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Mechanisms and complexities underlying the cancer cell immune evasion and its therapeutic implications

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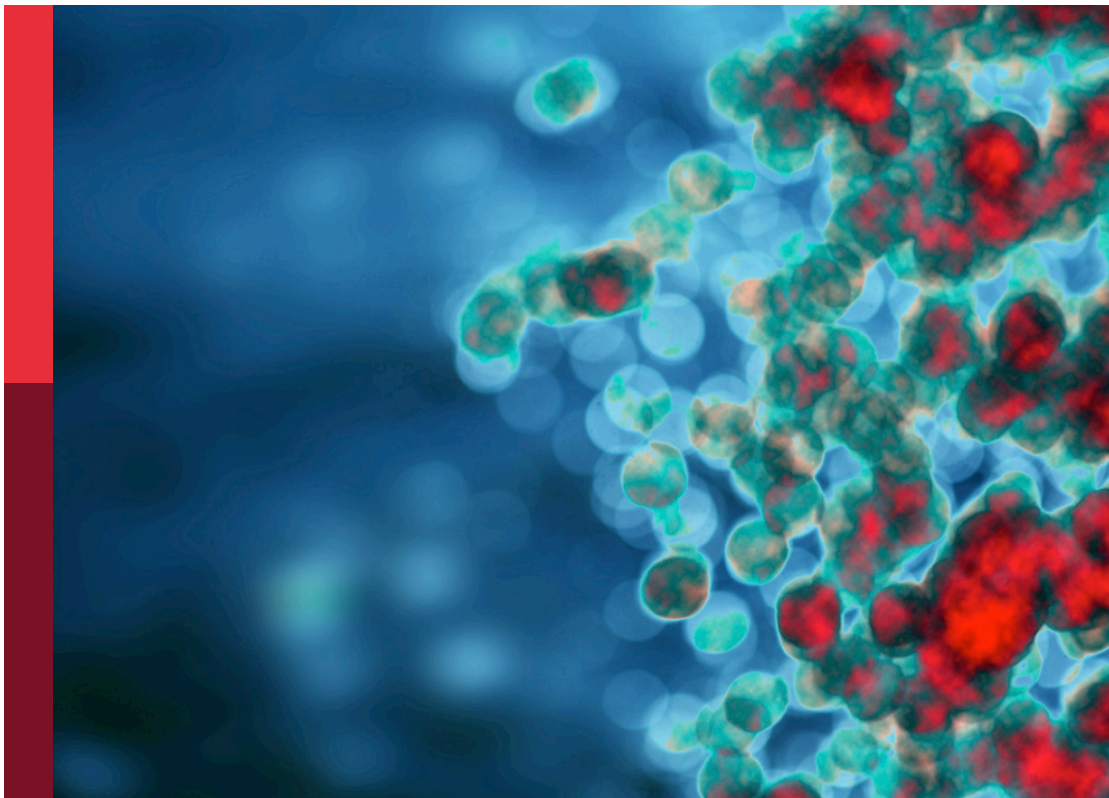

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Mechanisms and complexities underlying the cancer cell immune evasion and its therapeutic implications

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Editorial: Mechanisms and complexities underlying the cancer cell immune evasion and its therapeutic implications

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Editorial on the Research Topic

Mechanisms and complexities underlying the cancer cell immune evasion and its therapeutic implications

Cancer immune evasion represents a central barrier to effective antitumor immunity and remains one of the most challenging hallmarks of cancer biology. The Research Topic "Mechanisms and Complexities Underlying the Cancer Cell Immune Evasion and its Therapeutic Implications" brings together diverse contributions that elucidate the cellular, molecular, and microenvironmental determinants of immune escape across malignancies. The collected works highlight how tumors exploit immunoregulatory pathways, remodel local immune niches, and shape therapeutic responses. Together, these articles provide an integrated understanding of cancer-mediated immune suppression and propose translational strategies to counteract it.

A major theme emerging from this topic is the central role of immunosuppressive cell populations. Liu et al. detail how regulatory T cells, myeloid-derived suppressor cells, leukemia-associated macrophages, and regulatory B cells orchestrate a profoundly immunosuppressive milieu in acute myeloid leukemia. Their review underscores the importance of targeting cellular recruitment and suppressive signaling pathways to restore effective anti-leukemic immunity. Wang et al. identify cytosolic thiouridylase CTU2 as a pan-cancer biomarker that modulates immune infiltration, tumor immunogenicity, and immunotherapy response. Their multitier analysis suggests that tRNA modification systems represent an underexplored axis of immune regulation. Complementing this, Chen et al. provide high-resolution insights into the heterogeneous immune microenvironment of colorectal cancer–origin ovarian metastases. Their genomic analyses reveal highly variable neoantigen loads, immune-desert phenotypes, and distinct metastatic routes, illustrating how spatial and clonal evolution shapes immune interactions and patient outcomes.

Few articles address specific signaling mechanisms and immunomodulatory pathways. Guo et al. review the inhibitory immune checkpoint TIM-3 in myelodysplastic syndromes,

emphasizing its dual roles in tumor cell regulation and immune remodeling. Similarly, Han et al. integrate CRISPR-based functional genomics with transcriptomics to identify MELK-driven pathways that govern tumor progression, mutation burden, and immune contexture in clear cell renal cell carcinoma.

Mahamed et al. describe how cancer-derived exosomes convey immunosuppressive cargo that alter multiple immune cell populations, while engineered immune-cell–derived exosomes may counteract these effects. Beyond molecular pathways, this review highlights emerging systemic regulators of immune escape. Chen et al. expand this perspective, illustrating how multimodal intercellular communication ranging from metabolic competition to extracellular vesicle exchange and stromal interactions collectively drives $CD8^-$ T-cell dysfunction across solid tumors.

Additional contributions broaden the conceptual landscape. Zhu et al. map the global bibliometric trends in tumor immune escape research, identifying shifting hotspots from classical checkpoint biology to metabolic reprogramming, microbiome interactions, and AI-driven immunotherapy prediction. Kovaleva et al. challenge the classical dichotomy of macrophage biology by showing that cytotoxic M1 macrophages may paradoxically promote tumor progression through selection pressure. Yan et al. provide a systematic-review demonstrating that bispecific antibodies combined with chemotherapy significantly improve survival outcomes in solid tumors, highlighting the translational potential of multi-target immunomodulation.

Collectively, all the articles in this Research Topic illustrate that immune evasion is not governed by a single pathway but emerges through complex, dynamic interactions between cancer cells, immune effectors, stromal elements, extracellular vesicles, and metabolic networks. These studies emphasize the need for integrated therapeutic strategies that target multiple axes of immune suppression at cellular, molecular, spatial, and metabolic levels.

As immunotherapies continue to evolve, a deeper mechanistic understanding of immune escape will be essential for improving patient outcomes, predicting response, and designing effective combination strategies. We thank all authors and reviewers for their valuable contributions and hope this Research Topic inspires further exploration into the intricacies of cancer–immune interactions and their therapeutic exploitation.

Author contributions

ZC: Writing – review & editing, Writing – original draft. TP: Writing – original draft, Writing – review & editing. AN: Writing – original draft, Writing – review & editing.

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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Bispecific antibodies combined with chemotherapy in solid tumor treatment, the path forward?

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Background: Bispecific antibodies (bsAbs) introduced a novel strategy in anticancer therapy when chemotherapy alone could not meet life expectancy. Nonetheless, the efficacy of monotherapy was limited, and the safety profile of bsAbs combined with chemotherapy remained uncertain.

Methods: Literature retrieval was carried out through PubMed, Embase, and Cochrane from inception to January, 2025. Progression-free survival (PFS), overall survival (OS), and overall response rate (ORR), along with adverse effects (AEs), were utilized to assess the efficacy and safety. Publication bias was calculated using Funnel plots and Egger's test. Heterogeneity was examined through subgroup and sensitivity analyses. The protocol was preregistered in the International Prospective Register of Systematic Reviews (CRD42025633628).

Results: A total of 8 eligible clinical studies with 2,495 patients were included. Compared with chemotherapy alone, bsAb+chemotherapy exhibited positive outcomes in PFS (hazard ratio (HR): 0.52; 95% confidence interval (CI): 0.44-0.60; p<0.01), OS (HR: 0.67, 95% CI: 0.57-0.77; p<0.01), and ORR (HR: 0.31, 95% CI: 0.16-0.47; p<0.01). Subgroup analysis revealed that female patients, Asian patients, those under 65 years of age, and patients treated with IgG-like bsAb were more likely to benefit from the survival advantages of bsAb+chemotherapy. Despite the occurrence of leukopenia, metabolism-related, and skin-related AEs, RR of AEs in other systems showed no statistical significance.

Conclusion: BsAb+chemotherapy was superior to chemotherapy alone, especially in female patients, Asian patients, those under 65 years of age, and patients receiving IgG-like bsAb. Additionally, while the AEs associated with bsAb +chemotherapy are generally manageable, there is still room for improvement.

Systematic review registration: https://www.crd.york.ac.uk/prospero/, identifier CRD42025633628.

KEYWORDS

bispecific antibody, chemotherapy, solid tumor, efficacy, safety, meta-analysis

Introduction

According to the latest estimates by GLOBOCAN, in 2022 the annual number of solid tumors globally reached 18.7 million, accounting for over 90% of all cancer cases globally (1). In the same year, approximately 9.7 million deaths were caused by solid tumors and the number continues to rise steadily. Chemotherapy has long been the backbone of treatment for solid tumors. However, chemotherapy alone is often limited by off-target toxicity, drug resistance, and immunosuppression, underscoring the need for more targeted and effective therapeutic strategies.

Bispecific antibodies (bsAbs) emerge as a game-changing approach in anticancer therapy by simultaneously binding to two antigens or two epitopes of the same antigen (2). This dual targeting capability enables bsAbs to bridge immune cells, such as T cells or natural killer (NK) cells, with tumor cells, facilitating immune cell activation and tumor elimination. BsAbs can be categorized into IgG-like and non-IgG-like formats. IgG-like bsAbs retain Fc regions, enabling effector functions like antibody-dependent cellular cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC), while non-IgG-like bsAbs often lack Fc regions, favoring smaller size and improved tissue penetration. By engaging multiple tumor-associated targets, bsAbs can enhance precision in tumor targeting, overcome tumor heterogeneity, and counteract immune evasion mechanisms (3). Additionally, bsAbs can be engineered to address key challenges in cancer treatment, such as drug resistance and the immunosuppressive tumor microenvironment. Beyond their standalone efficacy, bsAbs are increasingly explored in combination with chemotherapy or other immunotherapies, offering the potential for longer-lasting disease control, improved survival outcomes, and the ability to overcome resistance observed with monotherapy. Although these new therapies provide additional options, they also carry specific and potential toxicities for patients. Most notable is the withdrawal from the European market of catumaxomab in 2017 (4).

To date, 11 bsAbs have been approved by the Food and Drug Administration (FDA), European Medicines Agency (EMA) or National Medical Products Administration (NMPA) for cancer treatment (5). However, the majority of these approvals are for hematologic malignancies, with only a handful target solid tumors (5). This may be explained by the poor penetration and trafficking of bsAbs, the inherent complexity of the solid tumor microenvironment, and the prevalence of immune evasion mechanisms in solid tumors (6, 7). Despite these challenges, bsAbs for solid tumors is predicted to have substantial market potential due to its wide mass foundation.

Overall, bsAbs+chemotherapy seems to be the path forward in the treatment of solid tumors. However, to the best of our knowledge, no systematic analysis has yet been conducted to substantiate this conclusion, particularly in comparison with the hematologic malignancies (8, 9). Furthermore, existing randomized control trails (RCTs) involve different kinds of bsAbs, various sample sizes, and diverse tumor types. Therefore, a meta-analysis of published RCTs was performed. The main objective of this study is to evaluate the efficacy and safety of bsAbs+chemotherapy for patients with solid tumors.

Methods

Literature search strategy

This meta-analysis was conducted following the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) guidelines. A thorough search was conducted on three databases, including PubMed, Embase, and Cochrane Library, from inception to January 2, 2025 by two independent investigators. Additional records identified through other sources including ClinicalTrials.gov, American Society of Clinical Oncology (ASCO), European Society for Medical Oncology (ESMO) and American Association for Cancer Research (AACR). Reference lists were reviewed for completeness to avoid missing relevant articles. Both MeSH terms and free terms were used. The MeSH terms used were as follows: “Bispecific Antibodies” and “Neoplasms”. The detailed search strategy in PubMed is presented in Supplementary Table 1. The protocol was preregistered in the International Prospective Register of Systematic Reviews (CRD42025633628).

Inclusion and exclusion criteria

The PICOS criteria were as follows: (1) Participants were patients with diagnosed solid tumor; (2) Intervention group was patients treated with bispecific antibody plus chemotherapy treatment; (3) Control group was patients treated with chemotherapy with or without placebo. (4) Outcomes included overall survival (OS) or progress-free survival (PFS), with or without overall response rate (ORR) and adverse events (AEs); (5) Study type was randomized clinical trials (RCTs).

The exclusion criteria were as follows: (1) studies that not reported specific data, including hazard ratio (HR) along with corresponding 95% confidence interval (CIs); (2) studies in which patients were diagnosed with hematological tumors; (3) studies without full-text; (4) studies that were single arms, reviews, observational studies, case reports, meta-analyses, letters, comments.

Two investigators independently lay down the inclusion and exclusion criteria. Any discrepancy would be addressed among three investigators.

Data extraction

Data were independently extracted and cross-checked by two investigators. The following characteristic information of the included studies was recorded: (1) Study characteristic: first author, publication year, location, follow-up, cancer type, intervention group, control group, phase, line, sample size,

median PFS and median OS, and drug target; (2) Study outcomes: effect estimates of OS, PFS, ORR, and AEs of all grade and ≥grade 3.

Quality assessment

Two researchers used the Cochrane Collaboration's tool to independently assess the quality of included RCTs. Briefly, each article was evaluated across 7 domains, including bias arising from intended the randomization process, bias due to allocation concealment, bias due to blinding of participants and personnel, bias due to blinding of outcome assessment, bias due to incomplete outcome data, bias due to selective reporting, and other bias. Each domain was judged as "low risk," "high risk," or "unclear risk" based on the criteria outlined in the Cochrane Handbook. Any discrepancies in their judgments were resolved through discussion and consensus.

Statistical analysis

Statistical analyses of study outcomes were performed and pooled as forest plots by Stata 18.0. The HR with 95% CI was used to assess the outcomes PFS and OS. HR<1 favored the intervention group, while HR>1 favored the control group. The Relative Risk (RR) with 95% CI was used to analyze ORR and AEs. For ORR and AEs, RR<1 indicated that the control group had a higher response rate and toxicity, while RR>1 indicated the opposite. Chi-square Q test and I^2 statistic was used to detect statistical heterogeneity. I^2<30% indicated low heterogeneity, 30%≤I^2 ≤ 60% represented moderate heterogeneity, and I^2>60% revealed high heterogeneity. Due to the clinical heterogeneity from diversity of tumor types and difference in intervention, the random-effects model was used for combined analysis. Furthermore, subgroup analysis was implemented to identify the factors contributing risk of bias. We also conducted the sensitivity analysis by sequential exclusion of included individual trial. Funnel plots and Egger's tests were also used to examine potential publication bias. All reported P-values were two-sided, with statistical significance defined as p<0.05.

Result

Literature search results

A total of 6,518 relevant articles were initially retrieved, and after removing duplicates, 3,045 articles remained. A preliminary review of titles, abstracts, and keywords led to the exclusion of 3,020 articles. The comprehensive reviews of the 25 surviving articles that might have qualified for inclusion were then conducted. Adhering to a rigorous screening process predicated on predetermined inclusion and exclusion criteria, 17 articles were excluded due to no results of interest, inappropriate criteria, duplicates, or no full-text available. Finally, 8 articles were deemed eligible and included in the meta-analysis (10–17). The detailed selection process is illustrated in Figure 1.

Basic characteristics of included studies

A total of 2,495 patients were enrolled in our study. The publication year ranged from 2018 to 2024, originating from Germany, Canada, The United States, and China. Among 8 eligible articles, four were conducted in single center and the remaining four were in multi-center. Five were used as 1 line therapy, one was performed as 1/2 line therapy, one was used as ≥2 line therapy. The median follow-up period ranged from 7.9 to 52.0 months. Overall, seven cancer types were identified in this review, incorporating gastric cancer (GC), peritoneal cancer (PC), metastatic pancreatic cancer, non-small cell lung cancer (NSCLC), gastric or gastroesophageal junction (G/GEJ) adenocarcinoma, biliary tract cancer, and cervical cancer (CC). The combination regimen included catumaxomab+5-fluorouracil, leucovorin, oxaliplatin, docetaxel (FLOT), istiratumab+nab-paclitaxel plus gemcitabine regime (NG), amivantamab+carboplatin plus pemetrexed regimen (CP), cadonilimab+capecitabine plus oxaliplatin regimen (XELOX), ivonescimab+CP, bintrafusp alfa+gemcitabine plus cisplatin regimen (GemCis), cadonilimab+cisplatin plus paclitaxel regimen (GP)/ paclitaxel plus carboplatin regimen (PCb). The detailed characteristics of included studies were shown in Table 1; Supplementary Table 2.

Efficacy

All of the eight articles reported HRs as PFS outcome. The pooled HR for PFS was 0.52 (95% CI: 0.44-0.60, Figure 2), with statistical significance (p<0.01) and moderate heterogeneity (I^2 = 36.29%). As for OS outcome, seven articles reported corresponding HRs. The pooled HR for OS was 0.67 (95% CI: 0.57-0.77, Figure 3), along with statistical significance (p<0.01) and low heterogeneity (I^2 = 0.0%). Seven articles reported ORR data, with a positive outcome (RR: 0.31; 95% CI: 0.16-0.47; p<0.01, Figure 4).

Subgroup analysis

Subgroup analysis was conducted to make a further exploration of combination regimen, mainly on age, brain metastasis, bsAb format, cancer type, Eastern Cooperative Oncology Group (ECOG) Performance Status (PS), metastasis, race, sex, and weight (Table 2).

BsAb+chemotherapy benefits patients both under (HR: 0.54; 95% CI: 0.39-0.74; p<0.01) or above the age of 65 years (HR: 0.61; 95% CI: 0.40-0.92; p=0.02) in terms of PFS. In terms of OS, bsAb +chemotherapy benefits patients under the age of 65 (HR: 0.64; 95% CI: 0.49-0.83; p<0.01), but for those above the age of 65, no marked survival benefit was observed (p=0.96). Totally, three articles focused on brain metastasis, and both metastasis group (HR: 0.52; 95% CI: 0.39-0.69; p<0.01) and non-metastasis group (HR: 0.42;

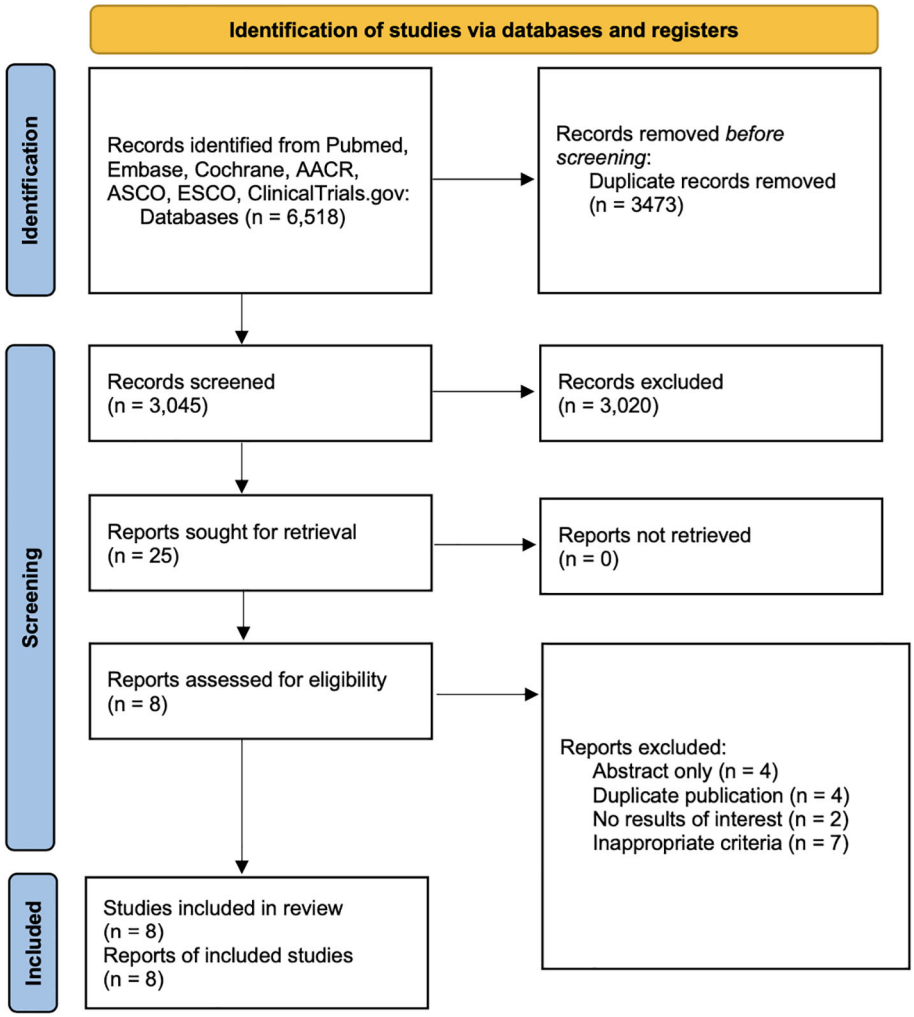

FIGURE 1
Flowchart of the study selection.

95% CI: 0.33-0.54; p<0.01) confirmed the superior PFS-related efficacy of bsAb+chemotherapy. As for bsAb format, both PFS-related (95% CI: 0.44-0.75; p<0.01) and OS-related benefit (95% CI: 0.58-0.93; p=0.01) were observed in patient treated with IgG-like bsAb (istiratumab, amivantamab, cadonilimab, ivonescimab) +chemotherapy. However, bsAb+chemotherapy failed to achieve better OS (p=0.63) or PFS (p=0.88) in patients receiving non-IgG-like bsAb (catumaxomab, bintrafusp alfa). When stratified by cancer type, two articles investigated on GC (HR: 0.55; 95% CI: 0.43-0.69; p<0.01) and three were on NSCLC (HR: 0.44; 95% CI: 0.38-0.53; p<0.01). Both cancer types exhibited statistical significance on PFS outcome. In terms of ECOG PS, PFS-related benefits were observed in patients with ECOG PS=1 (HR: 0.54; 95% CI: 0.45-0.66; p<0.01). No statistical difference was observed in OS benefit regarding ECOG PS (ECOG PS=1 (p=0.25), ECOG PS=0 (p=0.95)). When stratified by race, both Asian group (HR: 0.57; 95% CI: 0.44-0.73; p<0.01) and non-Asian group (HR: 0.59; 95% CI: 0.38-0.91; p=0.02) demonstrated PFS benefits. In terms of OS, Asian group (HR: 0.64; 95% CI: 0.54, 0.76; p<0.01) showed survival benefits, but there is no statistical difference between bsAb +chemotherapy and chemotherapy for non-Asian group (p=0.44). Regarding sex, a synthesized estimate from five studies on female indicated better prognosis on patients with bsAb+chemotherapy in terms of PFS (HR: 0.53; 95% CI: 0.39-0.73; p<0.01) and OS (HR: 0.70; 95% CI: 0.55-0.89; p<0.01), while male group failed to exhibit therapeutic superiority in terms of PFS (p=0.06) and OS (p=0.95). When stratified by weight, PFS-related benefit was observed in both <80 kg group (HR: 0.46; 95% CI: 0.37-0.57; p<0.01) and ≥80 kg group (HR: 0.38; 95% CI: 0.19-0.70; P<0.01).

Safety

The safety profile of combination regimen was illustrated in Table 3; Supplementary Table 3. It was carried out in digestive system, hematological system, liver function, metabolism, renal function, skin, and others. When exploring the incidence of severe side effects, we subsequently performed high-grade AEs (grade≥3).

RR of all grade AEs of digestive system revealed no statistical significance: abdominal pain (p=0.40), constipation (p=0.55),

TABLE 1 Characteristic of included studies.

Author, year	Country	Design	Line[1]	Cancer type	Follow-up, months	Sample size (M/F)	Race (non-A/A)	I/C	No. of patients	Median PFS, months	Median OS, months
Knödler M, 2018 (10)	Germany	Single center	NA	GC and PC	52	31 (17/14)	31/0	I: Catumaxomab+ FLOT	15	6.7	13.2
								C: FLOT	16	5.4	13.0
Kundranda M, 2020 (11)	Canada	Multi-center	1	Metastatic pancreatic cancer	NA	88 (46/42)	88/0	I: Istiratumab+NG	43	3.6	8.9
								C: Placebo+NG	45	7.3	11.7
Zhou C, 2023 (12)	US	Multi-center	1	NSCLC	14.9	308 (130/178)	117/186	I: amivantamab+CP	153	11.4	NA
								C: CP	155	6.7	24.4
Ji J, 2024 (17)	China	Single center	1	G/GEJ adenocarcinoma	18.6	610 (474/136)	0/610	I: Cadonilimab+XELOX	305	7.0	15.0
								C: Placebo+XELOX	305	5.3	10.8
Fang W, 2024 (13)	China	Single center	≥2	NSCLC	7.9	322 (156/166)	0/322	I: Ivonescimab+CP	161	7.2	NA
								C: Placebo+CP	161	7.1	NA
Oh DY, 2024 (14)	US	Multi-center	1	Biliary tract cancer	18.7	297 (151/146)	114/183	I: Bintrafusp alfa+GemCis	73	5.5	11.5
								C: Placebo+GemCis	77	5.6	11.5
Passaro A, 2024 (15)	US	Multi-center	1/2	NSCLC	8.7	394 (238/156)	204/190	I: Amivantamab+CP	131	8.2	6.3
								C: CP	263	4.2	4.2
Wu X, 2024 (16)	China	Single center	1	CC	25.6	445 (0/445)	0/445	I: Cadonilimab+GP/PCb (+bevacizumab)	222	12.7	NA
								C: Placebo+GP/PCb (+bevacizumab)	223	8.1	22.8

US, the United States; NA, no available; GC, gastric cancer; PC, peritoneal carcinoma; NSCLC, non-small cell lung cancer; G/GEJ, gastric or gastroesophageal junction; CC, cervical cancer; I, intervention group; C, control group; FLOT, 5-fluorouracil, leucovorin, oxaliplatin, docetaxel; NG, nab-paclitaxel plus gemcitabine regimen; CP, carboplatin plus pemetrexed regimen; XELOX, capecitabine plus oxaliplatin regimen; GemCis, gemcitabine plus cisplatin regimen; GP, cisplatin plus paclitaxel regimen; PCb, paclitaxel plus carboplatin regimen; PFS, progress-free survival; OS, overall survival; M, male; F, female; non-A, non-Asian people; A, Asian people.

[1]treatment line.

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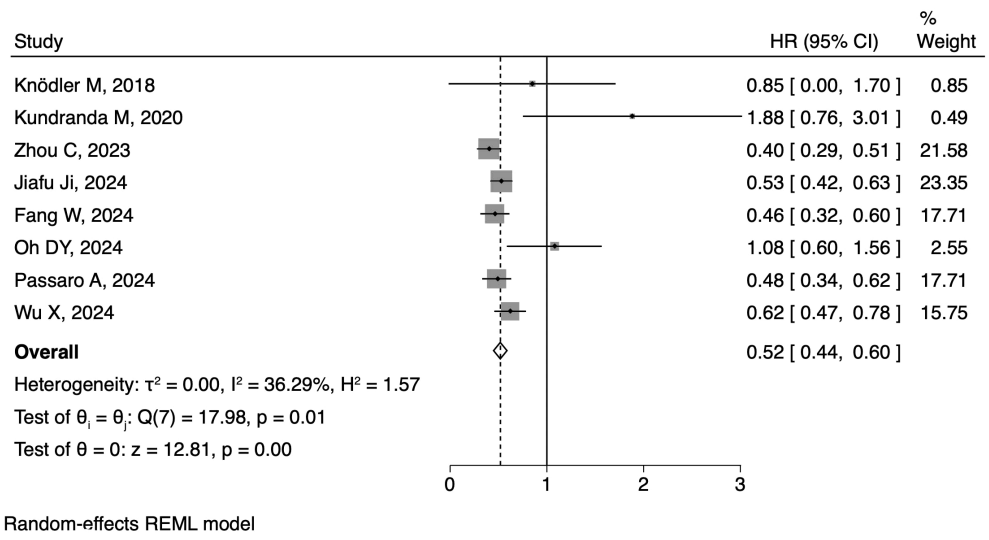

FIGURE 2
Forest plot of the meta-analysis on PFS.

diarrhea (p=0.09), nausea (p=0.91), stomatitis (p=0.06), and vomiting (p=0.24). RR of grade ≥3 AEs of digestive system also showed no statistical significance: abdominal pain (p=0.18), constipation (p=0.82), diarrhea (p=0.23), nausea (p=0.80), stomatitis (p=0.28), and vomiting (p=0.10). In hematological system, despite leukopenia (RR: 2.31, 95% CI: 1.47-3.63; p<0.01), RR of grade ≥3 AEs revealed no statistical significance: anemia (p=0.52), neutrophil decrease (p=0.21), neutropenia (p=0.15), platelet decrease (p=0.95), and white blood cell (WBC) decrease (p=0.46). RR of AEs of all grade revealed no statistical significance: anemia (p=0.43), leukopenia (p=0.38), neutrophil decrease (p=0.24), neutropenia (p=0.60), platelet decrease (p=0.77), and WBC decrease (p=0.26). No statistical significance in AEs of all grade or grade ≥3 was found in liver function and renal function. In metabolism, higher incidence of hypoproteinemia (RR: 3.23; 95% CI: 1.19-8.77; p=0.02) and hypokalemia (RR: 1.65; 95% CI: 1.09-2.48; p=0.02) was observed in all grade AEs. When it comes to severe metabolic disorders (grade ≥3), elevated incidence of hypoproteinemia (RR: 7.81; 95% CI: 1.33-45.91; p=0.02), hyperglycemia (RR: 3.29; 95% CI: 1.05-10.35; p=0.04) and hypokalemia (RR: 2.07; 95% CI: 1.07-4.00; p=0.03) was identified in combination arm. As for skin-related AEs, higher incidence of dermatitis acneiform (RR: 7.46; 95% CI: 4.20, 13.26; p<0.01), paronychia (RR: 23.02; 95% CI: 2.42-218.70; p=0.01), and rash (RR: 3.25; 95% CI: 1.90-5.54; p<0.01) was found in all grade AEs. When it comes to severe (grade ≥3) skin toxicity, patients with combination treatment tended to have elevated risk of dermatitis acneiform (RR: 16.51; 95% CI: 2.16-26.29; p=0.01), paronychia (RR:

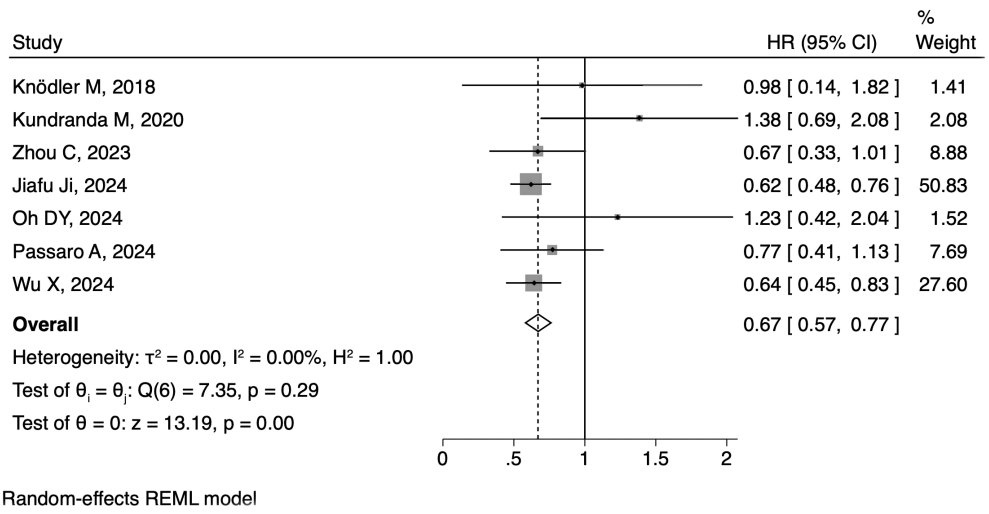

FIGURE 3
Forest plot of the meta-analysis on OS.

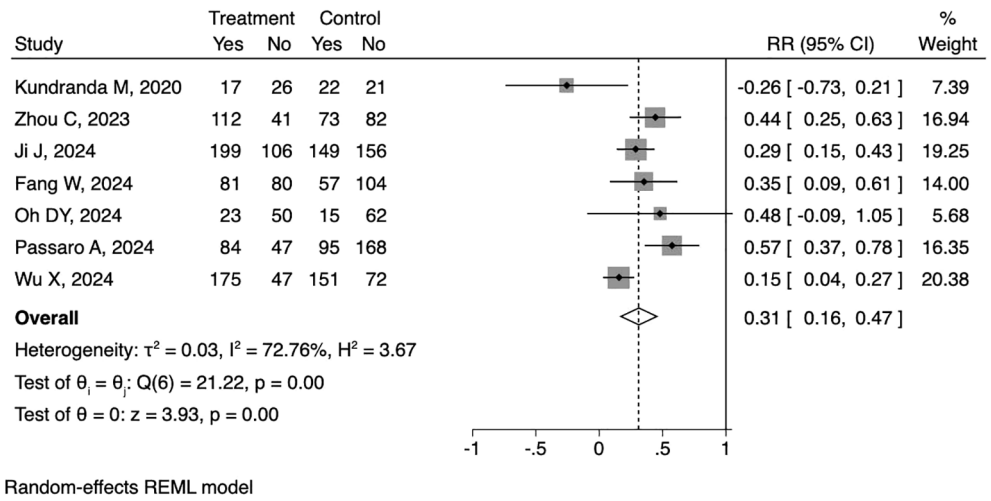

FIGURE 4
Forest plot of the meta-analysis on ORR.

16.95; 95% CI: 2.20-130.80; p=0.01) and rash (RR: 9.35; 95% CI: 3.05-28.64; p<0.01). Additionally, increments in asthenia (RR: 1.84; 95% CI: 1.10, 3.06; p=0.02), infusion-related reaction (RR: 25.15; 95% CI: 6.82-92.78; p<0.01), and weight decreased (RR: 1.42; 95% CI: 1.02-1.98; p=0.04) were observed in all grade AEs. And for grade ≥3 AEs, incidence of severe infusion-related reaction (RR: 15.53; 95% CI: 2.91-82.83; p<0.01) tended to be elevated. No statistical significance in grade ≥3 AEs was found in asthenia (p=0.48), fatigue (p=0.64) and weight decreased (p=0.11).

Quality assessment

The individual evaluation of each article included in this meta-analysis is depicted in Supplementary Figure 1; Figure 2. Seven articles showed a low risk of bias while one was considered as moderate reliability, specifically in domain D5.

Publication bias and sensitivity analysis

The funnel plots on PFS (Supplementary Figure 3) and ORR (Supplementary Figure 4) were symmetrical, suggesting no signs of publication bias. And the one on OS outcome was slightly asymmetrical (Supplementary Figure 5), indicating a potential presence of publication bias. Egger's test was performed to further assess publication bias. No significant publication bias was observed for PFS (p = 0.111) or ORR (p = 0.567). A statistically significant Egger's test result (p = 0.047) suggested the presence of potential publication bias for OS. Sensitivity analysis was performed to evaluate the reliability of the findings. No statistically significant changes in the overall results were observed after removing each included study, thus confirming the reliability and validity of our findings.

Discussion

This is the first meta-analysis to show that adding bsAbs to chemotherapy significantly and clinically meaningfully improved PFS and OS in solid tumors. In addition, treatment with bsAbs +chemotherapy was associated with a higher ORR, which led to a longer duration of response than with chemotherapy alone. Mechanistically, bsAbs can simultaneously engage multiple tumor-associated targets, overcoming resistance mechanisms that rely on specific molecular alterations within the tumor (5). Chemotherapy, in turn, provides activity against other resistance mechanisms that are independent of these specific pathways (18). When combined, this approach offers broad coverage against the diverse and polyclonal resistance that emerges as the tumor progresses, thereby enhancing the overall therapeutic effectiveness.

As a new kind of immunotherapy, bsAbs has achieved significant success in the field of hematologic malignancies like leukemia and lymphoma, attaining survival rates that were once considered unreachable (19–21). Nevertheless, according to the International Agency for Research on Cancer, solid tumor occurrences constituted over 90% of all cancer diagnoses, significantly surpassing the rates of leukemia and lymphoma (1). Unfortunately, the bsAbs which are effective for leukemia and lymphoma have exhibited unexpectedly low clinical response rates and unsatisfactory efficacy in treating solid tumors featuring specific microenvironments in tumor tissues (22). Although the clinical outcome of bsAbs is less favorable in solid tumors when compare with hematologic malignancies (23, 24), an increasing number of bsAbs targeted solid tumors have been approved and abundant clinical trials are underway. Presently, the main challenges for bsAbs in solid tumors are tumor microenvironment complexity and immune evasion (25). Concretely speaking, while hematologic tumors involve targets expressed on B-cells or bone marrow cells, T-cell-mediated damage to these cells is reversible because hematopoietic

TABLE 2 Subgroup analysis of PFS and OS.

Subgroup	PFS				OS			
	No. of studies	No. of cases	HR (95% CI)	p[1]	No. of studies	No. of cases	HR (95% CI)	p[1]
Age								
<65 yrs	5	1,174	0.54 (0.39, 0.74)	<0.01	5	482	0.64 (0.49, 0.83)	<0.01
≥65 yrs	5	592	0.61 (0.40, 0.92)	0.02	5	425	0.97 (0.34, 2.76)	0.96
Brain metastasis								
Yes	3	321	0.52 (0.39, 0.69)	<0.01	NA	NA	NA	NA
No	3	703	0.42 (0.33, 0.54)	<0.01	NA	NA	NA	NA
bsAb format								
Non-IgG-like	2	328	1.03 (0.70, 1.52)	0.88	2	328	1.13 (0.69, 1.83)	0.63
IgG-like	6	2,167	0.57 (0.44, 0.75)	<0.01	5	1,845	0.74 (0.58, 0.93)	0.01
Cancer type								
GC	2	641	0.55 (0.43, 0.69)	<0.01	2	641	0.67 (0.48, 0.92)	0.01
NSCLC	3	1,024	0.44 (0.38, 0.53)	<0.01	2	1,024	0.72 (0.52, 1.00)	0.05
ECOG PS								
0	5	617	0.60 (0.36, 1.00)	0.05	2	610	1.03 (0.40, 2.67)	0.95
1	5	1,149	0.54 (0.45, 0.66)	<0.01	2	297	0.72 (0.41, 1.26)	0.25
Race								
Asian	6	1,936	0.57 (0.44, 0.73)	<0.01	3	1,238	0.64 (0.54, 0.76)	<0.01
Non-Asian	4	466	0.59 (0.38, 0.91)	0.02	2	145	1.77 (0.42, 7.39)	0.44
Sex								
Female	5	1,091	0.53 (0.39, 0.73)	<0.01	3	1,065	0.70 (0.55, 0.89)	<0.01
Male	4	675	0.63 (0.38, 1.03)	0.06	2	287	0.97 (0.31, 3.00)	0.95
Weight								
<80 kg	2	599	0.46 (0.37, 0.57)	<0.01	NA	NA	NA	NA
≥80 kg	2	103	0.38 (0.19, 0.70)	<0.01	NA	NA	NA	NA

yrs, years; GC, gastric cancer; NSCLC, non-small cell lung cancer; ECOG PS, Eastern Cooperative Oncology Group Performance Status; No, number; PFS, progression-free survival; HR, hazard ratio; OS, overall survival; NA, not available.
[1]p<0.05 indicates significant.

stem cells can replenish the lost cells, minimizing systemic impact. Solid tumors, however, are expressed on normal cells, and if T cells kill them, they will cause irreversible damage to the body's function. Additionally, cold tumors present a further obstacle, as their dense extracellular matrix forms a physical barrier that prevents immune cell infiltration (26). Moreover, immunosuppressive cytokines such as TGF-β and CXCL12 in the tumor microenvironment inhibit T-cell penetration and activity (27), further hindering the effectiveness of bsAbs in these tumors. Chemotherapy can play a crucial role in overcoming these challenges and enhancing the effectiveness of bsAbs in solid tumors. Chemotherapy has been shown to modify the tumor microenvironment in ways that can make it more responsive to immune-based therapies like bsAbs (28). Specifically, chemotherapy can reduce the tumor cell burden, improve vascularization, and help normalize the tumor vasculature, facilitating better immune cell infiltration. This normalization of the microenvironment can reduce the physical barriers, such as the dense extracellular matrix, that typically prevent immune cells from effectively reaching and attacking the tumor (29). Additionally, chemotherapy can induce immunogenic cell death (ICD), which releases tumor antigens and enhances the presentation of these antigens by dendritic cells (30). This process primes the immune system, making the tumor more recognizable to T cells and increasing the potential for immune-mediated tumor destruction. Together, chemotherapy and bsAbs may work synergistically to overcome the key obstacles posed by the tumor microenvironment, offering a promising strategy to improve clinical outcomes in solid tumors. While challenges remain, ongoing research and clinical trials continue to explore ways to refine and optimize this combination approach, with the potential to significantly improve survival rates for patients with solid tumors (31).

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TABLE 3 Treatment-related common adverse events in this meta-analysis.

Adverse events		RR (95% CI)					
		No. of studies	All Grade	P value	No. of studies	Grade≥3	P value
Digestive system	Abdominal pain	2	0.73 (0.35, 1.52)	0.40	2	0.32 (0.06, 1.71)	0.18
	Constipation	6	1.13 (0.76, 1.69)	0.55	2	1.38 (0.09, 21.35)	0.82
	Diarrhea	5	1.60 (0.93, 2.74)	0.09	5	1.72 (0.71, 4.15)	0.23
	Nausea	6	1.02 (0.73, 1.42)	0.91	5	1.14 (0.42, 3.14)	0.80
	Stomatitis	4	3.26 (0.97, 10.92)	0.06	4	2.36 (0.49, 11.33)	0.28
	Vomiting	7	1.22 (0.88, 1.68)	0.24	7	2.15 (0.87, 5.30)	0.10
Hematological system	Anemia	7	1.10 (0.86, 1.41)	0.43	7	0.93 (0.76, 1.15)	0.52
	Leukopenia	3	1.40 (0.66, 2.98)	0.38	3	2.31 (1.47, 3.63)	<0.01
	NE decrease	2	0.67 (0.35, 1.31)	0.24	2	0.61 (0.27, 1.34)	0.21
	Neutropenia	6	1.07 (0.82, 1.40)	0.60	6	1.29 (0.91, 1.83)	0.15
	PLT decrease	6	1.06 (0.72, 1.56)	0.77	5	0.98 (0.50, 1.92)	0.95
	WBC decrease	3	0.89 (0.73, 1.09)	0.26	3	0.83 (0.51, 1.36)	0.46
Liver function	ALT increased	4	1.54 (0.81, 2.94)	0.27	4	1.44 (0.75, 2.76)	0.27
	AST increased	4	1.54 (0.79, 3.02)	0.20	4	1.50 (0.53, 4.26)	0.44
	GGT	2	2.67 (0.82, 8.72)	0.10	2	1.52 (0.22, 10.26)	0.67
Metabolism	HYPE	3	3.23 (1.19, 8.77)	0.02	2	7.81 (1.33, 45.91)	0.02
	HyperG	2	2.33 (0.97, 5.59)	0.06	2	3.29 (1.05, 10.35)	0.04
	HypoK	5	1.65 (1.09, 2.48)	0.02	5	2.07 (1.07, 4.00)	0.03
Renal function	P-Edema	3	3.86 (0.60, 24.73)	0.15	3	3.35 (0.62, 18.10)	0.16
	Proteinuria	2	1.34 (0.98, 1.83)	0.06	2	1.30 (0.43, 3.91)	0.64
Skin	DA	2	7.46 (4.20, 13.26)	<0.01	2	16.51 (2.16, 126.29)	0.01
	Paronychia	2	23.02 (2.42, 218.70)	0.01	2	16.95 (2.20, 130.80)	0.01
	Pyrexia	5	1.78 (0.79, 4.01)	0.17	3	1.00 (0.17, 5.90)	1.00
	Rash	6	3.25 (1.90, 5.54)	<0.01	5	9.35 (3.05, 28.64)	<0.01
Others	Asthenia	4	1.84 (1.10, 3.06)	0.02	4	1.38 (0.57, 3.30)	0.48
	Fatigue	7	1.09 (0.71, 1.68)	0.70	7	0.84 (0.41, 1.73)	0.64
	IRR	4	25.15 (6.82, 92.78)	<0.01	3	15.53 (2.91, 82.83)	<0.01
	Weight decreased	3	1.42 (1.02, 1.98)	0.04	3	2.89 (0.78, 10.67)	0.11

NE, neutrophil; PLT, platelet; WBC, white blood cell; ALT, alaninetransaminase; AST, aspartate aminotransferase; GGT, γ-glutamyltransferase; HYPE, hypoproteinemia; HyperG, hyperglycemia; HypoK, hypokalemia; P-Edema, peripheral edema; DA, dermatitis acneiform; IRR, infusion-related reaction; RR, relative risk.

Through subgroup analysis, female patients with solid tumors demonstrated better survival outcomes when receiving bsAbs +chemotherapy, which corroborates the findings of Thieblemont C (32) and Michael J (20). They found subgroup involving female showed a trend toward a higher percentage with a complete response. This may be explained by the generally stronger immune responses in females, attributed to hormonal influences (33). Mechanistically, estrogen enhances immune cell activity through multiple pathways: it promotes the proliferation and activation of T cells by upregulating the expression of cytokines such as IL-2 and IFN-γ; enhances the antigen-presenting capacity of dendritic cells by increasing the expression of co-stimulatory molecules like CD80 and CD86; and boosts the cytotoxic activity of natural killer (NK) cells by upregulating perforin and granzyme production (34, 35).

Additionally, due to a higher body fat percentage, certain chemotherapy agents are metabolized differently in women, which may optimize the synergistic effect when combined with bsAbs, leading to better therapeutic outcomes (2, 36). As for races, our study suggested Asian patients with solid tumors experienced better survival benefits when treated with bsAbs+chemotherapy. Ethnic differences in somatic mutations such as STK11, TP53 and EGFR may account for the differences of outcome for Asian and non-Asian patients receiving immunotherapy (37). For example, the mutation rate of STK11 differs among Asian (1.6%) and non-Asian patients (12.3%), which was reported previously to affect efficacy of immune checkpoint inhibitors (38, 39). Additionally, ethnicity may act as a key factor that influence the metabolism of chemotherapy agents and monoclonal antibodies (40). For example, low ERCC1 expression (common in Asian populations) is generally associated with better chemotherapy response to DNA-damaging agents like cisplatin (41) (42). Therefore, it is reasonable to assume that Asian populations may have higher drug exposure, potentially leading to more favorable outcomes when combining bsAbs with chemotherapy. Research has shown that the efficacy of certain bsAbs depends on immune function, which is influenced by age and physical status (43, 44). Our study demonstrated that in patients with an ECOG performance status of 1, bsAbs+chemotherapy demonstrated PFS benefits, suggesting better tolerance. However, caution is needed when making this inference, as OS was not affected. Conversely, in patients under the age of 65, bsAbs combined with chemotherapy showed OS benefits, indicating that this combination may be more suitable for frontline therapy. This age-related difference in outcomes may be partly explained by immune senescence, which can limit the effectiveness of these therapies in older patients. Immune senescence is characterized by a decline in immune function, including reduced T-cell diversity, impaired antigen presentation, and accumulation of senescent immune cells, all of which weaken the immune system's ability to mount an effective anti-tumor response (45). In younger patients, a more robust immune system may better synergize with bsAbs and chemotherapy, enhancing tumor cell killing and prolonging survival. In contrast, older patients often exhibit a less responsive immune microenvironment, which may diminish the therapeutic benefits of bsAbs and chemotherapy combinations (46).

