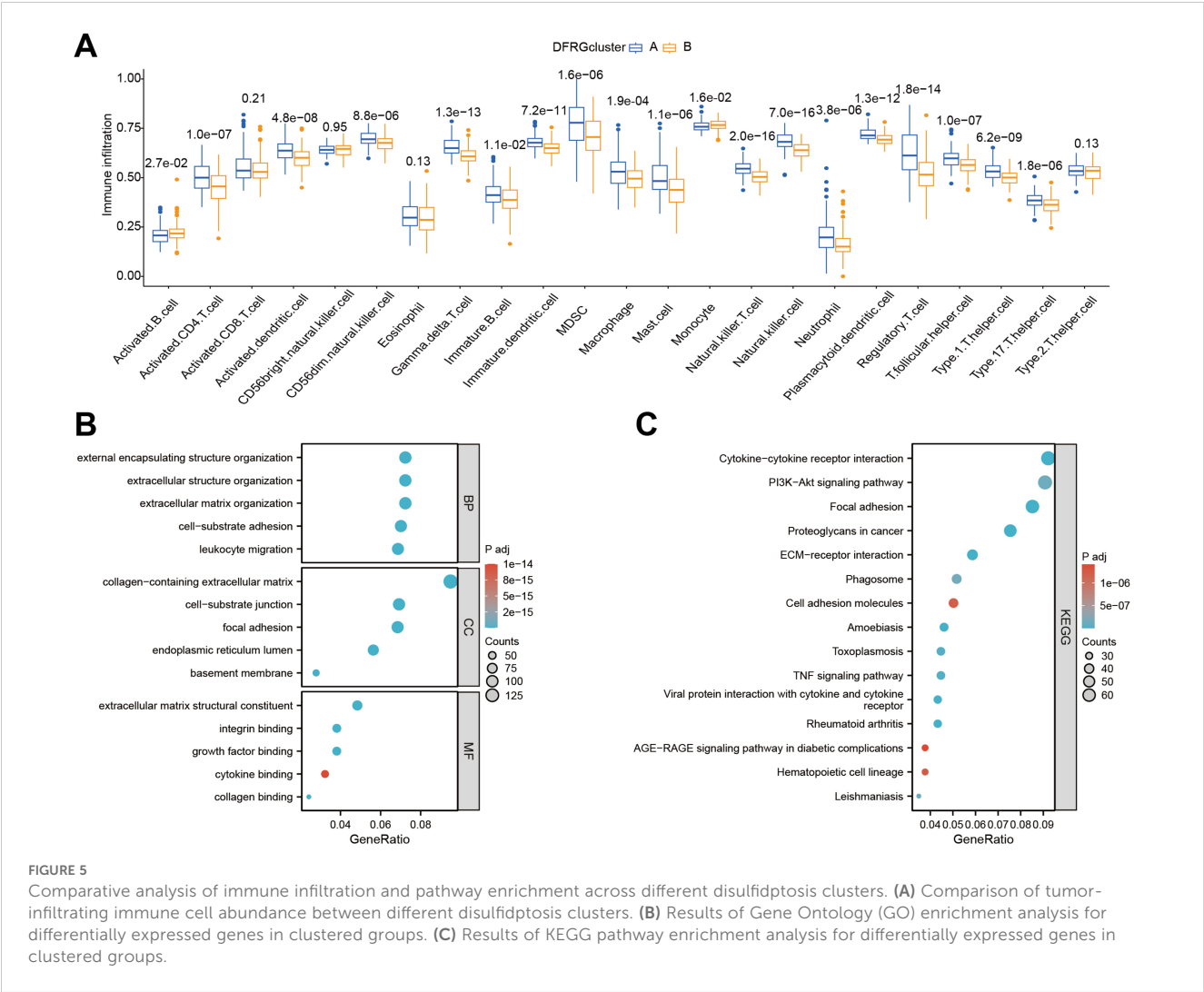


FIGURE 4
Comparison of clinical characteristics and KEGG and GO enrichment in each distinct cluster. **(A)** The heat map shows the differences in clinical features and DFRGs expression levels between DFRGcluster A and DFRGcluster B. **(B)** KEGG pathway enrichment by GSVA. **(C)** GO enrichment by GSVA.

their interrelationships using GEPIA. Comparative expression levels of SPAG4 mRNA and protein in GBM tumor and adjacent normal tissues are displayed in **Figures 9E, F**. Representative immunohistochemistry (IHC) images in **Figure 9G** visually capture the differential expression of SPAG4 in GBM tissues compared to adjacent normal tissues. The relative expression levels of SPAG4 mRNA in HEB, U87, and U251 cell lines are presented in **Figure 9H**. Immunoblotting in **Figures 9I, J** effectively confirms the knockdown efficiency of siRNAs targeting SPAG4 in the U87 cell line. Furthermore, depletion of SPAG4 is shown to elevate oxidative stress levels in U87 and U251 cell lines, as evidenced in **Figures 9K, L**. Wound-healing assays in **Figures 9M, N** demonstrate delayed healing in U87 and U251 cell lines upon SPAG4 depletion. Importantly, knockdown of SPAG4 rescues the invasive phenotype of U87 and U251 cell lines, as depicted in **Figures 9O, P**.

3.9 SPAG4 facilitates GBM biological function through fat acid metabolism

To delve deeper into the mechanisms by which SPAG4 enhances the proliferative and migratory phenotypes of GBM, we utilized single-cell sequencing data from the GSE24103 dataset, isolating cells with high SPAG4 expression. Subsequent Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analyses revealed significant enrichment in biological processes related to fatty acid metabolism (BP), cellular components associated with lipid droplets (CC), and molecular functions involving fatty acid binding (MF). Notably, the KEGG pathway enrichment pointed towards Lipid Metabolism and Atherosclerosis (**Figure 10A**). These findings suggest a correlation between SPAG4 and fatty acid metabolism. To



substantiate our hypothesis, we conducted an analysis on GEPIA, examining the correlation between SPAG4 and the significantly enriched genes CD36, SLC43A3, SLC2A1, and MYD88 within the aforementioned pathways. The results indicated a significant positive correlation (Figure 10B). Furthermore, Oil-red staining experiments demonstrated that overexpression of SPAG4 significantly increased the content of fatty acids in U87 and U251 cells, whereas SPAG4 knockout markedly decreased fatty acid content (Figure 10C, D). Based on these findings, we hypothesize that SPAG4 may modulate the biological functions of GBM by regulating fatty acid metabolism. To test this hypothesis, we treated SPAG4-overexpressing cells with the fatty acid oxidation inhibitor Orlistat. The results showed that the migratory (Figure 10E), invasive (Figure 10F), anti-apoptotic (Figure 10G), and proliferative (Figure 10H) capabilities of these cells were significantly affected. This comprehensive analysis provides compelling evidence that SPAG4 could play a pivotal role in GBM biology through its influence on fatty acid metabolism.

3.10 *In vivo* validation of SPAG4's mitigating effect on GBM

To evaluate the impact of SPAG4 modulation on U87-MG cell behavior in subcutaneous xenograft assays and to explore its correlation with immune-related markers, we performed subcutaneous xenografts. Our findings revealed significant effects of SPAG4 on the growth and characteristics of U87-MG cells *in vivo*. Notably, a substantial reduction in tumor volume was observed 28 days post-treatment with sh-SPAG4 compared to the sh-GFP group (Figure 11A). In further studies to elucidate the role of SPAG4 within the immune microenvironment, we utilized the GEPIA database. Our analysis indicated positive correlations between SPAG4 and MRC1 ($R=0.26$, $p\text{-value}=1.5\text{e-}5$, Figure 11B) as well as CD47 ($R=0.33$, $p\text{-value}=1.8\text{e-}5$, Figure 11C) in GBM. By downregulating SPAG4 in a mouse GBM xenograft model using sh-Spag4, we observed a significant increase in the M1 macrophage (iNOS) phenotype and a decrease in the M2 macrophage (CD206) phenotype compared to the sh-GFP group (Figures 11D, E). Based

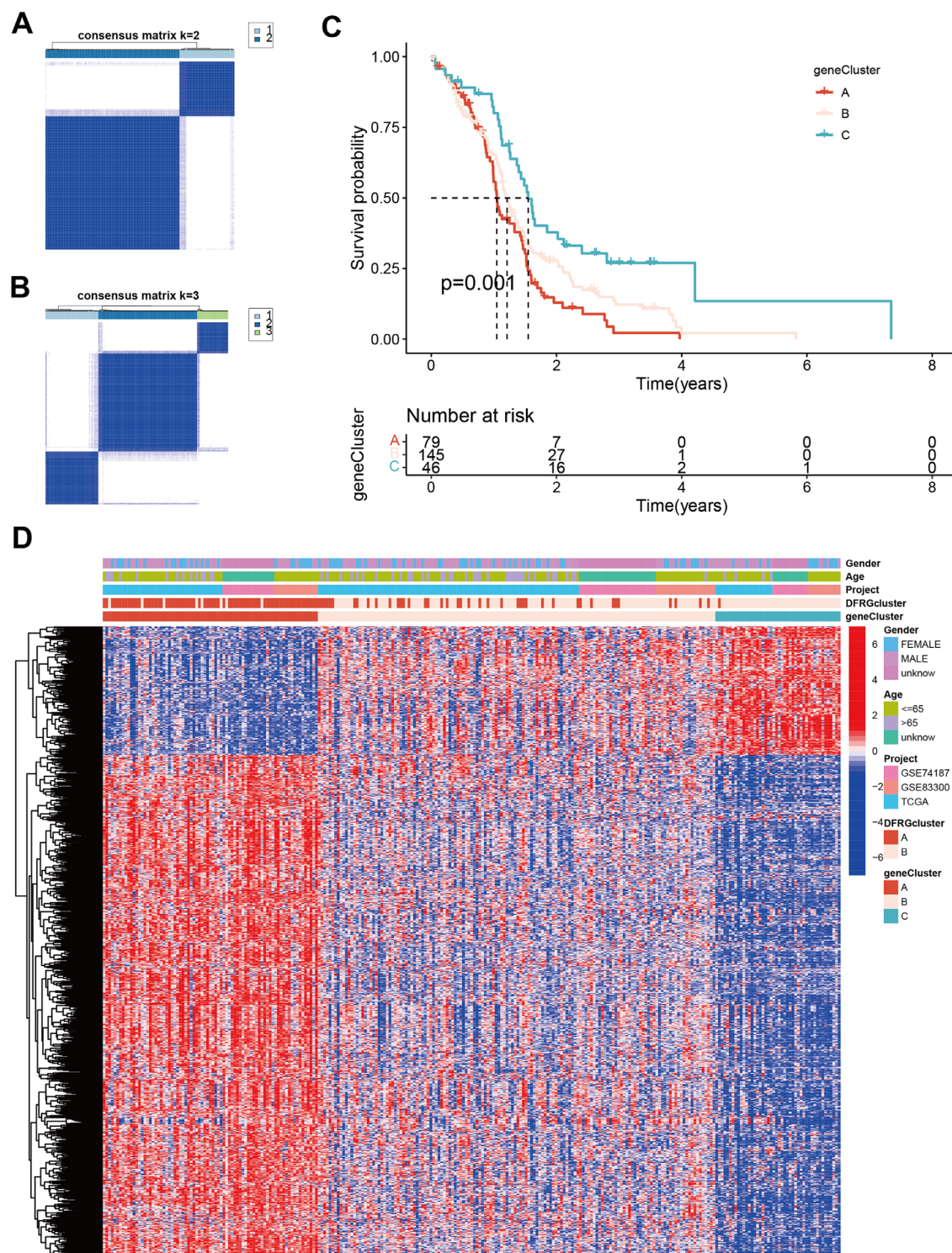


FIGURE 6

Identification of gene subtypes and comparison of clinical outcomes and characteristics and DFRGs expression levels between the two gene subtypes. **(A)** Unsupervised clustering analysis of prognostic DEGs between the two disulfidptosis subtypes. **(B, A)** Unsupervised clustering analysis of prognostic DEGs between the three disulfidptosis subtypes. **(C)** Kaplan-Meier curves for OS of the three gene subtypes (chi-square test, $p = 0.001$). **(D)** The heat map shows the differences in clinical features, and DEGs expression levels between geneCluster A, B, and C.

on these observations, we concluded that SPAG4 likely regulates the immune microenvironment in GBM. Additionally, we hypothesized that SPAG4 might enhance CD47 expression through the promotion of fatty acid oxidation. Overexpression of

SPAG4 via lentivirus in combination with the fatty acid synthase inhibitor Orlistat was used to test this hypothesis. The results demonstrated a significant increase in CD47 levels in the SPAG4-OE group, which was abrogated by Orlistat treatment (Figure 11F).

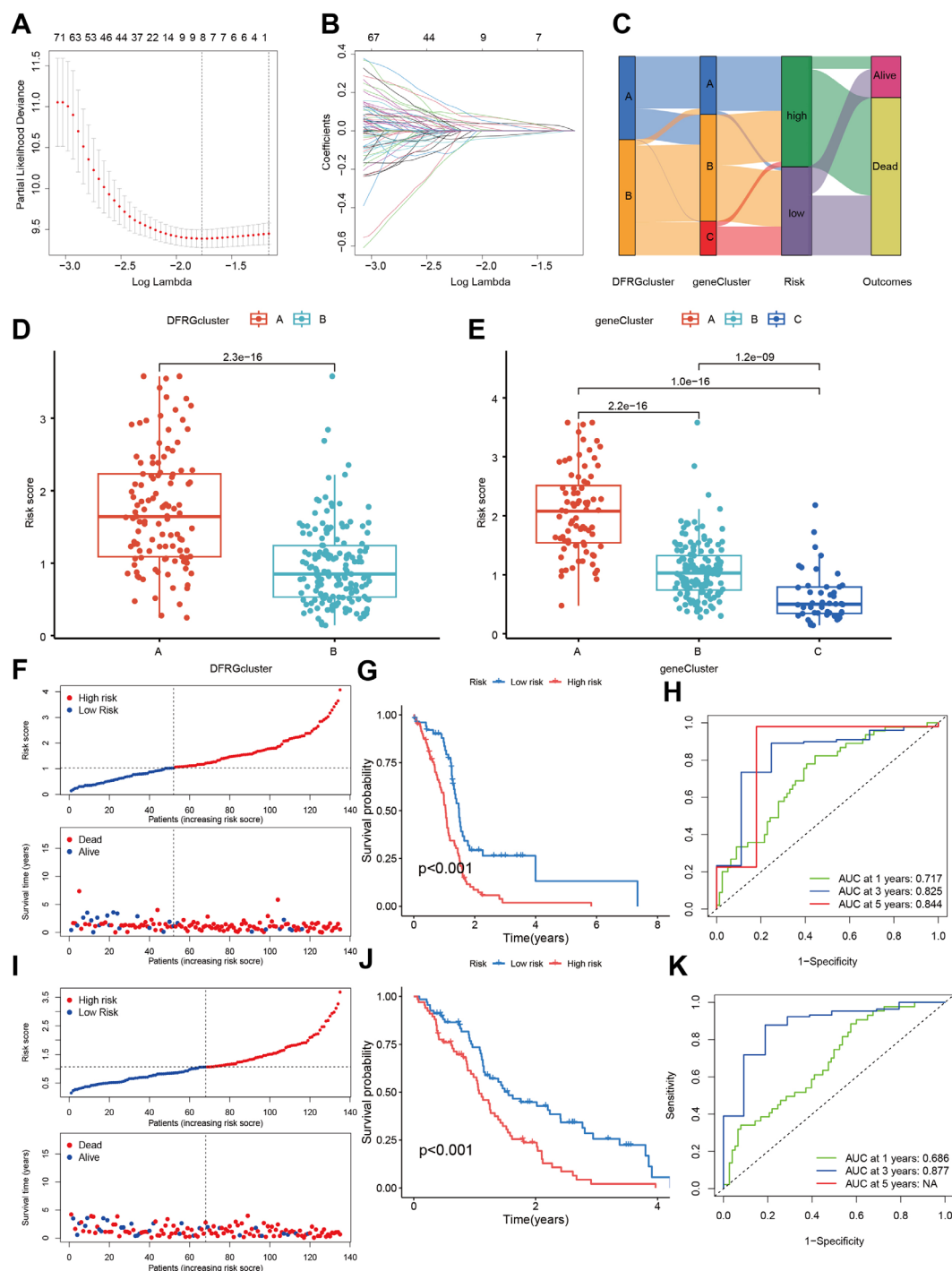
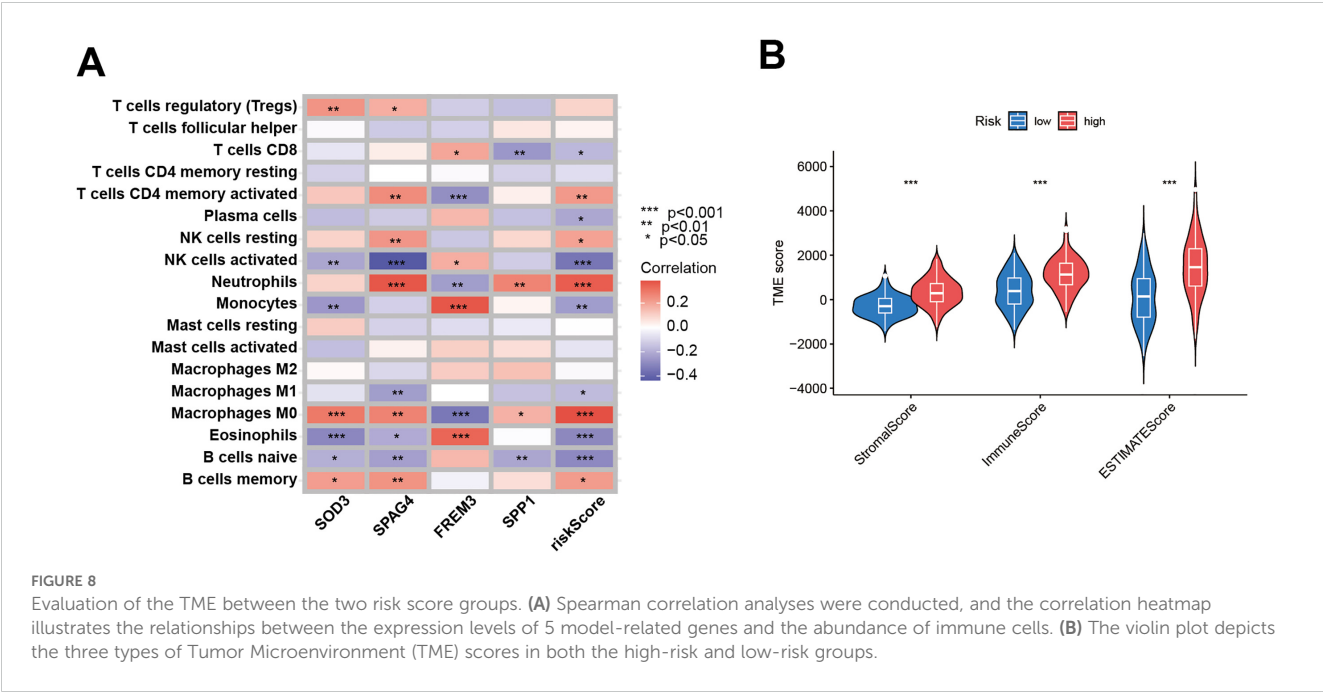


FIGURE 7

Construction and validation of a risk score model in the training and testing sets. (A, B) Utilizing LASSO regression analysis, we identified candidate prognostic genes along with the partial likelihood deviance associated with these prognostic genes. (C) An alluvial diagram illustrates the distribution of subtypes and the corresponding prognosis in GBM patients. (D) The risk scores demonstrate a distinction between the two DFRG subtypes. (E) Similar distinctions in risk scores are observed among the three gene subtypes. (F) The risk score for each patient was calculated using the formula: Risk Score = $SOD3 \times 0.1779 + SPAG4 \times 0.2525 + FREM3 \times -0.1837 + SPP1 \times 0.1810$. A ranked dot plot visualizes the distribution of risk scores, while a scatter plot depicts the survival status of patients in the training set. (G) Kaplan-Meier curves for overall survival (OS) reveal significant differences between the two risk groups (chi-square test, $p < 0.001$). (H) ROC curves assess the sensitivity and specificity of predicting 0.5-, 1.0-, and 1.5-year survival based on the risk score. (I) Another ranked dot plot illustrates risk score distribution, and a corresponding scatter plot depicts patients' survival status in the validation set. (J) Kaplan-Meier curves for OS in the validation set display significant differences between the two risk groups (chi-square test, $p < 0.001$). (K) ROC curves assess the sensitivity and specificity of predicting 0.5-, 1.0-, and 1.5-year survival based on the risk score.



Furthermore, we investigated whether SPAG4 regulates macrophage phagocytosis via fatty acid metabolism. Our data indicated that the phagocytosis rate in the SPAG4-OE group was significantly reduced compared to the Control group, and Orlistat intervention significantly enhanced the phagocytosis rate in SPAG4-OE (Figure 11G). We also explored the possibility that SPAG4 reduces macrophage phagocytosis by upregulating CD47. Our findings showed that the phagocytosis rate in the SPAG4-OE group was significantly decreased compared to the Control group, and treatment with anti-CD47 significantly increased the phagocytosis rate in SPAG4-OE (Figure 11H, I). In summary, our research concludes that in GBM, SPAG4 modulates fatty acid metabolism, increases CD47 levels, and consequently evades macrophage phagocytosis, facilitating immune evasion.

4 Discussion

Disulfidptosis could instigate a cascade of redox deficiencies and induce cell death. Moreover, the generation of immune responses by immune cells is significantly dependent on the occurrence of redox reactions (7, 8). Interestingly, the redox state constitutes a crucial mechanism in the initiation and progression of tumors (9). Therefore, inducing dual sulfur death holds the potential to be an effective therapeutic strategy for tumors, presenting expansive prospects for research. Extensive research has indicated a close association between the treatment response and prognosis of GBM patients and the infiltration status of immune cells (10–12). However, current research on the association between disulfidptosis and immune-related aspects in GBM is relatively limited. Therefore, this study sought to explore and analyze this relationship utilizing data from TCGA and GEO databases.

Based on the disulfidptosis-related genes (DFRGs) reported by Liu et al., this study selected a subset of 23 DFRGs most strongly associated with disulfidptosis. Among these, 19 DFRGs exhibited mutations, and positive correlations were observed among the DFRGs, suggesting potential cooperative interactions between different DFRGs. Clustering analysis of these DFRGs identified two subtypes, DFRGs A and B, within GBM patients. Notably, the expression of DFRGs was more abundant in the A subtype compared to the B subtype, and patients in the A subtype demonstrated a poorer prognosis than those in the B subtype, suggesting an association between DFRGs and adverse outcomes in GBM patients. Moreover, we conducted a correlation analysis of single-cell sequencing data and observed a significant association between these DFRGs and the progression of GBM. Further analysis revealed higher proportions of immune-infiltrating cells, including CD4+ T cells, dendritic cells, natural killer T cells, regulatory T cells, and helper T cells, in patients with the A subtype of GBM. Previous research has indicated that activated CD8+ T cells contribute to generating a sustained and effective anti-tumor immune response. Dendritic cells and natural killer T cells play crucial surveillance roles *in vivo*, and their dysregulation can facilitate GBM progression. On the other hand, the absence of regulatory T cells and helper T cells may lead to a decline in antigen presentation effectiveness, promoting immune escape. Both the findings from this study and the literature suggest that patients with the A subtype of GBM may have a better prognosis. This implies that these molecular subtypes exhibit distinct immune cell infiltration characteristics and pathways of functional enrichment, and DFRGs may impact the occurrence and development of GBM through mechanisms such as signaling pathway stimulation and regulation of immune cell infiltration in the tumor microenvironment.

SPAG4, a protein associated with sperm function, has recently been implicated in the regulation of Reactive Oxygen Species (ROS) in the context of cancer cell biology (13). According to the literature,

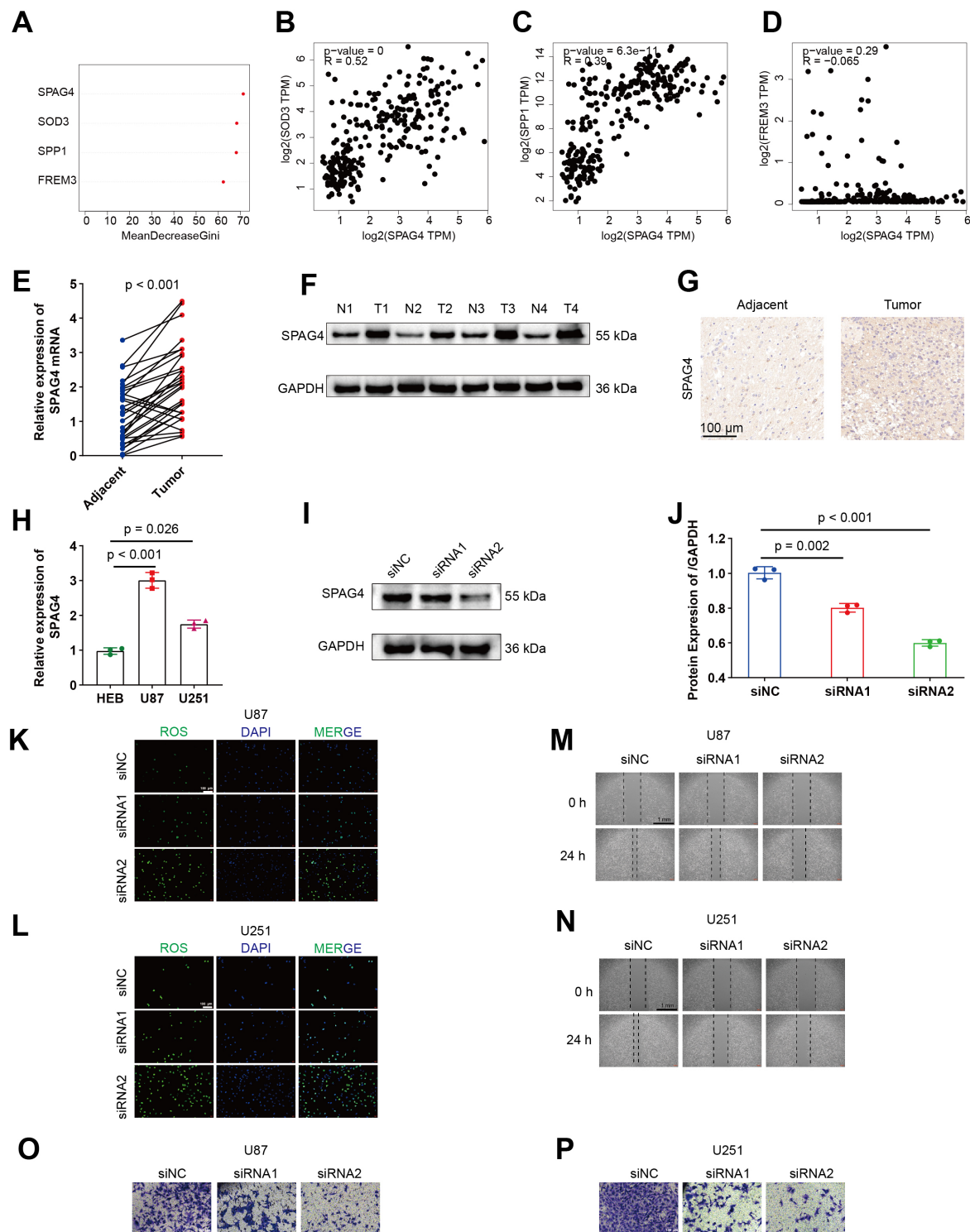


FIGURE 9

SPAG4 enhances the migratory and invasive capacities of GBM cells by modulating ROS levels. (A) Assessment of the relative significance of five model genes. (B–D) Spearman correlations between SPAG4 and SOD3, SPP1, and FREM3 using GEPIA. (E–F) Comparative expression levels of SPAG4 mRNA and protein in GBM tumor and adjacent normal tissues. (G) Representative IHC images illustrating SPAG4 expression in GBM tissues and adjacent normal tissues. (H) Relative expression levels of SPAG4 mRNA in HEB, U87, and U251 cell lines. (I–J) Immunoblotting validating the efficacy of siRNAs targeting SPAG4 in the U87 cell line. (K–L) Depletion of SPAG4 induces an increase in oxidative stress levels in U87 and U251 cell lines. (M–N) Wound-healing assays reveal delayed healing in U87 and U251 cell lines upon SPAG4 depletion. (O–P) Knockdown of SPAG4 rescues the invasive phenotype of U87 and U251 cell lines.

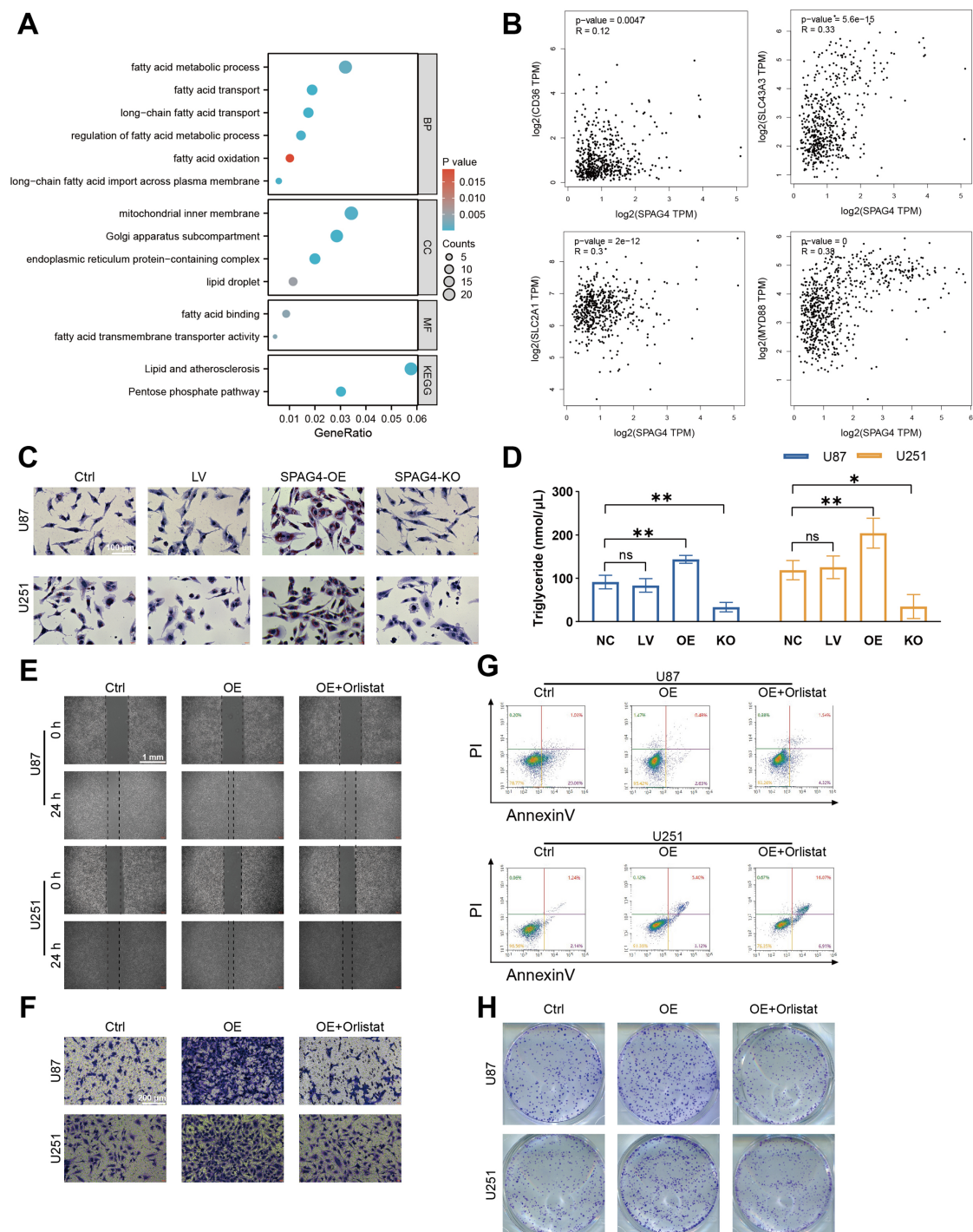


FIGURE 10

SPAG4 facilitates glioblastoma multiforme (GBM) proliferation, migration, invasion, and apoptosis inhibition through fatty acid metabolism.

(A) Enriched pathways in GBM cells positive for SPAG4, sourced from the single-cell RNA-sequencing dataset GSE24103. (B) Spearman correlation analysis of SPAG4 with CD36, SLC43A3, SLC2A1, and MYD88 using the GEPIA database. (C) Oil Red O staining of U87 and U251 cells in control, empty vector lentiviral (LV), SPAG4-overexpressing (OE), and SPAG4-knockout (KO) groups (n=3 per group). (D) Measurement of intracellular triglyceride levels in U87 and U251 cells in control, empty vector LV, SPAG4-OE, and SPAG4-knockout groups (n=3 per group). (E) Wound healing assay conducted on U87 and U251 cells in control, SPAG4-OE, and SPAG4-OE plus Orlistat-treated groups (n=3 per group). (F) Invasion assay performed with U87 and U251 cells in control, SPAG4-OE, and SPAG4-OE plus Orlistat-treated groups (n=3 per group). (G) Flow cytometry apoptosis assay on U87 and U251 cells in control, SPAG4-OE, and SPAG4-OE plus Orlistat-treated groups (n=3 per group). (H) Clonogenic assay of U87 and U251 cells in control, SPAG4-OE, and SPAG4-OE plus Orlistat-treated groups (n=3 per group). ns means no significance, *P < 0.05 and **P < 0.01.

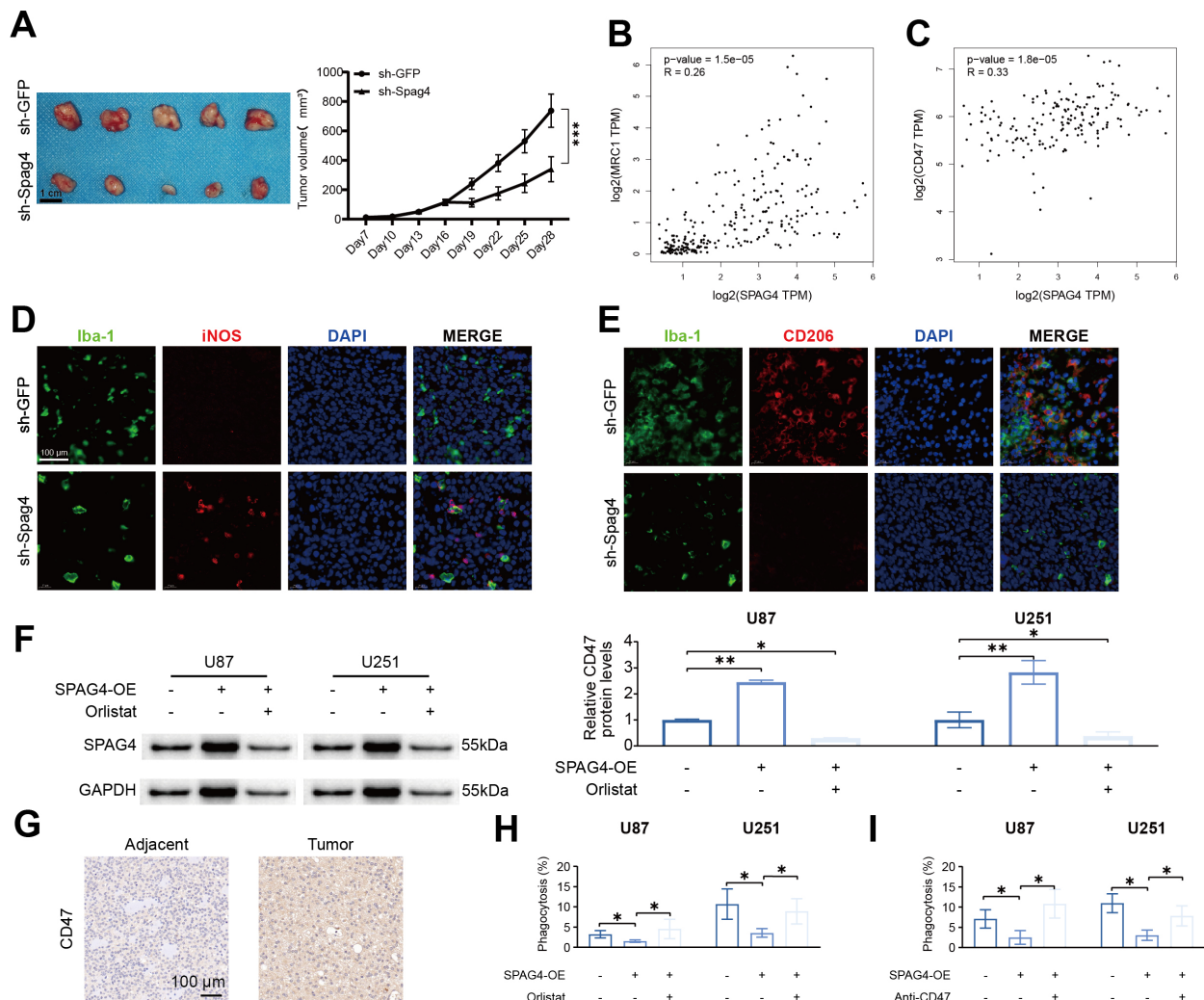


FIGURE 11

SPAG4 enhances glioblastoma immune evasion by upregulating CD47 via fatty acid metabolism. (A) Subcutaneous xenograft assays were performed utilizing U87-MG cells treated with sh-GFP or sh-Spag4 (the experiment spanned 30 days). Tumor volume following sh-GFP or sh-Spag4 treatment was monitored (n=5 per group). (B) Spearman correlation analysis between SPAG4 and MRC1. (C) Spearman correlation analysis between SPAG4 and CD47. (D) Immunofluorescence staining outcomes for Iba-1/iNOS in subcutaneous xenograft tumors from mice treated with sh-GFP and sh-Spag4. (E) Immunofluorescence staining results for Iba-1/CD206 in subcutaneous xenograft tumors from mice treated with sh-GFP and sh-Spag4. (F) Western blot (WB) analysis to determine CD47 expression in U87 and U251 cells in control, SPAG4-overexpressing (SPAG4-OE), and SPAG4-OE plus Orlistat-treated groups (n=3 per group). (G) Immunohistochemical assessment of CD47 expression levels in glioblastoma and adjacent tissues. (H) Phagocytosis assay conducted with U87 and U251 cells in control, SPAG4-OE, and SPAG4-OE plus Orlistat-treated groups (n=3 per group). (I) Phagocytosis assay performed with U87 and U251 cells in control, SPAG4-OE, and SPAG4-OE plus Anti-CD47 treated groups (n=3 per group). *P < 0.05 and **P < 0.01.

SPAG4 interacts with lamin A/C, modulating the nuclear translocation and transcriptional activity of SREBP1, a key regulator of lipogenic pathways. In our study, SPAG4 has been found to enhance the levels of fatty acids in U87 and U251 cell lines. Recent study indicates that fatty acid metabolism in GBM contributes to immune evasion, with one mechanism being the upregulation of CD47 expression (14). Our experimental findings suggest that SPAG4 enhances CD47 expression via fatty acid pathways, thereby reducing macrophage phagocytosis. These results offer novel insights into the immunotherapy of GBM.

In conclusion, Disulfidptosis, as a novel form of cell death, exhibits a close association between its gene expression, the prognosis of GBM patients, and their response to immune therapy. Therefore, targeted therapy focusing on DFRG may offer a novel avenue for diagnosis and treatment in pancreatic cancer. However, limitations such as a relatively small sample size in this study necessitate validation in different cohorts. Furthermore, additional prospective clinical research and fundamental studies are required to further refine and validate these findings.

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 humans were approved by Ethics Committee of The First Affiliated Hospital of Hainan Medical University. The studies were conducted in accordance with the local legislation and institutional requirements. The participants provided their written informed consent to participate in this study. The animal study was approved by Ethics Committee of The First Affiliated Hospital of Hainan Medical University. The study was conducted in accordance with the local legislation and institutional requirements. 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

SC: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Software, Validation, Writing – original draft, Writing – review & editing. MZ: Conceptualization, Data curation, Formal analysis, Methodology, Resources, Software, Validation, Writing – original draft. TC: Formal analysis, Investigation, Methodology, Resources, Software, Validation, Writing – original draft. HD: Writing – original draft, Formal analysis, Investigation, Methodology, Software, Visualization. JL: Writing – original draft, Investigation, Methodology, Software. FY: Writing – original draft, Formal analysis, Validation, Visualization. RW: Writing – original draft, Formal analysis, Methodology, Software, Validation, Visualization. LY: Validation, Writing – original draft, Writing – review & editing, Conceptualization, Data curation, Methodology, Project administration, Resources, Software, Supervision. KY: Conceptualization, Data curation, Formal analysis, Funding acquisition, Methodology, Project administration, Resources,

Software, Supervision, Validation, Writing – original draft, Writing – review & editing.

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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.2024.1462064/full#supplementary-material>

SUPPLEMENTARY FIGURE 1

(A) 25 disulfidptosis-related candidate genes (DFRGs). (B) Utilizing the criteria of $|\log FC| > 1$ and adjusted P.Value < 0.05 , we identified a total of 217 upregulated and 149 downregulated differentially expressed genes, encompassing 23 DFRGs.

SUPPLEMENTARY FIGURE 2

Box plot shows the differences of DFRGs expression levels among geneCluster A, B and C.

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Protective effects of YCHD on the autoimmune hepatitis mice model induced by Ad-CYP2D6 through modulating the Th1/Treg ratio and intestinal flora

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Background: Autoimmune hepatitis (AIH) is a chronic liver inflammatory disease mediated by autoimmune reactions, the pathogenesis of AIH is probably related to the imbalance of intestinal flora. Yinchenhao decoction (YCHD) has been used to relieve AIH. However, the mechanisms underpinning YCHD's hepatoprotective effects with the gut microbita have not been fully revealed.

Objective: To explore the potential mechanism of YCHD in treating AIH based on changes in the intestinal flora and Th1/Treg ratio in the spleen and hepatic hilar lymph nodes.

Methods: The AIH mice model induced by the adenovirus vectors that overexpress human cytochrome P450 family 2 subfamily D member 6 (Ad-CYP2D6) was established (untreated group). One week after the Ad-CYP2D6 injection, the AIH model mice were treated by administering YCHD by gavage for 14 days (YCHD-treated group). The therapeutic efficacy of YCHD on AIH was evaluated by detecting the histopathological changes of the liver, serum transaminases (ALT and AST), inflammatory factors (TNF- α , IL-17 and IFN- γ), and autoantibodies (including LKM-1 and LC-1). The ratio of Th1 to Treg within the spleen and hepatic hilar lymph nodes of the mice was detected by flow cytometry. The changes in the species and abundance of intestinal flora and intestinal flora metabolites were analyzed via 16S rRNA gene sequencing and gas chromatography-mass spectrometry (GC/MS) to reveal the protective mechanism of YCHD on liver injury.

Result: YCHD decreased the transaminase activity (AST and ALT), the content of autoantibodies (LC-1 and LKM-1), and the serum TNF- α , IL-12, and IL-17 levels in AIH mice. The degree of inflammatory infiltration in the YCHD-treated group was significantly less than that in the untreated group. YCHD can effectively reverse the abundance and diversity of intestinal flora in AIH mice and affect the release of short-chain fatty acids (SCFAs), especially butyric acid. Moreover, the flow cytometry results showed that YCHD could also decline the ratio of Th1/Treg, which probably be induced by SCFAs via the G protein-coupled receptor (GPR).

Conclusion: YCHD may affect the release of SCFAs by regulating the intestinal microbiota, thereby affecting the differentiation of Th1 and Treg, and achieving the effect of alleviating liver damage.

KEYWORDS

autoimmune hepatitis, Yinchenhao decoction, CYP2D6, Th1, Treg, intestinal flora

1 Introduction

Autoimmune hepatitis (AIH) is a progressive inflammatory liver disease, characterized by interface hepatitis, lymphocyte infiltration, as well as positive serum transaminase, immunoglobulin G, and serum autoantibodies (1). The incidence of AIH is 0.40/100000 people ~ 2.39/100000 people, and the prevalence rate is 4.8/100000 people ~ 42.9/100000 people (2). At the present stage, the pathogenesis of AIH is still in the exploratory stage, and the risk factors for AIH mainly include the genetic and environmental factors, such as human leukocyte antigen gene, virus, parasite, alcohol, drugs, intestinal flora, etc (3). In addition, gender and age are also vital factors in the onset of AIH with the condition affecting women four times more frequently than men (4–6). The existing treatment regimens mainly rely on non-specific immunosuppression to delay disease progression and prolong survival, but there are still some patients who fail to benefit from the current standard treatment (7).

The intestinal microbiota is a complex and huge micro-ecosystem in the human body and plays a significant role in keeping health by modulating intestinal epithelial development, inducing innate immunity, and regulating human metabolism processes (8, 9). The intestinal flora is a key factor in keeping the balance between intestinal and systemic immune responses. In addition, the intestinal flora metabolites also have a close relationship with immunologic homeostasis. In recent years, an increasing body of research has demonstrated a significant correlation between the occurrence of AIH and disorder within the intestinal microbiota (10, 11). Through 16S rRNA gene sequencing, it was found that the species and abundance of intestinal flora in AIH patients changed significantly, including the increase of *Veillonella*, *Klebsiella*, *Streptococcus*, and *Lactobacillus*, and the decrease of *Clostridium*, *Rumen cocci*, *Vibrio*, *Bacteroides*, and *Fecal cocci* (12). It is indicated that regulating intestinal flora may be a new way to treat AIH.

Yinchenhao decoction (YCHD) was recorded by Shanghanlun in the Han Dynasty of China (150–215 A.D.), which was composed of the *Artemisia caruifolia* Buch, *Gardenia jasminoides* Ellis, and *Rheum officinale* Baill. Modern pharmacological studies also have shown that YCHD has hepatoprotective and cholagogic effects (13). Although YCHD is frequently used to treat liver disorders in clinical practice (14), its hepatoprotective mechanisms are still unclear, especially for the relationship between YCHD and gut microbiota. Therefore, in

this study, the Gas Chromatography-Mass Spectrometer (GC/MS) coupled with 16S rRNA gene sequencing techniques were performed to study the effects of YCHD in treating AIH by detecting the changes in the species and abundance of intestinal flora and intestinal flora metabolites, with an AIH mice model induced by the adenovirus vectors that overexpress human cytochrome P450 family 2 subfamily D member 6 (Ad-CYP2D6), to reveal the uncovered intrinsic interaction between YCHD, AIH, and intestinal microflora (15).

2 Materials and methods

2.1 Experimental animal

24 specific pathogen-free (SPF) male C57BL/6 mice (6–8 weeks old, 18–20g) were supplied by SiPeiFu Co., Ltd. and raised in the Animal Laboratory of Animal Center of Shanxi University of Traditional Chinese Medicine at the conditions of $24.0 \pm 2.0^\circ$, $55\% \pm 5\%$ humidity, 12 hours light-dark cycle, free drinking water and diet. All the operations of experimental animals in this study have been approved by the Ethics Committee of Shanxi University of Traditional Chinese Medicine (2019LL41).

2.2 YCHD preparation

According to the the research of Shen H et al. (16) and Zhao X et al. (17), YCHD was prepared in the following way: 180g *Artemisia caruifolia* Buch, 120g *Gardenia jasminoides* Ellis, and 60g *Rheum officinale* Baill (all the Chinese medicinal materials were bought from Beijing Tongrentang) were soaked in 1.38L distilled water for 50 minutes and boiled for 30 minutes, the liquid was filtered out. 1.38L distilled water was added to the remaining residue of Chinese medicinal materials and boiled for 30 minutes again. Finally, two parts of the YCHD solution were mixed and concentrated to 50ml.