Subgroup analysis has also suggested that patients with solid tumors were more likely to receive survival benefits when treated with IgG-like bsAbs in combination with chemotherapy. Similar results were demonstrated in a nonrandomized controlled trial conducted by Birrer, M (47), who found that bintrafusp alfa, an IgG-like bsAbs, demonstrated clinical activity in patients with recurrent or metastatic cervical cancer. BsAbs are typically categorized into two types: IgG-like and non-IgG-like. IgG-like BsAbs are designed to mimic the structure of natural immunoglobulins (IgG), consisting of two heavy chains and two light chains. With a large molecular weight, IgG-like format containing Fc domains. Due to the presence of the Fc region, it can exhibit improving stability of the molecule and extending the half-life of the bsAbs, allowing for less frequent dosing (48, 49). Moreover, the Fc region is formed by the CH2 and CH3 domains of the heavy chains. It enables binding to Fc receptors on immune cells, facilitating ADCC and CDC (50). This dual mechanism enhances the immune system's ability to target and eliminate tumor cells. In contrast, non-IgG-like bispecific antibodies lack the Fc region. They often consist of two single-chain variable fragments (scFvs) connected by a flexible peptide linker. Although absence of the Fc region leads to a shorter half-life, necessitating more frequent dosing, their smaller size allows for better tissue penetration, which can be advantageous in treating tumors with dense stroma or those located in hard-to-reach areas[35]. In summary, the choice between IgG-like and non-IgG-like bsAbs for solid tumor therapy depends on factors such as tumor type, location, and the desired immune response. Ongoing research aims to optimize these antibodies to balance tissue penetration with effective immune engagement (51). Taken together, there is still development space in bsAbs+chemotherapy application.

The overall safety of bsAbs+chemotherapy is acceptable as it did not increase the risk of most AEs involving the liver function, renal function, digestive system, and hematological system. Nevertheless, it's important to note that adding bsAbs to chemotherapy does give rise to certain AEs that warrant attention. Leukopenia was significantly predisposed to occur in all grades. Since leukopenic individuals are more prone to severe, rapidly progressing infections that are often harder to treat, close monitoring of routine blood parameters following medication administration is essential. BsAbs +chemotherapy also increased the incidence of asthenia, weight decreased, and Infusion-related reaction, but these AEs can be effectively controlled by appropriated supportive care. The majority of AEs were driven by skin-related bsAbs toxic effects, such as dermatitis acneiform, paronychia, and rash, as well as reversible metabolic effects, including hypoproteinemia, hyperglycemia, and hypokalemia, often associated with chemotherapy. Nonetheless, these skin-related and metabolism related AEs are generally manageable with standard topical or systemic therapies. Intriguingly, clinical trials have shown that cancer patients who developed skin rash exhibited improved survival benefits compared with those without such skin reactions (52, 53). This underscores the possibility that immune-related skin rash might serve as a prognostic factor in patients with solid tumors. An alternative way to address the concerns of toxicity associated with bsAbs+chemotherapy may be to employ antibody-drug conjugates (ADCs), which induce less off-target toxicities by delivering cytotoxic payloads directly to tumor cells. Preclinical studies suggest that ADCs can induce immunogenic cell death (ICD), which enhances anti-tumor immune responses and may synergize with immunotherapy (54). However, research on the combination of ADCs with bsAbs remains limited (55), and further studies are needed to explore the potential synergies and safety profile of this approach. Overall, the AEs associated with bsAbs+chemotherapy are manageable but there is still a need for improvement and a necessity for close monitoring during therapy.

In our study, solid tumor was innovatively separated from the wide range of application areas of bsAbs+chemotherapy. Furthermore, efficacy and safety were analyzed from its components, targets and other multiple factors as well as multiple systems involving tumor and adverse reactions. As it should be, the limitation of this study was acknowledged. First, the data were aggregated at the study level instead of the individual level, which restricted our ability to examine more granular details. Additionally, the relatively small sample sizes within

each subgroup may contribute to a reduction in the reliability of the results. This highlights the need for future research to involve multi-center, long-term RCTs to strengthen the evidence base.

Conclusion

Generally, the combination of bsAb and chemotherapy could be a promising treatment option. Specifically, Asian patients, female patients, those under 65 years of age, and individuals treated with IgG-like bsAbs may benefit most from this combination. Meanwhile, potential toxicity on leukopenia, metabolism, and skin were also observed in patients, suggesting management of adverse events was of vital importance.

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.

Author contributions

YY: Conceptualization, Data curation, Formal Analysis, Writing – review & editing. JY: Data curation, Methodology, Software, Writing – original draft. YP: Investigation, Software, Writing – original draft. CZ: Data curation, Investigation, Writing – original draft. XL: Formal Analysis, Methodology, Writing – original draft. LS: Supervision, Writing – review & editing, Funding acquisition, Validation. QS: Conceptualization, Project administration, 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.

Generative AI statement

The author(s) declare that no Generative AI was used in the creation of this manuscript.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2025.1568724/full#supplementary-material

SUPPLEMENTARY FIGURE 1
Risk of bias summary.

SUPPLEMENTARY FIGURE 2
Risk of bias graph.

SUPPLEMENTARY FIGURE 3
Funnel plots of PFS. The symmetrical distribution of the data points around the vertical line indicates no significant publication bias for PFS.

SUPPLEMENTARY FIGURE 4
Funnel plots of ORR. The symmetrical distribution of the data points around the vertical line suggests no significant publication bias for ORR.

SUPPLEMENTARY FIGURE 5
Funnel plots of OS. The slight asymmetry in the distribution of the data points suggests potential publication bias for OS.

SUPPLEMENTARY TABLE 1
Search Strategy.

SUPPLEMENTARY TABLE 2
Further Characteristics of the included trials.

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Pan-cancer analysis identifies tRNA modification enzyme CTU2 as a novel tumor biomarker and its role in immune microenvironment

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Background: Recent studies have highlighted dysregulated tRNA modifications in the reprogramming of tumor translation. Cytosolic thiouridylase subunit 2 (CTU2) is an essential and conserved enzyme that modifies tRNA at the wobble position. However, the relationship between CTU2 expression and various cancer types remains insufficiently explored.

Methods: Pan-cancer data from TCGA, GEO, and CPTAC were used to analyze CTU2 expression and its prognostic value. Single-cell and spatial transcriptomic analyses were performed to identify CTU2's cell-type labels and distribution. The TCGA microRNA database was used to explore the expression patterns of CTU2-modified tRNAs and their prognostic significance. TIMER2.0, ESTIMATE, and TIP were employed to analyze the correlation between CTU2 expression, immune infiltration, and immunotherapy response. GSEA and Depmap databases were conducted to explore signaling pathways related to CTU2 expression. Drug sensitivity related to CTU2 was assessed using CMap and GDSC-V2. The oncogenic roles of CTU2 were validated in vitro and in vivo. Genomic alterations, public ChIP-seq data, dual-luciferase assays, and EMSA were employed to investigate the upstream regulatory mechanisms regulating CTU2.

Results: CTU2 and its modified tRNA, particularly tRNA-Lys-TTT, are differentially expressed across various tumor types, suggesting their potential as prognostic biomarkers. Abnormal CTU2 expression in tumors is associated with alterations in immune cell infiltration, immune evasion, and immunotherapy response. CTU2 may contribute to several key cancer-related pathways and biological processes. Mechanistically, CTU2 overexpression is likely driven by DNA copy number amplification and DNA methylation alterations. USF1 has been identified as one of the transcription factors regulating CTU2.

Conclusions: CTU2 may serve as a valuable prognostic and immunotherapeutic biomarker across multiple cancer types, providing new insights into tumor treatment strategies and immune evasion from the perspective of tRNA modifications.

KEYWORDS

CTU2, tRNA modification, pan-cancer, immune microenvironment, prognosis, USF1

1 Introduction

tRNAs, once viewed as static adaptors transporting amino acids and interpreting mRNA codons (1, 2), are now recognized for their dynamic roles in regulating gene expression and translation (3–6). A recent study reveals that tRNAs act as 'accomplices' in dysregulated translation systems. Specifically, tRNA-Glu-TTC is significantly upregulated in highly invasive breast cancer cells, and its overexpression enhances the translation of mRNAs with complementary codons (GAA, which base-pair with TTC). This upregulation increases the translation efficiency of exosome component 2 (EXOSC2) and GRIP1-associated protein 1 (GRIPAP1), both of which are enriched in GAA codons within their coding regions, positioning them as key downstream mediators of the pro-metastatic effects of tRNA-Glu-TTC overexpression. These findings emphasize the role of codon-biased translation, driven by upregulated tRNAs, in promoting the synthesis of oncoproteins (7).

tRNA modifications are essential for proper tRNA folding, aminoacylation, stability, and mRNA decoding, ensuring optimized translation (8, 9). Recent studies have revealed that tRNA modifications can significantly influence the decoding capability of tRNA, promote its codon-biased translation, and play an active role in the dynamic regulation of gene expression (8, 10). Modifications in the tRNA anticodon loop are crucial for modulating tRNA decoding ability, as abnormal modifications directly affect the pairing between the tRNA anticodon and the mRNA codon (11, 12). CTU2 catalyzes the critical final 2-thiolation step necessary for the mcm^5s^2U cascade modification at the first position of the tRNA anticodon (position 34) in the anticodon loop of tRNAs (13). Notably, the first position of the tRNA anticodon, known as the wobble position, exhibits non-Watson-Crick base pairing with the third nucleotide of the codon. For instance, the unmodified base uridine (U) at the first anticodon site can pair not only with codon adenine (A) but also with guanine (G) and cytosine (C) (10, 14). This non-complementary pairing is relaxed and unstable, increasing the likelihood of frameshift errors during translation. In contrast, the 5-methoxycarbonylmethyl-2-thiouridine (mcm^5s^2U) modification strictly regulates and stabilizes the complementary base pairing between U and A, occurring exclusively in three specific tRNAs (tRNA-Glu-TTC, tRNA-Lys-TTT, and tRNA-Gln-TTG), where the 34th position is U (in the DNA sequence, this corresponds to thymine, T) (13, 15, 16). While wobble pairing expands the decoding capacity of tRNAs, the mcm^5s^2U modification restricts strict complementary pairing between the anticodon (TTC, TTT, TTG) and their corresponding U34 codons (GAA, AAA, and CAA) (13, 17). Thus, CTU2-mediated mcm^5s^2U modification is crucial for maintaining the accuracy and fidelity of translation.

CTU2-mediated mcm^5s^2U modification is crucial for maintaining the accuracy and fidelity of translation across various organisms (13, 18–20). In the nematode and fission yeast, CTU2 knockout causes thermosensitive viability loss, accompanied by significant aberrant development, which could result from both misreading and frameshifting during translation (13). It has been reported to regulate plant immunity through translation reprogramming (18). In Arabidopsis, mutations in the CTU2 homolog lead to loss of tRNA thiolation, reducing translation of Non-expressor of Pathogenesis-Related genes 1 (NPR1), the salicylic acid receptor, and compromising salicylic acid signaling. In the Magnaporthe oryzae model system, the absence of CTU2 results in a reduction in translation elongation at AAA/CAA/GAA codons, without affecting their synonymous codons (21). This leads to a decrease in the levels of key proteins enriched in U34 codons, which are crucial for appressorium development and function.

CTU2 has increasingly been shown to play a role in the progression of various tumors (16, 20, 22–24). For instance, CTU2 levels are elevated in breast tumors and support metastasis. Mechanistically, CTU2 promotes cellular invasion through codon-biased translation of DEK (a DNA-binding oncoprotein), whose coding region is rich in U34 codons, thereby enhancing Internal Ribosome Entry Site (IRES)-dependent translation of the pro-invasive transcription factor Lymphoid enhancer-binding factor 1 (LEF1) (16). Furthermore, studies have found that CTU2 is highly expressed in BRAFV600E-expressing melanoma cells, potentially promoting glycolysis by codon-biased regulation of HIF1α mRNA translation, which is rich in U34 codons, and maintaining high levels of HIF1α protein. This may contribute to melanoma's acquired resistance to MAPK therapeutic agents (22). Recent research has elucidated the role of CTU2 in hepatocellular carcinoma development and its upstream transcriptional regulatory mechanisms, identifying it as a Liver X receptor (LXR) target gene. Mechanistically, CTU2 enhances lipogenesis by directly promoting the synthesis of lipogenic proteins, providing a novel mechanism for LXR-mediated lipid synthesis regulation (25).

Given the emerging novel role of tRNA in actively regulating gene expression and the crucial role of CTU2-mediated mcm^5s^2U tRNA modification, a comprehensive analysis of CTU2 in multiple cancers is extremely necessary.

2 Materials and methods

2.1 Pan-cancer data collection and processing

Phenotype data of pan-cancer in The Cancer Genome Atlas (TCGA) and normal tissues in Genotype-Tissue Expression (GTEx) database were downloaded from the UCSC Xena Browser (https://xenabrowser.net/). The Gene Expression Omnibus (GEO) database (https://www.ncbi.nlm.nih.gov/geo/) was used to obtain GSE115002 (26), GSE39582 (27), GSE161533 (28), GSE16449 (29), GSE36376 (30), GSE10927 (31), GSE50428 (32), GSE36376 (33), and GSE75037 (34). The proteomics data of multiple cancer types were obtained from the Clinical Proteomic Tumor Analysis Consortium (CPTAC) database (https://proteomics.cancer.gov/programs/cptac). Immunohistochemistry (IHC) images showing CTU2 expression in normal and cancer tissues were retrieved from the Human Protein Atlas (HPA) database (https://www.proteinatlas.org/).

The cBioPortal for Cancer Genomics (http://www.cbioportal.org) was used as a source of merged CTU2 methylation data. UALCAN (http://ualcan.path.uab.edu/analysis.html) was used to explore the promoter DNA methylation levels in CTU2 in normal and pan-cancer tissues. The $\log_2$ (TPM + 0.001) transformed normalized expression profiles, copy number variations on gene expression were estimated using the GISTIC2.0 method.

2.2 Single-cell expression and spatial transcriptomes analysis of CTU2

The single-cell expression levels of CTU2 across various pan-cancer tissues using the Tumor Immune Single-cell Hub (TISCH) database (http://tisch.comp-genomics.org/home/), which also provided UMAP plots illustrating CTU2 expression patterns across different cell types. Spatial transcriptome data were obtained from the 10xGenomics website, BRCA (GSE210616) and PAAD (GSE211895). The Spatial-FeaturePlot function from the Seurat package was used to visualize enrichment scores for each cell type.

2.3 Prognosis analysis

The survival information of pan-cancer, including overall survival (OS), progression-free interval (PFI), disease-free interval (DFI) and disease-specific survival (DSS), was downloaded from the TCGA database. The R packages 'survival' and 'survminer' were used to perform Cox analysis and to generate Kaplan-Meier (KM) survival curves to analyze the association between the expression of CTU2 and patient prognosis.

2.4 Immune-related analysis

The ESTIMATE algorithm (https://bioinformatics.mdanderson.org/*estimate*/) was used to compute Immune, Stromal, and ESTIMATE score values for 33 cancer types (35). Utilizing the TIMER2.0 (http://timer.cistrome.org/), we investigated the abundance of various cell types within the tumor microenvironment across 33 cancer types. A total of 11 immune checkpoint genes (including PDCD1, CTLA4, VSIR, HAVCR2, LAG3, TIGIT, SIRPA, BTLA, SIGLEC7, LILRB2, and LILRB4) were extracted from TCGA datasets for correlation analysis of immune checkpoint genes (36). In addition, CTU2 was analyzed in relation to tumor immunity in the following areas, including immune activation, chemokines, chemokine receptors, and major histocompatibility complex (MHC). All gene markers were obtained from previous studies (36–38). The impact of CTU2 expression level on the status of anti-cancer immunity was analyzed in 33 cancer types using the Tracking Tumor Immuno phenotype (TIP) database (http://biocc.hrbmu.edu.cn/TIP). The TIDE website (http://tide.dfci.harvard.edu) was used to retrieve the TIDE score for each patient.

2.5 Drug sensitivity analysis

The Genomics of Drug Sensitivity in Cancer (GDSC) database, established by the Sanger Research Institute, gathers data on how tumor cells respond to various drugs (39). The 'oncoPredict' tool utilized the GDSC V2 database to assess the drug sensitivity of samples in both the training and validation datasets (40). The CMAP_gene_signatures. RData file, which contains 1288 compounds-related signatures, was downloaded from https://www.pmgenomics.ca/bhklab/sites/default/files/downloads, and used for calculating the matching score. We constructed a gene-related signature consisting of the 150 most significantly upregulated and the 150 most significantly downregulated genes, determined by comparing patients with high and low gene expression in tumors. Using the optimal feature matching method XSum (eXtreme Sum), we compared the gene-related features with cMAP gene features to obtain similarity scores for 1,288 compounds. The analysis process was followed the methodology outlined in previous publications (41, 42).

2.6 Gene Set Enrichment Analysis (GSEA) and correlation analysis

To evaluate the biological function of a single gene in tumors, Pearson's correlation analysis was performed to examine the relationship between CTU2 expression and other mRNAs using

TCGA transcriptome data. Genes with the highest correlation with CTU2 expression were selected for enrichment analysis. GSEA was conducted using the R package 'clusterProfiler', based on predefined gene sets from the Molecular Signatures Database v5.0 (http://software.broadinstitute.org/gsea/msigdb/index.jsp). For this study, the 'c2.cp.kegg.v7.5.1.entrez.gmt' and 'c5.go.bp.v7.5.1.entrez.gmt' collection sets were utilized in the GSEA.

2.7 DepMap (The Cancer Dependency Map) analysis

For a diverse set of pan-cancer cell lines, gene-level essentiality scores (obtained from CRISPR knockout and RNAi knockdown screens) were extracted from the from the DepMap Public 21Q3 dataset using the DepMap portal (depmap.org/portal). For REACTOME gene sets (acquired from MSigDB v7.4), Student's t-tests were performed to compare the false discovery rate (FDR) values of genes within each gene set to those outside it. The gene set dependency score was computed by multiplying the FDR value for each gene set by the sign of its corresponding t-statistic.

2.8 Cell culture

Given the expression and prognostic significance of CTU2 across various cancer types, particularly considering the high incidence and mortality of kidney renal clear cell carcinoma (KIRC) and liver hepatocellular carcinoma (LIHC), representative cell lines from these malignancies were selected for functional validation. The human liver cancer cell line Huh-7, human renal clear cell carcinoma cell line 786-O and murine liver cancer cell line Hepa1–6 were obtained from the American Type Culture Collection. Both cell lines were cultured in complete DMEM medium (Thermo Scientific, Waltham), supplemented with 10% fetal bovine serum (FBS, Gibco, Thermo Scientific, Waltham), at 37°C in a 5% CO2 incubator.

2.9 Stable cell line construction

The shRNA sequences targeting human CTU2 gene, following the sequences shRNA-1: GTTCCTTCTGTCTTCACACCA; and shRNA-2: GAAGTGTGTGAAGTGCAAGGA, were obtained from Genechem (Shanghai, China) and were constructed into lentiviruses backbone plasmid. The shRNA sequences targeting mouse CTU2 gene are described in refs (22). A scrambled non-specific control shRNA sequence was also cloned into the same vector and used as a control. Huh-7, 786-O and Hepa1–6 cell lines were planted in six-well plates 24 h before transfection at the cell density of 2×10^5 cells/well. Lentivirus packaging was carried out following previously established protocols (43). Stable cell lines were generated by infecting cell cultures with lentivirus.

2.10 Colony formation assay

After the stable CTU2 knockdown cell lines were successfully constructed, the cells were seeded in six-well plates at densities of 1500 cells/well, and the cells were cultured for 2 weeks. Finally, the cells were fixed with 4% para-formaldehyde and stained with crystal violet, and colonies containing more than 50 cells were counted and analyzed.

2.11 Western blotting

The cells were lysed with ice-cold RIPA lysis buffer (Servicebio, China) containing protease inhibitors and centrifuged at 4°C (12,000 rpm, 20 min). The protein supernatant was then quantified using a BCA protein assay kit (Biyuntian, China). Following protein denaturation, 30 μg of protein was separated by SDS-PAGE on 10% gels and transferred to a PVDF membrane (Millipore, USA). After blocking with 5% skim milk in TBS-T, the membrane was incubated overnight at 4°C with the following antibodies: anti-CTU2 (ab177160, 1:1000), anti-USF1 (ab125020, 1:1000), and anti-GAPDH (Proteintech, 60004-1-Ig). The membrane was then incubated with goat anti-rabbit (Proteintech, RGAR001, 1:5000) or mouse IgG secondary antibodies (Proteintech, RGAM001, 1:5000) for 1 hour. Following this, the membranes were washed three times with TBS-T (5 min per wash) and visualized using an enhanced chemiluminescence substrate.

2.12 EdU proliferation assay

EdU detection was performed using the EdU Imaging Kits (APEXBIO, K1076, USA) according to the manufacturer's protocol. Briefly, cells were incubated with 10 μM EdU for 1 hour, then trypsinized, washed with PBS, fixed with 4% paraformaldehyde (PFA) for 20 minutes, and permeabilized with 0.1% Triton X-100 for 20 minutes. The single-cell suspensions were washed twice with PBS and incubated with the appropriate EdU flow cytometry antibodies for 30 minutes in the dark at room temperature. The EdU-positive rate was calculated as follows: EdU-positive rate = (EdU-positive cell count/(EdU-positive cell count + EdU-negative cell count)) × 100%.

2.13 Flow cytometric analysis of cell apoptosis

For apoptosis assays, the Alexa Fluor 488 Annexin V/PI Cell Apoptosis Kit (Vazyme, A211-01, China) was used according to the manufacturer's instructions. The established stable cell lines were digested with EDTA-free trypsin, washed with PBS, and stained with Annexin V-Alexa Fluor 488 (FITC) and propidium iodide (PI) as recommended. Flow cytometry was then performed according to the manufacturer's guidelines, and the proportion of apoptotic cells (early apoptosis plus late apoptosis) was calculated.

2.14 Luciferase reporter assay

CTU2 wild-type and mutant dual-luciferase reporter gene plasmids were constructed based on the base sequence by You Bao Biotechnology (Changsha, China). The dual-luciferase reporter assay was conducted using the Dual-Luciferase Reporter Assay System (Vazyme, DD1205, China). Cells were plated in 12-well plates at a density of 2×10^5 cells per well and transfected with Lipofectamine 3000. After 24 hours of transfection, Firefly and Renilla luciferase activities were measured according to the manufacturer's instructions. Firefly luciferase activities were normalized to Renilla luciferase activities, and the ratio of Firefly to Renilla luminescence was calculated.

2.15 Migration and invasion assays

Cell migration and invasion assays were conducted using Transwell chambers (8-μm pore size, Corning, USA). The lower compartment of the Transwell chamber was filled with 600 μl DMEM containing 10% FBS, and a 100 μl serum-free cell suspension containing 8×10^4 cells was seeded into the upper chamber. For the invasion assay, matrigel-coated invasion chambers were utilized to evaluate cell invasion.

2.16 *In vivo* LIHC murine models

All animal experiments in this study were performed in accordance with the guidelines for the welfare and ethics of experimental animals of Zhejiang University with the approval of the Animal Experimental Ethics Committee of Zhejiang University. Female nude mice (BALB/c, 6 weeks old) were obtained from GemPharmatech (Jiangsu, China) and housed in a specific-pathogen-free (SPF) animal facility. For the subcutaneous tumor xenograft models, mice were randomly assigned to three groups (6 mice per group): shNC, shCTU2-1, and shCTU2-2. Each nude mouse received a subcutaneous inoculation of 1 × 10^7 cells (100 μL) in the right hind limb. Tumor size was measured using Vernier calipers every five days, and tumor volume was calculated as V = (Length × Width^2)/2. Mice were euthanized when the maximum tumor volume reached 1500 mm^3, and tumors were harvested, weighed, and imaged.

An orthotopic LIHC tumor model was established by implanting 5×10^6 Hepa1–6 cells directly into the liver of C57BL/6 male mice (6–8 weeks old, GemPharmatech). Three weeks after inoculation, the mice were euthanized, and the tumor nodules in the liver were quantified and measured.

2.17 Flow cytometry analysis of orthotopic LIHC tumor nodules

Single-cell suspensions were generated from orthotopic liver of tumor-bearing mice. The following anti-mouse antibodies were used: FITC-Anti-CD11b (cat# 101205), BV605-Anti-Gr-1 (cat# 563299), APC-Cy7-Anti-MHC-II (cat# 107629), BV421-Anti-CD11c (cat# 117329), Percp-Cy5.5-Anti-CD8 (cat# 100733) and APC-Anti-PD-1 (cat# 100733) was purchased from Biolegend (San Diego, CA). Cells were analyzed using with CyAnADP analyzer (Beckman Coulter).

2.18 APM-dPAGE and Northern blot

To isolate single tRNA Lys, small RNAs (≤200 nt) were extracted using the MiPure cell miRNA Kit (Vazyme, RC201, China). The presence of the mcm^5s^2U modification in tRNAs was confirmed by observing reduced electrophoretic mobility in a 10% polyacrylamide gel containing 0.05 mg/ml [(N acryloyl amino) phenyl] mercuric chloride (APM) and 7 M urea, were performed as described (44). Subsequently, the APM-PAGE gels were transferred onto positively charged Nylon membranes (Roche, USA). Membranes containing tRNA were hybridized with DIG-labeled probes synthesized by Sangon Biotech (Shanghai, China), following the sequences: TAAAAGTCTGATGCTCTACC. The RNA from hydrogen peroxide (H2O2) pre-treatment served as a negative control for desulfurization.

2.19 Electrophoretic Mobility Shift Assay (EMSA)

Nuclear extracts from huh-7 cells were prepared using the Nuclear Extract Kit (Active Motif, CA) according to the manufacturer's instructions. The DNA-binding activity of USF1 in the nuclear extracts was assessed using the Light-Shift EMSA Optimization and Control Kit (Thermo Scientific, USA). A biotin-labeled wild-type oligonucleotide probe corresponding to the USF1 E-box motif was designed as follows: 5'- GGGCGGGCGCGCTCA CGTGTGGCCGCAGCTG-3'. Additionally, an unlabeled wild-type probe (without biotin) was designed and used in the competition reaction. A mutated E-box motif probe, also unlabeled, was constructed with the following sequence: 5'-GGGCGGGCGC GCTAAAAAAAGGCCGCAGCTG-3'. The DNA-protein complexes were resolved by electrophoresis on a 6% polyacrylamide gel, followed by visualization and analysis of band shifts via autoradiography.

2.20 Statistical analysis

Correlation analysis was performed using Spearman's rank correlation. The *in vitro* experiments were conducted in triplicate. All statistical analyses were carried out using GraphPad Prism 7.0, SPSS (version 22.0), or R software (version 4.1.2). *P value* of < 0.05 was considered statistically significant. Statistical significance is indicated as follows: ns (not significant), $^*P < 0.05$, $^{**}P < 0.01$ and $^{***}P<0.001$.

3 Results

3.1 CTU2 is upregulated across multiple cancer types

Initially, the TCGA and GTEx databases were utilized for a comprehensive pan-cancer analysis of CTU2 mRNA expression profiles. This investigation revealed significant differential expression of CTU2 across 24 cancer types (Figure 1A), with fold changes exceeding 2 in diffuse large B-cell lymphoma (DLBC), thymoma (THYM), cholangiocarcinoma (CHOL), and glioblastoma multiforme (GBM) (Supplementary Table S1). Paired Student's t-test further demonstrated a significant increase in CTU2 expression in multiple tumor tissues compared to adjacent normal tissues (Supplementary Figures S1A–1M). Analysis of seven GEO datasets confirmed elevated CTU2 expression in breast cancer (BRCA), colon adenocarcinoma (COAD), LIHC, non-small cell lung cancer (NSCLC) (Figures 1B–E), esophageal cancer (ESCA), KIRC, and adrenocortical carcinoma (ACC) (Supplementary Figures S1N–P). Consistently, immunohistochemical data from the HPA databases confirmed increased CTU2 protein levels in BRCA, COAD, LIHC, and lung adenocarcinoma (LUAD) (Figure 1F). At the protein level, CTU2 was upregulated in 9 datasets across 8 cancer types in the CPTAC database, including clear cell renal cell carcinoma (CCRCC), COAD, GBM, hepatocellular carcinoma (HCC), head and neck squamous cell carcinoma (HNSC), lung squamous cell carcinoma (LSCC), LUAD, and pancreatic ductal adenocarcinoma (PDA) (Figure 1G).

3.2 Overall landscapes of single-cell expression levels and spatial transcriptomics of CTU2

We analyzed the TISCH database to illustrate the landscape of CTU2 single-cell expression. Among 98 single-cell sequencing datasets, we found that CTU2 expression is predominantly observed in the malignant cell types of most tumors (Figure 2A, red arrow). We randomly selected common tumor types for specific analysis, and the UMAP plots of BRCA, NSCLC, and pancreatic adenocarcinoma (PAAD) datasets intuitively showed that CTU2 is mainly expressed in malignant cells (Figures 2B–D). Specifically, in BRCA (GSE136206), UMAP plots (Figure 2B, left panel) revealed CTU2 expression in various cell types, including malignant cells, endothelial cells, fibroblasts, monocytes, macrophages, $CD4^+$ T cells, $CD8^+$ T cells, natural killer (NK) cells, and T-proliferating cells, with particularly high expression levels observed in malignant cells (Figure 2B, right panel).

Unlike single-cell sequencing, spatial transcriptomics preserves spatial information while providing insights into gene expression, cell types, and tissue context. Next, we utilized spatial transcriptome data to further assess the spatial distribution of CTU2 and malignant cells in BRCA, lung squamous cell carcinoma (LUSC), and PAAD. Spatial infiltration heatmaps revealed that different sequencing spots were annotated with distinct cell types, including malignant cells, fibroblasts, and key immune cells ($CD4^+$ T cells, $CD8^+$ T cells, NK cells, B cells, and dendritic cells) (Figures 2E–G, upper panel). Spearman correlation analysis demonstrated a significant positive correlation between CTU2 expression and tumor cell density in specific regions, indicating that $CTU2^+$ cells were primarily clustered in regions populated by malignant cells (Figures 2E–G, lower panel). In the spatial transcriptomics data of LIHC and skin cutaneous melanoma (SKCM), CTU2 is also primarily expressed in tumor tissue regions (Supplementary Figure S2). These results emphasize that CTU2 is mainly expressed by tumor cells in pan-cancer and its potential as a therapeutic target.

3.3 Prognostic role of CTU2 in human cancers

Univariate Cox regression analyses revealed that high CTU2 mRNA expression was significantly associated with OS and DSS across multiple cancers, particularly in ACC, KIRC, lower-grade glioma (LGG), mesothelioma (MESO), and sarcoma (SARC) (Figure 3A). These associations were further supported by DFI and PFI analyses, primarily in LIHC and SARC (Supplementary Figure S3A). Kaplan-Meier curves indicated that elevated CTU2 mRNA expression correlated with poor prognosis in ACC, KIRC, LGG, LIHC, SARC, uveal melanoma (UVM), thyroid cancer (THCA) and LUSC (Supplementary Figures S3B, C). Similarly, CPTAC data indicated that high CTU2 protein levels correlated with poor prognosis in BRCA, LIHC, LUAD, and KIRC (Supplementary Figure S3D). Multiple GEO datasets from the TIDE website further validated poor prognosis in patients with high CTU2 mRNA levels in BRCA, COAD, DLBC, LUAD, SARC, and melanoma (Figure 3B). ROC curve analysis demonstrated that CTU2 has high diagnostic accuracy (AUC > 0.8) for eight cancer types, including READ, LUSC, LUAD, kidney renal papillary cell carcinoma (KIRP), KIRC, kidney chromophobe (KICH), COAD, and bladder urothelial carcinoma (BLCA) (Figure 3C). Integrating TCGA and GTEx data further supported CTU2's diagnostic potential in pheochromocytoma and paraganglioma (PCPG), PAAD, HNSC, and CHOL (Figure 3C).

We also analyzed clinical phenotype data from TCGA to investigate CTU2 mRNA expression patterns across different clinical stages and their association with clinical features in various cancers. CTU2 mRNA levels increased with advancing clinical stage in cancers such as BRCA, HNSC, KIRC, KIRP, LIHC, LUSC, and testicular germ cell tumors (TGCT) (Figure 3D). The CPTAC database indicates that in BRCA, CCRCC, LSCC, and LUAD, CTU2 protein levels are elevated in Stage IV (late-stage) compared to earlier stages (Stage I) (Supplementary Figure S3E). We further examined CTU2 expression across different molecular tumor subtypes and found distinct gene expression profiles for specific cancers (Supplementary Figure S3F). Additionally, the expression of CTU2 was found to be correlated with T stage, N stage, and M stage in various cancers (Supplementary Figures S4A–H). These findings suggest that CTU2 could be a significant and potential tumor marker across multiple cancers.

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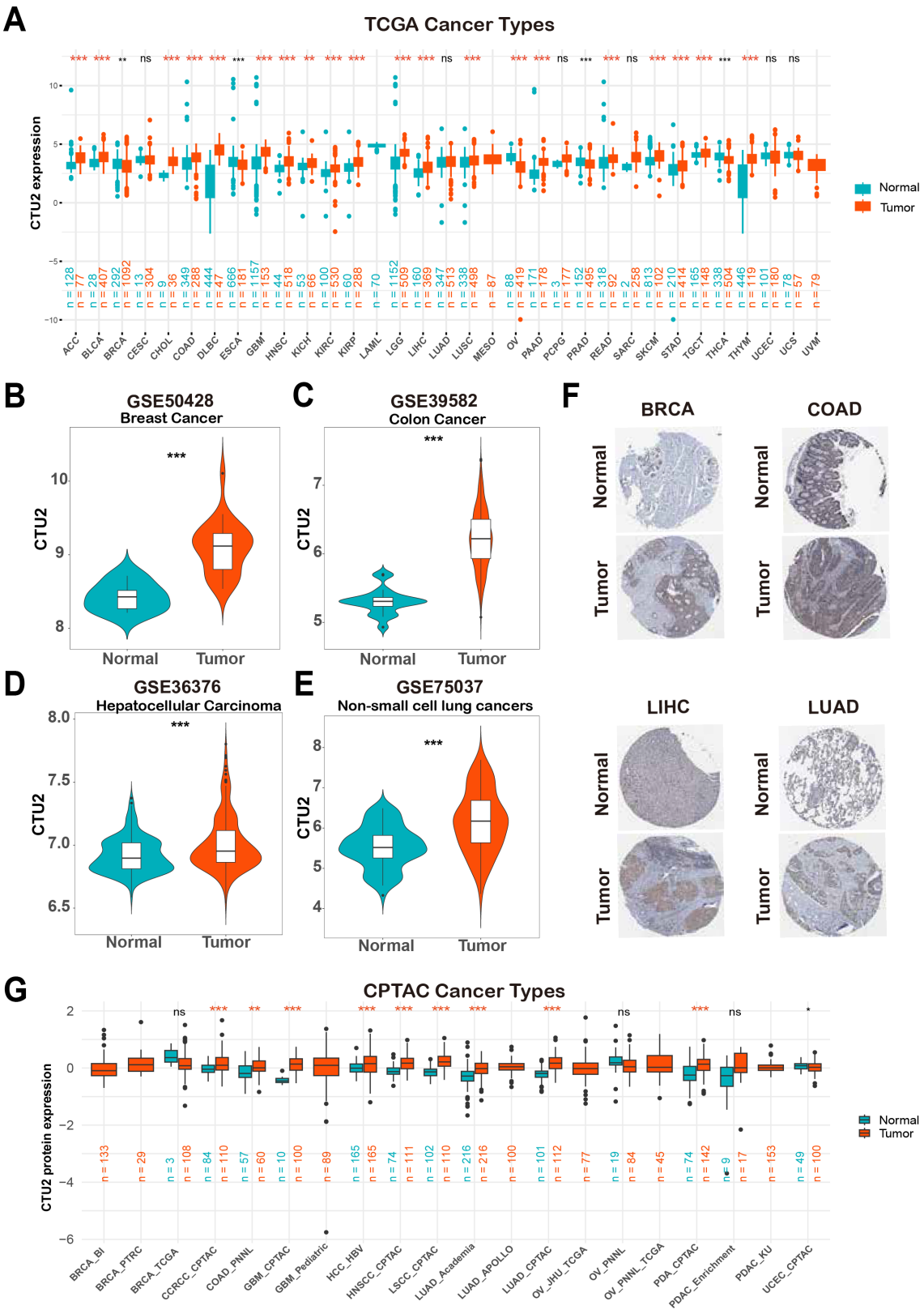

FIGURE 1
Upregulation of CTU2 across multiple cancer types. **(A)** Analysis of CTU2 mRNA expression across 33 cancer types using the TCGA and GTEx databases; **(B–E)** Differential CTU2 mRNA expression in various cancer GEO datasets; **(F)** Representative images of CTU2 protein expression in normal and tumor tissues of the breast, colon, liver, and lung from the HPA database; **(G)** CTU2 protein expression analysis in 12 cancer types using data from the CPTAC database. The red asterisk (*) indicates a significant upregulation. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, and ns (not significant).

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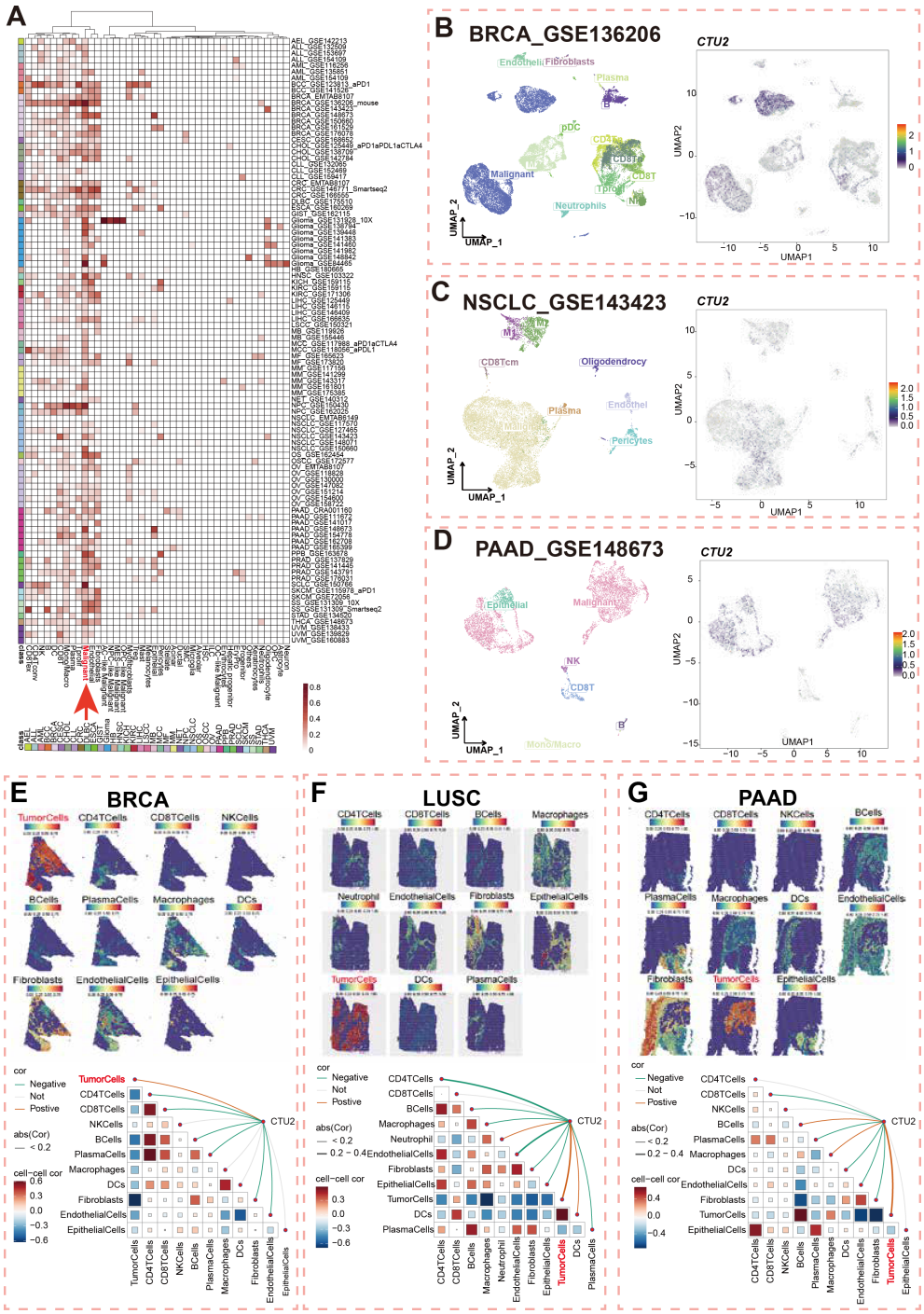

FIGURE 2
Single-cell and Spatial transcriptomics of CTU2 expression across multiple cancer types. **(A)** Cluster heatmaps showing the mRNA expression pattern of CTU2 in different cell types across different tumor types; **(B)** Umap plots displaying the clustering of different cell types (left panel) and CTU2 expression level (right panel) in BRCA **(B)**, NSCLC **(C)**, and PAAD tissues; Upper Spatial transcriptomics deconvolution maps visualize cell localization in BRCA **(E)**, LUSC **(F)** and PAAD **(G)**. Color ranging from blue to red represents the abundance of that cell type within the spot. Lower correlation analysis calculates the relationships between cell abundances and CTU2 expression levels. Red lines indicate positive correlations, green lines denote negative correlations, and gray lines represent non-significant correlations. The thickness of the lines reflects the absolute value of the correlation coefficients. The correlation in triangular regions is represented by the color intensity and size of the squares: red indicates a positive correlation, blue indicates a negative correlation and darker colors signify more significant *p-values*. Larger squares correspond to greater absolute values of the correlation coefficients.

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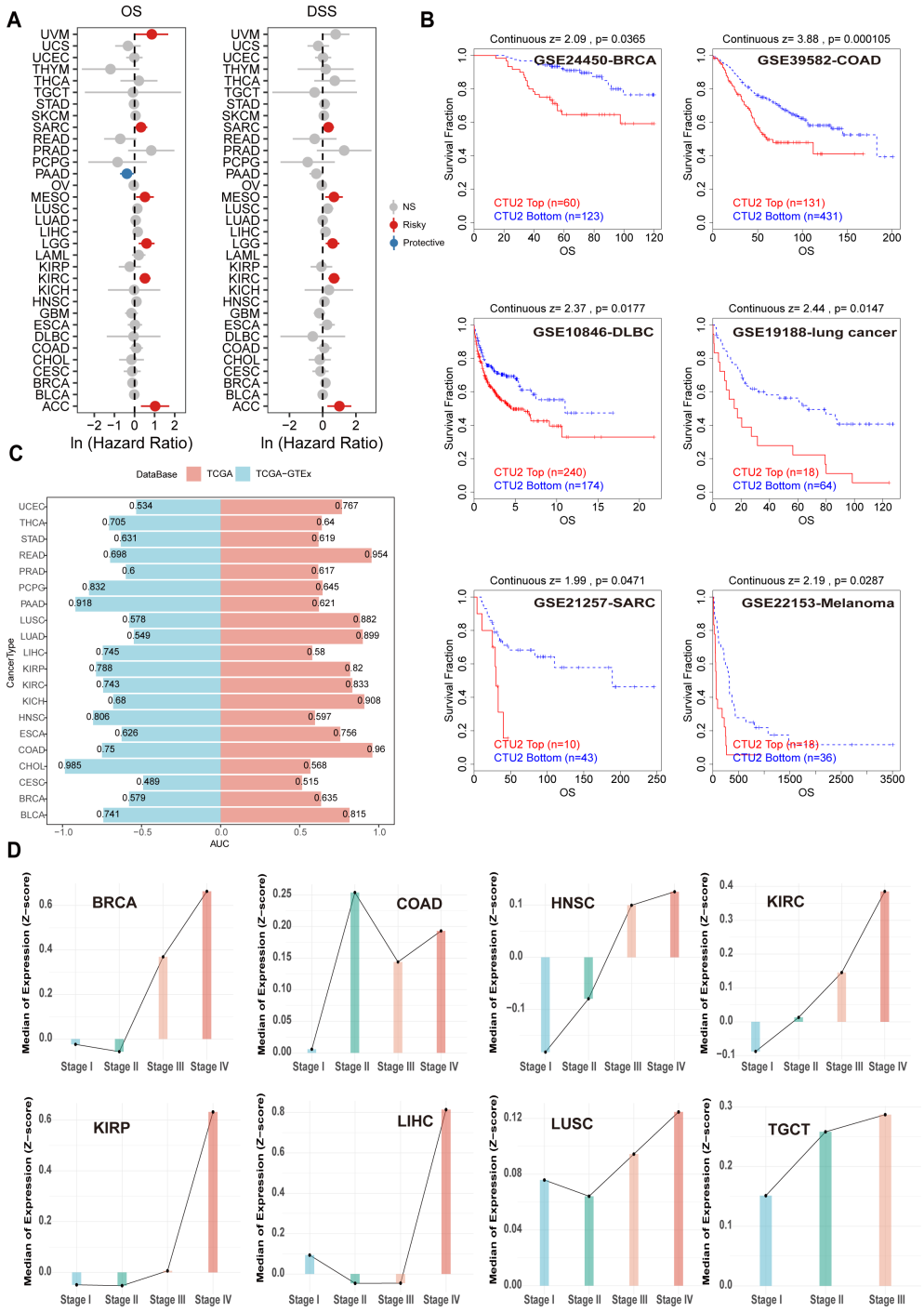

FIGURE 3
Correlation between CTU2 expression and pan-cancer prognosis and diagnosis. **(A)** OS and DSS associated with CTU2 expression in 33 cancer types from TCGA; **(B)** Kaplan-Meier analysis of OS based on CTU2 mRNA expression across multiple tumors using the TIDE tool; **(C)** AUC values from receiver operating ROC analysis; blue indicates the TCGA-GTEx cohort, while red represents the TCGA cohort; **(D)** CTU2 mRNA expression across different tumor stages in various cancers.

3.4 Alterations of CTU2 modified tRNA expression across cancer types

Given the role of CTU2 across cancers, we next map the expression profile of its modified tRNAs in a pan-cancer context. High-throughput quantification of tRNAs is challenging due to extensive post-transcriptional modifications and complex secondary structures. To overcome this, as reported in the literature, we utilized microRNA-sequencing data from the TCGA database, which includes data from approximately 10,000 patients, as an alternative method for quantifying tRNA expression (Supplementary Table S2). The mcm^{5}s^{2}U modification, mediated

by CTU2 at the wobble position, restricts and constrains the strict complementary pairing between the anticodon (tRNA-Glu-TTC, tRNA-Lys-TTT, tRNA-Gln-TTG) and its corresponding codon (GAA, AAA, CAA), despite the wobble pairing expands the decoding ability of tRNAs (8).

We first examined differential expression of the three modified tRNAs and their isoforms between paired tumor and normal samples, finding that tRNA-Lys-TTT (Figure 4A) and its isoforms (Figure 4B) were highly expressed in multiple cancer types, notably in KICH, uterine corpus endometrial carcinoma (UCEC), BRCA, KIRC, ESCA, and KIRP. Further correlation analysis revealed a significant positive association between CTU2 expression and the expression of multiple isoforms of tRNA-Lys-TTT across various tumors, especially in BRCA, LIHC, stomach cancer (STAD), OV and TGCT (Figure 4C). The tRNA mcm^5s^2U modification, a form of thiouridine modification, was evaluated by electrophoretic mobility retardation using Northern blot (45, 46). *In vitro* results confirmed that CTU2 knockdown reduced mcm^5s^2U modification levels on tRNA-Lys-TTT in LIHC (huh-7) cells and KIRC (786-O), as indicated by decreased thiolation of the target tRNA (Figure 4D). We also found that tRNA-Lys-TTT expression was linked to OS and DSS in multiple tumors (Figure 4E). These findings suggest that tRNA-Lys-TTT expression could serve as a prognostic marker, with KIRC as an example (Figure 4F). Thus, not only does CTU2 contribute to cancer progression, but its modified tRNA is also linked to poor prognosis in various tumors.

3.5 Impact of CTU2 expression on the tumor microenvironment in pan-cancer

Firstly, we utilized the ESTIMATE database to investigate the impact of CTU2 expression on immune cell infiltration in human cancers (Supplementary Table S3). It is worth noting that in most tumors, including COAD, GBM, HNSC, LGG, and SKCM, high CTU2 expression was associated with lower immune scores, suggesting that elevated CTU2 expression in these tumors may indicate reduced immune infiltration. Conversely, in BRCA and UCEC, high CTU2 expression was correlated with higher immune scores, implying greater immune infiltration (Supplementary Figure S5A, Figure 5A). We also utilized the TIMER 2.0 database to explore the correlation between CTU2 expression and the infiltration of specific immune cell types across various cancers. Our analysis revealed that, in most tumor types, tumor CTU2 expression is negatively correlated with the infiltration of major immune cell subtypes, such as $CD8^+$ T cells and DC cells (Figure 5B). These findings suggested that CTU2 expression in tumor cells may play a role in modulating the migration and infiltration of immune cells, potentially influencing the response to immunotherapy in human cancers.

3.6 Predictive potential of CTU2 in cancer immunotherapy response

Given the prognostic significance of CTU2 in immune infiltration, we proceeded to investigate its predictive impact on cancer immunotherapy response. We first investigated the predictive value of CTU2 in real-world immunotherapy response by incorporating data from two independent immunotherapy studies (GSE91061-melanoma; RCC-Braun_2020) (Figures 6A, B). We found that melanoma and kidney cancer patients with high CTU2 expression had poorer survival prognosis and lower response rates to anti-PD-1 immunotherapy (Figures 6A, B). However, patients with low CTU2 levels demonstrated a higher likelihood of responding to immunotherapy, as evidenced by improved prognosis in melanoma and renal cell carcinoma when treated with anti-PD-1 therapy, compared to those with high CTU2 levels (Figures 6A, B).

Higher TIDE prediction scores indicate a greater likelihood of immune evasion, suggesting that patients are less likely to benefit from immune checkpoint inhibition therapy (ICI therapy) (47, 48). In the TCGA dataset, high CTU2 expression was associated with higher TIDE scores, particularly in ACC, BLCA, cervical squamous cell carcinoma and endocervical adenocarcinoma (CESC), HNSC, ESCA, KIRC, LIHC, LGG, PCPG, SKCM, STAD, THCA, UCEC, and KIRP (Figure 6C, Supplementary Figure S6). Subsequently, we analyzed the comprehensive mechanism of tumor immune dysfunction and exclusion using the TIDE database. Our findings revealed that high CTU2 expression was associated with increased infiltration of myeloid-derived suppressor cells (MDSCs) and elevated T-cell exclusion scores across multiple cancers, including ACC, BLCA, CESC, DLBC, ESCA, HNSC, KIRC, KIRP, LGG, LIHC, LUAD, LUSC, PCPG, STAD, THCA, and UCEC (Figure 6C). The above results suggest that in most tumors, high CTU2 expression may be associated with an immunosuppressive microenvironment.

To validate this, we conducted *in vivo* experiments and found that knocking down CTU2 expression in Hepa1-6 (mouse liver cancer cell line) significantly reduced the number of tumor lesions in liver cancer orthotopic models (Figures 6D, E). Moreover, flow cytometric analysis of liver tumor lesions from the two groups showed that, compared to the NC group, the CTU2 knockdown group exhibited a more active immune microenvironment. This was evidenced by a significant reduction in MDSC numbers, an increase in $CD8^+$ T cells and DC cells, along with a decrease in the number of exhausted $CD8^+$ T cells (PD-1 high) (Figures 6F–I). Activity scores of the cancer-immunity cycles from the TIP database were downloaded and assessed (Supplementary Table S4). In addition, as shown in Figures 7A, B, the expression of CTU2 affects the tumor immune cycle response differently across various cancers. Additionally, CTU2 expression shows differential correlations with the expression of several key immune checkpoints (Supplementary Figure S5B) and various immunomodulators in different tumors (Supplementary Figure S7).

To further explore the relationship between tumor CTU2 expression and immune microenvironment infiltration, we analyzed the KIRC single-cell dataset (GSE207493) and the LIHC single-cell dataset (GSE202642). Based on the mRNA expression levels of CTU2 in malignant tumor cells, we classified the tumor cells into two groups: those with high CTU2 expression and those with low CTU2 expression. GSEA was then performed on the differentially expressed genes. Notably, we observed strikingly

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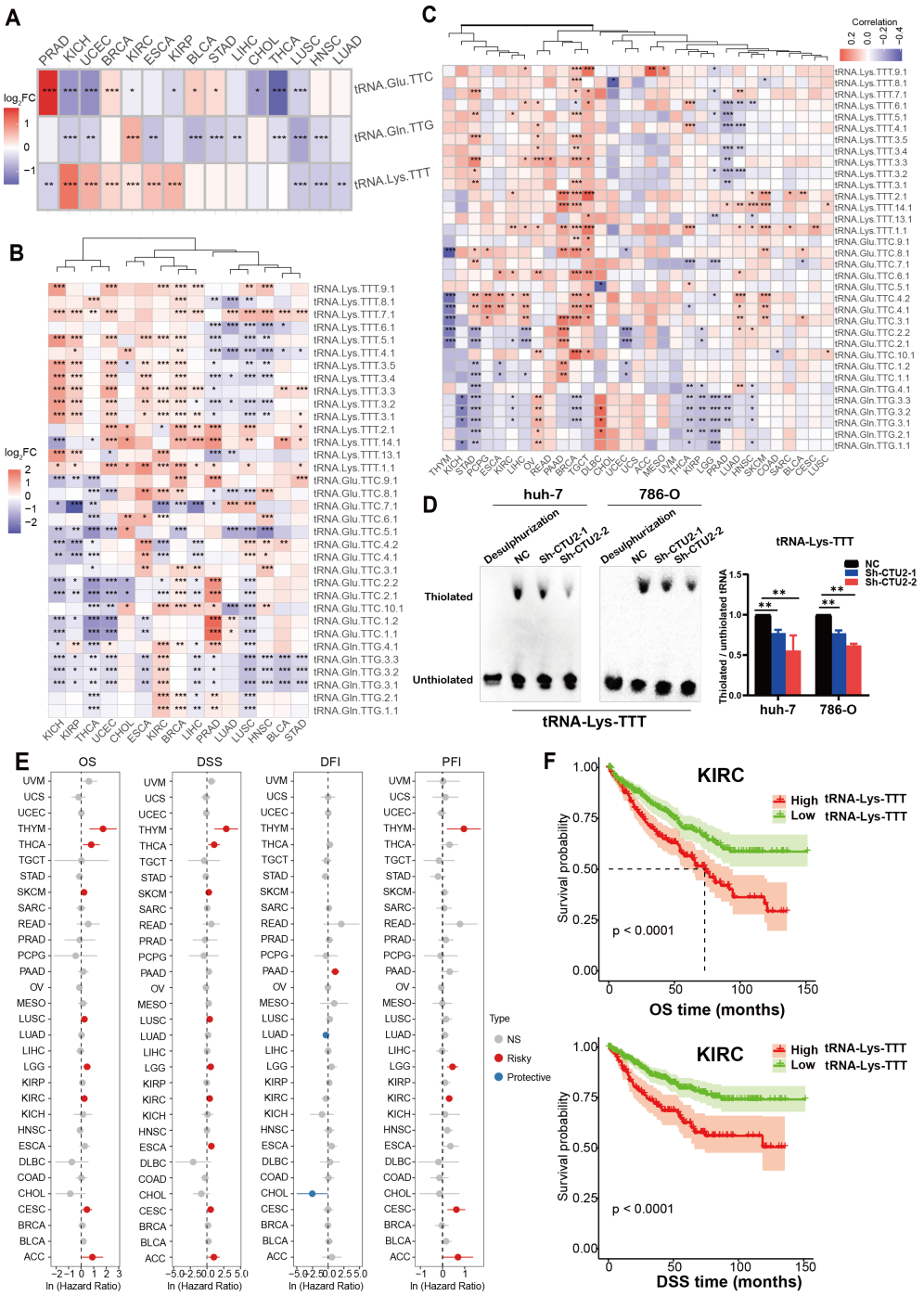

FIGURE 4
Expression characteristics of CTU2 specific-modified tRNAs in different cancer types. **(A)** Expression characteristics of the three specific-modified tRNAs in different cancer types, with colors ranging from blue to red representing the log_2FC values; **(B)** Expression characteristics of the tRNA isoforms in different cancer types, with colors ranging from blue to red representing the log_2FC values; **(C)** Heatmap showcases the specific-modified tRNAs correlated with CTU2 based on correlation analysis; **(D)** Northern blot analysis was performed to assess the mcm^5s^2U modification of tRNA-Lys-TTT in CTU2 knockdown and control huh-7 and 786-O cells (slow-migration band indicates thiolated tRNA). No retarded band was observed after desulphurization. The mcm^5s^2U modification level was normalized as the ratio of thiolated to unthiolated tRNA. The graph on the right represents the statistical analysis of gray values. The experiment was repeated independently three times; **(E)** OS, DSS, DFI and PFI of tRNA-Lys-TTT in 33 TCGA cancer types; **(F)** Kaplan-Meier analysis of OS and DSS for tRNA-Lys-TTT expression in KIRC. $^*P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$.

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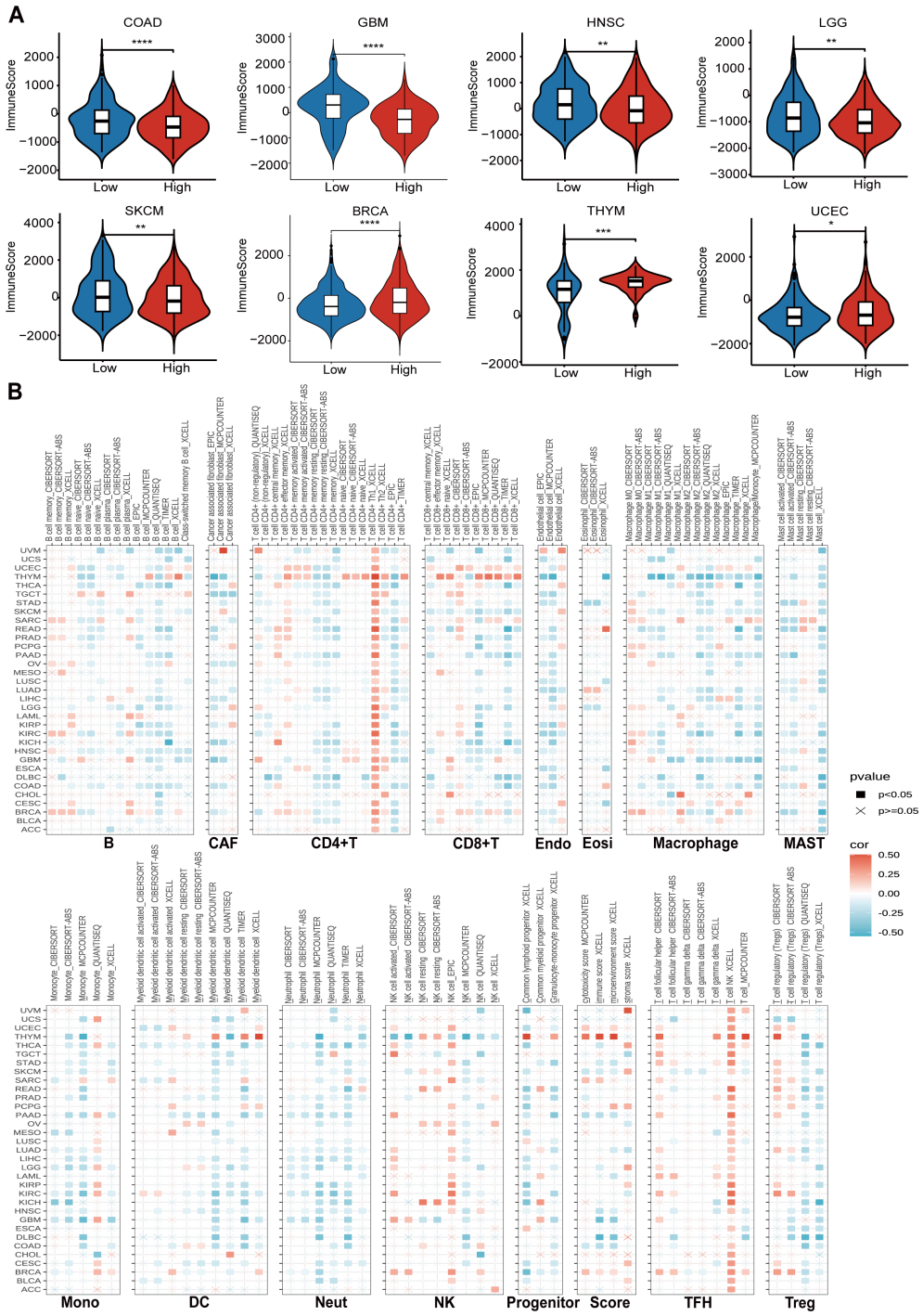

FIGURE 5
CTU2 contributes to diverse immune cell infiltration in various types of cancer. **(A)** Boxplots show the comparison of immune scores between CTU2-high and CTU2-low patients, distinguished by the median; **(B)** Cluster heatmaps display the correlation between CTU2 expressions and the degree of infiltration by B, cancer-associated fibroblast (CAF), $CD4^+T$, $CD8^+T$, endothelial (Endo), eosinophil (Eosi), macrophage, MAST, monocyte (Mono), DC, neutrophil (Neut), NK, progenitors, TFH, and Treg. *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$.

similar pathway enrichment patterns across these different cancer types (Figure 7C). Tumor cells with high CTU2 expression, compared to those with low CTU2 expression, exhibited negative enrichment in immune response-related pathways, including Regulation of T Cell Activation, Antigen Processing and Presentation of Peptide Antigen via MHC Class I, Macrophage Activation, and B Cell Immune Response. Additionally, negative enrichment was observed in cell adhesion-related pathways. In contrast, we observed a significant positive enrichment in translation-related pathways, such as tRNA wobble modification and ribosome assembly, as well as in mitochondrial energy metabolism and cellular inflammatory responses. Furthermore,

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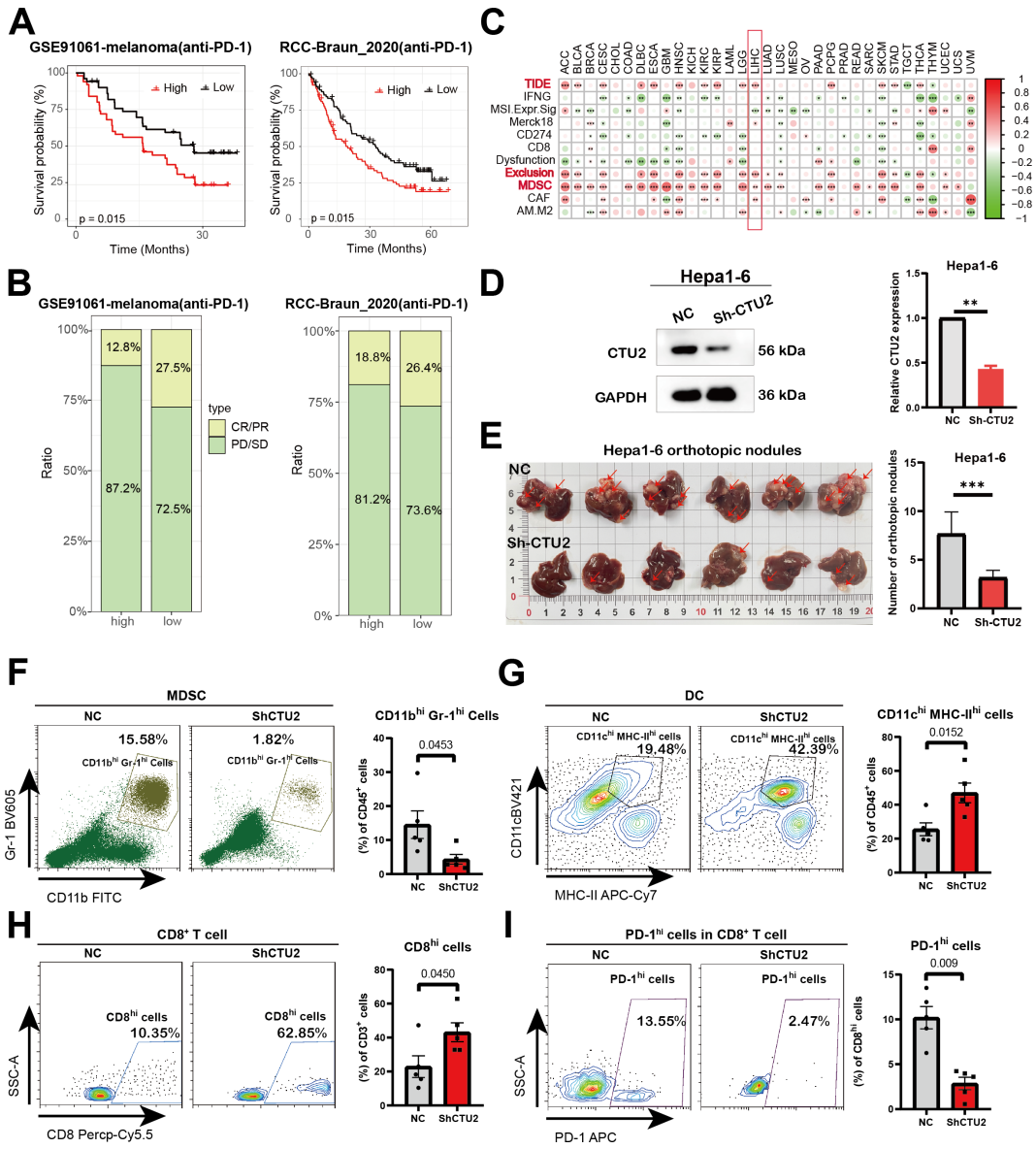

FIGURE 6
Influence of CTU2 expression on anti-tumor immunity and immunotherapy response. **(A)** Predictive values of CTU2 expression on OS of melanoma (left) and renal cell carcinoma (right) patients in anti-PD-1 immunotherapy; **(B)** Response rate of immunotherapy in melanoma (left) and renal cell carcinoma (right) patients, PD means progressive disease, SD means stable disease, CR means complete response, and PR refers to partial response; **(C)** The correlation heatmap shows the correlation between CTU2 expression and TIDE scores with the TIDE tool; **(D)** The results of western blotting confirmed the knockdown effect of CTU2 in Hepa1-6. The grey value of the CTU2 protein levels was normalized to that of the corresponding GAPDH (right panel). The experiment was independently repeated three times (***P value*< 0.01); **(E)** Representative pictures of Hepa1–6 liver orthotopic tumor lesions. Quantification of Hepa1–6 liver orthotopic tumor lesions (n = 6, ****P value*< 0.001) was listed in the right panel; **(F-I)** Left: Representative flow cytometry plots of MDSC cells, $CD8^+$ T cells, DC cells, and exhausted $CD8^+$ T cells (PD-1 high). Right: Statistical quantification of cell numbers (n = 5, *P* value<0.05 were considered statistically significant).

heatmaps were generated to display the differential expression of key molecules involved in the aforementioned functional pathways between tumor cells with high CTU2 expression and those with low CTU2 expression (Figure 7D). For instance, molecules associated with antigen presentation, such as those processed and presented by antigen-presenting HLA, were found to be expressed at lower levels in tumor cells with high CTU2 expression. In conclusion, the above results, from multiple perspectives, indicate the significant potential of CTU2 in tumors for immunotherapy response, particularly in immune evasion, suggesting its promising utility as a biomarker for cancer immunotherapy.

3.7 CTU2 functions as an oncogene across various cancer types

To anticipate the potential roles and underlying mechanisms of CTU2 in pan-cancer, GSEA was employed to enrich CTU2-

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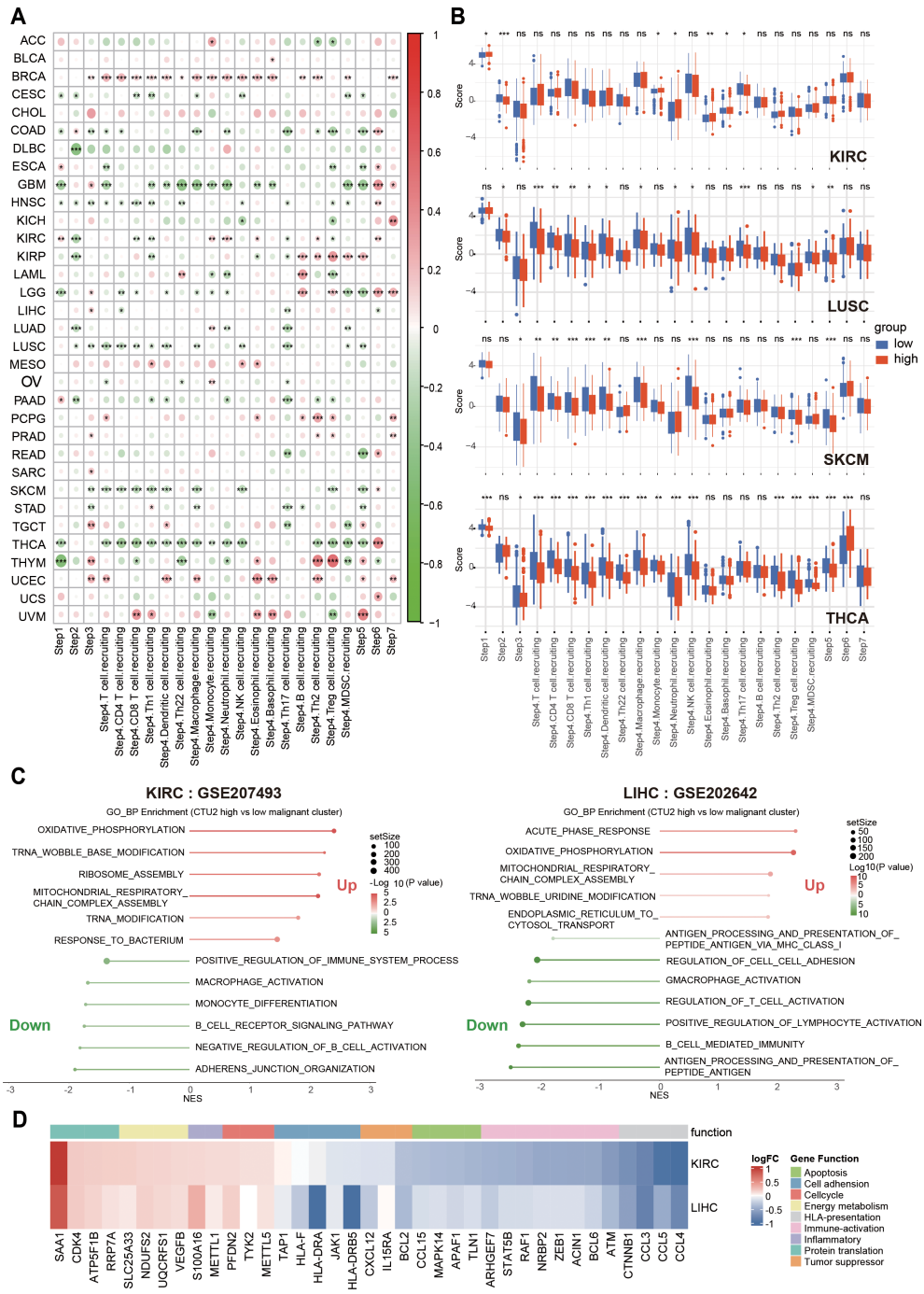

FIGURE 7
Correlation between CTU2 expression, cancer-immunity cycles, immune suppression, and cancer-related biological processes. **(A)** The correlation heatmap shows the correlation between CTU2 expression and the activity scores of the cancer-immunity cycles; **(B)** Boxplots show the differences in activity scores of the cancer-immunity cycles between CTU2 high-expressing and CTU2 low-expressing tumors in KIRC, LUSC, SKCM, and THCA. **(C)** GSEA pathway enrichment analysis of differentially expressed genes between high CTU2 expression and low CTU2 expression malignant tumor cells; **(D)** Heatmap showing differential expression of key genes involved in important biological function pathways between malignant tumor cells with high CTU2 expression and those with low CTU2 expression. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$ and ns (not significant).

associated Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways and Gene Ontology (GO) biological processes. Numerous cancer-related pathways were notably enriched (Supplementary Figure S8A), including cell cycle (Supplementary Figure S9A), DNA replication (Supplementary Figure S9B), base excision repair (Supplementary Figure S9C), nucleotide excision repair (Supplementary Figure S9D), spliceosome (Supplementary Figure S9E), and proteasome (Supplementary Figure S9F), along with focal adhesion and cell adhesion molecules. In addition, pathways involved in protein folding, tRNA metabolic progress, and tRNA

modification were also significantly enriched in this analysis, highlighting the significant role of CTU2 in tRNA physiological function and protein synthesis (Supplementary Figure S8B). While, the majority of cell-matrix adhesion-related genes were negatively correlated with CTU2, especially in CESC, DLBC, LGG, READ, and TGCT (Supplementary Figure S8A). The correlation analysis unveiled that CTU2 expression was additionally linked to various well-known oncogenes (Supplementary Figure S8C), including E2F transcription factor family members (Supplementary Figures S9G, H) and cell division cycle (CDC) protein (CCD45, CDC20) (Supplementary Figures S9I, J), and PLK1. Furthermore, the correlation analysis indicated that majority of genes linked to DNA replication and Base excision repair pathways exhibited positive correlations with CTU2 expression in KIRC (Supplementary Figures S10A, B) and LIHC (Supplementary Figures S10C, D). These findings suggest that targeting CTU2 and its associated pathways could be a viable strategy for developing new cancer therapies.

To further investigate the direct role of CTU2 in tumor cell function, we supplemented our analysis with data from the DepMap database. The DepMap database integrates data from thousands of cancer cell lines, known as the Cancer Cell Line Encyclopedia (CCLE), and conducts large-scale loss-of-function screens using CRISPR interference (CRISPRi) or RNA interference (RNAi) to evaluate gene essentiality. Specifically, when the loss or reduction of a gene significantly affects cell viability or fitness, the more negative the gene effect score, the stronger the gene dependency. As shown in Supplementary Figure 11A, knockdown or knockout of CTU2 impaired the proliferation of various cancer cell lines, with the gene effect scores being negative in nearly all of the cell lines, indicating a crucial gene dependency on CTU2 in the majority of cancer cells (Supplementary Figure S11B).

To further investigate the potential biological functions of CTU2 in pan-cancer, we examined whether cancer cell lines expressing high levels of CTU2 differ functionally from those with low levels. Functional enrichment analysis revealed a positive correlation between CTU2 expression and gene dependency in pathways involved in translation and tRNA aminoacylation (that is, higher CTU2 expression correlates with stronger dependency of these genes for cell survival) (Supplementary Figures S11C, D). This suggests that cancer cell lines with elevated CTU2 may regulate translation across multiple cancer types, which is consistent with the results shown in Supplementary Figure 8. Interestingly, we also observed a negative correlation between CTU2 expression and the gene dependency of canonical tumor suppressor genes, such as PTEN and RUNX3 (Supplementary Figures S11C, D), with these genes becoming less essential in CCLE-included cancer cell lines overexpressing CTU2.

3.8 CTU2 knockdown suppresses cell proliferation and migration

To further validate the functional role of CTU2 predicted by multi-omics analyses in tumors, we constructed CTU2 stably knockdown cells using the LIHC cell line (huh-7) and the KIRC cell line (786-O), and the efficiency of CTU2 knockdown was confirmed by Western blot (Figure 8A). Clone formation assays showed that CTU2 knockdown significantly inhibited the clone formation of huh-7 and 786-O (Figure 8B). Flow cytometric analysis revealed that compared with the cells transfected with empty vector (shNC) in both cell types, inhibition of CTU2 expression reduced the number of EdU-positive S phase cells (Figure 8C) and increased the proportion of apoptotic cells (early apoptosis plus late apoptosis) (Figure 8D). Additionally, transwell migration and invasion assays indicated that CTU2 knockdown inhibited cell migration and invasion in both cell lines (Figures 8E, F). To go a step further, we performed subcutaneous tumor experiments by huh-7 cells to explore the effects of CTU2 on the tumorigenic ability *in vivo*. Consistent with the *in vivo* results, CTU2 silencing inhibited subcutaneous huh-7 xenograft growth in nude mice (Figures 8G, H). Altogether, results from *in vitro* and *in vivo* were consistent with the findings from prognostic analyses and gene set enrichment analysis, indicating that CTU2 may serve as an oncogene in cancer.

3.9 Drug sensitivity analysis identifies potential compounds targeting CTU2 in pan-cancer

To identify potential therapeutic strategies targeting the tumor-promoting effects mediated by CTU2, we conducted a CMap analysis and developed a CTU2-related gene signature. This signature was created by selecting the top 150 significantly upregulated and 150 significantly downregulated genes from comparisons between CTU2-high and CTU2-low expressing patients across various cancer types. We employed the eXtreme-Sum (XSum) method, an optimized signature matching approach, to align the CTU2-related signature with CMap gene signatures. This analysis identified 1,288 compounds with similarity scores. Heatmap clustering analysis revealed 19 compounds with the top three lowest scores across 31 cancer types (Figure 9A). Notably, MS-275, STOCK1N.35874, and NU.1025 consistently exhibited significantly lower scores across multiple cancer types, suggesting their potential to counteract the pro-oncogenic effects of CTU2. Particularly, MS-275, a histone deacetylase (HDAC) inhibitor, targets HDAC enzymes and has shown anti-tumor effects in cancers such as leukemia, COAD, uveal melanoma, ESCA, BRCA, and HNSC. In 2024, after completing Phase III clinical trials (NCT03538171), it was approved for treating locally advanced or metastatic breast cancer, highlighting its potential in targeting CTU2-associated tumor progression (49). Additionally, using the 'OncoPredict' package and the GDSCv2 database, we assessed the sensitivity of 198 anti-tumor drugs (Supplementary Table S5). This analysis identified several drugs, such as Docetaxel_1007 (Figure 9B), Dactolisib_1057 (Figure 9C), Lapatinib_1558 (Figure 9D), and Tamoxifen_1199 (Figure 9E), with their sensitivity correlating with CTU2 expression levels, demonstrating a cancer-type-dependent response.

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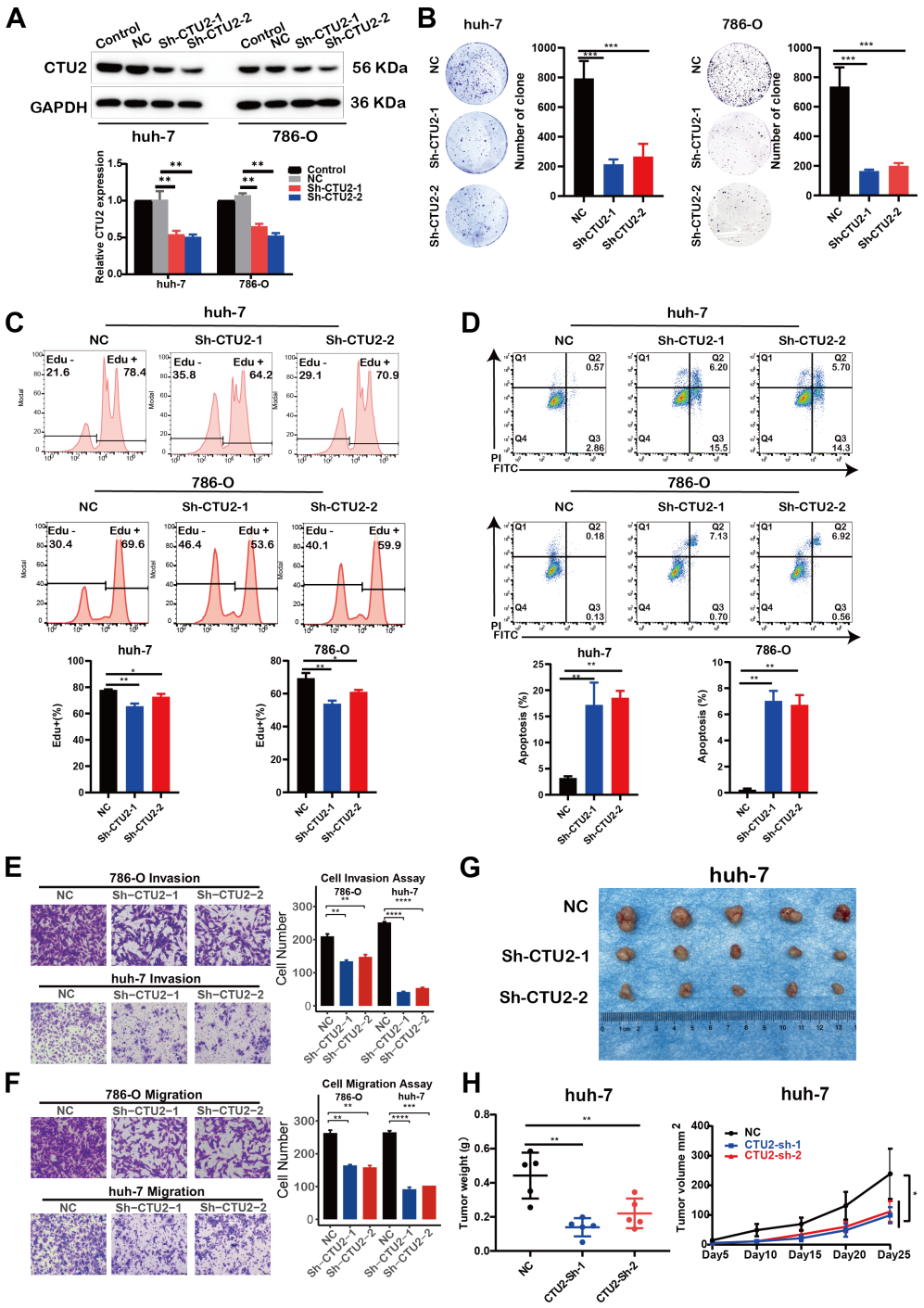

FIGURE 8
CTU2 knockdown impairs LIHC and KIRC progression *in vivo* and *in vitro*. **(A)** Western blot confirming CTU2 knockdown in huh-7 and 786-O. Control (untransfected wild-type cells), NC (lentiviral empty vector group), Sh-CTU2 (lentivirus-mediated CTU2 knockdown). The lower graph shows the grey value of CTU2 protein levels, normalized to the corresponding GAPDH levels. The experiment was independently repeated three times (**P < 0.01); **(B)** Colony-formation assay of CTU2 knockdown and control huh-7 and 786-O, representative images (left panel), and the quantitative analysis (right panel, ***P< 0.001); **(C)** EdU proliferation assay (upper) and the quantitative analysis (lower) of CTU2 knockdown and control cells (*P<0.05, **P < 0.01); **(D)** AnnexinV/PI apoptosis assay (upper) and the quantitative analysis (lower) of CTU2 knockdown and control cells (*P<0.05, **P < 0.01); **(E)** Matrigel invasion assay of CTU2 knockdown and control 786-O (upper) and huh-7 (lower) cell, representative images (left panel), and quantification analysis (right panel, **P < 0.01, ***P< 0.001, ****P< 0.0001); **(F)** Transwell cell migration analysis of CTU2 knockdown and control 786-O (upper) and huh-7 (lower) cell, representative images (left panel), and quantification analysis (right panel); **(G)** Representative picture of tumors in xenograft nude mice model subcutaneously implanted with CTU2 knockdown and control huh-7 cells; **(H)** Xenograft tumor weigh (left, n = 5, **P < 0.01) and xenograft tumor growth curve (right, n = 5, *P < 0.05).

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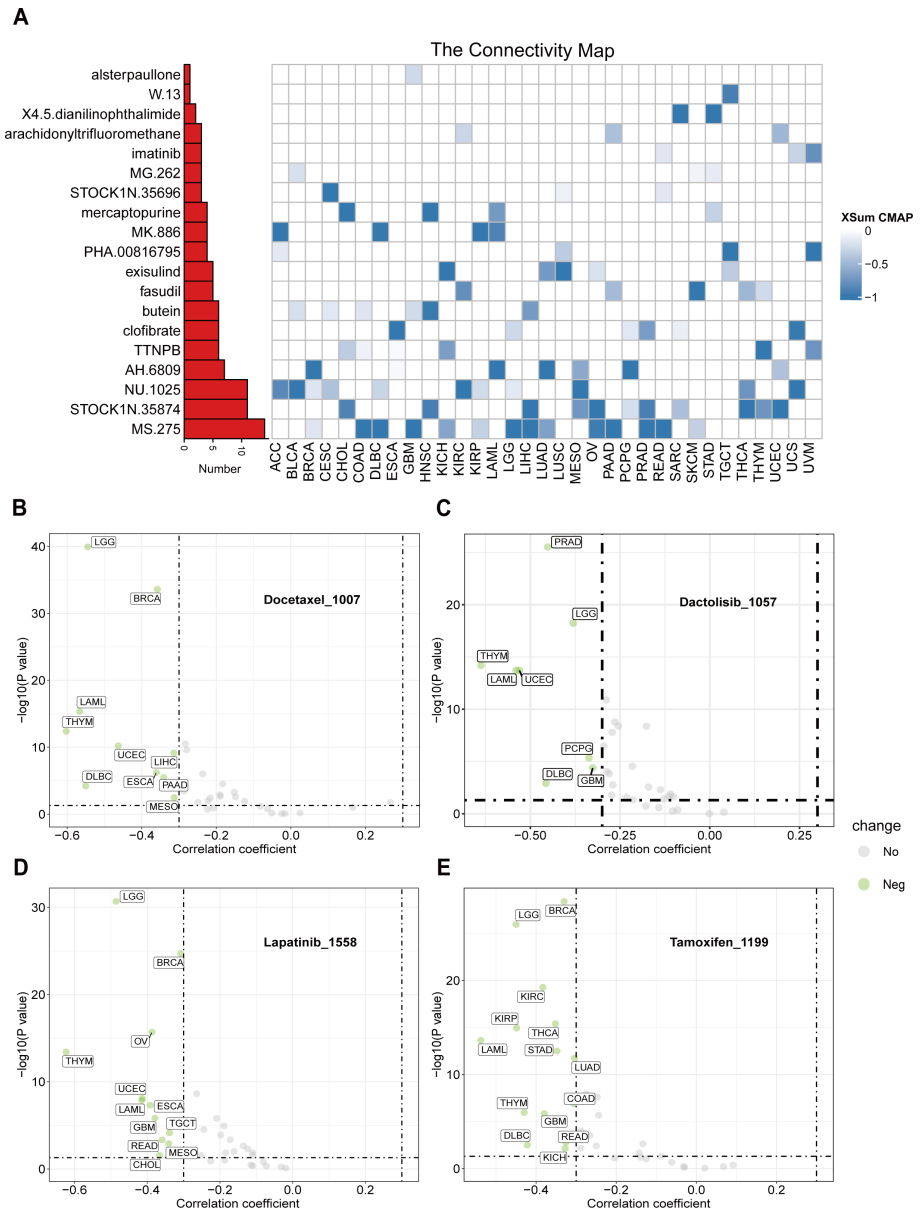

FIGURE 9
CTU2 is linked to the sensitivity of antitumor drugs across 33 cancer types. **(A)** A heatmap presentation shows the 19 candidate compounds that may target CTU2 based on the connectivity map analysis in 33 cancer types. The color codes from white to blue represent the XSum score from 0 to -1, respectively; Based on the 'oncoPredict' package, scatter plots present the Spearman correlation analysis results between CTU2 expression and drug sensitivity in **(B)** Docetaxel_1007, **(C)** Dactolisib_1057, **(D)** Lapatinib_1558, and **(E)** Tamoxifen_1199.

3.10 Copy Number Variation (CNV) and DNA methylation alterations of CTU2 across different human cancers

In order to uncover the mechanism underlying the elevated expression of CTU2, we conducted analyses on copy number variation of the CTU2 gene and DNA methylation alteration in the CTU2 promotor region. With regards to copy number variation, a higher prevalence of copy number gains was observed in CTU2 genes across various cancers such as ACC, KIRC, KIRP, and others (Figure 10A). Additionally, a significant positive correlation (Spearman r > 0.3; $P < 0.05$) was detected between CTU2 mRNA expression and copy number variation in the majority of tumor types (Figure 10B). We then investigated the differential promoter DNA methylation status of CTU2 between cancer and adjacent normal tissues by using UALCAN (Figure 10C). CTU2 had lower DNA methylation levels in BLCA, COAD, HNSC, LIHC, LUAD, LUSC, PRAD, READ, TGCT, THCA and UCEC tissues compared to adjacent normal tissues. To establish a connection between promoter DNA methylation levels and CTU2 expression, we conducted a correlation analysis between DNA methylation states and CTU2 expression (Figure 10D). A notable negative correlation was observed between DNA methylation and CTU2 expression in PRAD, TGCT, BLCA, BRCA, UCEC, SKCM, SARC, STAD, and

KIRC (-0.3 < Spearman r < -0.1). Hence, the abnormal increase in CTU2 mRNA expression in certain cancers likely stems from both CNV alterations and reduced DNA methylation levels.

3.11 CTU2 is regulated by the transcription factor USF1

Finally, given the prognostic significance of CTU2, we performed promoter sequence analysis and used established transcription factor prediction tools, including ENCODE, hTF-target, and KnockTF, to identify potential upstream regulators of CTU2 expression. From these analyses and the correlation results of CTU2 in LIHC and KIRC datasets, we identified one common transcription factor, upstream transcription factor 1 (USF1) (Figure 10E). Correlation analysis showed a highly significant positive correlation between CTU2 and USF1 in the majority of TCGA datasets (Figures 10F, G). Consistent with these findings, USF1 knockdown led to a decrease in CTU2 expression in huh-7 (Figure 10H) and 786-O cell lines (Supplementary Figure S12). Further analysis of eight published USF1 ChIP-seq profiles available in the Cistrome Data Browser revealed high ChIP-seq binding peaks of USF1 at consistent locations within the CTU2 promoter regions (Figure 10I).

Additionally, USF1 DNA-binding motif prediction within the CTU2 promoter, conducted using JASPAR, confirmed the presence of conserved E-box binding sites for USF1 around the transcription start site (TSS). We constructed wild-type CTU2 promoter luciferase plasmids and plasmids containing mutations in the predicted USF1 binding sites (Figure 10J, left). Luciferase assays demonstrated that USF1 knockdown significantly reduced the relative luciferase activity of the CTU2-WT vector, while having minimal impact on the CTU2-mutated vector (Figure 10J, right). To further investigate the transcriptional regulation of CTU2 by USF1, we conducted an EMSA to assess binding of USF1 to the E-box motif in the CTU2 promoter (Figure 10K). Using a wild-type oligonucleotide probe and nuclear extracts from Huh-7 cells, we observed a reduction in protein-DNA binding upon USF1 knockdown (lane 6). However, overexpression of USF1 in knockdown cells partially restored the binding shift (lane 7). Furthermore, USF1 overexpression alone enhanced the protein-DNA binding shift compared to the empty vector control, indicating increased binding to the CTU2 promoter DNA. In summary, our findings suggest that CTU2 may be regulated by the transcription factor USF1.

4 Discussion

The traditional view posited that tRNAs were abundant, readily available, and merely passive participants in mRNA decoding and protein translation. However, accumulating evidence indicates that tRNA expression is cell-specific, tissue-specific, disease-specific, and temporally regulated (50, 51). The regulation of mRNA translation is a critical process in cancer initiation and progression, and aberrant modifications of tRNAs can affect translation in three primary ways: aberrant modifications in the anticodon that directly restrict or expand decoding functions; aberrant modifications in the tRNA body that alter its folding characteristics or structural stability; and aberrant modifications that alter charging specificity (52).

Recent studies have demonstrated that CTU2 is significantly overexpressed in breast cancer (16), drug-resistant melanoma (22), and activated T cells (53), where it drives mcm^5s^2U-modified tRNAs to decode U34 codons, selectively upregulating the translation efficiency of metastasis-related LEF1, glycolysis-related HIF1α, and stress-responsive transcription factor Atf4, all of which feature gene coding regions rich in U34 codons. It is evident that CTU2-mediated mcm^5s^2U modification primarily regulates tRNA decoding functions, thereby influencing the translation of functional genes (12). In contrast, recent studies on the highly discussed methylation modifications, such as m^6A, m^5C, and m^1A, primarily occurring in messenger RNA (mRNA), microRNA (miRNA), and long non-coding RNA (lncRNA), mainly affect RNA stability, splicing, and decay, which is a form of regulation at the transcriptional level (54, 55). While tRNA also undergoes methylation modifications such as m^7G and m^3C, these are predominantly located in the tRNA body and similarly mainly influence tRNA stability (8). According to the central dogma of molecular biology, genetic information flows from DNA to RNA to protein, with proteins acting as the direct and final executors of gene function (56). However, therapeutic strategies targeting the tumor translation machinery remain scarce (57). Therefore, this study systematically analyzes the expression, prognostic relevance, and functions of CTU2 across various cancer types, aiming to provide a potential intervention strategy for tumors through CTU2-mediated tRNA mcm^5s^2U modification.

Changes in expression levels within tumor tissues are essential for genes to perform significant regulatory functions. Through analysis of TCGA data, we found that CTU2 expression varied significantly across various tumors compared to the corresponding paracancerous tissues. Subsequently, Clinicopathological staging analysis, OS analysis, and DSS analysis also revealed a close correlation between CTU2 expression and the clinical prognosis of various cancers, particularly in KIRC and LIHC. The drug sensitivity data from the GDSC database and DNA methylation data from cBioPortal and UALCAN further support the important role of CTU2 in various cancers.

The results of all the aforementioned analyses suggest that CTU2 is a critical diagnostic and therapeutic target for a variety of cancers. We believe that developing specific inhibitors or activators targeting CTU2 could significantly improve the disease progression and prognosis for cancer patients. Notably, in recent years, tRNA therapies have regained attention and achieved remarkable progress (58, 59). Therefore, developing tRNA-based therapies targeting the tRNAs modified by CTU2 may also be a viable approach. In addition, tumor immunotherapy also has been an effective treatment against tumors. We have been identifying biomarkers that activate the tumor immune response and facilitate immune evasion. To our excitement, pan-cancer analysis results have unveiled that CTU2 might play a pivotal role in the immune response across a spectrum of cancers. Chemokines, a group of relatively small molecular-weight secreted proteins, drive the movement and function of immune cells by interacting with chemokine receptors (60). The MHC, well-known for its role in antigen presentation and

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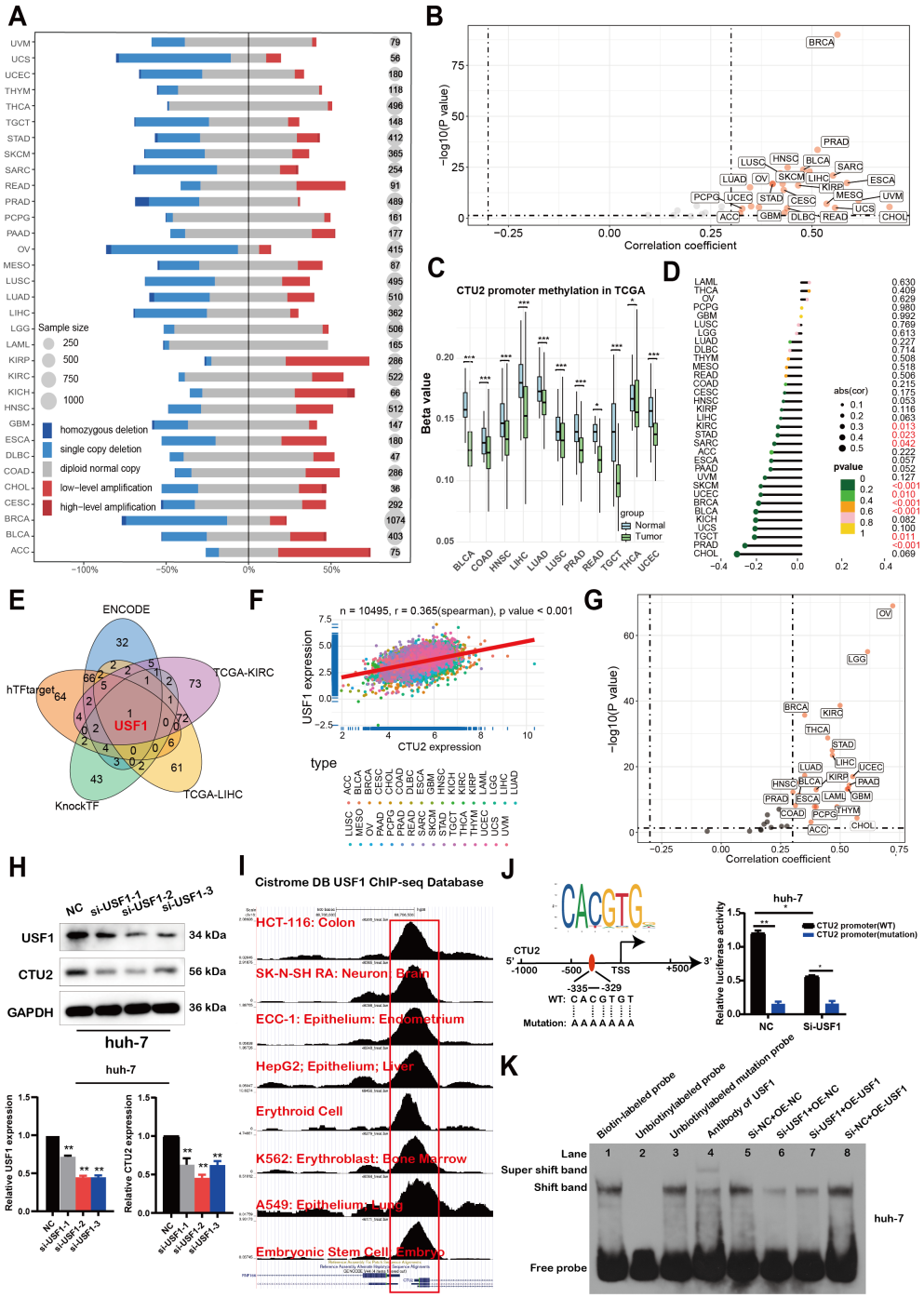

FIGURE 10
The mechanisms of upstream regulation of CTU2 expression in tumors. **(A)** DNA copy number variation analysis in 33 cancer types; **(B)** Scatter plot showing the results of Pearson correlation analysis in pan-cancer; **(C)** DNA methylation beta values ranging from 0 (unmethylated) to 1 (fully methylated) were determined by UALCAN; **(D)** Lollipop charts were used to visualize correlations between DNA methylation and mRNA expression of CTU2 (*P-value* < 0.05, marked in red font, shows statistical significance); **(E)** CTU2 upstream transcription factors prediction based on three web tools and correlation analysis; **(F, G)** Correlation analysis between CTU2 and USF1 expression in TCGA; **(H)** Western blot analysis confirmed USF1 knockdown and its effect on CTU2 expression in huh-7 cells. The lower graphs show the grey values of USF1 and CTU2 protein levels, normalized to the corresponding GAPDH levels. The experiment was independently repeated three times. The asterisk (*) indicates a statistically significant difference compared with NC, ***P* < 0.01; **(I)** ChIP-Seq data from the Cistrome Data Browser database to show the USF1 binding peaks of CTU2 promoter regions; **(J)** The cartoon shows the sequence logo of the USF1 potential binding site generated using JASPAR software (upper panel, http://jaspar.genereg.net/), with wild-type (WT) and mutated (Mutation) recognition sites of USF1 in the CTU2 promoter region depicted in the lower panel (left part). Luciferase assays demonstrated that USF1-mediated CTU2 promoter activity was significantly reduced following USF1 knockdown (right part); **(K)** EMSA analysis to evaluate the binding of the USF1 to the E-box motif in the CTU2 promoter under varying conditions of USF1 protein expression inhibition.

processing, is essential for initiating immune responses against a variety of human diseases (61). Our co-expression analysis has revealed a close association between CTU2 and the expression of these genes involved in chemokines, chemokine receptors, and MHC across different cancers, strongly suggesting that CTU2 could be indispensable for immunotherapy in diverse tumor types.