2.3 AIH mice model

Regarding the research of Müller P et al. (18) and Holdener M et al. (19), we selected different concentrations of Ad-CYP2D6

(Shanghai Genechem Co., Ltd.) and different induction times to establish AIH mouse model. Based on the serum transaminase level and the morphological changes in the liver, we finally determined the injection dose of the Ad-CYP2D6 ($100\mu\text{l } 1 \times 10^9$ pfu/ml injected via the tail vein) and the time of induction after injection (7 days). The method was shown in [Supplementary Material S1](#), and the experimental results were exhibited in [Supplementary Figures S1 and S2](#).

2.4 Grouping and drug administration

After a week of adaptive feeding, 24 male C57BL/6 mice were randomly divided into a control group, an untreated group, and a YCHD-treated group ($n=8$). For the mice in the untreated and YCHD-treated group, $100\mu\text{l } 1 \times 10^9$ pfu/ml Ad-CYP2D6 was injected via the tail vein under sterile conditions. According to the research of Cai FF et al., the mice of the YCHD-treated group were administrated with YCHD on the 7 days after being injected with the Ad-CYP2D6, at the dosage of 10ml/kg per day for 14 days (13). The intragastric dose of YCHD was calculated with human and animal body surface coefficient conversion algorithm. At the same time, the mice in both the untreated and control groups were given the same amount of normal saline.

2.5 Sample collection

All mice were anesthetized with pentobarbital after fasting for 12 hours. The peripheral blood of mice was collected and centrifuged at 1006 g for 20 minutes at 4°C to obtain the serum. The supernatant was stored at -80°C for biochemical analysis. The liver and spleen were separated and rinsed with normal saline. After being weighed, part of the liver and spleen tissue was immediately placed into the 4% paraformaldehyde solution for histopathology analysis, and the rest was prepared for T-cell subtype analysis with the flow cytometer. The hepatic hilar lymph nodes of mice in each group were collected for Flow cytometry. The small intestine contents were collected in a sterile tube and stored at -80°C for the subsequent detection of intestinal flora and its metabolites.

2.6 Liver index and HE stain

The liver index was calculated using the formula: Liver index = liver weight (mg)/body weight (g) to present the liver's gross morphology change. The histopathological changes of the liver were examined by hematoxylin-eosin (HE) stain with the conventional method, and the necrotic area of liver tissue and the degree of lymphocyte infiltration were observed under a light microscope.

2.7 Biochemistry detection

The serum of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) (105-020579-00 and 105-020580-00,

Mindray) were detected by BS-240VET Automatic biochemical analyzer for animals (Mindray animal care, China).

2.8 ELISA

The contents of serum inflammatory factors, including tumor necrosis factor- α (TNF- α), interferon- γ (INF- γ), and interleukin-17 (IL-17) (MM-0132M1, MM-0182M1, and MM-0170M1, MEIMIAN), and autoantibodies, involving anti-liver-kidney microsomal antibody-1 (LKM-1) and anti-liver cytosol type 1 (LC-1) (MM-47349M1 and MM-46546M1, MEIMIAN), were detected with relevant ELISA kits. All operations were performed according to the kit instructions. Finally, the A51119600 full-wavelength enzyme labeling instrument (Thermo Scientific, USA) was used to determine the absorbance value at the wavelength 450nm.

2.9 Flow cytometry

After being removed under an aseptic condition, the spleen and hepatic hilar lymph nodes were washed with normal saline and the excess connective tissue was removed. Then, they were transferred to a metal filter for grinding. The spleen cell suspension was collected and the supernatant was discarded after being centrifuged at 500g for 5 minutes at 4°C . The 3ml red blood cell lysis diluent ($10\times$) was added to the cell pellet for lysing on ice for 3 minutes and then 3ml PBS was added to terminate the lysis. The cell suspension was centrifuged at 500g for 5 minutes at 4°C and the cell concentration was adjusted to 3×10^6 cells/ml. The hepatic lymph node cell suspension was collected in the centrifuge tube, and the supernatant was discarded after 500g centrifugation for 5 minutes at 4°C and the cell concentration was also adjusted to 3×10^6 cells/ml. For Th1 detection, $200\mu\text{l}$ cell suspension was placed in the 96-well plate and incubated with 50ng/ml Phorbol 12-myristate 13-acetate (PMA) (HY-18739, MCE), $1\mu\text{g/ml}$ Ionomycin (HY-13434, MCE), and $10\mu\text{g/ml}$ Brefeldin A (BFA) (HY-16592, MCE) at 37° for 5 hours. Cells were blocked with $50\mu\text{l}$ blocking buffer (2% goat serum, 2% fetal bovine serum, and 96% PBS) for 30 minutes at 4° , $5\mu\text{l}$ PerCP Anti-Mouse CD3 Antibody (E-AB-F1013F, Elabscience) and $0.5\mu\text{l}$ FITC anti-mouse CD4 (B374032, BioLegend) were added for surface staining (incubated at 4°C for 40 minutes). After that, cells were fixed and permeabilized with the eBioscienceT Foxp3/Transcription FactorTM Fixation Buffer (00-5523-00, Thermo), respectively. $50\mu\text{l}$ blocking solution, $5\mu\text{l}$ PE anti-mouse CD25 (101904, BioLegend), and $2.5\mu\text{l}$ APC Rat Anti-Mouse IFN- γ (2010378, BD) were added to perform intracellular staining (incubated at 4°C for 40 minutes). The CD3⁺CD4⁺IFN- γ ⁺ Th1 population in the spleen and hepatic lymph nodes was detected by BD Accuric6 flow cytometer.

For Treg detection, $0.5\mu\text{l}$ FITC anti-mouse CD4 antibody and $5\mu\text{l}$ PE anti-mouse CD25 antibody were added to $50\mu\text{l}$ cell suspension for surface staining (incubating at 4°C for 40 minutes) after blocking. The eBioscienceT Foxp3/Transcription FactorTM Fixation Buffer (00-5523-00, Thermo) was added fixing and permeabilization. Another $50\mu\text{l}$ blocking solution with $5\mu\text{l}$ Alexa Fluor[®] 647 anti-mouse FOXP3 (320014, BioLegend) was used for intracellular staining (incubating at 4°C for 40 minutes). Then the

CD3⁺CD4⁺CD25⁺Foxp3⁺ Treg population in the spleen and hepatic lymph nodes was also detected by BD AccuriC6 flow cytometer. All the data were analyzed using Flowjo10.8.1.

2.10 16S rRNA gene sequencing analysis

PCR amplification of the bacterial 16S rRNA genes V3–V4 region was performed by using the forward primer 338F (5'-ACTCCTACGGGAGGCAGCA-3') and the reverse primer 806R (5'-GGACTACHVGGGTWTCTAAT-3'). Sample-specific 7-bp barcodes were incorporated into the primers for multiplex sequencing. Thermal cycling consisted of initial denaturation at 98°C for 5 minutes, followed by 25 cycles consisting of denaturation at 98°C for 30 seconds, annealing at 53°C for 30 seconds, and extension at 72°C for 45 seconds, with a final extension of 5 minutes at 72°C. PCR amplicons were purified with Vazyme VAHTSTM DNA Clean Beads (Vazyme, Nanjing, China) and quantified by using the Quant-iT PicoGreen dsDNA Assay Kit (Invitrogen, Carlsbad, CA, USA). After the individual quantification step, amplicons were pooled in equal amounts, and pair-end 2×250 bp sequencing was performed using the Illumina NovaSeq platform with NovaSeq 6000 SP Reagent Kit (Shanghai Personal Biotechnology Co., Ltd) (20).

2.11 Target metabolites-short chain fatty acid analysis

Extraction of metabolites: The fecal samples of three groups of mice (n=8) were thawed on ice and then put into a 2ml centrifuge tube. 900μl 0.5% phosphoric acid was added to the sample for re-suspension. The mixed liquid was centrifuged at 17949g for 10 minutes. 800μl supernatant was extracted and mixed with the same amount of ethyl acetate. The mixture was stirred for 2 minutes and then centrifuged at 14000g for 10 minutes. 600μl upper organic phase was extracted and mixed with 4-methyl pentanoic acid (the final concentration was 500μM). All the samples were separated by Agilent DB-WAX capillary column (30 m × 0.25 mm ID×0.25 μm). Programmed Heating: Initial temperature of 90°C, then 10°C/min to 120°C, continue to 5°C/min to 150°C, and finally 25°C/min to 250°C for 2 minutes. The Agilent 7890A/5975C gas-mass spectrometer was used for mass spectrometry (See [Supplementary Table S1](#) for details). The chromatographic peak area and retention time were extracted with MSDChemStation software. The content of short-chain fatty acids (SCFAs) in the sample was calculated according to the standard curve. Finally, the relationship between intestinal flora and SCFAs was comprehensively analyzed on the cloud platform (<https://www.genesccloud.cn/Home>).

2.12 Statistical analysis

All values are expressed as the means ± SD using the One-way analysis of variance (ANOVA). The statistical analyses were

conducted with SPSS Statistics 27 software, and *P* value < 0.05 was considered as the difference is significant.

3 Results

3.1 Alleviated liver injury in the YCHD-treated group of mice

As shown in [Figure 1](#), compared with the control group, the liver of mice in the untreated group was enlarged and exhibited a dark red color, and the liver index was higher, however, the liver of mice in the YCHD-treated group was similar to that of the control group, and the liver index was lower than that in the untreated group. The results of HE staining showed inflammatory cell infiltration and massive necrosis of hepatocytes could be found in the untreated group, whereas, the necrotic area and infiltration of inflammatory cells in the YCHD-treated group were alleviated compared to the untreated group. The levels of serum ALT and AST ([Figure 2A](#)), LC-1 and LKM-1 ([Figure 2B](#)), IFN-γ, TNF-α, and IL-17 ([Figure 2C](#)) in the untreated group were significantly higher than those in the control group, whereas, these factors declined in the YCHD-treated group compared to the untreated group.

3.2 Declined Th1/Treg ratio in the YCHD-treated group of mice

Compared to the control group, the Th1 and Treg population was significantly increased in the spleen of mice, and the ratio of Th1/Treg in the untreated group was higher than that in the control group, while the Th1 population and Th1/Treg ratio in the YCHD-treated group significantly decreased compared to the untreated group ([Figures 3A–C](#)). In the hepatic hilar lymph nodes, similar changes in the frequency of Th1 and Treg population, and Th1/Treg ratio were found ([Figures 3D–F](#)).

3.3 Reserved gut microbiota dysbiosis in the YCHD-treated group of mice

The 16S rDNA V3–V4 variable regions of fecal bacteria in 24 samples were sequenced by the Illumina MiSeq platform. The dilution curves of all samples tend to smooth after reaching a certain value ([Figure 4A](#)), indicating that the amount of sequencing data in this study is sufficient. Based on the Chao1 estimator and Shannon diversity index, YCHD effectively improved the species diversity of AIH mice ([Figure 4B](#)). According to the Operational Taxonomic Unit (OTU) abundance information, 3999, 2881, and 3223 OTU were identified respectively in the control group, untreated group, and YCHD-treated group through the Venn diagram analysis ([Figure 4C](#)). Principal coordinate analysis (PCoA) was used to analyze the differences between groups. The points, which represented the data of a mouse's intestinal flora in different groups, tended to cluster in their respective communities of each group ([Figure 4D](#)), indicating the characteristic difference of the bacterial community constituted in each group was significant.

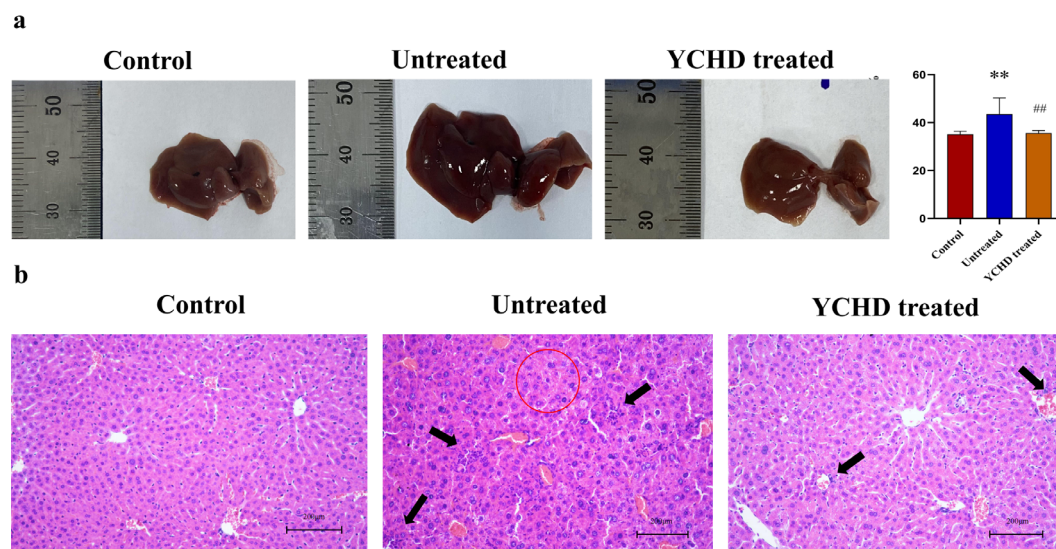


FIGURE 1

Pathological changes of the liver in each group. (A) Gross observation of the liver. The changes in the liver were represented by the liver index. (B) HE stain. The red circles are the sites of hepatocyte necrosis, and the black arrows point to the sites of inflammatory infiltrates. Data was expressed as mean $\pm$ S.D, $n=8$. ** $P<0.01$, compared to the control group, ## $P<0.01$, compared to the untreated group.

3.4 Altered gut microbiota composition in the YCHD-treated group of mice

At the genus level, the top 10 microflora in abundance of all groups were exhibited in Figure 5A. Compared to the control group, the relative abundances of *Klebsiella*, *Rummeliibacillus*, *Odoribacter*,

Coprococcus, and *Lactobacillus* increased in the untreated group, while the relative abundances of *Corynebacterium*, *Acinetobacter*, *Allobaculum*, and *Adlercreutzia* decreased. After being treated with YCHD, the relative abundance of *Klebsiella*, *Rummeliibacillus*, *Adlercreutzia*, *Corynebacterium*, *Acinetobacter*, and *Lactobacillus* decreased in the YCHD-treated group, while the relative abundance

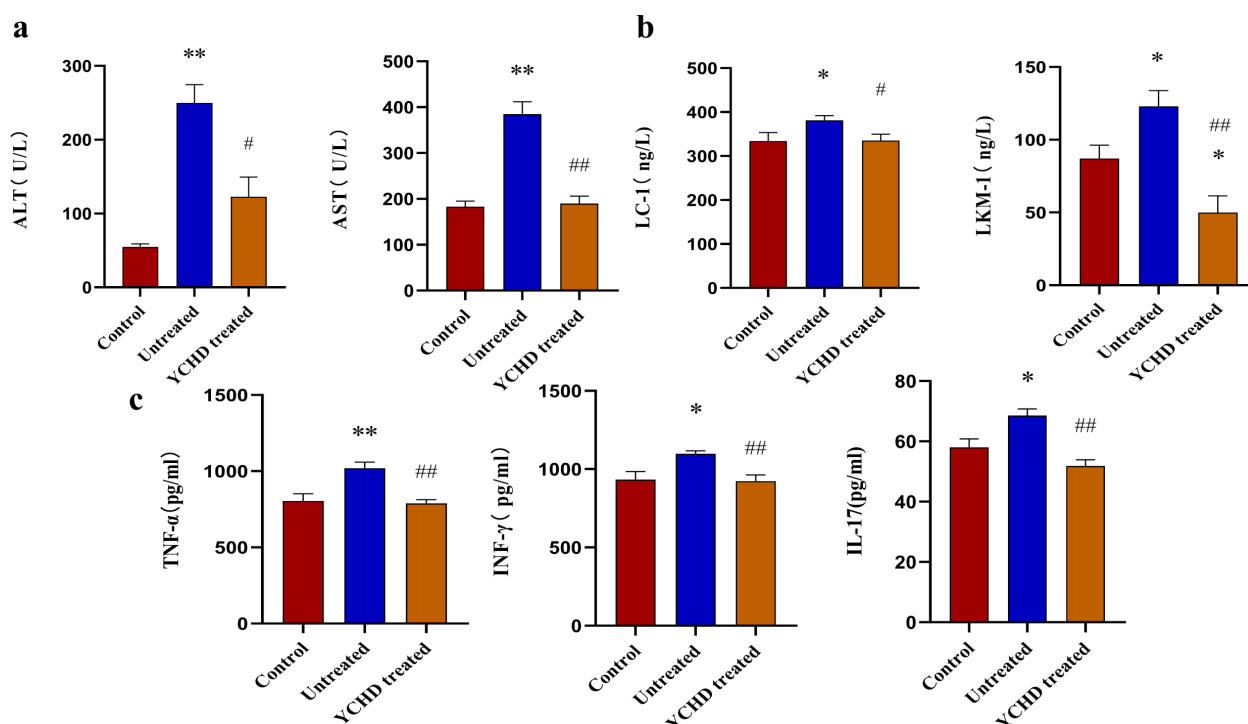


FIGURE 2

Serum levels of transaminase, autoantibodies, and inflammatory factors detected with the ELISA method. (A) Comparison of serum levels of AST and ALT in mice of each group. (B) Comparison of serum levels of LC-1 and LKM-1 in mice of each group. (C) Comparison of TNF- α , IFN- γ , and IL-17 levels in the serum of mice in each group. Data was expressed as mean $\pm$ S.D, $n=8$. * $P<0.01$, ** $P<0.01$, compared to the control group; # $P<0.05$, ## $P<0.01$, compared to the untreated group.

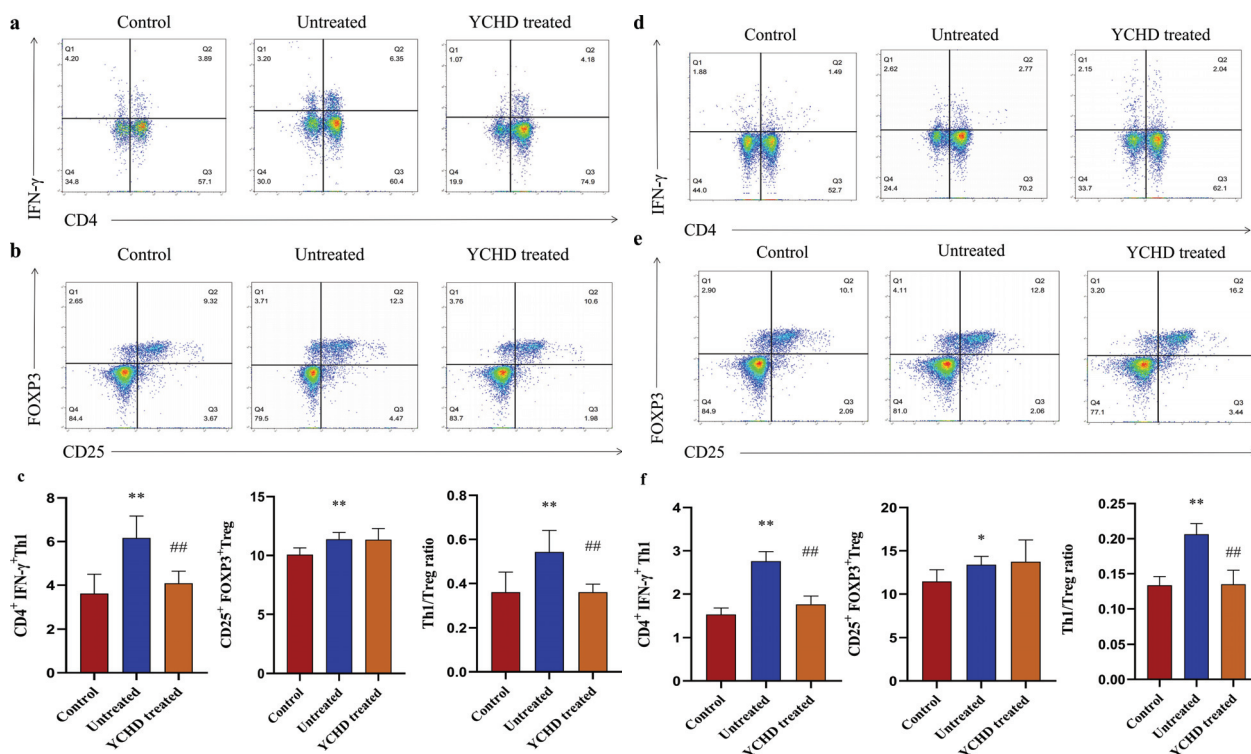


FIGURE 3

Effect of YCHD on Th1 and Treg cell differentiation. Lymphocytes isolated from the spleen and hepatic lymph nodes of the C57BL/6 mice were stimulated with or without PMA (50ng/ml), ionomycin (1 μ g/ml), and BFA (10 μ g/ml) for 5h at 37°. (A, B) The gating strategy of Th1 and Treg cells isolated from the spleen defined as CD3⁺CD4⁺IFN- γ ⁺ cells and CD3⁺CD4⁺CD25⁺FOXP3⁺ cells and the representative dot plots from each group are shown. (C) Frequency Th1 and Treg cells in the spleen of mice (n=8) in each group were analyzed by flow cytometry. (D, E) The Th1 and Treg population in the hepatic hilar lymph nodes was analyzed by staining the cell surface CD4 antigen and IFN- γ antigen, as well as by staining of cell surface CD4 antigen, CD25 antigen, and intracellular Foxp3, in the CD3⁺ T cells population. (F) Frequency Th1 and Treg cells in the hepatic hilar lymph nodes of mice (n=8) in each group were also analyzed by flow cytometry. *P<0.05, **P<0.01, compared with the control group. ##P<0.01, compared with the untreated group.

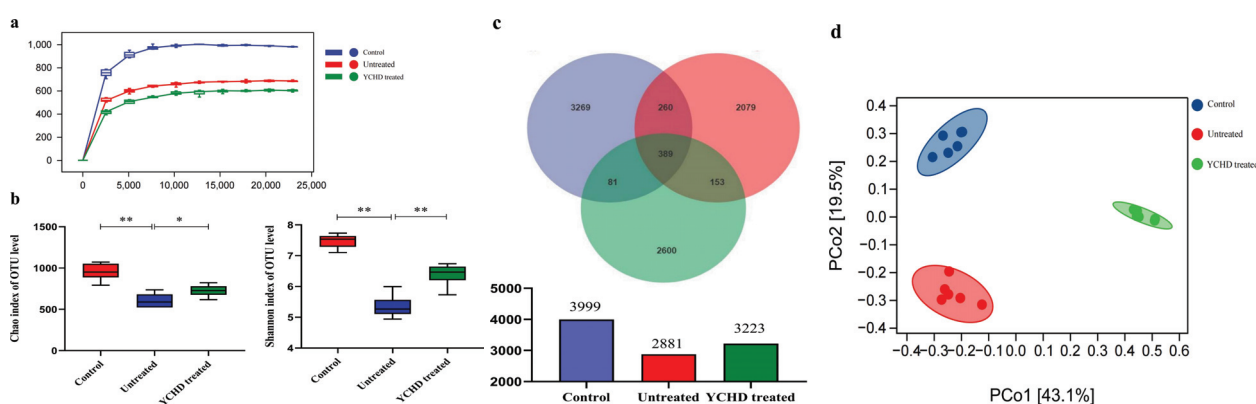


FIGURE 4

Effect of YCHD on intestinal microflora diversity in AIH mice. (A) Sparse curve. The abscissa is the depth of the test (read count), and the ordinate is the numerical value of the diversity index. The dilution curves tend to smooth after reaching a certain value indicating the amount of sequencing data in this study is sufficient. (B) The diversity of the gut microbiomes was analyzed with Chao and Shannon indices. The more of the OUT, the more the diversity of the gut microbiomes. (C) OTU characteristics among the three groups. Venn plots illustrate the overlap and differentiation of OTUs in the gut microbiota among samples. (D) Results of Principal coordinate analysis (PCoA). Significant discriminant taxon nodes of the control, untreated, and YCHD-treated groups are represented by blue, red, and green, respectively. The shorter the distance between samples in the same group, and the more significant the separation between samples in different groups, that indicated the more experimental reliability and rationality of sample selection. Data was expressed as mean $\pm$ S.D, n=6. **P<0.01, compared to the control group; *P<0.05, **P<0.01, compared to the untreated group.

of *Odoribacter*, *Coprococcus*, *Allobaculum*, and *Akkermansia* increased (Figures 5B, C). The above data suggested that YCHD improved the species composition of the intestinal microbiota of AIH mice.

3.5 Altered gut microbiota taxon in the YCHD-treated group of mice

To obtain more specific information about the effects of different groups on each taxon, the Linear discriminant analysis Effect Size (LEfSe) and generated linear discriminant analysis (LDA) scores were performed. The LDA value greater than 3.16 was used as the screening criterion to determine the abundance of microorganisms in the group. *Actinobacteria* were more common in the control group, while *Klebsiella* and *Lactobacillales* were dominant in the AIH group. *Verrucomicrobia* and *Bifidobacterium* accounted for a large proportion of the YCHD-treated group (Figure 6).

3.6 Elevated SCFA contents in the YCHD-treated group of mice

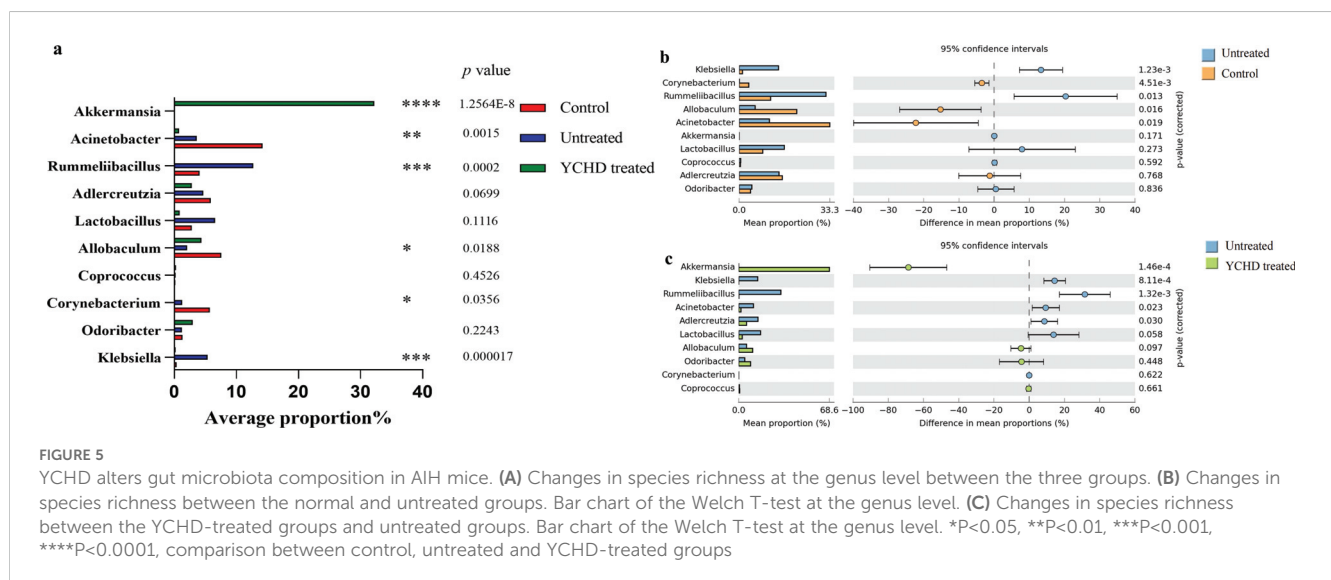
The SCFAs in intestinal contents were detected by the GC-MS method, and the results are listed in Figure 7. Overall, the intestinal total SCFA content of AIH mice was significantly lower than that of the control group, while compared to the untreated group, the intestinal total SCFA content of the YCHD-treated group was slightly increased. Compared with the untreated group, the butyric acid and acetic acid content in the YCHD-treated group was increased, however, the difference in acetic acid content between these two groups was not statistically significant. At the same time, the contents of isobutyric acid, valeric acid, and isovaleric acid in the YCHD-treated group were significantly reduced, while the differences in the contents of propionic acid and caproic acid were not statistically significant (See Supplementary Table S2 for details). Moreover, the Pearson correlation analysis was carried out to reveal the relationship

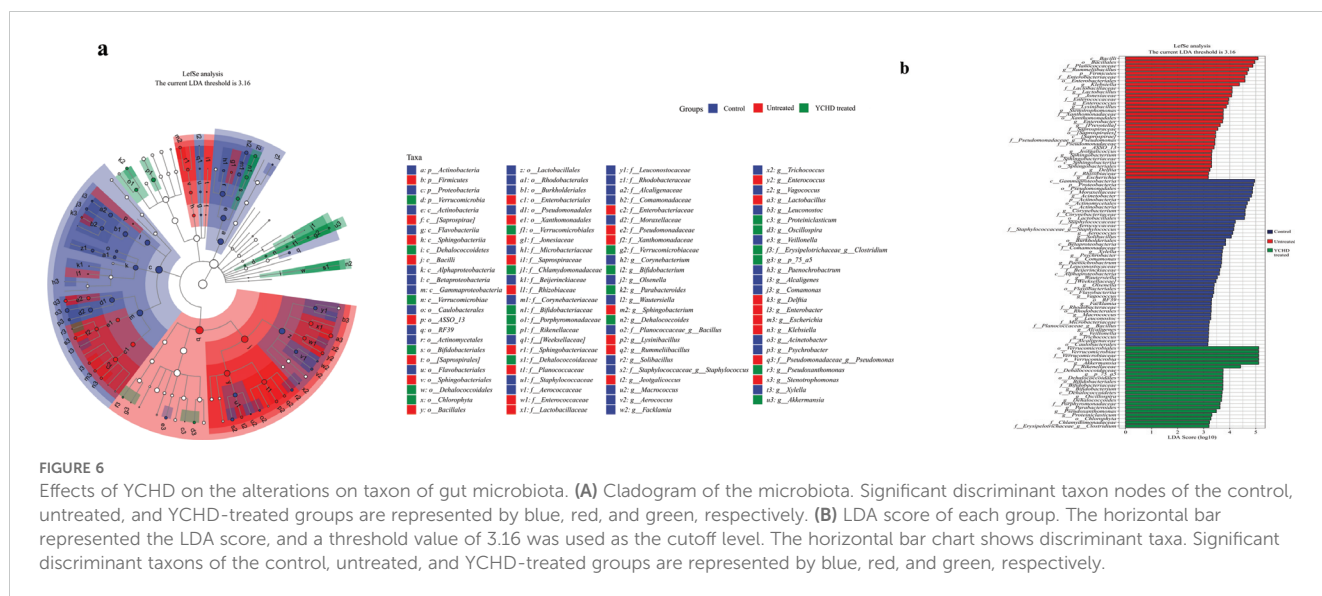
between intestinal microbial composition and SCFA content. The results shown that the abundance of *Corynebacterium* and *Klebsiella* was positively correlated with the content of acetic acid and the content of isobutyric acid, valeric acid, isovaleric acid, respectively. However, the abundance of *Klebsiella* was negatively correlated with the content of butyric acid (Supplementary Figure S3).

4 Discussion

The liver and intestines are important parts of the digestive system, engaging in highly complex physiological processes. Under healthy conditions, the intestinal epithelium can protect the body from intestinal microorganisms and their metabolites through a natural barrier including tight junctions, antibacterial molecules, and a mucus layer (21). When the intestinal mucosal barrier is damaged, some pathogenic bacteria will seep out of the intestinal cavity into other organ systems, thus endangering the host's health. In addition, intestinal flora is involved in metabolism as an important factor in environmental factors, and the disorder of intestinal flora is not only the key factor of intestinal immune response but also an important factor affecting systemic immune response (22, 23). The liver is thought to be the first organ to be exposed to harmful substances in the intestines. Some pathogen-associated molecular models (PAMPs) or metabolites of intestinal flora can enter the hepatic portal circulatory system through the damaged intestinal mucosal barrier, which can cause an inflammatory cascade effect. It will lead to acute injury of hepatocytes and strongly activate the lymphocytes (24, 25). Under certain conditions, it will also stimulate hepatic stellate cells and promote the occurrence and development of hepatic fibrosis (26).

Based on the research of Cheng Z et al. (12), the disorder of intestinal flora is closely related to the pathogenesis of AIH. As shown in Figure 8, the dysregulation of the microbial region system leads to the disruption of the intestinal barrier, which results in some intestinal microorganisms transferring from the intestine to the liver. Some of these intestinal microorganisms can be regarded as a

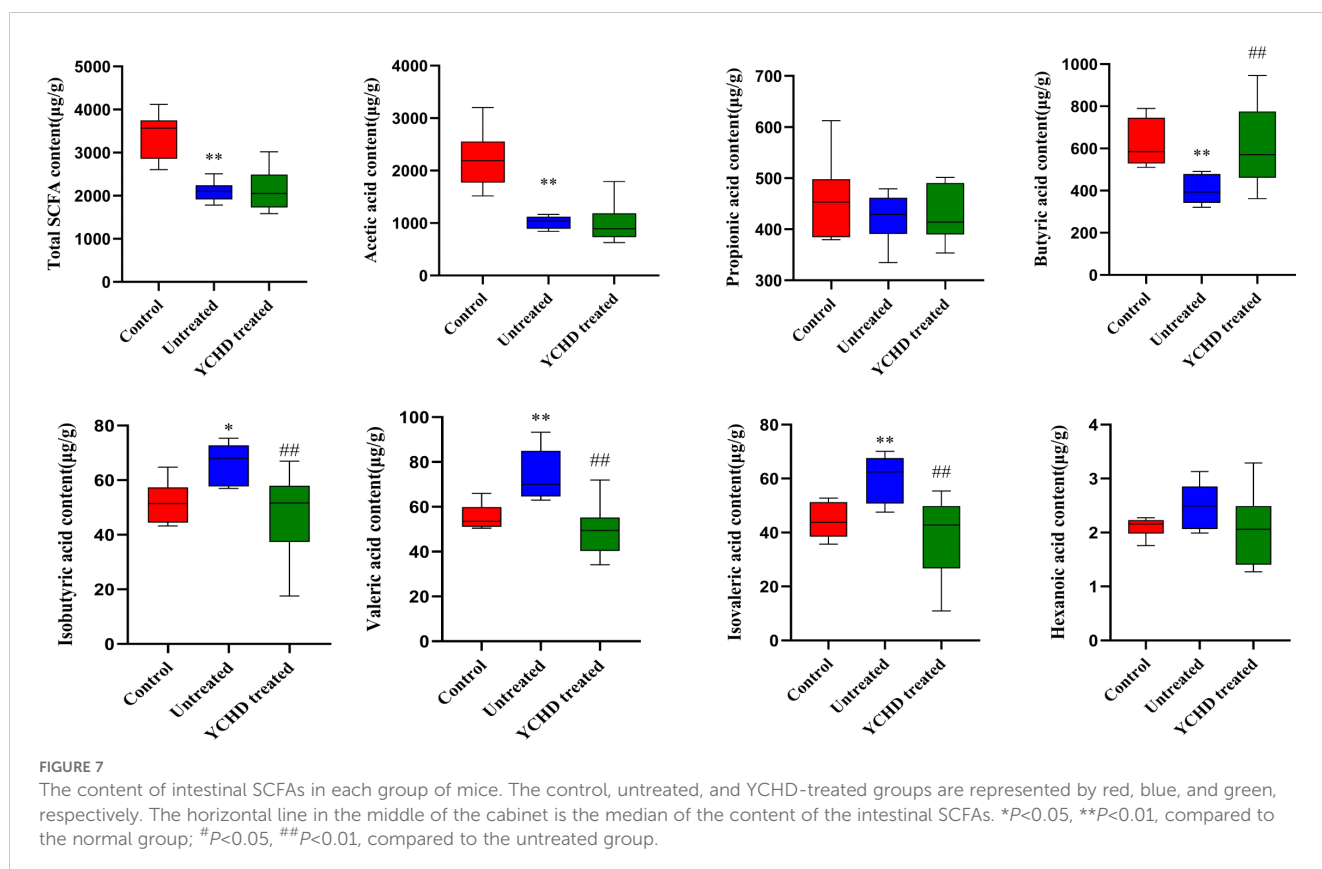




continuous reservoir of antigens. After the intestinal barrier is damaged, the intestinal flora can continue to present antigens, which may disrupt the dynamic balance of immunity homeostasis in the liver and result in the continuous occurrence of autoimmune reactions. Some studies have shown that the intestinal microbial composition of patients with AIH changed significantly, the abundance of *Clostridium*, *Rumen cocci*, *Vibrio*, *Bacteroides*, and *fecal cocci* declined, while the abundance of *Veillonella*, *Klebsiella*, *Streptococcus*, and *Lactobacillus* elevated (22). The diversity and total

load of the intestinal flora of the new HLA-DR3⁺ mouse model immunized with human CYP2D6 were significantly lower than that of healthy mice (27). All these prove that intestinal flora may be involved in the pathogenesis of AIH, and suggest that regulating the homeostasis of intestinal flora may be a new strategy for treating AIH.

The balance of Treg cells and Th1 cells plays an important role in maintaining immune homeostasis and is closely related to the occurrence and development of numerous autoimmune diseases. AIH is characterized by Th1-biased immune responses, and Th1



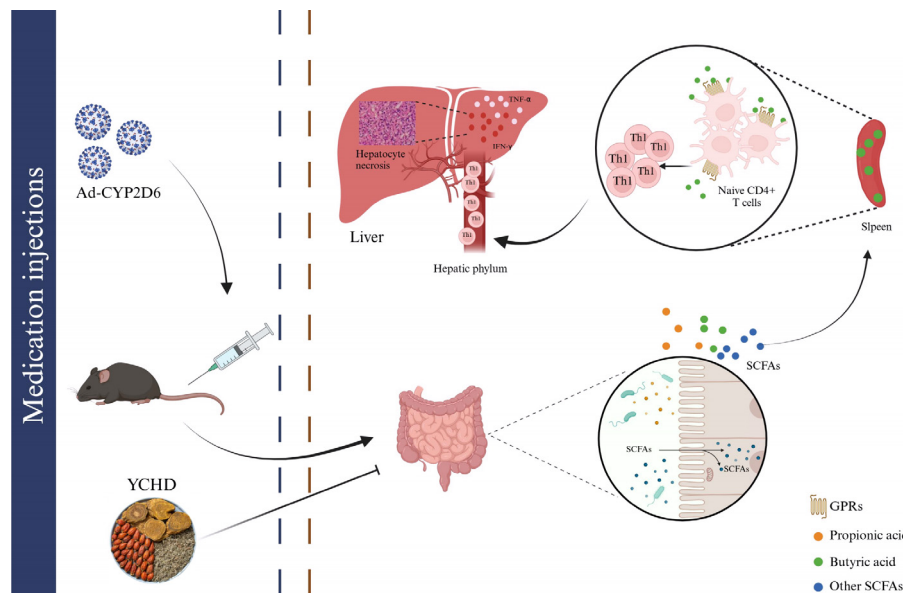


FIGURE 8

The mechanism of intestinal flora participating in the pathogenesis of AIH by regulating the differentiation of immune cells. Intestinal microbiota disturbances can be observed in AIH mice. Changes in the diversity and richness of species in the gut microbiota can affect the formation of SCFAs, such as butyric acid, propionic acid, and others. The reduction of butyric acid content reduced their inhibitory effect on Th1 differentiation, and the abnormally differentiated Th1 entered the liver through the hilar lymph nodes to induce damage of the liver by releasing inflammatory factors such as TNF- α and IFN- γ . YCHD can treat AIH by blocking the above effects.

cells, responsible for TNF- α and IFN- γ , have been reported to be highly represented in the liver inflammatory infiltrates of AIH patients (28, 29). Studies have shown that the occurrence of AIH may be related to defects in the number and function of Treg cells. The immunosuppressive function of Treg cells is mainly dependent on the continuous expression of the transcription factor forkhead box protein 3 (Foxp3) and the production of immunosuppressive cytokines including IL-10 and TGF- β (30). Alterations in the Th1/Treg balance may be responsible for AIH progression. However, the regulatory mechanism of T cell subset differentiation in the development of AIH is not fully understood.

YCHD is the representative Chinese herbal compound prescription for treating AIH in ancient and modern Chinese medicine clinical practice. However, it is still difficult to fully explain the pharmacodynamic mechanism of the liver-protecting effect for YCHD at present. In this study, except for confirming its hepatoprotective effect by detecting the morphological and serological changes (including transaminase, inflammatory cytokine, and autoantibody), we found that YCHD can reverse the ratio of Th1/Treg in the spleen of the AIH model mice (Figure 3). Moreover, YCHD can also regulate the species diversity of intestinal flora of the AIH model mice based on the diversity indexes, including the Chao1 estimator and Shannon diversity index (Figure 4B). According to previous studies, the depletion of beneficial bacteria and expansion of potential pathobionts are associated with AIH disease status (25). Therefore, we also analyzed the effects of YCHD on the composition of intestinal microflora at the genus level. According to the results exhibited in Figure 5, it was indicated that *Klebsiella*, *Rummeliibacillus*, *Lactobacillus*, *Allobaculum*,

especially *Akkermansia* may have a close relationship with the hepatoprotective activity of YCHD because the abundance of these gut flora was significantly altered compared to the untreated group after intragastric administration of this herbal compound. It has been reported that *Akkermansia* is a new probiotic that plays a protective role in improving liver disease, and it may maintain the intestinal barrier and inhibit inflammation by regulating the release of SCFAs that affect the differentiation of Treg cells, or affecting other types of signaling pathways to protect the liver (31–34).

At the same time, we also found that YCHD can influence the production of intestinal flora metabolites, such as SCFAs. Based on the results of GC-MS analysis listed in Figure 7, the contents of isobutyric acid, valeric acid, and isovaleric acid were decreased, while the contents of acetic acid, butyric acid, and propionic acid were increased in the YCHD-treated group. It has been found that SCFAs, especially butyric acid, serve as key messengers facilitating communication between the intestinal microbiome and the immune system (12). SCFAs bind histone deacetylase (HDAC) or G protein-coupled receptor (GPR) signals, including GPR41, GPR43, and GPR109A expressed in the intestinal epithelium and immune cells, to modulate the intestinal epithelial barrier function as well as maintaining the homeostasis of mucosal and systemic immunity (35–38). GPR41 exists widely in a variety of tissues, while GPR43 is mainly expressed in lymphoid tissues and various immune cells. GPR41 and GPR43 can bind to acetate, propionic acid, and butyric acid, while GPR109a can be activated by butyric acid. Since SCFAs can enter the blood circulation, they also have a wider range of systemic effects. An increase in Foxp3⁺Tregs was observed in mice given SCFAs (39). Moreover, SCFAs can also

influence the differentiation of naive T cells into effector T cell subsets, such as helper Th1 and Th17 cells. SCFAs can promote the microbiota's antigen-specific IL-10 production in Th1 cells through GPR43 and induce the differentiation of Th1 and Th17 cells upon exposure to immunological challenges. Butyric acid is one of the representative SCFAs, and we have found that YCHD can affect butyric acid production in AIH model mice in this study. Butyric acid is absorbed mainly by the intestinal epithelium. The anti-inflammatory effect of butyrate is achieved by directly affecting the differentiation of intestinal epithelial cells, phagocytes, B cells, and plasma cells as well as Tregs and effector T cells (40). It can induce the activation of NLRP3 inflammatory bodies and the secretion of IL-18 by colonic epithelial cells, promote the recruitment of neutrophils to inflammatory sites, and enhance the differentiation of Foxp3⁺ Tregs, which can then inhibit the activation of effector T cells (41–43). Most important of all, it is found that butyric acid can not only promote the differentiation of Treg but also have a certain effect on the differentiation of Th1. For instance, butyrate induces differentiation of intestinal Treg cells by promoting histone H3 acetylation of Foxp3 promoter and other conserved non-coding region Foxp3 genes (39). Butyrate also accelerates the oxidation of fatty acids by converting to the downstream activation of butyl-CoA and carnitine palmitoyltransferase 1A. It has been shown that this up-regulation of fatty acid oxidation promotes the differentiation of Treg *in vitro* (43). And, it has been reported that butyric acid can promote Tregs amplification and increase the expression of IL-10 by GPR109A-expressed macrophages and dendritic cells (44, 45). At the same time, it has also been proved that butyrate upregulates the expression of IL-10 in Th1 cells isolated from healthy individuals and IBD individuals through the GPR43-mediated transcription factor BLIMP1 (46). Meanwhile, excessive concentrations of butyrate and butyric acid can promote the differentiation of Th1 cells by up-regulating T-bet expression and down-regulating the expression of ROR γ t and other Th17 cell-related transcription factors (47, 48). In this study, we found that the effect of butyric acid on Treg differentiation may be higher than that of Th1 differentiation. However, the role of butyric acid in the differentiation of different lymphocytes still needs to be further studied. Moreover, some studies have shown that propionic acid can directly inhibit the production of IL-17 by $\gamma\delta$ T cells through an HDAC-dependent mechanism, and inhibit the secretion of IL-17 in human $\gamma\delta$ T cells from patients with IBD (49). Although we found the change in propionic acid content could play a positive role in protecting the liver injury, it still needs to be further investigated. To sum up, it was indicated that YCHD may affect the content of SCFAs (including butyric acid and propionic acid) by regulating the abundance and diversity of intestinal flora and then regulating the immune homeostasis of the body by reversing the ratio of Th1/Tregs. This is summarized in Figure 8.

By mass spectrometry, the main active components of YCHD include 7-methoxycoumarin, quercetin, Rhein, Genipin, and emodin-8- β -D glucoside (the experimental results are published separately) (50). According to related studies, quercetin may be a key ingredient in regulating intestinal flora and immune response by

YCHD (51–53). Quercetin is a kind of natural flavonoid widely found in flowers, leaves, and fruits of plants, which is mostly in the form of glycosides (54). A large number of studies have shown that quercetin has anti-inflammatory, antioxidant, anti-allergic, anti-virus, and other biological activities (55, 56). Studies have shown that polyphenols can regulate intestinal microorganisms, and quercetin may be one of the main components (57–60). Yu et al. reported that quercetin significantly changed the composition of intestinal flora in Wistar rats (61). At the phylum level, the relative abundance of *Chlamydia* and *Cyanobacteria* increased significantly, while the relative abundance of *Bacteroides* decreased. At the genus level, the relative abundance of *Candidatus Arthromitus*, *Lactococcus*, *Bacillus subtilis* and *Rumencocci* which are beneficial to host health increased significantly. Gwiazdowska et al. showed that the inhibitory effect of quercetin on *Bifidobacterium* cultured *in vitro* was dose-dependent and with the strength of inhibition intensifying as the dose increases (62). Therefore, quercetin in YCHD may be an important substance for its hepatoprotective and immunomodulatory effects through the “intestine-liver” axis. However, the above mechanism needs to be verified by further experiments.

5 Conclusion

In this study, we found that YCHD has a positive effect on liver injury in the Ad-CYP2D6-induced AIH mice model. Its therapeutic mechanism may be related to regulating the abundance and diversity of intestinal flora of mice, which will subsequently affect the release of SCFAs, such as butyric acid. SCFAs will bind to the GPR receptor on the surface of T cells, thereby affecting T cell differentiation, and ultimately achieving the effect of alleviating liver damage.

Data availability statement

The original contributions presented in the study are publicly available. This data can be found here: NCBI repository, accession number PRJNA1172145.

Ethics statement

The animal study was approved by Ethics Committee of Shanxi University of Traditional Chinese Medicine. The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

JW: Data curation, Writing – original draft, Methodology. SL: Writing – review & editing. DG: Writing – review & editing. NY: Writing – review & editing. YL: Funding acquisition, Supervision, Writing – review & editing.

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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.2024.1488125/full#supplementary-material>

SUPPLEMENTARY MATERIAL S1

The specific method of model building. Regarding the study of Müller P et al. (18), we selected 100μl different concentrations (0.5×10^9 pfu/ml, 1×10^9 pfu/ml, and 2×10^9 pfu/ml) of Ad-CYP2D6 to establish the AIH mice model via tail intravenous injection under sterile conditions. 7 days after intravenous administration, compared with the control group, the content of serum transaminase and the degree of infiltration of inflammatory cells in liver tissue were not significantly changed in the 0.5×10^9 pfu/ml group but significantly changed in the 1×10^9 pfu/ml and 2×10^9 pfu/ml group. However, the mortality in the 2×10^9 pfu/ml group was higher than that in the 1×10^9 pfu/ml group (shown in **Supplementary Figure S1**). Therefore, 100μl 1×10^9 pfu/ml was finally determined as the optimal dosage of Ad-CYP2D6 to establish AIH mice for the next experiment. According to the research of Müller P et al. (18) and Holdener M et al. (19), we also explored the induction time of the mouse model. On the 3, 7, and 14 days after the Ad-CYP2D6 injection (100μl 1×10^9 pfu/ml), the content of serum transaminase and the morphological changes in the liver of the mice were detected. The results showed that on the 3 days of Ad-CYP2D6 injection, the aminotransferase levels of the mice did not show significant changes, and no obvious inflammatory cell infiltration or liver tissue necrosis were found under microscopic observation. However, on the 7 and 14 days after Ad-CYP2D6 injection, the aminotransferase levels of the mice were significantly increased, and a large number of inflammatory factors infiltrated and some hepatocytes necrosis were observed under the microscope. Since there was no significant difference in liver function between the mice on day 7 and day 14, we chose day 7 after Ad-CYP2D6 injection as the time point for model success (shown in **Supplementary Figure S2**).

SUPPLEMENTARY FIGURE 1

Effect of different Ad-CYP2D6 concentrations on establishing the AIH mice model. (A) The mortality of mice in different concentration of Ad-CYP2D6 group. (B) Comparison of serum levels of AST and ALT in mice of low, medium, and high concentrations. (C) HE stain. The black arrows point to the sites of inflammatory infiltrates. Data was expressed as mean $\pm$ S.D, n=5. ** $P < 0.01$, compared to the control group.

SUPPLEMENTARY FIGURE 2

Effect of different induction times after Ad-CYP2D6 injection on establishing the AIH mice model. (A) Comparison of serum levels of AST and ALT in mice of 3 days, 7 days and 14 days after Ad-CYP2D6 injection. (B) HE stain. The black arrows point to the sites of inflammatory infiltrates. Data was expressed as mean $\pm$ S.D, n=5. * $P < 0.05$, compared to the control group, # $P > 0.05$, compared to the 7 day.

SUPPLEMENTARY FIGURE 3

Pearson correlation analysis between the contents of SCFAs and the abundance of intestinal microbiota in each group. Each box represented the correlation between different samples. The red box indicates positive correlation and the blue box represents negative correlation. The redder or bluer the box, the higher the relevance.

SUPPLEMENTARY TABLE 1

Specific temperature descriptions used for mass spectrometry.

SUPPLEMENTARY TABLE 2

Content of specific SCFA in different group.

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A novel anti-HER2 monoclonal antibody IAH0968 in HER2-positive heavily pretreated solid tumors: results from a phase Ia/Ib first-in-human, open-label, single center study

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Background: IAH0968 is an afucosylated anti-epidermal growth factor receptor 2 (HER2) monoclonal antibody which improved the activity of antibody-dependent cellular cytotoxicity (ADCC) and superior anti-tumor efficacy.

Methods: To determine the maximum tolerated dose (MTD) with dose-limiting toxicity (DLT), a single institution, phase Ia/Ib study was undertaken, using 3 + 3 design. The primary endpoints were safety, tolerability and preliminary clinical activity. Eighteen patients were evaluable for safety and fifteen patients were suitable for efficacy analysis. Dose escalations were 6 mg/kg ($N = 2$), 10 mg/kg ($N = 7$), 15 mg/kg ($N = 5$), and tolerable up to 20 mg/kg ($N = 4$).

Results: Only one DLT was found at dosage 10 mg/kg, and no MTD was reached. The most common Grade 3 treatment-related adverse events (TRAEs) were hypokalemia (5.6%), supraventricular tachycardia (5.6%), interval extension of QTC (5.6%), and infusion reaction (5.6%). Grade 4 TRAE was arrhythmia (5.6%). No serious TRAE or Grade 5 was reported. 22.2% of patients had a TRAE leading to dose adjustment and 16.7% of patients had a TRAE resulting in discontinuation of IAH0968. After a median follow-up of 9.7 months (range, 3.7 - 22.0), the objective response rate (ORR) was 13.3% (2/15), the disease control rate (DCR) was 53.3% (8/15), and median progression-free survival (mPFS) was 4.2 months (95% CI: 1.4 - 7.7), and the median duration of disease control (DDC) was 6.3 months (95% CI: 2.9–not reached), with 4/15 responses ongoing.

Conclusions: In HER2-positive heavily pretreated metastatic patients, IAH0968 demonstrated promising clinical activity with durable responses and tolerable safety profiles.

Clinical trial registration: [ClinicalTrials.gov](https://clinicaltrials.gov), identifier NCT04934514.

KEYWORDS

HER2, IAH0968, clinical study, safety, first-in-human

1 Introduction

Receptor tyrosine-protein kinase erbB-2, also known as HER2, is a member of the EGFR family of receptor tyrosine kinases. Heterodimerization of this receptor with other members of the EGFR family, typically owing to HER2 overexpression, results in the autophosphorylation of tyrosine residues within the cytoplasmic domain of the heterodimer and initiates a variety of signaling pathways leading to cellular proliferation and tumorigenesis (1). The activation of HER2 signaling observed in approximately 20% of breast cancers, and overexpression of HER2 has also been described in a variety of other solid tumors, including gastric and gastroesophageal junction cancers, biliary tract cancer (BTC), colorectal cancer (CRC), non-small-cell lung cancer and bladder cancer, with incidences varying from greater than 50% of uterine cancers to around 2% of non-small-cell lung cancers (1). Compared with other HER-2-negative subtypes, subtypes of HER2-positive cancer share a common mechanism of carcinogenesis characterized by decreased apoptosis and enhanced cell proliferation, mobility, invasiveness, metastasis, and angiogenesis (2). Furthermore, HER2-positive breast cancer is highly immunogenic and aggressive with poor clinical outcomes and high resistance to chemotherapy (3).

HER2-targeted therapies are effective in people with breast cancer and gastric cancer harboring HER2 overexpression and/or amplification. Trastuzumab is the first anti-HER2 monoclonal antibody which is effective in the treatment of breast cancer overexpressing HER2 and is a standard option together with chemotherapy in the treatment of HER2-positive metastatic gastric cancer (4–6). In light of the successes of treatment with trastuzumab and other agents in women with HER2-positive breast cancer, interest has emerged in exploring the efficacy of HER2-targeted therapies. In addition to directly inhibiting tumor cell signaling, antibody-dependent cellular cytotoxicity (ADCC) plays a key role in the mechanism of action of trastuzumab (7). The activity of ADCC can be modulated by the FcγRIIIa polymorphisms expressed on immune effector cells. A number of studies documented a correlation between the objective response rate (ORR) of trastuzumab in humans and their allotype of high-affinity (158 V) or low-affinity (158 F) polymorphic forms of FcγRIIIa (8). Amino acid mutagenesis and glycoform engineering have been used to enhance the binding affinity of Fc fragment to Fcγ

receptors to enhance the ADCC activities of antibodies (9–14). ADCC-enhanced anti-HER2 monoclonal antibody is produced by five amino acid mutagenesis and has been approved on by FDA (15). Lack of fucose on human IgG1 oligosaccharide was found to enhance the binding to Fcγ receptor IIIa (FcγRIIIa) and thereby improves (> 50-fold) ADCC (16). Hence, de-fucosylation of trastuzumab can be one of the most promising technologies to improve clinical efficacy and solve the ineffective and resistant of trastuzumab problems. Furthermore, HER2 alterations exist in various other solid tumors, some of which have limited therapeutic options. Therefore, it remains necessary to develop a safe and effective HER2-targeting therapy for the unmet clinical needs.

IAH0968 is an afucosylated anti-HER2 monoclonal antibody which is produced by FUT8-deficient CHO cell line with 100% fucose removal. IAH0968 has shown the same binding properties to HER2 as trastuzumab and enhanced binding affinity to FcγRIIIa allotypes, improved the activity of ADCC and superior anti-tumor efficacy (on-line [Supplementary Table S1](#), [Supplementary Figures S1](#), [S2](#)). In cynomolgus monkey studies, IAH0968 showed an excellent safety profile similar to that of trastuzumab. The safety and superior efficacy of IAH0968 supports the clinical studies of IAH0968 in a wider patient population besides breast and gastric cancer, including trastuzumab resistant and ineffective patients.

The present first-in-human study in HER2-positive patients with advanced malignant solid tumors was undertaken to assess the safety, tolerability, PK and preliminary clinical activity of the drug as well as to establish the recommended dose of IAH0968 for Phase II/III trials.

2 Methods

2.1 Study design and treatment

This was a single institution, phase Ia/Ib study, using 3 + 3 design including dose increase (Phase Ia) and dose expansion (Phase Ib) aiming to determine the maximum tolerated dose (MTD) based on the proportion of patients with dose-limiting toxicities (DLTs) and explore preliminary antitumor activity. This study is registered on [ClinicalTrials.gov](https://clinicaltrials.gov) (NCT04934514). The primary endpoints were

safety, tolerability and preliminary clinical activity of IAH0968. Secondary endpoints included pharmacokinetic (PK), recommended phase II dose (RP2D), and anti-drug antibody (ADA). Doses escalation were 6mg/kg (N=2), 10mg/kg (N=7), 15mg/kg (N=5), and tolerable up to 20mg/kg (N=4).

2.2 Patient eligibility

Eligible patients with confirmed diagnosis of advanced, unresectable, and/or metastatic HER2 overexpression solid tumor were recruited. Heavily pretreated solid tumors are defined as late stage solid tumor subjects who have failed standard treatment. Standard treatment failure refers to disease progression during or after the last treatment, inability to tolerate toxic side effects during treatment (hematological toxicity ≥ 4 or non-hematological toxicity ≥ 3 after previous standard treatment), disease progression or recurrence during neoadjuvant/adjuvant therapy or within 6 months after the end of treatment. HER2 overexpression was determined by immunohistochemical analysis of tumor tissue as 3+ or 2+ amplification by Fluorescence *in situ* hybridization (FISH). Pertinent eligibility criteria included: age ≥ 18 years; Eastern Cooperative Oncology Group Performance Status (ECOG PS) of 0/1; recovered from previous systemic therapy-related toxicity; at least one measurable lesion according to Response Evaluation Criteria in Solid Tumors (RECIST) version 1.1; a life expectancy of at least 12 weeks, and adequate organ function.

2.3 Assessment

Adverse events (AEs) were assessed and graded by the investigators according to the Common Terminology Criteria for Adverse Events (CTCAE) v5.0. Tumor imaging was performed at baseline, and every two cycles thereafter. Tumor response was assessed by the investigators according to the RECIST, Version 1.1. Antitumor activity assessment was defined as (1) overall response rate (ORR), the proportion of patients who displayed partial response (PR) or complete response (CR); (2) disease control rate (DCR), the proportion of patients who displayed PR or CR or stable disease (SD); (3) progression-free survival (PFS); (4) duration of disease control (DDC); (5) overall survival (OS). DLTs were graded by the CTCAE v5.0 during the MTD evaluation period. MTD was defined as the highest dose with $< 25\%$ risk of the true DLT rate > 0.33 during evaluation.

2.4 Pharmacokinetics and pharmacodynamics

Blood for single PK analysis was collected at pre-dose, 1 hour post-dose and 0, 2, 4, 8, 12, 24, 48, 72, 120, 168, 240, 312, 384 and 480 hours of the end of the first infusion. Thereafter, samples were collected at pre-dose and 0 hour of the second to fifth infusion. Besides, blood was collected at pre-dose, 1 hour post dose and 0, 2, 4, 8, 12, 24, 48, 72, 120, 168, 240, 312, 384 and 480 hours of the end

of the sixth infusion. IAH0968 serum levels were measured with an enzyme-linked immunosorbent assay (ELISA) assay, based on the specific recognition of HER2 by IAH0968. The capture agent was recombinant Human HER2 Protein (Sino Biological, 10004-H02H), coated onto a microplate. Samples were pipetted into the wells and any IAH0968 present will be bound by the coating reagent. After washing away any unbound substances, detection reagent (Biotin-HER2) was added into well to bind with IAH0968. After washing away any unbound detection reagent, streptavidin HRP (Jackson, 016-030-084) was added into well to bind with detection reagent. Following a wash to remove any unbound SA-HRP, a substrate solution was added to the wells and color develops in proportion to the amount of IAH0968 bound in the initial step. The color development was stopped and the intensity of the color was measured. The lower limit of quantification of the assay was 62.5ng/mL. PK parameters were derived from the individual patient serum concentration time profiles after the first and sixth infusion using non-compartmental methods. The maximum (C_{max}) and minimum (C_{min}) serum concentration after administration were directly taken from analytical data. Dose linearity and proportionality of the PK parameters, C_{max}, C_{min}, area under the plasma concentration time curve from time 0 to infinity (AUC_{0-∞}) and area under the plasma concentration time curve from time 0 to the last measured concentration (AUC_{0-last}) were investigated over the dose range, based on the individual values by linear regression analysis. A trough level 63.25μg/mL of the drug was set as target for the study, based on previous research that defines 17.75~63.25×10⁻³μg/mL as the EC₅₀ for anti-proliferative effects and ADCC. Accumulation of IAH0968 was assessed by dividing the C_{max} and AUC_{0-last} after the sixth dose by the C_{max} and AUC_{0-last} after the first dose.

2.5 Statistical analyses

Patients who received at least one dose of IAH0968 were conducted for safety assessments. Three participants withdrew from the study due to infusion reactions and missed the efficacy analysis. The KaplanMeier method was used to assess PFS and time-to-progression. Cox proportional hazards models were used to estimate the hazard ratio (HR) and 95% confidence intervals (95% CI). All data tests were two-sided testing. Fisher's exact testing was used to compare the response rate in subgroups. All statistics were analyzed with SAS software (Version 9.4, SAS Institute Inc, Cary, NC).

3 Results

3.1 Patient characteristics

From July 2021 to November 2022, a total of 18 participants were enrolled into the present trial. Their baseline characteristics are shown in [Table 1](#). There were seven males (38.9%) and 11 females (61.1%), with a median age of 52.5 (range 26–70) years. All the participants had an ECOG PS of 1. The primary cancer sites included breast (8/18, 44.4%), colon (3/18, 16.7%), stomach (2/18,

TABLE 1 Patient demographics and baseline characteristics.

Characteristic		Total (N=18)
Median age, years (range)		52.5 (26~70)
Sex (%)	Male	7 (38.9)
	Female	11 (61.1)
ECOG performance status (%)	0	0 (0)
	1	18 (100.0)
Primary site (%)	Breast	8 (44.4)
	Colon	3 (16.7)
	Rectum	2 (11.1)
	Stomach	2 (11.1)
	Biliary tract	2 (11.1)
HER2 status (%)	Pancreas	1 (5.6)
	IHC 1+ FISH+	1 (5.6)
	IHC 2+ FISH+	3 (16.7)
	IHC 3+	14 (77.8)
FISH status (%)	Positive	4 (22.2)
	Unknown	14 (77.8)
Metastatic site (%)	Lymph node	10 (55.6)
	Liver	4 (22.2)
	Lung	8 (44.4)
	Bone	2 (11.1)
	Abdominal cavity	4 (22.2)
	Pleura	1 (5.6)
Number of metastatic sites (%)	Brian	1 (5.6)
	1	11 (61.1)
	2	2 (11.1)
Number of lesions (%)	>2	5 (27.8)
	<3	4 (22.2)
Number of previous lines of therapy (%)	≥3	14 (77.8)
	≤2	5 (27.8)
Previous trastuzumab treatment (%)	>3	13 (72.2)
	Yes	14 (77.8)
	No	4 (22.2)

IHC, immunohistochemistry; FISH, fluorescence *in situ* hybridization.

11.1%), rectum (2/18, 11.1%), biliary tract (2/18, 11.1%), and pancreas (1/18, 5.6%). Fourteen patients (77.8%) underwent prior trastuzumab-containing therapy. Five (27.8%) were treated with either one or two lines of therapy and thirteen of whom had three or more lines. All of patients had at least one dose of IAH0968, of whom two patients received 6 mg/kg, seven received 10 mg/kg, five

received 15 mg/kg, and four received 20 mg/kg. The median treatment dosage number was two (range 1–21) cycles. The numbers of dosage in different groups are listed in Table 2.

3.2 Safety analysis

Among all eighteen participants who received at least one dose of IAH0968, 18 (100%) experienced at least one treatment-related adverse event (TRAE), mostly categorized as Grades 1 to 2 (Table 3). All four doses were well tolerated. Three patients (16.7%) experienced Grade 3 TRAE, including hypokalemia (5.6%), supraventricular tachycardia (5.6%), QTC interval extension (5.6%), and infusion reaction (5.6%). One subject experienced two TRAE instances at Grade 3. Only one patient (5.6%) experienced Grade 4 TRAE with arrhythmia (10 mg/kg). No serious TRAE or Grade 5 instances were reported, and there were no drug-related deaths. 22.2% (4/18) of patients had a TRAE leading to dose adjustment and 16.7% (3/18) of patients had a TRAE leading to discontinuation of IAH0968. The incidence of infusion reaction was 44.4% (8/18), including two patients with Grade 1, five patients with Grade 2, and one patient with Grade 3. Only one DLT was found at a dosage of 10 mg/kg, and no MTD was reached. The recommended phase-2 dose was set to 10 mg/kg and 15 mg/kg Q3W.

3.3 Efficacy analysis

Three subjects withdrew from the study due to infusion reactions and failed efficacy evaluation. In 15 subjects, the ORR was 13.3% (2/15), DCR was 53.3% (8/15) (Table 4). Notably, for patients with heavily pretreated HER2+ CRC and BTC (4 received 15 mg/kg and 1 received 10 mg/kg), the ORR was 40% (2/5), and the DCR was 80% (4/5). The best tumor change in sum of diameters for target lesions in all cohorts is shown in Figure 1 (N = 14). With a median follow-up of 9.7 months (range, 3.7 - 22.0) among all eighteen participants, the mPFS was 4.2 months (95% CI: 1.4 - 7.7, Figure 2), and mDDC was 6.3 months (95% CI: 2.9–not reached). The OS data are not yet sufficiently mature to report at time of writing. At the time of the data cut-off date of January 15, 2023, there are still four patients remaining on the treatment described herein, and 14 patients discontinued treatment due to disease progression (N = 9), TRAEs (N = 4), and death (N = 1).

3.4 PK evaluation

PK parameters are listed in Supplementary Table S2. Mean $T_{1/2}$ for doses 6~20 mg/kg IAH0968 ranged from 105 to 118 hours, and CL was from 25.59 to 31.55 mL/h. The volume of distribution (V_{z-obs}) ranged from 4110.16 mL to 5395.64 mL. A dose for C_{max} , AUC0~last and AUC0~inf showed a linear increase over the whole IAH0968 dose ranging from 6 to 20 mg/kg. IAH0968 trough level (C_{min}) by the end of the first cycle was 3.70~14.10 µg/mL from doses ranging from 6 to 20 mg/kg and the EC50 of ADCC activity of IAH0968 was

TABLE 2 Dosage numbers in different groups.

		6mg/kg (N=2)	10mg/kg (N=7)	15mg/kg (N=5)	20mg/kg (N=4)	Total (N=18)
Dose number	N (Nmiss)	2(0)	7(0)	5(0)	4(0)	18(0)
	Mean $\pm$ SD	1.5 $\pm$ 0.71	7.3 $\pm$ 7.91	5.8 $\pm$ 4.02	2.5 $\pm$ 1.29	5.2 $\pm$ 5.61
	Median (Q1~Q3)	1.5 (1.0~2.0)	2.0 (2.0~13.0)	8.0 (2.0~8.0)	2.5 (1.5~3.5)	2.0 (2.0~8.0)
	Min~Max	1~2	1~22	1~10	1~4	1~22

17.75~63.25 $\times 10^{-3}$ $\mu\text{g/mL}$ (Supplementary Figure S1). The accumulation factor of IAH0968 was between 0.93 and 1.13, which was assessed by dividing C_{max} and AUC0~last after the sixth dose by C_{max} and AUC0~last after the first dose. No significant accumulation was observed based on the available data (data not shown).

3.5 Immunogenicity evaluation

Samples were screened for ADAs before each infusion of IAH0968 with an electrochemiluminescence assay in bridging format using MSD technology. Briefly, the samples (positive controls, negative controls and study samples) were diluted 1:10 in dilution buffer and treated with 300-mM acetic acid to dissociate complexes of ADA and IAH0968. Then the samples were mixed with Tris-base, added into MSD plates coated by IAH0968, and Ruthenylated-IAH0968 were added to the plate. After 2 $\times$ MSD read buffer was added and chemiluminescence was measured; the measured light signal was proportional to the concentration of ADA against IAH0968 in the samples. No ADAs were detected at the tested time points except for 1/15 ADA positive at the pre-test (data not shown).

4 Discussion

This first-in-human clinical study showed that IAH0968 was well tolerated with a comparable safety profile and encouraging preliminary anti-tumor activity in participants with metastatic HER2-positive solid tumors who had progressed on at least one prior line of anti-HER2 therapy in the metastatic setting. Most AEs experienced were mild or moderate, with only four patients (22.2%) experiencing Grade 3/4 AEs related to IAH0968. Moreover, no Grade 5 AEs occurred. There was no difference in safety among the four dosage groups. The confirmed ORR was 13.3%, and DCR was 53.3%. With a median follow-up of 9.7 months, the mPFS was 4.2 months, and mDDC was 6.3 months.

HER2 is overexpressed in subsets of patients with solid tumors beyond breast cancer and gastric cancer. Therefore, HER2 is an interesting therapeutic target for the development of new drug development, including more potent anti-HER2 antibodies or other methods of targeting HER2. Fc receptor is important for engagement of ADCC against tumor cells, with promising levels of activity reported even among patients with trastuzumab-resistant disease. HER2-targeted antibody-drug conjugate (ADC) and

bispecific antibodies are examples of other HER2-targeted agents with potentially more potent anti-tumor activity than trastuzumab. ARX788 is an ADC consisting of HER2-targeted monoclonal antibody conjugated with the AS269 cytotoxic payload, existing promising anti-tumor activity with an ORR of 37.9% and 55.2% DCR (17). Besides, clinical trials with a novel monoclonal antibody margetuximab demonstrated its efficacy in treating HER2-positive breast cancer (18). A humanized, bispecific monoclonal antibody directed against two non-overlapping domains of HER2 named Zanidatamab exerted anti-tumor activity across a range of solid tumors with expression or amplification of HER2 (19). In addition, the HER2 bispecific antibody (KN026) achieved comparable efficacy as trastuzumab and pertuzumab doublet even in the more heavily pretreated patients (20). These Phase 1 data reported here showed that IAH0968 and trastuzumab have comparable PK, safety, tolerance, and immunogenicity. In the present study, all patients were heavily pretreated with a variety of tumor types, and the limited number of patients with heavily advanced disease at the time of enrolment might restrain the therapeutic efficacy. Although all enrolled participants have received anti-HER2 therapies including 77.8% (14/18) with trastuzumab resistance, IAH0968 monotherapy still achieved promising efficacy with an ORR of 13.3% and 53.3% DCR, and an even higher 40% ORR and 80% DCR in patients with heavily pretreated HER2+ CRC and BTC. Therefore, it provides treatment options for patients who have failed to benefit from standard anti-HER2 therapy. These results support that HER2 is an actionable target in various cancer histologies, including BTC and CRC. Considering the limited and heterogenous population in our study cohort, the efficacy is still needed to be validated in future randomized controlled trials for combination treatment.

One of the primary adverse effects of trastuzumab is the increasing risk of cardiac dysfunction, and cardiotoxicity is a typically reversible reduction in left ventricular ejection fraction, and 10.9% patients (19/173) experienced grade 3 cardiac toxicity (21). In this study, IAH0968 monotherapy induced Grade 3/4 cardiac disorders in two patients, including one patient at 10 mg/kg (in the arm) undergoing Grade 3 supraventricular tachycardia and Grade 4 arrhythmia, and the other one at 10 mg/kg (in the arm) developing Grade 3 QTC interval extension. None of the participants experienced reduction in left ventricular ejection fraction. Only one DLT was found at a dosage of 10 mg/kg, and no MTD was reached. Finally, the IAH0968 dose reached 20 mg/kg with sustained anti-tumor activity.

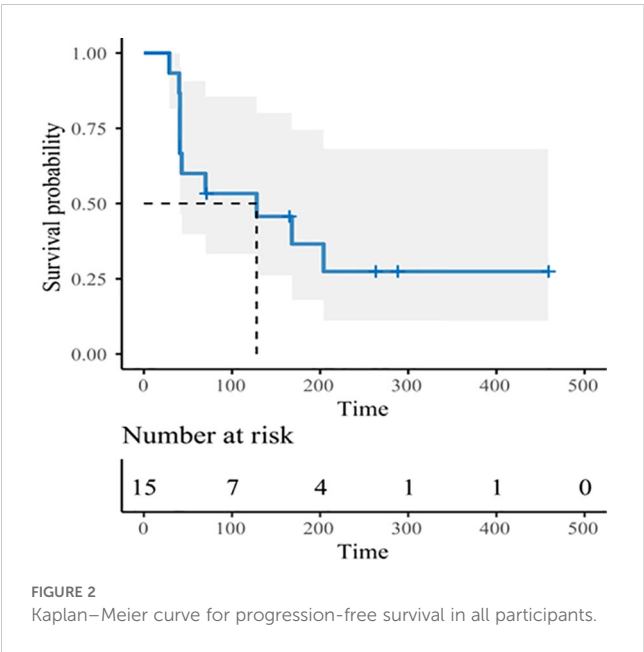
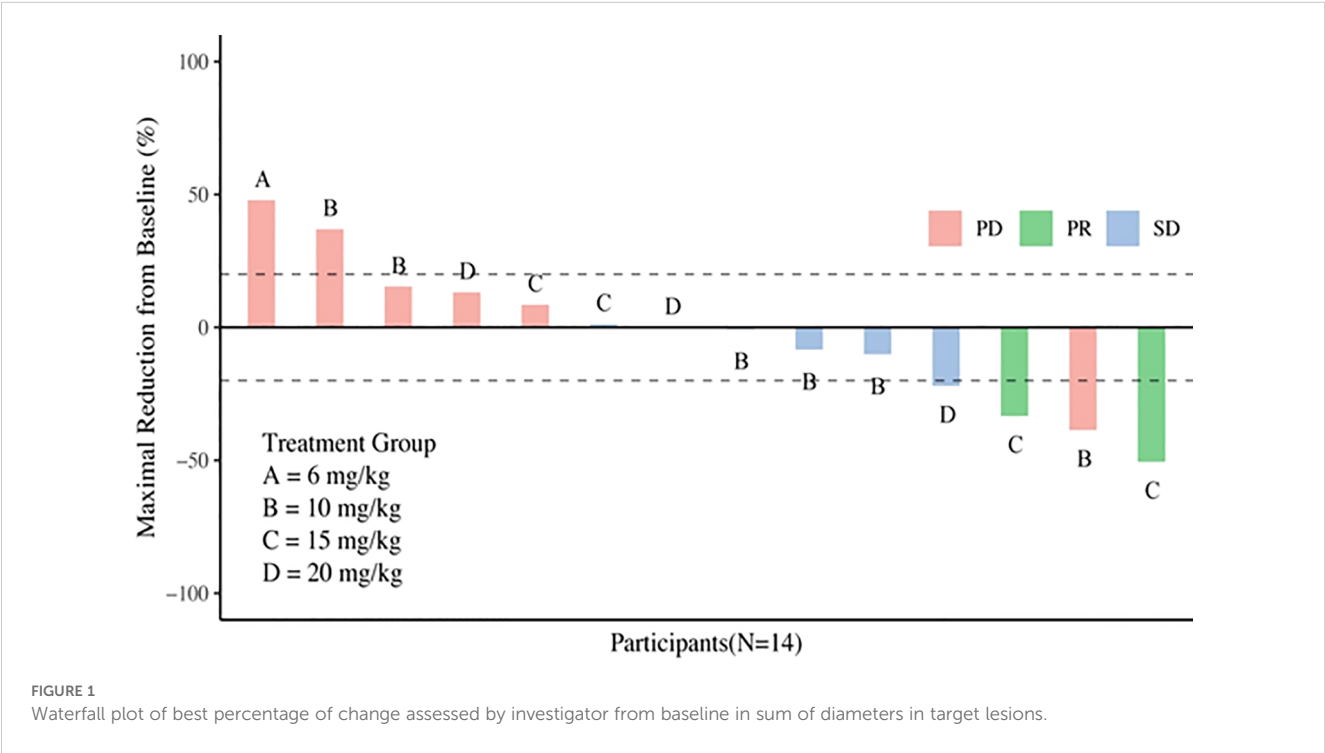
TABLE 3 Most common treatment-related adverse events (any occurred in 10% or more patients and any grade 3/4).

TRAEs	6mg/kg (N=2)			10mg/kg (N=7)			15mg/kg (N=5)			20mg/kg (N=4)			Any (N=18)		
	Grade 3	Grade 4	Any	Grade 3	Grade 4	Any	Grade 3	Grade 4	Any	Grade 3	Grade 4	Any	Grade 3	Grade 4	Any
Any adverse event (%)	0	0	2 (100.0)	2 (28.6)	1 (14.3)	7 (100.0)	0	0	5 (100.0)	1 (25)	0	4 (100.0)	3 (16.7)	1 (5.6)	18 (100.0)
Blood and lymphatic system disorders (%)															
Anemia	0	0	0	0	0	5 (71.4)	0	0	5 (100.0)	0	0	2 (50.0)	0	0	12 (66.7)
White blood cell count decreased	0	0	0	0	0	3 (42.9)	0	0	1 (20.0)	0	0	0	0	0	4 (22.2)
Neutrophil cell count decreased	0	0	0	0	0	2 (28.6)	0	0	0	0	0	0	0	0	2 (11.1)
Platelet count decreased	0	0	0	0	0	2 (28.6)	0	0	0	0	0	1 (25.0)	0	0	3 (16.7)
Gastrointestinal disorders (%)															
Diarrhea	0	0	0	0	0	0	0	0	2 (40.0)	0	0	1 (25.0)	0	0	3 (16.7)
Constipation	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Liver disorder (%)															
Aspartate aminotransferase increased	0	0	0	0	0	1 (14.3)	0	0	1 (20.0)	0	0	2 (50.0)	0	0	4 (22.2)
Alanine aminotransferase increased	0	0	1 (50.0)	0	0	1 (14.3)	0	0	2 (40.0)	0	0	2 (50.0)	0	0	6 (33.3)
γ-glutamyltransferase increased	0	0	0	0	0	0	0	0	1 (20.0)	0	0	1 (25.0)	0	0	2 (11.1)
Blood bilirubin increased	0	0	1 (50.0)	0	0	0	0	0	0	0	0	0	0	0	1 (5.6)
Cardiac disorder (%)															
Arrhythmia	0	0	0	0	1 (14.3)	1 (14.3)	0	0	1 (20.0)	0	0	0	0	1 (5.6)	2 (11.1)
supraventricular tachycardia	0	0	0	1 (14.3)	0	1 (14.3)	0	0	0	0	0	0	1 (5.6)	0	1 (5.6)
QTC interval extension	0	0	0	1 (14.3)	0	1 (14.3)	0	0	0	0	0	0	1 (5.6)	0	1 (5.6)
Others (%)															
Infusion reaction (%)	0	0	1 (50.0)	0	0	3 (42.9)	0	0	3 (60.0)	1 (25.0)	0	2 (50.0)	1 (5.6)	0	9 (50.0)
Fever (%)	0	0	0	0	0	1 (14.3)	0	0	1 (20.0)	0	0	0	0	0	2 (11.1)
hypokalemia (%)	0	0	2 (100.0)	1 (14.3)	0	1 (14.3)	0	0	0	0	0	0	1 (5.6)	0	3 (16.7)

TABLE 4 Summary of confirmed responses in 15 participants.

Overall response (%)	6mg/kg (N=1)	10mg/kg (N=7)	15mg/kg (N=4)	20mg/kg (N=3)	Total (N=15)
CR	0	0	0	0	0
PR	0	0	2 (50.0)	0	2 (13.3)
SD	0	3 (42.9)	1 (25.0)	2 (66.7)	6 (40.0)
PD	1 (100.0)	4 (57.1)	1 (25.0)	1 (33.3)	7 (46.7)
ORR (95% CI)					13.3 (1.7-40.5)
DCR (95% CI)					53.3 (26.6-78.7)

Tumor response was confirmed by investigators. ORR includes complete response and partial response. Disease control rates include complete response, partial response, and stable disease ≥ 12 weeks.



While various anti-HER2-directed therapies have shown variable success in a large set of HER2-positive carcinomas, they always face the problems of drug resistance including both primary and acquired resistance. Genetic aberrations in downstream signaling and genomic alterations beyond ERBB2 might compensate for HER2 suppression (22, 23). In addition, loss of HER2 expression after failure of trastuzumab-containing chemotherapy concerns for one source of acquired resistance mechanisms (24). Therefore, reassessing the HER2 status following first-line trastuzumab before second-line trials is necessary by repeat biopsy or ctDNA. This study did not evaluate the HER2 status before IAH0968 monotherapy. Therefore, whether the absence of HER2 status affects the efficacy of IAH0968 therapy remains unclear. In summary, a deeper understanding of the molecular environment will lead to a greater understanding of the mechanisms of to HER2-directed therapies, and further evidence of combining approaches to target compensatory pathways with anti-HER2 agents is worth exploring. Besides, more translational research is required to reveal the promising biomarkers in predicting better response to HER2-targeted agents.

There are three limitations in this study. The major limitation is the small sample size. The second limitation is the HER2 status was not re-evaluated. Furthermore, the study design omits collection of blood and tissue samples for exploratory analysis.

In conclusion, IAH0968 monotherapy demonstrated tolerable safety and promising anti-tumor activity with a confirmed ORR of 13.3% and DCR of 53.3%. Notably, for patients with heavily pretreated HER2+ CRC and BTC, the ORR was 40%, and the DCR was 80%. Furthermore, the mPFS was 4.2 months, and mDDC was 6.3 months. Based on these encouraging results, a randomized controlled, open-label phase II/III study to evaluate the efficacy of IAH0968 combined with chemotherapy as first-line treatment with HER2-positive advanced colorectal cancer is ongoing (Chinadrugtrials.org.cn: NCT05991518).

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 humans were approved by ethics committee of the First Hospital of China Medical University (2020YL080). The studies were conducted in accordance with the local legislation and institutional requirements. Written informed consent for participation in this study was provided by the participants' legal guardians/next of kin.

Author contributions

NS: Conceptualization, Data curation, Investigation, Project administration, Writing – original draft, Writing – review & editing. YT: Conceptualization, Investigation, Project administration, Resources, Writing – review & editing. JS: Conceptualization, Project administration, Resources, Writing – review & editing. ZT: Investigation, Project administration, Resources, Writing – review & editing. BJ: Data curation, Investigation, Project administration, Writing – review & editing. JQ: Investigation, Project administration, Resources, Writing – review & editing. LiZ: Investigation, Project administration, Resources, Writing – review & editing. PY: Investigation, Project administration, Resources, Writing – review & editing. LeZ: Investigation, Project administration, Resources, Writing – review & editing. JW: Investigation, Project administration, Resources, Writing – review & editing. AL: Investigation, Project administration, Resources, Writing – review & editing. LT: Investigation, Project administration, Resources, Writing – review & editing. SJ: Resources, Investigation, Supervision, Validation, Writing – review & editing. YaL: Resources, Supervision, Investigation, Validation, Writing – review & editing. LY: Conceptualization, Funding acquisition, Resources, Project administration, Visualization, Writing – review & editing. XJ: Supervision, Conceptualization, Funding acquisition, Project administration, Resources, Writing –

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Conflict of interest

Authors LY, XJ and TX are employees of SUNHO CHINA BIOPHARMACEUTICAL CO., LTD.

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.

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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.2024.1481326/full#supplementary-material>

SUPPLEMENTARY FIGURE S1

Enhanced ADCC activity of IAH0968 with two FcγRIIIa allotypes. The NK92-MI-CD16a (158 V/V allele) and NK92-MI-CD16a (158F/V allele) cells were used as effector cells and HER2-expressed BT474 cell was used as target cell. The ratio of effector cells and target cell (E:T) was 5:1. The specific lysis was evaluated using an LDH release assay (CytoTox 96 Non-Radioactive Cytotoxicity Assay; Promega) according to the manufacturer's protocol. The percentage of specific lysis was calculated: 100 x (experimental release - spontaneous release)/(maximum release - spontaneous release).

SUPPLEMENTARY FIGURE S2

Superior *in vivo* efficacy of IAH0968 compared with trastuzumab. Established human breast cancer cells BT474 xenografts in female BALB/c nude mice were treated with vehicle (black), 5mg/kg trastuzumab(green) and three

doses of IAH0968, 1mg/kg, 5mg/kg, and 30mg/kg by intraperitoneal administration biweekly for 4 weeks. Dosing was started at the average tumor size of approximately 190 mm³. Tumor volume was measured for each group.

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Mixed radiation with different doses induces CCL17 to recruit CD8⁺T cell to exert anti-tumor effects in non-small cell lung cancer

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Background: Different doses of radiotherapy (RT) exert diverse effects on tumor immunity, although the precise irradiation method remains unknown. This study sought to elucidate the influence of combining different doses of RT with immune checkpoint inhibitors (ICIs) on the infiltration of CD8⁺T cells within tumors, thereby augmenting the anti-tumor response.

Methods: Constructing a mouse model featuring bilateral lung cancer tumors subjected to high and low dose irradiation, the analysis of RNA transcriptome sequencing data and immunohistochemical validation for tumors exposed to various dosages guided the selection of the optimal low-dose irradiation scheme. Subsequently, upon the integration of immune checkpoint inhibitors (ICIs) therapy, the infiltration of immune cells within the tumor was ascertained via immunohistochemistry (IHC) and flow cytometry (FCM). Finally, through bioinformatics analysis and experimental verification, potential strategies to bolster the anti-tumor immune response were investigated.

Results: In comparison to the administration of 20Gy alone to the primary tumor, supplementing with 6Gy directed at the abscopal tumor produces a more pronounced abscopal response. The synergy of 20Gy, 6Gy, and ICIs markedly boosts the efficiency of ICIs. According to the findings from IHC and FCM studies, the triple therapy group exhibits a heightened infiltration of immune cells into the tumor, largely attributable to the augmented expression of CCL17 within the tumor under these irradiation regimens, which subsequently draws CD8⁺ T cells to infiltrate the tumor site, exerting cytotoxic effects.

Conclusion: Our study shows that the combined application of 20Gy and 6Gy can enhance the infiltration of tumor CD8⁺T cells in mice and improve the effectiveness of immunotherapy.

KEYWORDS

lung cancer, radiotherapy, animal research, immunotherapy, combination therapy

Introduction

Approximately 70% of cancer patients receive radiation therapy, which is regarded as one of the primary treatment modalities for cancer (1). Radiotherapy (RT) primarily exerts its therapeutic effect by inducing acute DNA damage, which triggers apoptosis or other forms of cell death in cancer cells (2). Technological advancements in RT, including four-dimensional computed tomography (4DCT), intensity-modulated RT (IMRT), respiratory gating techniques, and stereotactic ablative body RT (SABR), have facilitated higher tumor doses while reducing exposure to surrounding tissues (3). Consequently, an increasing number of patients are benefiting from these improvements (4). However, the efficacy of RT as a localized treatment remains limited, thereby constraining its application in the management of metastatic illness. In 1953, Mole and his colleagues were the first to define radiation-induced systemic antitumor effects that could shrink tumor lesions in irradiated areas and unirradiated regions (5). This phenomenon, known as the abscopal effect, offers a novel therapeutic approach for managing metastatic diseases, such as lung cancer that has spread to the brain, bone, adrenal glands, and face, as well as areas unsuitable for high doses of radiation therapy. Nevertheless, reports of distant effects caused by radiation therapy alone remain rare (6).

These observed medical phenomena prompted a search for methods to enhance the far-reaching effects of radiation therapy. In recent years, there has been increasing emphasis on the integration of immunotherapy with RT (7). A growing body of clinical trial- and pre-clinical data has demonstrated that immunotherapies significantly improve the radio-induced distancing effect (8–10). Research into the combined use of RT with anti-CTLA-4 and anti-PD-L1 antibodies in melanoma revealed that 26% of cases exhibited this distancing effect (11). While these findings suggest an enhancement of the distancing effect, the benefits appear to be limited to a minority of patients, with the majority still demonstrating poor therapeutic responses. The manifestation of distant effects can be influenced by various factors, including RT dosage and segmentation mode, immunotherapy modalities, the timing of interventions, and tumor type (12, 13). Consequently, it is imperative to explore novel strategies to optimize these combinatorial approaches. Further investigation is warranted to elucidate the mechanisms underlying the variability in treatment responses and to develop more effective therapeutic protocols that can benefit a broader patient population.

High-dose RT (HDRT) has been shown to elicit a vaccination-like response, to alter cancer cell characteristics to enhance their susceptibility to T cell-mediated attack, and to modify the tumor microenvironment to facilitate immune cell infiltration (14). However, for patients with recurrent and metastatic disease, the application of large-field, high-dose irradiation is often impractical. In contrast, low-dose RT (LDRT) plays a crucial role in modulating the tumor-immune microenvironment by promoting immune effector cell infiltration and mitigating the immunosuppressive effects of RT (15). Thus, utilizing a combination of RT regimens may be the optimal approach to induce antitumor immune response.

Lung cancer remains the leading cause of cancer-related mortality worldwide and ranks as the second most prevalent malignancy, with an estimated 2.2 million new cases and 1.8 million fatalities annually (16). Non-small cell lung cancer (NSCLC) accounts for 85% of lung cancer cases, representing the predominant pathological lung cancer subtype (17). Despite advances in treatment, NSCLC-related mortality continues to rise. A significant clinical challenge is that upon initial diagnosis, 75% of NSCLC patients present with metastatic disease, and the five-year survival rate for these patients ranges between 2.8% to 14.6% (18, 19). For patients with advanced stage III or IV lung cancer patients who are ineligible for surgical intervention, the standard treatment regimen comprises combined chemotherapy and RT (20). Over the past decade, the advent of immunotherapy has significantly improved outcomes for locally advanced NSCLC (through consolidation durvalumab following deterministic chemoradiotherapy) and advanced NSCLC (via immune checkpoint inhibitor monotherapy or in combination with chemotherapy) (21, 22). Nonetheless, these approaches have not yielded favorable results in patients with multiple metastases (23). Currently, combination RT and immunotherapy is primarily used in advanced non-metastatic clinical cases, with emerging evidence supporting its efficacy (24, 25). However, the response rate remains limited to 41.7% (26), underscoring the need for further optimization. Consequently, there is an urgent need to explore novel therapeutic strategies that can efficiently integrate RT and immunotherapy, maximizing their anticancer properties.

In this work, we observed several interesting phenomena. When administering a lethal radiation dose to primary metastases in NSCLC patients, we noted that other metastases along the treatment pathway received a lower doses. Subsequently, these patients received immunotherapy. Surprisingly, the metastases that received low-dose radiation showed significant regression following immunotherapy, while other sites remained unchanged. Based on these observations, we established a mixed irradiation model, applying a dose of 20 Gy to one tumor site and a dose less than 20 Gy to another site. Our findings revealed that the combination of 20 Gy and 6 Gy irradiation was optimal, enhancing the efficacy of immune checkpoint inhibitors (ICI) treatment. Specifically, 6 Gy treatment resulted in increased CD8⁺ T-cell infiltration at the tumor site, which correlated with elevated expression of the chemokine, CCL17. Our research demonstrates that the application of this mixed irradiation model significantly enhances the CD8⁺ T-cell infiltration in murine tumors and boost the efficacy of immunotherapy. These findings provide valuable insights into potential strategies for improving the outcomes of combined radiotherapy and immunotherapy in advanced NSCLC patients.

Materials and methods

Clinical data

Case 1: A 61-year-old male patient was diagnosed with upper left lung cancer in December 2020. In August 2021, he developed

metastases in the nasal region, left face, and right upper limb. Subsequently, in December 2021 the patient started treatment at the Fourth Affiliated Hospital of Soochow University, where he received nasal RT in combination with natalizumab.

Case 2: A 71-year-old male patient presented with a diagnosis of squamous cell carcinoma of the upper lobe of the left lung in May 2024. Positron Emission Tomography-Computed Tomography (PET-CT) imaging revealed increased Fluorodeoxyglucose (FDG) uptake in the left hilum lymph node (station 10), indicating a potential for metastatic involvement. Following the diagnostic workup, the patient underwent Stereotactic Body Radiation Therapy (SBRT) targeting the lesion in the left upper lobe. This was subsequently followed by three cycles of combination therapy, encompassing both chemotherapy and immunotherapy.

Case 3: A 59-year-old male patient was diagnosed with squamous cell carcinoma of the upper lobe of the right lung in July 2024. PET-CT imaging demonstrated increased FDG metabolism in the right upper tracheal lymph node (station 2R), hinting at metastatic involvement. Subsequently, the patient underwent SBRT to address the lesion in the upper lobe of the right lung. This therapeutic intervention was followed by three cycles of chemotherapy combined with immunotherapy.

Cell lines

The human T lymphocyte Jurkat cell line and the human H1299 NSCLC cell line were purchased from Shanghai Mingjin Biolo Gy Co., Ltd. (Shanghai, China), while the human A549 NSCLC cell line was purchased from the American Type Culture Collection (ATCC). The lines and mouse lung cancer cells (LLC) mouse lung cancer cell line was provided by Nanjing University. LLC and A549 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM; eallbio, China) containing 10% fetal bovine serum (FBS) and 1% penicillin–streptomycin. Jurkat and H1299 cells were cultured in RPMI 1640 medium (eallbio, China) containing 10% FBS and 1% penicillin–streptomycin. All cells were maintained at 37°C in a humidified atmosphere containing 5% CO₂.

Radiation parameters

LLC, H1299, and A549 cells were seeded into T25 culture flasks (Corning, USA). When cell density reached 75%–85%, the cells were exposed to X-ray irradiation doses of 3, 6, 9, and 12 Gy using a linear accelerator (Elekta Limited, Sweden) at a dose rate of 3 Gy per minute. During irradiation, a 1.5-cm-thick bolus was used to adjust the radiation distribution, with the T25 flask positioned on the treatment couch. The radiation properties were as follows: 6-MV photon beam energy, 100 cm source-surface distance, 10×10 cm² radiation field size, and 180° gantry angle. Dosimetry was performed using a cylindrical ionization chamber prior to irradiation.

Tumor models

Male C57BL/6 mice, aged six weeks and weighing 18 ± 2 g, were obtained from Shanghai SLAC Laboratory Animal Co., Ltd. (Shanghai, China) and maintained in a pathogen-free environment. To establish bilateral tumors, 4×10⁶ LLC cells were injected subcutaneously into the left hind limb to create the primary tumor, while an additional 4×10⁶ LLC tumor cells were implanted in the right limb to form the secondary tumor. When the secondary tumor reached approximately 500 mm³ in size, mice bearing bilateral tumors were randomly assigned to various treatment groups.

Tumor therapy

Tumor progression was monitored and quantified using previously described methods. Prior to radiation therapy, each mouse was shielded with a lead plate to ensure that only the targeted tumor was exposed to radiation. An Elekta Synergy instrument (Elekta limited, Sweden) was used to administer the radiation treatment. An anti-PD-L1 antibody (Bioxcell, USA) was administered via intraperitoneal injection, with an initial dose of 200 µg followed by subsequent doses of 100 µg every three days for a total of five injections. An anti-CTLA4 (Bioxcell, USA) was also administered intraperitoneally at a dose of 100 µg per mouse every three days for a total of five injections. In accordance with ethical standards, mice were euthanized when the implanted tumors reached a volume of 2000 mm³.