In terms of function and mechanism, GSEA revealed that CTU2 may contribute to numerous critical cancer-related pathways and biological processes. Specifically, CTU2 was found to have significant effects on the cell cycle and DNA replication. Combined with our analytical results, CTU2 exhibited notable regulatory roles in KIRC and LIHC, both in terms of differential expression analysis and prognosis. Therefore, we selected CTU2 for further investigation in KIRC and LIHC to validate our analytical findings. Experiments *in vitro* further confirmed that CTU2 promotes cancer behavior by enhancing cell proliferation and migration. Mechanistically, multi-omics analysis revealed that CTU2 upregulation is regulated by DNA copy number amplification and promoter methylation modifications. Notably, the transcription factor USF1 was identified as a regulator of CTU2 expression and has been confirmed to be an oncogene widely expressed in multiple cancer types (62–64).

5 Conclusions

In summary, this work demonstrated that high CTU2 expression in patients is significantly associated with poor prognosis and highlighted its potential as a biomarker for modulating immune cell infiltration, particularly in immune evasion processes, potentially influencing the response to immunotherapy in human cancers. Furthermore, CTU2-modified tRNA-Lys-TTT correlates with unfavorable outcomes across various tumor types. We validated its regulatory functions in KIRC and LIHC. Mechanistically, the amplification of copy number variation, hypomethylation of the promoter, and transcriptional regulation by USF1 may drive CTU2 expression in tumors. Overall, this study provided a comprehensive overview of genetic landscape of CTU2 across cancer types, providing new insights and support for the role of tRNA modification enzymes in cancer therapy.

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

Ethical approval was not required for the studies on humans in accordance with the local legislation and institutional requirements because only commercially available established cell lines were used. The animal study was approved by Zhejiang University School of Medicine Ethics Committee. The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

JJW: Conceptualization, Data curation, Formal Analysis, Writing – original draft. CG: Data curation, Formal Analysis, Validation, Writing – original draft. JZ: Data curation, Formal Analysis, Writing – original draft. HL: Data curation, Formal Analysis, Validation, Writing – original draft. SD: Conceptualization, Supervision, Validation, Writing – review & editing. JWW: Conceptualization, Project administration, Supervision, Validation, 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.

Generative AI statement

The author(s) declare that no Generative AI was used in the creation of this manuscript.

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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.2025.1547794/full#supplementary-material

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The heterogeneity of genomic alterations, metastatic patterns and immune microenvironment in metastatic ovarian cancer originating from colorectal cancer

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Purpose: The ovarian metastases originating from colorectal cancer (CRCOM) develops rapidly and lethally. Previously, the genetic alterations and metastatic pathway in CRCOM were not well understood. The aim of this study is to explore the special molecular phenotype and dissemination patterns of CRCOM.

Methods: The whole-exome sequencing (WES) was performed on 65 matched tissue samples from 11 CRCOM patients, including 11 primary colorectal cancer (CRC) with 11 matched normal tissues, and 43 multi-site metastases (including 15 CRCOMs and 4 patients had bilateral ovarian metastases (OMs). Genetic landscape, neoantigens, tumor clonal origin and spread of CRCOMs were analyzed. TCGA-COAD dataset combined with our data were used for survival analysis and validation of the findings.

Results: There was significant intertumoral heterogeneity among patients with CRCOM and intra-tumoral heterogeneity among multiorgan metastases. 19 genes were inferred as the potential driver genes of CRCOM. USP7 and RPA1 were HRD-related mutations and potential to serve as predictive biomarkers in OM. The putative neoantigen number of the primary CRC and OM varies widely among patients. The OM showed an immune desert state, extremely deficient in each subtype of immune cells. According to COSMIC signatures features, the CRCOM patients were divided into two groups, which are different in overall survival (OS) (median OS, 720 days vs 360 days, P = 0.074) and genetic alterations. Two metastatic patterns of CRCOM were summarized, which were primary CRC to OM, and metastases to metastases (including lymph node metastases (LNM) to OM, peritoneal metastases (PM) to OM, and other metastases to OM). Interestingly, the sources of bilateral OM might be different in the two patients.

Conclusion: This study presents a better understanding the heterogeneity of the genetic characterizations and metastatic pattern in CRCOM. The subtypes of CRCOM with USP7 mutation, more copy number alterations, lower neoantigens, and immunoscore have a worse prognosis.

KEYWORDS

ovarian metastases, colorectal cancer, whole exome sequencing, genetic alterations, phylogenetic tree analysis, metastatic pattern, neoantigen

Highlights

- CRCOM was classified into two subtypes, indicating the heterogeneity of CRCOM patients. The subtypes with USP7 mutation and more copy number alterations had a worse prognosis, and lower neoantigen numbers and immunoscore.
- The metastasis pathways of CRCOMs can be classified into two categories: one pattern is direct metastasis of the primary lesions to the ovary; Another pattern may be from other metastatic sites to the ovary.
- It was discovered that the metastatic pathways of bilateral ovarian metastases of colorectal cancer may be different.

1 Introduction

Colorectal cancer (CRC) is one of the digestive system malignant tumors with the highest incidence in the population, and its mortality rate ranks the top three among all malignant tumors (1). With the development of detection and treatment of CRC, the survival time of CRC patients has been prolonged, however, distant metastasis is still a big challenge (2). About 2-9% of female CRC patients were combined with ovarian metastases (OM) at initial diagnosis, as well as 0.4-7% of female CRC patients with metachronous OM (3–6). The incidence of colorectal cancer with ovarian metastases (CRCOM) has been rising in recent years due to the development of imaging techniques for metastatic colorectal cancer (6). OM often occurs in young female CRC patients (7), meanwhile, CRCOM is progressing rapidly and relatively resistant to chemotherapy (8–10). Compared with primary CRC and other distant metastases, there are fewer effective treatments for CRCOM due to the special molecular characteristics and unclear evolutionary relationship between OM and primary CRC (10, 11). Despite receiving active treatment with surgery, chemotherapy, and immunotherapy, the median overall survival time of patients with CRCOM was only 10.0 months (4, 7) (less than 30 months reported by CALGB 80405 (12), a large clinical trial of CRC patients with distant metastases). Given its potential impact on patient care, a better understanding of the special molecular phenotype and metastatic pathways of CRCOM could prolong the survival time and improve the quality of life among these patients.

Researchers have proposed various mechanisms in primary CRC metastasizing to distant organs. According to anatomy, regional lymph nodes (RLN) are the first step after cancer cells detach from the primary tumor and then distant metastasis (13–16), but a part of patients with CRCOM didn't have lymph node metastases (LNM). Some studies have shown that OM originated from the implantation metastasis of primary CRC (10, 17). Primary CRC cells penetrate the serosal layer and fall off into the peritoneal cavity or ascites, eventually reaching the ovarian capsule through intestinal peristalsis and gravity, and then developing into OM (18). However, it was found that the infiltration depth of primary CRC did not reach the serosal layer and the metastases were located in the ovarian stroma rather than on the ovarian surface in some patients with CRCOM. In addition, although the metastases were large, the capsule was intact. Other scholars believed that peritoneal metastases (PM) were an important source of OM because the ovary and peritoneum have similar biological behaviors and most patients with CRCOM also experienced PM (19, 20).

With the progress of whole exon sequencing (WES), some researchers have illustrated that distant metastasis may be spread from one or more subclones in any cancer site, including primary cancer and metastatic cancer (21, 22), and suggested that genetic divergence and heterogeneity of metastatic cancer (23, 24). Cancer cells, tumor microenvironment, signaling pathways, and special molecules related to cancer metastasis constantly adjust and change to promote the invasion and growth of cancer cells (25–27). Thereafter, these cancer cells continue to evolve and acquire private mutations, thus metastasizing to other organs and forming metastases (28, 29). To date, most studies focused on the

Abbreviations: CRCOM, Ovarian metastases originating from colorectal cancer; WES, Whole-exome sequencing; CRC, colorectal cancer; OM, Ovarian metastases; OS, overall survival; RLN, regional lymph nodes; LNM, lymph node metastases; PM, peritoneal metastases; LM, liver metastases; SpM, spleen metastasis; OMM, omentum metastases; TD, tumor deposits; FFPE, formalin-fixed paraffin-embedded; CNAs, Copy number alterations; CCF, cancer cell fraction; MSS, microsatellite stable; TMB, tumor mutation burden; SNVs, single-nucleotide variation; DSB, double-strand break-repair; NMF, Non-negative matrix factorization; HRD, homologous recombination deficiency; USP, ubiquitin-specific proteases; DDR, DNA damage response.

relationship between primary CRC and distant metastasis by using single pairing, for example, primary CRC paired with brain metastases or liver metastases. It remains lacking in the integrated metastatic evolution of multiple metastases from CRC, especially OM, which is associated with poorer prognosis relative to other organ metastases such as liver or lung metastasis.

In this study, we performed WES on 65 samples, including matched primary CRC, normal tissues, and multiorgan metastases, from 11 patients with CRCOM. We are the first to characterize the molecular phenotype and the clonal evolution pattern of CRC with OM using comprehensive genetic sequencing. The purpose of our study was to investigate the mysterious nature of CRCOM and identify the CRCOM with distinct molecular and clinical features that capture the clinical heterogeneity in CRCOM and could direct future therapy development.

2 Methods

2.1 Patients and specimens

The study protocol was reviewed and approved by the Ethics Committee of the Second Affiliated Hospital of Zhejiang University School of Medicine (SAHZU). We collected 65 tissue samples from 11 patients with the microsatellite-stable (MSS) CRC at SAHZU from 2016 to 2018. All the primary and metastatic tumors were collected from these patients, including 11 primary CRC and 11 matched normal tissues, 10 paracolic lymphonode metastases (LNM), 3 liver metastases (LM), 5 omentum metastases (OMM), 8 peritoneal metastases (PM), 1 spleen metastasis (SpM), 2 tumor deposits (TD) and 15 CRCOMs. Patients 1, 4, 8, and 10 had bilateral OM, while the remaining 7 patients had unilateral OM.

HE-stained sections from each sample were reviewed to confirm that the tumor specimen was histologically consistent with metastatic CRC (>40% tumor cells) and that the adjacent tissue specimen contained no tumor cells by two independent pathologies.

2.2 Whole exome sequencing

Genomic DNA from formalin-fixed paraffin-embedded (FFPE) samples was extracted using QIAamp DNA FFPE Tissue Kit (Qiagen), and fragmented by M220 Focused ultrasonicator (Covaris) into ~250 bp. The whole genome library was prepared using KAPA Hyper Prep Kit (KAPA Biosystems). Exome capture was performed using the Illumina Rapid Capture Extended Exome Kit (Illumina Inc.). Enriched libraries were sequenced using the Illumina HiSeq 2500 platform as paired 125-bp reads, to reach the mean coverage of ~80X for the normal control and ~250X for the tumor samples. Raw VCF data has been deposited in the Genome Sequence Archive in the National Genomics Data Center, China National Center for Bioinformation/Beijing Institute of Genomics, Chinese Academy of Sciences, under accession number GVM000406 (Project: PRJCA011872). The median depths of whole-exome sequencing coverage across all tumor and normal colon tissues were 219× (43× to 661×) and 223× (100× to 665×), respectively, both of which were deeper than those from the whole-exome dataset in TCGA-COAD (Supplementary Data 6).

2.3 Single nucleotide variation

Paired-end sequencing data from WES were aligned to the reference human genome (Homo_sapiens_assembly38.fasta) using the Burrows-Wheeler Aligner with default parameters(bwa-mem). Alignment results (BAM files) were further processed for de-duplication, base quality recalibration, and indel realignment using the Picard tools (http://picard.sourceforge.net/) and Genome Analysis Toolkit (GATK4.0). Point mutations were called using Mutect2. All variants (single nucleotide variants, SNVs) were annotated using the Ensembl Variant Effect Predictor v89 (https://www.ensembl.org/info/docs/tools/vep/) and ANNONAR (https://annovar.openbioinformatics.org/en/latest/) incorporating COSMIC v90, dbSNP build 146, Exome Aggregation Consortium (Exac03) and clinvar_20190305 annotations. For SNVs, we used maftools tools (R packages) to plot the summary of SNVs, which displays a number of variants in each sample as a stacked barplot and variant types as a boxplot summarized by Variant_Classification, and to draw a waterfall plot (Oncoplots).

2.4 Copy number alterations

Sequenza (v3.0.0 R packages) was used to call CNAs while considering both ploidy and cellularity. Briefly, we used BAM files from the WES data of each tumor and the paired normal samples as input to calculate the depth ratio, which was normalized based on both GC content bias and the data ratio. To acquire segmented copy numbers and estimate cellularity and ploidy. For each tumor sample, the copy numbers of segments were then divided by ploidy following log2 transformation. Copy number gains and losses were analyzed by GISTIC2.0. Among these gains and losses, amplifications were defined as four or more copies more than the ploidy, whereas deletions were defined as total deletion of the segment. Finally, CNA visualization was by Integrative Genomics Viewer (IGV) (http://www.broadinstitute.org/igv/).

2.5 Phylogenetic trees

The cancer cell fraction (CCF) of somatic mutations across all regions in each patient was estimated by PyClone (v0.13.0), a hierarchical Bayesian model incorporating local CNAs and SNVs. We also included mutations that were not located in exome regions to improve the sensitivity of the analysis.

Next, ClonEvol packages (R3.6) were used for phylogenetic inference from CCF subclones and the following visualization.

Briefly, this tool first enumerates all trees independently for each sample and then tries to build a 'consensus' tree model that fits multiple samples from a single patient at once. We successfully obtained consensus models in 11 patients and constructed phylogenetic trees accordingly.

MEGA 11(Molecular Evolutionary Genetics Analysis)is an open-source software that integrates sequence alignment, sequence analysis, and phylogenetic tree construction (30).

2.6 Potential driver genes in CRCOM

MutSig2CV (31), dNdScv (32), and OncodriveCLUST (33) were used to generate potential driver genes. Of the three computational tools, dNdScv, MuSig2CV, and OncodriveCLUST are all based on mutation frequency; MutSig2CV was used to identify genes that were mutated more often than expected by chance given the background mutation processes. The dNdScv is a group of maximum-likelihood dN/dS methods designed to quantify selection in cancer and somatic evolution, and uses trinucleotide context-dependent substitution matrices to avoid common mutation biases affecting dN/dS. OncodriveCLUST is based on the fact that most of the variants in cancer-causing genes are enriched at a few specific loci (aka hot spots) and takes advantage of such positions to identify cancer genes. It could detect genes with a significant bias toward mutation clustering in specific protein regions using silent mutations as a background mutation model. Genes were deemed significant at a q-value of 0.1. Collectively, we used candidate genes identified in either method or merged them.

The unsupervised clustering was performed by using the hclust function (the agglomeration method is "ward. D2") in R software (Version 4.0.2).

2.7 Putative neoantigens identification and prediction

The OptiType algorithm was utilized for HLA typing (34). Non-silent mutations were employed to create a list of mutant peptides, each approximately 9–11 amino acids long, with the altered residues represented in each position. NetMHCpan (v4.0) was then applied to predict the binding affinities of both the mutant and corresponding wild-type peptides to the patient's germline HLA alleles (35). Neoantigen candidates were identified based on a predicted mutant peptide binding affinity of less than 500 nmol/L and a rank of less than 2.

2.8 Immunohistochemistry

Paraffin-embedded slides of primary CRC and OM were stained by labelling the CD3+ (BOSTER, No. PB0112), CD8+ (BOSTER, No. PB0235) T cells and CD20+ (BOSTER, No. PB0028) B cell with specific antibodies. All the slides were stained with hematoxylin and eosin (HE). The CD3+, CD8+, and CD20+ stained cells were executed by a pathologist. The hot spots with positive staining were obtained. Computer-assisted calculations of the density of the positively stained immune cells were performed using Image J software (National Institute of Health, Bethesda, MD, USA).

2.9 Statistical analysis

Statistical analyses were performed with SPSS 22.0 and GraphPad Prism software. Continuous variables were analyzed by the student's t-test, one-way ANOVA, or two-way ANOVA test. Survival and univariate analysis were determined by Kaplan–Meier analysis, and statistical analysis was calculated with the log-rank test. All statistical analyses were two-sided, and P value <0.05 was considered statistically significant.

3 Result

3.1 Information of samples

The workflow was presented in Figure 1A. 11 patients with CRCOM who underwent primary and metastatic surgery in our

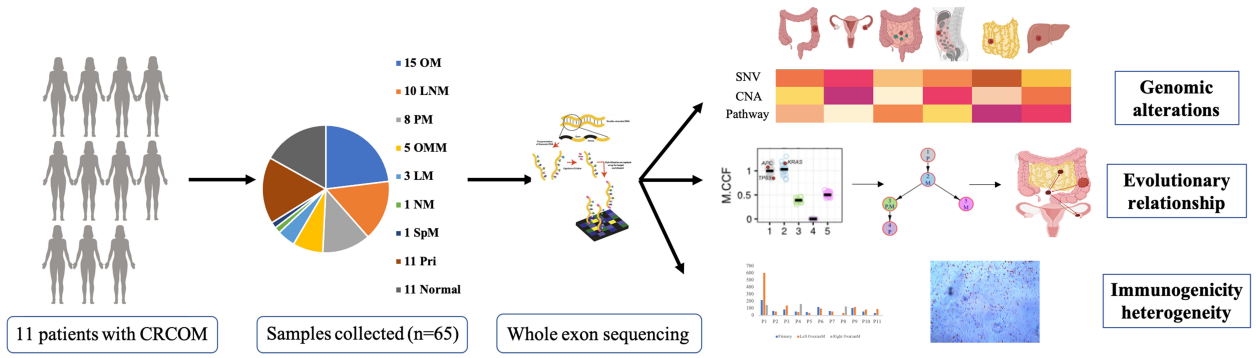

FIGURE 1
Study design. The flow chart showed the design and workflow of our research.

hospital were included in this study. A total of 65 patient-matched samples were collected, including 11 primary CRC and 11 matched normal tissues, 10 paracolic LNMs, 3 liver metastases (LM), 5 omentum metastases (OMM), 8 PMs, 1 spleen metastasis (SpM), 1 nodule metastasis (NM) and 15 OMs. Patient 1 (P1), P4, P8, and P10 had bilateral OM, while the remaining 7 patients had unilateral OM. The basic information on CRCOM patients and samples is shown in Table 1. The average age of our cohort was 46 years old (range 28-60). There were 4 cases of right colon cancer and 5 cases of left colon cancer, 2 cases of rectal cancer. Moreover, most of the patients presented with histologically confirmed adenocarcinoma whereas only one patient was presented with signet ring cell carcinoma. All patients are microsatellite stable (MSS). The median overall survival time of patients with CRCOM was 12 months.

3.2 Genomic alterations across CRCOM

We performed whole-exome sequencing (WES) and the average sequencing depth of tumor and normal samples was 145x (range 49x-289x) (Supplementary Data 1). The mean tumor mutation burden (TMB) for primary tumors and ovarian metastases was 10.73 and 6.46 mutations per megabase, respectively (Supplementary Figure 1, Supplementary Data 2). We calculated the mutated genes of all samples and the top 20 alteration spectrums of primary CRC and OM are shown in Figure 2A (Supplementary Data 3). Among the top 20 genes with the highest alteration rates, TP53 (55%), KRAS (36%), and APC (27%) were the 3 genes with the highest alteration rates in CRC primary CRC. APC (47%), TTN (40%), and TP53 (33%) were the 3 genes with the highest alteration rates in OM. We also marked the known 47 CRC driver genes based on the list from the COSMIC Cancer Gene Census in primary CRC and OM, respectively. CRC driver genes with high alteration rates in primary CRC and ovarian metastasis, include APC (27% *vs* 47%), KRAS (36% *vs* 27%), and TP53 (55% *vs* 33%). AXIN1, BRAF, HIF1A, KZF3, and RSPO3 alternated only in OM, and SMAD4 mutated only in primary CRC (Figure 2B). We used three different tools (OncodriveCLUST, MutSigCV2.0 and dNd Scv) to identify the potential driver genes mutated in CRCOM (Supplementary Data 4), and summarized a list of 19 potential driver genes (including KRAS, TP53, APC, BRAF, RNF43, PCDHB12, ACVR2A, ZNF160, ZNF716, STOML1, SMIM3, NLGN1, DMD, LRP2, FAT4, ARID1A, NCOR1, RPTOR, SMAD3, MUC16). The well-known driver genes for CRC, such as TP53, NRAS, APC, and KRAS, were also mutated in our cohort. We also found that the mutation rate of several genes (including RPTOR, LRP2, NLGN1, and ZNF160) in OM was higher than that in primary CRC (Figure 2C).

The single-nucleotide variations (SNVs) displayed a preponderance of C > T transitions in primary CRC and OM. SNVs displayed considerable variations across and within patients, indicating intratumor heterogeneity. The SNV pattern in the P2, P3, P5, P6, P7, and P9, is similar between the primary and metastatic lesions, except for Patient 11. For P1 and P8 with bilateral ovarian metastasis, the SNV pattern is also highly similar between bilateral ovarian metastasis, but not similar in P4 and P10 (Figure 2D). The contributions of various known signatures to each sample are demonstrated in Figure 2E (Supplementary Data 5). Signature 1,6 and 30 were prevalent in CRC primary CRC and OM. Signature 4 was only prevalent in primary CRC, while Signature 3,11 and 12 were only prevalent in OM. Signature 3 was identified in 60% (9/15) of ovarian metastases, indicating that DNA double-strand break-repair (DSB) was highly involved in the etiology of CRCOM. To further determine the changes in Signature 3, we analyzed the DSB-related genes and found the mutation of USP7 (20% *vs* 9%), the rate of deletion in TP53BP1 (60 *vs* 45%), and RPA1 (93 *vs* 64%) were higher in OM (Figure 2F).

Based on the similarity between the COSMIC signatures features and the average link of the CRC primary lesions, non-negative matrix factorization (NMF) hierarchical clustering was performed on 11 patients. The patients were divided into two groups, the NMF_cos1 group including patient 1,3,5,6,7,9 and 10, as well as NMF_cos2 group including patient 2,4,8 and 11 (Figure 3A). Unsupervised clustering was also performed on all OMs, and patient 2,4,8 and 11 can also be clustered in one group (Supplementary Figure 2A). Survival analysis showed that there was a difference in overall survival (OS) between the two groups of patients (median OS, 720 days *vs* 360 days, $P = 0.074$) (Supplementary Figure 2B). To further explore the reason about CRC patients with OM in NMF_cos1 have better prognosis, we analyzed the genomic heterogeneity among different cluster samples. The known genes in CRC were frequently mutated both in primary CRC of NMF_cos1 or NMF_cos2, including KRAS, TP53, APC, PCDHB12, ZNF160, LRP2, FAT4, MUC16, and ARID1A, however, the mutation rates of these genes were different. As for OM, we found the rate of mutation of TP53 was higher in NMF_cos2. Besides, RNF43 and DMD are mutated only in primary CRC and OM of NMF_cos1 (Figure 3B). Significant heterogeneity was observed in two clusters since the median of tumor mutation burden (TMB) of primary CRC in NMF_cos 1 was 8.12/MB, which is greater than NMF_cos2 (3.55/MB, $P = 0.028$) (Figure 3C). The homologous recombination (HRD-score) was higher both in primary CRC and OM in NMF_cos1 than NMF_cos2 (Supplementary Figure 2C). The different SNVs and signatures are shown in (Supplementary Figure 3). To further determine the changes in genome segments of two clusters, we analyzed copy number alterations (CNA) in two clusters using Gistic 2.0. However, we don't detect any significant CNA in NMF_cos1. The significant focal deletion of 17p11.2 and 18p11.31 are detected in all OM of NMF_cos2 (Figure 3D). We calculated the significantly different genes of OM between NMF_cos1 and NMF_cos2, and we found USP7 was significantly higher in NMF_cos2 (0 *vs* 3, $P = 0.0439$) (Figure 3E, Supplementary Data 5). We also collected the data of CRC patients in TCGA and found the mutation of USP7 is associated with poor DFS (Supplementary Figure 2D).

TABLE 1 Clinical information of patients with CRCOM in our hospital.

Patient ID	Age	Tumor location	Pathology	Grade	DMR	OS	Time of OM	tumor size	Normal	Primary CRC	LNM	OM	PM	LM	OMM	Other metastasis	Samples number
P1	53	Rectum	Adenocarcinoma	Moderately	MSS	12	Synchronous	3*2*1.5	Yes	Yes	Yes	Bilateral	Yes	Yes	No		7
P2	43	Left colon	Adenocarcinoma	Moderately	MSS	12	Metachronous	left: 11*6*3.5 right: 11*8*6	Yes	Yes	Yes	Unilateral	Yes	Yes	No		6
P3	37	Right colon	Adenocarcinoma	Moderately	MSS	39	Synchronous	18*12*7	Yes	Yes	Yes	Unilateral	Yes	No	No	Spleen metastasis	6
P4	44	Left colon	Adenocarcinoma	Moderately	MSS	9	Synchronous	left: 8*5*5 right: 20*10*8	Yes	Yes	Yes	Bilateral	No	No	No		5
P5	42	Right colon	Adenocarcinoma	Moderately	MSS	24	Synchronous	6.5*4.5*4.2	Yes	Yes	Yes	Unilateral	Yes	No	No		5
P6	72	Left colon	Adenocarcinoma	Moderately	MSS	9	Synchronous	16*12.5*7	Yes	Yes	Yes	Unilateral	Yes	Yes	Yes		7
P7	28	Left colon	Signet-ring cell carcinoma	Signet-ring cell carcinoma	MSS	16	Metachronous	10*7*5	Yes	Yes	Yes	Unilateral	Yes	No	Yes		6
P8	47	Rectum	Adenocarcinoma	Moderately	MSS	20	Synchronous	left: 3*2.2*1.5 right: 4*4*1.7	Yes	Yes	Yes	Bilateral	No	No	Yes		6
P9	52	Right colon	Adenocarcinoma	Moderately to poorly	MSS	40	Metachronous	10*8*5	Yes	Yes	Yes	Unilateral	Yes	No	Yes		6
P10	60	Left colon	Adenocarcinoma	Moderately to poorly	MSS	11	Metachronous	left: 6*7*6 right: 5*6*4	Yes	Yes	No	Bilateral	Yes	No	Yes		6
P11	28	Right colon	Adenocarcinoma	Moderately	MSS	6	Synchronous	20*16*9	Yes	Yes	Yes	Unilateral	No	No	No	Tumors deposits	5
Summary									11	11	10	15	8	3	5	2	65

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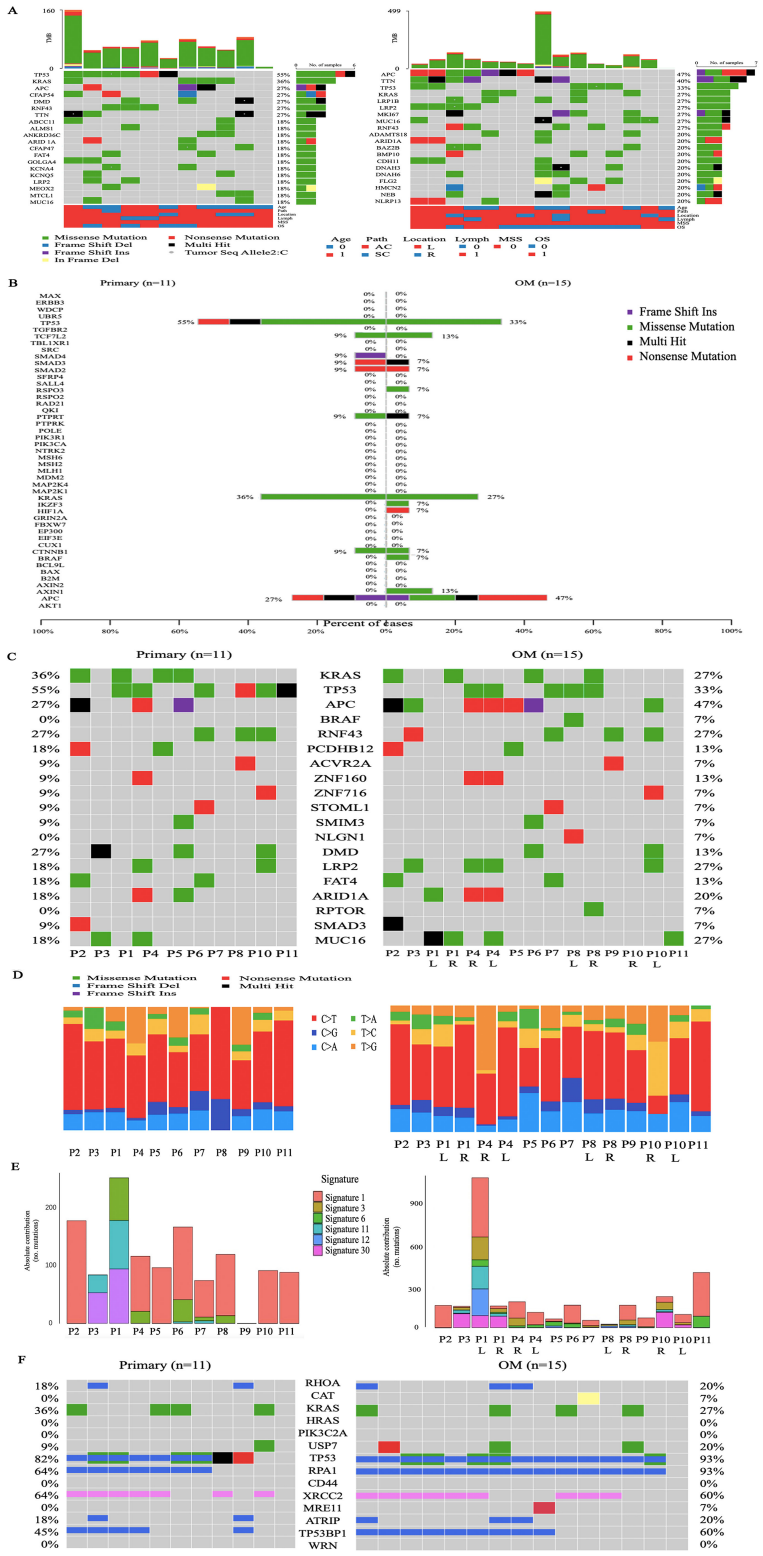

FIGURE 2
Genomic alterations across CRCOM. **(A)** The top 20 alteration spectrums of primary CRC and OM, the demographic and clinical information of the 11 patients was shown in the bottom. **(B)** The mutation of known 47 CRC driver genes in primary CRC and OM. Driver gene identification is based on the COSMIC Cancer Gene Census. **(C)** The list of 19 potential driver genes was identified by using OncodriveCLUST, MutSigCV2.0 and dNd Scv. **(D)** The single-nucleotide variations (SNVs) in primary CRC and OM. **(E)** The contributions of various signatures in primary CRC and OM based on the COSMIC Mutational Signatures database. **(F)** The mutations of DSB-related genes in primary CRC and OM.

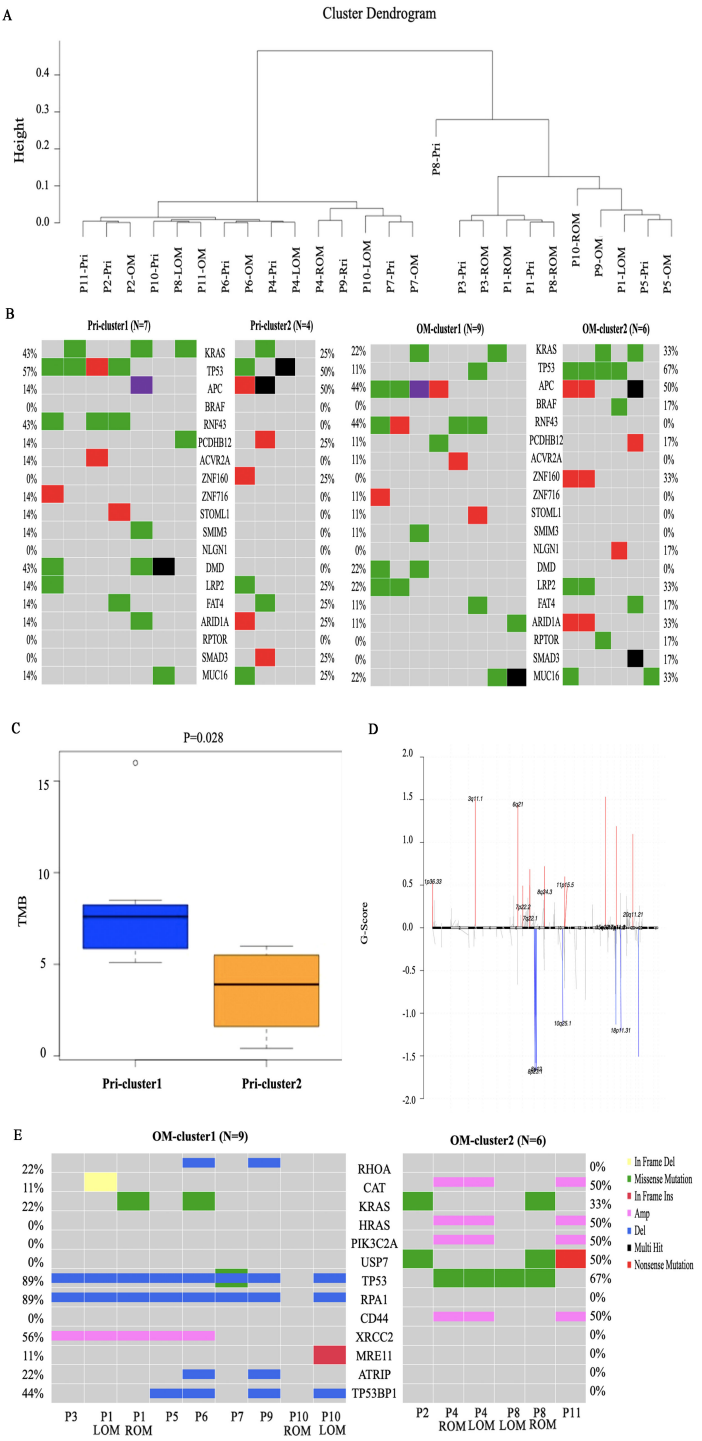

FIGURE 3
Genomic heterogeneity among different clusters. **(A)** The unsupervised clustering of all 11 patients is based on COSMIC Mutational Signatures. **(B)** The mutation of the list of 19 potential driver genes in primary CRC and OM of two clusters. **(C)** The tumor mutation burden (TMB) of primary CRC in two clusters. **(D)** The copy number alterations in OM of NMF_cos2. **(E)** The significantly different genes of OM between NMF_cos1 and NMF_cos2.

3.3 Clonal origin and spread of CRC with OM

The main goal of our study is to illuminate the evolutionary relationship between primary CRC and OM. ClonEvol and MEGA 11 are used to build the phylogenetic tree of the CRCOM in each case (Supplementary Figure 4, Supplementary Data 6). We observed diverse evolutional patterns between primary CRC and OM. Firstly, OM derived from primary CRC is the main seeding model to describe metastasis dissemination. In P9, MEGA showed that the

genetic distance of OM is closer to primary CRC. Similarly, ClonEvol showed that OM may come from primary CRC (Figure 4). The same phenomenon was found in P2, P4, P5, P6, and P11. Interestingly, there were bilateral OM in P4, however, the ROM and LOM are both seeded by primary CRC. Secondly, the lymphatic origin of CRCOM has been evidenced in ROM of P1, LOM of P8, P3, and P7. Thirdly, we first analyzed the metastatic pathway of P1, who harbored bilateral OMs, LNM, PM, and primary CRC. We portrayed the potential metastatic map of P1 and we speculated that the origin of LOM and ROM in P1 was different. LOM was directly derived from the primary CRC. However, ROM was derived from lymph nodes. The same phenomenon was found in P8 and P10, who have bilateral OMs derived from different organs. In P10, the genetic distance from LOM to ROM was shorter than the distance from primary CRC or other metastasis, so we speculate that ROM from primary CRC and LOM from ROM. MEGA 11 showed a long genetic distance exists between LOM and ROM. ROM is closer to omentum metastasis while LOM showed a closer genetic distance to primary CRC. Clonevol also showed that right ovary metastasis could come from omentum metastasis, while LOM derived from primary CRC.

3.4 Immunogenicity heterogeneity across and within individuals

Surgical resection and chemotherapy are the major choices for CRC patients with OM, immunotherapy is rarely applied in the treatment of CRCOM. We performed neoantigen number prediction to provide new insights into immunotherapy delivery in CRCOM. The predicted neoantigen number of each sample is shown in Figure 5A. The neoantigens of primary CRC and OM vary widely among patients, as well as a large difference in neoantigen between primary CRC and OM in the same patient. The predicted neoantigen number of primary CRC was higher than that of OM in NMF_cos1 patients. For NMF_cos2 patients (P2,4,8, and 11), the predicted neoantigen number of primary CRC and OM was lower than that of NMF_cos1 (P1, 3, 5, 6, 7, 9, and 10), suggesting neoantigens may be able to predict the infiltrating state and immune integral in tumor tissue (Figure 5B). The immunoscore is based on the infiltrating density of $CD3^+$ and $CD8^+$ TILs, and is used to predict the prognosis of patients with stage II and III colon cancer and has independent prognostic value. We compare the immune status of primary CRC and OM focus in 4 patients with

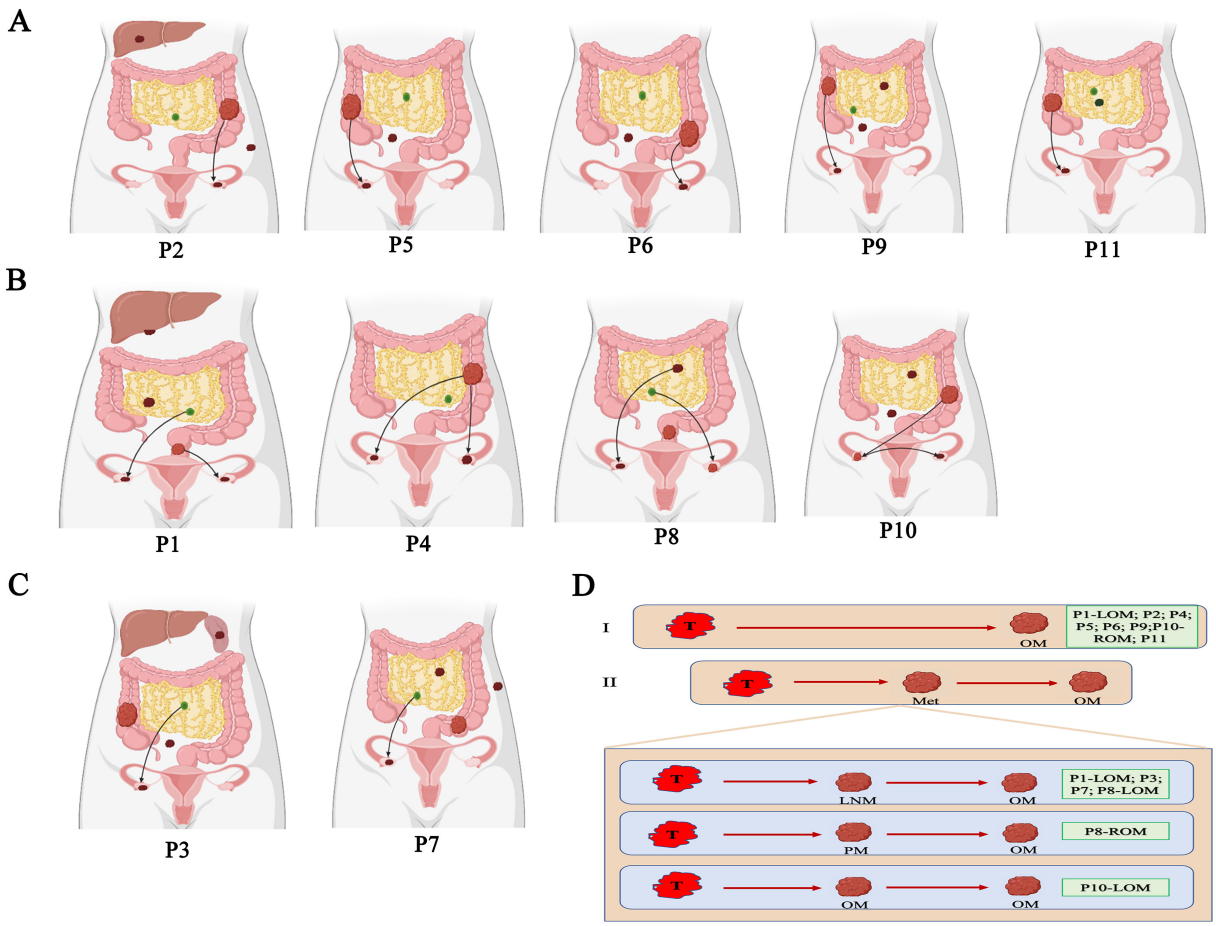

FIGURE 4
Parsimonious metastatic map and two modes of metastasis in CRCOM. **(A)** Primary CRC-seeding-OM models in CRCOM (including P2, P5, P6 and P9). **(B)** The models of bilateral OM (including P1, P4, P8 and P10). **(C)** LNM- seeding-OM models in CRCOM (including P3 and P7) **(D)** two modes of metastasis in CRCOM: primary CRC to OM and metastases to metastases (including LNM to OM, PM to OM, and OM to OM).

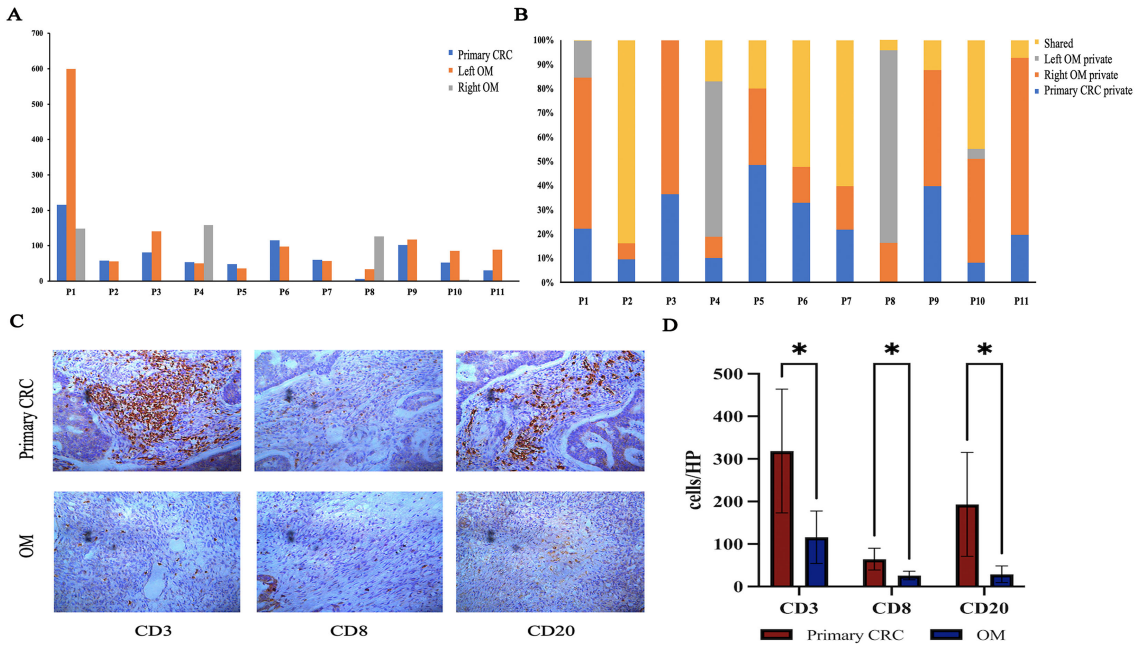

FIGURE 5
Immunogenicity heterogeneity across primary CRC and OM. **(A)** Predictive neoantigens numbers of CRC primary and OM. **(B)** Distributions of predicted neoantigens in each patient. **(C)** Image of primary CRC and OM tumor center with CD3+/CD8+/CD20+ staining. **(D)** The density was calculated as the number of positive cells/HP. (*represents P <.05).

CRCOM. The OM showed an immune desert state, extremely deficient in each subtype of immune cells (Figure 5C). Compared with the primary lesions, the infiltration of CD3+ T cells, CD8+ T cells, and CD20+ B cells, associated with better prognosis, were substantially lower in OM (Figure 5E).

4 Discussion

Although the diagnosis and treatment of metastatic CRC have been improved in recent years, CRCOM is still a big challenge for clinicians and cancer workers due to its special phenotype and unclear evolutionary relationship (36). Understanding the special phenotype and evolutionary relationship of CRCOM is important for the diagnosis and treatment of patients with CRCOM. Previous studies always explored the metastatic evolution in metastatic CRC by using single pairing (21, 22), however, it has been proved that metastatic CRC is a systemic disease with multi-organ involvement (37). In this research, we collected all the multi-site metastases in CRCOM patients, including OM, paired primary CRC, PM, LNM, LM, and so on. A total of 54 tumor samples and 11 normal tissues in 11 patients were collected to identify possible biological differences in OM, primary CRC, and other metastases, as well as portray a detailed metastatic map of CRCOM.

Based on our data, the significant genomic heterogeneity between primary CRC and OM has been evidenced. The most frequently mutated gene is TP53 in primary CRC while APC is in OM. Only 14/47 known driver genes were mutated in our study and 5 known driver genes were only mutated in OM, which implies CRC with OM may have unique mutation features. It will promote the progression of CRC due to the accumulation of mutations, which are the essential component of the signaling pathway in regulating cellular replication (38). SNVs displayed considerable variations across and within patients, also indicating intratumor heterogeneity. Previous research has shown the heterogeneity among metastases was minimal (39), however, we also found significant inter-metastatic heterogeneity between bilateral OM of P4 and P10 in our cohort, which might be explained by the multiple metastases rather than a single pairing in each patient. These results showed that the CRC cells must adapt molecular characteristics to escape from primary CRC and form CRCOM by interacting with tissue microenvironments across the ovary.

There is emerging evidence about the predictive role of homologous recombination deficiency (HRD) in multiple cancers (40). The mechanism of HRD is complex, as reflected by the variable definitions between studies. BRCA1/2 alterations are currently the main biomarkers of HRD (41). However, many tumors with phenotypic signatures consistent with HRD did not harbor BRCA1/2 mutations. There has been increased recognition of the role of other HRD-related mutations beyond BRCA and PALB2 and their potential to serve as predictive biomarkers (42). In this study, we found that signature 3 (associated with homologous recombination, HR) was identified in most CRC patients with OM, and the HRD-score was higher both in primary CRC and OM in NMF_cos1 than NMF_cos2. Cancers exhibiting HRD frequently demonstrate increased susceptibility to precision therapeutics, particularly poly-ADP-ribose polymerase inhibitors (PARPi). Given the restricted treatment alternatives for CRCOM, we will

focus on investigating necessitate precision stratification strategies to delineate patient subgroups that may derive clinical benefit from PARPi, particularly those refractories to immune checkpoint blockade or conventional chemotherapeutic regimens. The mutation of USP7 and RPA1 is higher in OM. We divided patients into two groups according to NMF hierarchical clustering, the patients in NMF_cos1 have better prognosis than NMF_cos2. Comparing the two clusters, we also found the mutation of USP7 only existed in NMF-cluster 2. Ubiquitin-specific protease 7 (USP7) is one of the most abundant ubiquitin-specific proteases (USP), and plays multifaceted roles in many cellular events, including the p53-dependent DNA damage response (DDR) pathway (43, 44). USP7 is also a master regulator of genomic integrity pathways (45). Recent study showed USP7 deubiquitylates and stabilizes DDX3X, augments Wnt/β-catenin signaling, thereby facilitating CRC tumorigenesis (46). USP7 is also identified as a crucial role on YAP in the regulation of CRC cell proliferation and tumor growth (47). Yang et al. also found that STAT3 bound to the promoter region of USP7 and inhibited its activity through recruiting HDAC1. As a result of the decline of USP7 expression, endogenous P53 protein level was decreased (48). In CRC, USP7 also plays a key role in regulating YY1 protein levels, which promote tumor development. By binding to 296–414 amino acid residues of YY1, USP7 weakened its ubiquitination and degradation of K63 linkage, thereby extending the functional lifespan of YY1 (49). Recent studies have shown that USP7 deubiquitination and stabilization of β - catenin promote the occurrence of CRC (50). According to a meta-analysis, which had a total of 1192 patients and assessed five types of cancer, the high-expression of USP7 may promote the progression of epithelial ovarian cancer (EOC) and predict unfavorable prognosis of EOC patients (51). There are studies indicated that USP7 emerges as a potential therapeutic target for cancers, as it plays an important role in the development of tumorigenesis by stabilizing multiple cancer-relevant proteins. Selective USP7 inhibitor (e.g., N-benzylpiperidinol derivatives, erteporfin (VP), and Compound P5091) showed efficacy in CRC models (52) (48) (47). We found the deletion of 17p11.2 and 18p11.31 in all OM of NMF_cos2. Therefore, the subtypes of CRCOM with USP7 mutations and more copy number alterations had a worse prognosis. This evidence suggests that targeting USP7 may have therapeutic potential in CRC with OM. The prospective trials are needed to determine whether targeting HRD pathways (e.g., PARP inhibitors in USP7-mutant cases) or modulating the immunosuppressive microenvironment could improve outcomes. We propose a precision medicine framework where CRCOM molecular subtyping guides second-line therapy selection post-standard chemotherapy, pending validation in interventional studies.

Exploring the evolutionary relationship between primary CRC and OM is vital to choosing the best treatment for CRCOM patients. A notable finding is that we observed the models of evolution in primary CRC could impact the metastatic model. We observed that the metastases were seeded from multiple late subclones of primary CRC, resulting in inter-metastatic heterogeneity across metastatic lesions. Identifying these subclones with metastatic capacity could be helpful in early diagnosis and potentially curative treatment for CRCOM. According to the pattern of the metastatic pathway in each CRCOM patient, we summarized two different modes of CRCOM, including primary CRC to OM, and other metastasis to OM. Firstly, our data supported primary CRC invaded the ovary directly in most cases, according to CRCOM derived from primary CRC in 9/15 cases. Some studies showed that hematogenous pathways were vital in CRCOM because both primary CRC and ovary are rich in blood vessels with frequent cancer embolus (53, 54). Besides, CRCOM was usually detected in young women, whose ovulatory cycle provided a suitable microenvironment for CRC cells to survive and invade (55, 56). Secondly, based on our data, the lymphatic origin of CRCOM has been evidenced in 4 patients, cancer cells first spread to adjacent lymph nodes and then metastasized through the lymphatic system to the ovary. Lymphatic origin was the widely accepted model in the CRC distant metastasis pathway, the presence of LNM is an important prognostic factor for CRC patients based on this model. Previous studies have shown that CRCOM was an independent risk factor for retroperitoneal lymph node recurrence ($P = 0.0012$) (57). They reviewed 105 CRC patients with PM who underwent surgery and HIPEC, of whom 62 patients also had OM. Retroperitoneal lymph node recurrence in CRC patients after surgery is a rare phenomenon, which only occurs in about 1% of patients, however, 29% of CRCOM patients in that study (57, 58). Lymph node dissection during primary CRC surgery may help prevent CRCOM. Identifying the LNM with high metastatic potential is crucial for the diagnosis and treatment of CRCOM since not all LNMs have the same metastatic potential. Besides, there were two patients with bilateral CRCOM, however, the sources of bilateral CRCOM were different in each patient. Thirdly, the evolutional patterns of P1, P8, and P10 also supported a model of metastasis-seeding-metastasis. In P4, ROM and LOM are seeded by different subclones in primary CRC, supporting polyclonal metastasis existing in the primary-seeding-metastasis model. Branched evolution has classically been viewed as the predominant evolution model in the process of tumor dissemination. These results showed that CRCOM is a complex process that may require the cooperation of multiple cells from different subclones, or occur during continuous evolution involving different clones. In conclusion, these results indicated that there were multiple metastasis pathways in the same CRCOM patients. Cancer cells from both primary CRC and other metastases could metastasize to the ovary and then form OM, and primary CRC and LNM were the important sources of CRCOM. More experimental and clinical studies are needed to verify the specific metastatic pathway and mechanism of CRCOM and then to apply them in developing precision therapy.

We offered novel insights for the immunotherapy administration in CRC with OM. There is emerging evidence that immune checkpoint inhibitors achieved considerable success in multiple malignancies, but this is less defined in CRCOM. We also observed that the multiple tumors within individuals were highly heterogeneous in neoantigen, while disparities exist between primary CRC and OM. The immunoscore provides a reliable

estimate of the risk of recurrence in patients with colon cancer. We assessed the immunoscore by quantifying the densities of CD3+ and cytotoxic CD8+ T cells in the tumor and in the invasive margin of patients with CRCOM and found the immunoscore of CRCOM is low. Our findings shed light on the application of ICIs (immune checkpoint inhibitors) on CRCOM and suggested that different strategies should be applied to primary CRC and OM. The selection of CD3, CD8, and CD20 was driven by their established prognostic value in CRC and technical feasibility for multi-sample cohort analysis (59, 60). These markers provide a foundational assessment of adaptive immune cell recruitment. While our study characterized the immune landscape using CD3, CD8, and CD20 as key markers for T-cell and B-cell infiltration, we recognize that additional markers (e.g., PD-1/PD-L1 for immune checkpoint activity, FOXP3 for Tregs, CD68/CD163 for macrophage polarization) are critical to fully dissect the immunosuppressive mechanisms in CRCOM. The absence of these analyses may limit our understanding of therapeutic vulnerabilities, such as potential responsiveness to immune checkpoint inhibitors.

This study has several limitations that warrant consideration. First, the small cohort size (n=11 patients, despite multi-site sampling of 65 tissues) may restrict the statistical power and generalizability of our findings, particularly for subgroup analyses such as bilateral ovarian metastases comparisons. Future validation in larger, independent cohorts is imperative to confirm the clinical relevance of the proposed molecular subtypes and metastatic patterns. Second, all samples were derived from a single tertiary hospital in China, which may introduce selection bias toward patients with specific clinical profiles and limit extrapolation to other populations or healthcare settings. We will recruit external validation using geographically diverse cohorts to assess the robustness of our observations in the future. Furthermore, the exclusively Chinese cohort raises concerns about genetic ancestry-specific effects, as known population differences in colorectal cancer driver mutations and immune microenvironment dynamics could influence CRCOM biology. Studies should include multi-ethnic cohorts to investigate potential ancestry-related differences in CRCOM biology and metastatic behavior in future. Lastly, the OMs were collected from secondary surgery in four patients, who have received adjuvant therapy. This might cause the accumulation of treatment-resistant mutations, however, previous research verified that adjuvant therapy didn't affect building phylogenetic tree (37). Future multi-center studies with ethnically diverse cohorts, complemented by mechanistic validations, are essential to address these limitations and advance CRCOM precision medicine.

In conclusion, we described the special molecular features of CRCOM by comparing paired primary CRC and multi-metastases. Our data indicated that there was significant intertumoral heterogeneity among patients with CRCOM, besides intratumoral heterogeneity among primary CRC, OM, and other metastatic lesions. 19 genes were inferred as the potential driver genes of CRCOM. Moreover, the USP7 was identified as the prognosis biomarkers in CRCOM. The subtypes of CRCOM with USP7 mutation, more copy number alterations, lower neoantigens and immunoscore have a worse prognosis. We also portrayed two metastatic patterns of CRCOM: primary CRC to OM and metastases to metastases (including LNM to OM, PM to OM, and other metastases to OM), and LNM was one of the important sources of CRCOM. Biopsy and sequencing of CRCOM should be applied to understand the dynamics of cancer evolution and choose a better treatment to improve the clinical outcomes of patients with CRCOM.

Data availability statement

Publicly available datasets were analyzed in this study. This data can be found here: The raw sequence data reported in this paper have been deposited in the Genome Sequence Archive in the National Genomics Data Center, China National Center for Bioinformation/ Beijing Institute of Genomics, Chinese Academy of Sciences, under accession number GVM000406 (Project: PRJCA011872).

Ethics statement

The studies involving humans were approved by the local institutional ethical committee of the Second Affiliated Hospital, Zhejiang University School of Medicine. The studies were conducted in accordance with the local legislation and institutional requirements. The ethics committee/institutional review board waived the requirement of written informed consent for participation from the participants or the participants' legal guardians/next of kin because This study utilizes anonymized, residual post-operative specimens collected during standard surgical procedures.

Author contributions

CC: Writing – original draft. JW: Writing – review & editing. BS: Writing – original draft, Methodology. YZ: Resources, Writing – original draft. XG: Writing – original draft, Data curation. ZG: Writing – original draft, Investigation. HG: Writing – original draft, Investigation. ZZ: Writing – original draft, Software. AZ: Methodology, Writing – original draft. YS: Writing – original draft, Methodology. YH: Writing – review & editing. LM: Methodology, Writing – review & editing. YL: Methodology, Writing – review & editing. KD: Writing – review & editing. DW: Writing – review & editing. LS: Writing – review & editing, Funding acquisition, Supervision.

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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.2025.1593439/full#supplementary-material

SUPPLEMENTARY FIGURE 1
The mean tumor mutation burden (TMB) for primary tumors and ovarian metastases.

SUPPLEMENTARY FIGURE 2
(A) Unsupervised clustering was also performed on all OMs. **(B)** Survival analysis in overall survival (OS) between the two groups of patients. **(C)** The homologous recombination (HRD-score) of two groups. **(D)** Survival analysis of the mutation of USP7 in TCGA.

SUPPLEMENTARY FIGURE 3
The different SNVs and signatures of the two groups.

SUPPLEMENTARY FIGURE 4
ClonEvol and MEGA 11 are used to build the phylogenetic tree of the CRCOM in each case.

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CRISPR/Cas9-based discovery of ccRCC therapeutic opportunities through molecular mechanism and immune microenvironment analysis

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Introduction: Clear cell renal cell carcinoma is a common and aggressive form of renal cell carcinoma. Its incidence continues to rise, and metastatic recurrence leads to poor clinical outcomes. Current prognostic biomarkers lack reliability. We integrated multi-omics data to discover key ccRCC genes and build a prognostic model to improve risk prediction and guide treatment decisions.

Methods: Our study integrated genome-wide CRISPR screening data from DepMap and transcriptomic profiles from TCGA to identify key genes associated with ccRCC pathogenesis. Initial screening identified 11 candidate genes through differential expression analysis and CRISPR functional validation. Using LASSO and Cox regression, we selected five key genes (GGT6, HAO2, SLPI, MELK, and EIF4A1) for model construction. The functional role of MELK was tested by knockdown experiments. Additional analyses included tumor mutation burden, immune microenvironment assessment, and drug response prediction.

Results: The model stratified patients into high-risk and low-risk groups with distinct survival outcomes. High-risk cases showed higher mutation loads, immunosuppressive features, and activated cytokine pathways, whereas low-risk cases displayed metabolic pathway activity. MELK knockdown reduced cancer cell proliferation and migration. High-risk patients exhibited better responses to targeted drugs such as pazopanib and sunitinib.

Discussion: Our study demonstrates the pivotal role of MELK in ccRCC progression. This multi-omics-driven model elucidates MELK-mediated mechanisms and their interactions with the tumor microenvironment, providing novel strategies for risk stratification and targeted therapy. Future studies will validate these findings in independent cohorts and investigate the regulatory networks of MELK to identify potential therapeutic targets.

KEYWORDS

CRISPR-Cas9 screening, ccRCC, prognostic model, MELK, immunotherapy

Introduction

Renal cell carcinoma (RCC) ranks among the most prevalent cancers in the urological system, with its incidence on the rise, representing approximately 2%–3% of malignant neoplasms in adults (1). RCC is a prevalent malignancy within the genitourinary tract, characterized by its aggressive nature and high fatality rate (2). Among RCC subgroups, clear cell Renal Cell Carcinoma (ccRCC) predominates histologically, representing about 75-80% among RCC diagnoses (3). Globally, approximately 400,000 RCC diagnoses are identified each year, with the United States contributing an estimated 82,000 cases in 2024 with ccRCC accounting for about 75%–80% of these cases. RCC is responsible for over 170,000 deaths annually. The vast majority of which were ccRCC, with around 15,000 deaths attributed to the disease.

ccRCC exhibits significant heterogeneity, a high propensity for metastasis, and a generally unfavorable prognosis (4). Despite surgical excision being the mainstay treatment for patients with localized ccRCC, a significant proportion 30–40% of these patients experience metastatic relapse after surgery during subsequent follow-up. As a result, early detection of metastatic propensity in ccRCC is crucial for enhancing the precision of prognostic predictions. At present, our knowledge of the pathogenesis of ccRCC remains incomplete, and reliable tumor biomarkers for predicting prognosis have yet to be established.

Recently, high-throughput screening initiatives, such as the DepMap project, have gained prominence. These projects leverage RNA interference silencing and CRISPR-Cas9 (Clustered Regularly Interspaced Short Palindromic Repeats-associated protein 9) knockout techniques to pinpoint possible essential genes vital to tumor survival, metastasis, or recurrence (5–7). Researchers have employed CRISPR technology to selectively knock out target genes, thereby exploring potential therapeutic strategies (8, 9). To systematically identify potential cancer biomarkers, the CRISPR-Cas9 system has been employed to screen essential genes regulating cancer cell growth and viability. To enhance the specificity of CRISPR-based screens, the CERES algorithm was developed to computationally correct copy number effects, thereby quantifying the median impact of core and dispensable genes on a for each individual cell line basis (10). Genes deemed essential in a limited number of cell lines are regarded as more promising therapeutic targets, since targeting these genes is less likely to induce off-tissue toxicity. In addition, studying the prognostic value of ccRCC can help urologists better treat patients.

By combining DepMap CRISPR screening and TCGA transcriptomic data, we identified five pivotal ccRCC-associated genes. Using LASSO and multivariate Cox regression, we developed a prognostic model and analyzed its relationships with tumor mutational burden (TMB), Tumor microenvironment (TME) immune infiltration, immunotherapy response, and chemotherapy efficacy. A clinical nomogram incorporating risk scores and clinical features was established for ccRCC prognosis prediction.

Method

Data collection and preprocessing

This study focuses on characterizing molecular biomarkers while investigating potential therapeutic targets for ccRCC. Utilizing TCGA database, gene expression profiles and clinical data from 537 ccRCC patients were analyzed. Differential expression analysis was conducted between matched tumor-normal tissue pairs from the TCGA cohort, with differentially expressed genes (DEGs) identified using a false discovery rate (FDR) threshold of less than 0.05 and a log2 fold change (log2FC) greater than 1 as the criteria for defining primary cancer-associated genes. Subsequently, the DepMap database contains gene dependency data from cancer cell lines, was employed in conjunction with CRISPR-Cas9 gene-editing technology to further validate the critical role of these genes in cancer cell survival. For this purpose, the CRISPR dataset from the 24Q4 release of the DepMap database was downloaded, and genes with Chronos scores below zero were identified as essential genes. By integrating the analytical results from TCGA and DepMap, the study successfully identified a group of core genes closely associated with ccRCC, which may serve as potential diagnostic markers and pharmacological targets for further in-depth analysis. External validation was performed using the GEO dataset GSE26909 (n=39), with risk scores calculated using the same coefficients derived from the TCGA cohort.

Identification of DEGs

After identifying 11 genes in ccRCC, we first analyzed their expression and copy number variation (CNV) profiles. A cutoff-based approach was applied, and heatmaps were generated using the “pheatmap” R package (11). Next, differential expression and co-expression analyses of these 11 genes were performed to assess their expression patterns. Boxplots were generated using the ‘ggpubr’ R package. (12).

Recognition of key genes in ccRCC

To identify survival-related genes in ccRCC, we conducted univariate, LASSO-penalized, and multivariate Cox proportional hazards regression analyses using R’s glmnet package to develop a prognostic prediction model (13–16). The heatmap illustrates the pattern of clinical feature distribution across patients in the high-risk and low-risk groups which was generated to visualize the expression patterns of DEGs across the patient samples. The expression data were normalized and log2-transformed to reduce skewness and improve comparability. Hierarchical clustering was performed on both genes and samples to group those with similar expression profiles. The chord diagram was generated to visualize regulatory or functional interactions between the top DEGs. The risk score for each patient was calculated using a linear combination

of the expression levels of the DEGs, weighted by their respective regression coefficients derived from multivariate Cox analysis. The formula is as follows:

$$\text{Riskscore} = \sum \text{iCoefficient (i)} * \text{Expression of gene(i)}$$

Differences in survival between risk strata were evaluated through Kaplan-Meier (KM) analysis performed with the "survival" R package (17, 18). Patients were dichotomized into high- and low-risk groups using the median risk score as the threshold. This cutoff was selected to ensure balanced group sizes and clinical interpretability. Time-dependent Receiver operating characteristic (ROC) analysis evaluated the gene risk model's performance using 1-year, 3-year, and 5-year follow-up data. We validated the optimal threshold value via principal component analysi (PCA) (19). Calibration curves approaching the 45-degree line indicated optimal predictive performance of the nomogram.

Consensus clustering analysis

This study investigates the application of clustering analysis in data classification through experiments, centered on the k-means partitioning method and its implementation in the R environment using the ConsensusClusterPlus tool (20). The experiment employed Euclidean distance as the similarity measure and incorporated the Partitioning Around Medoids (PAM) algorithm to perform clustering analysis on the dataset, ranging from 2 to 9 clusters. the study constructed a reliable consensus matrix, significantly reducing inter-cluster overlap and achieving efficient data classification. This analysis was implemented using the R package ConsensusClusterPlus.

Predictive nomogram with interactive dynamic features

We developed the prognostic nomogram with the "rms" package (21) and implemented an interactive web calculator using "shiny" and "DynNom" packages (22, 23) for real-time survival probability estimation. The model's predictive performance was validated through calibration plots comparing observed KM versus predicted 1-year, 3-year, and 5-year survival outcomes.

TMB calculation

TMB was quantified based on the count including nonsynonymous single nucleotide variants and insertion-deletion alterations per megabase. Leveraging the "maftools" R package, we derived TMB values for our predictive model (24).

Function enrichment analysis

Gene Ontology (GO), Kyoto Encyclopedia of Genes and Genomes (KEGG), and Gene Set Enrichment Analysis (GSEA) were performed using the R packages clusterProfiler and GOplot to identify biological functions and pathways associated with cancer essentiality in high-risk vs low-risk groups (25). Results were visualized with ggplot2 (26).

Drug sensitivity analysis

We conducted a drug sensitivity analysis aimed at evaluating the impact of various compounds on specific cell lines. For this purpose, we utilized the "limma", "ggpubr" and the "pRRophetic" R package for our analysis, with the selection threshold set at $p < 0.05$ and $q < 1$ (27).

Investigation of immune cell infiltration

Immune cell infiltration profiles were analyzed using complementary approaches: ssGSEA via the GSVA package quantified 22 immune cell subtypes, while CIBERSORT assessed immune infiltration patterns and their association with immune checkpoints across risk groups.

Cell culture

The ccRCC cell lines 786O, 769P, and Caki-1 were obtained from the American Type Culture Collection (ATCC). The cells were cultured in RPMI-1640 medium containing 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin. All reagents were purchased from Gibco (Invitrogen-Gibco). Cells were incubated at 37°C with 5% CO_2 in a humidified environment.

Human specimens

This study was conducted at the Second Affiliated Hospital of Harbin Medical University to provide a scientific basis for ccRCC early detection and therapy. Tumor and adjacent normal tissues (0.5 cm^3 each) were collected from surgically treated ccRCC patients. The study was approved by the hospital's Ethics Committee, after obtaining participant consent. Formalin-fixed paraffin-embedded specimens were prepared for immunohistochemistry, and clinical data were verified by two board-certified surgeons.

Western blot

Cells were lysed in RIPA buffer containing protease inhibitors (Seven, China), collected by scraping (BIOFIL), and quantified by BCA (Beyotime). Proteins were separated by 10% SDS-PAGE, transferred to PVDF membranes (Millipore), and incubated with specific primary antibodies at 4°C for 12-16 hours followed by HRP-secondary antibodies (RT, 1 h) were detected by chemiluminescence (Tanon).

Cell colony formation analyze

Cells were harvested in RIPA/protease inhibitor cocktail (Seven, China), collected by scraping (BIOFIL), and quantified by BCA (Beyotime). Proteins were resolved on 10% SDS-PAGE, transferred to PVDF membranes, and immunoblotted with primary antibodies (4° C, overnight) and HRP-secondaries (RT, 1 h), followed by chemiluminescent detection (Tanon). Following distilled water washes and air-drying, colonies (≥50 cells) were microscopically counted to calculate formation rates, with images captured for analysis.

Transwell assay

Cells (5×10^4 ccRCC) were seeded in serum-free 8 μm Transwell chambers (Corning), with 600 μL complete medium in the lower compartment. Following a 24-hour incubation period, non-invasive cells were gently eliminated. Transmigrated cells underwent fixation using 4% paraformaldehyde, labeled with 0.5% crystal violet solution, and quantified by light microscopy.

Statistical analysis

The experiments were repeated independently a minimum of three replicates and presented as mean values ± SD. All statistical evaluations and computations were conducted using R software (4.4.0). Statistical significance was determined using unpaired t-tests and two-factor variance analyses (GraphPad Prism 8). Threshold for statistical significance was set at $p<0.05$.

Result

Identification of 11 important DEGs in ccRCC

The complete analytical workflow is presented (Figure 1). Initially, essential genes that significantly impact cell viability in ccRCC cell lines were identified based on genome-wide CRISPR knockout screening data from the DepMap database. Subsequently, DEGs in ccRCC tumors compared to adjacent normal tissues were detected via TCGA transcriptomic data mining. By integrating these two datasets (Figures 2A), we identified 11 key genes exhibiting significant difference in ccRCC (Figures 2B, C). Further analysis revealed that these genes commonly exhibit CNVs, predominantly characterized by copy number losses (Figure 2D). Additionally, the correlations among these 11 DEGs are shown in Figure 2E. Most importantly, we successfully identified 11 crucial DEGs for further in-depth analysis.

The construction and evaluation of the prognostic model

Through univariate Cox regression analysis of the 11 candidate genes, we identified 7 genes that exhibited stronger associations with the prognosis of ccRCC. Subsequently, we employed the k-means clustering algorithm to perform grouping experiments on these 7 genes. The results demonstrated that the clustering performance was most stable when k=2 (Supplementary Figures S1A–D). UAMP revealed distinct gene expression patterns between cluster 1 and cluster 2 (Supplementary Figure S1E). Additionally, the Kaplan–Meier analysis demonstrated significantly better OS in cluster 2 compared to cluster 1 among ccRCC patients (Supplementary Figure S1F). The findings not only confirmed the classification of ccRCC patients into two subgroups but also revealed notable disparities in their OS. Pronounced differences in expression patterns between the two gene groups with high internal consistency. In the initial stage of our analysis, we performed univariate Cox regression on the 11 DEGs (Figure 3A). Subsequently, we applied LASSO regression to further refine the gene set (Figures 3B, C). Intriguingly, 7 genes were retained based on partial likelihood minimization and were subsequently applied in constructing the risk prediction model. Then we utilize multivariate Cox regression analysis, ultimately screening out 5 core genes: GGT6 (95% CI = 0.62-0.99, $p = 0.041$), HAO2 (95% CI = 0.78-0.97, $p = 0.013$), SLPI (95% CI = 1.03-1.18, $p = 0.006$), MELK (95% CI = 1.11-1.92, $p = 0.006$), and EIF4A1 (95% CI = 1.14-1.69, $p = 0.001$). These genes showed significant correlations with the OS (Figures 3D, E). The correlations between these DEGs are displayed (Figure 3F).

Clinical evaluation based on a risk score-derived prognostic model

We built a risk score model from the transcriptional signatures of the five genes, dividing patients into high-risk and low-risk groups. Through heatmap analysis (Supplementary Figure S2A), we revealed potential associations between risk scores of ccRCC and clinical characteristics of patients. The heatmap results demonstrated a positive correlation between elevated risk scores and poor prognosis. To further quantify these relationships, we constructed scatter plots using the Wilcoxon signed-rank test (Supplementary Figures S2B–G). It indicated that ccRCC risk stratification exhibited a strong positive association with clinical stage, N stage, T stage, M stage, gender, and tumor grade ($p < 0.05$). However, no statistically significant correlation was observed between age and ccRCC risk scores (Supplementary Figure S2H). In summary, the ccRCC risk score serves as a robust indicator for evaluating tumor malignancy, with predictive efficacy independent of age.

Prognostic stratification and risk assessment

KM analysis confirmed a worse prognosis in high-risk versus low-risk patients (Figure 4A). Additionally, the prognostic value of our model was examined using ROC curve methodology (Figure 4B). The model demonstrated strong predictive accuracy with 1-year, 3-year, and 5-year AUCs of 0.711, 0.673, and 0.706, confirming its robust prognostic value. It's displays the risk score distribution across high-

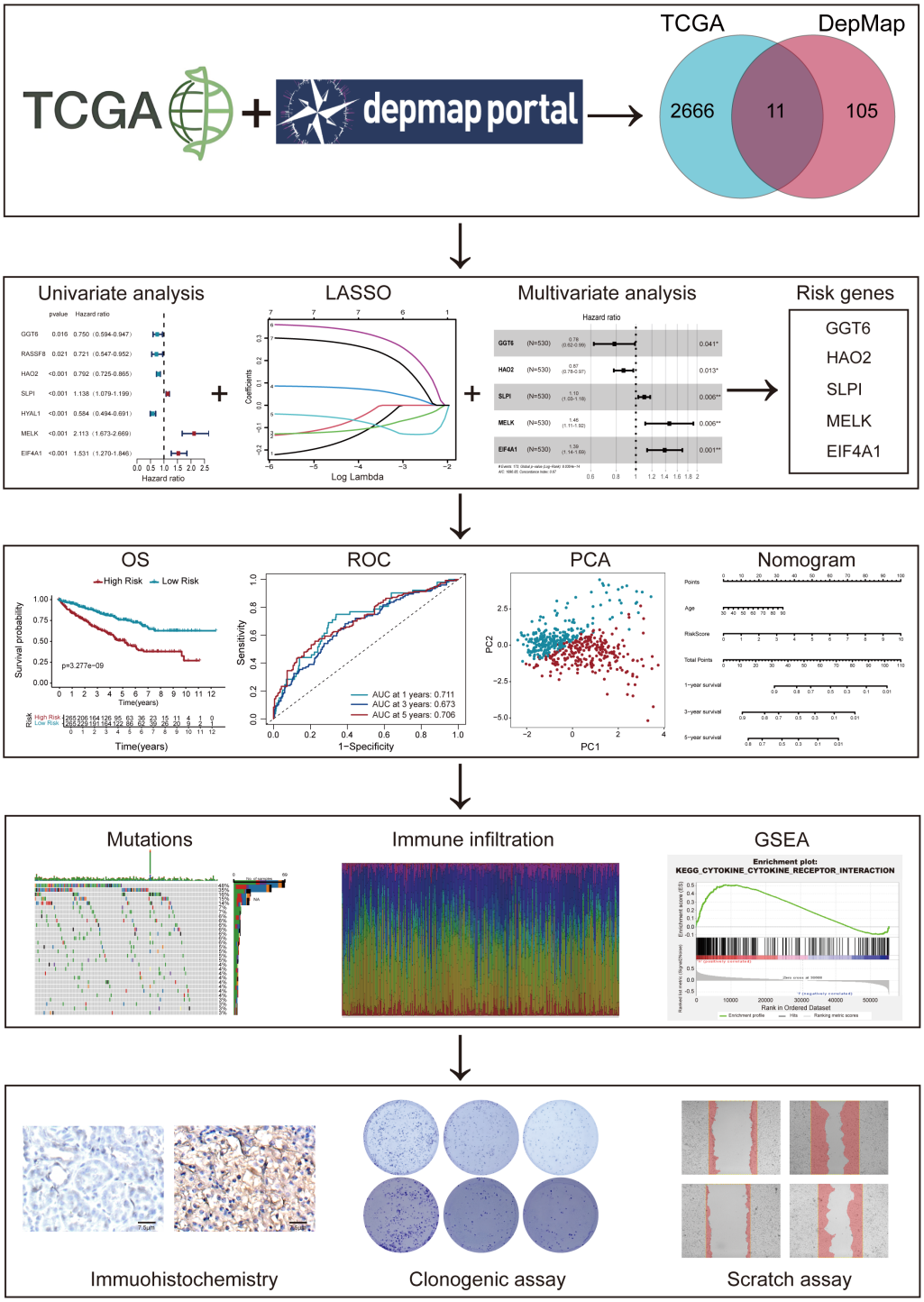

FIGURE 1
The flowchart and graphic abstract of this study.

and low-risk groups (Figure 4C). indicating a direct relationship between rising risk scores and mortality probability (Figure 4D). Furthermore, PCA was employed to classify ccRCC samples into distinct groups. PCA results distinctly stratified ccRCC samples into high-risk and low-risk groups, reaffirming the significant prognostic differentiation of ccRCC patients based on our risk model (Figures 4E). To further validate our prognostic model, we applied it to an independent GEO dataset (GSE26909, n=39). Consistent with TCGA results, the model significantly stratified patients into high- and low-risk groups (Figure 4F), confirming its generalizability.

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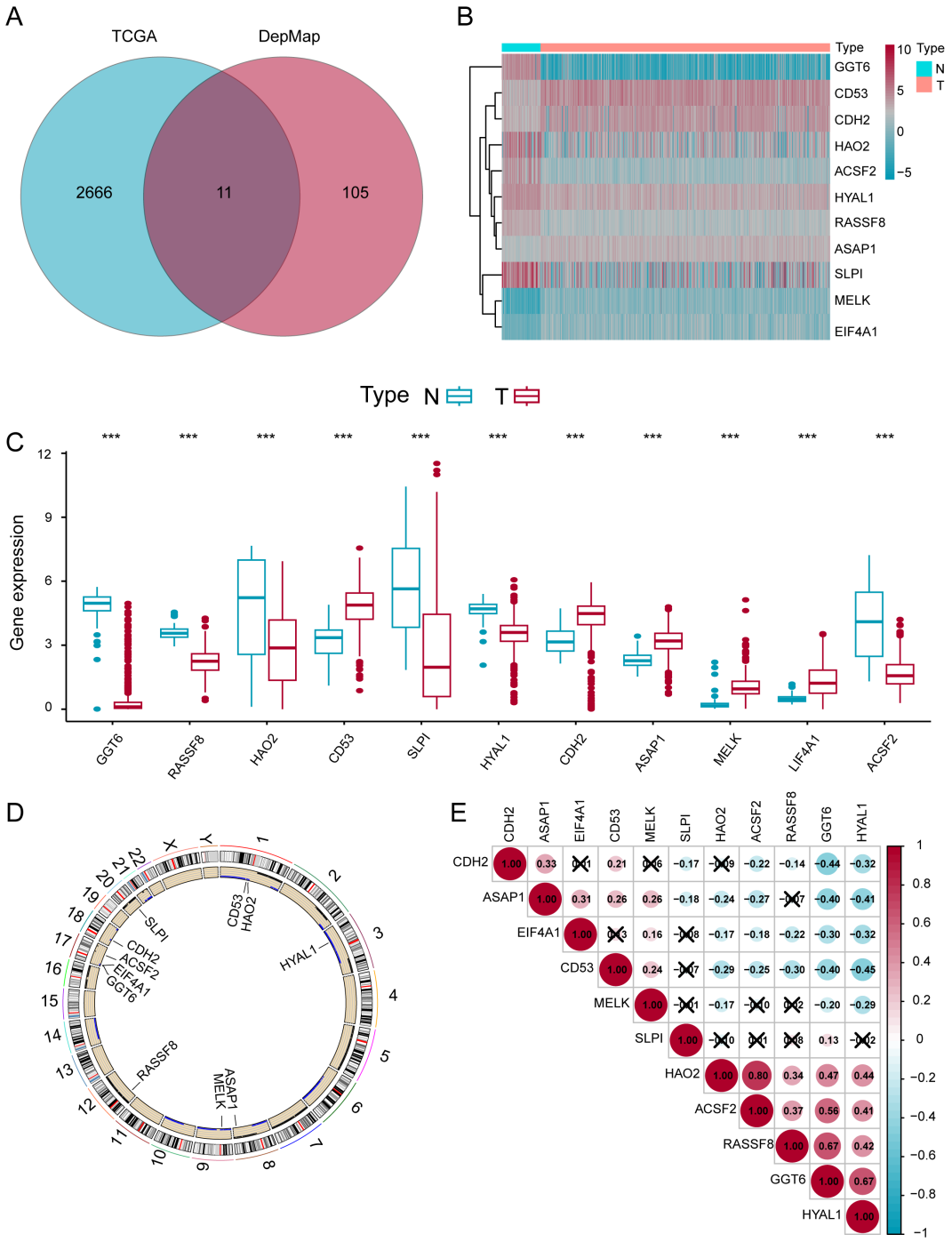

FIGURE 2
Identification of 11 Important DEGs in ccRCC. **(A)** Venn diagram of genes in the TCGA and DEPMap datasets. **(B)** Expression heatmap of the eleven genes in normal versus tumor samples. **(C)** Differential expression levels of the eleven genes in normal and tumor samples. **(D)** Locations of the DEGs on chromosomes. **(E)** Expression correlation analysis of the eleven DEGs. *p < 0.05; **p < 0.01; ***p < 0.001.

Formulation and evaluation of the nomogram

Univariate and multivariate Cox proportional hazards models were utilized to evaluate the risk score's independence as a prognostic indicator for ccRCC (Figures 5A, B). Notably, while age did not show a significant correlation with the risk score (Supplementary Figure S2H), multivariate Cox regression analysis confirmed its independent prognostic value for overall survival. Therefore, we included age in the nomogram and considered potential confounding factors, such as treatment tolerance and comorbidities, which may independently affect patient prognosis regardless of molecular risk stratification. Based on significant *p*-values from multivariate Cox regression, we constructed a nomogram as a quantitative method to predict OS in

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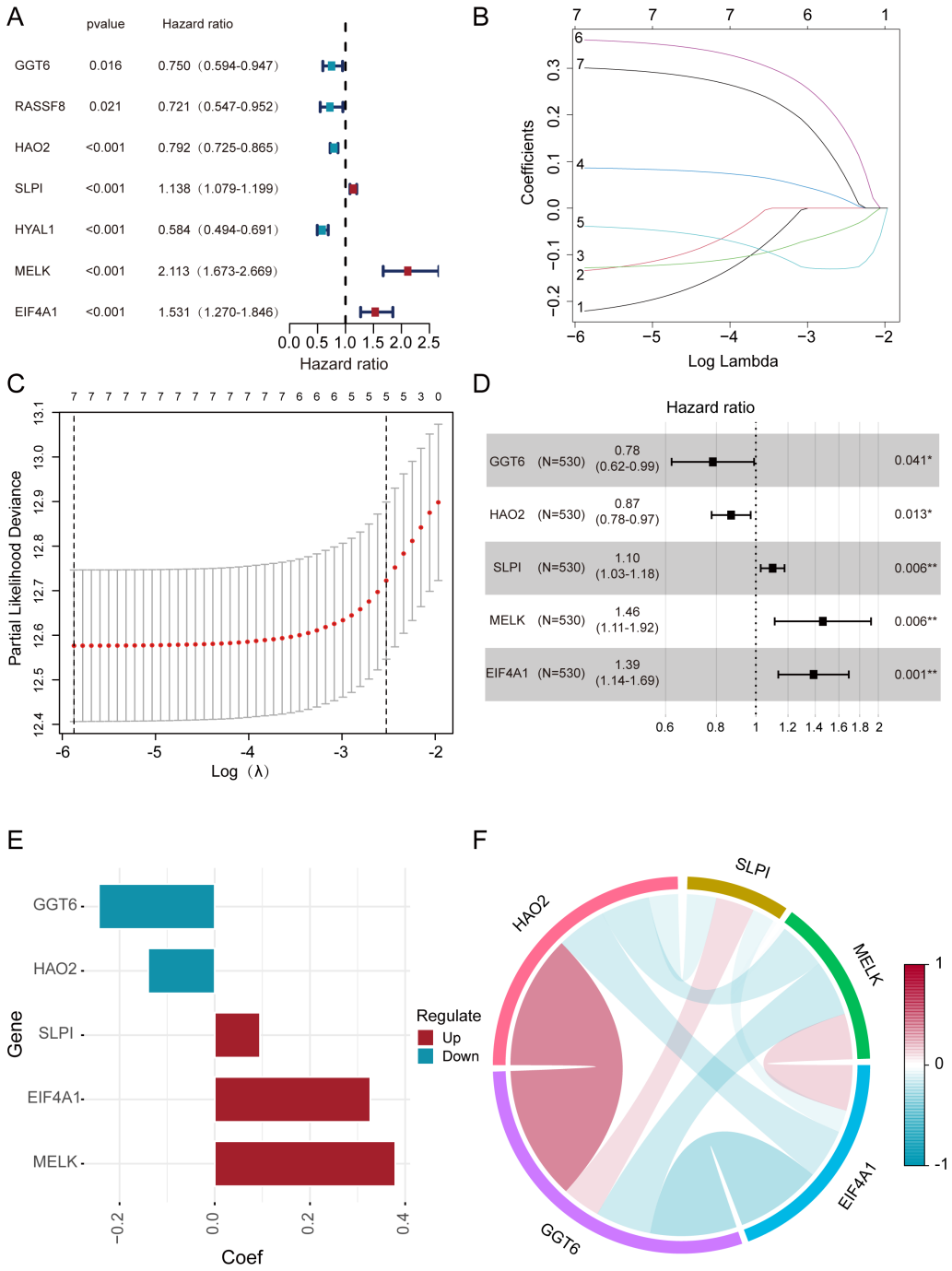

FIGURE 3
The construction and evaluation of the prognostic models. **(A)** Univariate Cox regression identifies 7 DEGs. **(B)** Coefficient trajectories of 7 DEGs in LASSO regression. **(C)** Optimal lambda selection in LASSO regression (10-fold CV). **(D, E)** Prognostic impact of 5 DEGs assessed by multivariate Cox regression. **(F)** Inter-gene correlations among the five DEGs.

ccRCC patients(Figure 5C). The predictive factors included in the nomogram were the risk score and age. The results showed that the risk score was the key prognostic indicator. Additionally, calibration curves for 1-year, 3-year, and 5-year predictions were generated, demonstrating that the model exhibited satisfactory predictive accuracy (Figures 5D–F). The data indicate this signature may serve as a dependable assessment method for OS prediction in ccRCC.

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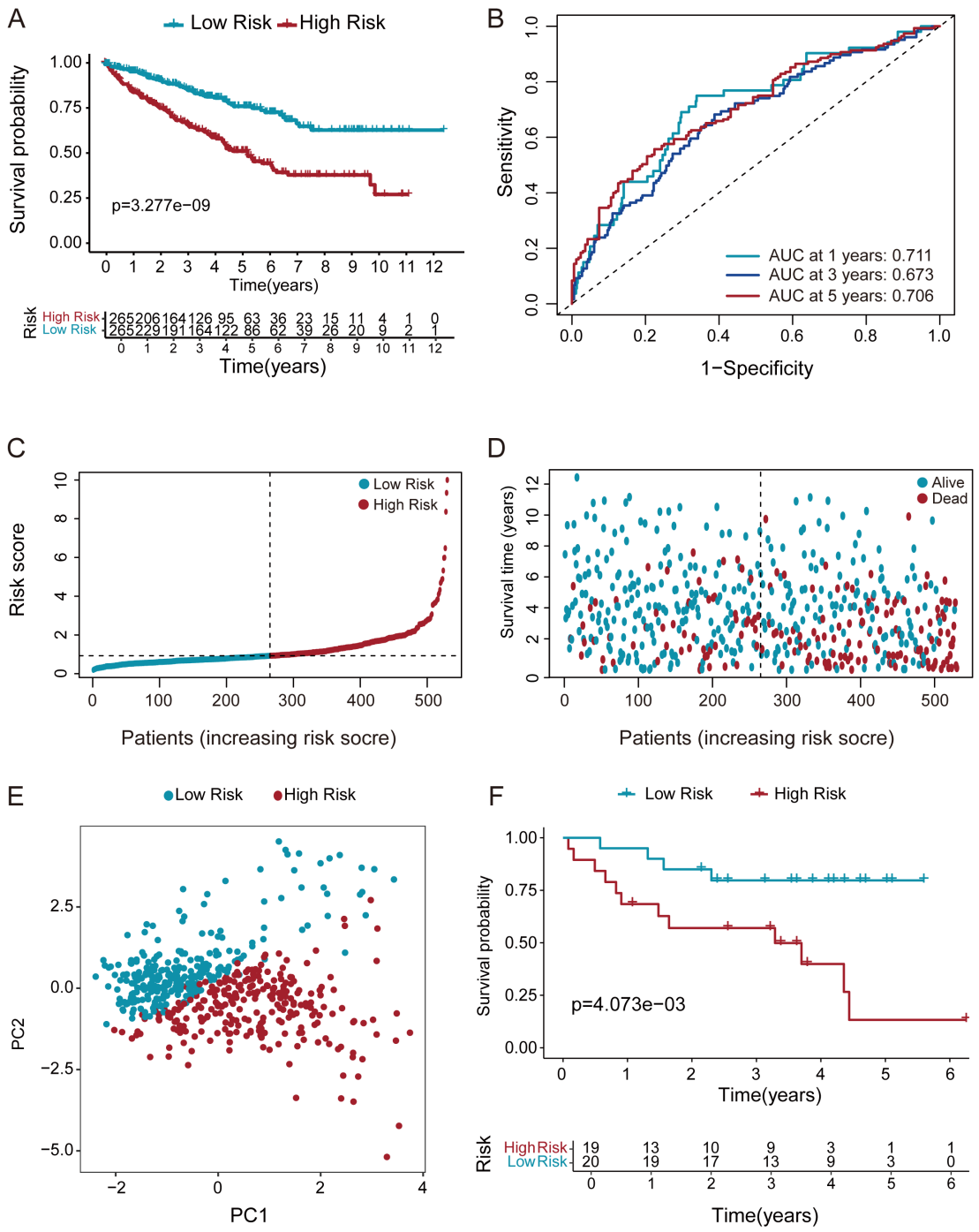

FIGURE 4
Multi method validation of risk score-derived prognostic models. **(A)** KM survival curves demonstrated markedly shorter overall survival in high-risk ccRCC patients relative to those in the low-risk group. **(B)** ROC analysis of the DEGs prognostic signature for predicting the 1/3/5-year survival. **(C, D)** Risk score stratification and survival duration distribution in ccRCC cohort. **(E)** PCA discriminates high- and low-risk groups using whole transcriptome data. **(F)** KM survival analysis of ccRCC patients stratified by risk score in the GEO validation cohort (GSE26909, n=39).

Investigating the relationship between TMB and risk scores

Subsequently, we focused on the potential value of TMB in tumor immunotherapy and its molecular characteristics. We analyzed genomic alteration landscapes in high-risk and low-risk groups risk scores from the TCGA database (Figures 6A, B). Survival curves stratified by TMB levels indicated that patients with low TMB exhibited improved clinical prognosis compared to those with high TMB (Figure 6C). Subgroup analysis revealed significant differences in mutation distribution and genetic features between high TMB groups(Figures 6D–F) and low TMB

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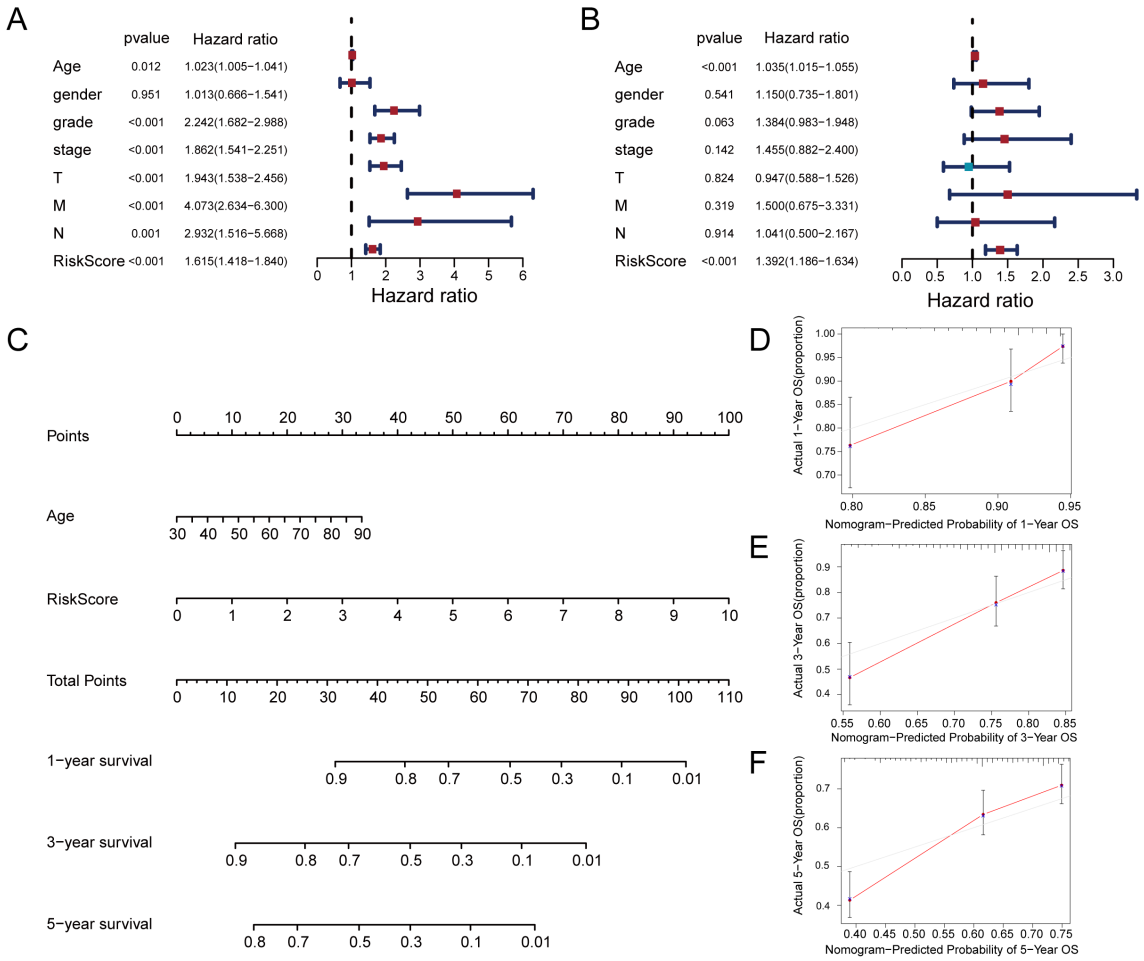

FIGURE 5
Construction of a nomogram for prediction prognosis. **(A)** Univariate Cox regression analysis identified grade, stage, T stage, M stage, and risk score as significant prognostic factors. **(B)** Multivariate Cox regression identifies risk score and age as independent prognostic predictors. **(C)** Prognostic nomogram incorporating risk score and age for ccRCC survival probability. **(D–F)** Calibration curves demonstrate the accuracy of 1-year, 3-year, and 5-year overall survival predictions.

groups(Figures 6G–I). Missense mutations predominated in both groups, while frameshift mutations demonstrated pronounced prevalence in the low TMB group, hinting at distinct functional impacts on tumor progression. Mutation distribution and gene characteristics also differed between TMB groups.