CD8A, PD-L1, and CTLA4 mRNA expression in different types of NSCLC in TIMER

The Tumor Immune Estimation Resource (TIMER) database contains 10,897 samples from 32 cancer types sourced from the Cancer Genome Atlas (TCGA). The differential expression of CD8A, PD-L1 (CD274), and CTLA4 between tumors and normal tissues was examined using the TCGA database, and the significance of the difference was determined using the Wilcoxon test. A p-value of less than 0.05 was considered significant.

Survival analysis of CCL17 in the Kaplan-Mayer plotter database

In this study, we utilized the Kaplan-Meier plotter database to investigate the relationship between the presence of CCL17 expression and survival duration in individuals with lung cancer. The database enables analysis of the impact of 54,000 genes (including mRNA, miRNA, and protein) on survival rates across 21 cancer types. Data for our analysis were obtained from the Gene Expression Omnibus (GEO), European Genome-phenome Archive (EGA), and TCGA databases, which provided comprehensive

information. To thoroughly examine the association between CCL17 expression in lung cancer and overall survival (OS), we calculated hazard ratios (HRs) with corresponding 95% confidence intervals (CI) and log-rank P-values (27). This approach allowed for a robust statistical assessment of the prognostic significance of CCL17 in lung cancer.

CCL17 expression and immune cell correlation in the TISIDB

The Tumor and Immune System Interaction Database (TISIDB) is a comprehensive resource designed to analyze relationships between tumors and various immune components, including tumor-infiltrating lymphocytes (TILs), immunomodulators, and major histocompatibility complexes (MHCs) (28). The TISIDB enables researchers to explore gene functions and their impact on tumor-immune interactions. We employed the Spearman correlation test to evaluate associations between prognostic genes and TILs. All hypothesis tests were conducted using two-sided considerations, with $P < 0.05$ considered statistically significant.

Flow cytometry

Tumor samples were excised, diced, and then suspended in phosphate buffered saline (PBS) to create a cell suspension. The tumor fragments were then digested with a combination of collagenase, deoxyribonuclease I, and hyaluronidase (Sigma, USA) for 50 minutes at 37°C in a temperature-controlled shaker. The resulting supernatant was filtered through a 70 µm mesh (BD Falcon, USA). Immune cells were isolated using a mouse tumor-infiltrating mononuclear cell isolation kit (Solar Bio, China) and resuspended in PBS at a concentration of 1×10^7 /mL. Subsequently, 100 µL of the cell suspension was stained with the fluorescently labeled anti-mouse antibodies: CD3-FITC, CD8-APC (all from Biolegend, USA) for 30 minutes on ice. After two PBS washes, the cells were resuspended for analysis using flow cytometry. These data were analyzed using NovoExpress software (Agilent, USA).

RNA sequencing analysis

Mice were euthanized by cervical dislocation, and tumors were excised, rinsed twice with DEPC-treated water, minced into small fragments, and transferred to cryostorage tubes. These samples were then stored in liquid nitrogen and sent to Shanghai OE Biotech Co., Ltd. (Shanghai, China) for sequencing. Libraries were sequenced on an Illumina NovaSeq 6000 platform, generating 150 bp paired-end reads. Approximately 50 million raw reads were obtained per sample. Raw reads in FASTQ format were processed using fastp (29) to remove low-quality reads, resulting in approximately 45 million clean reads per sample for subsequent analyses. Clean reads were mapped to the reference genome using HISAT2 (30). FPKM values for each gene were calculated, and read counts were obtained using HTSeq-count (31). Principal

Component Analysis (PCA) was performed using R (v3.2.0) to evaluate biological replication of samples. Differential expression analysis was conducted using DESeq2 (32), with a threshold of $q\text{-value} < 0.05$ and fold change > 1 or < 0.5 for significantly differentially expressed genes (DEGs). Hierarchical cluster analysis of DEGs was performed using R (v3.2.0) to illustrate gene expression patterns across different groups and samples. A radar map of the top 30 genes was generated using the R package ggadar to visualize up- and downregulated DEGs. Gene Ontology (GO), KEGG pathway, Reactome, and WikiPathways enrichment analyses of DEGs were performed using R (v3.2.0) based on the hypergeometric distribution to identify significantly enriched terms. Column diagrams, chord diagrams, and bubble plots were generated to visualize significant enrichment terms. Gene Set Enrichment Analysis (GSEA) was conducted using GSEA software (33). This analysis employed predefined gene sets, with genes ranked according to their differential expression between two sample types. The analysis then tested whether the predefined gene sets were enriched at the top or bottom of the ranking list.

Immunostaining

Tumor samples were fixed in 10% neutral buffered formalin, embedded in paraffin wax, and subsequently stained with hematoxylin and eosin (H&E) or subjected to immunohistochemistry. For immunostaining, tumor sections were deparaffinized, rehydrated, and subjected to antigen retrieval. Sections were then incubated overnight at 4°C with primary antibodies: rabbit anti-CD8 (1:100, Bioss, China), mouse anti-PD-L1 (1:200, Protein, China), mouse anti-CTLA-4 (1:50, Santa Cruz, China), and rabbit anti-CCL17 (1:200, Abcam, UK). Following primary antibody incubation, sections were treated with corresponding secondary antibodies (Genentech, USA). Immunoreactivity was then visualized using diaminobenzidine (DAB) as the chromogen. For quantification, multiple high-power fields were randomly selected from each section, and positively stained cells were counted per unit area.

ELISA

A549 cells, irradiated and unirradiated at various doses, were resuspended and plated in 24-well plates with 300 µL of complete medium. After 3 days, the supernatant was collected and CCL17 expression levels were evaluated using ELISA kits (Mlbio, China) according to the manufacturer's instructions.

qRT-PCR

Total RNA was extracted using TRIzol reagent (TaKaRa, Japan). Complementary DNA (cDNA) was synthesized using the Prime-Script RT reagent kit (CWbio, China). Radiation-induced mRNA expression was detected using UltraSYBR mixture (CWbio, China). Actin levels were used as a reference for normalization. The experiment was performed in triplicate, with RT-PCR reactions

repeated independently three times. Primer sequences are provided in the [Supplementary Table](#).

Migration assays

Jurkat cells (2×10^5 cells/mL) were plated in 200 μ L of serum-free medium (HyClone, USA) in the upper chamber of a 5 μ m Transwell insert (Corning, USA). The lower chamber contained 500 μ L of medium supplemented with 10% FBS (Albino, China) and either CCL17 chemokines (5 μ g/mL) (Sangon, China) or the cell supernatant from irradiated (9 Gy) A549 and H1299 cells, with or without anti-CCL17 mAb (R&D Systems, USA). After a 3-hour incubation, migrated cells were fixed with 4% paraformaldehyde, stained with 0.1% crystal violet for 10 minutes, and washed thrice with PBS. Quantification was performed by analyzing five randomly selected fields.

Statistical analysis

Data were analyzed using SPSS 22.0 and GraphPad Prism 9.4. Results are expressed as mean $\pm$ standard deviation (SD). Two-tailed Student's t-test was used for comparisons between two groups, while one-way analysis of variance (ANOVA) was employed for comparisons among multiple groups. Results with $P < 0.05$ were considered statistically significant.

Results

Combined RT and immunotherapy induces tumor shrinkage

Case 1 included a 61-year-old man with squamous cell carcinoma of the lung with nasal metastasis. The patient presented to the hospital with primary lung cancer of the left upper lung (December 2020). Later, the patient developed nasal metastasis (August 2021). Nasal radiotherapy (60 Gy/30 f) (December 2021) was performed on the patient. During radiotherapy, the patient's left face and left jaw developed new tumors from the previous ones ([Figure 1A](#)). Due to the large irradiation range of the patient's face, radiotherapy was not performed for the new tumors considering the patient's facial injuries and other problems. Low-dose radiotherapy (≈ 20 Gy/30 f) was administered to the left facial tumor in the irradiation path of the nasal tumor, and the nasal tumor significantly shrank after the end of radiotherapy, but the left facial tumor did not shrink. Two cycles of anti-PD-L1 (Etrilizumab) therapy (January 2022) were subsequently performed, and the new tumors on the left face significantly subsided (April 2022) ([Figure 1B](#)). However, no significant shrinkage was observed in the lung and left mandibular areas that were not irradiated ([Figures 1C, D](#)).

Case 2 involved a 71-year-old male who was diagnosed with squamous cell carcinoma of the upper left lung lobe in May 2024. PET-CT imaging revealed increased FDG uptake in the left hilar

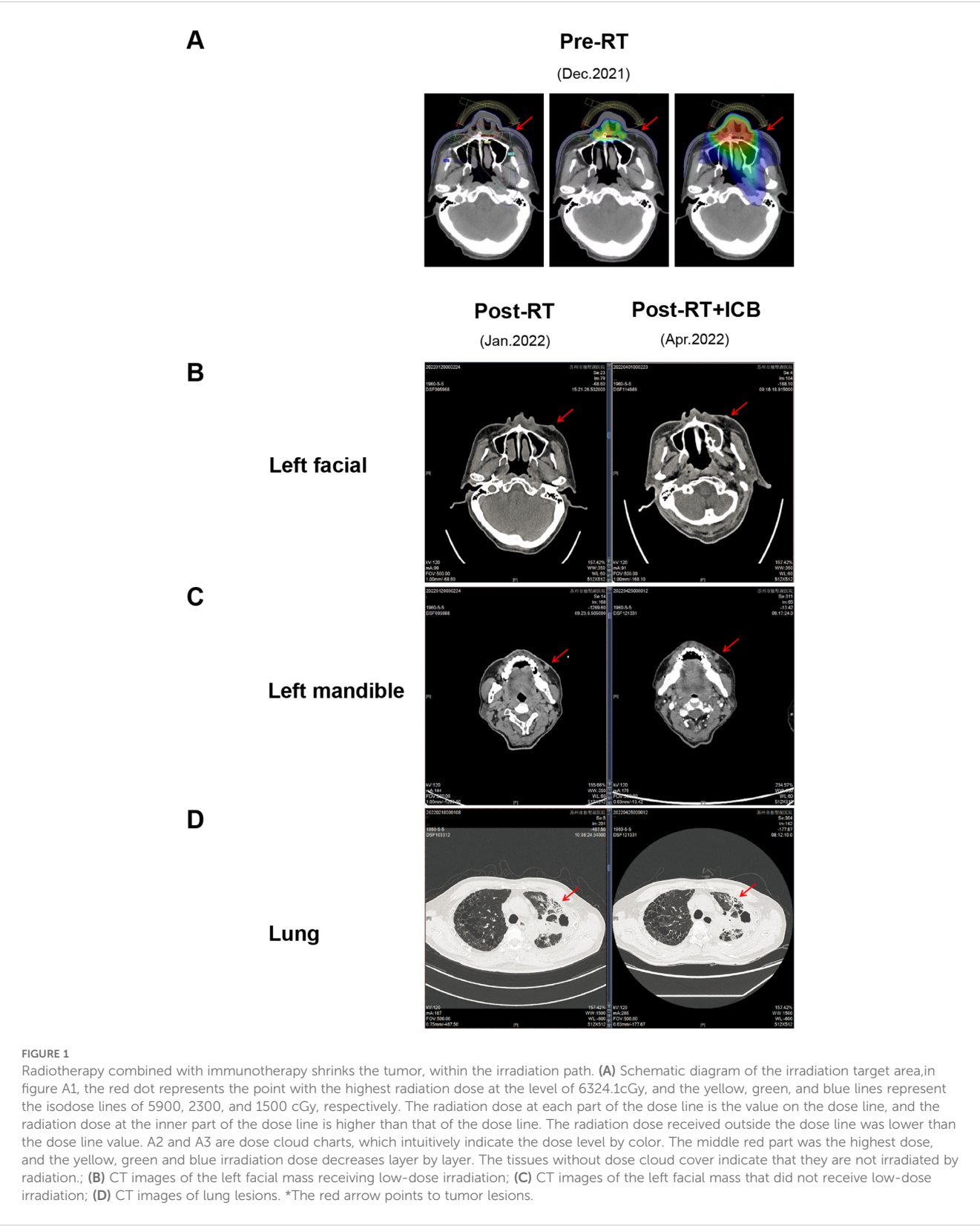
lymph node (station 10), suggesting tumor metastasis to this location ([Supplementary Figure S1A](#)). Subsequently, in June 2024, the patient underwent SBRT with the upper left lung lobe tumor receiving a dose of 24 Gy/3f, while the adjacent 10L lymph node received a low dose of 5 Gy ([Supplementary Figure S1B](#)). After completing three cycles of chemotherapy combined with immunotherapy, a CT scan showed a reduction in the size of the upper left lung tumor and alleviation of atelectasis ([Supplementary Figure S2](#)). Following this, the patient underwent radical surgery, and postoperative pathology confirmed a complete pathological response in the hilar lymph nodes at station 10.

In Case 3, the patient is a 59-year-old male who was diagnosed with squamous cell carcinoma of the upper right lung lobe in July 2024. PET-CT imaging revealed increased FDG metabolism in the right upper paratracheal lymph node (station 2R), suggesting a possibility of metastasis ([Supplementary Figure S3A](#)). Subsequently, starting from August 2024, the lesion in the upper right lung lobe underwent SBRT with a prescribed dose of 24 Gy delivered in 3 fractions. The adjacent 2R lymph node received a low dose of 5 Gy for the majority of its volume, while the more distant cephalic portion of the lesion received only a dose of 3 Gy ([Supplementary Figure S3B](#)). Following three cycles of chemotherapy combined with immunotherapy, a CT scan demonstrated a partial response of the tumor lesion ([Supplementary Figure S4](#)). Later, in October 2024, a lymph node biopsy was performed, and postoperative pathology indicated a complete pathological response in the lymph nodes of station 2R.

These cases suggest that the combination therapy of HDRT + LDRT + ICI may further activate the immune response and enhance anti-tumor efficacy. Therefore, we intend to explore whether this combination therapy mode can be used in some special cases of patients with multiple tumors or multiple metastases that are not suitable for large-area irradiation.

Mixed-dose radiation therapy inhibits tumor growth

To investigate the effects of diverse irradiation doses on tumor growth inhibition and immune cell recruitment, we developed a bilateral LLC lung cancer model in C57BL/6J mice. To minimize radiation-induced damage to normal tissues, tumor cells were subcutaneously implanted into both lower limbs. The left limb received a uniform dose of 20 Gy, designated as the "primary tumor," while the right limb was irradiated with varying doses of 3, 6, 9, or 12 Gy, termed the "secondary tumor" ([Figure 2A](#)). Tumor size was measured every other day following the initiation of irradiation. Our results demonstrated that the curative dose of 20 Gy effectively suppressed the growth of the "primary tumor" across all groups, with no significant differences in tumor size ([Figures 2B, C](#)). By day 9 post-irradiation, the average tumor volume in the "secondary tumor" groups receiving 3, 6, 9, and 12 Gy was reduced compared to the non-irradiated group, with the 20 + 6 Gy group exhibiting the most pronounced tumor inhibition ([Figures 2B, D](#)). To assess treatment-related toxicities, we monitored changes in mouse body weight and found no significant differences among the groups ([Figure 2E](#)). In



conclusion, combined treatments using various irradiation dose regimens demonstrated inhibitory effects on tumor growth in mice, with the 20 + 6 Gy group exerting the most substantial influence. Notably, this treatment approach did not induce significant toxic side effects in the mice.

The 20 + 6 Gy group demonstrates optimal RT efficacy

To investigate gene expression in the “secondary tumor” post-irradiation, we collected mouse tumor samples for RNA sequencing.

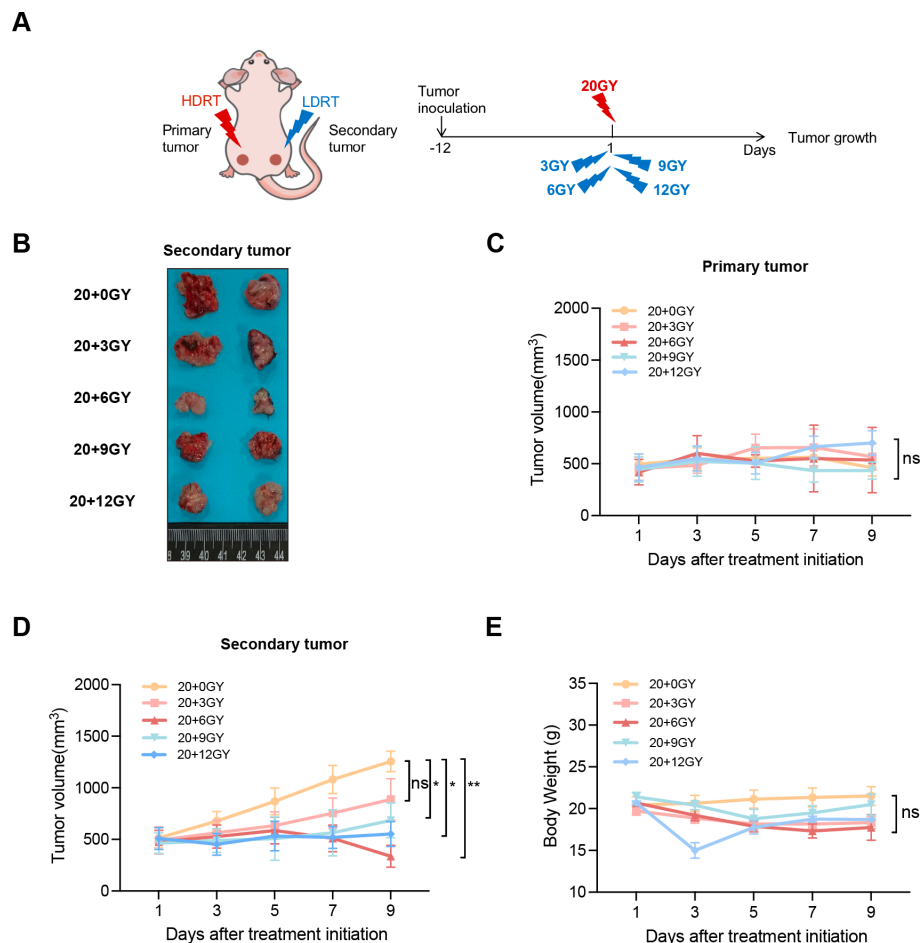
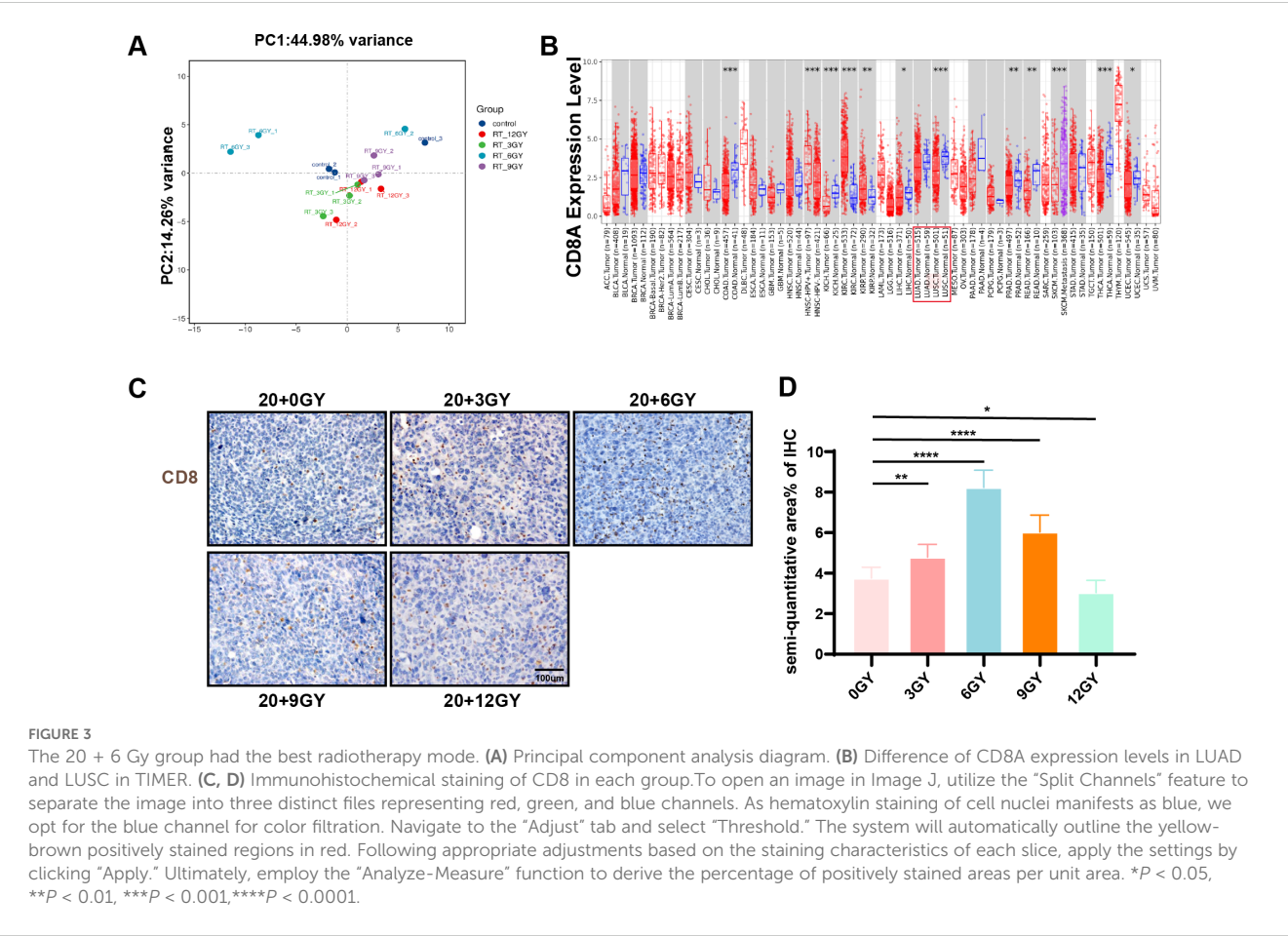


FIGURE 2

The combination of mixed dose radiation therapy mode inhibits tumor growth. (A) Treatment scheme. (B) Secondary tumor physical image. (C, D) In the mouse model constructed by LLC (n = 6–8 mice/group/time point, one-way analysis of variance), 4×10^6 LLC cells were injected subcutaneously into the left hind limb to create the primary tumor, and an additional 4×10^6 LLC tumor cells were implanted in the right limb to form the secondary tumor. The tumor volume was measured at intervals of one day commencing from seven days after tumor implantation. primary tumor (C) and secondary tumor (D) tumor growth curves. (E) Weight gain curve of irradiated mice. ns $P > 0.05$, * $P < 0.05$, ** $P < 0.01$.

Transcriptomic analysis was performed on the sequencing data, integrating gene detection values to generate expression box plots and conduct PCA (Figure 3A). While reproducibility across groups was generally satisfactory, occasional biased data points were observed. Consequently, we selected data points exhibiting reasonable repeatability between sample groups for subsequent analysis. Our findings revealed that the 20 + 6 Gy treatment regimen induced the most pronounced variations in gene expression within the “secondary tumor.” KEGG pathway enrichment analysis of DEGs across groups (Supplementary Figure S5A) indicated that the majority of these genes were primarily implicated in immune system functions. Given that CD8⁺ T cells are central to antineoplastic immunity, we focused our analysis on CD8 expression across various tumors. Using the TIMER database, we assessed CD8 expression between TCGA tumors and adjacent normal tissues. Notably, CD8 expression was significantly reduced in lung adenocarcinoma (LUAD) and lung squamous cell carcinoma (LUSC) tissues compared to adjacent normal tissues (Figure 3B). Additionally, CTLA4 expression was increased, while PD-L1

expression decreased in LUAD and LUSC tissues (Supplementary Figures S5B, D). CD8⁺ T-cell infiltration is frequently associated with favorable prognosis in solid tumors (34). Using the Kaplan-Meier plotter database, we investigated the correlation between these factors and lung cancer patient survival, finding that PD-L1 and CTLA4 did not significantly affect OS (Supplementary Figures S5C, E). Integrating transcriptomic analysis results, we performed pathway enrichment on immune system-related DEGs across groups (Supplementary Figure S5F). The 20 + 6 Gy group exhibited the highest number of upregulated differential genes. Further examination via the GEPIA database revealed that the majority of these upregulated genes were expressed at higher levels in normal lung tissues, suggesting that changes in the 6 Gy irradiated group might potentially improve lung cancer prognosis. Immunohistochemical staining for CD8 on tumor samples demonstrated that CD8⁺ T-cell infiltration was most pronounced in the 20 + 6 Gy group (Figures 3C, D). These findings confirm that the 20 + 6 Gy regimen represents the optimal combined irradiation dose for effectively recruiting CD8⁺ T cells.



Combined treatment with 20 + 6 Gy irradiation and ICIs enhances tumor suppression and immune activation

To further validate the efficacy of combined RT and immunotherapy in tumor treatment, we incorporated two ICIs, anti-PD-L1 and anti-CTLA4, with RT in the 20 + 6 Gy group (Figure 4A). Nine days post-irradiation, the average tumor volume of the "secondary tumor" was significantly reduced in the 20 + 6 Gy + α PD-L1 group (293 mm³) and the 20 + 6 Gy + α PD-L1 + α CTLA4 group (235 mm³) compared to the 20 + 6 Gy group (395 mm³) (Figures 4B, C). Notably, the combination of both ICIs resulted in more pronounced tumor suppression, with statistically significant differences observed. These findings demonstrate that LDRT combined with ICI treatment further enhances the inhibitory effect on mouse tumors without inducing apparent adverse effects. To assess tumor-immune infiltration, we analyzed CD8⁺ cell populations within CD3⁺ cells as characteristic markers for infiltrating T-cell populations. In the 20 + 6 Gy, 20 + 6 Gy + α PD-L1, and 20 + 6 Gy + α PD-L1 + α CTLA4 groups, the percentages of CD8⁺ T cells were 2.12%, 11.13%, and 20.69%, respectively (Figures 4D, E). Immunohistochemical staining of mouse tumors corroborated these findings, revealing increased infiltration of CD8⁺ T cells in tumors treated with combined RT and ICIs compared to those treated with RT alone (Figures 4F, G). These results suggest

that combined treatment with 20 + 6 Gy irradiation and ICIs can further enhance tumor inhibition and immune activation within the tumor microenvironment in mice.

The 20 + 6 Gy irradiation regimen enhances intracellular CCL17 secretion and correlates with improved prognosis in NSCLC

To elucidate the mechanism by which the 20 + 6 Gy group maximally activates the immune system, we conducted a comprehensive analysis of tumor RNA sequencing results. Among the DEGs related to the immune system in the 20 + 6 Gy group, 23 genes were upregulated and two were downregulated. We analyzed the expression of these genes in cancerous and adjacent normal tissues using the TIMER database, categorizing upregulated genes highly expressed in adjacent normal tissues as "up genes," suggesting their potential to improve patient prognosis. Through rigorous screening, we identified 19 "up genes" (Figure 5A). Analysis of differences in expression levels across experimental and control groups (Figure 5B) revealed CCL17 as the most significantly upregulated gene in the 20 + 6 Gy group, prompting its selection as a potential target for antitumor effects in subsequent studies. Using the TIMER database, we analyzed CCL17 expression

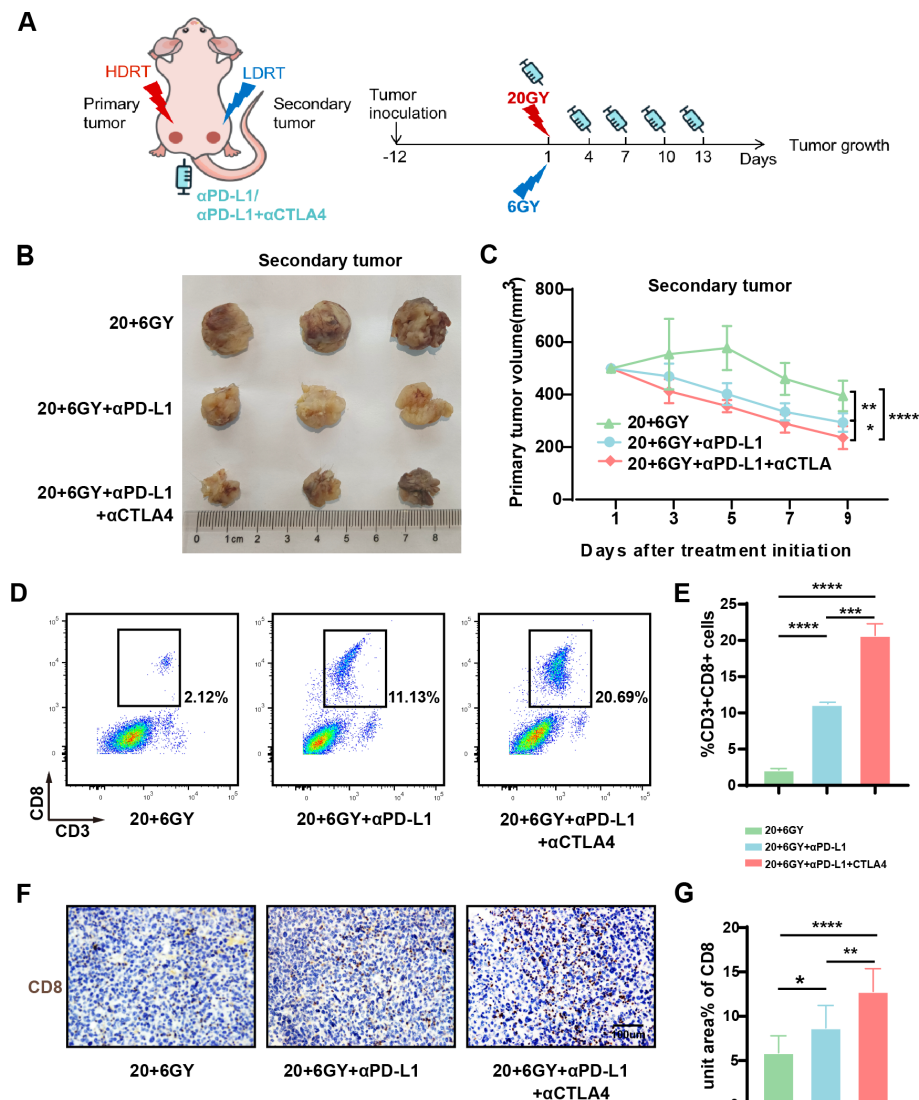


FIGURE 4

The combination of mixed dose radiation therapy mode combined with ICIs further inhibit tumor growth and activate the tumor immune microenvironment. (A) Treatment scheme. (B) Secondary tumor physical image. (C) Secondary tumor growth curve ($n = 7-9$ mice/group/time point, one-way analysis of variance). (D, E) Flow cytometry analysis of CD8⁺ T (CD3⁺CD8⁺ cells) in the tumors of each group ($n = 3$ mice/group, one-way analysis of variance); (F, G) Immunohistochemical maps of CD8 in the tumors of mice in each group. To open an image in Image J, utilize the "Split Channels" feature to separate the image into three distinct files representing red, green, and blue channels. As hematoxylin staining of cell nuclei manifests as blue, we opt for the blue channel for color filtration. Navigate to the "Adjust" tab and select "Threshold." The system will automatically outline the yellow-brown positively stained regions in red. Following appropriate adjustments based on the staining characteristics of each slice, apply the settings by clicking "Apply." Ultimately, employ the "Analyze-Measure" function to derive the percentage of positively stained areas per unit area. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$.

across all TCGA tumor types, focusing on the contrast between tumor tissues and adjacent normal tissues (Figure 5C). CCL17 expression was significantly lower in LUAD and LUSC tissues compared to adjacent normal tissues ($p < 0.0001$). Investigation of the relationship between CCL17 expression levels and survival outcomes in lung cancer patients using the Kaplan-Meier mapper database demonstrated that high CCL17 expression was significantly associated with favorable prognosis (OS, HR=0.79 (0.70-0.89), $p = 0.00012$) (Figure 5D). Furthermore, exploration of the correlation between CCL17 expression and key pathways in NSCLC patients (Figures 5E, F) revealed a positive correlation with pro-inflammatory pathways and a significant negative correlation

with tumor proliferation pathways. These findings suggest that in patients with high expression and favorable prognosis, CCL17 likely exerts dual effects of promoting inflammation and inhibiting tumor proliferation.

CCL17 promotes T-cell infiltration and activation in tumors

When investigating the correlation between CCL17 expression and immune cell infiltration in tumors, initial analysis using the TISIDB database revealed a positive

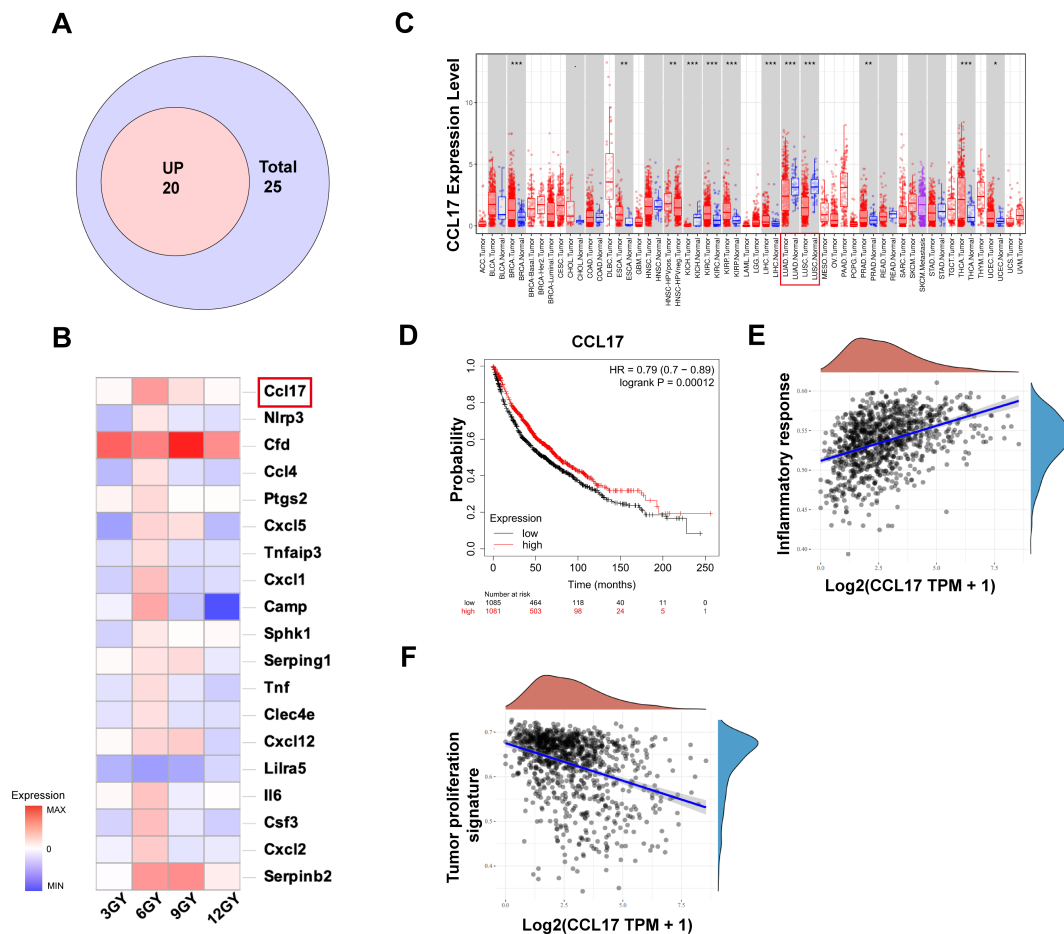


FIGURE 5

The combination of mixed dose radiation therapy mode increases intracellular CCL17 secretion, and its high expression is associated with good prognosis in non-small cell lung cancer. (A) The Venn diagram of the “up” gene. (B) Visualization analysis of differentially expressed genes in different dosage groups compared to the 20 + 0 Gy group. (C) Differences in the expression levels of CCL17 in LUAD and LUSC in IMER database; (D) Kaplan–Meier plotter database CCL17 and overall survival of patients with lung cancer were analyzed and progression-free survival curves. (E) Association of CCL17 with pro-inflammatory pathways in non-small cell lung cancer. (F) Correlation of CCL17 with pro-inflammatory pathways in non-small cell lung cancer. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

correlation between CCL17 expression and CD4 and CD8 expression (Supplementary Figure S6A). To validate these findings, we performed immunohistochemistry and multiple immunofluorescence staining for CD8 and CCL17 on tumor sections from the initial mouse group (Figure 6A–C, 3G, Supplementary Figure S6B). The 20 + 6 Gy group showed the highest expression of CD8 and CCL17 compared to other groups, with an increased CD8⁺ T-cell population. These results suggest that increased CCL17 production may facilitate CD8⁺ T-cell infiltration in the tumor-immune microenvironment. We hypothesize that CCL17 exerts a chemotactic function on T cells within the tumor-immune microenvironment, attracting activated CD8⁺ T cells to the tumor site to elicit antitumor effects. Previous *in vivo* studies have demonstrated that CCL17 enhances T-cell infiltration into the tumor microenvironment. We subsequently validated these findings through *in vitro* experiments using two common NSCLC cell LLC. Irradiated progeny cells were exposed to doses of 3, 6, 9, and 12 Gy, and their survival was monitored (Supplementary Figures S6C–E).

ELISA analysis of irradiated progeny cell supernatants revealed an overall increase in CCL17 secretion compared to unirradiated tumor cells. However, this increase peaked at a specific dose before decreasing, indicating that CCL17 expression is not linearly dependent on radiation dose. Notably, CCL17 levels in LLC supernatants peaked at 6 Gy, which is consistent with *in vivo* data, while A549 and H1299 cell supernatants showed peak CCL17 levels at 9 Gy. To investigate T-cell activation, Jurkat cells were cultured with varying concentrations of CCL17 cytokines. After 72 hours, qPCR analysis of T-cell activation markers (IL-2, granzyme, and perforin) revealed a significant increase in Jurkat cell activation, up to 7-fold, following CCL17 introduction (Figures 6D–F). Co-culture of Jurkat cells with irradiated and unirradiated lung cancer cells for 72 hours further enhanced T-cell activation (Figures 6G–I). Moreover, transwell assays demonstrated that CCL17 enhanced Jurkat cell migration (Figure 6J), while the addition of a CCL17 neutralizing antibody to A549/9 Gy and H1299/9 Gy supernatants inhibited this migration (Figures 6K, L).

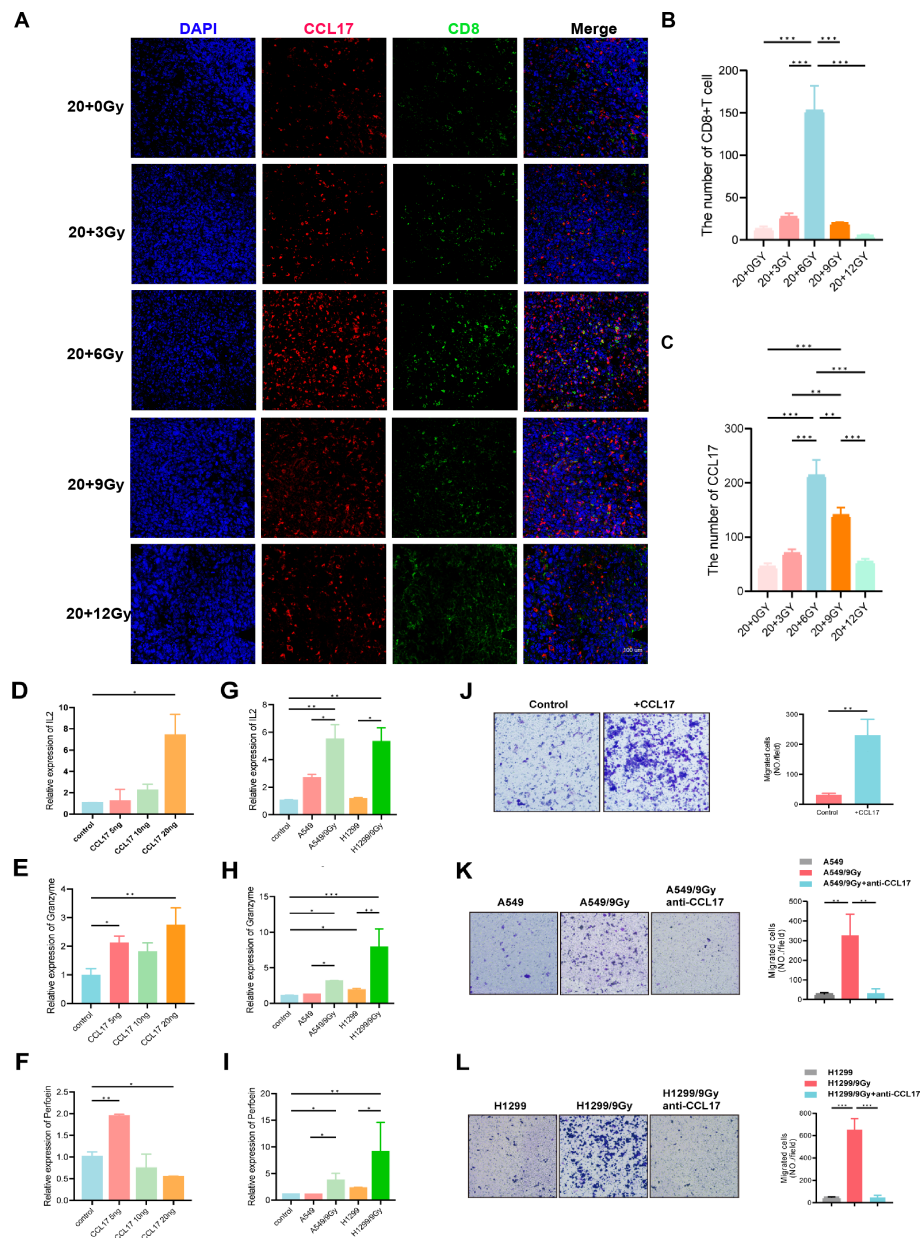


FIGURE 6

CCL17 promotes T-cell infiltration and activation in tumors. (A–C) Multiple immunofluorescence of CD8 and CCL17. (D–F) A total of 400,000 Jurkat T cells were inoculated into 6-well plates, and different concentrations of CCL17 cytokines were added to the medium. RNA was extracted after 72 h of culture. The T-cell activation indices IL-2, granzyme and perforin were determined by qPCR. (G–I) A total of 400,000 Jurkat T cells were inoculated into 6-well plates, and Jurkat T cells were cocultured with non-small cell lung cancer cells with different irradiation treatments. RNA was extracted 72 hours later. The T-cell activation indices IL-2, granzyme and perforin were determined by qPCR. (J) The migration ability of Jurkat cells was measured by adding CCL17 chemokines to the downward chamber. (K, L) The supernatant of A549/9Gy or H1299/9Gy cells was added to the lower chamber, and anti-CCL17 was added to detect the migration ability of Jurkat cells. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

To summarize, bioinformatic analysis revealed a positive correlation between CD4 and CD8 expression and CCL17. In addition, immunohistochemistry and multiple immunofluorescence studies demonstrated that increased CCL17 expression in tumors corresponded with increased CD8 infiltration. Further, *in vitro* studies confirmed substantial CCL17 chemokine secretion from precursor cells, which stimulated T-cell activation and migration.

Discussion

Moore initially described systemic antitumor effects caused by radiation in 1953, associating them with stimulation of systemic immunity (35). However, distant reactions in clinical settings have been comparatively infrequent (26). Several factors, including radiation dosage, segmentation, chemotherapy, and the host's immune microenvironment, appear to influence the induction of

distant responses (36). Consequently, an appropriate RT combination and an associated regimen (i.e., radiation dosage and pattern) is required to achieve a successful response in distant areas. For combination therapy with different irradiation doses, we utilized an *in vivo* model of mouse lung cancer cells (LLC) to select the optimal combination dose. Our approach activates adaptive immunity, specifically cytotoxic CD8⁺ T cells, and potentiates systemic effects using ICIs like PD-L1 inhibitors and CTLA4 blockers.

Most lung cancer patients present with metastases at diagnosis, indicating advanced-stage cancer with a remarkably low 5-year survival rate (18, 19). High tumor load makes HDRT administration to all lesions impractical due to potential toxicity. For individuals with intact immune systems, applying relatively low radiation doses to small lesions may boost antitumor defenses (37). However, exactly how the doses should be combined has not been reported. Our data showed the most effective treatment involved 6 Gy irradiation. Wang et al. also found that 6 Gy irradiation upregulates tumor-infiltration molecules like intracellular adhesion molecule-1 (ICAM-1) or FAS in HCT-15 cells, supporting a possible synergistic enhancement effect of RT. In addition, 6 Gy irradiation has also been shown to activate the immune microenvironment in solid tumors (38). Typically, a cold tumor that has not been irradiated will exhibit a low density of activated CD8⁺ T cells and non-self CD8⁺ T cells in the primary tumor. By various means, we verified that the combined irradiation pattern of 20 + 6 Gy enhances CD8⁺ T-cell activation at the primary tumor site. In addition, we found that CCL17 expression was upregulated in 20 + 6 Gy irradiated mouse tumors. CCL17, a potent chemokine produced by thymus and antigen-presenting cells, binds to the CCR4 receptor and plays a crucial role in CD8⁺ T-cell function and migration (39). Interestingly, Ye et al. hypothesize that the OS of patients that show upregulated CCL17 expression is higher than patients with lower CCL17 expression, especially for patients with early (stages I and II) LUAD (40). Additionally, CCL17 can potentially act as an anticancer agent by introducing TILs into tumors (41). Valente et al. suggest that cross-talk between iNKT cells and CD8⁺ T cells in the spleen requires the IL-4/CCL17 axis for the generation of short-lived effector cells (42). In addition, other studies indicate that dendritic cell-produced IL-12 augments CD8⁺ T-cell activation through the production of the chemokines, CCL1 and CCL17. Taken together, these data reflect the pleiotropic role of CCL17 and its ligand, CCR4, in immune responses (43). By analyzing public databases, we show that tumors demonstrate decreased CD8⁺ T-cell densities and decreased CCL17 expression compared to normal tissues. In addition, we show that the mechanism by which tumors facilitate the involvement of non-natural CD8⁺ T cells and enhance tumor resistance following 6 Gy treatment is through the release of CCL17. Further investigations are required to determine the potential collaborative role of other factors in promoting the activation and migration of CD8⁺ T cells.

Recent immunotherapy research for NSCLC has shown progress, but low objective remission rates (ORRs) remain a drawback. First-line monotherapy achieves an ORR of 45%, whereas second-line therapy only results in a 20% ORR. In

addition, immunotherapy carries a risk of hyper- (6%–29%) and pseudo progression (4.5%) (44). Earlier research on NSCLC has indicated that the use of ICIs along with RT improves the response to immunotherapy and extends the OS period (26). Our data further demonstrated that PD-L1 and anti-CTLA4 combined with RT significantly inhibited tumor growth. Despite this, the clinical applicability of this treatment strategy is yet to be determined.

In this study, we explored the effects of different radiation levels on the immune microenvironment through various dose combinations. Investigating different ways of boosting the immune system in cancer is crucial for minimizing radiation-induced harm and enhancing immunotherapy efficacy. Nonetheless, the uniform radiation distribution observed in this work is somewhat tedious, and forthcoming research may favor the use of aggregate accumulated dosage of multi-fraction treatment. Importantly, our study was limited to animal experiments, and future clinical trials are crucial. Further investigation is required to evaluate the frequency of this reaction in alternative cancer models and to ascertain the respective functions of the natural and adaptive immune systems in these impacts.

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 humans were approved by Institutional Review Board of the Fourth Affiliated Hospital of Soochow University. The studies were conducted in accordance with the local legislation and institutional requirements. The participants provided their written informed consent to participate in this study. The animal study was approved by Institutional Animal Care and Use Committee of the Affiliated Hospital of Jiangnan University. The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

LH: Data curation, Formal analysis, Investigation, Methodology, Validation, Writing – original draft, Software. DW: Writing – review & editing, Data curation, Formal analysis, Validation. MX: Investigation, Project administration, Software, Supervision, Visualization, Writing – review & editing. DQ: Software, Supervision, Writing – review & editing. YC: Investigation, Supervision, Writing – review & editing. XW: Investigation, Methodology, Writing – review & editing. LM: Investigation, Methodology, Writing – review & editing. JT: Investigation, Methodology, Writing – review & editing. ZH: Conceptualization, Funding acquisition, Methodology, Supervision, Writing – review & editing. YY: Conceptualization, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Supervision, Validation, Visualization,

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Supplementary material

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Big lessons from the little *Akkermansia muciniphila* in hepatocellular carcinoma

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Hepatocellular carcinoma (HCC) is the most frequently occurring type of liver tumor and is considered one of the most common primary malignant neoplasms. The prognosis for HCC is dismal because of its complicated etiology and high level of medication resistance. Immunotherapy is presently regarded as one of the most effective therapeutic options for HCC; nevertheless, because of the disturbance of intestinal flora, immunotherapy shows low antitumor efficacy. An increasing body of research indicates that intestinal flora, particularly *Akkermansia muciniphila* (*A. muciniphila*), is vital for the treatment of tumors. Studies have demonstrated that the diminished effectiveness of immunotherapy in cancer patients is associated with a reduction in *A. muciniphila* levels, suggesting that increasing *A. muciniphila* levels significantly enhance the efficacy of immunotherapy. *A. muciniphila* functions as a gut probiotic and can treat and prevent a wide range of illnesses, including cancer. Consequently, preserving *A. muciniphila* abundance is enough to prevent and lower the danger of developing cancer disorders. In this review, we critically evaluate the current body of research on *A. muciniphila*, with a primary focus on its biological properties and functions. The different illnesses that *A. muciniphila* treats were then discussed, particularly the way it works with liver cancer. This review aims to give a novel treatment plan for patients with HCC as well as a theoretical foundation for improving HCC immunotherapy.

KEYWORDS

hepatocellular carcinoma, *Akkermansia muciniphila*, immunotherapy, traditional Chinese medicine, intestinal flora

1 Introduction

Liver cancer is the most prevalent type of primary tumor, ranking third in cancer mortality and sixth in incidence, with hepatocellular carcinoma (HCC) being its main type (1). Presently, hepatectomy is the main radical treatment for patients with early liver cancer, and radiofrequency ablation can be selected for patients who cannot be operated (2). Transcatheter arterial chemoembolization (TACE) is mainly used in patients with mid-term HCC (2). When TACE treatment fails in monkeys, systemic treatment will be

recommended (3). Systemic treatment is suitable for most patients with HCC in intermediate and advanced stages. Current first-line treatment includes atezolizumab combined with bevacizumab, sorafenib, and lenvatinib (2). In recent years, the advent of immunotherapies, including immune checkpoint inhibitors (ICIs), has brought new hope to cancer patients, such as anti-programmed cell death 1 (PD-1)/L1 antibodies, anticytotoxic T lymphocyte-associated antigen-4 (CTLA-4) antibodies, and anti-lymphocyte-activating gene-3 (LAG3) antibodies (4–6). A study reported that the median overall survival (mOS) of atezolizumab combined with bevacizumab was longer than that of sorafenib (7). Although much work has been done to enhance responses and eliminate toxicities, the response of HCC patients to ICI remains unsatisfactory.

In recent years, there is growing evidence that there is a correlation between intestinal flora and the efficacy of tumor immunotherapy (5, 8). Studies have shown that after receiving ICI treatment, analysis of fecal samples from patients has found a decrease in the diversity and richness of intestinal flora, which is associated with low response rates and survival rates in patients (9, 10). For example, disorders of intestinal flora may promote tumor progression, through mechanisms including the production of toxic metabolites, induction of an inflammatory environment, and suppression of antitumor immunity (10). Thus, in mouse models of tumors, mice receiving ICI had reduced antitumor effects after antibiotic treatment (11). However, intestinal flora regulates the efficacy and toxicity of cancer immunotherapy, and the most researched is improving immunotherapy and its associated side effects (10). The most renowned study involved the transplantation of fecal matter from patients who had shown a positive response to immunotherapy into germ-free mice, revealing that these mice exhibited enhanced immunity, significantly inhibited tumor growth, and enhanced the effectiveness of anti-PD-L1 therapy (12, 13). Therefore, targeted regulation of intestinal flora is one of the effective ways to improve the efficacy of immunotherapy in tumors.

Abbreviations: AmEVs, *A. muciniphila* extracellular vesicles; AmTARS, *A. muciniphila* threonyl-tRNA synthetase; CCL4, carbon tetrachloride; CREB, cAMP response element-binding; DCA, deoxycholic acid; DEN, N-nitrosodiethylamine; ERIC-PCR, enterobacterial repetitive intergenic consensus; FUBP1, far upstream element binding protein 1; GLP-1, glucagon-like peptide-1; GPR43, G-protein-coupled receptor 43; H3K14ac, acetyltransferase on Lys14 of histone H3; HFD, high-fat diet; HSCs, hepatic stellate cells; HSP70, heat shock protein 70; ICAM-2, intercellular adhesion molecule 2; ICI, immune checkpoint inhibitor; ISC, intestinal stem cell; LPS, lipopolysaccharide; MAMPs, microbe-associated molecular patterns; mOS, median overall survival; Muc2, mucin 2; NAFLD, non-alcoholic fatty liver disease; NASH, non-alcoholic steatohepatitis; NKT, natural killer T; NLRP3, NLR family pyrin domain 3; NSCLC, non-small cell lung cancer; ROS, reactive oxygen species; SCFAs, short-chain fatty acids; SASP, senescence-associated secretory phenotype; TACE, transcatheter arterial chemoembolization; T2D, type 2 diabetes; TLR2, Toll-like receptor 2; TME, tumor microenvironment; TME, tumor microenvironment.

Akkermansia muciniphila (*A. muciniphila*), a bacterium that inhabits the intestinal mucus layer, is recognized as the most prevalent lytic bacteria in the intestines of healthy hosts and plays a crucial role in maintaining the intestinal barrier, promoting mucus production, and regulating mucus layer thickness (14, 15). The specific mechanism is that *A. muciniphila* reduces intestinal permeability, increases the expression of tight junction proteins, and increases the number of goblet cells that produce mucins (16–18). Furthermore, *A. muciniphila* has been implicated in the progression of various diseases, including inflammatory bowel diseases (19, 20), aging-related conditions (21), obesity (22, 23), type 2 diabetes (T2D) (24, 25), cardiovascular disorders (26, 27), non-alcoholic fatty liver disease (NAFLD) (28, 29), and cancers (11). Another study showed that whether *A. muciniphila* is live or pasteurized, oral administration is safe for human volunteers (30). In 2021, the European Food Safety Authority (EFSA) affirmed the safety of pasteurized *A. muciniphila* in adults and authorized its usage as a food supplement (31). Consequently, elucidating the molecular and immune response mechanisms of *A. muciniphila* in disease contexts, as well as determining safe supplementation strategies for *A. muciniphila* during disease states, is crucial for the exploration of innovative therapeutic approaches aimed at enhancing prevention and treatment outcomes.

This review seeks to clarify the connection between *A. muciniphila* and HCC, particularly in its role as an adjuvant in immunotherapy, while also elucidating the possible mechanisms behind the antitumor effects of *A. muciniphila*. Therefore, this review seeks to propose an innovative treatment strategy for patients with HCC while providing a theoretical framework to enhance immunotherapy approaches for HCC.

2 The background introduction of *A. muciniphila*

A. muciniphila, a common intestinal anaerobic bacterium, was first successfully isolated in 2004 (15). It is widely distributed in the intestines of humans and animals and has the largest number in the cecum, accounting for 3%–5% of the human microbiome (15, 32). *A. muciniphila* relies solely on mucin as its primary source of carbon, nitrogen, and energy, thereby supplying energy to epithelial cells (33). The main function of *A. muciniphila* involves the breakdown of mucus, facilitated by a range of mucolytic enzymes encoded in its genetic material (34, 35). In addition, *A. muciniphila* and its functional active components, including metabolites, metabolic enzymes, and outer membrane proteins, effectively improve intestinal barrier function by regulating metabolism and enhancing tight junctions (36–38). Interestingly, *A. muciniphila* commenced colonization and exhibited rapid growth and enrichment in the human intestines during infancy (39). However, its abundance progressively declined with aging and the progression of disease (39, 40). Therefore, *A. muciniphila* supplementation has demonstrated therapeutic benefits for a range of diseases, including liver diseases, colitis, obesity, and diabetes (40). At present, there are many traditional Chinese medicine formulas, individual Chinese medicinal materials, and

extracts to increase the number of *A. muciniphila*, including Si-Ni-San (41), Liangxue guyuan yishen decoction (42), wolfberry (43), berberine (44), and *Dendrobium officinale* polysaccharide (45). In summary, increasing the abundance of *A. muciniphila* has become one of the effective treatment measures for many diseases.

2.1 Biological characteristics of *A. muciniphila*

Akkermansia muciniphila is classified within the *Akkermansia* genus of the *Verrucomicrobia* (15, 34). Moreover, in addition to *A. muciniphila*, this study also includes other species within the genus *Akkermansia*, such as *massiliensis* (46), *biwaensis* (47), *ignis* (48), *durhamii*, and others with less precise descriptions (49). This bacterium is characterized as gram-negative, non-motile, and non-spore-forming, and the long axis of the cell changes with the medium. In the mucin medium, the strain exhibits a diameter of 640 nm and a length of 690 nm; however, in brain-heart infusion (BHI), the diameter increases to 830 nm with a corresponding length of 1 μ m (15). It predominantly exists as individual cells or in pairs, with chain formation being a rare occurrence (15). Interestingly, the strictly anaerobic *A. muciniphila* demonstrates a notable capacity for survival when subjected to atmospheric conditions. After 24 h in a standard air environment, its survival rate is observed to be 25%, while after 48 h, this rate declines significantly to merely 1% (33). Studies have demonstrated that *A. muciniphila* can be cultured in various media, including BHI, porcine mucin, human mucin combined with BHI medium, Colombia medium, and tryptic soy broth (15, 50–52). The medium utilized by our research group comprised 2.45 g of BHI, 0.3 g of mucin, 100 ml of a 0.3% L-cysteine solution, and autoclaved distilled water, which were mixed uniformly (43). Furthermore, *A. muciniphila* is cultivated at temperatures ranging from 20°C to 40°C and a pH of 5.5 to 8.0, with optimal growth conditions identified as 37°C and a pH of 6.5 (15). Notably, the bacterial growth is inhibited when the pH falls below 5.5 or exceeds 8.0. In the agar base medium, the *A. muciniphila* colony displayed a circular morphology, appeared white in color, and had a diameter of 0.7 mm (15).

A. muciniphila is recognized as one of the most prevalent bacterial species, comprising over 1% of the overall fecal microbiota (53). Fluctuations in its abundance are closely associated with various factors including age, diet, body weight, disease states, and immune status (54). Similar to other intestinal bacteria, *A. muciniphila* predominantly colonizes the outer layer of intestinal mucus; its development is maintained by carbon and nitrogen produced by goblet cells to maintain the dynamic balance of the mucus layer (55–57). A study published in 2007 revealed that *A. muciniphila* is capable of colonizing the intestines of infants early in life, achieving levels comparable to those found in adults within 1 year, and subsequently experiencing a gradual decline in abundance among the elderly population (39). Interestingly, recent studies have revealed that *A. muciniphila* is not only present in the human intestine but is also detected in the nasopharynx and breast milk, where it utilizes human milk oligosaccharides as a carbon source (58–60). This finding offers robust evidence supporting the early

colonization of *A. muciniphila* within the intestinal microbiota. Furthermore, *A. muciniphila* is not only colonized in the human intestines but is also present in the intestinal microbiota of various other animals, including rats (61), mice (62), horses (63), rex rabbits (64), guinea pigs (65), calves (66), rock ptarmigans (67), zebrafish (68), and Burmese pythons (69). These findings demonstrate the extensive colonization of *A. muciniphila* across various animal models and provide foundational research to support its applications.

The initial gene sequencing of *A. muciniphila* ATCC BAA-835 was accomplished in 2011, revealing a complete genome comprising a circular chromosome of 2,664,102 bp and encompassing a total of 2,176 predicted protein-coding sequences, which collectively exhibit an overall coding capacity of 88.8% (35). Twenty-two *A. muciniphila* species were isolated from the feces of people in southern China, which had more than 99% consistency with strain ATCC BAA-835, and 12 subtypes were distinguished by enterobacterial repetitive intergenic consensus (ERIC-PCR) (70). Additionally, *A. muciniphila* in the human gut utilized over 2,000 genomes from humans and various other animals for a comprehensive population genomic analysis, resulting in the delineation of five distinct candidate species (71). In 2017, a total of 39 *A. muciniphila* samples derived from human and mice feces were sequenced to elucidate the genomic structure, leading to the identification of three distinct species-level *A. muciniphila* populations (AmI, AmII, and AmIII) through phylogenetic analysis (72). Furthermore, through an integrative approach combining genomic, molecular biology, and traditional microbiological methods, *A. muciniphila* has identified at least four species-level groups within its systems (AmI to AmIV) exhibiting different functional characteristics (73). Subsequent investigations indicated that the AmII strain possessed a gene responsible for vitamin B₁₂ synthesis. In the absence of vitamin B₁₂, the AmII strain was able to produce acetate and propionate, whereas the AmI strain produced acetate and succinate. Interestingly, the abundance of AmI strains was greatest among children and adolescents; however, antibiotic-treated mice exhibited a higher quantity of AmII and AmIV strains compared to AmI strains (74).

2.2 Active compounds and metabolites obtained from *A. muciniphila*

In the contemporary research landscape, an escalating volume of investigations has been centered on *A. muciniphila* and its specific derivatives, encompassing pasteurized *A. muciniphila*, the Amuc series, extracellular vesicles (AmEVs), short-chain fatty acids (SCFAs), and metabolic enzymes (Figure 1).

2.2.1 The living and pasteurized *A. muciniphila*

Current research indicates that live *A. muciniphila* modulates the intestinal immune response and fortifies intestinal barrier function, participating in the immune system and enhancing the intestinal barrier through the production of SCFAs and acetate (16, 75). Furthermore, due to its capacity to degrade intestinal mucus, *A. muciniphila* may exert an indirect influence on both innate and

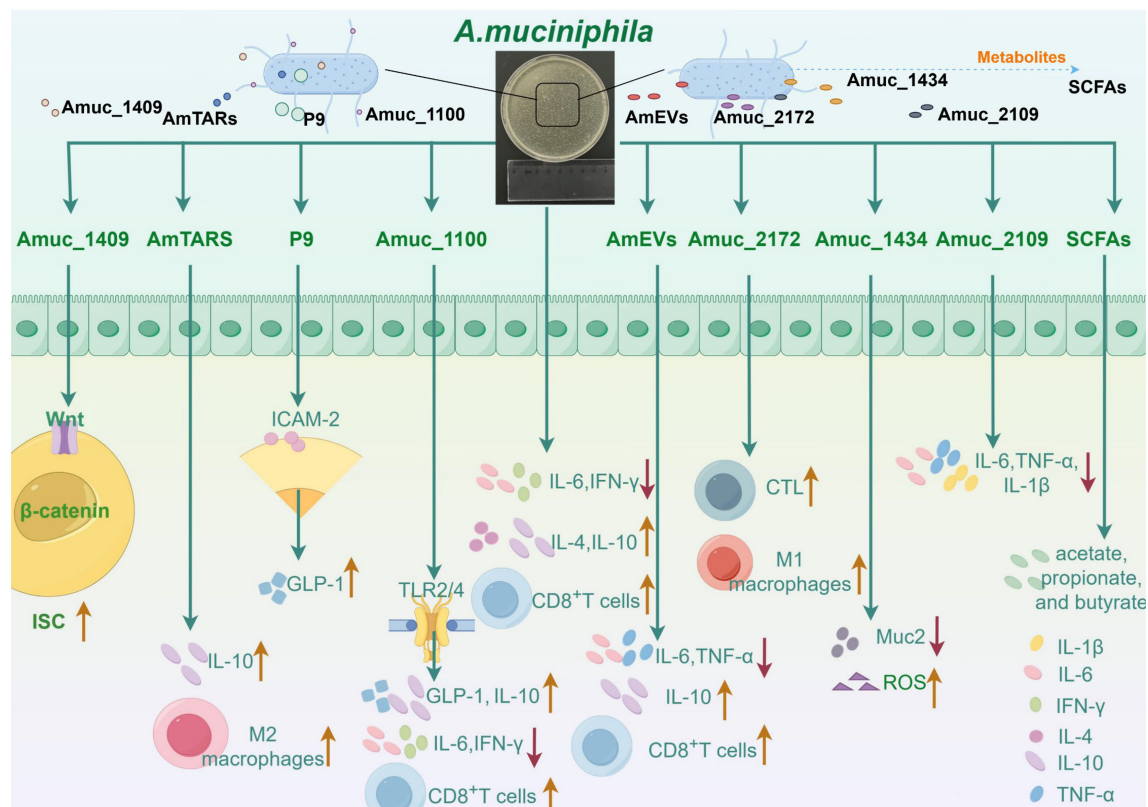


FIGURE 1

The ways in which *Akkermansia muciniphila* and its active proteins interact with the immune system are complex and varied. *Akkermansia muciniphila* has been shown to reduce the levels of interleukin-6 (IL-6) and interferon- γ (IFN- γ) in mice with abdominal aortic aneurysms. Moreover, *A. muciniphila* simultaneously increased the concentrations of interleukin-4 (IL-4) and interleukin-10 (IL-10), thereby enhancing the capacity of A549 and NCI-H1395 cells to recruit CD8⁺ T cells. Amuc_1100 mitigates inflammatory responses by decreasing IFN- γ and IL-6 levels in both the spleen and pancreas. Furthermore, Amuc_1100 interacts with Toll-like receptor 2 (TLR2) and modestly promotes glucagon-like peptide-1 (GLP-1) production by L cells, thus reinforcing gut barrier function. It also plays a role in the host intestinal immune response through activation of TLR2 and TLR4, leading to increased IL-10 production. Additionally, Amuc_1100 exhibits potent antitumor effects by augmenting both the quantity and functionality of CD8⁺ T cells. Studies suggest that P9 interacts with intercellular adhesion molecule 2 (ICAM-2), leading to the activation of the glucagon-like peptide-1 receptor (GLP-1R) pathway, which in turn enhances GLP-1 production. Moreover, AmTARS facilitates M2 macrophage polarization while specifically interacting with TLR2 to induce IL-10 production. Furthermore, Amuc_1409 regulates intestinal stem cell (ISC) activity via Wnt/ β -catenin signal transduction pathways. Extracellular vesicles derived from *A. muciniphila* (AmEVs) significantly lower serum TNF- α and IL-6 levels while elevating IL-10 concentrations, thereby ameliorating liver damage and fibrosis in murine models with hepatic injury. Studies have demonstrated that these extracellular vesicles can serve as an immunotherapeutic agent against prostate cancer progression primarily through activating effector states in CD8⁺ T cells alongside promoting macrophage polarization toward an M1 phenotype. Furthermore, Amuc_2172 enhances transcriptional expression as well as secretion levels of heat shock protein 70 (HSP70), subsequently increasing CD8 cytotoxic T lymphocyte (CTL) numbers and activities within colorectal cancer contexts. In another investigation, it was revealed that Amuc_2172 induces M1 macrophage polarization; however, this effect is reversed by puerarin treatment which improves ulcerative colitis outcomes. Lastly, Amuc_1434 promotes apoptosis along with degradation processes involving mucin 2 (Muc2) within LS174T cells while inducing intracellular reactive oxygen species (ROS). Recent findings suggest that Amuc_2109 reduces TNF- α , IL-1 β , and IL-6 levels alongside downregulating NLR family pyrin domain 3 (NLRP3) expression while enhancing gut barrier integrity—thereby improving DSS-induced colitis symptoms. Short-chain fatty acids (SCFAs)—the primary metabolites produced by *A. muciniphila*—are predominantly composed of acetate, propionate, and butyrate.

adaptive immune responses (76). *A. muciniphila* is frequently correlated with inflammation-related cytokines. Studies reported that during liver injury treatment, *A. muciniphila* combined with inosine increased the number of regulatory T (Treg) but reduced T helper (Th) 1 (77). During lactation, *A. muciniphila* levels in breast milk are positively correlated with TNF- α and IFN- γ levels but negatively correlated with interleukin-10 (IL-10) and interleukin-4 (IL-4) levels (59). In contrast, *A. muciniphila* was observed to decrease the expression of interleukin-33 (IL-33) and the levels of interleukin-6 (IL-6) and IFN- γ in mice with abdominal aortic aneurysm, while simultaneously increasing the concentrations of IL-4 and IL-10 (26). Additionally, our previous research

demonstrated that during dihydroartemisinin (DHA) treatment, *A. muciniphila* increased the population of CD8⁺ T cells in both the spleen and tumor niche of liver cancer model mice (78). Similarly, *A. muciniphila* enhances the capacity of A549 and NCI-H1395 cells to recruit CD8⁺ T cells and augments the cytotoxicity of CD8⁺ T cells (79). Interestingly, in Lewis lung cancer mouse models treated with anti-PD-1 therapy, *A. muciniphila* activates the MHC-II-pDC pathway, reduces the expression of CXCL3 in cancer-associated fibroblasts, stimulates CD8⁺ T-cell activation, and enhances their functionality compared to pasteurized *A. muciniphila* and Amuc_1100 (80). The combination of *A. muciniphila* and cisplatin inhibits the growth of lung cancer in mice, with the underlying

mechanism involving an increase in serum expression of IFN- γ , IL-6, and TNF- α and a reduction in the number of Treg cells (81). In another study, *A. muciniphila* improved the inflammation of chronic colitis induced by dextran sulfate sodium (DSS) in mice but did not increase the ability of Treg cells to differentiate from the CD4⁺ T-cell population (82). Treatment with *A. muciniphila* alleviates liver tissue damage by upregulating serotonin (5-HT) expression and enhances cognitive function through the upregulation of brain-derived neurotrophic factor (BDNF) expression in the gut–liver–brain axis (83). Currently, *A. muciniphila* supplementation has been established as a novel therapeutic strategy across multiple disease models and is considered safe for oral administration in humans, thereby earning recognition as a new generation of probiotic bacteria.

Additionally, the EFSA has verified the safety of pasteurized *A. muciniphila* as a new food item. Numerous studies indicate that live *A. muciniphila* exhibits comparable therapeutic effects to those of pasteurized *A. muciniphila*, with some evidence suggesting that the latter may even demonstrate superior efficacy (84–87). For instance, both pasteurized *A. muciniphila* and live *A. muciniphila* restore damaged intestinal structure, improve liver function, and reduce the number of Treg cells in the spleen, but pasteurized *A. muciniphila* is more effective at improving glucose tolerance (87). Pasteurized *A. muciniphila* significantly reduced fat accumulation and dyslipidemia, thereby ameliorating obesity-related comorbidities (30). Additionally, another study demonstrated that pasteurized *A. muciniphila* enhances GLP-1 production to improve glucose homeostasis and alleviate symptoms associated with T2D in mice (24). Interestingly, pasteurized *A. muciniphila* improves colitis-related tumorigenesis by regulating CD8 cytotoxic T lymphocytes (CTLs) (88). A recent report showed that pasteurized *A. muciniphila* improved symptoms associated with irritable bowel syndrome in mice by reducing colon hypersensitivity reactions while enhancing intestinal barrier function (89). These lines of evidence suggest that pasteurized *A. muciniphila* also has a therapeutic effect on some diseases and is hopeful of clinical application.

2.2.2 The outer membrane protein of *A. muciniphila*

Amuc_1100, a particular outer membrane protein extracted from *A. muciniphila*, was first isolated and characterized in 2017 (30). It has been demonstrated to interact with Toll-like receptor 2 (TLR2) and slightly promote glucagon-like peptide-1 (GLP-1) production via L cells, thereby enhancing intestinal microecology and improving metabolic outcomes in murine models of obesity and diabetes (30, 90). Furthermore, Amuc_1100 participates in the host intestinal immune response by activating TLR2 and TLR4 to produce IL-10 (91). Amuc_1100 demonstrates an antitumor effect in lung adenocarcinoma by inhibiting the JAK/STAT signaling pathway and enhancing both the quantity and activity of CD8⁺ T cells (79). Both *A. muciniphila* and Amuc_1100 ameliorate colitis by diminishing the infiltration of macrophages and CTLs in the colon; however, they also impede tumor progression by enhancing the population of CTLs within this tissue (88). Simultaneously, *A. muciniphila* and Amuc_1100 downregulate the expression of nodule receptor protein 3 (NLRP3) and TLR4/nuclear factor κ B (NF- κ B), inhibiting the

production of inflammatory cytokines and thereby improving NAFLD (28). Furthermore, Amuc_1100 pretreatment attenuates the inflammatory response of acute pancreatitis by inhibiting the NF- κ B signaling pathway, reducing the levels of proinflammatory cytokines, including TNF- α , IL-1 β , IFN- γ , and IL-6, in both the spleen and pancreas, and decreasing the inflammatory infiltration of Ly6C⁺ macrophages and neutrophils within the spleen (92). Moreover, Amuc_1100 has been demonstrated to promote the proliferation of anti-inflammatory M2 macrophages while reducing the number of proinflammatory M1 macrophages in periodontitis, particularly by increasing IL-10 levels and decreasing TNF- α levels (93). Interestingly, a nanodrug formulation containing Amuc_1100 has been demonstrated to mitigate doxorubicin-induced cardiotoxicity by enhancing the intestinal barrier function, enhancing the synthesis of butyric and valeric acids, and ultimately regulating lymphocyte homeostasis in both the spleen and heart (94). Therefore, Amuc_1100 could become a new option for metabolic diseases, inflammation-related diseases, and immunotherapy. Furthermore, in 2022, research demonstrated that a phospholipid derived from the cell membranes of *A. muciniphila* exhibits immunomodulatory properties and facilitates the release of TNF- α and IL-6 (95). These findings are solely derived from *in-vitro* trials, and the outcomes of *in-vivo* research remain ambiguous; therefore, an in-depth investigation is required to verify these findings.

2.2.3 The secreted protein of *A. muciniphila*

P9, an 84-kDa protein, was initially identified as being released through *A. muciniphila*, which significantly stimulates GLP-1 production and enhances thermogenesis in brown adipose tissue, thereby ameliorating glucose regulation in mice fed a high-fat diet (HFD) (96). Moreover, the underlying mechanism involves the binding of P9 to intercellular adhesion molecule 2 (ICAM-2), leading to the activation of the glucagon-like peptide-1 receptor (GLP-1R) pathway and IL-6. In a distinct study, recombinant *Lactococcus lactis* NZP9 was shown to secrete P9 heterologously, thereby stimulating NCI-H716 cells to produce GLP-1 as well (97). Furthermore, threonyl-tRNA synthetase (AmTARS) derived from *A. muciniphila* serves an essential function in monitoring and regulating immune response. AmTARS facilitates the polarization of M2 macrophages and engages in specific interactions with TLR2 (98). This interaction focuses on cAMP response element-binding (CREB), ultimately resulting in the production of IL-10. Concurrently, AmTARS promotes the restoration of IL-10-positive macrophages, elevates serum IL-10 levels, and mitigates inflammation in colitis mice (98). In a related study published this year, it was demonstrated that the protein Amuc_1409, secreted by *A. muciniphila*, serves an essential function in enhancing intestinal health through the modulation of intestinal stem cell (ISC) activity (52). Mechanistically, Amuc_1409 promotes the dissociation of the E-cadherin/ β -catenin complex via its interaction with E-cadherin, thereby activating Wnt/ β -catenin signaling and regulating ISC activity (52). Notably, Amuc_1409 significantly increases ISC populations across diverse experimental models, including isolated intestinal organoids and instances of chemically induced intestinal damage as well as natural aging in male mice.

2.2.4 The extracellular vesicles of *A. muciniphila*

AmEVs, another component of *A. muciniphila*, demonstrate decreased levels in T2D patients and DSS mice models (99). Furthermore, in HFD-induced diabetic mice, AmEVs enhanced intestinal tight junction function and reduced the intestinal permeability of lipopolysaccharide (LPS)-treated Caco-2 cells (99). Similarly, AmEVs restored the gut microbiota of HFD/carbon tetrachloride (CCL4) mice to levels comparable to those of normal mice, improved intestinal permeability, reduced serum levels of TNF- α and IL-6, and elevated IL-10, thereby ameliorating liver damage and fibrosis in HFD/CCL4 mice (100). AmEVs can function as immunotherapeutics to impede the progression of prostate cancer, primarily by activating the effector state of CD8⁺ T cells and facilitating the polarization of macrophages toward the M1 phenotype (101). AmEVs have also demonstrated protective effects against hypertension; specifically, they enhance the expression of genes involved in vasoconstriction and vasodilation within the kidney, while simultaneously increasing the number of Treg cells and tending to reduce the production of proinflammatory cytokines (102, 103). On the other hand, AmEVs have also demonstrated potential for treating preeclampsia (PE) through AmEV transfer from the gastrointestinal tract to the placenta (104). Molecular investigations indicate that the therapeutic efficacy of AmEVs in managing PE is mediated through activation of the epidermal growth factor receptor (EGFR)-phosphoinositide 3-kinases (PI3K)/protein kinase B (AKT) signaling, thereby regulating trophoblast invasion into spiral arteries (SpA). Moreover, Amuc_2172, a recently identified probiotic enzyme derived from *A. muciniphila*, is present in AmEVs secreted by this bacterium. Research has demonstrated that Amuc_2172 functions as an acetyltransferase on Lys14 of histone H3 (H3K14ac), thereby enhancing the transcription and secretion of heat shock protein 70 (HSP70) and subsequently increasing both the quantity and activity of CTLs in colorectal cancer (CRC) (105). Finally, the researchers developed a bioengineering method to prepare macrophage membrane-encapsulated coated Amuc_2172 (CoAmuc_2172), which was proven to be a safe and reliable CRC treatment strategy, improving the clinical transformation value of the research. In another study, Amuc_2172 was shown to induce polarization in M1 macrophages, but puerarin reversed this change, thereby improving ulcerative colitis (106). Similarly, Amuc_1434, which is also derived from AmEVs, has been reported to enhance the adhesion of the colon cancer cell line LS174T that expresses high levels of mucin 2 (Muc2) and also facilitated the degradation of Muc2 in colon cancer cells (107). Subsequently, in-depth studies demonstrated that Amuc_1434 inhibits the viability of LS174T cells by the apoptotic pathway (108). The specific mechanism is that Amuc_1434 inhibited the proliferation and blocked the cell cycle of LS174T cells, promoted the apoptosis of CRC cell LS174T cells, and also induced the production of intracellular reactive oxygen species (ROS), damaged the mitochondrial membrane potential, and caused mitochondrial dysfunction of LS174T cells (108). Although AmEVs have demonstrated significant therapeutic potential for hypertension, gastrointestinal disorders, and tumor immunotherapy, its cultivation remains constrained as a derivative of *A. muciniphila*, with the inability

to scale up production being a primary factor that impedes its development.

2.2.5 The metabolites of *A. muciniphila*

Amuc_2109, a β -N-acetylhexosaminidase, is classified within the GH family 3 enzymes, secreted via *A. muciniphila*. Furthermore, Amuc_2109 exhibits enzymatic activity at pH levels above 4.0 and temperatures ranging from 20°C to 80°C, demonstrating its high survival rate in the intestinal environment (109). A recent study demonstrated that Amuc_2109 reduces the levels of TNF- α , IL-1 β , and IL-6, as well as NLRP3 expression, while enhancing intestinal barrier function and improving DSS-induced colitis (110). SCFAs are the primary intestinal metabolites of *A. muciniphila*, with acetate, propionate, and butyrate as its principal components (111). SCFAs have been demonstrated to alleviate intestinal inflammation and cognitive dysfunction (112). *A. muciniphila* elevates serum levels of acetate and butyrate in sleep-deprived mice, while a mixture of SCFAs containing acetate and butyrate inhibits microglial synaptic phagocytosis and neuronal synapse loss, thereby ameliorating cognitive dysfunction induced by sleep deprivation (113). In addition, recent studies have shown that acetic acid derived from *A. muciniphila* plays an anti-aging role not only in *Caenorhabditis elegans* but also in naturally aging mice (114). On the other hand, studies have shown that acetic acid supports the growth and proliferation of tumor cells, leads to reduced infiltration of CD8⁺ T cells in tumor tissues, and promotes immune escape of tumor cells (115). Fucose fermentation enhances the production and metabolism of propionic acid in *A. muciniphila*, thereby facilitating the intestinal stem cell-mediated development of the intestinal epithelium (116). Other studies have shown that propionic acid plays an anti-inflammatory role and enhances intestinal barrier function by binding to the G-protein-coupled receptor 43 (GPR43) receptor (117, 118). Furthermore, the supplementation of butyric acid significantly mitigates pathological damage to lung tissue and improves the inflammatory response in mice with acute lung injury by restoring intestinal butyric acid production (119). Notably, *A. muciniphila* possesses the capability to synthesize γ -aminobutyric acid (GABA) in acidic environments, primarily through its genome encoding glutamate decarboxylase (120). In summary, the identification of these metabolites from *A. muciniphila* clarifies the relationship between *A. muciniphila* and health, elucidates the molecular mechanisms underlying its effects, and serves as a reference for future investigations into other probiotics.

2.3 Substances in traditional Chinese medicine that increase the abundance of *A. muciniphila*

Traditional Chinese medicine is abundant in natural bioactive compounds and demonstrates remarkable therapeutic efficacy by holistically regulating the homeostasis of various organs within the body (Table 1). Notably, numerous studies have indicated that traditional Chinese medicine can effectively restore intestinal barrier function and maintain microecological balance, mainly by

growing the levels of *A. muciniphila* (121–123). Our prior research has shown that the number of *A. muciniphila* can be enhanced by 4.1-fold when cultured in a wolfberry decoction, and *in-vivo* experiments have further certified that wolfberry supplementation increases the abundance of *A. muciniphila* in both male and female mice (43). Further investigations demonstrated that a synergistic combination of wolfberry, yams, and chrysanthemums markedly increased the levels of *A. muciniphila* both *in vivo* and *in vitro*, with their polysaccharide mixtures displaying a similar effect (124). Furthermore, polysaccharides derived from *D. officinale*, *Atractylodes chinensis*, and *Poria cocos* promote the proliferation of intestinal microorganisms, including *Akkermansia*, *Lactobacillus*, and *Bifidobacterium* (45, 125, 126). In a mouse model of drug-induced liver injury, we similarly demonstrated that wolfberry significantly augmented the abundance of *A. muciniphila* by approximately twofold (127). Recent studies further demonstrated that wolfberry, *Platycodon grandiflorus* root extract, and *Polygonatum sibiricum* rhizome aqueous extracts enhance intestinal barrier function by elevating the abundance of *A. muciniphila* in HFD rats (128–130). *Hypericum perforatum* L. enhances the gut microbiome by elevating the levels of *A. muciniphila*, thereby contributing to an improvement in depressive symptoms (131). Interestingly, berberine is a promising prebiotic that indirectly elevates *A. muciniphila* levels by stimulating mucin secretion in the gut (44). Furthermore, traditional Chinese medicine prescriptions also address diseases associated with disruptions in intestinal homeostasis by enhancing the abundance of *A. muciniphila* (42, 132–135). Research has demonstrated that both Jinqi Jiangtang tablets and

Shouhuitongbian can ameliorate T2D by enhancing the population of beneficial bacteria, particularly *A. muciniphila* (134, 135). Furthermore, the synergistic effect of *Astragalus membranaceus* and *Salvia miltiorrhiza* enhanced the number of *A. muciniphila* and improved diabetic nephropathy (122). Zexie-Baizhu decoction boosts the number of beneficial bacteria, especially *A. muciniphila*; regulates the composition of intestinal flora; and improves NAFLD (133). Shuangshen granules also increase the number of beneficial bacteria in the intestine, mainly *A. muciniphila* and *Limosilactobacillus reuteri*, while inhibiting the NF-κB pathway and thus inhibiting lung metastasis (132). The predominant dosage forms of traditional Chinese medicine are primarily oral. Upon entering the intestine, the active components of traditional Chinese medicine effectively interact with intestinal flora, which in turn influences the metabolic transformation of these compounds. This interaction may enhance the absorption of active ingredients, improve bioavailability, bolster intestinal barrier function, promote probiotic proliferation, and inhibit the growth of pathogenic bacteria. These mechanisms may constitute the primary pathways through which traditional Chinese medicine exerts its therapeutic effects. These results indicate the promise of traditional Chinese medicine as a prebiotic and provide new perspectives on its molecular mechanisms. In addition to the aforementioned factors, dietary interventions can also enhance the levels of *Akkermansia*, including dietary polyphenols and FODMAP (136). FODMAP refers to fermentable oligosaccharides, disaccharides, monosaccharides, and polyols, which are small carbohydrates that many people cannot digest. A randomized controlled trial

TABLE 1 Traditional Chinese medicine improves the abundance of *A. muciniphila*.

Drug	Research subject	Changes in flora	Ref.
Wolfberry	Male and female mice	<i>A. muciniphila</i> ↑	(43)
Wolfberry, yams, and chrysanthemums	Acetaminophen-induced liver injury in mice	<i>A. muciniphila</i> ↑	(124)
<i>Dendrobium officinale</i> polysaccharide	Cognitive dysfunction mice	<i>Akkermansia</i> , <i>Lactobacillus</i> , and <i>Bifidobacterium</i> ↑	(45)
<i>Atractylodes chinensis</i> polysaccharide	Alcohol-induced intestinal injury in rats	<i>A. muciniphila</i> ↑	(125)
<i>Poria cocos</i> polysaccharide	Male mice	<i>Akkermansia</i> , <i>Lactobacillus</i> , and <i>Bifidobacterium</i> ↑	(126)
<i>Platycodon grandiflorus</i> and <i>Polygonatum sibiricum</i>	High-fat diet-fed rats	<i>A. muciniphila</i> ↑	(129, 130)
<i>Hypericum perforatum</i> L.	Chronic restraint stress (CRS)-induced depression mice	<i>A. muciniphila</i> ↑	(131)
Berberine	High-fat diet-fed Institute of Cancer Research mice	<i>A. muciniphila</i> ↑	(44)
Jinqi Jiangtang tablet	Type 2 diabetes mellitus mice	<i>A. muciniphila</i> ↑, <i>Desulfovibrio</i> ↓	(134)
Shouhuitongbian	High-fat diet-fed mice and db/db mice	<i>Akkermansia</i> and <i>Parabacteroides</i> ↑	(135)
<i>Astragalus membranaceus</i> and <i>Salvia miltiorrhiza</i> in combination	Diabetic nephropathy rat model	<i>Akkermansia</i> , <i>A. muciniphila</i> , <i>Lactobacillus</i> , and <i>Lactobacillus murinus</i> ↑	(122)
Zexie-Baizhu decoction	High-fat diet-fed mice	<i>A. muciniphila</i> ↑	(133)
Shuangshen granules	Lewis lung cancer mouse model	<i>A. muciniphila</i> and <i>Limosilactobacillus reuteri</i> ↑	(132)

“↑” indicates that the abundance of relevant bacterial groups is increased. “↓” indicates that the abundance of relevant bacterial groups is reduced.

demonstrated that oats possess prebiotic potential, significantly increasing the abundance of *A. muciniphila*, *Dialister*, *Butyrivibrio*, and *Paraprevotella* while concurrently reducing total cholesterol levels (137). Furthermore, a diverse array of compounds has been shown to significantly promote the growth of *A. muciniphila* in axenic culture, including metformin (138), tryptophan (139), fructo-oligosaccharides (22, 140), and betaine (141). Interestingly, the study demonstrated that the combination of probiotics *Lactobacillus rhamnosus* (*L. rhamnosus*) LMG S-28148 and *Bifidobacterium animalis* (*B. animalis*) LMG P-28149 significantly enhanced the abundance of *A. muciniphila*, likely attributable primarily to the influence of the latter (142). In contrast, the administration of *Bifidobacterium bifidum* G9-1 inhibited the growth of *A. muciniphila*, thus preventing the reduction of jejunal goblet cells in a mouse model of small intestinal loss (143). Noteworthy, broad-spectrum antibiotic treatment also increases the amount of *A. muciniphila* in the gut, especially vancomycin (144–146). While these findings collectively demonstrate an enhancement in the numbers of *A. muciniphila*, the underlying mechanisms of action differ among them, necessitating further investigation to determine any potential commonalities.

3 *A. muciniphila* and HCC

Intestinal flora constitutes a crucial component of the human intestinal microbial ecosystem and serves an essential function in liver physiology and pathology via the gut–liver axis (147). Numerous studies have shown that intestinal flora facilitates the onset and progression of HCC by influencing intestinal barrier function, exacerbating liver inflammation, and undermining antitumor responses. The primary factors implicated in this process include dysbiosis, increased intestinal permeability, microbe-associated molecular patterns (MAMPs), and their metabolites (147, 148). Therefore, dysregulation of the intestinal microbiota is a key factor in promoting the progression of HCC.

3.1 Changes in intestinal flora in HCC

Intestinal permeability is compromised at all stages of HCC development, allowing MAMPs to translocate from the intestine into the liver via the portal vein. This process promotes the onset and progression of HCC through signaling pathways mediated by receptors on hepatocyte surfaces (149). Furthermore, as HCC progresses, the biosynthetic pathways of LPS are upregulated, leading to a gradual increase in serum LPS levels during liver injury, which correlates with the Th1/Th17 proinflammatory phenotype (150). LPS, a common proinflammatory MAMP, constitutes the principal element of the outer membrane. LPS translocates into the bloodstream as a result of alterations in intestinal permeability, subsequently binding to TLR4 on hepatocyte surfaces. This interaction triggers the production of numerous inflammatory cytokines, thereby exacerbating the occurrence of liver cancer and facilitating hepatic cell regeneration (151–153). Studies found that high levels of LPS were detected in the

peripheral blood of mice with N-nitrosodiethylamine (DEN)-induced HCC, and long-term application of low-toxic doses of LPS increased the volume and number of tumors in HCC (154). In another study, serum endotoxin levels were used as a relevant diagnostic biomarker for NAFLD and were used to detect and determine disease stage (155). The use of antibiotic regimens to reduce LPS or knock out the *TLR4* gene on hepatocytes in animal models of HCC to inhibit HCC growth confirmed the important role of the LPS–TLR4 axis in the occurrence and development of HCC (154). Metagenomic sequencing has elucidated alterations in the intestinal microbiota of patients with chronic liver disease and HCC, characterized by an addition in pathogenic bacteria and a reduction in beneficial bacterial populations (156, 157). For instance, among patients with liver cirrhosis, there was a notable rise in the abundance of *Enterobacter* within pathogenic taxa, while the numbers of *Lactobacillus* and *Akkermansia*, among beneficial taxa, were diminished (158, 159). Similarly, the abundance of *Phascolarctobacterium*, *Gemella*, *Enterococcus*, and *Streptococcus* increased in the intestinal flora of patients with HCC, and the levels of *Collinsella* and *Akkermansia* reduced (159). A separate study demonstrated that *A. muciniphila* was significantly diminished in both patients with non-alcoholic steatohepatitis (NASH)-associated HCC and in murine models (160). However, increased abundance of *A. muciniphila* has been reported in patients with esophageal adenocarcinoma and CRC (161–163). The level of *A. muciniphila* in tumor patients changes depending on the tumor site, but this does not affect the beneficial bacterial status of *A. muciniphila*. Supplementing *A. muciniphila* still inhibits tumor growth in HCC and CRC. In HCC induced by NASH, the proliferation of *Clostridium* in the intestine results in an increased synthesis of the bacterial metabolite deoxycholic acid (DCA) in the bloodstream. DCA primarily induces the production of the senescence-associated secretory phenotype (SASP) in hepatic stellate cells (HSCs) via enterohepatic circulation, which subsequently secretes various inflammatory and protumor factors within the liver or activates the mTOR signaling pathway, thereby facilitating the progression of NASH-induced HCC (149, 164). In addition to DCA, SCFAs not only serve as an energy source for intestinal epithelial cells but also modulate the permeability of the intestinal mucosa, resulting in increased harmful leakage within the intestine and thereby facilitating the progression of HCC (165, 166). Consequently, intestinal flora is anticipated to emerge as one of the most promising therapeutic targets for HCC. Finally, metagenomic analysis elucidated the correlation between species diversity and abundance within the intestinal microbiota and clinical responses as well as adverse reactions to immunotherapy. This study confirmed the potential of intestinal microbiota as a biomarker for liver cancer immunotherapy and identified new targets for modulating immunotherapy responses and mitigating adverse effects (167) (Figure 2).

3.2 *A. muciniphila* potentiates the efficacy of immunotherapy in HCC

Recent investigations indicate that the presence of specific intestinal microbiota and diverse bacterial populations is essential for effective antitumor immunotherapy (5, 167, 168). *A. muciniphila*

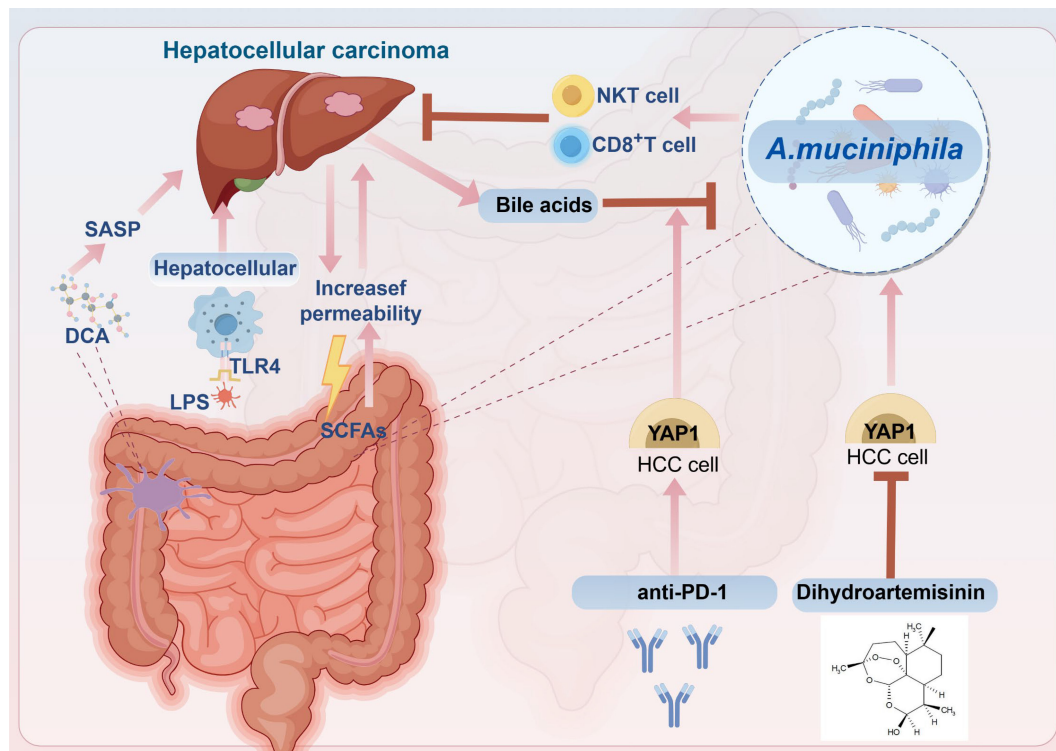


FIGURE 2

The correlation between hepatocellular carcinoma (HCC) and intestinal flora and the mechanism of *A. muciniphila* in treating HCC. Studies have shown that HCC promotes the increase of intestinal permeability, resulting in the production and release of large quantities of lipopolysaccharide (LPS). LPS binds to Toll-like receptor 4 (TLR4) on the surface of hepatocytes, leading to the production of various proinflammatory cytokines, thus promoting the occurrence and development of HCC. In addition, deoxycholic acid (DCA) derived from intestinal bacteria promotes the production of senescence-associated secretory phenotype (SASP), which leads to the progression of HCC. In our previous study, we found that anti-PD-1 promotes the expression of YAP1, thereby synergizing with bile acids to inhibit the growth of *A. muciniphila*, but dihydroartemisinin (DHA) can inhibit the expression of YAP1, thereby increasing the abundance of *A. muciniphila*, and the activity and number of CD8⁺ T cells sensitize the antitumor effect of anti-PD-1 in HCC.

has been demonstrated to enhance the response of tumor immunotherapy by inducing immunoglobulin G1 antibodies and promoting antigen-specific T-cell activation (169). Notably, research has shown that *A. muciniphila* is associated with favorable therapeutic outcomes in immunotherapy across various solid tumors, including HCC (11, 170), non-small cell lung cancer (NSCLC) (171), metastatic renal cell carcinoma (172), and CRC (173). Research has demonstrated that the response to ICIs influences the variety and structure of gut microbiota in fecal samples (11). Mice that responded improved the antitumor effectiveness of anti-PD-1 treatment, whereas FMT from non-responding mice did not yield comparable effects. Furthermore, oral administration of *A. muciniphila* improves the efficacy of anti-PD-1 in ICI non-responders, suggesting that *A. muciniphila* may play a potential role in regulating the clinical efficacy of ICIs (11). Therefore, increasing the abundance of *A. muciniphila* in the intestine or supplementing *A. muciniphila* appropriately is expected to become a new strategy for adjuvant HCC immunotherapy. Moreover, *A. muciniphila* effectively inhibited the progression of NASH to HCC, primarily by enhancing liver natural killer T (NKT) cell populations and reducing macrophage infiltration; additionally, *A. muciniphila* facilitated the cytotoxic activity of NKT cells against HepG2 cells (160). Furthermore, *A. muciniphila* enhances the efficacy of anti-PD-1 therapy in HCC mouse models by promoting apoptosis of HCC

tumor cells and increasing the proportion of CD8⁺ T cells within the tumor microenvironment (TME) (170). In general, *A. muciniphila* exerts antitumor effects in HCC mainly by enhancing the number and activity of tumor killer cells and inhibiting the infiltration of immunosuppressive cells.