Prognostic model using immune cells and drug sensitivity

TME has been shown to have a critical impact on the progression and treatment of various cancers. By constructing an immune cell atlas of the TME, we systematically analyzed the infiltration patterns of 22 immune cell subsets in ccRCC (Figure 7A). Our findings revealed that immune cell populations including dendritic cells, M1 macrophages, mast cells, and monocytes exhibited significant anti-tumor activity, with their abundance positively correlated with improved patient prognosis (Figures 7B, C). In contrast, neutrophils, memory T cells, regulatory T cells, follicular helper T cells, M0 macrophages, activated mast cells demonstrated pro-tumor characteristics, and elevated infiltration levels correlated significantly with adverse clinical outcomes (Figures 7D–F). Further analysis using the ESTIMATE algorithm evaluated immune cell infiltration in the TME of ccRCC patients (Figure 7G). The results showed a marked reduction in anti-tumor immune cells and a concomitant increase in immunosuppressive cell infiltration in high-risk TME. Based on these derivations, we assessed the therapeutic efficacy of three targeted agents pazopanib, sunitinib, and temsirolimus in high-risk and low-risk group (Figures 7H–J). The research indicate that these agents show significantly higher drug sensitivity and improved treatment outcomes in low-risk patients. These findings indicate that our model is closely associated with tumor-infiltrating immune cells and drug sensitivity, providing valuable insights for the development of targeted immunotherapies in ccRCC.

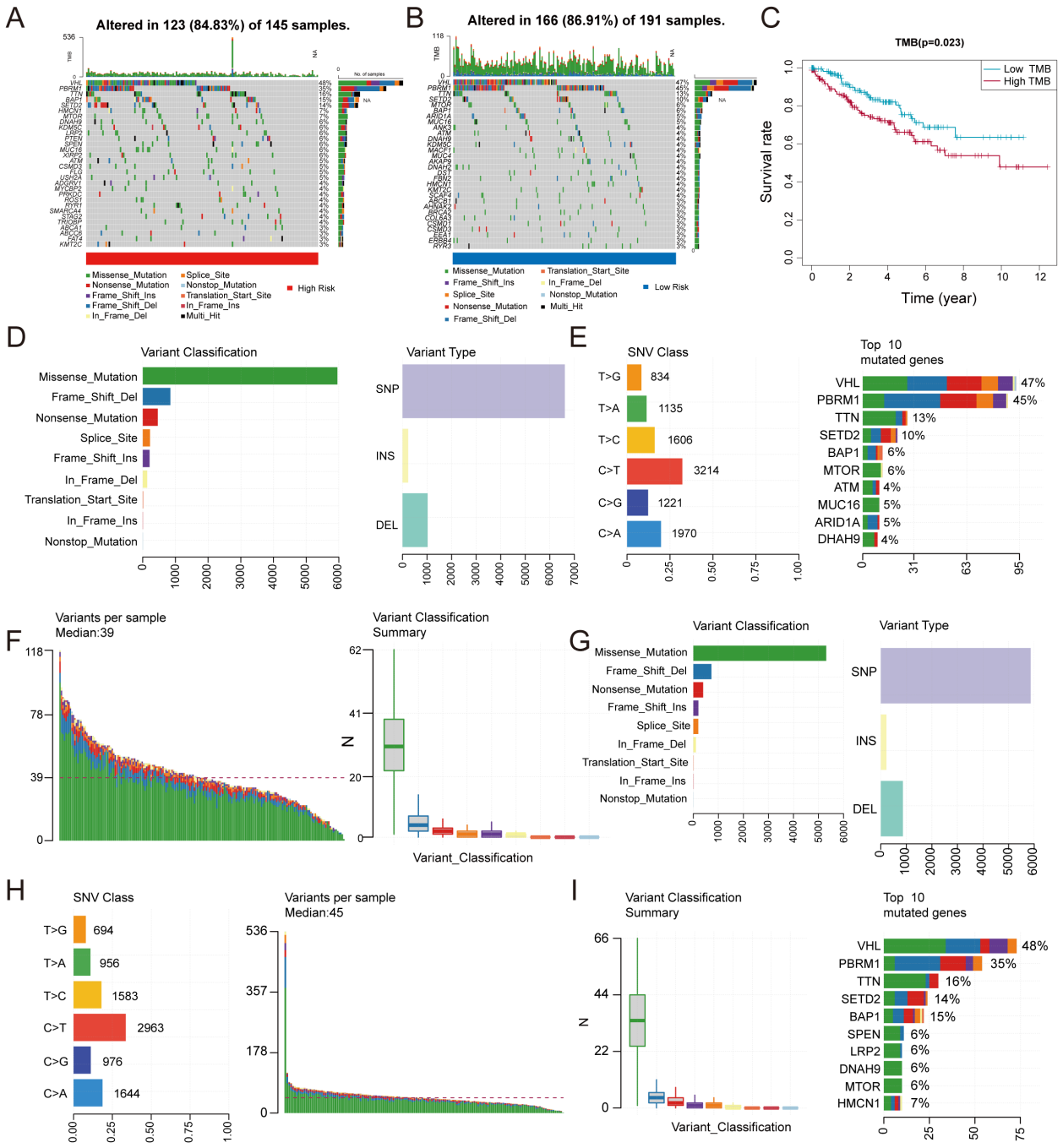

FIGURE 6
Correlation between TMB and risk score. **(A, B)** Comparative mutation landscapes in high-risk **(A)** and low-risk **(B)** groups. **(C)** Survival outcomes stratified by TMB levels. **(D–I)** Variant type distributions are shown for high-risk **(D–F)** and low-risk **(G–I)** patients.

Enrichment analysis of the prognostic model

To further annotate the functional enrichments in the high-risk and low-risk groups, we performed GSEA to identify significantly enriched signaling pathways (Figures 8A–F). The high-risk group showed prominent enrichment in the "Cytokine-cytokine receptor interaction" pathway, while the low-risk group exhibited significant enrichment in metabolic pathways including fatty acid, propanoate, and branched-chain amino acid degradation. KEGG and GO analyses (Figures 8G, H) were performed to explore the molecular mechanisms of the five prognosis-related genes. KEGG pathway analysis indicated significant enrichments in pathways including Phagosome, Carbon metabolism, Diabetic cardiomyopathy. These findings suggest that the prognosis of RCC patients may be influenced by the aforementioned biological functions and signaling pathways. GO analysis highlighted enrichment in cell adhesion regulation, energy metabolism, and extracellular matrix components.

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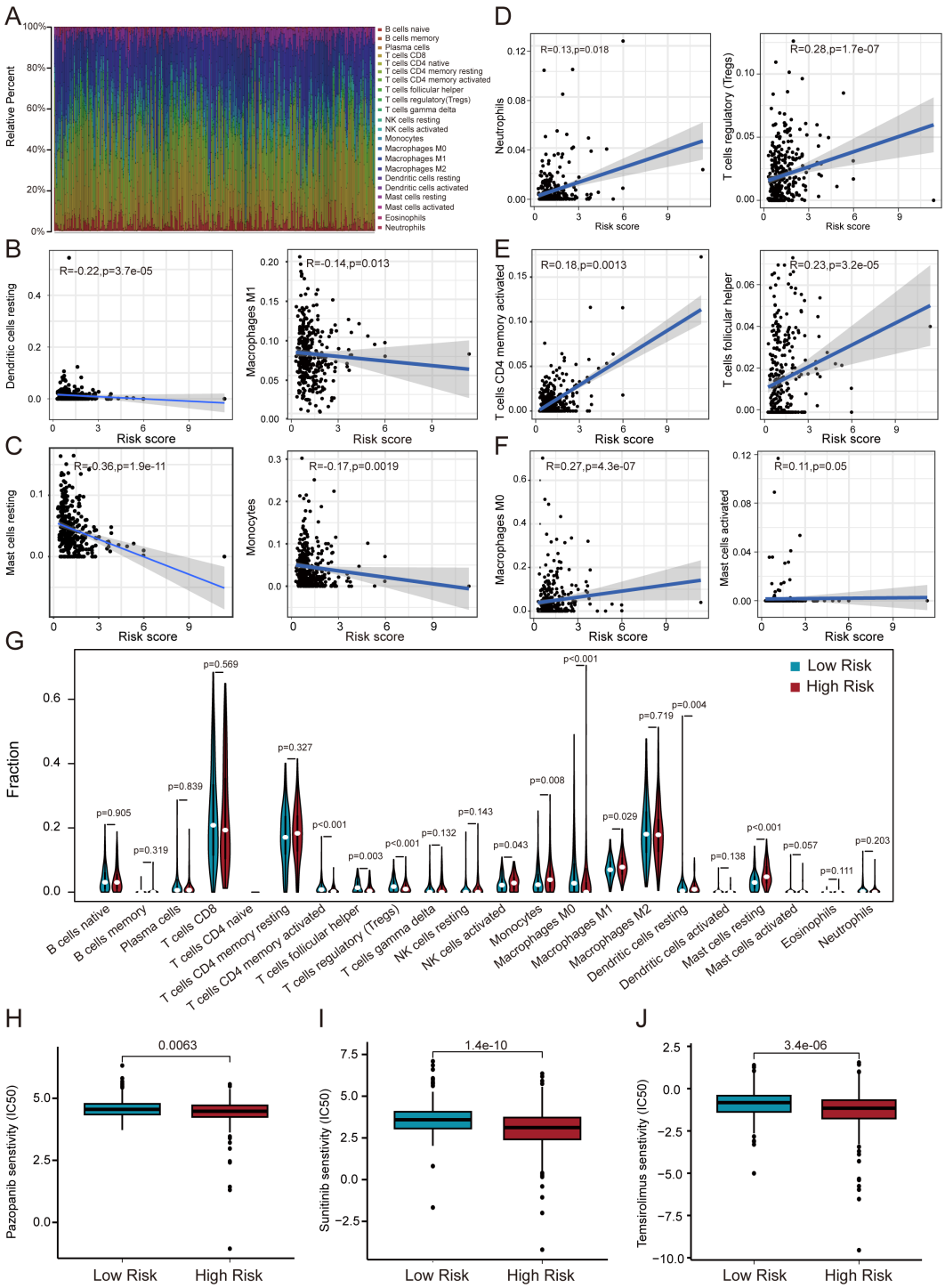

FIGURE 7
Correlation of immune microenvironment with risk score. **(A)** Immune cell infiltration landscape in ccRCC revealed by CIBERSORT. **(B–F)** Linear regression models demonstrate risk score-dependent immune cell infiltration patterns. **(G)** Differential immune cell distribution between risk groups. **(H–J)** Risk-stratified therapeutic sensitivity to pazopanib, sunitinib, and temsirolimus.

High MELK expression is associated with poor prognosis in patients with ccRCC

Based on existing studies, both MELK and EIF4A1 are highly expressed in tumor cells, and high EIF4A1 expression has been confirmed to correlate with poor patient prognosis (28). Elevated MELK (HR=1.46) and EIF4A1 (HR=1.39) expression predicted adverse outcomes, with MELK showing the highest risk association. Based on our analysis, high MELK expression levels correlated with adverse clinical outcomes (Figure 9A). IHC staining

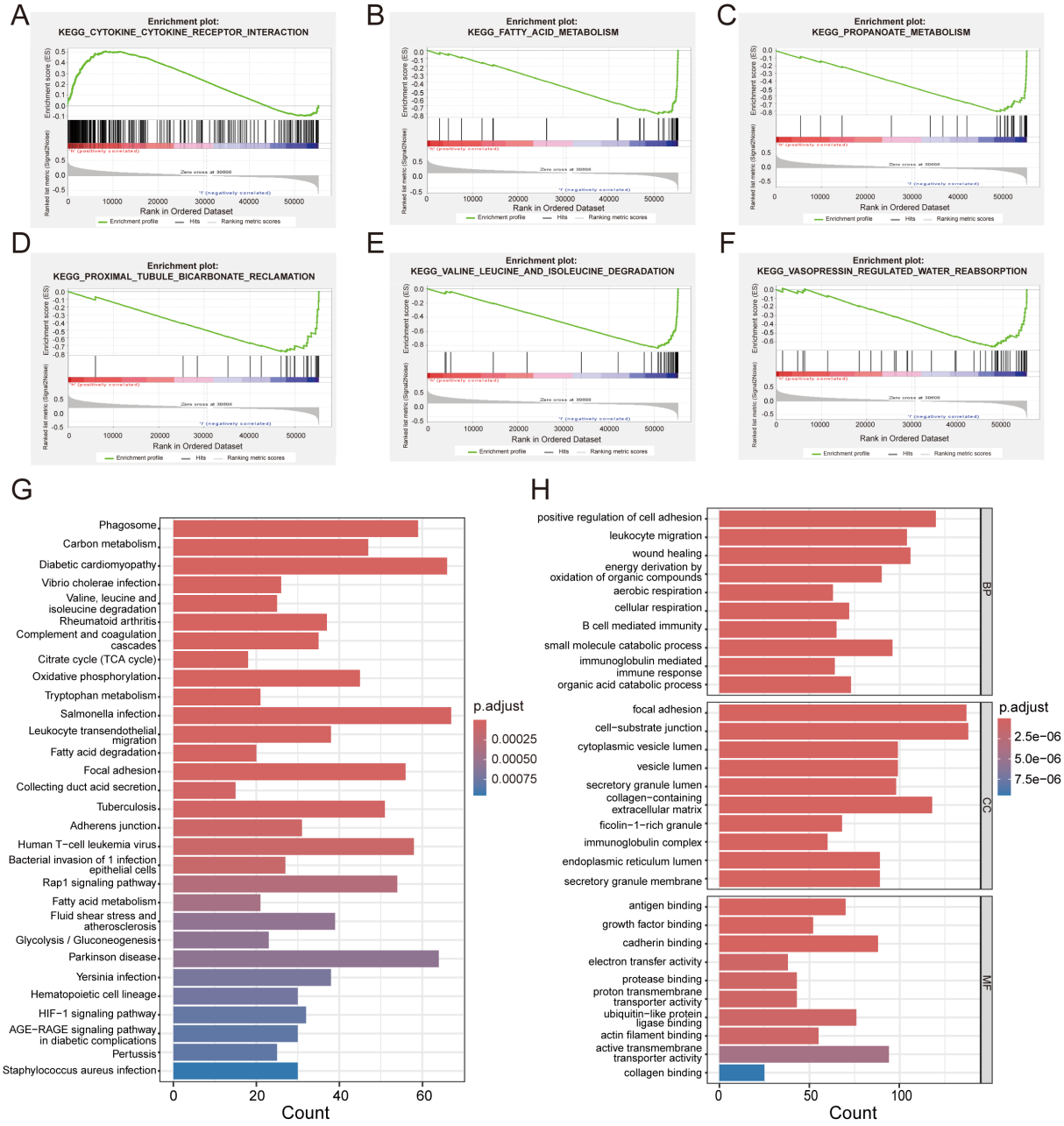

FIGURE 8
Functional enrichment and GSEA analysis. **(A)** Significantly enriched biological pathways in high-risk patients. **(B–F)** Distinct biological pathway enrichment profile in low-risk cohort. **(G)** GO analysis reveals key biological processes of DEGs. **(H)** KEGG pathway enrichment landscape of DEGs.

further demonstrated that MELK expression was higher in tumor tissues than in normal adjacent tissues (NAT) (Figures 9B, C), confirming that MELK levels are elevated in tumor tissues. Moreover, MELK levels increased significantly with tumor progression, showing higher expression in advanced-stage compared to early-stage ccRCC (Supplementary Figures S3A–E). Patients in the high-risk category demonstrated markedly elevated MELK expression compared to their low-risk counterparts. (Supplementary Figure S3F). MELK upregulation represents a potential prognostic marker in ccRCC. We selected three ccRCC cell lines (786-O, 769-P, and Caki-1) and transfected these cells with MELK-specific siRNA plasmids. Successful knockdown of MELK was confirmed by Western blotting (Figure 9D). MELK knockdown substantially inhibited colony formation and cell proliferation (Figure 9E). The results of migration assays demonstrated that relative counts of migrating cells were significantly reduced in MELK knockdown groups (Figures 9F–I). This indicates that MELK knockdown significantly suppresses the migratory abilities of 786-O, 769-P, and Caki-1 cells. Collectively, our clinical and experimental data establish MELK as a critical oncogenic driver in ccRCC, whose overexpression correlates with advanced tumor progression, poor prognosis, and enhanced malignant

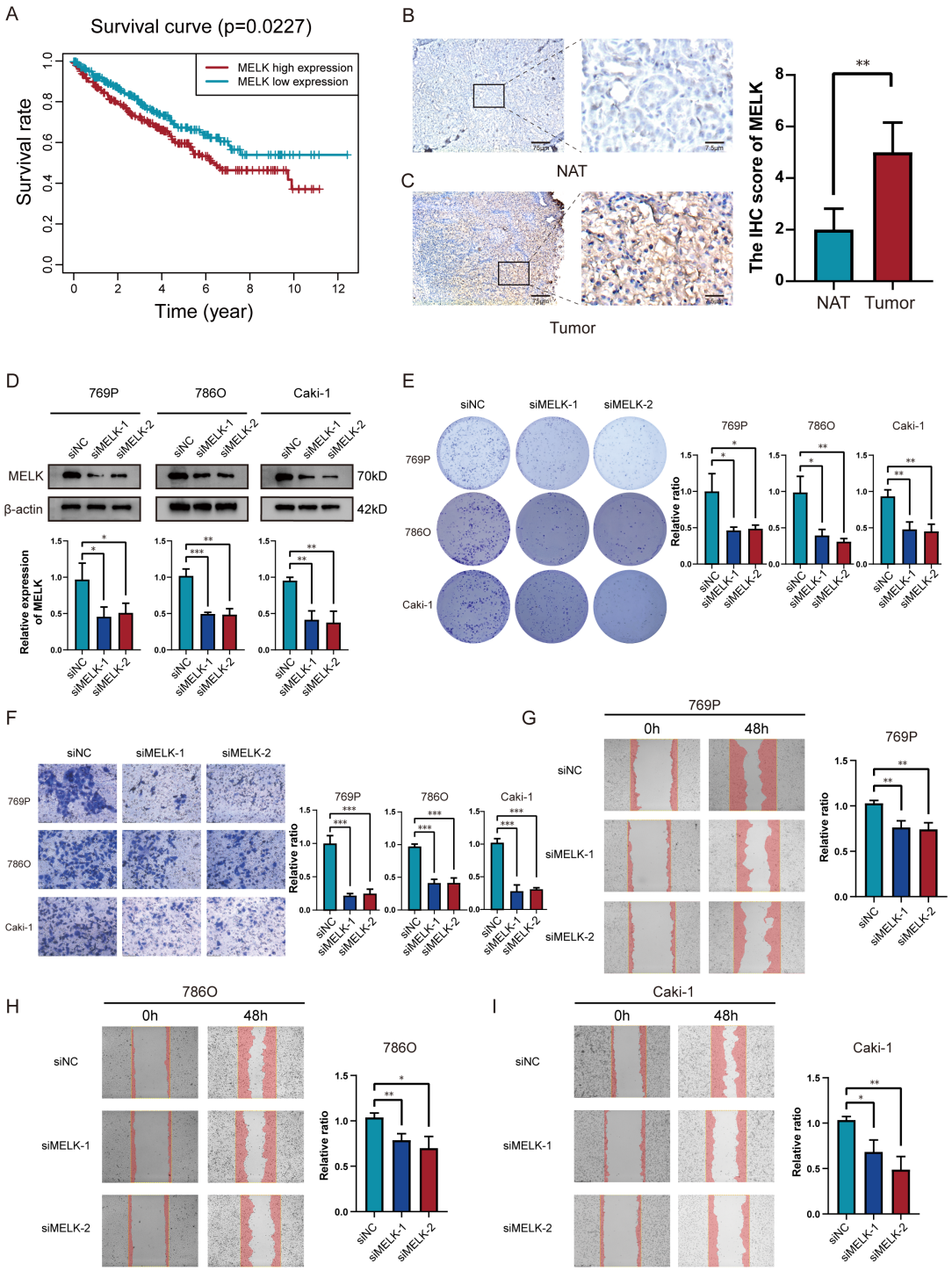

FIGURE 9
MELK is a poor prognostic marker in ccRCC. **(A)** Significant variations in overall survival between ccRCC patients with high and low MELK expression. **(B, C)** Immunohistochemical evidence of MELK overexpression in tumor tissues versus NAT. **(D)** Successful MELK knockdown confirmed by western blot across 769P, 786O and Caki-1 cell lines. **(E)** Silencing MELK suppressed proliferation abilities in 769P, 786O and Caki-1 cells. **(F–I)** Silencing MELK suppressed migration abilities as measured via transwell assay **(F)** and scratch assay **(G–I)** in 769P, 786O and Caki-1 cells. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

phenotypes, while its knockdown potently suppresses tumor aggressiveness, highlighting its potential as both a prognostic biomarker and therapeutic target.

Discussion

As the predominant pathological category of renal carcinoma, ccRCC is notable for substantial heterogeneity and aggressive progression. Despite recent advancements in therapeutic strategies, the prognosis for ccRCC remains poor, particularly for advanced-stage patients (29, 30). Identifying key prognostic genes and constructing robust prognostic models are therefore critical for improving survival rates and guiding personalized treatment (31). Current ccRCC risk stratification methods primarily rely on clinical and pathological features, lacking consideration of tumor molecular mechanisms and the immune microenvironment. This limits their predictive accuracy and ability to provide personalized treatment recommendations. Our study integrates CRISPR-Cas9 gene-editing data from DepMap and transcriptome data from TCGA to construct a prognostic model, which has been further validated in an independent GEO cohort (GSE29609). This model not only enhances the accuracy of risk stratification but also offers more precise clinical guidance through drug sensitivity analysis. The consistent performance across multiple datasets (TCGA and GEO) demonstrates its robustness and generalizability. It helps optimize treatment plans, improve therapeutic outcomes, and reduce medical costs. The DepMap database, a comprehensive resource cataloging genetic dependencies in cancer cell lines, facilitated the identification of genes essential for ccRCC survival through CRISPR-Cas9 knockout screening. By leveraging DepMap's Chronos scores we prioritized genes with significant functional relevance, ensuring that findings were grounded in both *in vitro* experimentation and clinical data (32). This dual-validation approach minimized false-positive results and enhanced the translational potential of the prognostic model. The development of genome-wide CRISPR-Cas9 loss-of-function screening represents a major breakthrough in biological research, offering a powerful tool to dissect gene function in tumorigenesis (33–37). Concurrently, TCGA project has unveiled the complex genomic landscape of ccRCC, including mutations, CNVs, dysregulated gene expression, and immune microenvironment alterations, laying the groundwork for novel diagnostic markers and therapeutic targets. This study integrates TCGA-derived ccRCC data with DepMap CRISPR-Cas9 screening to identify prognostic genes and construct a predictive model, thereby advancing precision medicine strategies for ccRCC.

From DepMap (CERES scores), we identified 116 ccRCC-essential proliferation genes, while TCGA-KIRC analysis uncovered 2,677 DEGs. Intersecting these datasets yielded 11 candidate genes. Subsequent univariate Cox and LASSO regression analyses narrowed the selection to five key genes—GGT6, HAO2, SLPI, MELK, and EIF4A1—whose expression patterns correlated strongly with tumor grade, clinical stage, and metastatic status. KM analysis revealed pronounced survival differences between gene-stratified high-risk and low-risk groups. ROC analysis confirmed the model's superior predictive accuracy compared to conventional clinical parameters (AUC >0.75 for 1–5-year survival), while its age independence underscored its applicability across diverse patient populations. The prognostic model, validated by nomogram calibration and marked survival differences between risk groups, exhibited exceptional performance. Notably, MELK and EIF4A1 were highly expressed in tumor cells. MELK, a serine/threonine kinase implicated in cancer stem cell maintenance and chemoresistance in multiple malignancies, was associated with poor prognosis (38). Similarly, EIF4A1, a translation initiation factor, may drive tumor proliferation by enhancing oncoprotein synthesis, a mechanism observed in other cancers (39).

Further analysis revealed interactions between risk scores and TMB, highlighting their combined prognostic value. Patients with low TMB exhibited improved clinical outcomes, while distinct mutational profiles between high-TMB and low-TMB groups (e.g., VHL mutations in high TMB vs. DNAH9 in low TMB) emphasized the genomic heterogeneity of ccRCC and the need for tailored therapies. TME analysis demonstrated that immune cell infiltration patterns significantly influenced disease progression and treatment response. Anti-tumor immune cells, such as dendritic cells and M1 macrophages, were enriched in low-risk groups, whereas neutrophils and regulatory T cells (Tregs) exhibited pro-tumor activity (40). The immunosuppressive TME in high-risk patients, marked by reduced anti-tumor immunity and increased immunosuppressive cell infiltration, underscores the therapeutic potential of targeting the TME. Drug sensitivity assays validated the model's clinical utility, revealing significant associations with pazopanib, sunitinib, and temsirolimus—agents targeting angiogenesis and mTOR pathways central to ccRCC treatment (41). Enrichment of "cytokine-cytokine receptor interaction" pathways in high-risk tumors further supports the potential of immunomodulatory therapies to counteract pro-tumor inflammation.

GSEA uncovered divergent signaling pathways between risk groups. High-risk patients exhibited enrichment in cytokine-related pathways linked to tumor progression and immune evasion, while low-risk patients showed metabolic pathway activation, suggesting metabolic reprogramming contributes to favorable outcomes. These findings deepen our understanding of ccRCC biology and highlight actionable therapeutic targets. For instance, HAO2, associated with fatty acid metabolism, underscores the role of metabolic dysregulation in driving tumor aggressiveness—a hallmark of ccRCC. HAO2 (glycine oxidase 2) is upregulated in ccRCC and involved in glycine oxidation, impacting cellular energy metabolism and oxidative stress response. Its overexpression may enhance tumor cell proliferation and survival by boosting energy metabolism and antioxidant capacity. Additionally, metabolic pathway alterations can influence immune cell infiltration in the tumor microenvironment, affecting tumor immune evasion (42).

This study establishes a multi-omics-driven prognostic framework for ccRCC, bridging genetic vulnerabilities with

clinical outcomes. The identified genes and pathways not only enhance our mechanistic understanding of ccRCC but also offer translatable strategies for risk stratification and therapeutic innovation. MELK has been pinpointed as a core gene within the constructed prognostic model, playing a pivotal role in the genesis and progression of ccRCC. As a member of the AMPK-related kinase family, MELK is overexpressed in various malignancies including breast cancer, hepatocellular carcinoma, and glioma, where it drives oncogenesis by regulating cell cycle progression, cancer stemness, and therapy resistance (43, 44). Previous studies have demonstrated that MELK is not only crucial for the development of breast and liver cancers, but also contributes to radio- and chemoresistance in patients with hepatocellular carcinoma and glioma (45). Given its oncogenic properties, MELK is currently being investigated as a potential therapeutic target, although its specific impact on ccRCC requires further elucidation. Further validation studies in independent cohorts are warranted to confirm these observations, elucidating downstream signaling mechanisms, and exploring targeted therapies against MELK and EIF4A1 to realize their clinical potential. Among the five prognostic genes, MELK emerged as a central player in ccRCC progression. Our functional studies demonstrated that MELK knockdown potently inhibited tumor cell proliferation, migration and invasion in ccRCC cell lines. These results corroborate prior findings in other cancers, where MELK overexpression promotes tumorigenesis via cell cycle regulation and DNA damage repair. The elevated MELK expression in advanced-stage tumors and its correlation with poor prognosis highlight its potential as a therapeutic target. Notably, the efficacy of pazopanib, sunitinib, and temsirolimus in high-risk tumors suggests that targeting MELK-related pathways may synergize with existing therapies to improve outcomes.

Despite these advances, Certain methodological constraints merit careful consideration. First, the reliance on TCGA data may introduce selection bias, and external validation in independent cohorts is essential to confirm the model's generalizability. Second, while *in vitro* experiments demonstrated MELK's functional role, *in vivo* studies and mechanistic investigations are needed to elucidate its downstream signaling networks. Third, the clinical utility of the nomogram requires prospective validation to assess its impact on therapeutic decision-making.

Future studies should focus on translating these findings into clinical practice. For instance, exploring small-molecule inhibitors targeting MELK or EIF4A1 may open new avenues for precision therapy. Additionally, integrating immune cell infiltration profiles with genomic data could refine immunotherapy selection, particularly for patients with high-risk scores and immunosuppressive TME features.

In conclusion, our study has developed a novel prognostic framework for ccRCC by integrating CRISPR-Cas9 screening data from DepMap and transcriptomic profiles from TCGA. This approach bridges genomic vulnerabilities with clinical outcomes, offering a more comprehensive understanding of ccRCC biology compared to previous models that rely solely on transcriptomic data. The identified genes and pathways not only enhance our insights into the disease but also provide actionable targets for risk stratification and therapeutic development. Furthermore, the identification of MELK as a key driver gene and its association with the immunosuppressive tumor microenvironment highlight new avenues for targeted therapy in high-risk patients. Future validation and functional studies will be critical to realizing the translational potential of these findings and further improving the reliability and clinical applicability of our model.

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 the Medical Ethics Committee of the Second Affiliated Hospital of Harbin 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.

Author contributions

BH: Conceptualization, Writing – review & editing, Writing – original draft, Investigation. WL: Data curation, Writing – original draft, Writing – review & editing. WW: Writing – original draft, Methodology, Writing – review & editing. ZL: Software, Investigation, Writing – review & editing. BY: Funding acquisition, Resources, Writing – review & editing. DL: Writing – review & editing, Conceptualization. YN: Formal Analysis, Writing – review & editing. TD: Data curation, Writing – review & editing. ZD: Investigation, Writing – review & editing. YZ: Methodology, Writing – review & editing. WZ: Writing – original draft, Supervision, Writing – review & editing. QL: Writing – review & editing, Writing – original draft, Supervision, Validation. XL: Writing – original draft, Writing – review & editing, Funding acquisition, Visualization.

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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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The author(s) declare that no Generative AI was used in the creation of this manuscript.

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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.2025.1619361/full#supplementary-material

SUPPLEMENTARY FIGURE 1
Consensus clusters by 7 DEGs. **(A)** Cumulative distribution function (CDF) plot demonstrating consensus clustering stability. **(B)** Delta area plot showing relative changes in CDF curve area for each k value. **(C)** Consensus clustering matrix identifies two distinct molecular subtypes (k=2). **(D)** Cluster stability assessment. **(E)** Transcriptome-wide UAMP reveals inter-cluster divergence. **(F)** Survival disparity between clusters by KM analysis.

SUPPLEMENTARY FIGURE 2
Clinical Evaluation Based on a Risk Score-Derived Prognostic Models. The heatmap **(A)** and scatter plots demonstrate association of the stage **(B)**, N stage **(C)**, T stage **(D)**, M stage **(E)**, gender **(F)**, grade **(G)**, and age **(H)** with the risk score.

SUPPLEMENTARY FIGURE 3
Expression levels and functions of the MELK gene. Box plots of MELK gene expression in different clinical stages. Scatter plots demonstrate that T stage **(A)**, N stage **(B)**, M stage **(C)**, grade **(D)**, and stage **(E)**.Violin plots show the differential expression of the MELK gene between the high - risk and low - risk groups **(F)**.

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Immunosuppressive cells in acute myeloid leukemia: mechanisms and therapeutic target

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Immunotherapy has emerged as a cornerstone strategy for augmenting therapeutic efficacy in acute myeloid leukemia (AML). The immunosuppressive AML microenvironment, characterized by profound immune dysfunction, critically impairs anti-leukemic immune surveillance. This immunologically hostile niche is principally governed by specialized immunosuppressive cell populations—notably regulatory T cells (Tregs), myeloid-derived suppressor cells (MDSCs), leukemia-associated macrophages (LAMs), and regulatory B cells (Bregs)—which collectively establish an immune-privileged sanctuary for leukemic cells. This review critically examines three fundamental aspects of these immunosuppressive regulators in AML pathogenesis: (1) their recruitment dynamics within the leukemic niche, (2) the molecular mechanisms underlying their immunosuppressive functions, and (3) current and emerging therapeutic approaches designed to neutralize their inhibitory effects. Through this comprehensive analysis, we aim to provide a mechanistic framework for developing more effective immunotherapeutic interventions against AML.

KEYWORDS

acute myeloid leukemia, regulatory T cells, regulatory B cells, myeloid-derived suppressor cells, leukemia-associated macrophages, leukemia-associated neutrophils

1 Introduction

Acute myeloid leukemia (AML) is a highly aggressive hematologic malignancy characterized by uncontrolled clonal proliferation of immature myeloid cells, resulting in the accumulation of abnormal blast cells in the bone marrow (BM) and impairment of normal hematopoietic function (1). AML is the most prevalent form of leukemia in adults, with an annual incidence rate of approximately 3 to 5 cases per 100,000 individuals (2–4).

AML patients typically have a poor prognosis, marked by a short survival time and unsatisfactory clinical outcomes. AML is a profoundly heterogeneous hematologic malignancy with multifaceted pathophysiology involving: genomic instability and mutational accumulation, oncogenic fusion events, epigenetic reprogramming, immune dysregulation and inflammatory cascades, apoptosis resistance mechanisms, metabolic pathway derangements, cellular senescence evasion, growth suppression circumvention, and sustained proliferative signaling (5–12).

Current AML treatment strategies include conventional chemotherapy, targeted therapies (FLT3/IDH/BCL-2 inhibitors), hematopoietic stem cell transplantation, and emerging immunotherapies (CAR-T, checkpoint inhibitors) with microenvironment-modulating approaches (13). Although advancements in treatment have led to improvements in AML prognosis, challenges such as chemoresistance, relapse, and refractory disease persist as significant barriers (14).

Emerging evidence underscores the pivotal role of bone marrow niche dysregulation in AML pathogenesis (9, 10, 15). During disease progression, the microenvironment undergoes profound cellular and functional remodeling, creating a permissive ecosystem that sustains leukemic cell survival (16). Notably, the AML microenvironment exhibits prominent immunosuppressive characteristics (17). Key immunosuppressive cell populations—including regulatory T cells (Tregs), myeloid-derived suppressor cells (MDSCs), leukemia-associated macrophages (LAMs), regulatory B cells (Bregs) and leukemia-associated neutrophils (LANs)—employ diverse mechanisms to facilitate immune evasion by leukemic cells. Therapeutic targeting of these immunosuppressive populations represents a promising strategic approach for AML immunotherapy. A comprehensive understanding of the regulatory networks of these immunosuppressive cells is crucial for developing novel immunotherapeutic strategies. This review provides a comprehensive analysis of the role and mechanisms of crucial immunosuppressive cells within the AML microenvironment, including Tregs, MDSCs, LAMs, Bregs and LANs, to serve as a reference for future research in this field.

2 The famous immunosuppressive cell: regulatory T cell

2.1 The phenotype of Treg

Tregs represent a heterogeneous population of T cells, exhibiting diverse origins, phenotypes, and effects. The traditional classification of Tregs comprises two primary subsets: thymic Tregs (tTregs), also referred to as natural Tregs (nTregs), and peripheral Tregs (pTregs), alternatively known as induced Tregs (iTregs) or adaptive Tregs (aTregs), depending on their distinct sources (18). In the thymus, a subset of CD4 single-positive autoreactive cells successfully undergo negative selection by expressing FOXP3, leading to their differentiation into thymic Tregs (tTregs). These tTregs make up approximately 5% to 10% of CD4+ T cells present in the peripheral blood (PB) (19, 20). pTregs are generated from naive CD4+ T cells in the peripheral tissues in response to various stimuli, including antigens, as well as factors like TGF-β and IL-2 (21, 22). Interestingly, Treg cells display a relatively anergic state and are unable to produce IL-2 due to the transcriptional repressive effects of FOXP3 (23), despite the fact that IL-2 is essential for the generation, survival, and activation of Tregs (24). Aside from the conventional CD4+ Treg cells mentioned previously, several other T cell subsets have been identified to possess immunosuppressive capabilities. These include CD8+ T cells (25), IL-17+ Treg cells (26), ICOS+ Treg cells (27), Type II NKT cells (28, 29), and γδT cells (30). A comprehensive summary detailing the phenotypes of T cells exhibiting regulatory properties can be found in Table 1.

Currently, the primary markers employed for the identification of conventional Tregs are $CD25^{high}$, $CD127^{low/-}$, and FOXP3+ (31). Furthermore, several supplementary molecules, including CD45RA (32), CD39/CD73 (33), CD26 (34), CD6 (35), NRP-1 (36), TIM-3 (37), and others (38), can serve as surface markers for Tregs.

2.2 Treg accumulation and its mechanisms in AML

Numerous studies have demonstrated an elevated frequency of Tregs in the BM and PB of AML patients. The heightened accumulation of Tregs within the AML microenvironment not only facilitates the development and advancement of AML but also amplifies treatment resistance and the likelihood of relapse.

2.2.1 Elevated Tregs observed in AML occurrence, drug resistance, and relapse

Elevated percentages of Tregs contribute to the establishment of an immunosuppressive microenvironment in AML, providing favorable conditions for the survival and proliferation of malignant AML cells. Consequently, this immunosuppressive milieu plays a facilitating role in the progression and pathogenesis of the disease. Wang et al. discovered that individuals newly diagnosed with AML exhibited an increased proportion of CD4 +$CD25^{high}$ Tregs in both PB and BM. Notably, these Tregs displayed a more robust state of renewal, characterized by heightened rates of proliferation and apoptosis, when compared to healthy donors (39). The elevated presence of Tregs in newly diagnosed AML patients results in a reduced ratio of Th17/Treg cells. This finding confirms the immunosuppressive polarization of the bone marrow microenvironment in AML (40). In the PB of AML patients, circulating T follicular regulatory cells (cTfr), defined as CD4+CXCR5+PD-1+FOXP3+, were elevated, indicating increased suppression of B cell responses (41). Additional studies have consistently identified greater proportions of Tregs in the BM and PB of patients diagnosed with AML compared to healthy control subjects (42, 43). These findings underscore the abundant presence of Tregs in AML and their role in establishing an immunosuppressive microenvironment. Contrary to previous beliefs, a recent report suggests that the proportion of Tregs in the BM is similar between individuals with AML and healthy donors. However, it was observed that AML patients exhibit

TABLE 1 Phenotypes of T cells with regulatory properties.

Cell type			Phenotype	Reference
CD4+	nTreg		CD4+CD25+FOXP3+CTLA-4+CD45RO+CD127^{low}	(225)
	iTreg	Th3	CD4+CD25±FOXP3±CD45RO+CTLA-4+	(226)
		Tr1	CD4+CD25±FOXP3±CD45RO+CTLA-4−	
		TGF-β/IL-10 double-positive Treg	CD4+CD25−FOXP3−	
		IL-17+ Treg	CD4+CCR9+CD25+CD127$^{dim/-}$	(227)
CD8+			CD8+FOXP3+	(228)
			CD8+CD103+	
			CD8+CD28−	
			CD8+CD122+CD49d+	
			CD8+CD122^{high}Ly49+	
γδT cell			FOXP3+TCRγδ+	(51, 52)
CD4-CD8- double negative Treg			TCRαβ+/γδ+CD3+CD4−CD8−NK1.1−	(229, 230)
Type II NKT cell			CD3+CD56+CD161+TCRγδ−TCRVα7.2−TCRVα24−	(29, 231)

higher proportions of effector Tregs (CD45RA− Tregs). Furthermore, the study found a significant increase in PD1 +/TIGIT+ Tregs in the BM of AML patients with a high leukemia burden (44). This suggests that the AML microenvironment may intensify the regulatory function of Tregs, and the number of Tregs present is influenced by the extent of leukemia burden.

In addition to the involvement in pathogenesis, Tregs have also been demonstrated a connection to chemotherapy resistance and disease relapse. Szczepanski et al. conducted a study that reaffirmed the observation of elevated percentages of Tregs and their suppressive activity in the PB of AML patients. Remarkably, the study found that patients with a lower frequency of Tregs at the time of diagnosis exhibited a more positive response to induction chemotherapy (45). Ersvaer et al. observed persistent high frequency of Tregs in AML patients both prior to chemotherapy and throughout the period of cytopenia induced by intensive chemotherapy. Additionally, these proportions remained elevated during the regeneration phase following treatment (46). Moreover, several other research groups have reported an increase in Treg expansion in the PB during the recovery of lymphocytes after intensive chemotherapy and during cytotoxic maintenance chemotherapy (47, 48). Several studies have indicated that patients with AML who achieved complete remission (CR) experienced a notable decrease in Treg frequency compared to those at the time of diagnosis (42, 49), and Zhang et al. further observed a sudden increase in Tregs during relapse, suggesting that monitoring Treg frequency after achieving CR could serve as a valuable predictor of relapse (49). Additionally, findings from a phase IV clinical trial (NCT01347996) revealed that the accumulation of Tregs in the PB as a result of immunotherapy with HDC/IL-2 is associated with the risk of relapse in AML. In cycle 3 of the treatment, a decrease in Treg accumulation was indicative of a lower risk of relapse, supporting the notion that the prolonged presence of Tregs may adversely affect the prognosis of AML (50). Strikingly, in Szczepanski's study, patients who achieved CR still maintained an increased frequency of Tregs, which was counterintuitive and inconsistent with the observations of other researchers. They proposed an interesting conclusion that Tregs are resistant to conventional chemotherapy (45). In addition to the conventional Tregs, studies have also shown that γδ Treg cells are increased in AML patients and correlated with unfavorable clinical outcomes (51, 52). Therefore, the assessment of Treg frequency holds considerable importance in understanding the progression of leukemia, treatment response, and prognosis in AML patients. A compilation of studies focusing on Treg accumulation in AML can be found in Supplementary Table S1.

2.2.2 Accumulation mechanisms of Tregs in AML microenvironment

Numerous studies have elucidated the mechanisms underlying the accumulation of Tregs within the microenvironment of AML. These well-established mechanisms encompass the secretion of specific factors, interactions between receptors and ligands, chemotactic effects, and metabolic advantages (Figure 1). Subsequently, we will delve into each of these mechanisms in detail.

Recent findings have revealed that extracellular vesicles (EVs) derived from AML cells and containing 4-1BBL play a pivotal role in augmenting the expression of FOXP3 and the effector phenotype in Tregs, thereby bolstering their activity. Treg cells actively internalize EVs carrying the costimulatory ligand 4-1BBL, resulting in the upregulation of STAT5 and the suppression of mTOR-S6 signaling. Consequently, this process promotes the immunosuppressive effector Treg cells (53). In addition, miR-21 originating from AML-derived EVs has been demonstrated to promote the expression of genes recognized as markers for Tregs and immunosuppression. These genes include *IL-10*, *FOXP3*,

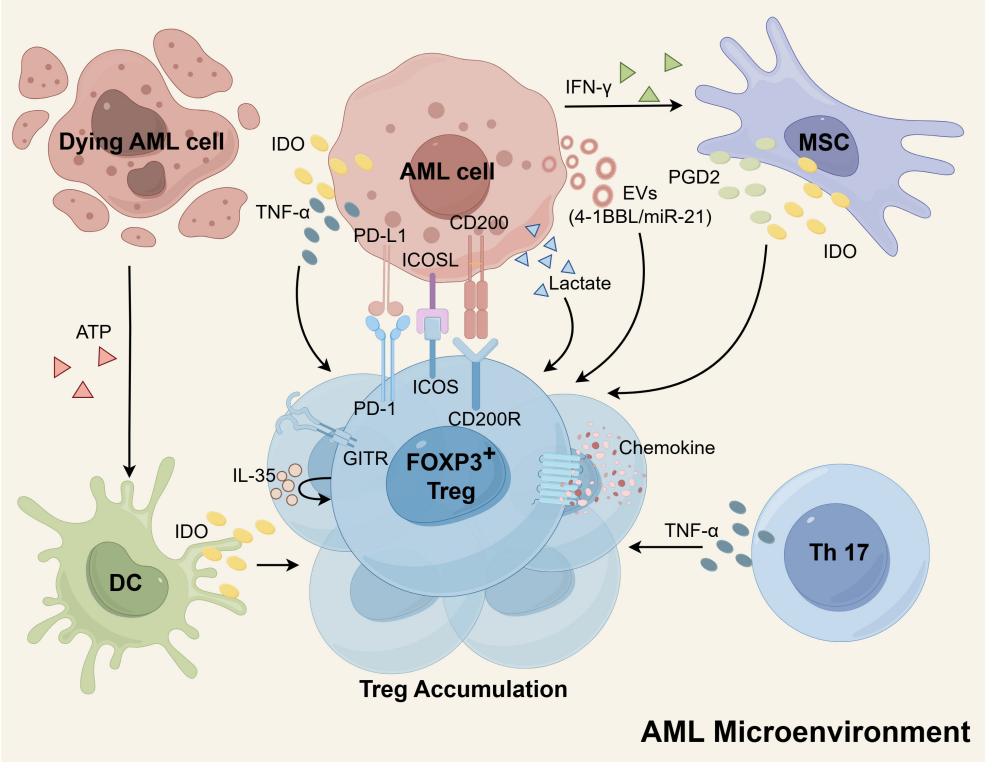

FIGURE 1
The mechanisms of Treg cells accumulation in the AML microenvironment. The secretion of EVs by AML cells plays a role in increasing Tregs, as these EVs contain molecules such as miR-21 and 4-1BBL that promote Treg expansion. Additionally, AML cells, DCs, and MSCs can produce IDO, which induces proliferation of Tregs. MSCs also release PGD2 to enhance Treg numbers. Both Th17 cells and AML cells express TNF-α, which supports the expansion of Tregs. Furthermore, Tregs themselves express high levels of IL-35, which can further amplify Treg proliferation. The interaction between AML cells and Tregs through receptor-ligand interactions, including PD-L1/PD-1, ICOSL/ICOS, and CD200/CD200R, also promotes Treg expansion. Tregs possess enhanced chemokine receptors, facilitating robust migration and contributing to their aggregation. Moreover, Tregs have a metabolic advantage as they can utilize lactate for metabolism, indirectly contributing to their accumulation. Schematic figure was drawn by Figdraw (www.figdraw.com).

CTLA-4, and others. Intriguingly, the transfer of miR-21 into leukemia-infiltrating T lymphocyte cells yielded the acquisition of a Treg cell phenotype, accompanied by a notable increase in FOXP3 levels in AML (54).

Indoleamine 2,3-dioxygenase (IDO) is an enzyme with immunomodulatory properties that facilitates the conversion of tryptophan (Trp) into kynurenines (Kyn). These Kyn metabolites have the ability to promote the generation of Treg (55). The generation of this inducible Treg can be significantly hindered by the IDO inhibitor, 1-methyl tryptophan (1-MT) (56, 57). Arandi et al. revealed that elevated expression of IDO in patients with AML may contribute to an increase in the number of Treg (58). Furthermore, *in vitro* studies have demonstrated the presence of functionally active IDO proteins within AML cells, which have the capability to stimulate the proliferation of Treg (56, 59). In a study by Curti et al., it was reported that a notable proportion of primary blast cells derived from adult patients with AML constitutively express the active form of IDO protein (60). Conversely, a multicenter study involving pediatric AML patients indicated that blast cells do not exhibit constitutive expression of IDO protein. However, functional IDO protein was found to be upregulated in approximately half of the AML samples in response to IFN-γ stimulation (61). IDO is an IFN-γ-inducible enzyme, whose expression is transcriptionally activated through the JAK-STAT1 signaling pathway in coordination with the transcription factor IRF1 (62). These studies suggest that regardless of whether IDO protein is constitutively expressed or induced, it is evident that AML cells have the capability to produce and release IDO protein. This leads to an elevation of IDO concentration within the microenvironment, consequently promoting the expansion of Treg. Additionally, dendritic cells (DCs) are known to express functional IDO protein, which can hinder the T-cell response by facilitating the expansion of Tregs (62). DCs derived from AML cells have been suggested as potential leukemia vaccines due to their increased immunogenicity. However, one challenge is that these DCs show upregulation of IDO, which can negatively impact immune responses by activating powerful Tregs (63). Clinical sample analysis has demonstrated that adenosine triphosphate (ATP) released by dying AML cells, specifically those targeted by chemotherapy, plays a role in the induction of Tregs. The release of ATP from AML cells treated with chemotherapy leads to the upregulation of IDO1 in DCs. These DCs, in turn, are fully capable of inducing Tregs through the IDO1 pathway *in vitro* (64). Moreover, bone marrow mesenchymal stem cells (MSCs)

derived from AML patients exhibited considerable upregulation of IDO and released heightened levels of PGD2. These factors collectively contributed to the expansion of Tregs (65, 66). PGD2 derived from MSCs engages the receptor CRTH2 on type 2 innate lymphoid cells (ILC2s) to promote the overproduction of IL-5, which specifically expands CD4+CD25+IL5Rα+ Tregs (66). Furthermore, experimental evidence has shown that the release of IFN-γ by AML cells *in vitro* triggers the upregulation of IDO expression in MSCs. Consequently, this upregulation contributes to the proliferation of Tregs (67, 68).