Similarly, supplementing *A. muciniphila* in the intestine enhanced the intestinal barrier function of mice, promoted intestinal homeostasis, improved antitumor immune function, and activated the activity of CD8⁺ T cells in the TME of ovarian cancer (174). Interestingly, fecal metagenomic analysis of patients with NSCLC or RCC who exhibited poor responses to anti-PD-1 antibody treatment revealed that those with partial response or stable disease had a higher abundance of *A. muciniphila* compared to patients experiencing disease progression (11). A recent study involving microbiome analysis of fecal samples from patients with advanced NSCLC demonstrated that *A. muciniphila* was associated with improved objective response rates and overall survival in multivariate analyses (175). Furthermore, utilizing 16S rDNA sequencing, it was observed that *A. muciniphila* facilitated an increase in its abundance within the bloodstream and tumor tissue, thereby inhibiting the progression of lung cancer in murine models (176). Another study demonstrated that the abundance of *A. muciniphila* is also reduced in patients with CRC. *A. muciniphila*

induces the polarization of M1 macrophages via the TLR2/NLRP3 pathway, thereby inhibiting the progression of CRC (177). Likewise, *A. muciniphila* inhibits tryptophan metabolism through the aryl hydrocarbon receptor (AhR)/ β -catenin signaling pathway to prevent the development of CRC (178). Moreover, the active components derived from *A. muciniphila* can also exhibit antitumor effects. AmEVs inhibit the growth of prostate cancer by enhancing the proportion of activated CD8⁺ T cells and M1 macrophages, while concurrently suppressing the polarization of M2 macrophages (101). Another *A. muciniphila*-derived component, Amuc_2172, inhibits the progression of CRC by enhancing the transcription and secretion of HSP70 to promote the immune response of CTLs (105). Both *A. muciniphila* and Amuc_1100 ameliorate colitis primarily by reducing the infiltration of macrophages and CTLs. Further investigations have demonstrated that they prevent colitis-related tumors by increasing the number of CTLs in the colon (88). Therefore, both *A. muciniphila* and its active compounds are candidate antitumor strategies, especially in tumor immunotherapy.

3.3 *A. muciniphila* potentiates the efficacy of chemotherapy drugs in cancer treatment

On the other hand, *A. muciniphila* can also improve the antitumor efficacy of chemotherapeutic drugs. For example, *A. muciniphila* enhances the antitumor effect of cisplatin in Lewis lung cancer mice, increasing the levels of IFN- γ , IL-6, and TNF- α by reducing the proportion of CD4⁺ CD25⁺ Foxp3⁺ Treg in the peripheral blood and spleen of mice (81). Studies have shown that after FOLFOX (oxaliplatin, fluorouracil, and calcium folinate) intervention, the abundance of *A. muciniphila* increases significantly and is positively correlated with treatment effect (179). It can also improve the antitumor effect of FOLFOX on colon cancer. Furthermore, pentadecanoic acid derived from *A. muciniphila* inhibits glycolysis by antagonizing the activity of far upstream element binding protein 1 (FUBP1), thereby enhancing the sensitivity of gastric cancer to oxaliplatin (180). These findings expand the potential applications of *A. muciniphila* and further substantiate its anticancer effects.

3.4 Traditional Chinese medicine combats tumors by enhancing the abundance of *A. muciniphila*

Likewise, interventions utilizing traditional Chinese medicine to enhance *A. muciniphila* colonization in the host represent a promising strategy for cancer treatment. Our previous study demonstrated that DHA inhibits YAP1 expression in a mouse model of liver cancer and enhances the accumulation of *A. muciniphila* to improve the therapeutic response to PD-1 inhibitor treatment (78). The combination of *A. muciniphila* with DHA, followed by its combination with the PD-1 inhibitor, resulted

in an increase in the quantity and activation of CD8⁺ T cells within liver tumors. Quercetin combined with anti-PD-1 antibodies increases the levels in *Dubosiella* and *Akkermansia* and improves intestinal flora and macrophage immunity, thereby reshaping the TME of HCC (181). Furthermore, Shuangshen particles inhibit lung metastasis of lung cancer by increasing the abundance of *A. muciniphila*, enhancing the polarization of tumor-associated macrophages (TAMs), and suppressing the activation of the NF- κ B signaling pathway (132). Similarly, another article showed that Huoxue Yiqi Recipe-2 increased *A. muciniphila* levels and thus enhanced the therapeutic effect of PD-L1 antibodies, thereby inhibiting the growth of lung cancer (182). Sini decoction inhibits the onset and progression of colitis-associated colon cancer in mice by upregulating the numbers of *A. muciniphila*, *Bifidobacterium* in the intestine, and CD8⁺ T cells, while downregulating CD4⁺ T cells, IL-6, and TNF- α (183). In addition, studies have shown that intervention with ginsenoside compound K restores intestinal flora to normal, partially increasing the abundance of *A. muciniphila* to prevent the progression of colitis-related colon cancer (184). It is reported that ginsenoside Rh4 also inhibits the progression of CRC by increasing the diversity of intestinal flora, especially the abundance of *A. muciniphila*, improving intestinal microbiota disorders and impaired intestinal barrier function caused by CRC (185). In short, traditional Chinese medicine and its active ingredients can treat cancer by increasing the colonization of *A. muciniphila*.

4 Conclusions

To sum up, as a standard for the next generation of probiotics, *A. muciniphila* is colonized in the intestinal mucus layer, participates in maintaining intestinal homeostasis and immune regulation to maintain host health, and is negatively related to the progress of various diseases, including metabolic-related diseases and tumors. On the contrary, studies have shown that *A. muciniphila* can also promote the progression of certain diseases. The mechanism behind this still needs further exploration and analysis to provide a reference for a detailed evaluation of *A. muciniphila* clinical application. In addition, the existing technology cannot cultivate *A. muciniphila* on a large scale. For *A. muciniphila* to be used on a large scale, the cultivation of *A. muciniphila* is a limitation. Although it has been shown that certain compounds, traditional Chinese medicines, and their monomers can enhance the levels of *A. muciniphila*, research on large-scale cultivation has not been reported, and the technology for large-scale preparation of *A. muciniphila* still needs further exploration. At present, most research on *A. muciniphila* focuses on *in-vitro* and rodent experiments, so clinical applications still require a lot of research, especially on side effects and safety. Although existing studies have displayed that oral *A. muciniphila* is safe and effective (30, 186, 187), it has not been officially approved as a food additive in China. In addition, the European Food Safety Authority has also verified the safety of pasteurized *A. muciniphila* as a new food.

There are many studies showing that live *A. muciniphila* has the same therapeutic effect as pasteurized *A. muciniphila*, and even pasteurized *A. muciniphila* shows better effects (84–87), and other compounds derived from *A. muciniphila* have also shown therapeutic effects on certain diseases, indicating that research on the active ingredients of *A. muciniphila* is promising and further studies are needed. In terms of tumor immunotherapy, *A. muciniphila* has demonstrated strong immune activity and sensitizing effect, which provides favorable evidence for the use of *A. muciniphila* in tumor treatment. Overall, *A. muciniphila* is a powerful target for disease intervention, especially in tumor immunotherapy.

Author contributions

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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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Case Report: Pembrolizumab in a patient with preexisting paraneoplastic dermatomyositis and sarcomatoid urothelial carcinoma. Searching for balance

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Dermatomyositis (DM) is an uncommon systemic autoimmune disorder classified as one of the idiopathic inflammatory myopathies (IIM). DM could also represent a manifestation of an underlying neoplasm with a relative risk of cancer globally ranging from 3% to 8%. Owing to the strong connection between immunosurveillance and cancer progression, the management of paraneoplastic DM represents a challenging issue. To complicate matters is the advent of cancer immunotherapy, that might interfere with self-tolerance with a true risk of previous autoimmune disorders re-exacerbation. We report the case of a 50-year-old patient with advanced urothelial bladder cancer and preexisting paraneoplastic DM treated with pembrolizumab. On the basis of our experience, previous paraneoplastic DM might not necessarily represent an absolute contraindication for ICIs treatment. Furthermore, this case might suggest a role of intravenous immunoglobulins (IVIG) in preventing DM reactivation, underling the importance of a multidisciplinary approach.

KEYWORDS

immunotherapy, paraneoplastic syndrome, autoimmune disease, dermatomyositis, urothelial carcinoma, pembrolizumab

Introduction

Immune checkpoint inhibitors (ICIs) represent a relatively new class of antineoplastic drugs approved to treat many cancer types, including urothelial carcinoma, with durable long-term responses and a favorable toxicity profile (1–3). ICIs have relocated the paradigm of cancer treatment acting through the stimulation of immune system to fight tumor cells. These monoclonal antibodies work by blocking interaction between a group of transmembrane molecules (as programmed cell death protein, PD-1) and their ligands (as PD-L1 and PD-L2),

normally responsible of T-cells exhaustion, immune response modulation and self-tolerance in order to maintain immunologic homeostasis (4). Due to their mechanism of action, ICIs show a very specific safety profile with a considerable variety of immune-related adverse events (irAEs) triggered by T-cells activation. irAEs management require a prompt identification and grading, an adequate diagnostic work-up and, most important, a specific treatment according to the severity of toxicity (5). On this basis, underlying autoimmune disorders (including autoimmune paraneoplastic syndrome) have constantly represented a factor precluding inclusion in cancer immunotherapy clinical trials. Therefore, real-world evidence is essential to acquire data regarding ICIs efficacy and safety in this specific population (6). Here we present the case of preexisting paraneoplastic DM in a patient with urothelial bladder cancer who received ICI (pembrolizumab) for advanced disease, with a review of other reported cases.

Case description

A 50-year-old man with no significant comorbidities and a smoking history of over 50 pack-years presented with 3 months progressive fatigue, muscles weakness and diffuse joint pain (especially in both hips and shoulders) associated with heliotrope rash (Figure 1A), photosensitivity with purplish sunburn reaction over décolleté and anterior chest (Figure 1B) and papules overlying Interphalangeal joints of the hands (Gottron's papules) (Figure 1C). Laboratory evaluation showed normocytic anemia, transaminitis and a creatine phosphokinase (CPK) level of 3500 units/L (normal 26–192 UI/L). Electromyography and muscle biopsy supported the preliminary diagnosis of inflammatory myopathy. Screening for specific antibodies of myositis were positive for anti-transcription intermediary factor 1 gamma (anti-TIF1- γ) corroborating the diagnosis of DM. The patient underwent whole-body computed tomography (CT) with IV contrast that was negative for neoplastic diseases. He received IV high-dose corticosteroids with no response. A 3-month second-line therapy course of IVIG at a dose of 2.0 g per kilogram of body weight were administrated leading to a prompt reduction of both CPK level and DM clinical manifestations. Prednisone was tapered over 8 weeks. About six months later the diagnosis of DM, the patient suddenly presented gross hematuria with severe anemia, hypotension and suprapubic pain, all coinciding with a mild exacerbation of cutaneous and muscular

manifestation of DM as heliotrope rash and muscles weakness and pain requiring an increase of daily prednisone. An abdominal CT with IV contrast showed irregular bladder wall thickening with foci of urothelial hyperenhancement and a vascularized mass along posterior wall, several enlarged pelvic lymph nodes, two suspected liver metastases in the right lobe and also a bone metastasis in left ischiopubic ramus. Bladder biopsy taken during cystoscopy demonstrated urothelial carcinoma. Cancer staging was completed with brain and thoracic CT, showing negative results, and 18F-fluorodeoxyglucose positron emission tomography (FDG-PET) that confirmed locoregional nodal, bone and liver metastasis. Due to intractable hematuria, the patient underwent palliative radical cystectomy, lymphadenectomy and Bricker-type cutaneous ureteral ileostomy, with diagnosis of sarcomatoid urothelial carcinoma infiltrating the entire bladder wall and local lymph nodes (stage pT4N2). One month after surgery, he was started on first-line chemotherapy with cisplatin and gemcitabine with concomitant administration of pegfilgrastim and dexamethasone during each cycle. We knew that our patient would be candidate to receive avelumab as maintenance therapy or pembrolizumab as second-line treatment subsequently chemotherapy; therefore, after multidisciplinary discussion involving rheumatologist and medical oncologist as well the patient itself, we decided to administrate another 3-month course IVIG preceding ICI initiation to reduce the risk of DM exacerbation as well as steroid sparing agent to avoid any potential loss of efficacy. In the course of chemotherapy DM symptoms and laboratory tests (CPK and inflammatory markers) gradually improved also due to IVIG administration and daily prednisone was gradually tapered to < 10 mg. A whole-body CT scan performed after four cycles revealed metastatic progression in bone (right ischiopubic ramus and iliac wing) and lungs with round sharply nodules of varying size throughout the pulmonary fields. Due to disease progression, the patient started pembrolizumab continuing with the administration of IVIG to prevent DM exacerbation. During immunotherapy the patient was closely monitored to assess the onset of irAEs: physical examination, kidney, liver and pancreatic function tests together with thyroid and pituitary gland function tests and CPK were performed before each ICI administration, while echocardiogram was done every 3 cycles. Pembrolizumab was well tolerated: the patient was totally asymptomatic and did not report any irAEs as well as any exacerbation of myopathy symptoms and skin manifestation related to DM. The first CT scan performed after the fourth cycle

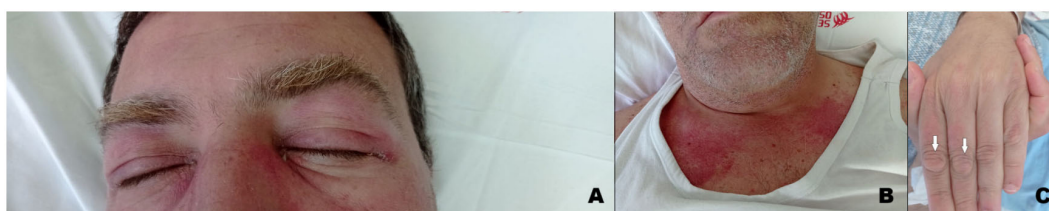


FIGURE 1

Clinical manifestation of dermatomyositis: (A). Violaceous rash over the eyelids with periorbital oedema (heliotrope rash). (B). Erythematous macules involving the anterior aspect of the neck and the upper chest (V sign). (C). Papules overlying Interphalangeal joints of the hand (Gottron's papules).

showed stable disease; on the assumption of good tolerance and efficacy, the patient continued pembrolizumab and concomitant IVIG administration. Unfortunately, six months after starting immunotherapy the patient re-experienced heliotrope rash around eyes and on both cheeks and erythema over chest and upper back, followed by muscle weakness of the extremities and myalgia without oculomotor involvement; serum CPK restarted growing. DM exacerbation required higher dose of steroids; at the same time, pembrolizumab was discontinued and a whole-body CT scan was performed showing cancer progression in liver. Therefore, the patient started treatment with enfortumab vedotin (EV) continuing with concomitant administration of high-dose corticosteroids and IVIG due to DM exacerbation. Two days after EV first administration, he experienced a severe worsening of skin manifestation involving all the extremities, extreme muscle weakness, difficulties in walking and swallowing. Supportive therapy with fluids, parenteral nutrition and intravenous higher dose of steroids were administered achieving a gradually improvement of clinical condition within four weeks. A whole-body CT scan showed further disease progression with new bone lesions in the T12-L2 vertebral bodies, lymph node metastasis in the mediastinal and abdominal fields and major metastatic dissemination in lungs. The patient received fourth line therapy with weekly paclitaxel but he passed away two months after starting chemotherapy.

Discussion

DM is an uncommon systemic autoimmune disorder of unknown pathogenesis and classified as one of the IIM. In adults, the disorder is characterized by chronic inflammation of the skin and muscles leading to rashes and progressive weakness, predominantly in proximal muscles. In 2017, the European League Against Rheumatism and the American College of Rheumatology (EULAR/ACR) released a set of criteria to help identify IIM and its major subgroups. According to this classification, a combination of clinical criteria, laboratory tests and muscle biopsy are used to calculate the probability of having IIM (7). The presence of characteristic skin findings in addition to symmetric muscle weakness should help to distinguish DM from other conditions (inclusion body myositis, drug-induced myopathy, hypothyroidism, polymyalgia rheumatica, muscular dystrophies, motor neuron disease, neuropathy, inherited metabolic myopathies and myasthenia gravis). Electromyography might help to differentiate DM from neuropathic causes of weakness. Muscle biopsy showing the hallmark pathological features of DM also helps to exclude other causes. Glucocorticoids are administered as first-line therapy, followed by various immunosuppressants; IVIG usually in combination with immunosuppressive drugs and has been recommended, also as a glucocorticoid-sparing agent in patients with this disorder. More recently, in a pivotal phase III, double blind, parallel group, randomized placebo-controlled trial, IVIG achieved a significant clinical response based on a composite score of disease activity compared to placebo (79% versus 44%) at 16 weeks (8).

The advantages of IVIG include safety in pregnancy, malignancy, and absence of infectious risk (9). DM could also represent a manifestation of an underlying neoplasm, appearing as a facultative paraneoplastic syndrome with a relative risk of cancer globally ranging from 3% to 8%. This association seems stronger in male and older (> 45 years) patients, while juvenile DM rarely constitutes a paraneoplastic condition. DM diagnosis might precede (with a higher risk in the first year after the onset), follow or be concomitant to tumor detection; therefore, a comprehensive cancer screening should be accomplished in all patients, although no specific guidelines are available to date (10). Since TIF1- γ antibody have been discovered in 83% of patients with paraneoplastic DM, Ferronato M et al. recently suggested the importance of TIF1- γ , a transcript regulator involved in DNA damage repair and tumor suppression, in the pathogenesis of cancer-associated DM acting as a tumor autoantigen. However, a genetic predisposition should not be ignored considering the strong association between this syndrome and specific HLA sequences (HLA-DRB1*0301 and HLA-DQA1*0501 in Caucasians), regardless of dysregulated expression of TIF1- γ (11). Many types of malignancy might be associated to this autoimmune disease, especially gastrointestinal, ovarian and lung carcinomas, with a significant heterogeneity reflecting cancer prevalence across different population (12). In 1982 Behan WMH et al. firstly reported a case of paraneoplastic DM in a 69 years old patient with localized bladder carcinoma and other concomitant autoimmune disorders (myasthenia gravis, Hashimoto's thyroiditis and pemphigoid), suggesting the importance of both disordered immunoregulation and genetic predisposition in the pathogenetic mechanisms (13). From then to 2023, at least 38 other cases of paraneoplastic DM in UC were published worldwide, with a median age of the patients equal to 67.5 years and a mighty predominance in males than females. In many cases UC was localized and treated with only surgical resection, and the most common histological type was transitional cell carcinoma; among patients tested for antibodies, only 20% was positive for antinuclear antibody (ANA) was positive (14, 15). Treatment of paraneoplastic DM is similar to non-cancer-associated forms although few peculiarity: (1) long-term immunosuppressive therapy might promote cancer progression and/or cancer relapse weakening immunosurveillance; (2) interactions between antineoplastic drugs and DM treatment are common at both pharmacokinetic (interference in hepatic metabolic pathways with increased toxicity) and pharmacodynamic (concomitant adverse effects) level; (3) antitumor agents might cause adverse events simulating DM symptoms (i.e. weakness for paclitaxel, skin rash for capecitabine/5-fluorouracil, etc.); (4) tumor response frequently leads to a concomitant improvement of paraneoplastic DM (16). Therefore, the management of cancer-related DM represents an arduous challenge requiring a multidisciplinary approach and a close collaboration between physicians of different specialties (*in primis* oncologist and rheumatologist). Furthermore, the advent of cancer immunotherapy in the treatment of a huge types of neoplasms raised the stakes. Due to their mechanism of action, ICI might interfere with self-tolerance inducing autoimmunity as an unpredictable side effect. Skin toxicity (including autoimmune disorders) represents the most expected reaction occurring in >

50% of treated patients although usually not severe (17). Other common irAEs includes endocrinopathies, hepatic and gastrointestinal toxicity and lung toxicity, while immuno-related myositis is a rare (< 1%) but potentially fatal complication of ICIs requiring rapid treatment with high-dose corticosteroids possibly followed by immunosuppression, plasmapheresis or IVIG in severe cases (18). Even though their uncommon occurrence, immuno-related DM have been already documented in different type of neoplasms (melanoma, small cell lung cancer, lung adenocarcinoma, renal-cell carcinoma, non-Hodgkin lymphoma) treated with different type of ICIs (ipilimumab, nivolumab, pembrolizumab, cabiralizumab + nivolumab). In all cases immunotherapy was discontinued and all patients required corticosteroids with or without other IVIG and immunosuppressive agents (19). DM in oncologic patients receiving ICIs might represent a paraneoplastic syndrome as well as a treatment-related adverse event. An early onset (antecedent to immunotherapy) and the presence of anti-TIF1- γ antibodies generally suggest the diagnosis of cancer-related DM, although a preexisting paraneoplastic syndrome might be worsened by antineoplastic drugs (20). Due to their safety profile, patients with underlying autoimmune disorders (including autoimmune paraneoplastic syndrome) were excluded from cancer immunotherapy pivotal trials. Therefore, data regarding ICIs efficacy and safety in this specific population remain uncertain and mainly resulting from real-world setting. In their retrospective study, Kehl KL et al. showed that immunotherapy can reactivate preexisting autoimmune disease (in about 40% of cases) but also promote the development of new autoimmune manifestations with a modest increase of hospitalization and corticosteroid treatment (21). Furthermore, a higher risk of toxicity with a shorter irAEs-free survival time was observed in another retrospective case series (6). Although prudence is recommended, previous autoimmune disorders might not necessarily represent an absolute contraindication for ICIs treatment whenever patients are

strictly monitored (6, 21). In our patient, paraneoplastic DM preceded the diagnosis of urothelial carcinoma figuring a cancer-related syndrome that required high dose corticosteroid therapy and IVIG. Since the presence of distant metastases, first line therapy was started after palliative cystectomy. During chemotherapy DM symptoms initially improved, presumably due to IVIG and steroid therapy administrated during each cycle of chemotherapy. Subsequently, pembrolizumab was well tolerated, probably due to IVIG treatment that contributed to avoid DM aggravation, even though disease response to treatment might also have a role. After 6 months, DM symptoms rapidly worsened requiring higher dose of steroids; at the same time CT scan showed cancer progression. Therefore, paraneoplastic DM and underlying neoplasm showed a parallel clinical course through entirely medical history, as represented in Figure 2: after an initial improvement due to high-dose steroid and IVIG, clinical manifestations of DM rapidly worsened concomitantly with cancer diagnosis; at this point, we observed another amelioration immediately after chemotherapy administration, and DM symptoms decreased until reaching a plateau; DM manifestations remained mild also during the first 6 months of immunotherapy and restarted to get worse concomitantly with liver metastasis growth. Pembrolizumab was discontinued owing to disease progression and not to toxicity, and the absence of other irAEs and ocular findings (oculomotor involvement is typically associated with immunotherapy-related myositis) seem to corroborate this hypothesis, although a role in autoimmune paraneoplastic syndrome exacerbation cannot be totally ruled out. To the best of our knowledge, there are only few other cases reporting administration of ICIs in patients with preexisting paraneoplastic DM (22–24) (Table 1). Estenaga A et al. described a 78-year-old patient treated with chemoimmunotherapy (cisplatin + etoposide + atezolizumab) for advanced small cell lung cancer, while Poli De Frias F et al. reported a case of gastric adenocarcinoma in a 49-year-old male patient that received chemotherapy + nivolumab as first line

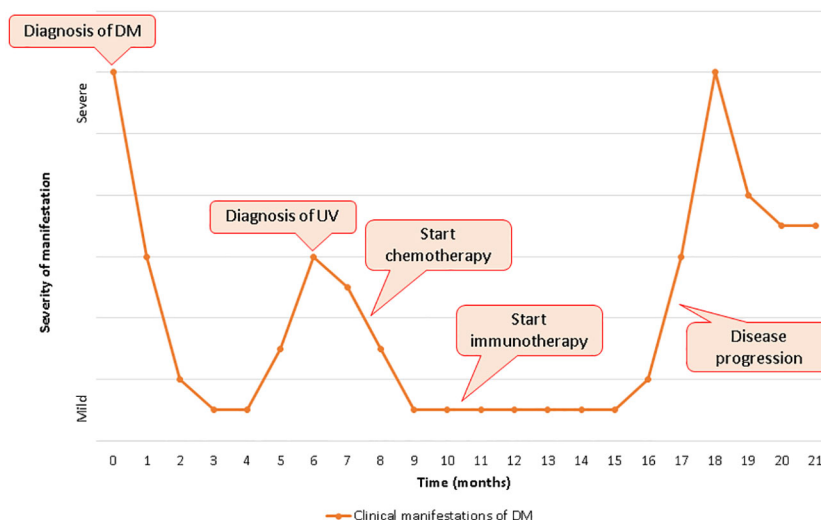


FIGURE 2

Clinical manifestation of dermatomyositis and clinical course of bladder carcinoma over time.

TABLE 1 Reported cases of immune checkpoint inhibitors in patients with preexisting paraneoplastic dermatomyositis.

Reference [year]	Study type	Age and gender	Time of DM onset	Cancer type and staging	Cancer treatment (with ICIs)	DM during cancer treatment	Treatment for DM	ICIs discontinuation
Estenaga A. et al. (22)	CR	78 yo M	Months before the cancer diagnosis	Advanced small-cell lung cancer	Cisplatin + Etoposide + Atezolizumab	Worsening	High-dose steroids, topical steroids	Yes
Poli De Frias F. et al. (23)	CR	49 yo M	Concomitant to cancer diagnosis	Locally advanced gastric adenocarcinoma	FLOT + Nivolumab (added from 3 rd cycle)	Worsening	High-dose steroids	No
Kim S et al. (24)	CR	78 yo M (patient A)	Concomitant to cancer diagnosis	Advanced small-cell lung cancer	Carboplatin + Etoposide + Atezolizumab	Improving	None	No
Kim S et al. (24)	CR	57 yo M (patient B)	Concomitant to cancer diagnosis	Advanced small-cell lung cancer	Carboplatin + Etoposide + Atezolizumab	Worsening (?)	Antibiotics	Yes

CR, case report; M, male, F, female; FLOT, fluorouracil, leucovorin, oxaliplatin, and docetaxel.

regimen. Both patients showed an exacerbation of paraneoplastic DM during ICIs administration demanding high-dose steroids; atezolizumab was permanently discontinued in the first case, while the other patient continued nivolumab in association with chemotherapy and then alone as maintenance therapy (22, 23). Kim S et al. presented two cases of metastatic small cell lung cancer patients receiving the same first line therapy (carboplatin + etoposide + atezolizumab) albeit with different outcomes. In the first patient systemic treatment achieved not only tumor response but also a concomitant improvement of muscle strength and skin manifestation attributable to cancer-related DM; the other case, conversely, developed severe skin changes and deterioration of general condition until death, suggesting a fatal irAEs rather than an aggravation of paraneoplastic DM (24). Finally, our patient showed a severe exacerbation of skin and muscular manifestation after the first administration of EV. This first-in-class Nectin-4-directed antibody-drug conjugate has been associated to dermatologic adverse reactions (even fatal events) due to Nectin-4 expression in epidermal keratinocytes and skin appendages (25). However, the concomitant presence of muscular weakness, dysphagia and severe fatigue might indicate an aggravation of paraneoplastic DM, potentially due to disease progression either than a treatment toxicity.

Conclusions

In conclusion, the role of ICIs administration in patients with preexisting cancer-related DM still appears challenging due to the higher risk of symptoms exacerbation and irAEs onset. In the absence of data coming from pivotal trials, real world experiences might help physicians in differential diagnosis between paraneoplastic syndrome and treatment-related adverse events occurring throughout systemic therapy with ICIs. Moreover, case reports focusing on this specific population might provide significant information regarding the management of

immunotherapy and cancer-related DM. On the basis of our experience and other reported cases, previous paraneoplastic DM might not necessarily represent an absolute contraindication for ICIs treatment, with a strong correlation between antitumor treatment efficacy and DM improvement. Obviously, patients require a strict monitoring and a prompt administration of high-dose steroids and eventually other immunosuppressive agents in case of DM worsening. Lastly, our case might suggest a role of IVIG administration (previously and simultaneously to immunotherapy) in preventing DM reactivation although further evidence is needed to routinely recommend this procedure.

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 requirement of ethical approval was waived by Comitato Etico Regione Marche (CERM) for the studies involving humans. The studies were conducted in accordance with the local legislation and institutional requirements. The participants provided their written informed consent to participate in this study. 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

MT: Conceptualization, Supervision, Writing – original draft, Writing – review & editing. GM: Writing – original draft, Writing –

review & editing. CG: Writing – original draft, Writing – review & editing. SA: Writing – original draft, Writing – review & editing. RB: Writing – original draft, Writing – review & editing.

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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

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The dual roles of human PYHIN family proteins in cancer: mechanisms and therapeutic implications

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The human PYHIN family proteins, including AIM2, IFI16, IFIX, and MND4, which are crucial cytosolic nucleic acid sensors. These proteins share a common structural feature, including signature N-terminal PYD domain and C-terminal HIN-200 domain, which enable them to recognize intracellular nucleic acids and assemble inflammasomes, triggering inflammatory responses and programmed cell death. Over the last decade, it has emerged that the PYHIN family proteins play multifaceted roles in cancer biology, with dualistic roles due to tumor heterogeneity and the tumor microenvironment's plasticity through dependent or independent of inflammasome mechanisms. Here, we discuss their ability to function as both a tumor suppressor and a tumor promoter of tumor progression emphasizes the need for further research to delineate the precise mechanisms by which these proteins operate in various cancer contexts. Understanding these dynamics could pave the way for novel therapeutic approaches that harness the dual nature of PYHIN family members to improve cancer treatment outcomes.

KEYWORDS

human PYHIN family, AIM2, IFI16, IFIX, MND4, inflammasome, tumor suppressor, tumor promoter

1 Introduction

The innate immune system is the first defense line that recognizes pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMP) by pattern recognition receptors (PRRs) against pathogen incursion. Membrane-bound PRRs include Toll-like receptors (TLR) and C-type lectin receptors (CLR), while cytoplasmic PRRs include NOD-like receptors (NLR), RIG-1 like receptors (retinoic acid-induced gene-I receptor, RLRs), and PYHIN (pyrin and HIN domain-containing proteins) family members (1). The specific ligands of these receptors and the mechanisms

of their signaling cascades have been relatively well explored. With the deepening of the research on immune response caused by DNA in cells, four members of human PYHIN family have been discovered, including absent in melanoma 2 (AIM2), IFN-inducible protein 16 (IFI16), IFN-inducible protein X (IFIX/PYHIN1), myeloid cell nuclear differentiation antigen (MNDA) (2), which have similar molecular structures featuring an N-terminal pyrin domain (PYD) and a C-terminal oligonucleotide-binding HIN-200 domain (hematopoietic, interferon-inducible nuclear protein with 200 amino acid repeat) (3). The PYD domain facilitates protein-protein interactions, particularly with the ASC (apoptosis-associated speck-like protein containing a caspase recruitment team), which is essential for inflammasome formation and activation. The HIN-200 domain, on the other hand, is responsible for binding to DNA, enabling these proteins to sense and respond to the presence of foreign or damaged nucleic acids. Functionally, human PYHIN family can be categorized into DNA sensors that mediate the production of type I IFN and those that mediate inflammasome activation. Despite their shared family lineage, these proteins exhibit distinct structural and functional differences that influence their roles in immunity.

Members of the human PYHIN family not only participate in the formation and activation of inflammasomes but may also function through non-inflammasome-dependent mechanisms influencing inflammation and tumor progression in different contexts. AIM2 and IFI16, for instance, are well-known for their roles in forming the inflammasome, which is crucial for the activation of caspase-1 and the subsequent release of pro-inflammatory cytokines like IL-1 β . The activation of AIM2 is closely associated with the onset and progression of various diseases, particularly in inflammatory bowel disease (IBD), where AIM2 expression is significantly upregulated, suggesting its potential role in disease progression (4). Studies have shown that AIM2 can promote tumor growth in KRAS-driven lung adenocarcinoma models, indicating its role in tumorigenesis independent of inflammasome activation. This process is particularly relevant in the context of cancers where inflammation is a driving factor. Several non-inflammasome-independent pathways involved in neoplastic development, including cellular senescence (5), regulation of gene expression (6, 7), modulation of immune signaling (8), DNA damage responses (9), and cell cycle regulation (10). Additionally, IFI16 has been shown to suppress the activation of other inflammasomes, such as AIM2 and NLRP3, thereby modulating inflammatory responses in a way that can either promote or inhibit tumor progression depending on the context (11). On the other hand, MNDA, another member of the PYHIN family, has been implicated in the regulation of gene transcription and immune responses. Its ability to bind DNA and regulate transcription in monocytes suggests that MNDA may also play a role in modulating inflammation and tumorigenesis through non-inflammasome pathways (12). The structural insights into MNDA's interaction with DNA reveal a unique binding mode that could influence its function in immune regulation and cancer biology.

The interplay between these PYHIN proteins and their mechanisms of action underscores the complexity of their roles in inflammation and tumor progression. While inflammasome activation is a well-characterized pathway, the non-inflammasome functions of these proteins are gaining recognition for their potential impact on tumor biology. Understanding these dual roles could provide new therapeutic avenues for targeting inflammation in cancer treatment, particularly in tumors characterized by aberrant immune responses. In this review, we commence with activation and immune regulation mechanisms of the human PYHIN family. Subsequent to this, our discussion centers on the diverse roles of AIM2, IFI16, IFIX, and MNDA, providing a comprehensive elucidation regarding their functions and mechanisms across various tumor types. Human HIN-200 proteins exhibit multifaceted roles in tumor biology in a context-dependent manner. Their intricate interplay in immunity and cancer progression paves the path for the potential as therapeutic targets.

2 Overview of human PYHIN family

2.1 Structure of human PYHIN family

The human PYHIN family proteins, including AIM2, IFI16, IFIX, and MNDA, share a common structural framework characterized by an N-terminal PYD domain and a C-terminal HIN-200 domain (Figure 1). These domains play critical roles in the proteins' ability to recognize intracellular nucleic acids and initiate immune responses. The PYD domain, also called Pyrin, PAAD or DAPIN, can interact with other PYD-containing family members or adapter protein ASC (13). The HIN domain consists of two consecutive oligonucleotide/oligosaccharide-binding (OB) folds and is classified into three subtypes: A, B, C (14). MNDA and IFIX contain a single type A HIN domain (HIN-A), IFI16 have one HIN-A and one HIN-B domain, while AIM2 possesses a single HIN-C domain. Through electrostatic attraction, the positively charged HIN domain senses and binds to the phosphoric groups of negatively charged single-stranded DNA (ssDNA) and double-stranded DNA (dsDNA) (14, 15).

First, from the perspective of the PYD domains, both AIM2 and IFI16's PYD domains are involved in interacting with ASC to form inflammasome which activates caspase-1 and induces cell death (16, 17). The PYD domain of IFI16 promotes DNA binding and aggregation after binding to DNA, forming signaling aggregates that enhance antiviral response (18). In contrast, the PYD domain of IFIX plays a crucial role in regulating cell proliferation and apoptosis, but its specific role in the formation of inflammasome is not yet clear (19). In terms of HIN domains, both AIM2 and IFI16's HIN domains can recognize dsDNA and bind to the sugar-phosphate backbone of DNA through electrostatic attraction (11, 20, 21). However, after binding to dsDNA, AIM2's HIN domain releases its self-repressive state, thereby promoting the assembly of

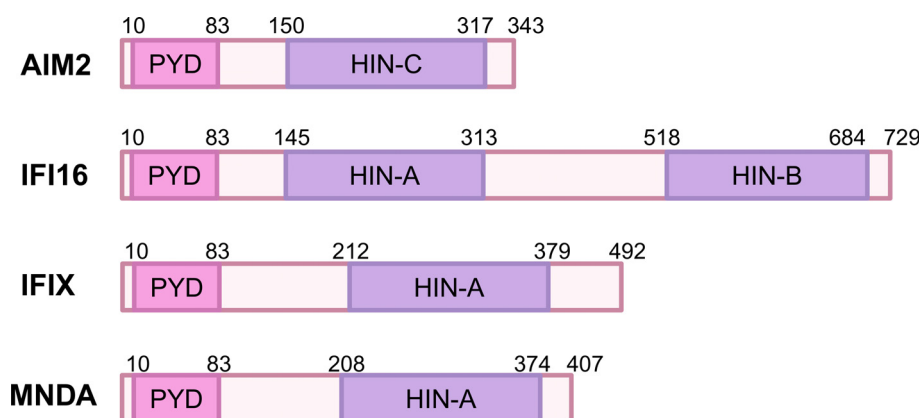


FIGURE 1

Domain organization of human PYHIN family proteins. The PYHIN protein has a conserved domain structure consisting of an N-terminal PYD and one or more C-terminal DNA binding HIN domains. PYD and HIN-200 are depicted in orange and red, respectively. The HIN domain is divided into three subtypes: HIN-A, HIN-B, and HIN-C. NCBI reference sequences are as follows: NP_004824.1 (AIM2); NP_001193496.1 (IFI16); NP_689714.2 (IFI16); NP_002423.1 (MNDA).

inflammasomes (22). In contrast, IFI16's HIN domain forms a polymer upon recognizing DNA, enhancing its ability to sense viruses (23). The MNDA's HIN domain also exhibits the capacity to engage with dsDNA through two distinct binding interfaces: AIM2 HIN-like DNA binding mode and a MNDA-specific DNA binding mode (12, 24), with the MNDA-specific mode differing from other HIN-200 proteins by being located opposite the AIM2-like binding interface, but its function within cells is primarily associated with regulating immune responses, and its role in inflammasomes remains unclear (24).

The differences in the PYD and HIN200 domains among AIM2, IFI16, IFIX, and MNDA not only reflect their evolutionary adaptations but also underscore their specialized functions in the immune system. Understanding these distinctions is crucial for developing targeted therapies that can modulate immune responses in various diseases, including infections, autoimmune disorders and cancers. The interplay between these proteins and their structural features continues to be an important area of research in immunology.

2.2 Distribution and function of human PYHIN family

Nucleic acid sensing by PYHIN family proteins includes dsDNA, ssDNA, and DNA-RNA hybrids, primarily derived from microbial pathogens and endogenous cancer or necrotic cells. During bacterial infections, IFN-inducible proteins like guanylate-binding proteins (GBPs) and immunity-related GTPase family member b10 (IRGB10) facilitate the release of bacterial DNA into the cytoplasm (25, 26). In cancer cells, DNA originating from cell apoptosis, active secretion, and exosome release is particularly prominent in cytoplasm due to genomic instability, necrosis. Furthermore, ionizing radiation and chemotherapeutic agents can

induce double-strand DNA breaks, with this DNA primarily located in the nucleus. In the study of intracellular DNA sensors, proteins such as AIM2, IFI16, IFI204, and MNDA are particularly important for their distribution in the cytoplasm and nucleus and their ability to recognize different DNAs (Figure 2). Except for AIM2, primarily cytoplasmic, other members contain at least one multipartite N-terminal nuclear localization signal (NLS) and can translocate from the nucleus to the cytoplasm under pathogen DNA stimulation (6, 27–29).

Human PYHIN family proteins initiate immune responses through two pathways: inflammasome assembly/activation and type I IFN production. One pathway is the responses of AIM2 and IFI16 to Kaposi sarcoma-associated herpesvirus (KSHV) and herpes simplex virus 1 (HSV-1) infections (30–32), which bind to the sugar backbone of cytosolic dsDNA through its positively charged HIN domain and then associates with ASC via PYD-PYD interactions, recruit pro-caspase-1 through caspase recruitment domain (CARD)-CARD interactions that converted into active caspase-1, and then it catalyzes the cleavage of pro-IL-1 β , pro-IL-18, and Gasdermin D (GSDMD), leading to the maturation and secretion of inflammatory factors IL-1 β , IL-18, and the formation of GSDMD pores on the cell membrane, inducing pyroptosis. The production of inflammatory factors and pyroptosis trigger a series of innate immune mechanisms to clear pathogens. However, the progress of immune responses can be deactivated by certain viral tegument proteins, such as pUL83 of human cytomegalovirus (HCMV) and VP22 of HSV-1 (33, 34). Furthermore, elevated levels of POP3 or IFI16 also suppress the activation of AIM2 inflammasomes, possibly due to competitive recruitment of ASC (35). Concurrently, the other pathway is responsible for IFI16 and IFIX distinctive as a dual sensor for both nuclear and cytoplasmic dsDNA to mediate type I IFN production through the stimulator of interferon genes (STING)-TANK binding kinase 1 (TBK1)-IFN regulatory factor 3 (IRF3)

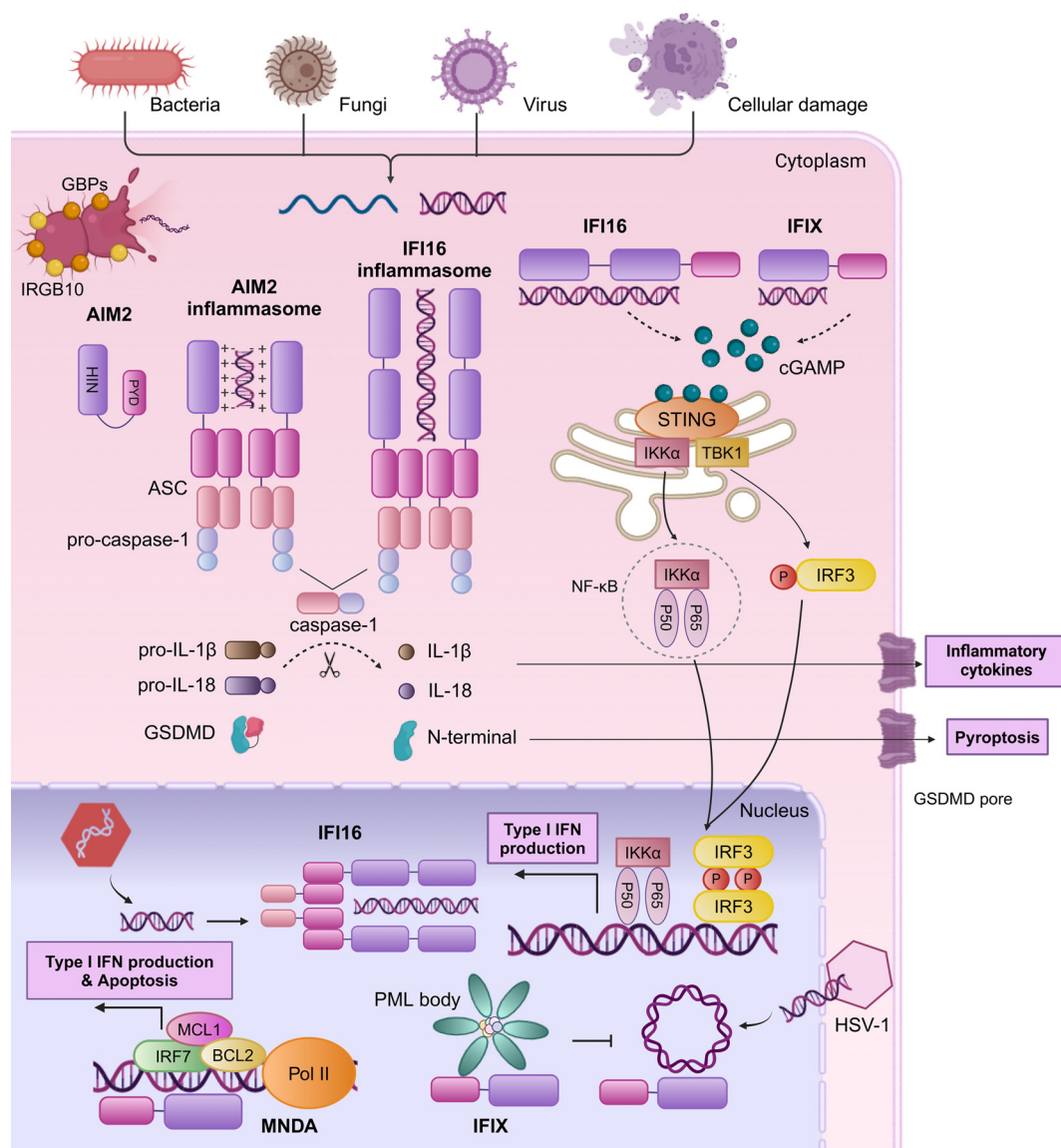


FIGURE 2

Human PYHIN family proteins sense dsDNA by mediating inflammasome complex and type I IFN signaling. AIM2 possesses a HIN domain that binds dsDNA and a PYD that interacts with the PYD of ASC. The CARD domain of ASC can then interact with the CARD domain of pro-caspase-1 to facilitate the maturation of IL-1 β , IL-18, and GSDMD, culminating in the secretion of inflammatory factors and induction of pyroptosis. Upon DNA encounter within the nucleus, IFI16 initiates the formation of an ASC-dependent inflammasome complex, facilitating its translocation to the cytoplasm. In the cytoplasm, the HIN domain of IFI16 binds dsDNA to activate the cGAS-STING pathway through the elevation of cGAMP levels. This activation leads to STING-mediated IRF3 phosphorylation and NF- κ B complex assembly, prompting their nuclear translocation to stimulate the expression of IFN- β and other immune-regulatory molecules. IFIX engages in a STING-TBK1-IRF3 response similar to IFI16 in the cytoplasm. During HSV-1 infection, IFIX recognizes and binds viral DNA within the nucleus, potentially through its recruitment to PML bodies. MNDA undergoes cleavage by caspase-1 during genotoxic reactions, leading to the translocation of the PYD fragment to the cytoplasm, where is hypothesized to promote inflammasome assembly (not illustrated). MNDA interacts with chromatin and RNA PolII to control the transcription of genes such as IRF7, MCL1, and BCL2, enhancing production of type I IFN and apoptosis.

signaling cascade. Ultimately, type I IFN plays a central role in cross-priming, T cell activation, and tumor regression. In HSV-1-infected cells, IFI16 binds to the viral DNA at the nuclear periphery (36). Subsequently, phosphorylation of the intrinsically disordered region leads to IFI16 oligomerization and the extension into a solid filamentous structure (36). Then, IFI16 translocates to the cytoplasm, resulting in the recruitment of IFI16-STING-mediated TBK1, the activation of the IRF3 and NF- κ B pathways, and the

generation of IFN- β (28). IFIX engages in a STING-TBK1-IRF3 response similar to IFI16 in the cytoplasm, playing a crucial role in DNA damage response pathways.

Beyond the inflammasome complex and type I IFN signaling, the human PYHIN family proteins have been found to engage in a variety of mechanisms. IFIX binds HSV-1 DNA substrates in a sequence-independent manner, potentially through its recruitment to subnuclear protein structures known as PML (promyelocytic

leukemia) bodies (37). MNDA interacts with chromatin and RNA polymerase II (Pol II) to control the transcription of genes such as IRF7, myeloid cell leukemia 1 (MCL1), and BCL2, enhancing type I IFN production (38) and promoting apoptosis in leukocytes and chronic lymphocytic leukemia (CLL) cells (6, 7). Additionally, IFI16 inhibits genes transcription by interfering with transcription factors (39), impeding their binding cognate promoters (40) or modulating chromatin structure (40–42). Specifically, IFI16 disrupts the binding of TATA-binding protein (TBP) and octamer-binding transcription factor 1 (Oct 1) to the HSV-1 promoter (40), and directly interacts with Sp1 to suppress HIV-1 transcription (39). Additionally, IFI16 induces the chromatinization of viral DNA, thereby suppressing the replication and expression of viral genes (43).

In summary, the distribution and functional differences of these DNA sensors enable them to play their respective roles under different physiological and pathological conditions. This complex regulatory mechanism allows cells to effectively respond to exogenous DNA threats and also provides new insights into host immune responses. These studies provide a theoretical foundation

for the future development of therapeutic strategies targeting viral infections, autoimmune diseases and cancers.

3 The dual role of AIM2 in cancer

3.1 The anti-tumor role of AIM2

3.1.1 AIM2 in glioblastoma multiforme

AIM2 is one of the tumor-associated antigens expressed by glioma cells, which specifically recognize and engage with the HLA-A1 locus (44). Due to this property, AIM2 is commonly applied to the monitoring test of new dendritic cell vaccine and immunotherapy which can attack and destroy tumor cells (45, 46). There was a significant inverse correlation between methylation levels of CpG loci and AIM2 gene expression in GBM, with distinct variations observed based on the tumor grade (47). Notably, AIM2 expression was an increase in spatial heterogeneity within GBM samples, with strong expression in tumor cores associated with reduced cell proliferation (Table 1) (48). In astrocytoma, the

TABLE 1 The role of AIM2 in tumor biology and the underlying mechanisms in various tumor types.

Tumor types	Molecular mechanisms of AIM2	System	Role of AIM2	References
GBM	Inhibition of cell proliferation independent of the inflammasome with IL-1 β secretion	Brain specimens from patients with epilepsy and U251 cells	Anti-tumor	(48)
SCC	Activation of NF- κ B signaling	OSCC tumors samples; human OSCC cell lines (Ca9-22, HO-1-u-1, HSC-2, HSC-3, HSC-4, SAS, HSQ-89, Sa-3)	Pro-tumor	(93)
	Promoting NF- κ B/BCL2A1/MAPK/c-Myc pathway	PSCC tumors samples; Pen12, 149rca and 156lm PSCC cell lines	Pro-tumor	(96)
	Activation of STAT1/NF- κ B and promotion of irradiation resistance, migration ability and PD-L1 expression	Oral cancer cell lines HSC2, HSC3, HSC4 and SAS	Pro-tumor	(95, 177)
	STAT1 mediated transcription and IL-17/ MAPK signaling	HNSCC specimens and HNSCC cell lines (2A3 LCC, CAL27, FADU and HEK 293 T, BEAS-2B)	Pro-tumor	(91)
NSCLC	Facilitation of calcium release and mitochondrial ROS generation, resulting in calpain activation and elevated IL-1 α concentrations	Lung samples of NSCLC patients; pDCs	Pro-tumor	(111)
	Fostering M2 polarization and PD-L1 expression through the JAK/STAT3 pathway	Lung samples of NSCLC patients, C57BL/6N mice	Pro-tumor	(108, 109)
	Regulation of cell-cycle, induction of G2/M phase arrest	BALB/c athymic nude mice, Human NSCLC A549, H460, H226 and 16HBE	Pro-tumor	(102, 178)
	Augmenting ROS generation and activating the MAPK/ERK signaling	Human H1975, H358, A549, H157, HCC827, H3255, and H460 NSCLC cell lines, Xenograft tumors	Pro-tumor	(103)
	Promoting PD-L1 expression and immune evasion of NSCLC via NF- κ B/STAT1 and JAK/STAT3 pathway	Lung adenocarcinoma cell lines A549, H1355, HCC827, and PC9; Raw264.7, LA795 cells and C57BL/6J-Aim2em1Cya mice	Pro-tumor	(101, 109)
	Inhibition of ferroptosis through STAT5B-ACSL4 axis	Lung cancer cell lines NCI-H1395, SK-MES-1, and NCI-H1975	Pro-tumor	(179)

(Continued)

TABLE 1 Continued

Tumor types	Molecular mechanisms of AIM2	System	Role of AIM2	References
BC	Inhibition of NF- κ B transcriptional function and reduced sensitivity to TNF α -driven NF- κ B activation	MCF-7, MDA-MB-231, MDA-MB-435, and MDA-MB-453 cells; tetracycline-inducible AIM2 Cell Lines	Anti-tumor	(51)
	Down-regulating Bcl-xl, up-regulating Bad and Bax, activating caspases that leads to the cleavage of PARP	MCF-7 tTA-AIM2 cells, MCF-7 tTA-Luc cells under Tet-Off model system	Anti-tumor	(52)
	Promoting the division of DFNA5 by activating caspase-3	BALB/c nude mice and MCF-7, MDA-MB-231 cell lines	Anti-tumor	(53)
HCC	Inhibiting the mTOR-S6K1 signaling pathway and in turn inhibiting HIF-1 α	BALB/c athymic nude mice and HCC cell lines (MHCC97H, MHCC97L, BEL7402, SMCC7721, HepG2 and HUH7)	Anti-tumor	(54)
	Promote autophagy and inhibiting M2 macrophage polarization	Normal hepatocyte(L-02) and HCC cells (Huh-7, Hep3B, and PLC/PRF/5); HepG2215 cells	Anti-tumor	(55, 59)
	Binding and reduction influenced by HBx; reduction of AIM2 stimulates EMT by elevating FN1 expression	Primary HCC patients and HCC cell lines (Bel-7402, SMMC-7721, Huh7 and Bel-7404)	Anti-tumor	(57)
GC	Inhibition the proliferation and migration of GC cells through the inhibition of Akt signaling pathway	GC specimens; SGC7901 GC cells	Anti-tumor	(63)
	Interaction with EB1 and connection of the cytokine-STAT3 signaling pathway	GC cell line xenografts and gp130 F/F mice	Pro-tumor	(118)
	Increase of the phosphorylation of p38, JNK and ERK and promotion of the MAPK signaling to promote proliferation and migration	BGC-823 and MGC-803 GC cells	Pro-tumor	(117)
RCC	Inhibition of cell invasion and metastasis via enhancing autophagy induction.	786-O and OSRC-2 RCC cells	Anti-tumor	(68)
	Promoting M1 polarization to inhibit malignant behaviors of tumor cells	BALB/c mice and RCC cell lines (HEK293T, L929)	Anti-tumor	(180)
	Inhibition of ferroptosis through FOXO3a-ACSL4 axis	RCC cell lines (786O, OSRC2, Caki-1, A498 and ACHN) and renal tubular epithelial cell line HK-2	Pro-tumor	(116)
CRC	Initiating G2/M cell cycle halt, protracted progression from G2 to M phase in the cellular cycle	HCT116 CRC cells	Anti-tumor	(10, 72, 76)
	Inhibition of cell growth and modulation of intestinal stem cell proliferation	Wild-type and AIM2-deficient mice, organoid models, and bone marrow chimera constructs	Anti-tumor	(75)
	Interaction with DNA-PK and Inhibition of Akt activation	Wild-type and AIM2-deleted mice, MEFs, BMDM models, and organoid systems	Anti-tumor	(74)
	Promoting apoptosis in CRC cells by suppressing PI3K/Akt pathway	HCT116 CRC cells	Anti-tumor	(72)
	Regulating Gli1 to inhibit proliferation and migration through the Akt/mTOR pathway	HCT116, SW480, SW620 and LoVo CRC cells	Anti-tumor	(73)
	Inhibition of BRAF-mutant CRC growth in a caspase-1-dependent manner	50 CRC patients and CRC cell lines (HCT29, HCT116, COLO205, and SW480)	Anti-tumor	(79)
	Inhibition of proliferation and migration via P38MAPK signaling	HCT116 CRC cells	Anti-tumor	(10)
PCa	Gathering of senescent cells in the prostate epithelium	Human prostate cancer cell lines (RWPE-1, RWPE-2, DU-145, PC-3, and LNCaP)	Anti-tumor	(86)
OS	Inhibition of PI3K/Akt/mTOR signaling pathway and EMT	OS cell lines C396, CAL-72 and MG-63	Anti-tumor	(88)

expression of AIM2 induced by the inflammatory agent Neamycin was accompanied by decreased cell proliferation and mitochondrial activity. In general, these observations indicated that AIM2 was independent of inflammasome function (48), suggesting that AIM2 might be a reasonable regulator of GBM cell proliferation.

3.1.2 AIM2 in EBV-associated nasopharyngeal carcinoma

Both AIM2 and IL-1 β exhibit elevated expression in EB virus-associated NPC, with significant correlations between their expression levels and patients' survival (49). The activation of the AIM2 inflammasome by viral stimuli and elements within tumor microenvironment (TME) facilitates the secretion of IL-1 β . Intriguingly, tumor-derived IL-1 β has been implicated in enhancing local tumor control and improved patient survival (49), indicating a paradoxical role for the tumor inflammasome in tumor suppression. The precise interplay between AIM2 and IL-1 β in NPC, particularly how they synergize to inhibit tumor progression, warrants further investigation.

3.1.3 AIM2 in squamous cell carcinoma

The prognostic significance of AIM2 in SCC is underscored by studies showing that low AIM2 expression is associated with poor

survival outcomes. In hypopharyngeal squamous cell carcinoma, for instance, the expression of AIM2 in tumor tissues was significantly lower than that in adjacent normal hypopharyngeal tissues. Low AIM2 levels combined with high expression of p-STAT3, correlate with increased lymph node metastasis and poor prognosis, highlighting its potential as a biomarker for disease progression (Table 1) (50). Therefore, AIM2 may represent a valuable prognostic marker and therapeutic target in SCC, warranting further investigation into its role in tumor progression and metastasis.

3.1.4 AIM2 in breast cancer

Research has illuminated that the expression of AIM2 can suppress the proliferation and tumorigenicity of human breast cancer cells through the negative regulation of the TNF- α /NF- κ B anti-apoptotic pathway (Figure 3, left panels) (51). Liu et al. revealed that the expression of AIM2 leads to the cytoplasmic translocation of cytochrome C, simultaneously with downregulation of the Bcl-xL, upregulation of Bax, culminating in the cleavage of PARP (Figure 3, left panels) (52). In addition, AIM2 enhances the cleavage of DFNA5 by activating caspase-3, and that docosahexaenoic acid (DHA) triggers pyroptosis in BC cells through the pivotal AIM2/Caspase-3/DFNA5 pathway (53). In

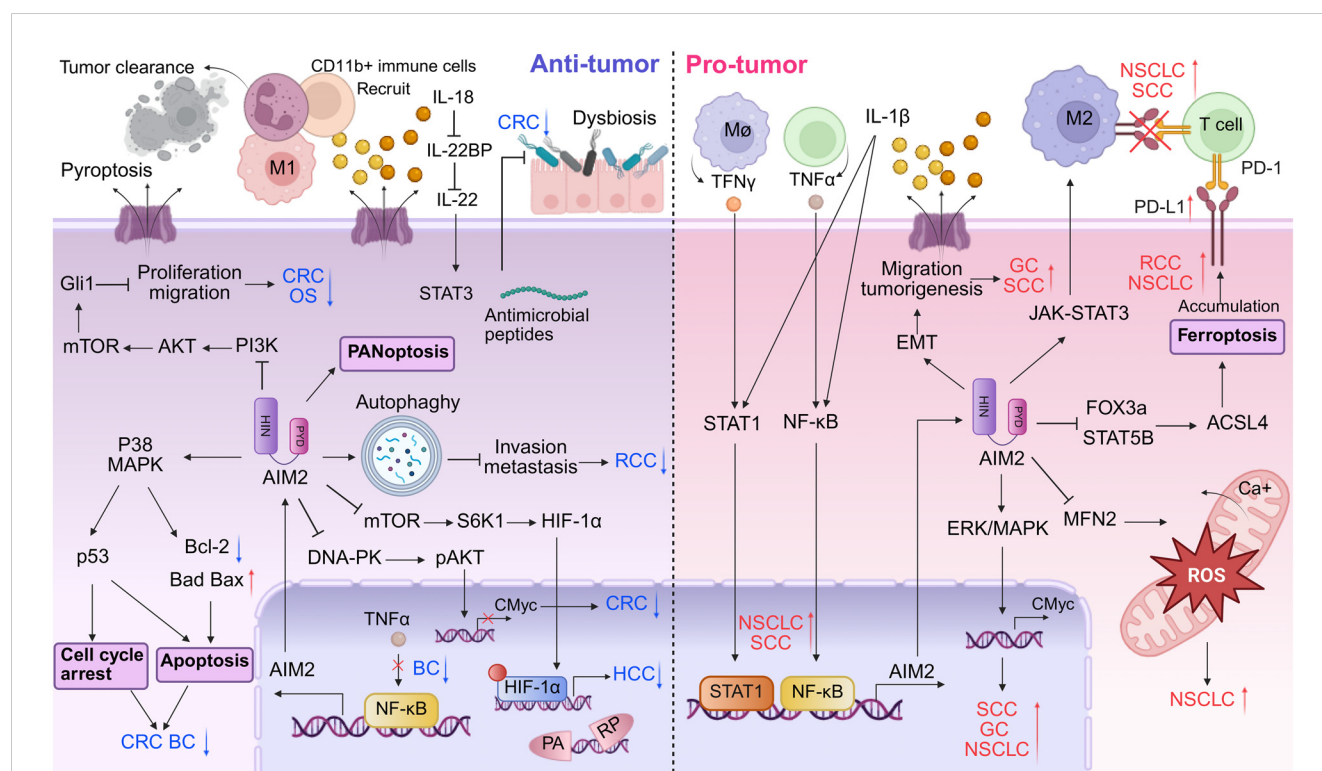


FIGURE 3

Dual roles of AIM2 in tumor microenvironment. AIM2 exhibits a bivalent role in tumor progression by modulating both pro- and anti-oncogenic activities within the immune landscape. In TME where AIM2 exerts tumor-suppressive functions, it facilitates the recruitment of inflammatory immune cells, such as T cells and NK cells, to promote tumor clearance. Furthermore, AIM2 enhances cellular death mechanisms including apoptosis, autophagy, and PANoptosis. AIM2 can also inhibit tumor growth via various inflammasome-independent pathways, notably in CRC. Conversely, in tumor contexts where AIM2 exerts oncogenic effects, it fosters an immunosuppressive environment marked by M2 macrophage polarization and upregulation of PD-L1, thereby dampening the immune response against tumors and promoting immune evasion. Additionally, AIM2 inflammasome-mediated release of pro-inflammatory cytokines like IL-1 β can activate signaling cascades that enhance tumor growth and metastasis. While cell death mechanisms such as ferroptosis are attenuated, processes that facilitate cell migration, including EMT, are augmented.

summary, these studies collectively shed light on AIM2's multifaceted role in the regulation of BC cells death, presenting new therapeutic opportunities in oncology.

3.1.5 AIM2 in hepatocellular carcinoma

In an intriguing analysis of the molecular landscape of liver cancer, the expression profile of AIM2 has emerged as a pivotal determinant of tumor progression. Notably, a significant downregulation of AIM2 expression was observed in HCC tissues (Figure 3, left panels and Table 1) (54), with this decremental trend closely associated with advanced stages of tumorigenesis. Further investigation revealed that exogenous overexpression of AIM2 in HCC cells exerted inhibitory effects on the mammalian target of rapamycin (mTOR)-S6K1 signaling pathway, a critical axis implicated in cellular proliferation, survival, and metabolism (54). Consequently, this upregulation of AIM2 led to a suppression of HCC cells proliferation, colony formation, and invasive capabilities, highlighting its tumor-suppressive role. The expression of caspase-1 and the levels of IL-1 β and IL-18 are positively correlated with AIM2 expression in HCC cells. Activation of the AIM2 inflammasome has been shown to promote autophagy and inhibit M2 macrophage polarization, potentially through the secretion of inflammatory cytokines (55). AIM2's role in amplifying autophagy also extends to mitigating age-related acute liver injury (56). Specifically, AIM2 has been implicated in regulating lipid peroxidation and oxidative stress in aged mice, highlighting its protective role in acute liver damage (56). Of particular note, the loss of AIM2 induced by Hepatitis B virus X protein (HBx) has been implicated in adverse clinical outcomes and facilitated HCC metastasis through the induction of EMT (57), a process pivotal for cancer cell dissemination and colonization at distant sites.

In the therapeutic realm, radiofrequency ablation (RFA) has demonstrated efficacy inhibit HCC tumor growth by harnessing the AIM2-mediated induction of pyroptosis, a lytic form of programmed cell death (58). This mechanism suggests that therapeutically targeting AIM2-driven inflammasome signaling pathways may offer a promising strategy to augment the therapeutic outcomes of RFA in HCC patients. Moreover, a novel mechanism has been unveiled wherein dihydroartemisinin, a compound derived from traditional medicinal plants, promotes the activation of the AIM2/caspase-1 inflammasome, thereby contributing to autophagy in HepG2215 cells (59). This discovery broadens the therapeutic horizon for HCC by suggesting potential synergistic effects between dihydroartemisinin and AIM2-mediated pathways in fostering cellular self-digestion, a process known to counteract tumorigenesis. In advanced HCC, a recent study has revealed that in HCC cells overexpressing DANSEIL3, sorafenib induces pyroptosis, apoptosis, and necroptosis (PANoptosis) rather than apoptosis (60). Unlike apoptosis, PANoptosis is mediated by AIM2 and leads to the release of pro-inflammatory cytokines, which enhances immunogenicity and transforms "immune-cold" tumors into "immune-hot" tumors, thereby increasing the efficacy of immunotherapy (61). This finding underscores the potential of targeting AIM2 to modulate PANoptosis as a novel strategy for enhancing immunotherapy in HCC.

Collectively, these findings underscore the multifaceted roles of AIM2 in HCC biology and its potential as a therapeutic target. Future studies exploring the intricate interplay between AIM2 and its downstream effectors, as well as the integration of AIM2-targeting strategies into existing treatment paradigms, hold promise for advancing the management of HCC.

3.1.6 AIM2 in gastric cancer

Reports on the role of AIM2 in GC have yielded mixed results and we will delve into its tumor-suppressing effects. Zhou et al. compared the samples of patients with early and advanced GC and demonstrated that AIM2 expression was notably lower in advanced GC tissues (62). Mechanistically, AIM2 exerts its anti-tumor effects by regulating the Akt signaling pathway. The knockdown of AIM2 in GC cells leads to increased cellular proliferation and migration, whereas its overexpression results in the opposite effects, highlighting its potential as a tumor suppressor in GC (Table 1) (63). Targeting the AIM2/Akt signaling axis could offer new avenues for GC treatment, providing a novel entry point for therapeutic intervention.

3.1.7 AIM2 in renal cell carcinoma

In the context of RCC, AIM2 has been recognized as a potential biomarker and therapeutic target. Chai et al. practiced combined molecularly targeted anti-cancer therapies and immunotherapies, developing nanoparticle systems to deliver AIM2 (64–66). Furthermore, the role of AIM2 in RCC is linked to its interaction with other molecular pathways. For instance, the Wnt/ β -catenin signaling pathway (67) and upregulating autophagy-related genes (Bcl-2, Beclin-1 and ATG-5) (Table 1) (68), have been implicated in RCC progression, and AIM2's influence on these pathways could provide insights into its anti-tumor role (Figure 3, left panels). The modulation of these pathways by AIM2 could potentially inhibit RCC cell proliferation, invasion, and migration, offering a novel therapeutic angle. In addition to its direct effects on tumor cells, AIM2's role in regulating immune responses is of particular interest. The cytokine interleukin-18 (IL-18), which has immunostimulatory effects, is negatively regulated by IL-18 binding protein (IL-18BP). A decoy-resistant form of IL-18 (DR-18) has been developed to enhance its anti-cancer efficacy. In RCC, DR-18 has been shown to enhance the activity of anti-CTLA-4 treatment, suggesting that AIM2's modulation of immune pathways could be leveraged to improve immunotherapy outcomes (69).

Overall, AIM2's involvement in RCC highlights the complexity of cancer biology and the need for integrated approaches to treatment. By targeting AIM2 and its associated pathways, there is potential to develop more effective therapies for RCC, improving patient outcomes and overcoming resistance to current treatments.

3.1.8 AIM2 in colorectal cancer

The AIM2 protein has emerged as a significant player in the context of CRC, particularly due to its role as a DNA sensor in the innate immune response. Recent studies have highlighted AIM2's potential as a tumor suppressor, demonstrating its involvement in regulating various cellular processes that are crucial for tumor

progression and metastasis. For instance, AIM2 has been shown to inhibit epithelial-mesenchymal transition (EMT) in CRC cells, a process that is often associated with increased invasiveness and metastatic potential. This suppression of EMT is mediated through the Akt signaling pathway and the inflammasome pathway, indicating that AIM2 may exert its anti-tumor effects by modulating critical signaling cascades involved in cancer cell behavior (Figure 3, left panels) (70–73). Several research revealed that AIM2 inhibits the proliferation of CRC cells by interacting with DNA-dependent protein kinase DNA-PK (74, 75) or triggering cell cycle arrest (Figure 3, left panels) (10, 72, 76). Furthermore, AIM2's expression levels have been correlated with patient prognosis in CRC. Low levels of AIM2 have been associated with poorer outcomes, suggesting that it may serve as a valuable prognostic marker (77, 78). In BRAF-mutant CRC, restoring AIM2 expression significantly inhibited tumor growth and induced necrotic cell death in a caspase-1 dependent manner, further underscoring its role as a tumor suppressor (Figure 3, left panels) (79). The interplay between AIM2 and the tumor microenvironment also warrants attention, as AIM2 has been implicated in enhancing immune responses against tumor cells, potentially through the activation of pro-inflammatory cytokines like IL-1 β (80).

In addition to its direct effects on tumor cells, AIM2's interaction with the gut microbiota has been identified as a critical factor in CRC susceptibility. Dysbiosis in the gut microbiota can exacerbate the tumorigenic effects of AIM2 deficiency, highlighting the complex relationship between host genetics, immune responses, and microbial influences in CRC development (74, 75). Moreover, the AIM2 inflammasome plays a central role in preventing gut microbiota dysbiosis and intestinal inflammation by modulating the STAT3 pathways and the expression of specific antimicrobial peptides (Figure 3, left panels) (81). This synergy suggests that therapeutic strategies aimed at modulating AIM2 expression or enhancing its activity could provide new avenues for CRC prevention and treatment. Moreover, the therapeutic potential of targeting AIM2 in CRC is supported by its ability to regulate the expression of key immune-related genes. AIM2 has been shown to mediate the expression of HLA-DRA and HLA-DRB, which are crucial for antigen presentation and immune surveillance (71). This indicates that AIM2 not only plays a role in tumor suppression but also in shaping the immune landscape of CRC, making it a promising target for immunotherapy approaches (Table 1).

Overall, AIM2 represents a multifaceted target in CRC, with its anti-tumor effects stemming from its ability to regulate cell signaling, influence the immune microenvironment, and interact with gut microbiota. Continued research into the mechanisms underlying AIM2's functions could pave the way for novel therapeutic strategies aimed at improving outcomes for CRC patients.

3.1.9 AIM2 in HPV-infected cervical cancer

HPV is a sexually transmitted DNA virus that has been irrevocably linked to the development of CCA (82). During the

early stages of tumorigenesis, AIM2 expression might ascend in response to viral infection and declined at a later stage due to various inhibitory mechanisms. SIRT1 is frequently overexpressed in HPV-infected CCA, with a negative regulatory effect on the transcription of the AIM2 gene. This regulation impaired the AIM2 inflammasome and enable HPV-infected CCA cells to evade antiviral immune responses (83). Furthermore, the knockout of SIRT1 has been shown to reactivate the AIM2 inflammasome, triggering the demise of CCA cells (83). Consequently, the interplay between SIRT1 and AIM2 may emerge as a promising therapeutic target for human CCA.

3.1.10 AIM2 in prostate cancer

Extensive research correlates inflammation with the PCa development (84, 85). Expression of AIM2 mRNA is elevated in benign prostatic hyperplasia compared to normal prostate tissue, yet it is significantly diminished in PCa cells (86). The loss of AIM2 expression in PCa cells is associated with increased tumor growth and metastasis, highlighting its importance in maintaining cellular homeostasis and preventing malignant transformation. In addition, IFN treatment upregulate AIM2 in PCa cell lines, activating the AIM2 inflammasome and promoting IL-1 β secretion (Table 1) (86). IL-1 β is also known to support the progression of bone colonization and metastasis of forefront cancer cells (87). Currently, there is an absence of direct evidence linking AIM2's inhibitory effects on PCa, and the underlying mechanisms remain elusive.

3.1.11 AIM2 in osteosarcoma

AIM2 is down-regulated in OS cell lines, and its overexpression has been shown to exert a suppressive effect on the proliferation and migration, while simultaneously promoting apoptosis (Table 1) (88). Furthermore, it has been observed that the overexpression of AIM2 leads to a significant downregulation of the phosphorylation levels of key proteins within the PI3K/Akt/mTOR signaling pathway (Figure 3, left panels) (89, 90). These findings not only corroborate the tumor-suppressive role of AIM2 but also shed light on the molecular pathways that may be targeted for therapeutic intervention in OS.

3.2 The pro-tumor role of AIM2

3.2.1 AIM2 in squamous cell carcinoma

Based on the bioinformatics analysis and immunohistochemistry, elevated levels of AIM2 are prevalent in primary oral squamous cell carcinoma (OSCC) tissues compared to adjacent normal tissues, which are positively associated with disease stage and HPV infection (91, 92). It has been noted that simultaneous high expression of AIM2 and IFI16 promoted p53-deficient OSCC cell proliferation by activating NF- κ B signaling pathway, while they inhibited wild-type p53 cells growth (93). These results indicated that AIM2 possess oncogenic properties in OSCC cells with p53 deletion (93, 94). AIM2 overexpression has been shown to increase tumor growth and invasion into lymphatic vessels by promoting

EMT that is a critical process for cancer metastasis, which lead to decreased survival rates in affected individuals (Figure 3, right panels) (92). AIM2 exerts pro-tumor effects activated by STAT1/NF- κ B transcription through the IL-17/MAPK pathway (Figure 3, right panels) (91). In turn, upregulated AIM2 further activates STAT1/NF- κ B signaling pathway through IL-1 β and induction of immune cell infiltration that produces IFN γ and TNF- α , to promote irradiation resistance, migration ability and PD-L1 expression in OSCC (Figure 3, right panels) (95). Additionally, AIM2 are emerging as a valuable indicator for predicting the efficacy of immune checkpoint inhibitors against refractory OSCC (95).

In penile squamous cell carcinoma (PSCC), the upregulation of AIM2 and BCL2A1 is associated with pN status and can serve as a novel molecular classifier for precise prognostic assessment (96). Silencing AIM2 inhibits the growth of cancerous epithelial cells via an anti-tumor inflammatory cytokine-independent pathway (96). Moreover, knockdown of AIM2 significantly reduces BCL2A1 expression through the NF- κ B pathway, thereby suppressing the MAPK/c-Myc signaling pathway (Figure 3, right panels and Table 1) (96).

The presence of AIM2 in cutaneous squamous cell carcinoma (cSCC) cells is more abundant than in normal skin tissues. Additionally, the reduction of AIM2 expression triggers cSCC cell apoptosis, diminishes cell invasiveness, and impedes cell vascularization (97). These findings indicate that AIM2 may function as a protumorigenic factor in cutaneous squamous cell carcinoma (cSCC), and that targeting AIM2 could potentially curtail tumor growth and invasive capacity. dihydroartemisinin, a compound with both antimalarial and antitumor capabilities (98), has been further discovered to exert effects by inhibiting the activation of AIM2 inflammasome pathway (99). These observations hint at the potential of AIM2 as a therapeutic target for SCC.

AIM2 has been identified as a potential biomarker for predicting tumor progression and patient outcomes in various SCC types. Its expression levels could serve as a prognostic factor, helping to stratify patients based on their risk of tumor progression and survival.

3.2.2 AIM2 in non-small cell lung cancer

Cancer-elicited inflammation is a critical factor in the carcinogenesis and metastasis of NSCLC. AIM2 expression is frequently dysregulated in NSCLC, with increased levels observed in tumor tissues compared to normal lung tissue reducing overall survival (100, 101). Different studies have drawn different conclusions about the pro-cancer effects of AIM2 independent or independent of inflammasomes activation (102–104). Upon exposure to noxious substances, the AIM2 inflammasome is activated, thereby inducing sustained oxidative stress and lung injury (105). Additionally, AIM2 might influence the tumor microenvironment by affecting immune cell infiltration and function. The activation of the AIM2 inflammasome is involved in the establishment of an immunosuppressive lung microenvironment (106, 107), the recruitment of T cells and

plasmacytoid dendritic cells (pDCs), as well as the polarization of tumor-associated macrophages (TAM) (Figure 3, right panels) (108, 109). AIM2 has also been observed to promote PD-L1 expression, suggesting that targeting AIM2 inflammasome therapy could potentially down-regulate PD-L1 expression, thereby counteracting the immune evasion in NSCLC (Figure 3, right panels) (101, 109, 110).

Research indicates that AIM2, predominantly localized within the mitochondria, exerts influences over mitochondrial morphology and reactive oxygen species (ROS) generation (103). AIM2 knockdown promoted mitochondrial derivation and fusion, whereas AIM2 upregulation promoted mitochondrial fission and fragmentation (103). Correspondingly, ROS produced by mitochondria fluctuate in response to changes in mitochondrial morphology, with, increased mitochondrial fission significantly heighten ROS levels in NSCLC cells (103, 111). Mitochondrial dynamics and functionality in mitochondria in cancer are pivotal to tumor progression, particularly through excessive production and alterations of ROS (Figure 3, right panels).

Intriguingly, AIM2 emerges as a distinctive biomarker of differential expression, uniquely identifying adenocarcinoma-like large cell lung cancer (112). There is an imperative for further research about the relationship between AIM2 and NSCLC (Table 1).

3.2.3 AIM2 in breast cancer

Extensive research has shown the role of pyroptosis in the occurrence, progression, and prognosis of BC. AIM2 and Z-deoxyribonucleic acid-binding protein 1 (ZBP1) were found to be important pyroptosis genes in DEGs, and significant differences in their expression in primary lesions and BMs were observed. Patients with a high expression of AIM2 had a worse prognosis than low expression, while patients with a high expression of ZBP1 had a better prognosis than low expression. AIM2 and ZBP1 increase immune cell infiltration and may be potential targets for predicting and treating triple-negative breast cancer (TNBC) patients with brain metastasis (BM) (113).

3.2.4 AIM2 in hepatocellular carcinoma

In the context of exploring the mechanisms underlying diethylnitrosamine (DEN)-induced HCC in murine models, recent research has highlighted the pivotal role of genetic inactivation of AIM2 in modulating liver pathology and tumorigenesis (114). Specifically, studies have demonstrated that the genetic inactivation of AIM2, but not NLRP3, results in a significant reduction in liver damage and the progression of HCC in this experimental paradigm. Notably, the deficiency of AIM2 appears to ameliorate the activation of the inflammasome, a critical component of innate immune responses, thereby mitigating liver inflammation and the proliferative responses that are integral to the initiation phase of HCC (114). These findings suggest a nuanced regulation of inflammatory pathways by AIM2, which may represent a promising therapeutic target for interrupting the carcinogenic process in DEN-induced liver cancer.

3.2.5 AIM2 in renal cell carcinoma

The oncogenic role of AIM2 in RCC has also been reported, highlighting its complex and paradoxical functions. In scenarios of acute tubular necrosis, the AIM2 inflammasome is capable of detecting DNA amidst necrotic remnants, thereby triggering an onset of necrotizing inflammation (115). Additionally, studies have shown that AIM2 can promote RCC progression and resistance to therapies such as sunitinib, a common treatment for RCC, independently of the inflammasome. Specifically, AIM2 has been found to reduce the susceptibility of RCC to sunitinib through the FOXO3a-ACSL4 axis-regulated ferroptosis, a type of programmed cell death distinct from apoptosis (Figure 3, right panels and Table 1) (116). Collectively, the intricate and context-dependent role of AIM2 in RCC underscores its potential as both a driver of tumorigenesis and a therapeutic target, underscoring further investigation into the mechanisms by which AIM2 modulates RCC biology.

3.2.6 AIM2 in gastric cancer

In a study leveraging the comprehensive resources of the TCGA database, coupled with primary GC samples from five patients, Feng et al. reported a significant upregulation of AIM2 expression within GC tissues (117). Their findings underscored the functional significance of AIM2, demonstrating that its knockdown effectively inhibited the invasive and migratory capabilities of GC cells by modulating and attenuating excessive MAPK signaling (Figure 3, right panels). This work contributes to a growing body of evidence indicating that AIM2 upregulation in GC tissues correlates adversely with patient survival outcomes. Further research has expanded our understanding of STAT3-mediated upregulation of the AIM2 interacting with microtubule-associated end-binding protein 1 (EB1) in GC, which facilitates epithelial cell migration and tumorigenesis, operating independently of inflammasome activation (Figure 3, right panels and Table 1) (118). These findings highlight AIM2 as a pivotal player in GC progression, linking innate immune responses and cellular migration to oncogenic processes. The identification of AIM2 as a key mediator in these pathways not only enhances our understanding of GC biology but also presents potential therapeutic targets for interventions aimed at mitigating GC progression and improving patient outcomes.

4 The dual role of IFI16 in cancer

4.1 The anti-tumor role of IFI16

4.1.1 IFI16 in squamous cell carcinoma

Restoration of IFI16 levels in head and neck squamous cell carcinoma (HNSCC) exhibits potent antitumor effects by inhibiting tumor growth and *in vitro* transforming activity, as well as augmenting doxorubicin-induced cell death via the accumulation of cells at the G2/M phase (Figure 4, left panels and Table 2) (119). Subsequent investigations have further elucidated the mechanisms underlying IFI16's antitumor activity *in vivo*. Specifically, IFI16

promotes apoptosis of tumor cells, inhibits neovascularization, and enhances the recruitment of CD68/CD14-positive macrophages through the release of chemotactic factors (Figure 4, left panels and Table 2) (120). Intriguingly, a correlation has been observed between IFI16 expression and the prognosis of HPV-positive HNSCC cases with low proliferative indices. Specifically, tumors positive for IFI16 staining and exhibiting high levels of the tumor suppressor protein pRb were associated with a more favorable prognosis compared to those with low IFI16 and pRb levels and a high proliferation index (121). This finding underscores the potential of IFI16 as a biomarker for predicting disease outcome in this subset of HNSCC patients. Moreover, an additional layer of complexity in the role of IFI16 in HNSCC has been revealed by studies indicating a negative correlation between IFI16 expression and lymph node metastasis, even in the absence of HPV infection (122). This observation suggests that IFI16 may play a protective role against lymphatic dissemination of HNSCC cells, irrespective of the HPV status of the tumor. Future research is needed to further elucidate the molecular mechanisms underlying IFI16's antitumor activities and to explore its clinical application in the management of HNSCC patients.

4.1.2 IFI16 in breast cancer

The tumor-suppressive functions of IFI16 are closely linked to its unique DNA-binding capacity, particularly its interactions with single-stranded DNA (ssDNA) and cruciform structures. Notably, clinical observations reveal reduced IFI16 expression levels in BC tissues, reinforcing its crucial role as a tumor suppressor intriguing DNA-binding capability of the IFI16 protein towards ssDNA and cruciform structures emerges as a pivotal aspect in its tumor suppressive functions (123). Interestingly, while IFI16 primarily exerts its tumor-suppressive effects through nuclear DNA surveillance, elevated serum levels of this protein have paradoxically been associated with poor prognosis in BC patients (124). This clinical paradox suggests that extracellular IFI16 may execute non-canonical biological functions distinct from its established nuclear tumor-suppressive mechanisms. The interaction between IFI16 and p53 is more intricate than previously understood. IFI16 is traditionally recognized for its role in disrupting apoptotic pathways by modulating both the p53/p21^{CIP1} and Rb/E2F signaling axes. Notably, it establishes a self-amplifying regulatory loop through p53-mediated induction of its own expression, collectively driving malignant transformation. This constitutes a component of a positive feedback loop between p53 and IFI16 (Figure 4, left panels). However, study indicates that IFI16 may suppress p53 transcriptional activity in MCF-7 and MDA-MB-231 cell lines, while paradoxically increasing the expression of p53 mRNA at the post-transcriptional level. This complexity suggests a nuanced role for IFI16 in modulating p53 function, with potential implications for cancer development and progression. IFI16's proteasomal degradation mediated by oncogenic factor HERC5 activates p53 transcription and enhances BC cell proliferation and migration (125). More recent insights have unveiled a novel facet of IFI16/IFI202 within TME, where it triggers tumor-promoting inflammation, thereby contributing to the formation of an immunosuppressive TME in BC (124). This finding expands our understanding of IFI16's multifaceted functions beyond its direct tumor suppressive activity. Triple-negative breast cancer (TNBC),

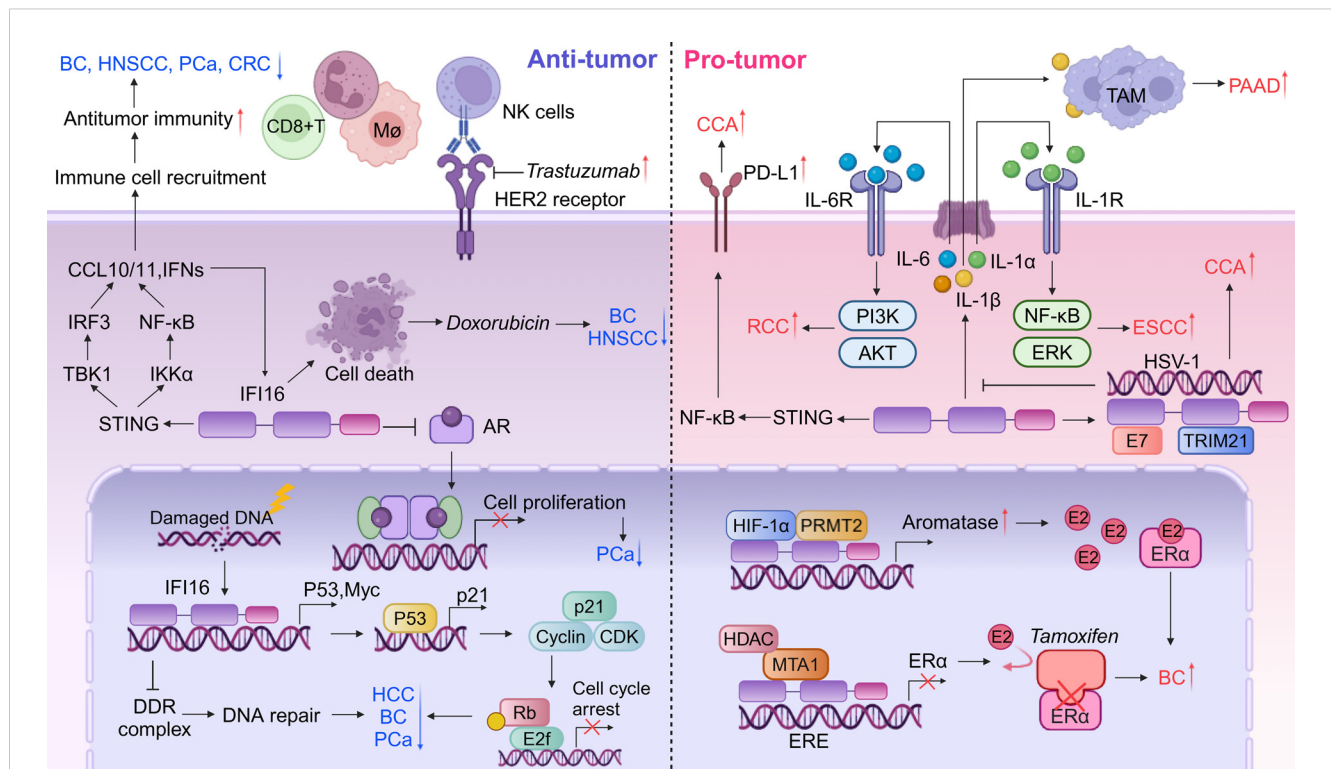


FIGURE 4

Dual roles of IFI16 in tumor microenvironment. IFI16 functions as a critical mediator in the tumor microenvironment, linking DNA damage to subsequent antitumor responses. It inhibits DNA repair processes, thereby promoting cell cycle arrest associated with cellular senescence and increasing cellular vulnerability to further DNA damage. The activation of the IFI16-STING pathway plays a central role in orchestrating antitumor immune responses, exhibiting tumor-suppressive properties and enhancing the chemosensitivity of cancer cells to therapeutic agents such as trastuzumab and doxorubicin. Conversely, IFI16 can also contribute to inflammasome formation, leading to the release of various inflammatory cytokines. These cytokines can activate intracellular signaling pathways in an autocrine manner or recruit TAMs, which may promote oncogenic progression and create an immunosuppressive microenvironment. Furthermore, IFI16 is involved in modulating hormone receptors, including AR and ER, thereby exerting diverse effects on tumor biology that range from suppression to promotion.

characterized by the absence of progesterone receptor, ER, and HER2 expression, is a heterogeneous and basal-like subtype. In TNBC patients, high expression levels of IFI16 have been notably associated with improved outcomes following chemotherapy treatments. Specifically, these high levels potentiate the inhibitory effects of doxorubicin on tumor growth *in vivo* (8). IFI16 amplifies the STING-induced type I IFN response by suppressing DNA repair and facilitating the translocation of DNA fragments into the cytoplasm, thereby intensifying the antitumor activity in TNBC (Figure 4, left panels). This suggests that IFI16 may serve as a sentinel and mediator of DNA damage, orchestrating subsequent antitumor responses within the TNBC microenvironment (8). Furthermore, for human epidermal growth factor receptor 2 (HER2)-positive BC, IFI16 is a key determinant in the response to monoclonal antibody therapy by activating the STING cascade (126). The reactivation of IFI16-directed immune responses has the potential to convert HER2-positive breast cancer into immunologically active 'hot tumors', thereby augmenting patient responsiveness to trastuzumab treatment (Figure 4, left panels and Table 2) (126).

Collectively, these findings underscore the nature of IFI16 in BC, with its tumor suppressive properties being counterbalanced by its potential to elicit an immunosuppressive TME.

4.1.3 IFI16 in hepatocellular carcinoma

IFI16 expression is significantly downregulated in HCC tissues, highlighting its potential role as a tumor suppressor. Restoration of IFI16 expression in HCC cells not only elicits p53/p21-dependent inhibition of tumor growth but also promotes the recruitment of inflammasomes, suggesting its multifaceted antitumor effects (Figure 4, left panels) (127). Furthermore, studies have demonstrated that IFI16 enhances chemosensitivity to cisplatin and increases susceptibility to DNA damage, thereby augmenting the efficacy of chemotherapy in HCC (9). Collectively, these findings underscore the importance of IFI16 as a tumor suppressor in HCC and its potential therapeutic implications (Table 2).

4.1.4 IFI16 in colorectal cancer

The phenotypic expression characterized by IFI16 negativity combined with Ki-67 positivity (IFI16-/Ki-67+) has been notably and positively correlated with advanced TNM staging in cancer patients, and it exhibits a borderline significant link to lymph node metastasis, as reported in a pivotal study (Table 2) (128, 129). Intriguingly, the expression levels of IFI16 did not display any statistically significant association with the infiltration of CD8+ TILs or the expression of PD-L1, implying that its impact on cancer

TABLE 2 The role of IFI16 in tumor biology and the underlying mechanisms in various tumor types.

Cancer types	Molecular mechanisms of IFI16	System	Role of IFI16	Reference
SCC	Increasing doxorubicin-induced cell death by enhancing G2/M phase arrest	HNSCC derived cell line HNO136,	Antitumor	(119, 120)
	Inducing IL-1 α to promote tumor progression through the Erk and NF- κ B signaling	Human ESCC cell lines TE-9, TE-10, and TE-11	Protumor	(126)
	Upregulating FGF protein to promote metastasis	Human ESCC cell lines (KYSE30, KYSE180, KYSE450) and high metastasis model 30M cells	Protumor	(138)
BC	Enhancing trastuzumab response in HER2-positive breast cancer by activating the STING cascade	HER2 + cell line (SKBR3, MDA-MB-361, BT-474), TNBC cell line (MDA-MB-231, MDA-MB-436), breast epithelial cell line (MCF10A)	Antitumor	(126)
	Mediating BC cell proliferation and migration via HERC 5 / IFI 16 / p53 signaling	MCF-7 and MDA-MB-231 BC cells	Antitumor	(125)
	Binding to toll-like receptor 2 and inducing macrophages to secrete proinflammatory cytokines	BC cell lines (MDA-MB-231, BT549, Hs578T, BT-474, MCF7, T47D,4T1, E0771, 67NR), monocytic cell line (THP-1), and kidney epithelial-like cell line (HEK293T)	Protumor	(124)
	Promoting aromatase expression and E2 production in the surrounding adipose tissue	MCF7, T47D, BT474 and THP1 cell line	Protumor	(139)
HCC	Inducing tumor inhibition via activation of p53 signals and inflammasome	SMMC-7721 and Huh7 HCC cells	Antitumor	(127, 143)
	Promoting DNA damage through interaction with IRF3 and reversing cisplatin resistance	Huh7 and HepG3 HCC cells	Antitumor	(9)
	Regulated by DLK1 to induce cell proliferation <i>in vitro</i>	SMMC-7721 and Huh7 HCC cells	Protumor	(141)
RCC	Promoting RCC progression through the IL6/PI3K/Akt axis	Human RCC cell lines (769-P, 786-O, ACHN, Caki-1, OS-RC-2) and human proximal tubular epithelial cell line (HK-2)	Protumor	(145)
CRC	Positively correlated with the expression of Ki67	CRC tissues and adjacent normal tissues	Antitumor	(129)
	Associated with the RAS pathway	HCT116 and RKO CRC cells	None	(131, 132)
PCa	Down-regulating AR expression and inhibiting cell proliferation	LNCaP PC cells	Antitumor	(134)
HPV-infected CCA	Regulating the expression of PD-L1 by promoting the activation of the STING-TBK1-NF- κ B pathway	Human HPV-positive cervical cancer cell lines (CaSki, SiHa, Hela) and HPV-negative cervical cancer cells (C33A)	Protumor	(148)

progression may be independent of these immunological factors. Factors causing DNA damage, such as oxidative stress, promote the deubiquitination of IFI16, thereby driving the STING-mediated antitumor immune response in CRC (Figure 4, left panels) (130). It is noteworthy that IFI16 also acts as an RAS pathway related protein, which frequently activates mutations in CRC (131, 132). Given the close association between RAS pathway activation and tumor proliferation as well as invasiveness, it warrants further investigation to elucidate its potential as a therapeutic target. Collectively, the findings from this research endeavor offer a fresh perspective on the functional role of IFI16 in the pathogenesis of CRC, particularly through its modulation of cancer cell proliferation. This novel insight underscores the potential significance of IFI16 as a biomarker and therapeutic target in CRC management.