In AML patients, abnormally high levels of TNF-α secreted by Th17 cells promote Treg proliferation through the TNF-α receptor 2 (TNFR2) pathway expressed by Tregs (69). Additionally, AML blast cells also generate significant quantities of TNF-α, which have the potential to induce the proliferation of Tregs by upregulating the expression of TNFR2 and FOXP3 on T cells (70, 71). Further research has shown that TNF-α binding to TNFR2 activates the p38 MAPK signaling pathway, which upregulates the surface expression of TNFR2 and Foxp3 on Tregs, thereby driving their proliferation and expansion (72, 73). Azacitidine combined with lenalidomide or panobinostat therapy can reduce TNFR2+ Tregs *in vivo*, which may contribute to the maintenance of clinical remission (70, 74). Previous reports indicate that azacitidine promotes Treg expansion by hypomethylation of the CpG island associated with the promoter of the FOXP3 gene (75, 76). This potentially contradictory finding can be explained by several reasons. First, the combined drugs, lenalidomide or panobinostat, might reverse this effect of azacitidine. *In vitro* studies have provided evidence that lenalidomide can decrease the expression of FOXP3 and inhibit the expansion of Tregs mediated by IL-2 (77). Similarly, studies have shown that administering low doses of panobinostat can lead to a reduction in FOXP3 expression and Treg frequency (78). Additionally, azacitidine treatment indirectly decreases TNFR2+ Tregs by reducing the population of residual blast cells, as blast cells secrete TNF to stimulate Treg expansion (70, 79). Furthermore, within the AML microenvironment, Tregs express elevated levels of IL-35, which can further contribute to the expansion of Tregs themselves (80).

The expansion of Tregs is facilitated by the interaction between AML cells and Treg cells through receptor-ligand interactions. This includes the interaction of PD-L1 (B7-H1) on the surface of AML cells with PD-1 on Tregs, as well as the ICOSL/ICOS and CD200/CD200R interactions. The expression of PD-L1 on AML cells increases the population of PD1+ Tregs and suppresses anti-leukemia immunity (81, 82). The PD-L1/PD-1 pathway has been found to have a role in driving the conversion of naive T cells into FOXP3+ Tregs by antagonizing the Akt-mTOR signaling pathway (82). Blocking the PD-L1/PD-1 signaling pathway using anti-PD-L1 antibodies has been shown to reduce Treg production and delay the progression of AML in mouse models (83, 84). Han et al. revealed that AML cells possess the ability to express ICOSL, which interacts with ICOS on the surface of Tregs and fosters their proliferation. Through the utilization of an antibody targeting ICOSL, they successfully impeded the generation of ICOS-positive Tregs and effectively retarded the advancement of AML in a murine model (85). Studies have reported that elevated levels of CD200 expression in AML blasts promote the induction of Tregs (86, 87). Inhibition of the interaction between CD200 and its receptor CD200R has been shown to decrease the intensity of FOXP3 (87). Research has demonstrated that the GITR plays a role in promoting the differentiation and expansion of Tregs (88). Furthermore, studies have indicated that surface expression of GITR is increased in Treg of AML patients (45). However, further studies are needed to determine if and how GITR can promote Treg accumulation in AML. Zhou et al. found that Gal-9 defective mice were more resistant to AML cells than wild-type mice, which was associated with less Treg accumulation, hinting that Gal-9 on AML cells may be engaged in expansion of Treg (89). The Gal-9/TIM-3 signaling pathway has been found to contribute to excessive proliferation and activation of Treg cells in chronic lymphocytic leukemia (CLL) (90). Additional evidence is required to determine if a similar role exists in AML.

The expression of chemokine receptors has been demonstrated to play a role in the excessive accumulation of Tregs (91, 92). Specifically in AML, there is an increased presence of TNFR2+ Tregs, which exhibit a heightened capacity for migration towards the BM (74). Additionally, study has reported that the frequencies of Tregs in the BM are significantly higher compared to PB in the same patients with AML (49). *In vitro* research has also demonstrated that AML-induced DCs exert a significant chemotactic effect on Tregs, which may contribute to the accumulation of Tregs at the site of leukemia (93). Tregs in AML have been shown to display strong migration towards the BM due to their increased expression of the chemokine receptor CXCR4 (43). It has been found that blocking the CCL3-CCR1/CCR5 and CXCL12-CXCR4 axes can slow down AML progression by inhibiting the migration of Tregs into the leukemic hematopoietic microenvironment (94).

Additionally, the metabolic profile of Tregs provides them with a competitive advantage, indirectly promoting aggregation. The hypermetabolic state of tumor cells creates a low-glucose and lactate-rich microenvironment, which is unfavorable for immune effector cells. Tregs possess the ability to reprogram their metabolic profile by regulation of FOXP3, thereby conferring upon them a metabolic edge and enhanced adaptive capacity within this environment (95). In the B16-F10 melanoma mouse model, tumor-infiltrating Treg cells have the capability to utilize lactate as a source of energy to sustain their proliferation and functional activity in a glucose-deficient environment (96). Consistent with this, higher lactate concentrations were observed in BM of AML (97). Zhang et al. reaffirmed the contribution of AML cells to the lactate-rich TME, and then they employed the lactate transporter inhibitor Syrosingopine to reduce lactate production, which resulted in a reduction of Treg. Based on these findings, the researchers concluded that lactate produced by AML cells actively promotes the aggregation of Treg cells (44). Additionally, Tregs in AML displayed an enrichment of pathways linked to fatty acid metabolism, providing further evidence that Tregs have the capacity to enhance energy production through the utilization of fatty acids present in their surrounding environment (98).

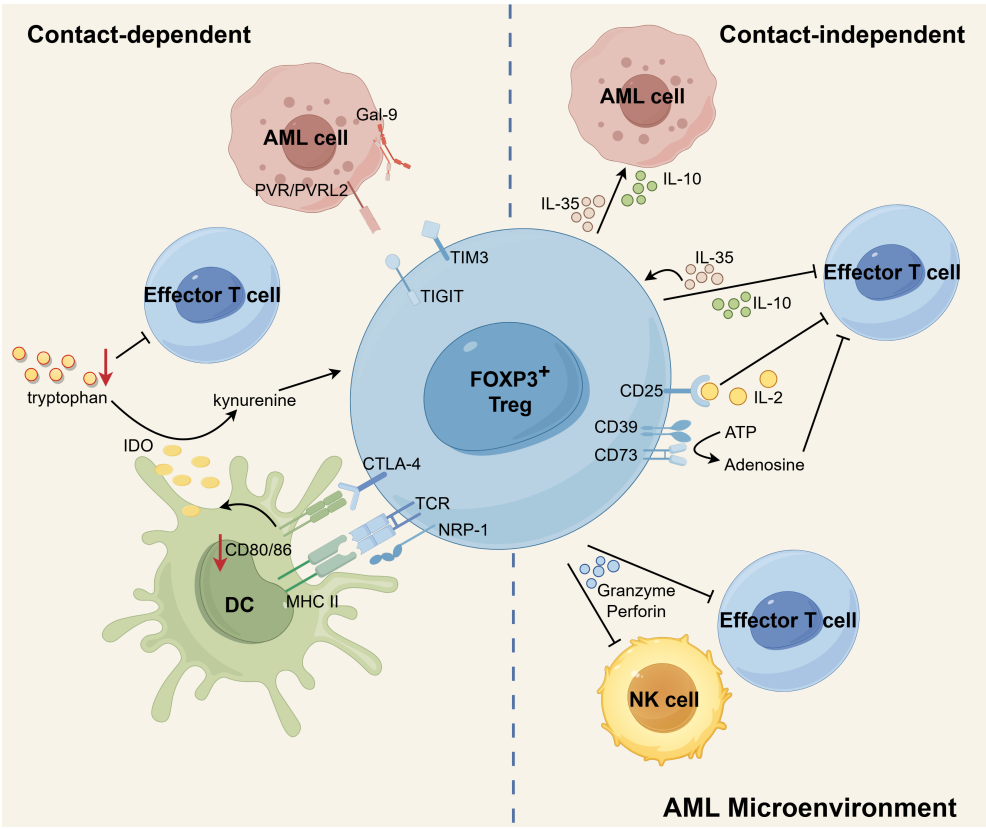

FIGURE 2
The immunosuppressive mechanisms of Tregs in AML microenvironment. CTLA-4 expressed by Tregs binds to CD80/86 on DCs, leading to inhibition of co-stimulation of Teffs, downregulation of CD80/86 on DCs, and elevated expression of IDO in DCs. By degrading tryptophan to kynurenines, IDO contributes to the induction of Tregs and the suppression of T-cell responses. Additionally, NRP1 prolongs the MHC-II molecule-dependent interactions between Tregs and DCs, which effectively restricts the recruitment of MHC-II peptides to immune synapses, ultimately inhibiting immune responses. Treg-derived IL-10 diminishes anti-leukemia immunity by suppressing the activity of Teffs. IL-35 released by Treg can suppress Teff functions and proliferation while also expanding a population of inducible Tregs. IL-10 and IL-35 also stimulate the proliferation of AML blasts. Additionally, Tregs can induce cell death in NK and Teff cells by utilizing granzyme and perforin. CD25, expressed on Tregs, allows for continuous uptake of IL-2, leading to the cytokine deprivation-induced apoptosis of Teff cells. Tregs express membrane surface enzymes CD39 and CD73, which can hydrolyze ATP to generate adenosine. Adenosine, in turn, inhibits cytokine production and proliferation of Teff cells, further contributing to the suppressive function of Tregs. In addition, the possible existence of TIGIT-PVR/PVRL2 and TIM3-Gal9 signaling pathways between Tregs and AML cells may contribute to a propensity for leukemia progression. Schematic figure was drawn by Figdraw (www.figdraw.com).

2.3 The immunosuppressive mechanisms of Treg in the AML microenvironment

Tregs play a pivotal role in the inhibition of immune effector cells, ultimately leading to the impairment of anti-leukemia immune responses in AML. Tregs achieve this immunosuppressive effect through two ways: cell-to-cell contact and contact-independent pathways (Figure 2). The contact-dependent mechanism primarily involves intricate receptor-ligand interactions between cells, while the contact-independent mechanism predominantly relies on cytokine secretion and other non-secretory means. Subsequently, this section will provide an elaborate elucidation of how Treg cells effectively suppress immune effector cells in AML by employing these two mechanisms.

2.3.1 Contact-dependent mechanism

Contact-dependent immunosuppression heavily relies on the interaction between surface molecules expressed by Tregs and other cells. Notably, investigations have revealed that Tregs in AML enhance the expression of specific suppressive surface molecules. In particular, Tregs derived from individuals with AML have demonstrated elevated levels of CTLA-4 expression (45, 50). The expression of CTLA-4 by Tregs hinders the co-stimulation of effector T cells (Teffs) by outcompeting CD28 for binding to CD80/86 on antigen-presenting cells (APCs) (99). Additionally, CTLA-4 on Tregs downregulates the expression of CD80/86 on DCs, thereby impeding the activation of Teffs (99, 100). Furthermore, the interaction between CTLA-4 and CD80/86 triggers an upregulation of IDO in DCs (62, 101). IDO, in turn, degrades tryptophan within the microenvironment, leading to the suppression of T-cell responses (102) and the generation of Tregs (55). In acute leukemia patients, there is an observed increase in the expression of NRP-1 on Tregs. Interestingly, the introduction of exogenous Sema3A, which serves as a ligand for NRP-1, can effectively downregulate NRP-1 expression on Tregs and facilitate the apoptosis of leukemia cells (103). Notably, NRP-1 is highly

expressed on intratumoral Tregs (104), and enables prolonged interactions between Tregs and DCs that are dependent on MHC-II molecules. This, in turn, restricts the recruitment of MHC-II peptide complexes to immune synapses, ultimately impeding immune responses (105).

There are some studies implicating that Tregs may interact with AML cells through TIGIT and TIM-3 to help them escape immune surveillance. TIGIT, as a co-inhibitory receptor, was found to be ubiquitously expressed on the Tregs in AML (44, 53). The activation of TIGIT signaling leads to the upregulation of suppressive genes (such as *Pdcd1*, *IL10*, *Prf1*, and *Havcr2*) in TIGIT-positive Tregs, resulting in the manifestation of a highly activated suppressive phenotype (106). Stamm et al. conducted a study demonstrating that AML cell lines and patient samples exhibit high expression levels of the TIGIT ligands, PVR and PVRL2, which correlates with a poor prognosis. They further revealed that blocking PVR/PVRL2 on AML cells or inhibiting TIGIT on immune cells enhances the anti-leukemic effects *in vitro* (107). Moreover, TIGIT+ Tregs were found to upregulate the expression of the co-inhibitory receptor TIM-3, suggesting a collaborative suppression of antitumor responses by TIM-3 and TIGIT (106). Indeed, it was observed that TIM-3+ Treg cells significantly increased in *de novo* AML patients (108). High levels of Gal-9 (the ligand of TIM-3) were also observed on leukemia blasts in AML samples (109, 110). Interestingly, TIM-3 is also expressed on leukemic stem cells in AML (111, 112), and even Gal-9 has been shown to be expressed on activated Treg (113). These studies illustrate that Gal-9 and TIM-3 may engage in complex interactions within the AML microenvironment.

2.3.2 Contact-independent mechanism

Cytokines, granzyme and perforin are involved in a contact-independent mechanism (40, 80, 114). Newly diagnosed AML patients have been found to exhibit heightened levels of Treg-associated cytokines, specifically IL-10 and IL-35 (115). The immunosuppressive factor IL-10, derived from Tregs, plays a crucial role in diminishing anti-tumor immune responses by suppressing the activity of Teffs and APCs (116). IL-35 has the ability to suppress the functions and proliferation of Teffs, while simultaneously promoting the expansion of inducible Tregs (117, 118). In the AML microenvironment, both IL-10 and IL-35 not only exert inhibitory effects on immune cells but also contribute to the stimulation of AML blast proliferation. The highly expressed cytokine IL-10 by Tregs has been shown to enhance the stemness of AML cells by activating the PI3K/AKT signaling pathway. In AML/ETO c-kit^{mut} (A/Ec) leukemia mice, blocking the IL10/IL10R/PI3K/AKT signaling pathway extended their survival and significantly reduced the stemness of A/Ec leukemia cells. Furthermore, a positive correlation was found between the proportion of Tregs and leukemia stem cells (LSCs) in patient samples. AML patients with high Treg infiltration also exhibited stronger activation of the PI3K/AKT pathway in CD34+ primary AML cells (119). Additionally, IL-35 has been shown to directly promote the proliferation of AML blasts and inhibit their apoptosis (80). The expression of perforin and granzyme B is upregulated in Tregs of patients with AML compared to healthy individuals. Additionally, Tregs in AML patients have been shown to exert immunosuppressive effects by utilizing perforin and granzyme B (45). Tregs have the ability to induce apoptosis in natural killer (NK) cells and CD8+ T cells by utilizing granzyme B and perforin. Research indicates that mice lacking granzyme B show improved efficacy in clearing AML cells in comparison to mice with intact granzyme B functionality. Moreover, when wild-type Treg cells are introduced into granzyme B-deficient mice, there is a discernible suppression of AML clearance (114).

In addition to the secretion, the uptake and enzymatic hydrolysis of factors from the microenvironment also occur independently of contact. The constitutive expression of CD25, which represents high affinity IL-2 receptors, allows Treg cells to continually absorb IL-2. This uptake of IL-2 leads to cytokine deprivation-induced apoptosis of Teff cells (120). Tregs constitutively express the membrane surface enzymes CD39 and CD73. These enzymes have the ability to hydrolyze ATP or ADP, resulting in the production of adenosine. Consequently, the levels of adenosine in the microenvironment are elevated. Adenosine, in turn, interacts with the adenosine receptor A2A on the surface of Teff cells, leading to the inhibition of cytokine production and proliferation (33). Indeed, study has shown that CD39 and CD73 are expressed on CD4+CD25^{high} Tregs isolated from patients with AML. Interestingly, Tregs obtained from AML patients have been shown to have a higher ability to hydrolyze ATP into adenosine compared to Tregs from healthy individuals (45).

2.4 Potential immunotherapy strategies targeting Treg in AML

Currently, immunotherapy for AML targeting Tregs represents an extremely promising treatment, with a main focus on reducing the number of Tregs (Table 2). Evidence suggests that the downregulation of Tregs coincides with an increase in antileukemic reactivity (121). The combination therapy of Ara-C, a CXCR4 inhibitor, and PD-L1 mAb has been shown to enhance the eradication of leukemic myeloid blast cells by effectively suppressing Tregs (122). In mouse models, it has been shown that the depletion of Tregs using anti-CD25 antibodies prior to DC vaccination against AML significantly enhances the immune response against leukemia. This approach facilitates the development of robust and long-lasting immune responses (123). The depletion of Tregs using anti-CD25 antibody (124) or interleukin-2 diphtheria toxin (IL-2DT) (NCT01106950) (125) prior to IL-2 administration has demonstrated enhanced antileukemic effects mediated by NK cells. Similarly, IL-2DT can eliminate Tregs, increasing the quantity of transferred cytotoxic T lymphocytes (CTL) at AML disease sites and reducing tumor burden (126). Clinical trials (NCT00675831, NCT00987987) have shown that a donor lymphocyte infusion depleted of CD25+ Tregs can lead to enhanced anti-tumor efficacy in patients with hematologic malignancies who have experienced relapse after undergoing allo-HSCT (127, 128). The safety and efficacy of the combined treatment

TABLE 2 AML treatment through reducing Treg numbers.

Target	Treatment	Study IDs	Research stage	Clinical outcomes	References
CD25	anti-CD25 Ab	——	preclinical phase	——	(123, 124)
	IL-2DT	NCT01106950	Phase II (Terminated)	Depletion of host Tregs with IL2DT improves efficacy of haploidentical NK cell therapy for refractory AML.	(125)
		——	preclinical phase	——	(126)
——	Treg-depleted donor lymphocytes infusion	NCT00675831	Phase I (Completed)	Treg-depleted donor lymphocytes infusion was associated with a better response rate and improved event-free survival.	(127)
		NCT00987987	Phase I/II (Completed)	Treg-depleted donor lymphocyte infusion safely induces graft-versus-host/tumor effects in alloreactivity-resistant patients.	(128)
		NCT01513109	Phase I/II (Unknown status)	——	——

strategy of infusion of Treg-depleted T lymphocytes and WT1 antigen-specific cancer immunotherapeutic in patients with WT1-positive AML are under evaluation (NCT01513109). Various targets highly expressed on Treg cells, including LAG3, TIM3, VISTA, TIGIT, OX40, ICOS, and chemokine receptors such as CCR4, CCR5, and CCR8, have been suggested as potential targets for eliminating Treg cells (116). These studies suggest that reducing the population of Treg cells may hold therapeutic benefits in the treatment of AML.

3 The other usual one: myeloid-derived suppressor cell

3.1 The phenotype of MDSC

The TME impedes the normal differentiation of hematopoietic stem cells, resulting in the emergence of a subset of immature and heterogeneous myeloid cells called MDSCs (129). MDSCs can be broadly classified into two main categories: monocytic MDSCs (M-MDSCs) and polymorphonuclear MDSCs (PMN-MDSCs). M-MDSCs are characterized as Lin−(CD3, CD19, CD56)CD11b+CD15−CD14+HLA-DR$^{low/-}$, while PMN-MDSCs are defined as Lin−CD11b+CD15+CD14−CD66b+HLA-DR$^{low/-}$ (130, 131). M-MDSCs exhibit phenotypic and morphological similarities to monocytes, while PMN-MDSCs share closer resemblance to neutrophils (129). In humans, M-MDSCs can be distinguished from monocytes by the absence of MHC class II molecules, and the population of PMN-MDSCs can be identified using LOX-1 as a marker to differentiate them from neutrophils (132, 133). PMN-MDSCs comprise the majority of MDSCs, accounting for more than 75% of the population, whereas M-MDSCs make up only 10-20% (133). However, it is important to highlight that M-MDSCs possess a higher immunosuppressive potential compared to PMN-MDSCs (133, 134). In recent years, researchers have identified a small population of human bone marrow progenitor and precursor cells that exhibit colony-forming activity. These cells, known as early myeloid-derived suppressor cells (eMDSCs), are characterized by their labeling as Lin−HLA-DR$^{low/-}$CD11b+CD14−CD15−CD33+ (131).

3.2 MDSC accumulation and its mechanisms in AML

Substantial evidence suggests that MDSCs are expanded in AML and significantly contributes to poor prognosis. Specifically, in C57BL/6 mice engrafted with TIB-49 AML, an expansion of CD11b+Gr11+ MDSCs was observed in both the BM and spleen (135). Clinical studies have demonstrated that adult patients with AML exhibit significantly elevated frequency of MDSCs in their BM. These MDSCs are identified by CD33^{high}CD11b+HLA-DR$^{low/neg}$. Importantly, it has been observed that the proportion of MDSCs decreased after patients achieve CR. Additionally, the frequency of MDSCs is positively correlated with minimal residual disease (MRD) levels, suggesting that these cells may impact the clinical course and prognosis of AML (136). Studies have provided evidence that circulating M-MDSCs are increased in individuals with AML. Moreover, the presence of elevated M-MDSC percentage has been associated with a low CR rate, a high relapse/refractory rate, and poor long-term survival in AML patients (137–139). In a monocentric prospective study on AML, two independent negative prognostic indicators for overall survival were identified: an initial peripheral percentage of M-MDSCs exceeding 0.55% of leukocytes at the time of diagnosis, and a subsequent decrease in the percentage of M-MDSCs following induction therapy (140). Research conducted by Hyun et al. demonstrated that AML patients with a heightened frequency of MDSC-like blasts, characterized by elevated levels of ARG-1 and iNOS, exhibited the ability to suppress T cell proliferation, thereby contributing to an unfavorable prognosis (141).

Extensive research has been conducted to investigate the mechanisms of MDSC accumulation. AML-derived EVs are an important factor contributing to the accumulation of MDSCs

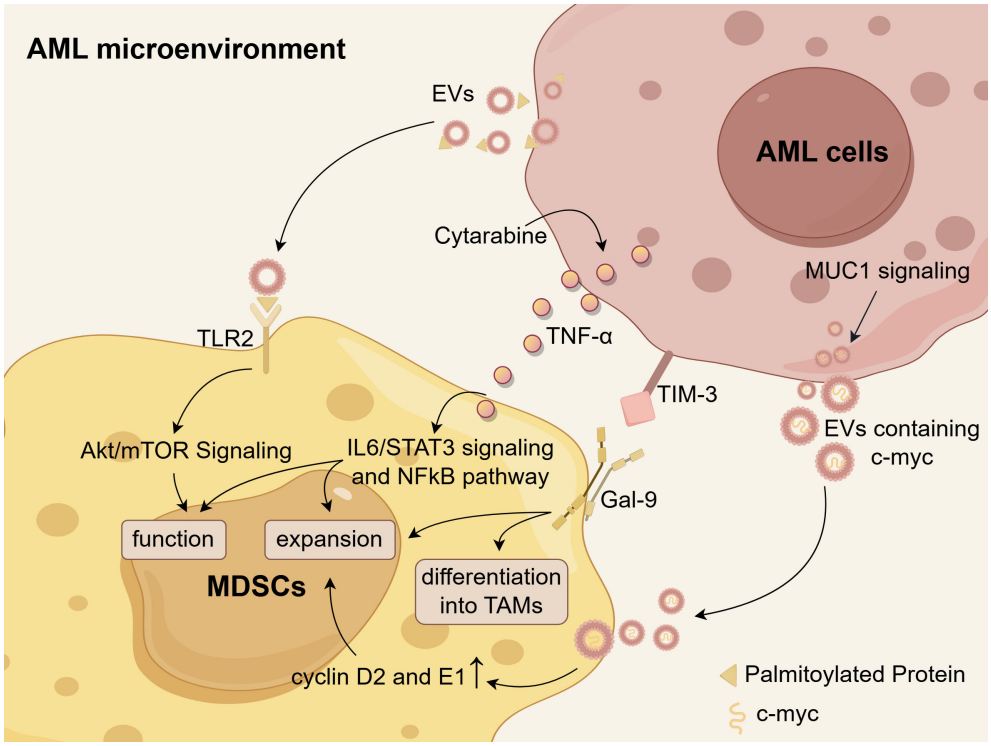

FIGURE 3
The mechanisms of MDSC accumulation in the AML microenvironment. Palmitoylated proteins present on the surface of AML-derived EVs activate TLR2, triggering the Akt/mTOR-dependent induction of MDSCs. Cytarabine-induced TNF-α secretion from AML cells leads to an expansion of MDSCs and enhances their functions and survival by activating IL-6/STAT3 signaling and NFκB pathways. AML cells secrete EVs containing c-myc in a MUC1-dependent manner, which facilitates MDSC proliferation through upregulation of cyclin D2 and E1. There is a hypothesis that the Tim-3/Gal-9 pathway may promote the expansion of MDSCs and their differentiation into TAMs in AML. Schematic figure was drawn by Figdraw (www.figdraw.com).

in AML. Specifically, palmitoylated proteins present on the surface of AML-EVs activate Toll-like receptor 2 (TLR2) of monocytes and trigger MDSC induction controlled by Akt/mTOR signaling pathway (142). Therefore, targeting protein palmitoylation could serve as a potential approach to disrupt the differentiation of MDSCs. Additionally, AML cells employ a MUC1-dependent mechanism to secrete EVs containing c-myc, when co-cultured with MDSCs. The presence of these EVs subsequently prompts the upregulation of cyclin D2 and cyclin E1 in MDSCs, suggesting that the c-myc-containing EVs potentially enhance MDSC proliferation (135). Cytarabine (Ara-C) treatment prompted AML cells to express and secret TNF-α, which subsequently facilitated the expansion of MDSCs and enhanced their function and survival through activating IL-6/STAT3 and NFκB pathways (143). Additionally, Gao et al. proposed the hypothesis that TIM-3 on AML stem cells interacts with Gal-9 on MDSCs, thereby promoting the expansion of MDSCs and their differentiation into tumor-associated macrophages (TAMs) (144). However, further research is necessary to validate this hypothesis. Theoretically, if the increase in MDSCs could be inhibited based on these mechanisms, it may offer a potential rescue strategy for AML patients. The mechanisms underlying MDSC accumulation within the AML microenvironment are illustrated in Figure 3.

3.3 The immunosuppressive mechanisms of MDSC in the AML microenvironment

MDSCs exhibit immunosuppressive activities that hinder effective anti-leukemic immune responses. MDSCs accumulated in the PB of AML patients exhibit high expression of VISTA, which is thought to be associated with the suppression of the T-cell response. Evidence suggests that VISTA exerts an inhibitory effect on the anti-leukemia T-cell response, as demonstrated by the effective reduction of MDSC-mediated CD8+ T-cell inhibition in AML following VISTA knockdown using specific siRNA (145). However, the precise mechanisms by which MDSCs operate within the AML microenvironment remain unclear at present, underscoring the urgent need for a more detailed investigation of their functional roles.

3.4 Potential immunotherapy strategies targeting MDSC in AML

Targeted intervention of MDSCs has the potential to attenuate their immunosuppressive capabilities and strengthen the immune response against leukemia. In an AML mouse model, Hwang et al.

TABLE 3 AML treatment through targeting MDSC.

Target	Treatment	Study IDs	Research stage	Clinical outcomes	References
CD33	CD33/CD3-bispecific T-cell engaging (BiTE®) antibody (AMG 330)	——	preclinical phase	——	(148)
	CD16/IL-15/CD33 tri-specific killer cell engager (GTB-3550 TriKE®)	NCT03214666	Phase I/II (Terminated)	Study terminated prematurely with no analyzable results.	——
CD123 and NKG2DL	123NL CAR-T	——	preclinical phase	——	(149)
——	guadecitabine (SGI-110)	——	preclinical phase	——	(150)
——	Combination therapy with Ara-C, CXCR4 inhibitor and PD-L1 mAb	——	preclinical phase	——	(122)
——	HDC and low-dose IL-2	NCT01347996	Phase IV (Completed)	Peripheral M-MDSCs were reduced during HDC/IL-2 therapy, heralding favorable clinical outcome.	(146)

demonstrated that a triple combination therapy consisting of Ara-C, a CXCR4 inhibitor, and a PD-L1 mAb resulted in a significant reduction of MDSCs and a potent eradication of leukemic myeloid blast cells (122). In addition, a clinical trial (NCT01347996) demonstrated a notable decrease in peripheral M-MDSCs among AML patients treated with histamine dihydrochloride (HDC) and low-dose IL-2 for relapse prevention, heralding a promising clinical outcome (146). Given the prevalent expression of CD33 on MDSCs, CD33 is frequently employed as a target of MDSCs (147). The CD33/CD3-bispecific T-cell engaging (BiTE®) antibody (AMG 330) exhibited notable efficacy in combating leukemia by specifically targeting CD33+ MDSCs in AML (148). A multicenter clinical trial (NCT03214666) is currently underway to investigate the potential of CD16/IL-15/CD33 tri-specific killer cell engager (GTB-3550 TriKE®) in targeting CD33+ MDSCs. The 123NL CAR-T therapy, which has been designed to target CD123 and NKG2DL, has demonstrated the ability to effectively eliminate M-MDSCs in AML (149). In a murine AML model, treatment with the hypomethylating agent guadecitabine (SGI-110) has been shown to reduce the MDSC burden, subsequently resulting in an increase proportion of functionally active leukemia-specific T cells (150). These studies suggest that targeted decrease of MDSCs is advantageous for the AML treatment. Immunotherapy strategies targeting MDSC in AML are summarized in Table 3.

4 The other developing one: leukemia-associated macrophage

4.1 The phenotype of LAM

Tumor-associated macrophages within the leukemia microenvironment, specifically referred to as LAMs, have been documented to play a significant role in the progression of leukemia. Macrophages can undergo polarization from the M0 state into classically activated (M1) macrophages, which demonstrate anti-leukemic and immunostimulatory capabilities, or alternatively activated (M2) macrophages, which exhibit pro-leukemic and immunosuppressive characteristics (151, 152). LAMs share functional characteristics with both M1- and M2-like macrophages. However, they predominantly align with the pro-leukemic properties of M2 macrophages (151, 153). M2 macrophages are characterized by the expression of surface markers such as CD163, CD206, and the M-CSF receptor CD115. Additionally, they secrete arginase II (Arg2), chitinase-3-like protein 1 (CHI3L1/YKL-40), and the anti-inflammatory cytokines IL-10 and TGF-β, which contribute to their immunosuppressive and tumor-promoting roles (154).

4.2 LAM accumulation and its mechanisms in AML

The expansion of M2-like LAMs in AML is a contributor to a negative prognosis. Al-Matary et al. demonstrated that M2-like macrophages were elevated in the BM of AML patients and mice (155). It has been observed that more M2-like LAMs are associated with a worse prognosis in AML patients (156, 157). Tian et al. found that the proportion and number of LAMs were higher in patients with refractory AML than in those who achieved CR (156). Consistent with this finding, a study by Brauneck et al. demonstrated an increased frequency of BM-infiltrating immunosuppressive M2 macrophages expressing TIGIT, TIM-3, and LAG-3 in patients with newly diagnosed and relapsed AML (157). Xu et al. reaffirmed that M2-like LAMs, characterized by CD206 positivity, are predominantly enriched within the AML microenvironment, and a high infiltration of M2 macrophages is correlated with adverse clinical outcomes (158). Patients with AML exhibiting elevated levels of CD163 transcripts demonstrated a diminished likelihood of survival (159). This finding aligns with

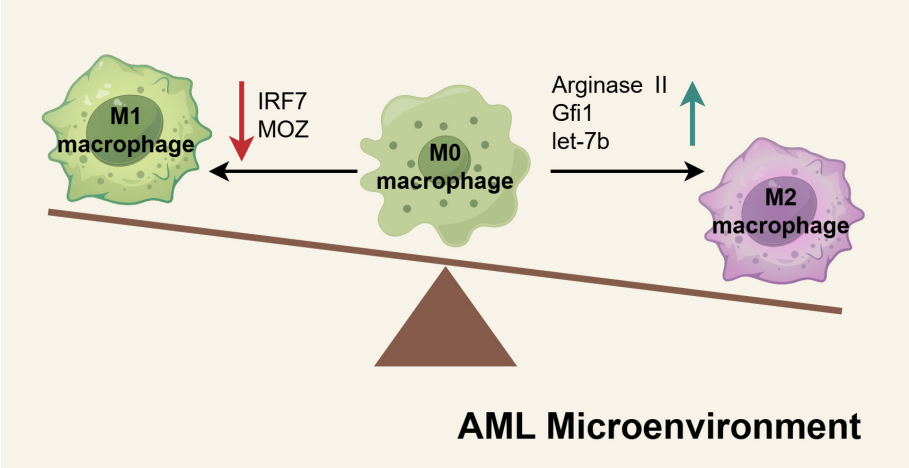

FIGURE 4
The mechanisms of M2-like LAMs accumulated in AML microenvironment. In the AML microenvironment, the factors regulating M1 or M2 macrophage polarization are dysregulated, creating an imbalance in macrophage differentiation. Pro-M1 factors such as IRF7 and MOZ are downregulated in AML macrophages, resulting in diminished M1 activation. Conversely, elevated levels of pro-M2 factors, including arginase II, Gfi1, and let-7b, drive increased polarization toward the M2 phenotype. These shifts culminate in the accumulation of M2-like macrophages within the AML microenvironment, fostering an immunosuppressive milieu that supports leukemia progression. Schematic figure was drawn by Figdraw (www.figdraw.com).

the results reported by Guo et al. through single-cell RNA sequencing, which identified a specific monocyte/macrophage cluster characterized by high CD163 expression that correlates with a reduced probability of survival in AML patients (160).

The mechanisms underlying the increase of M2-like LAMs has been comprehensively investigated. There is increasing evidence that the factors influencing M1 and M2 characteristics are imbalanced within the AML microenvironment, resulting in a greater accumulation of M2-like LAMs (Figure 4). Using *in vitro* and *in vivo* models, Mussai et al. provided the first reports demonstrating that the secretion of arginase II by AML blasts induces the polarization of monocytes into an immunosuppressive M2-like phenotype, marked by the increased expression of CD206 (161). The transcription factor Gfi1 expression was about two-fold upregulated in LAMs of AML compared to non-leukemic macrophages, and it promote the polarization of macrophages to a leukemia-supporting state (155). Recently, Tian et al. identified let-7b as a potential aberrant gene implicated in conferring M2-like characteristics and demonstrated its significant upregulation in LAMs from refractory AML mice. Knockdown of let-7b in LAMs was shown to suppress AML progression by reprogramming LAMs toward an M1-like phenotype, mediated through the activation of the Toll-like receptor and NF-κB signaling pathways (156). Jiang et al. discovered that low levels of MOZ correlate with poor prognosis in AML. They observed that the loss of MOZ led to reduced M1 activation in macrophages and heightened resistance to chemotherapeutic agents (162). Similarly, IRF7, a key contributor to M1 polarization, was found to be underexpressed in the more immunosuppressive phenotype of spleen-derived LAMs. IRF7 promotes M1 characteristics by activating the SAPK/JNK pathway in macrophages, and stimulation of this pathway was shown to significantly extend the survival duration of AML mice (159).

4.3 The immunosuppressive mechanisms of LAM in the AML microenvironment

The interplay between LAMs and AML blasts enhances AML cell survival. M2-like macrophages secrete soluble factors such as CCL2 and CXCL8, which activate pro-survival pathways and suppress apoptosis in leukemic blasts (151). Williams et al. show that M2-like macrophages protect the U937 and THP-1 AML cell lines against daunorubicin-induced apoptosis (163). While the role of TAMs in solid tumors has been extensively studied (164), the significance of LAMs in leukemia has only recently gained attention due to the unique and heterogeneous nature of leukemic microenvironments. Overall, the precise mechanisms by which LAMs influence AML remain poorly understood.

4.4 Potential immunotherapy strategies targeting LAM in AML

To counteract the immunosuppressive and leukemia-promoting effects mediated by M2-like LAMs in AML, current effective strategies primarily focus on depletion and reprogramming. In a mouse model of MLL-AF9-driven AML, Keech et al. demonstrated that targeted depletion of CD169+/SIGLEC1+ macrophages via diphtheria toxin injection significantly extended median survival in mice treated with cytarabine and doxorubicin (165). Furthermore, the 123NL CAR-T therapy designed to target CD123 and NKG2DL, has proven effective in eliminating M2 macrophages in AML (149). Experimental evidence indicates that knockdown of let-7b in LAMs causes M1-like polarization, thereby significantly inhibiting the progression of AML in a mouse model driven by MLL-AF9 (156). Additionally, Liu et al. revealed that chenodeoxycholic acid (CDCA) inhibited the

TABLE 4 AML treatment through targeting LAM.

Therapeutic strategies	Treatment	Research stage	References
Depletion of LAMs	specific depletion of CD169+ macrophages	preclinical phase	(165)
	123NL CAR-T therapy	preclinical phase	(149)
Reprogramming LAMs	knockdown of let-7b	preclinical phase	(156)
	chenodeoxycholic acid (CDCA)	preclinical phase	(166)
	blockade of TIGIT	preclinical phase	(157)

polarization of M2-like LAMs and curtailed their proliferation-promoting effects on AML cells (166). Moreover, *in vitro* blockade of TIGIT reprograms M2 LAMs toward an M1 phenotype and enhances anti-CD47-mediated phagocytosis of AML cells (157). Immunotherapy strategies targeting LAMs in AML are comprehensively summarized in Table 4.

5 The other emerging one: regulatory B cell

As early as the 1970s, researchers proposed that certain B cells could exert immunosuppressive function by secreting inhibitory cytokines (167). In 2002, Mizoguchi identified a subset of B cells characterized by up-regulation of CD1d in the mesenteric lymph nodes of intestinal inflammation murine models, which inhibited the progression of enteritis by producing IL-10, and defined this group of B cells with immunomodulatory functions as regulatory B cells (Breg) (168). Currently, the origin and development of Breg cells are poorly understood. It is widely accepted that immature and mature B cells, as well as plasmablasts, can differentiate into Breg cells under appropriate stimulation and timing, resulting in a heterogeneous Breg population (169). Several different subtypes of Breg cells have been identified in humans and mice, though specific biomarkers for Breg cell activation have yet to be established (169, 170). The phenotypes of major Breg subsets are summarized in Table 5. The most well-characterized human Breg phenotypes include CD19 +CD24^{high}CD38^{high} (171) and CD19+CD24^{high}CD27+ (172).

The absence of definitive biomarkers for Breg cells considerably impedes research advancements, particularly in the context of AML, where investigations remain markedly constrained. Wan et al. demonstrated a significant elevation in the proportion of CD19+CD24^{high}CD38^{high} Breg cells within the BM of AML patients (43). This aligns with the findings of Lv et al., who observed an elevated frequency of Breg cells in both PB and BM of AML patients compared to healthy controls, and this increased frequency was associated with a shorter overall survival (173). However, a subsequent study by Dong et al. found that patients with newly diagnosed AML exhibited a significantly lower Breg frequency in PB than healthy controls (174). Interestingly, all three studies utilized CD19, CD24, and CD38 as markers to define Breg cells, yet their results exhibited notable inconsistencies. Wan's study enrolled 45 patients, Lv's included 46, and Dong's involved 40. This divergence may be attributed to their relatively limited sample sizes. Furthermore, the inclusion of samples from both PB and BM sources could have introduced variability, potentially compromising the accuracy of the findings. To resolve this controversy, more extensive, well-replicated studies are imperative. Shi et al. demonstrated that PD-L1 expression was elevated on Breg cells from AML patients, with higher PD-L1 levels correlating with poorer prognosis (175). Research on Breg in AML is indeed quite scarce, underscoring both the significance and urgency of this investigative focus.

6 The other newly identified one: leukemia-associated neutrophils

Within the microenvironment of AML, leukemia/tumor-associated neutrophils (LANs/TANs) have emerged as a critical cellular component with increasingly recognized pathophysiological significance.

TANs are neutrophils recruited to tumor sites via chemokines (including CXCL1, CXCL2, and IL-8) secreted by tumor cells and stromal cells. Functionally, TANs can be polarized into anti-tumor N1 and pro-tumor N2 phenotypes. N1-type TANs are characterized by high expression of ICAM-1 and CD95, exerting anti-tumor effects through the release of ROS and cytokines such as IFN-γ. In contrast, N2-type TANs exhibit elevated expression of CCL2, IL-8, and ARG1, promoting tumor progression via angiogenesis induction, extracellular matrix remodeling, and immunosuppressive microenvironment formation (176).

In AML, LANs' functional role is unclear, but an FGFR1-driven murine model revealed leukemogenesis polarizes neutrophils into six subsets (notably Ly6g+ and Camk1d+), which upregulate MMP8/9 to migrate from bone marrow to blood and differentiate into PMN-MDSCs; MMP inhibition with Ilomastat blocked migration and improved survival, while clinical data linked high MMP8 to poor AML outcomes, highlighting MMP8 as a potential therapeutic target to disrupt immune evasion (177).

7 Discussions and future prospects

The immunosuppressive role in AML orchestrated by immunosuppressive cells persists as a critical impediment to eliciting a robust anti-leukemic immune response. Despite significant advancements in understanding these cells, the development of viable therapeutic strategies remains an ongoing challenge, requiring further innovation and exploration.

TABLE 5 Phenotypes of Breg subsets in humans and mice.

Breg type	Human	Mouse	Reference
B10 cells	$CD24^{hi}CD27+$	$CD19+CD5+CD1d^{hi}$	(172, 232)
T2-MZP cells	——	$CD19+CD21^{hi}CD23^{hi}CD24^{hi}$	(233)
Plasma cells	——	$CD138+MHC\text{-}11^{lo}B220+$	(234)
MZ cells	——	$CD19+CD21^{hi}CD23-$	(235)
Tim-1+ B cells	——	Tim-1+CD19+	(236)
Plasmablasts	$CD19+CD27^{int}CD38+$	$CD138+CD44^{hi}$	(237)
Immature cells	$CD19+CD24^{hi}CD38^{hi}$	——	(171)
Br1 cells	$CD19+CD25^{hi}CD71^{hi}$	——	(238)
GrB+ B cell	CD19+CD38+CD1d+IgM+CD147+	——	(239)
CD9+	CD19+CD9+	CD19+CD9+	(240)

7.1 Tregs in AML: current understanding and future directions

While research on Tregs in AML remains challenging, the function mechanisms of Treg in the solid tumor have been more clearly elucidated. Tumor-infiltrating Tregs heightened activation and potent immunosuppressive capabilities, characterized by elevated expression of LAG-3, LFA-1, TGF-β, EVs, and others (116). LAG-3 expressed on the surface of Treg could bind with a high affinity to MHC class II molecules on the surface of DCs, effectively inhibiting the maturation and immunostimulatory capacity of DCs (178). Treg-expressed LFA-1 has been shown to be involved in downregulating CD80/86 on DCs (179). TGF-β produced by intratumoral Tregs directly inhibited proliferation and differentiation of immunocompetent cells (180). Contrary to observations in solid tumors, AML demonstrates distinct TGF-β dynamics, with studies reporting either unchanged or reduced TGF-β levels in AML patients (115, 174). The underlying mechanisms for this differential expression remain unclear and warrant further investigation. Through gap junctions, Tregs deliver substantial quantities of cAMP to Teff cells, inducing metabolic interference that culminates in Teff suppression and apoptosis (181, 182). Additionally, recent studies have identified a novel suppression mechanism involving Treg-derived EVs. These EVs serve as bioactive carriers of proteins, lipids, and nucleic acids, orchestrating intercellular communication networks and modulating anti-tumor immunity (183). It was demonstrated that EVs derived from natural CD8+CD25+ Treg cells, containing LAMP-1 and CD9, were observed to significantly inhibit CTL responses and anti-tumor immunity in a B16 melanoma model (184). While established mechanisms of Treg-mediated immunosuppression in solid tumors provide a valuable framework for investigating their role in AML, critical distinctions must be acknowledged. The TME exhibits remarkable complexity, with Treg populations demonstrating substantial functional and phenotypic heterogeneity that varies significantly across different tumor subtypes (185). Additionally, emerging evidence suggests that Tregs may develop distinct functional properties within the unique leukemic microenvironment. TIGIT was ubiquitously expressed on the Tregs in AML (44, 53), and its ligands PVR and PVRL2 have been reported to be highly expressed on AML cell lines and patient samples (107). Moreover, antibody blockade of PVR or PVRL2 on AML cell lines or primary AML cells or TIGIT blockade on immune cells could enhance the anti-leukemic effects (107). It is possible that TIGIT on Treg cells may engage with PVR/PVRL2 on AML cells, thereby protecting leukemic cells from immune attack. However, there is no direct evidence so far. Further research is needed to determine the function of these molecules. A marked increase in TIM-3+ Treg cell populations was observed among *de novo* AML cases (108). Previous studies have reported that TIM-3+ Tregs in CLL drive immunosuppression via its ligand soluble Gal-9 (90). High levels of Gal-9 expression were also observed on blasts in primary AML samples (109, 110). Whether a similar situation exists in AML requires further study. Interestingly, TIM-3 is also expressed on AML stem cells (111, 112), and even Gal-9 has been shown to be expressed on activated Treg (113). These studies illustrate that the interaction between Gal-9 and TIM-3 in the AML immune microenvironment is complex and needs further exploration.

In AML treatment strategies, therapeutic depletion of Tregs can potentiate antileukemic immunity and improve clinical outcomes. However, any pharmacological approaches to reduce Treg frequency should be carefully optimized to mitigate potential adverse effects, including autoimmune reactions or uncontrolled inflammatory responses resulting from Treg dysregulation. Given the pivotal role of Treg homeostasis, targeting the molecular mechanisms underlying their accumulation represents a promising therapeutic avenue. Disrupting these pathways—such as with the IDO inhibitor 1-MT, which has demonstrated efficacy in suppressing Treg expansion—could offer a novel and clinically viable strategy for AML immunotherapy (56, 57). To facilitate clinical translation, rigorous evaluation of therapeutic feasibility remains essential, alongside the development of novel agents with optimized efficacy and safety profiles. Alternatively, attenuating Treg functionality represents a viable strategy to counteract the immunosuppressive AML microenvironment. OX40 activation has been shown to diminish Treg-mediated immunosuppression (186, 187), and targeting other immune checkpoint proteins and kinase signaling pathways in Tregs similarly disrupts their suppressive capacity (188). However, most investigations remain confined to preclinical studies or solid tumor trials, with AML-specific research notably limited. To realize effective Treg-targeted therapies in AML and maximize clinical benefits, comprehensive mechanistic elucidation and dedicated clinical validation are urgently required.

7.2 MDSCs in AML: current understanding and future directions

Similarly, insights into MDSC biology in AML may benefit greatly from an understanding of its mode of function in solid tumors and pan-cancer models. In TME, MDSCs highly express arginase-1 (ARG-1) (189) and inducible nitric oxide synthase (iNOS) (190), and transfer the metabolite methylglyoxal to CD8+ T cells (191), all of which degrade L-arginine and thus prevent T cell proliferation (192). In addition, MDSCs suppress T-cell activation by depleting cystine and cysteine (193). Within the TME, M-MDSCs exhibit heightened glucose uptake and consumption, thereby disrupting the metabolic activity of neighboring immune cells (194). Notably, in breast cancer models, MDSC-mediated tryptophan catabolism via IDO has been shown to drive Treg expansion while concurrently inducing T-cell autophagy, cell cycle arrest, and cell death (195). Adenosine production by CD39/CD73-expressing MDSCs further potentiates their expansion and enhances immunosuppressive activity in lung cancer models (196, 197). The immunosuppressive capacity of MDSCs is mediated through excessive generation of reactive oxygen species (ROS) (198, 199), nitric oxide (NO), and peroxynitrite (PNT) (200), which collectively impair T-cell function. Additionally, tumor-infiltrating MDSCs engage with T cells through multiple immune checkpoint interactions—including PD-L1/PD-1, Gal-9/TIM-3, CD80\CD86/CTLA-4, CD155/TIGIT, VISTA/VISTAL, and FasL/Fas—inducing T-cell anergy and apoptosis (201). In murine tumor models, tumor-expanded MDSCs can suppress NK cell function via membrane-bound TGF-β1 (202). However, the existence and relative contribution of these MDSC-mediated immunosuppressive mechanisms in AML remain unclear and warrant further investigation.

Therapeutic targeting of MDSCs represents a promising strategy to augment anti-leukemic immunity through multiple approaches: inhibiting their generation, promoting differentiation into immunocompetent mature cells, suppressing their immunosuppressive activity, or selectively depleting MDSC populations (203, 204). AML-derived EVs, characterized by surface palmitoylated proteins or c-Myc cargo, potently drive MDSC expansion (135, 142). EV inhibition represents a theoretically viable approach to curtail MDSC generation, and experimental validation remains essential. In addition, reprogramming existing MDSCs into immunocompetent mature cells serves as an alternative strategy. Preclinical studies demonstrate that all-trans retinoic acid (ATRA) effectively reprogram MDSCs into mature APCs, thereby restoring T-cell functionality in both renal carcinoma and pulmonary malignancy models (203, 205). Thus, pharmacological induction of MDSC differentiation into non-immunosuppressive myeloid lineages represents a viable therapeutic strategy for AML. The suppression of MDSC activity may be based on its immunosuppressive mechanisms, such as the reduction of ROS and NO production. Targeting depletion of MDSCs through agents like gemtuzumab ozogamicin (GO) has demonstrated significant clinical potential. As a CD33-directed antibody-drug conjugate (ADC) approved for CD33+ AML treatment, GO has shown both efficacy and a manageable safety profile in multiple clinical trials (206). The constitutive expression of CD33 across MDSC subtypes makes it an attractive therapeutic target, with a study by Fultang et al. demonstrating GO's ability to increase MDSC death, consequently restoring T-cell response and enhancing tumor cell clearance (147). This study encompassed multiple tumor subtypes; however, AML samples were not included, warranting further investigation in the AML context. These findings provide a strong rationale for developing novel MDSC-targeted therapies in AML, potentially leading to significant advances in treatment outcomes.