4.1.5 IFI16 in prostate cancer

IFI16 has emerged as a pivotal regulator of cellular senescence and growth arrest in PCa cell lines. By upregulating p21WAF1 and inhibiting E2F-stimulated transcription, IFI16 suppresses cellular proliferation (133). Elevated levels of IFI16 in prostate epithelial cells (PrECs) contribute to senescence-associated growth arrest, suggesting its role in PCa suppression. IFI16 binds to the androgen receptor (AR) in a ligand-dependent manner, specifically through the AR's DNA-binding domain (DBD) (Figure 4, left panels and Table 2) (134). Re-expression of IFI16 in LNCaP cells, which lack endogenous IFI16, downregulates AR expression and inhibits the expression of AR target genes, leading to decreased cell viability and apoptosis (135). These findings support the hypothesis that histone deacetylase-dependent transcriptional silencing of IFI16

contributes to PCa development. Importantly, the re-expression of IFI16 orthologs in immunocompetent mice abrogates tumorigenesis and activates anti-tumor immunity, indicating that early epigenetic changes may influence tumorigenesis by targeting co-located genes (Figure 4, left panels) (136). Hypomethylation domains are detectable in blood samples enriched for metastatic circulating tumor cells, providing insights into the potential mechanisms underlying PCa progression.

4.1.6 IFI16 in osteosarcoma

In human OS, the expression level of IFI16 is markedly reduced compared to that in normal bone tissue. Overexpression of IFI16 has been demonstrated to modulate the expression of several key genes involved in cell cycle regulation and oncogenesis. Specifically, it upregulates p21 while downregulating cyclin E, cyclin D1, as well as the oncogenes c-Myc and Ras (137). In the context of viral infections, loss of IFI16 has been shown to impact chromatin labeling in OS cells, characterized by increased labeling of active chromatin and decreased labeling of viral and cellular gene promoters that inhibit chromatin activity (40). These findings suggest that IFI16 plays a pivotal role in histone modification and the regulation of gene expression in OS cells, potentially influencing the epigenetic landscape and DNA accessibility for transcriptional regulation.

4.2 The pro-tumor role of IFI16

4.2.1 IFI16 in squamous cell carcinoma

In ESCC, the role of IFI16 appears to contrast with its function in HNSCC. Specifically, high expression levels of IFI16 within ESCC tissues have been associated with unfavorable prognostic outcomes and increased macrophage infiltration, indicating its potential as a marker of aggressive disease (126). Mechanistically, IFI16-induced IL-1 α has been shown to exacerbate the malignant phenotype of ESCC cells, with particular impacts on cell survival and migration, thus contributing to disease progression (Figure 4, right panels) (126). Furthermore, emerging evidence suggests that IFI16 promotes ESCC metastasis by upregulating the expression of fibroblast growth factor (FGF) proteins, which are known to play crucial roles in cancer cell migration, invasion, and angiogenesis (138). These findings (Table 2) underscore the complex and context-dependent roles of IFI16 in different cancer types and highlight its potential as a therapeutic target in ESCC.

4.2.2 IFI16 in breast cancer

In the context of estrogen receptor (ER)-positive BC, IFI16 plays a pivotal role in augmenting tumor growth by facilitating the transcriptional activation of aromatase and subsequent E2 production in adipose tissue (Figure 4, right panels and Table 2) (139). Intriguingly, a stable knockdown of IFI16 or MTA1 in an MDA-MB-231-derived line significantly sensitized these cells to tamoxifen-induced growth inhibition. These findings collectively suggest that the MTA1-TFAP2C or MTA1-IFI16 complex may be involved in the epigenetic regulation of ESR1 expression, thereby

influencing the chemosensitivity of BC tumors to tamoxifen therapy (140).

4.2.3 IFI16 in hepatocellular carcinoma

Delta-like 1 (DLK1) upregulated the expression of IFI16 and its promoter transcriptional activity, is accompanied by a decrease in p21^{WAF1/CIP1} levels and subsequent acceleration of the cell cycle, suggesting that IFI16 may serve as a pivotal downstream target of DLK1 in promoting HCC cell proliferation (Table 2) (141). Furthermore, the chromatin-bound localization of IFI16 has been implicated in the progression of HCC, indicating its role as a potential biomarker or therapeutic target in this disease (142). Of note, Nutlin-3, a DNA damage agent, has been found to alter the subcellular localization of chromatin-bound IFI16 in HCC cells *in vitro*. This re-localization appears to be regulated in a p53-dependent manner, suggesting that Nutlin-3 may exert its effects on HCC cells through modulation of the p53-IFI16 axis (143). These findings provide new insights into the complex regulatory networks governing HCC progression and highlight the potential of targeting IFI16 and its interactions with other proteins, such as p53 and DLK1, for therapeutic intervention in this disease.

4.2.4 IFI16 in renal cell carcinoma

Elevated levels of IFI16 have been observed to positively correlate with several key clinicopathological features of RCC, including lymphatic metastasis, tumor stage, and histopathological grade. Notably, high IFI16 expression has been linked to adverse patient prognosis, with studies indicating that such expression is predictive of worse overall survival outcomes (144). At the molecular level, IFI16 has been shown to play a pivotal role in promoting clear cell RCC (ccRCC) progression. Specifically, IFI16 stimulates the transcription and translation of IL-6, which in turn activates the PI3K/Akt signaling pathway and induces EMT (Figure 4, right panels). Indeed, studies have demonstrated that IFI16-induced EMT promotes the progression of clear cell RCC (ccRCC) (Table 2) (145). Together, these findings provide new insights into the mechanisms underlying RCC progression and highlight the potential of targeting IFI16-related pathways for therapeutic intervention in this disease.

4.2.5 IFI16 in pancreatic adenocarcinoma

Overexpression of IFI16 has been implicated in the activation of the inflammasome, leading to the induction of IL-1 β production in the tumor microenvironment of PAAD. This IL-1 β production, in turn, mediates the maturation, proliferation, and migration of tumor-associated macrophages (TAMs), ultimately promoting the growth and progression of PAAD tumors. Notably, downregulation of IFI16 has been shown to reduce the infiltration of TAMs in the PAAD microenvironment when treated with gemcitabine, and to enhance the sensitivity of gemcitabine in these tumors (Figure 4, right panels) (146). Taken together, these findings suggest that IFI16 may emerge as a pivotal target for the development of novel therapeutic strategies aimed at inhibiting PAAD progression and improving treatment efficacy.

4.2.6 IFI16 in HPV-infected cervical cancer

The expression of IFI16 is significantly higher in HPV-positive lesions compared to HPV-negative lesions (147), and positively correlated with HPV infection (148). IFI16 has been implicated in positively regulating the expression of PD-L1 via the STING-TBK1-NF- κ B signaling pathway, thereby fostering the progression of CCA, as evidenced in a recent study (Figure 4, right panels and Table 2) (148). This revelation underscores the intricate roles that IFI16 plays in the oncogenic processes of CCA and suggests its potential as a novel therapeutic target for immunotherapy in the future. Furthermore, intriguing interactions between the HPV E7 oncogene and both IFI16 and TRIM21 have been documented (149). Specifically, E7 interacts with these proteins, leading to the recruitment of the E3 ligase TRIM21, which in turn ubiquitinates and degrades the IFI16 inflammasome (Figure 4, right panels). This process results in the inhibition of cell pyroptosis and enables cancer cells to evade immune surveillance (149). These findings warrant further investigation into the multifaceted roles of IFI16 in CCA progression and highlight its potential as a key player in the modulation of immune responses in this malignancy.

5 The anti-tumor role of IFIX

5.1 IFIX in breast cancer

In the context of breast tumorigenesis, the expression of IFIX is frequently downregulated, serving as a pivotal tumor suppressor. Despite its recognized role, the intricate mechanisms underlying IFIX's antitumor effects remain an area of ongoing research. Several plausible pathways through which IFIX exerts its suppressive influence on BC progression can be delineated as follows: Firstly, IFIX α 1 exhibits an autonomous tumor-suppressive function that transcends the classical regulatory pathways involving the pRb and p53. Specifically, IFIX α 1 upregulates the expression of p21CIP1, a cyclin-dependent kinase (CDK) inhibitor. This upregulation leads to a reduction in the kinase activities of Cdk2 and p34Cdc2, the latter being a crucial CDK controlling the G2/M transition phase of the cell cycle. Consequently, the activation of p34Cdc2, which is essential for the progression through S-phase and the G2/M transition, is impeded, resulting in the accumulation of cells in these phases (150). Secondly, IFIX negatively regulates HDM2 (also known as MDM2), a protein implicated in oncogenesis. HDM2 functions by inhibiting the activity of p53 through binding to its N-terminus. Following DNA damage, an intriguing shift occurs in the ubiquitination process mediated by MDM2's E3 ubiquitin ligase activity. Specifically, ubiquitination is partly redirected from p53 to MDMX, thereby facilitating p53 activation. This intricate p53/MDMX feedback loop plays a critical role in maintaining appropriate p53 levels within the cell (19). Thirdly, IFIX α is also implicated in the downregulation of histone deacetylase 1 (HDAC1) protein via a ubiquitin-proteasome pathway. This downregulation has indirect consequences on BC cell behavior, specifically

promoting the transactivation of maspin in MDA-MB-468 cells (151). The modulation of HDAC1 by IFIX α represents an additional layer of complexity in its anti-tumor mechanisms, highlighting its multifaceted role in BC suppression. Collectively, these findings underscore the significant anti-tumor potential of IFIX in BC and suggest multiple mechanisms through which it exerts its suppressive effects.

5.2 IFIX in oral squamous cell carcinoma

The recently discovered IFIX has emerged as a pivotal tumor suppressor in breast cancer. However, our understanding of IFIX's role in OSCC remains relatively nascent. Intriguingly, studies have demonstrated that the overexpression of IFIX potently inhibits the invasive capabilities of human CAL-27 cells. This inhibitory effect is mechanistically underpinned by the stabilization of the cytoskeleton, achieved through the modulation of various cytokeratin (152). Additionally, IFIX downregulates paxillin, an intracellular adaptor protein known to facilitate tumor invasion (152). Notably, the stabilization of the cytoskeleton is closely linked to the suppression of EMT, which involves extensive cytoskeletal reorganization to enable cancer cells to acquire migratory and invasive properties. In this context, IFIX has been shown to inhibit EMT by mediating the Wnt/ β -catenin signaling pathway through the upregulation of naked cuticle 2 (NKD2), a known inhibitor of this pathway in CAL-27 cells (153). By suppressing Wnt/ β -catenin signaling, IFI16 prevents the transcriptional activation of EMT-inducing genes, thereby maintaining epithelial characteristics and reducing tumor aggressiveness (153). Collectively, these findings contribute novel and fundamental insights into the tumor-suppressive functions of IFIX, particularly its role in stabilizing the cytoskeleton of cancer cells and preventing the transition to a mesenchymal phenotype.

6 The dual role of MNDA in cancer

6.1 The anti-tumor role of MNDA in myelodysplastic syndromes

The human MNDA, a nuclear protein specific to myelomonocytic cells, is regulated by interferon alpha in a cell-specific manner. MNDA influences the function of ubiquitous proteins like nucleolin in a cell/lineage- and differentiation-specific context (154). Elucidating MNDA's protein binding could advance our understanding of its function and the roles of other interferon-inducible gene family members. MNDA interacts with multiple proteins regulating gene transcription, exhibiting unique expression patterns across cell lineages and mediating interferon effects (155). Notably, MNDA alters the expression of a hematopoiesis-essential gene (155). Reduced MNDA transcripts are observed in both familial and sporadic MDS cases (156, 157),

impacting programmed cell death in myeloid progenitor cells and enhancing tumor necrosis factor-related apoptosis inducing ligand (TRAIL)-induced cell death sensitivity in granulocyte-macrophage progenitors (158). Lower MNDA expression in granulocytes and blasts distinguishes MDS from non-MDS, establishing it as an independent marker for dyspoiesis assessment and a potential addition to flow cytometric (FCM)-based quantitative panels (159). In summary, MNDA's multifaceted role in regulating cellular processes, particularly within the myelomonocytic lineage, and its potential implications in diseases such as MDS, underscore the necessity for further investigation into its protein interactions and functional mechanisms. Such endeavors could significantly contribute to advancing our understanding of hematopoiesis and the pathobiology of MDS.

6.2 The pro-tumor role of MNDA in marginal zone lymphoma

In recent scientific inquiries, the expression profile of MNDA has emerged as a significant biomarker in the context of various B-cell lymphomas. MNDA is constitutively expressed in a distinct subset of marginal zone B cells within normal tissue, underscoring its physiological role in B-cell biology. Notably, this expression pattern extends to specific lymphoma subtypes, particularly those originating from the marginal zone, such as mucosa-associated lymphoid tissue (MALT) lymphoma, splenic marginal-zone lymphoma (SMZL), and nodal marginal zone lymphoma (NMZL). The preferential expression of MNDA in these lymphomas, coupled with its rarity in follicular lymphoma (FL), positions it as a valuable diagnostic tool in differentiating NMZL from FL (160). Moreover, MNDA's elevated expression in MZL further enhances its utility in distinguishing MZL from reactive lymphoid hyperplasia (RLH) and FL. However, its discriminatory power wanes when distinguishing MZL from mantle-cell lymphoma (MCL) and chronic lymphocytic leukemia/small lymphocytic lymphoma (CLL/SLL) (161). Intriguingly, within diffuse large B-cell lymphoma (DLBCL), MNDA expression is more prevalent in the non-germinal center B-cell (non-GCB) subtype and the BCL2/MYC double-expression group, displaying a correlation with CD5 expression—a finding that merits further exploration. The clinical implications of MNDA expression and its potential prognostic significance in these lymphomas remain incompletely understood and warrant thorough elucidation. Interestingly, MNDA expression does not serve as an effective identifier in cases characterized by MYD88 p.Leu265Pro mutations or specific SMZL-related genetic aberrations (162), indicating that its diagnostic utility is context-dependent. A novel insight comes from the VR09 cell line, derived from an atypical, non-CLL B-cell chronic lymphoproliferative disorder with plasmacytic features. This cell line exhibits an activated B-cell maturation phenotype with secretory differentiation, harboring an episomal EBV genome, trisomy of chromosome 12, and a wild-type

p53 status, among other genetic features. Notably, it also displays MNDA expression, suggesting that this EBV-positive cell line could serve as a valuable model for further characterizing *in vivo* activated DLBCL with plasmacytic characteristics (163).

In summary, MNDA expression profiling has emerged as a promising diagnostic aid in the differentiation of specific B-cell lymphoma subtypes, particularly within the context of MZL and certain DLBCL subsets. However, its application should be nuanced, considering its variable expression across different lymphoma entities and its limitations in certain genetic contexts. Future studies are needed to fully elucidate the clinical relevance and prognostic implications of MNDA expression in these lymphomas.

6.3 The anti-tumor role of MNDA in osteosarcoma

MNDA was highly expressed in normal bone tissue, but was significantly lower in OS cells (164). MNDA overexpression effectively inhibited proliferation induced apoptosis and reduced migration/invasiveness in Saos-2 cells (164). To further explore the potential mechanisms underlying MNDA's tumor-suppressive effects, recent studies have identified hsa-miR-889-3p as a key regulator. This microRNA could promote the proliferation of OS through inhibiting MNDA expression, which provides a potential therapeutic strategy in treatment for OS (165). In the tumor microenvironment of OS, MNDA+ macrophages could also contribute to disease progression by enhancing osteoclast activity, leading to bone destruction and the release of growth factors that promote tumor cell proliferation and invasion (166). Therefore, targeting MNDA+ macrophages, restoring MNDA expression in OS cells, or inhibiting hsa-miR-889-3p could represent promising therapeutic strategies for OS, particularly in preventing bone destruction and tumor progression.

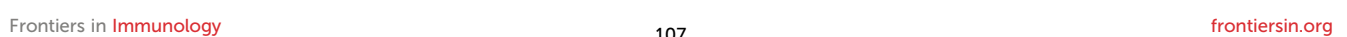
6.4 The pro-tumor role of MNDA in hepatocellular carcinoma

MNDA, predominantly expressed in tumor-associated M2 macrophages, critically orchestrates the crosstalk between M2 macrophages and HCC cells through exosome-mediated transfer of pro-metastatic proteins (167). Mechanistically, MNDA enhances M2 macrophage polarization by upregulating key polarization enhancers such as TGFB1, CSF1, and S100A4. Furthermore, MNDA drives the selective packaging of metastasis-promoting proteins, including NRP2, TIMP1, ITGB2, and ITGAM, into exosomes secreted by M2 macrophages. M2-derived exosomes robustly enhance HCC cell migration, an effect attenuated by MNDA knockdown (167). Clinically, high MNDA expression correlates with poor prognosis in HCC patients, highlighting its potential as a therapeutic target. Targeting MNDA or its exosomal

both AIM2 and NLRP3 inflammasomes (133). Furthermore, in the context of PANoptosis, an inflammatory cell death pathway involving AIM2 and other inflammasomes, IFI16 emerges as a key regulatory factor (169). The complex interplay between AIM2 and IFI16 inflammasomes and its implications for disease pathogenesis warrant further investigation, as the relationship between the PYHIN family and cancer holds significant, yet untapped, potential.

Beyond their canonical roles in inflammasome formation, PYHIN family proteins exhibit diverse inflammasome-independent functions in cancer. For instance, AIM2 regulates intestinal stem cell proliferation and gut microbiota, influencing CRC pathogenesis (74, 75). IFI16, by sequestering transcription factors and modulating the expression of viral and cellular genes, impacts cancer progression (170). Furthermore, IFIX's ability to detect viral DNA and induce an interferon response hints at its potential protective role against virus-induced cancers (126, 133). MNDA, typically linked to hematological disorders characterized by apoptotic imbalances, has been implicated in promoting OS metastasis (167), suggesting that the PYHIN family may have novel roles in a broader range of tumors. The development of broader and more effective anticancer drugs is a promising avenue.

Current strategies focus on molecularly tailored interventions against PYHIN family members through multiple modalities. Small-molecule inhibitors, like quercetin and Rg1, suppress inflammasome activity, mitigating treatment-related toxicity from radiotherapy or chemotherapy (171, 172), while PYHIN agonists, such as deoxyribonuclease 1 like 3 (DNASE1L3) and dihydroartemisinin,



may enhance therapeutic efficacy in specific contexts (53, 60, 173). Tissue-targeted viral and non-viral gene delivery systems enable precise modulation of PYHIN expression to inhibit tumor progression (65, 174). Combination approaches integrating DNA sensor targeting with immune therapy or radiation therapy demonstrate synergistic antitumor effects, creating a promising therapeutic blueprint (58, 148, 175, 176). However, challenges remain, such as systemic toxicity of inflammasome suppression, which may compromise innate immunity or exacerbate off-target effects. Optimized combinatorial regimens balancing inflammasome modulation with conventional therapies, coupled with dynamically responsive drug delivery platforms, are essential to maximize therapeutic windows and minimize adverse outcomes.

Despite the significant progress made in understanding the roles of PYHIN family proteins in cancer, several key questions remain unanswered. For instance, future research should focus on elucidating the precise mechanisms underlying the dual roles of AIM2 and IFI16 in various cancers, as well as the interactions and crosstalk among PYHIN family members. Additionally, the potential synergistic effects of targeting multiple PYHIN family members or combining PYHIN-targeted therapies with other treatment modalities (such as immunotherapy, chemotherapy, or radiation therapy) need to be explored. Clinical trials are essential to validate the efficacy and safety of these therapies in patients with different cancer types. In short, although further work is needed, the PYHIN family proteins represent promising targets for cancer therapy, and continued research in this area holds great potential for improving patient outcomes.

Author contributions

SZ: Conceptualization, Investigation, Writing – original draft, Writing – review & editing. ZZ: Investigation, Writing – original draft, Supervision. YL: Investigation, Visualization, Writing – original draft. DW: Investigation, Visualization, Writing – original draft. QX: Resources, Supervision, Writing – original draft. HP: Conceptualization, Funding acquisition, Investigation, Project administration, Supervision, Writing – original draft, Writing – review & editing.

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Tislelizumab-induced hemophagocytic lymphohistiocytosis in a patient with microsatellite instability-high colon cancer and coexisting systemic lupus erythematosus: a case report and literature review

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Immune checkpoint inhibitors (ICIs) have profoundly transformed the treatment landscape for microsatellite instability-high (MSI-H) colorectal cancer (CRC). However, immune-related adverse events (irAEs) remain a common and unpredictable complication among patients undergoing ICI therapy. Hemophagocytic lymphohistiocytosis (HLH), a rare and life-threatening irAE, is triggered by hyperactivated immune cells and excessive secretion of proinflammatory cytokines. We report a case of an MSI-H female patient with a history of systemic lupus erythematosus (SLE) who developed HLH after treatment with the anti-programmed cell death protein 1 (PD-1) antibody tislelizumab. After treated with tislelizumab 31 days, the patient presented with fever, pancytopenia, hemophagocytosis in bone marrow, elevated ferritin and triglyceride levels, and decreased fibrinogen. A diagnosis of HLH was confirmed with an H-score of 264. Despite steroid therapy, the patient's HLH progressed rapidly. Etoposide was deemed intolerable, and tocilizumab and immunoglobulin were declined due to financial constraints. Regrettably, the patient succumbed to HLH within 16 days of diagnosis. This is the first reported case of ICI-induced HLH in an MSI-H colon cancer patient with a history of SLE, prompting an analysis of the potential mechanisms underlying the induction of HLH in this case. Clinicians should be vigilant for the development of HLH during ICI treatment and initiate combination therapy as early as possible upon onset of HLH.

KEYWORDS

MSI-H, PD-1, colon cancer, SLE, HLH

Introduction

Colorectal cancer (CRC) is the third most common cancer worldwide with an estimated incidence of over 1.9 million new cases annually, and it is the second leading cause of cancer-related mortality with 904,000 deaths (1). Approximately 20% of patients present at an advanced stage without the opportunity for surgery (2). Fluorouracil-based and platinum-based combinations are frequently utilized as chemotherapeutic agents for these advanced patients (3). Targeted agents such as bevacizumab or cetuximab, depending on the RAS gene mutational status and tumor location, are combined to augment the anti-tumor effect of chemotherapy agents (4). Nevertheless, the prognosis for these patients remains bleak (5). Immune checkpoint inhibitors (ICIs) targeting the programmed cell death protein 1 (PD-1) and cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) have made significant breakthroughs in cancer treatment. Following their initial approval for melanoma treatment, ICIs have been widely applied in adjuvant treatment for various types of cancer (6). The KEYNOTE-177 trial showed that an anti-PD-1 antibody of pembrolizumab significantly improved the progression-free survival (PFS) compared to chemotherapy (16.5 months versus 8.2 months) as a first-line treatment in advanced CRC patients with microsatellite instability-high (MSI-H) or mismatch repair-deficient (dMMR) (7). Based on the result of KEYNOTE-177, pembrolizumab was approved by Food and Drug Administration (FDA) as the first-line treatment for MSI-H advanced CRC patients.

ICIs are believed to primarily exert anti-tumor effects by activating immune responses, particularly T lymphocytes (8). However, as a result of antigens shared between the tumor tissue and the target organ, the activated immune response may lead to T cell cross-reactivity, thereby resulting in the development of immune-related adverse events (irAEs) (9). The incidence of irAEs for ICI treatment of MSI-H colorectal cancer is approximately 31%, with 9% classified as grade 3–4 irAEs. These frequently include hypothyroidism, colitis, hyperthyroidism, pneumonitis and adrenal insufficiency (7). Nevertheless, hemophagocytic lymphohistiocytosis (HLH) caused by ICIs in MSI-H colorectal cancer patients has not been reported yet.

HLH is a rare syndrome characterized by high fever, cytopenia, hepatosplenomegaly, elevated levels of liver enzymes, and high serum levels of ferritin and triglyceride (10). The disease commonly results from the hyper-activation of immune cells, leading to excessive pro-inflammatory cytokine secretion, progressive tissue injury, multi-organ failure, and ultimately mortality (11). HLH is classified as primary or acquired based on its etiology, with the latter typically triggered by malignancies, severe acute infections, autoimmune diseases and exposure to drugs including ICIs (12). A study indicates that 5.7% of all HLH cases are associated with the application of ICIs (13). However, cancer patients with ICI-related HLH had a history of autoimmune disease were rarely reported. Here, we presented a case of HLH following treatment with an anti-PD-1 antibody of tislelizumab in a patient with MSI-H colon cancer and co-existing systemic lupus erythematosus (SLE).

Case report

A 68-year-old Chinese female was admitted to the Fourth Hospital of Hebei Medical University on January 15, 2024, presenting with a complaint of appetite loss persisting for over a month. Notably, her medical history included a three-year diagnosis of SLE. Upon admission, the patient was administered a daily dosage of 3 mg of methylprednisolone and 0.1 g of hydroxychloroquine, maintaining her SLE in a stable state. Twenty days prior to admission, a colonoscopy revealed an ulcerative mass in the ascending colon, causing lumen narrowing (Figure 1A). Pathological and immunohistochemical analyses confirmed the tumor as a poorly differentiated adenocarcinoma (Figure 1B) with MSI-H, characterized by PMS2 (-), MLH1 (-), MSH2 (+), and MSH6 (+) (Figures 1C–F). An enhanced computed tomography (CT) scan demonstrated ascending colon wall thickening accompanied by enlarged peripheral lymph nodes (Figure 2A), alongside suspected malignant lesions and multiple metastatic nodules in the pelvic and abdominal cavities, leading to a diagnosis of Stage IV colon carcinoma. Laboratory tests indicated a negative anti-dsDNA antibody, while the patient tested positive for ANA (1:320) with TOPO I and anti-Sm antibody. Additional laboratory findings revealed complement C3 was 0.69g/L (normal range: 0.7–1.4g/L), complement C4 0.12g/L (normal range: 0.1–0.4g/L), white blood cells (WBC) $9.01 \times 10^{12}/L$ (normal range: $3.8\text{--}5.1 \times 10^{12}/L$), hemoglobin 81.7g/L (normal range: 115–150g/L), platelets $238 \times 10^9/L$ (normal range: $125\text{--}350 \times 10^9/L$), D-dimer 0.193 mg/l (normal range: <0.243mg/L), triglyceride 1.98 mmol/L (normal range: 0.3–1.7mmol/L), and C-reactive protein (CRP) 82.3mg/L (normal range: 0–6.0mg/L). Routine urine analysis was within normal limits. Based on the patient's clinical manifestations and laboratory results, the SLEDAI-2000 score was calculated as 0, confirming her SLE was in a stable phase. Given the patient's stable SLE, she received tislelizumab - the only PD-1 monoclonal antibody with government-sponsored medical insurance coverage for MSI-H solid tumor indications in China, (200 mg intravenously once every 3 weeks) on January 19, 2024, and was successfully discharged post-treatment. The patient returned to the Fourth Hospital of Hebei Medical University for the next treatment cycle on February 17, 2024. Since her last discharge, she experienced intermittent appetite loss and fatigue. Physical examination found no throat congestion or swelling, and cardiopulmonary and abdominal assessments revealed no significant abnormalities. The blood counts showed WBC was $3.23 \times 10^{12}/L$, hemoglobin of 75 g/l and platelets $12 \times 10^9/L$. Her D-dimer was 4.782 mg/l, triglyceride 2.78mmol/L, fibrinogen 0.81 g/L (normal range: 2.38–4.98g/L), ferritin $\geq 2000\text{ng/L}$ (normal range: 13.0–150), C3 0.46g/L, and CRP 71mg/L. A follow-up CT scan (Figure 2B) showed persistent thickening of the ascending colon wall, but with a reduction in the number of abdominal and pelvic nodules, suggesting a partial response to colon cancer treatment. However, due to the patient's predisposition to HLH induced by ICI, administration of tislelizumab was discontinued. Although a bone marrow aspiration was proposed, the patient declined. Consequently, the patient was treated with daily 40 mg of methylprednisolone, along

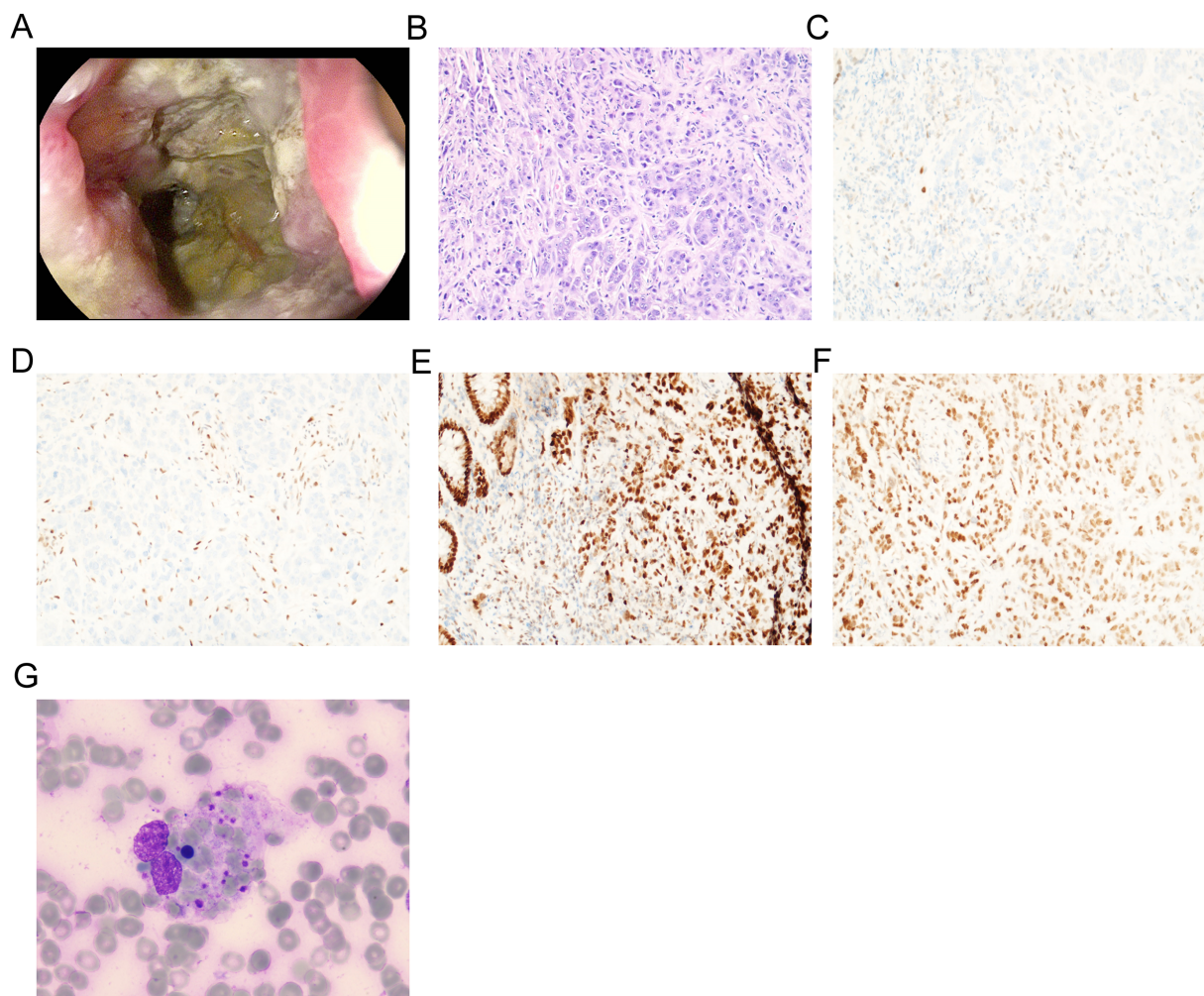


FIGURE 1

Gastroscopy and biopsy of the patient. **(A)** The colonoscopy examination revealed an ulcerative mass in the ascending colon; **(B)** The histopathological examination of biopsies sampled revealed poorly differentiated adenocarcinoma (stained using the H&E method, magnification, x200); **(C)** PMS2(-) by immunohistochemistry (magnification, x200); **(D)** MLH1(-) by immunohistochemistry (magnification, x200); **(E)** MSH2(+) by immunohistochemistry (magnification, x200); **(F)** MSH6(+) by immunohistochemistry (magnification, x200); **(G)** Bone marrow biopsy of the patient (magnification, x400).

with platelet and red blood cell transfusions, and symptomatic and supportive care. Three days later, the patient developed a non-infectious fever, peaking at 38.5°C. Following repeated and emphatic recommendations, the patient underwent a bone marrow aspiration, revealing mixed anemia and an irritable bone marrow image with prominent hemophagocytosis (**Figure 1G**). Based on these findings, the patient met the diagnostic criteria for HLH-2004, with an H-score of 264. Given her poor physical condition, chemotherapy with etoposide and cyclophosphamide was deemed unsuitable, and she refused cytokine antagonists and immunoglobulin due to financial constraints. The patient was then treated with 80 mg of methylprednisolone daily for an additional four days, but her condition failed to improve. Ultimately, the patient opted to discontinue treatment and passed away ten days after discharge. The treatment process of the patient is depicted in **Figure 3**.

Discussion

Patients with cancer and concurrent autoimmune diseases (ADs) including SLE, have historically been excluded from ICIs clinical trials. This exclusion primarily stems from concerns that ADs may be exacerbated, leading to an increased risk of severe irAEs. Nevertheless, in clinical practice, an increasing number of such patients are being encountered, and evidence suggests that ICIs treatment can be both safe and effective for them. A retrospective study evaluated the therapeutic outcomes of 85 individuals diagnosed with both cancer and ADs who received anti-PD-1/PD-L1 antibodies therapy. Their outcomes were compared with those of patients without ADs. Although a higher proportion of AD patients experienced irAEs of any grade (65.9%

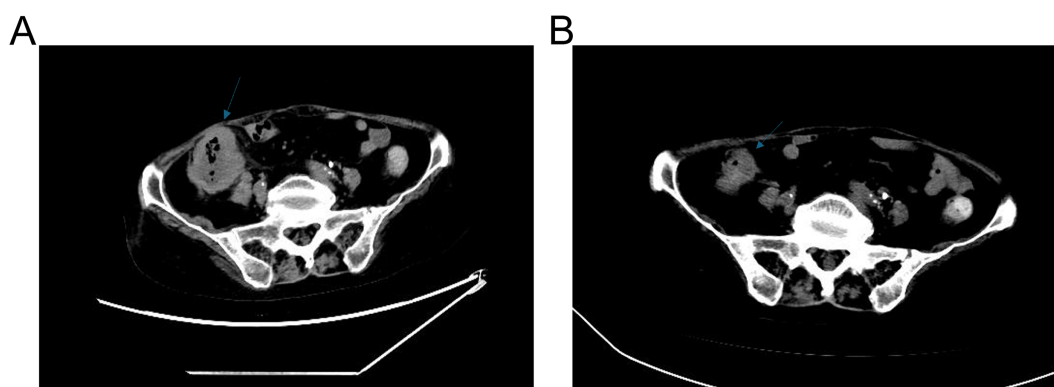


FIGURE 2
CT of the patient. (A) Abdominal CT scan prior to the treatment of tislelizumab. (B) Abdominal CT scan after the treatment of tislelizumab.

compared to 39.9%), no significant difference was observed in the incidence of grade 3–4 events between the two groups. Furthermore, no notable differences were identified in prognosis (14). Three additional studies have similarly demonstrated the safety of ICIs in patients with cancer and coexisting ADs. In these studies, only 20%–40% of patients experienced worsening of their autoimmune conditions, and all adverse effects were manageable. Discontinuation of ICI therapy was rare, and tumor responses were reported (15–19). A systematic review evaluated the use of ICIs in 123 cancer patients with a history of ADs (20). Overall, 92 patients (75%) experienced irAEs. Among them, 50 patients (41%) had exacerbations of pre-existing ADs, 31 patients (25%) developed new irAEs, and 11 patients (9%) experienced both types of events.

Based on these findings, the following conclusions can be drawn: 1) Patients with ADs including SLE, experience a higher overall incidence of irAEs than those without ADs; however, there is no significant difference in the occurrence of high-grade (grade 3–4) events. 2) The most common types of irAEs involve exacerbations of underlying ADs. This may result from PD-1/PD-L1 inhibition-induced activation of T cells and other immune cells, which leads to the release of pro-inflammatory cytokines (21, 22), potentially triggering off-target inflammation and irAEs (23). 3) Flare-ups of ADs generally do not necessitate discontinuation of ICIs therapy and

can be effectively managed. 4) The effectiveness of ICIs appears similar in patients with and without ADs (24). Despite this evidence, patients with cancer and ADs currently lack standardized treatment guidelines and reliable methods for assessing individual benefit-risk ratios. This gap represents a significant unmet need in oncology. Therefore, prospective studies are urgently needed to establish optimal strategies for administering ICIs in this patient population.

The H-score, which integrates various clinical indicators such as recognized immunosuppression, elevated temperature, and organomegaly; laboratory findings encompassing triglycerides, ferritin, serum glutamic oxaloacetic transaminase levels, fibrinogen levels, and cytopenia; as well as a specific cytological criterion (the presence of hemophagocytosis in bone marrow aspirate), is extensively employed for diagnosing HLH (25). A score exceeding 250, accompanied by a clinical presentation devoid of detectable infection, strongly indicates HLH. The patient exhibited a clinical profile characterized by fever, pancytopenia, hemophagocytosis in the bone marrow, elevated ferritin and triglyceride levels, and decreased fibrinogen, collectively pointing towards a diagnosis of HLH. The onset of HLH can be precipitated by multiple external factors, including malignancies, severe acute infections, autoimmune diseases, and exposure to ICIs (26). Hematological malignancies are the most common malignancies associated with HLH, whereas solid

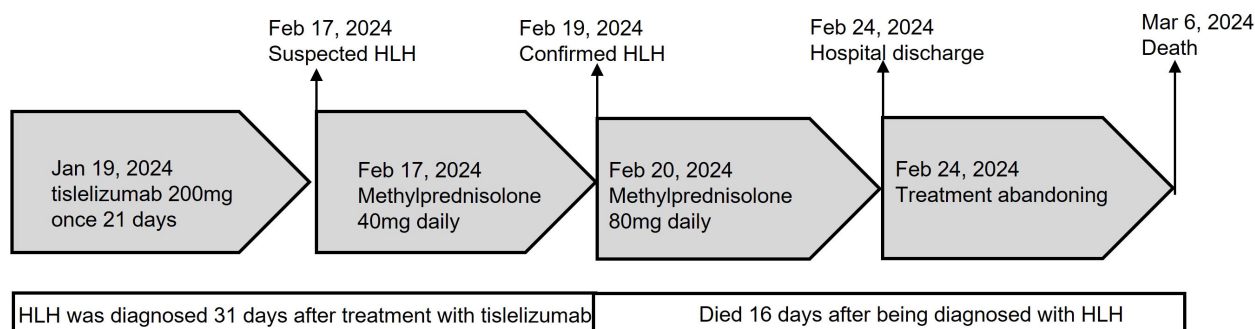


FIGURE 3
Treatment summary of the patient.

TABLE 1 ICI-related HLH reported in the literature in patients with cancer.

Study References	Malignancy type and stage	Age and sex	ICI	ADs	Time To HLH (days)
(48)	RCC, IV	54, M	Nivolumab + ipilimumab	None	6
(49)	LUAD, IV	78, M	Pembrolizumab	None	7
(50)	LUSC, IIIB	78, M	Pembrolizumab	None	10
(51)	Melanoma, IV	35, F	Ipilimumab + nivolumab	None	21
(52)	NSCLC, IV	63, F	Nivolumab	None	22
(53)	Choroidal melanoma, IV	65, F	Ipilimumab	None	42
(54)	Glioblastoma	74, M	Nivolumab	None	47
(55)	Melanoma, IV	42, M	Ipilimumab + nivolumab	None	51
(56)	Melanoma, IV	52, F	Ipilimumab	None	56
(57)	LUAD, IIIB	52, F	Nivolumab	None	56
(58)	Melanoma, IV	57, F	Ipilimumab + nivolumab	None	63
(55)	Melanoma, IV	36, M	Nivolumab	None	78
(55)	Melanoma, IV	32, M	Ipilimumab + nivolumab	None	91
(59)	Breast cancer, IV	58, F	Pembrolizumab	None	93
(60)	Melanoma, IV	58, M	Pembrolizumab	None	136
(61)	NSCLC, IV	60, M	Pembrolizumab	None	186
(61)	NSCLC, IV	60, M	Pembrolizumab	None	210
(62)	Kaposi sarcoma	85, M	Nivolumab	None	240
(63)	Bladder, IV	76, M	Pembrolizumab	None	270
(64)	OPSCC, IV	61, M	Pembrolizumab	None	277
(47)	Thymic carcinoma, IV	50, F	Pembrolizumab	Sjögren's syndrome	7
(44)	CESC, IVB	73, F	Pembrolizumab	Psoriasis	12
(65)	Melanoma, IV	69, F	Ipilimumab + nivolumab	Sarcoidosis	22
(66)	LUSC, IIIB	74, M	Pembrolizumab	Rheumatoid arthritis	27
(67)	LUAD, IV	65, F	Atezolizumab	Antinuclear antibody positive	37
(68)	Melanoma, IV	26, F	Ipilimumab + nivolumab	Immune thyroiditis	70
(45)	Gastric cancer, IV	79, M	Nivolumab	Ankylosing spondylitis	143
(46)	Thymic carcinoma, IV	49, M	Pembrolizumab	Psoriasis	365

ICIs, Immune checkpoint inhibitors; HLH, Haemophagocytic lymphohistiocytosis; M, Male; F, Female; RCC, Renal cell carcinoma; LUAD, Lung adenocarcinoma; LUSC, Lung squamous cell carcinoma; NSCLC, Non-small cell lung cancer; OPSCC, Oropharyngeal squamous cell carcinoma; ITP, Immune thrombocytopenic purpura; CESC, Cervical squamous carcinoma.

tumors are infrequently reported (27). Notably, no cases have established a direct link between colon cancer and the initiation of HLH.

During ICIs treatment, the inhibition of the PD-1/PD-L1 signaling pathway disrupts the normal inhibitory control that negatively regulates T-cell function. This leads to hyperactivation

of cytotoxic T lymphocytes (CTLs) and excessive secretion of pro-inflammatory cytokines in healthy tissues (9, 28). HLH, a rare and severe irAE, can be triggered by hyperactivated immune cells and oversecretion of pro-inflammatory cytokines (12). Pathological features of HLH reveal abundant infiltration of immune cells, including CTLs and macrophages, along with oversecretion of

pro-inflammatory cytokines such as interferon (IFN)- γ , tumor necrosis factor (TNF)- α , interleukin (IL)-1 β , IL-6, IL-10, and IL-18 (29, 30). The molecular mechanisms underlying HLH are not fully understood. However, some studies suggest that excessive CTLs secrete significant amounts of IFN- γ , which potently stimulates macrophages. These macrophages then release large quantities of IL-1 β , IL-6, IL-18, and TNF- α , potentially leading to tissue injury (31). Subsequent tissue damage produces high levels of IL-1 β and IL-33, which continue to activate macrophages (30, 32). Additionally, IL-18, in combination with IL-12, is believed to facilitate further activation of CTLs and their production of IFN- γ (33, 34). On the other hand, IFN- γ enhances the expression of major histocompatibility complex (MHC) I on immune cells, thereby amplifying the feed-forward loops (12). This cytokine storm may exacerbate hematopoietic failure, contribute to various clinical manifestations of HLH, and worsen the disease (31). An HLH animal model suggests that IFN- γ contributes to anemia via inhibiting erythropoiesis and promoting hemophagocytosis (35). Another animal model indicates that release of IFN- γ leads to splenomegaly in HLH (12, 36). Furthermore, ferritin secreted by macrophages elevates plasminogen activator levels and triggers hyperfibrinolysis (37). Other pro-inflammatory cytokines, such as TNF- α , IL-1 β and IL-6, are thought to be associated with fever and multi-organ dysfunction in HLH (38). Considering the critical role of cytokines in the pathogenesis of HLH, we strongly recommended that the patient undergo cytokine testing at the time of HLA diagnosis. However, the patient's family declined the examination for economic reasons, as it was not covered by government-sponsored medical insurance.

To evaluate the impact of ADs on the onset time of ICIs-related HLH, we identified 28 cases of ICIs-related HLH from 25 articles with sufficient clinical data. The cohort characteristics are summarized in Table 1. Our analysis reveals that 8 patients had concomitant ADs. The median time from ICI infusion to HLH onset was 59.5 days in patients without ADs, compared to 32 days in those with ADs, suggesting ADs may accelerate the occurrence of ICI-related HLH. This temporal disparity could be attributed to pre-existing cytokine storm and excessive immune cell infiltration in ADs (21, 22). Specifically, in SLE, impaired antigen clearance triggers aberrant activation of lymphocytes and macrophages, along with excessive pro-inflammatory cytokine production. Such a hyperinflammatory milieu primes the immune system, increasing susceptibility to rapid HLH progression upon ICI exposure (39, 40). Additionally, MSI-H status, a feature of this case, confers high tumor mutational burden due to defective mismatch repair. This generates abundant neoantigens that persistently activate CD8⁺ T cells and natural killer cells, sustaining immune hyperactivation and providing a pathological basis for HLH (41). The synergy between SLE-driven autoimmunity and MSI-H immunogenicity establishes a “dual-engine” immune stimulation mechanism. This likely ignited a cascading inflammatory response, culminating in fulminant HLH within 31 days in our patient. Despite these insights, our

conclusions require cautious interpretation due to clinical heterogeneity: variations in ADs subtypes, malignancy types, and ICI regimens across the cohort may confound onset-time comparisons. Moreover, our patient's non-adherence to regular hematological monitoring outside the hospital, along with initial refusal of bone marrow aspiration may obscure the true HLH onset timeline.

Although the Society for Immunotherapy of Cancer (SITC) classifies HLH as a fatal irAE, it fails to provide specific treatment recommendations (42). Typical management guidelines for HLH propose potential benefits from therapeutic interventions such as steroids, immunosuppressive agents like cyclophosphamide and etoposide, intravenous immunoglobulin, and the anti-IL-6 receptor antibody tocilizumab (12, 43). In this particular case, despite attempting steroid therapy, the patient's condition rapidly deteriorated. Given the patient's frail physical state, chemotherapy was deemed unfeasible, and etoposide was consequently not recommended. Notably, a study has reported a low mortality rate of 1 in 10 HLH cases treated with tocilizumab (44). However, due to financial constraints, the patient declined both tocilizumab and immunoglobulin treatments. Ultimately, the patient succumbed to HLH within 16 days of diagnosis.

While previous reports have associated irAE-related HLH with pre-existing autoimmune disorders, including ankylosing spondylitis, psoriasis, and Sjögren's syndrome (44–47), this represents the first instance of irAE-related HLH occurring in conjunction with SLE. The patient's unsuccessful treatment outcome was attributed to the rapid progression of the disease, exacerbated by an excessive number of precursors, and further complicated by the patient's non-compliance, including the refusal of timely evaluations and the use of a cytokine inhibitor proven effective against cytokine storms. In conclusion, the coexistence of MSI-H tumors and pre-existing SLE may exacerbate the prognosis of irAE-related HLH, suggesting a potential interplay between these factors. Clinicians should maintain heightened vigilance regarding the potential emergence of HLH in patients with MSI-H cancers and SLE undergoing ICIs therapy. It is crucial to promptly initiate a combination of other pharmacological agents and steroid therapy upon the onset of HLH.

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

PJ: Project administration, Writing – original draft. SW: Investigation, Project administration, Writing – original draft. JW: Investigation, Project administration, Writing – original draft. YZ: Project administration, Writing – original draft. JH: Writing – original draft. ZS: Conceptualization, Funding acquisition, Supervision, Writing – review & editing.

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