7.3 LAMs in AML: current understanding and future directions

The AML microenvironment is characterized by significant infiltration of M2-like LAMs, which actively support leukemic cell survival and disease progression. These cells represent the leukemic counterpart of TAMs observed in solid malignancies. TAMs exhibit pro-tumorigenic properties through multiple mechanisms: (1) direct promotion of malignant cell proliferation and metastasis, (2) suppression of T cell-mediated anti-tumor immunity, and (3) facilitation of angiogenic processes. TAMs can facilitate the proliferation of tumor cells by producing growth factors, cytokines, and chemokines, including FGF-2, TGF-β, PDGF, IL-10, CXCL, and so on (207). Evidence demonstrates that TAMs significantly enhance osteosarcoma metastasis and invasion through activating the COX-2/STAT3 axis and epithelial-mesenchymal transition (208). TAMs suppress antitumor immunity by inhibiting T cells, B cells, NK cells, and DCs, while promoting Tregs, Th17, γδT cells, MDSCs, angiogenesis, and metastasis (207). TAMs can induce tumor angiogenesis through the secretion of cytokines, including VEGF, COX-2, and PDGF (209). Building on the well-characterized role of TAMs in solid tumors, investigating LAMs in AML represents a promising research direction.

Given the established pro-tumor functions of TAMs, targeting LAMs may offer novel therapeutic strategies to disrupt AML progression and improve treatment outcomes. Therapeutic reprogramming of LAMs from a pro-tumorigenic to an anti-tumor M1-like phenotype emerges as a promising strategy for AML treatment. Experimental evidence demonstrates that let-7b knockdown in LAMs induces M1-like polarization, resulting in significant suppression of AML progression and extended survival in MLL-AF9-driven murine leukemia models (156). RNA-seq profiling of AML patient-derived LAMs identified let-7b as a potential target, though its downstream mechanisms remain undefined. Future work should characterize let-7b effector pathways and assess whether targeting either the microRNA itself or its products offer therapeutic benefit in AML.

7.4 Bregs in AML: current understanding and future directions

Breg cells have been found to be increased in AML and are thought to be involved in the negative immunoregulation of the hematopoietic microenvironment of AML. However, so far, no specific marker has been identified for Breg cells to define their phenotype. These findings suggest that Breg cells may not represent a distinct lineage, but rather reflect a functional state adopted by B cells at various developmental stages in response to microenvironmental stimuli (169). Nevertheless, the possibility remains that specific Breg markers exist but were not identified in the current study. Further investigation is required to fully elucidate the origin, developmental pathways, and phenotypic characteristics of Breg cells. While their phenotype remains incompletely defined, their functional significance in immune regulation has become increasingly evident. Breg research in solid malignancies has revealed their critical immunosuppressive role, with IL-10 emerging as the prototypical functional marker of Breg (168, 210). Recent advances have revealed that Breg cells employ a broader immunomodulatory factor to mediate immune suppression, including TGF-β, IL-35, CD1d and PD-L1 (211). Breg cells suppress immune responses by inhibiting CD4+ T cell proliferation and cytokine secretion (212), while also blocking TNF-α production in monocyte-macrophages (172). Given the nascent state of Breg research in AML, systematic efforts are needed to map their ontogeny, functional heterogeneity, and clinical relevance. Such studies could unlock Breg-targeted therapies to complement existing AML immunotherapies.

7.5 LANs in AML: current understanding and future directions

While research on LANs in AML remains limited, their mechanistic roles in CLL have been well characterized (213). In CLL, LANs promote leukemic cell proliferation and survival via IL-17/IL-6 secretion while fostering immunosuppression through T-cell inhibition. Notably, LANs enhance bone marrow homing and maintain leukemic stemness via the CXCR4/CXCL12 axis (213, 214). These findings offer valuable insights for AML research, particularly regarding LANs-leukemic stem cell crosstalk and the therapeutic potential of modulating LANs polarization. Key unresolved questions include (1): spatiotemporal dynamics of LANs subsets in AML progression, and (2) mechanistic interactions between LANs and the leukemic stem cell niche. Addressing these gaps could advance precision immunotherapy strategies for AML.

7.6 The likely coordinated network of immunosuppressive cells in AML

The development of an immunosuppressive microenvironment in AML involves a coordinated interplay of multiple regulatory cell populations. While studies have individually characterized the leukemia-promoting effects of Tregs, MDSCs, LAMs, and Bregs, accumulating evidence suggests these cells function synergistically to establish a potent immunosuppressive network that facilitates immune evasion and disease progression (Figure 5). As demonstrated by Flores-Borja et al., CD19+CD24^{high}CD38^{high} Bregs in healthy individuals can induce regulatory properties in CD4+CD25− T cells through IL-10-dependent mechanisms (212). However, in the study by Wan et al., the researchers observed that Bregs from healthy controls failed to promote the conversion of CD4+CD25− T cells into CD4+CD25+FOXP3+ Tregs, irrespective of whether the T cells originated from healthy individuals or AML patients. In contrast, BM-derived CD19+CD24^{high}CD38^{high} Bregs of AML patients possessed this conversion capability. Furthermore, this conversion appeared to be primarily mediated through direct cell-to-cell contact, as cytokine profiling revealed no significant alterations in the expression levels of soluble factors (43). More investigations are required to elucidate the precise mechanisms underlying Treg and Breg interactions within the AML microenvironment. In the TME, it has been demonstrated that Bregs promote Treg tumorigenicity through secretion of IL-21, IL-35, and TGF-β (215). Emerging evidence demonstrates functional reciprocity between Tregs and MDSCs across diverse tumor models. This bidirectional crosstalk establishes self-reinforcing immunosuppressive circuits, wherein factors (such as TGF-β, IL-10) produced by each population reciprocally stimulate expansion and activation, thereby amplifying immune suppression within the TME (216). M-MDSCs in CLL exhibit elevated IDO expression, which drives enhanced Treg differentiation (217). In breast cancer, MDSCs promote the development of PD-L1+ Bregs through PD-1/PD-L1-mediated activation of the PI3K/AKT/NF-κB signaling axis in B lymphocytes (218). MDSCs can drive macrophage polarization toward an immunosuppressive M2-like phenotype via IL-10 secretion, thereby facilitating solid tumor progression (219). Additionally, M2 cells secrete CCL2 into the TME to recruit MDSCs and Tregs (220). A reciprocal regulatory axis further connects M2-polarized macrophages and Tregs within the TME (215). Tregs promote monocyte differentiation into M2 macrophages through the release of IL-10, VEGF and STAT3 signaling (215, 221). In turn, M2 cells secrete IL-6 (222) and IL-10 (223) to activate Tregs. M2 cells release CCL22 and recruit more CCR4-expressing Tregs to infiltrate the tumor microenvironment (224). Evidences suggests a coordinated network of immunosuppressive cells collectively fosters tumor progression in AML and other malignancies. While these cooperative mechanisms remain incompletely characterized, their systematic investigation represents a crucial frontier in tumor. A comprehensive elucidation of these cellular interactions potentially informing novel immunomodulatory approaches for AML.

7.7 Advantages and challenges of targeting immunosuppressive cells

7.7.1 Advantages of targeting Tregs in AML immunotherapy

Targeting Tregs in AML immunotherapy offers multiple benefits. Depleting Tregs via anti-CD25 antibodies, IL-2DT, or

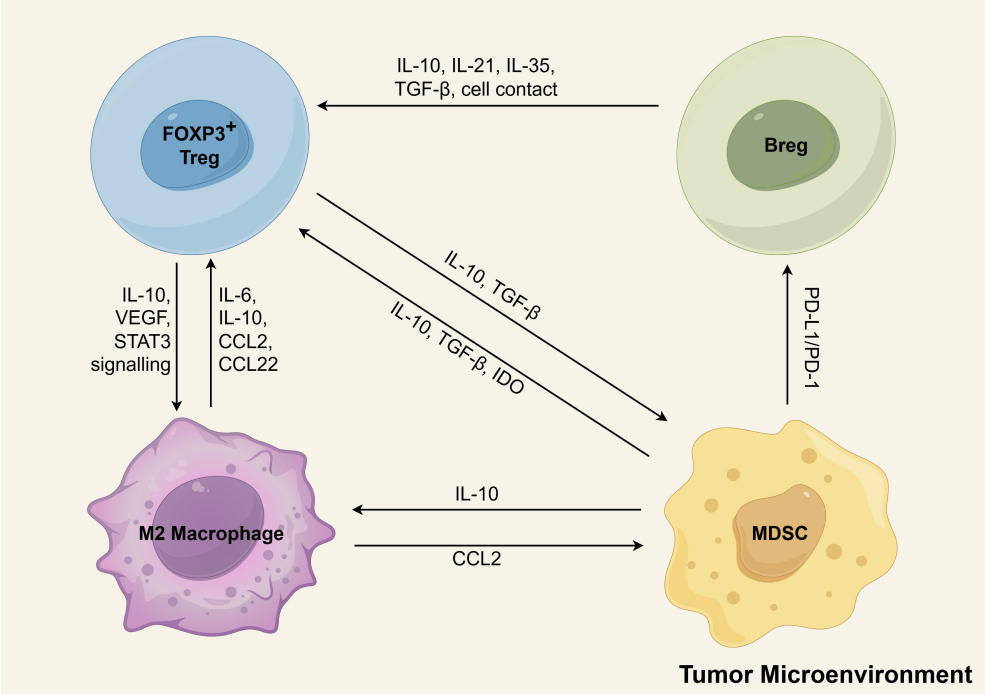

FIGURE 5
The positive feedback loops of immunosuppressive cells in tumor microenvironment. Tregs, MDSCs, M2 macrophages, and Bregs form interlinked positive feedback loops that reinforce immune suppression and drive immune evasion and AML progression. Key interactions include: (1) Breg-mediated enhancement of Treg function via IL-10, IL-21, IL-35, TGF-β, and direct cell contact; (2) Treg-induced monocyte-to-M2 differentiation through IL-10, VEGF, and STAT3 signaling; (3) M2 macrophage secretion of IL-6/IL-10 for Treg activation and CCL2/CCL22 for Treg recruitment; (4) Reciprocal TGF-β/IL-10-mediated activation between Tregs and MDSCs; (5) MDSC-driven Treg differentiation via IDO upregulation; (6) PD-1/PD-L1-dependent MDSC induction of PD-L1+ Bregs; and (7) IL-10-mediated MDSC promotion of M2 polarization. M2-derived CCL2 further recruits MDSCs to the TME. Schematic figure was drawn by Figdraw (www.figdraw.com).

CXCR4 inhibitors significantly enhances NK/CTL-mediated antileukemic activity, with preclinical studies demonstrating durable immune responses (124–126). Combination therapies (e.g., Treg depletion with DC vaccines) synergistically improve leukemic cell clearance (123), while clinical trials show that Treg-depleted donor lymphocyte infusions boost graft-versus-leukemia effects post-allo-HSCT (127, 128). Multiple targetable markers (LAG3/TIM3/CCR family) enable precise interventions, and existing regimens (e.g., IL-2DT) exhibit acceptable safety profiles (116).

7.7.2 Challenges of targeting Tregs in AML immunotherapy

This approach faces critical limitations. Systemic Treg depletion risks triggering GVHD or autoimmune toxicity, and non-specific agents like CXCR4 inhibitors may compromise effector T cells. Tumor microenvironment complexity leads to compensatory immunosuppression (e.g., MDSC expansion) and drug delivery barriers, while Treg populations often rebound post-treatment. Clinical translation remains challenging, with current efficacy largely confined to murine models or post-transplant settings, limited responses in advanced AML, and a lack of predictive biomarkers for personalized therapy. These hurdles underscore the need for more precise Treg-targeting strategies and optimized combination regimens.

7.7.3 Advantages of targeting MDSCs in AML immunotherapy

Targeting MDSCs in AML presents multiple therapeutic benefits, including the ability to reverse immunosuppression and restore anti-leukemic immune responses through various approaches such as CXCR4 inhibition (122), CD33-targeting agents (e.g., BiTE® antibodies AMG 330 and TriKE® engagers GTB-3550) (148, 149), and hypomethylating agents (150). These strategies have demonstrated efficacy in reducing MDSC populations and enhancing T-cell function in both preclinical models and early clinical trials. Additionally, combination therapies integrating MDSC-targeted interventions with chemotherapy or immune checkpoint blockade show synergistic effects, improving leukemic cell clearance and potentially overcoming treatment resistance (122).

7.7.4 Challenges of targeting MDSCs in AML immunotherapy

However, MDSC-targeted therapies face significant hurdles, including the heterogeneity of MDSC subsets (e.g., M-MDSCs vs.

PMN-MDSCs) with distinct immunosuppressive mechanisms, complicating broad-spectrum targeting. CD33-directed therapies may also deplete normal myeloid cells, leading to myelosuppression and infection risks. Furthermore, while preclinical studies are promising, clinical translation remains inconsistent, with variable patient responses and a lack of standardized biomarkers for patient selection. The tumor microenvironment's adaptability, including compensatory recruitment of alternative immunosuppressive cells, further limits sustained efficacy, underscoring the need for more precise and combination-based strategies.

7.7.5 Advantages of targeting LAMs in AML immunotherapy

Targeting LAMs in AML offers several therapeutic advantages. First, strategies such as CD169+/SIGLEC1+ macrophage depletion (165) and CD123/NKG2DL-targeted CAR-T therapy (149) have demonstrated significant efficacy in disrupting the immunosuppressive tumor microenvironment and directly eliminating pro-leukemic M2-like LAMs, leading to improved survival in preclinical models. Second, innovative approaches like TIGIT blockade (157) and let-7b knockdown (156) not only reduce M2 polarization but also actively reprogram LAMs toward anti-tumor M1 phenotypes, enhancing phagocytic activity and synergizing with therapies like anti-CD47. These dual-action mechanisms provide a multifaceted attack against AML progression while potentially restoring immune surveillance.

7.7.6 Challenges of targeting LAMs in AML immunotherapy

Despite these advantages, LAM-targeted therapies face notable limitations. A major concern is the risk of off-target effects, as broad macrophage depletion may damage beneficial tissue-resident macrophages, potentially leading to unintended toxicity. Additionally, the plasticity of LAM phenotypes poses a challenge, as reprogrammed M1-like macrophages can revert to immunosuppressive M2 states under persistent tumor microenvironment pressures, undermining long-term therapeutic efficacy. Finally, while preclinical models (e.g., MLL-AF9-driven AML) show promise, translating these findings to human patients remains difficult due to the heterogeneity of LAM populations in AML and the lack of validated biomarkers for patient stratification. These hurdles highlight the need for more selective targeting strategies and robust combination approaches to maximize clinical benefit.

8 Conclusion

The therapeutic landscape of AML has been reshaped by immunotherapy advances, yet clinical outcomes remain suboptimal for most patients, with limited agents specifically targeting immunosuppressive cells. Critical challenges endure in characterizing these inhibitory immune populations, as key molecular signatures for distinct subsets remain undefined. While preclinical studies constitute most current research, few therapeutic strategies have advanced to clinical testing, highlighting crucial unmet needs in bridging the laboratory-to-clinic translation gap for immunotherapeutic development.

Author contributions

ML: Conceptualization, Visualization, Writing – original draft, Writing – review & editing. MY: Visualization, Writing – original draft, Writing – review & editing. YQ: Writing – review & editing. YM: Data curation, Writing – original draft, Writing – review & editing. QG: Conceptualization, Funding acquisition, Methodology, Supervision, Writing – review & editing. LG: Formal Analysis, Investigation, Writing – review & editing. CL: Conceptualization, Methodology, Writing – review & editing. WL: Conceptualization, Funding acquisition, Supervision, Writing – original draft, Writing – review & editing. LX: Conceptualization, Methodology, Supervision, Writing – review & editing. YY: Funding acquisition, 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.

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The author(s) declare that no Generative AI was used in the creation of this manuscript.

Publisher's note

Supplementary material

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M1 macrophages – unexpected contribution to tumor progression

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The anti-tumor role of the immune system has long been associated with interferon-γ-mediated activation of immune cells and their ability to recognize and eliminate transformed cells. Fundamental principles of tumor immunoediting describe a dynamic interplay between the immune system and neoplastic cells, wherein immune pressure can paradoxically shape tumor evolution. Within this context, macrophages, natural killer cells, and T lymphocytes are central effectors of anti-tumor immunity. Traditionally, macrophages exhibiting M1 phenotype are characterized by high cytotoxic potential and considered important contributors to tumor eradication. In contrast, M2-polarized tumor-associated macrophages are associated with immune suppression and tumor progression. However, recent evidence challenges this binary paradigm. It is increasingly evident that M1 macrophages, while initially exerting anti-tumor effects, can also promote tumor progression by applying sustained cytotoxic pressure that selects for more malignant and immune-resistant tumor clones. This phenomenon represents an unexpected and overlooked contribution of cytotoxic macrophages to tumor progression. In this review, we examine the complex, context-dependent function of M1 macrophages and reassess current strategies aimed at enhancing their cytotoxicity. While such approaches may offer short-term benefits, they risk driving clonal selection of aggressive, immune-evasive tumor cells. Therefore, we propose a paradigm shift: instead of promoting M1 polarization alone, therapeutic strategies should consider the broader consequences of macrophage–tumor interactions. A nuanced understanding of macrophage plasticity and tumor dynamics is essential for designing effective immunotherapies. Recognizing the paradoxical role of M1 macrophages is critical to avoiding unintended support of tumor evolution and improving treatment outcomes.

KEYWORDS

macrophage, inflammation, tumor, innate immunity, immunotherapy, ADCC, ADCP

Introduction

Experimental evidence confirms that the tumor stroma is an essential component of malignant neoplasms and plays a critical role in disease progression. It is primarily composed of various mesenchymal cell types, including fibroblasts, endothelial cells, and a broad spectrum of immune cells (1). In the early stages of tumor development, immune cells within the stroma may exert anti-tumor effects. However, as the tumor evolves, these cells often undergo phenotypic shifts toward immunosuppressive profiles, ultimately promoting tumor growth and dissemination. The immune infiltrate within tumors is highly heterogeneous, comprising T lymphocytes, neutrophils, macrophages, myeloid-derived cells, natural killer (NK) cells, and dendritic cells. This dynamic and complex cellular network highlights the intricacies of tumor immunology and underscores the importance of immune regulation in cancer progression (2).

At the onset of carcinogenesis, immune response is activated, specifically aimed at suppressing tumor growth and eradicating malignantly transformed cells. Macrophages, are the crucial players in this defense mechanism. During this early stage of tumor development, the tumor cells express a broad spectrum of protein and non-protein antigens. These antigens, can be recognized by macrophages, and include well-documented tumor-associated proteins from the MAGE, GAGE, and BAGE families, glycoproteins such as gp100, NY-ESO-1, HER-2/neu, MUC1, WT-1, and some others (3). Despite this antigenic diversity, the intrinsic heterogeneity of the tumor often results in a variable immunogenic profile among its cells. Not all malignant cells demonstrate a high level of immunogenicity, complicating the ability of immune system to uniformly detect and eliminate them. The tumor survival strategies are sophisticated, involving the emergence and selective proliferation of cells with diminished or absent expression of these tumor antigens. These cells effectively evade immune surveillance by camouflaging themselves within the normal cellular landscape of the body. This evasion is not just a passive process but a dynamic adaptation that challenges the capacity of the immune system to maintain systematic surveillance and effective tumor control.

In addition to passive mechanisms of evasion from immunological surveillance, tumor cells are capable of activating more direct methods. During tumor development, due to mutagenesis, tumor cells start to express both surface and soluble molecules that modify the activation characteristics of immune system cells. For instance, the expression of cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4) on the surface of a tumor cell results in the inhibition of the cytotoxic activity of T-cells (4). Malignant cells produce interleukins like IL-6, IL-13, IL-2, and IL-12, which shift cytotoxic macrophages to an immunoregulatory phenotype (5). These macrophages, in turn, begin to produce factors that promote tumor progression, such as transforming growth factor beta (TGF-β) and vascular endothelial growth factor (VEGF) (6). Therefore, within the tumor microenvironment, cells exhibiting an immunosuppressive phenotype develop, thereby promoting the progression of the disease. These cells not only subvert immune detection but also reprogram the local immune environment to support tumor cells growth and proliferation, thereby advancing the complexity and severity of the tumor.

The cells of the tumor microenvironment are categorized into two groups based on their functions. The first group includes cytotoxic cells (dendritic cells, pro-inflammatory macrophages (M1), CD8+ and CD4 + T-lymphocytes, B-lymphocytes, and NK cells), which contribute to the suppression of tumor progression. In contrast, regulatory T-cells (Treg) and immunosuppressive macrophages (M2) reduce the effectiveness of the immune response by limiting the activation of lymphocytes and specific immune reactions. These dynamics illustrate the complex interplay within the tumor microenvironment, where various cell types either combat or facilitate the progression of the tumor, significantly influencing the overall outcome of the disease.

Macrophages are multifunctional cells whose phenotype develops under the influence of the surrounding cytokine environment. In the context of a tumor, due to the action of cytokines and growth factors produced by tumor cells, an immunosuppressive phenotype of macrophages - M2 is developed. These tumor-associated macrophages (TAMs) contribute to the progression of the tumor and increase its malignant potential (7). Furthermore, it is known that increased infiltration of M2 in the tumor stroma is a marker of poor prognosis for most solid tumors (8). This relationship highlights the critical role of the tumor microenvironment in shaping the behavior of TAMs, directly impacting the aggressiveness and clinical outcomes of the disease.

Macrophage features and functions

These discoveries have prompted a deeper exploration into macrophage biology, particularly their functional diversity in different pathological settings. In cancer, the dual nature of macrophage phenotypes has become a focal point of research, as their influence over tumor progression or suppression hinges on the microenvironment. While M1 macrophages demonstrate cytotoxic activity capable of targeting tumor cells, their presence in certain contexts can paradoxically contribute to tumor evolution by exerting selective pressure. This underscores the crucial need for therapeutic strategies that carefully consider the full spectrum of macrophage functions.

The last decade has seen a significant shift in our understanding of the origins of tissue macrophages. Studies using animal models have revealed that most tissue macrophages actually form during embryonic development. These resident macrophages typically originate from hematopoietic precursor cells located in specialized sites such as the yolk sac, fetal liver, and bone marrow. It was observed that while these embryonically derived macrophages are maintained throughout life in some tissues, in others, particularly under conditions of inflammation or as the organism ages, macrophages differentiated from circulating monocytes become the predominant population (9, 10).

The implications of these findings are profound, indicating that macrophages are not a uniform cell population but are instead

highly diverse. The local microenvironment significantly influences their phenotype and functions, leading to a complex landscape of macrophage activity within different tissues. This variability is crucial for understanding the role of macrophages in health and disease, including their involvement in tissue repair, inflammation, and immune surveillance. This evolving paradigm enhances our ability to target specific macrophage populations for therapeutic interventions in diseases such as cancer, autoimmune disorders, and chronic inflammatory conditions.

Macrophage dichotomy

There are at least two principal types of macrophage activation within the immune system: classical (M1) and alternative (M2) (11, 12). The classical or pro-inflammatory phenotype is initiated primarily in response to cytokines secreted by Th1 type T-cells, such as interferon-gamma (IFN-γ) and tumor necrosis factor (TNF). Additionally, components of bacterial cell walls like lipopolysaccharide (LPS) and muramyl dipeptide (MDP), as well as other pathogen-associated molecular patterns (PAMPs), also trigger M1 activation (13). M1 macrophages are integral to the inflammatory process, not only participating actively in immune defense mechanisms but also possessing cytotoxic capabilities that can directly target and destroy tumor cells (14). They are key producers of a wide array of effector molecules and pro-inflammatory cytokines.

M1 macrophages: inflammatory and cytotoxic function

The classical activation pathway endows M1 macrophages with enhanced expression of class II major histocompatibility complex receptors (HLA-DR) and inducible nitric oxide synthase (iNOS), both critical for their role in antigen presentation and microbial killing (15, 16).

Additionally, markers commonly associated with M1 macrophages include CD11c, CD86, and the phosphorylated form of STAT1 (pSTAT1). pSTAT1 acts as a transcription factor that regulates genes essential for the cytotoxic functions of macrophages, influencing their ability to respond to infectious threats and malignantly transformed cells effectively. Through these mechanisms, M1 macrophages contribute significantly to the body's first line of defense, orchestrating both innate and adaptive immune responses (16).

While M1 macrophages were initially known as activated macrophages (17), M2 macrophages described decades later (18) have gained more attention due to their role in supporting tumors. In response to cytokines secreted by Th2 type T-cells (IL-4, IL-13, IL-33, IL-10, IL-21), as well as other mediators such as TGF-β, vitamin D3, and glucocorticoids, the immunosuppressive M2 phenotype of macrophages is established (11). These M2 macrophages are crucial for maintaining tissue homeostasis and possess notable anti-inflammatory functions, which are essential in tissue repair and regeneration (13). Additionally, they have a significant role in promoting tumor growth by creating an environment that supports tumor survival and expansion. The development of the M2 macrophage phenotype is largely mediated through the activation of the transcription factor STAT6 (19), which orchestrates a network of genes responsible for their immunosuppressive and tissue repairing functions.

M2 macrophages: immunoregulatory and pro-tumor functions

M2 macrophages are characterized by an enhanced production of anti-inflammatory cytokines such as IL-10 and TGF-β, and growth factors like VEGF, which are crucial for angiogenesis and tissue repair. These macrophages also exhibit a reduced secretion of IL-12, supporting their role in damping inflammatory responses. The expression of surface markers like mannose receptor-1 (CD206) and scavenger receptors (CD204 and CD163) is markedly increased in M2 macrophages, aiding in the clearance of debris and dead cells, thereby maintaining homeostasis (20, 21).

M2 macrophages are not a uniform population but consist of distinct subtypes—M2a, M2b, M2c, and M2d—each induced by different stimuli and performing specific roles in immune regulation and tumor progression (22). M2a macrophages are generated in response to IL-4 and IL-13 and are primarily involved in tissue repair and fibrosis and contribute to tumor dissemination (23). M2b macrophages are induced by immune complexes in combination with TLR agonists or IL-1β. These cells display a mixed cytokine profile, simultaneously producing pro-inflammatory (e.g., IL-1β, TNF) and anti-inflammatory (e.g., IL-10) mediators. Their immunoregulatory nature allows them to suppress adaptive immune responses while maintaining chronic inflammation that favors tumor development (23). M2c macrophages arise under the influence of IL-10, TGF-β, or glucocorticoids and are strongly immunosuppressive. They are involved in matrix deposition, clearance of apoptotic cells, and promotion of tumor tolerance. Their high expression of CD163 and MerTK receptors aligns them closely with the phenotype of tumor-associated macrophages found in various cancer types (23, 24). M2d macrophages, often equated with tumor-associated macrophages (TAMs), are induced by IL-6 and adenosine signaling within the tumor microenvironment. They are potent promoters of angiogenesis, mainly through VEGF production, and they suppress anti-tumor immune responses by inhibiting cytotoxic T-cell function and promoting regulatory T-cell expansion (22).

Cytotoxic functions of macrophages

Contact-independent mechanism

The cytotoxic activity of macrophages enables these cells to destroy tumor cells through both direct and indirect mechanisms. The primary mechanisms of direct cytotoxic activity include phagocytosis, the production of pro-inflammatory cytokines, and

mediators of inflammation such as nitric oxide and reactive oxygen species, which trigger processes of programmed cell death in the target cells (25). Additionally, macrophages attract cells of the adaptive immune system, such as T-cells, to the site of inflammation.

The mechanism of macrophage cytotoxic activity can be classified into contact-dependent and contact-independent interactions with the target cell. The initiation of contact-independent cytotoxic activity by macrophages primarily occurs in response to soluble factors (cytokines) produced by T-lymphocytes following interactions of T-cells with antigen-presenting cells or mitogens, such as phytohemagglutinin (PHA) and concanavalin A (Con A) (26). Well-known cytokines include Macrophage Activation Factor (MAF) and Macrophage Migration Inhibitory Factor (MIF) produced by T-cells regardless of contact with the tumor cell (27). The interaction with cytokines leads to macrophage activation. Cytotoxic activity is conducted without direct physical contact through secreted soluble factors by macrophages, such as cytokines, chemokines, as well as reactive oxygen and nitrogen species, which lead to the death of the target cells (28).

This complex interplay not only facilitates the elimination of tumor cells but also significantly impacts the microenvironment by modulating inflammatory responses and orchestrating the recruitment and activation of other immune cells. This nuanced role of macrophages highlights their importance in both innate and adaptive immune responses, making them a crucial target for therapeutic strategies aimed at enhancing anti-tumor immunity.

Contact-dependent mechanism

The contact-dependent mechanism can occur via antibodies bound to the surface of the target cell (antibody-dependent cellular cytotoxicity, ADCC), as well as without the involvement of antibodies. In the context of anti-tumor immune response, macrophage-mediated ADCC plays a central role in cytotoxic activity. Moreover, this mechanism mediates the action of many immunotherapeutic drugs based on monoclonal antibodies (mAbs) (29, 30). ADCC by macrophages is primarily carried out through phagocytosis. This process is initiated by the binding of Fc receptors on the surface of macrophages to antibodies on the surface of malignantly transformed cells. This mechanism can be enhanced by the action of certain cytokines and mAbs. For instance, it is known that IL-15, IL-21, IL-18, IL-2, and antibodies to CD137, CD96, TIGIT, KIR, PD-1 possess this activity (31). There is evidence that cytokines and mAbs act synergistically in the context of anti-tumor therapy. For example, IL-15 enhances the efficacy of mAbs against CD20 and CD52 (32). It is known that the number of engaged Fc receptors on the surface of macrophages directly correlates with the effectiveness of ADCC in the context of tumor cells (33).

The data presented in the scientific literature about the mechanism of antibody-independent cytotoxic activity of macrophages are fragmented. It is known that this process also requires opsonization of the target cell. In this case, complement factors act as opsonins. The opsonization of tumor cells with the complement component C3, along with the generation of pro-inflammatory mediators C3a and C5a, activates the cytotoxic activity of macrophages. The C3 components of the complement on the surface of the tumor cell are recognized by macrophages through complement receptors CR3 and CR4 (CRs), which results in increased FcγR-mediated phagocytic activity (34). There is evidence that the C9 factor plays an important role in complement-mediated cytotoxic activity of macrophages in the context of non-small cell lung cancer (35).

Antibody-independent cytotoxic activity of macrophages can be enhanced by the action of IFN-γ, bacterial products such as LPS, MDP, and other PAMPs. The action of IFNγ is mediated by the phosphorylation of the transcription factor STAT1, which initiates the transcription of about 200 genes, most of which are associated with inflammation (36). In response to the interaction of TLRs on the surface of macrophages with PAMP, a cascade of reactions is triggered, leading to an increase in the cytotoxic activity of the immune cell. For instance, LPS, by binding to TLR4, initiates a cascade of reactions that activate the transcription factor NFkB, resulting in the activation of transcription of genes for pro-inflammatory cytokines such as TNF, IL-1β, IL-6, IL-12, IL-27, as well as nitric oxide synthase (NOS2), and others (37). The bacterial cell wall component MDP activates macrophages by binding to another receptor, NOD2. This interaction also activates NF-κB, subsequently enhancing the cytotoxic potential of the effector cell.

These insights into the antibody-independent cytotoxic mechanisms underscore the sophisticated nature of macrophage activation and their crucial role in innate immunity. By harnessing such pathways, macrophages are capable of directly combating pathogenic and cancerous cells without the direct need for antibody mediation, marking them as key players in the body's defense system against a variety of threats.

Macrophage-derived anti-tumor factors

Thus, the primary function of macrophages in the context of malignant neoplasms is anti-tumor. Recruited monocytes primarily differentiate into M1 macrophages and produce a range of inflammatory mediators that activate the immune response. Some of these mediators initiate feedback loops. For example, IL-12 produced by M1 macrophages stimulates NK cells and dendritic cells to secrete IFN-γ, which enhances the cytotoxic potential of macrophages, including an increase in the production of reactive oxygen species and nitric oxide (NO). These compounds lead to the activation of apoptosis in the target cell. One of the primary targets of reactive oxygen species within cells, including malignantly transformed ones, are lysosomes. Oxidation causes destabilization of the lysosomal membrane, leading to the release of lysosomal enzymes and damage to the cell. In response, the cell activates the process of autophagy as a defense mechanism; however, prolonged oxidative stress leads to what is known as autophagic cell death, which is currently classified as a type of programmed cell death (38).

Programmed cell death processes in tumor cells are also triggered in response to other inflammatory cytokines such as TNF, IL-1β, MCP-1 (monocyte chemotactic protein 1), and others. ADCP (antibody-dependent cellular phagocytosis) is accompanied by the presentation of tumor antigens to T-cells and the activation of an adaptive anti-tumor immune response. Activated lymphocytes proliferate, forming tumor-specific clones and infiltrating the tumor, thus forming an adaptive anti-tumor immunity (38). M1 macrophages can inhibit tumor development significantly through phagocytosis and the presentation of antigens on their surface, recruiting CD8+ T-cells and cytotoxic NK cells (39).

Recent studies have also highlighted the significant impact of intracellular molecules, such as microRNAs (miRNAs), on the polarization of macrophages. For instance, miR-720 is known to push M2 macrophages towards an M1 phenotype while simultaneously inhibiting their phagocytic activity, suggesting a complex regulatory mechanism that balances pro- and anti-inflammatory responses (40). Similarly, miR-127 enhances the expression of pro-inflammatory cytokines like IL-6 and IL-1β, suggesting its potential role in promoting an M1 phenotype, which is geared towards fighting infections and tumor cells (41). Moreover, miRNAs such as miR-23a/27a/24–2 are actively involved in reprogramming macrophages towards an M1 phenotype, thereby supporting anti-tumor activity. miR-23a, by interacting with the NF-κB pathway inhibitor A20, not only promotes the expression of inflammatory cytokines but also disrupts the immunosuppressive signaling pathways, typically prevalent in M2 macrophages, through inhibition of the JAK1/STAT6 pathway. miR-27a exerts similar effects by targeting regulatory factors like IRF4 and the PPARγ receptor, further demonstrating the intricate network of gene regulation involved in macrophage polarization (42).

These mechanisms illustrate the critical roles that M1 macrophages play not only in direct tumor cell elimination but also in orchestrating a broader immune response against tumors. Their ability to present antigens and recruit other immune cells underscores the importance of macrophages in the development of effective anti-cancer strategies, highlighting potential therapeutic targets for enhancing anti-tumor immunity.

Macrophage-based therapeutic approaches

Based on these observations, numerous therapeutic approaches have been developed to reprogram pro-tumoral M2 macrophages into inflammatory M1 cells within the tumor microenvironment. One approach involves the use of pattern-recognition receptor agonists: TLR7/8 ligands (43), TLR3/5/9 agonists delivered by ROS-inducing micelles (44), ferritin (45) or liposomal systems that trigger NF-κB/IRF cascades in F4/80$^+$ cells inside the tumor. These treatments promote the expression of iNOS and IL-12, leading to enhanced antitumor immunity in murine models (45–48). Another strategy relies on metabolic reprogramming of TAMs, including pH-responsive micelles or exosomes that silence STAT6 (49) and miR-155 conjugated graphene quantum dots (50) re-educating resident TAMs. Additional approaches include checkpoint-targeted and vesicle-based systems such as dual-inhibitor supramolecules (CSF-1R + SHP2), SIRPα-blocking magnetic nanoparticles (51), and hybrid nanovesicles that fuse M1-derived membranes with CD47-targeting modules (52).

Clinical translation of these approaches was, however, limited. In patients with melanoma, non-small cell lung cancer, and renal cell carcinoma, CSF-1R blockade—alone or in combination with CD40 agonists and nivolumab—failed to induce durable M2-to-M1 repolarization and resulted in low objective response rates (53, 54). Broad myeloid-targeted combinations, such as CSF-1R with CCR2/5 and CXCR2 inhibitors, were similarly ineffective, as compensatory immunosuppressive myeloid populations rapidly re-emerged (55). Moreover, the multi-kinase CSF-1R inhibitor pexidartinib caused off-target depletion of dendritic cells and liver toxicity when combined with durvalumab, yielding only limited partial response rate in advanced colorectal and pancreatic cancers (56).

Pro-tumor function of M1 macrophages

Inflammatory factors

The cytotoxic activity of type I macrophages may paradoxically facilitate tumor progression. Reactive oxygen species (ROS), nitric oxide (NO), and a spectrum of pro-inflammatory cytokines such as IL-6, TNF, and IFN-γ can exert mutagenic effects on tumor cells and their surrounding microenvironment (57, 58). These inflammatory mediators, while intended to combat tumor cells, can unintentionally promote genetic mutations that lead to enhanced tumor survival and adaptation. Furthermore, certain chemokines produced by cytotoxic macrophages serve as chemoattractants for regulatory T-cells (Tregs), which are known to suppress anti-tumor immune responses and thus facilitate tumor progression (59).

TNF, in particular, plays a critical role in promoting tumor angiogenesis, proliferation, invasion, and metastasis (60). This cytokine activates the NF-κB signaling pathway within tumor cells, leading to increased tumor cell survival and proliferation. Notably, TNF exposure results in a loss of gp100 protein expression in melanoma cells, while simultaneously elevating levels of the neurotrophin receptor (NGFR) (61). Since gp100 is a recognized target for immune attack and NGFR is linked with tumor aggressiveness, this shift could lead to decreased immune surveillance and increased tumor malignancy. Moreover, NGFR's role in inactivating the tumor suppressor gene p53 further underscores its contribution to tumor growth and resistance to cell death (62).

TNF also attracts endothelial cells, fibroblasts, and pericytes to the tumor site, facilitating the formation of a supportive tumor microenvironment that is conducive to further growth and spread. The production of matrix metalloproteinases by cytotoxic macrophages, often seen in high levels within the tumor microenvironment, aids in breaking down extracellular matrix

barriers, thus enabling tumor invasion and metastasis (63). Additionally, the presence of IFN-γ induces macrophages to express indoleamine 2,3-dioxygenase (IDO), which suppresses cytotoxic T-lymphocyte activity, further dampening the immune response against tumor cells (64, 65).

The enduring M1/M2 paradigm maintains that while M1 macrophages are typically anti-tumoral, M2 macrophages generally promote tumor growth. This dichotomy underscores the dualistic nature of macrophage function in cancer biology (66). Current research continues to explore macrophage reprogramming strategies, aiming to convert pro-tumoral M2 macrophages back into anti-tumoral M1 types, thus enhancing the overall effectiveness of anti-cancer therapies (67). Nevertheless, emerging studies challenge this binary classification, revealing scenarios where M1 macrophages unintentionally support tumor growth, highlighting the complexity and dynamic behavior of these immune cells within different tumor contexts. This evolving understanding necessitates a more nuanced approach in leveraging macrophages in cancer therapy, ensuring that interventions precisely target the multifaceted roles these cells play in tumor progression.

Experimental evidence

For instance, it has been demonstrated that conditioned medium from M1 macrophages can stimulate the invasive capacity of pancreatic adenocarcinoma cells, as shown by increased migration and invasion of MiaPaCa-2 and HPAF-II cells in response to GM-CSF–polarized M1 macrophages derived from human blood monocytes (68). In hepatocellular carcinoma (HCC), exposure of monocytes to HCC-conditioned medium induced an M1-like phenotype that paradoxically promoted tumor growth *in vivo* by suppressing tumor-specific T cells; notably, this effect was reversed by PD-L1 blockade (69). Moreover, M1 macrophages generated by stimulation of U937 cells with IFN-γ and LPS were shown to enhance proliferation and invasion while reducing apoptosis in HepG2 and SMMC-7721 hepatocellular carcinoma cells (70). In a melanoma model it was demonstrated that the conditioned medium from M1 macrophages can stimulate the invasive capability of tumor cells through activation of the TNFR–NF-κB signaling pathway (71). It has also been shown that the conditioned medium from M1 macrophages promotes the proliferation of hepatocellular carcinoma cells via the NF-κB signaling pathway (72). Furthermore, the conditioned medium from M1 macrophages has been found to stimulate the proliferative ability of gastric cancer cells (73). Recently, it has been shown that M1 macrophages enhance the survival and invasion of squamous cell carcinoma cells of the oral mucosa by activating ErbB2 (74). Table 1 provides an overview of studies discussed.

The findings from these studies highlight a complex paradox where M1 macrophages, traditionally considered as anti-tumoral, can under certain conditions promote tumor progression. This phenomenon may be explained by the multifaceted nature of the cytokine and chemokine profiles secreted by M1 macrophages, which, while aimed at fighting infections and tumors, can inadvertently provide growth factors and survival signals to cancer cells. The local tumor environment also plays a critical role in dictating the effects of these signals, with certain cancer types possibly more predisposed to exploit the inflammatory milieu to their advantage.

These insights underscore the need for a deeper understanding of the tumor microenvironment and the interplay between immune cells and cancer cells. This knowledge is crucial for designing targeted therapies that can modulate the tumor-promoting effects of M1 macrophages or potentially harness their anti-tumoral capabilities more effectively. As research progresses, strategies may need to be tailored to not only enhance the cytotoxic functions of M1 macrophages but also mitigate their potential to

TABLE 1 Summary of studies demonstrating pro-tumorigenic effects of M1 macrophages.

Publication	M1-activation strategy	Cancer model tested	Pro-tumor read-out demonstrated
Salmiheima 2016 (68)	Human blood monocytes stimulated with GM-CSF	Pancreatic-adenocarcinoma cell lines MiaPaCa-2, HPAF-II	Increased tumor-cell migration/invasion
Kuang 2009 (69)	Human monocytes exposed to HCC-conditioned medium.	Human HCC samples; HepG2 xenografts in NOD/SCID mice.	M1 suppressed tumor-specific T-cells and accelerated tumor growth *in vivo*; PD-L1 blockade reversed.
Xie 2016 (70)	U937 stimulated with IFN-γ, LPS	HepG2, SMMC-7721 HCC cells.	Increased proliferation, invasion; reduced apoptosis.
Kainulainen 2022 (71)	THP-1 stimulated with PMA, IFN-γ, LPS	Melanoma lines MV3, A375	Increased invasion.
Sharen 2022 (72)	THP-1 stimulated with PMA, IFN-γ, LPS.	Hepatocellular carcinoma cells HepG2, SNU-182	Increased proliferation, clonogenicity, radio/chemo-resistance.
Zhou 2018 (73)	THP-1 stimulated with PMA, LPS	Gastric-cancer lines BGC823, MKN28	Accelerated proliferation.
Lv 2022 (74)	THP-1 stimulated with PMA, IFN-γ, LPS.	OSCC lines SCC25, CAL27; nude-mouse xenografts.	Increased proliferation, colony-formation, migration/invasion
Podlesnaya 2022 (75)	THP-1 stimulated with PMA, IFN-γ, LPS.	Cell lines PC3 (prostate), H1299 (lung)	Resistance to macrophage killing, increased proliferation, migration
Kovaleva 2022 (76)	THP-1 stimulated with PMA, IFN-γ, LPS.	H1975 (lung), nude-mouse xenografts.	Increased proliferation *in vitro* and *in vivo*, increased tumor size with vascularization

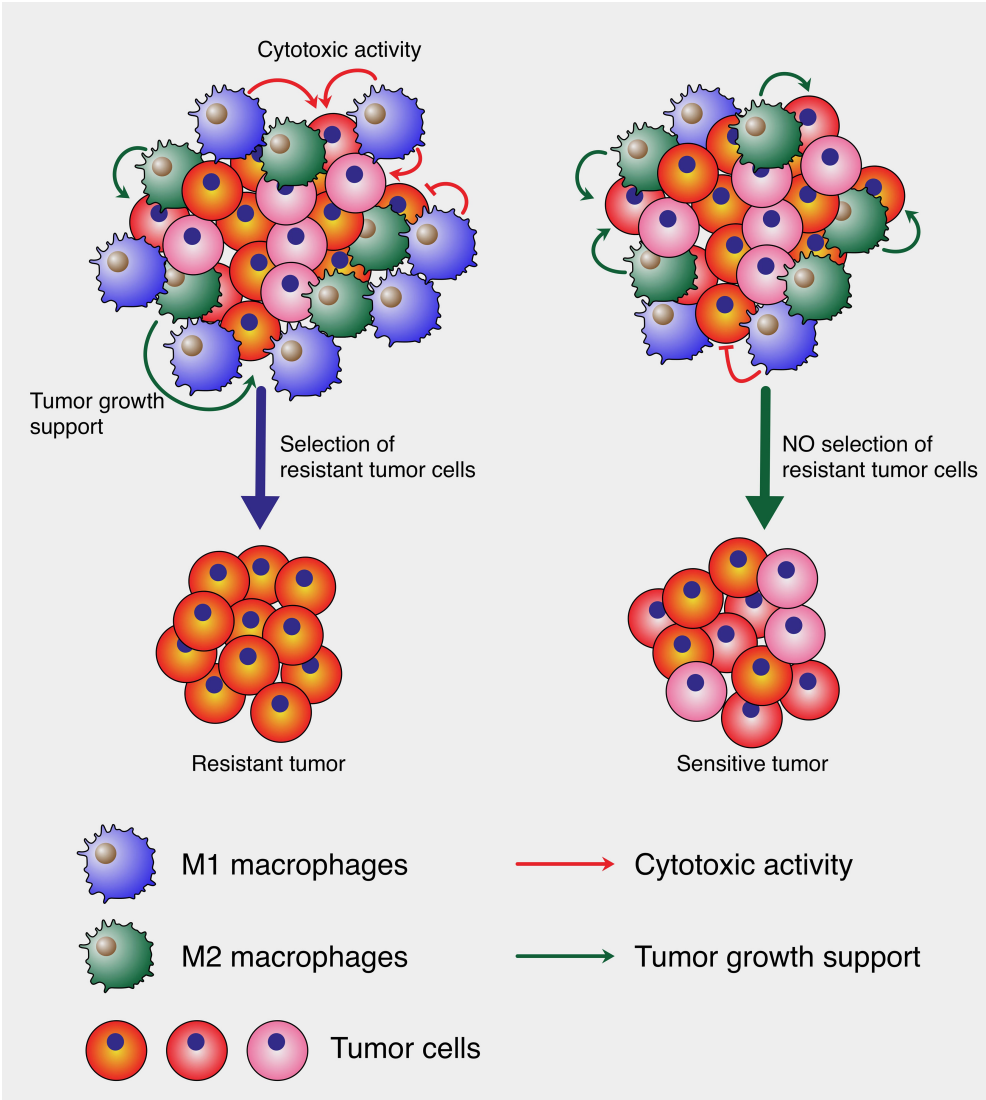

FIGURE 1
Cytotoxic macrophage-mediated selection of tumor cells. The left panel depicts a heterogeneous tumor microenvironment where large amount of M1 macrophages exert cytotoxic pressure (red arrows), leading to the elimination of sensitive tumor cells and the survival of resistant clones, culminating in the emergence of a resistant tumor. Concurrently, M2 macrophages support tumor growth (green arrows). In contrast, the right panel shows a balanced microenvironment where such selective pressure is low, allowing for the persistence of a mixed tumor cell population without the dominance of resistant clones, resulting in a sensitive tumor phenotype. This model illustrates how cytotoxic M1 can paradoxically contribute to tumor evolution.

support tumor growth, ensuring that therapeutic interventions are both precise and effective in combating cancer.

Previous research on the resident microbiome and tumor stroma prompted us to explore the potential pro-tumoral role of type 1 activated macrophages (M1). We hypothesized that under certain conditions M1 macrophages may contribute to tumor progression by cytotoxic pressure that selects more malignant, resistant tumor clones. To test this hypothesis, we created a unique *in vitro* model in which tumor cell lines of various origins (lung, prostate, kidney, breast) were repeatedly exposed to macrophages stimulated with the TLR4 ligand lipopolysaccharide (LPS), leading to the development of tumor cell sublines resistant to macrophage cytotoxicity (75). This model enabled us to characterize tumor cells that acquired resistance to macrophage cytotoxicity. Our findings revealed that these resistant sublines exhibited several features associated with increased malignancy, including accelerated proliferation, enhanced tumor growth *in vivo*, increased vascularization, and perineural invasion (76). Transcriptomic analysis further identified signaling pathways and gene expression changes potentially underlying this acquired resistance. While these results suggest that inflammatory macrophages may act as a selective pressure favoring immune-evasive and more aggressive tumor phenotypes, we acknowledge that this does not establish a direct mechanistic link between M1

macrophages and immune escape. These findings support the idea that pro-inflammatory macrophages may contribute to tumor evolution by selecting clones capable of resisting their cytotoxic effects, although further studies are needed to clarify the precise mechanisms involved in this process.

Possible mechanisms

An analysis of the transcriptome of the derivative cell lines compared to the originals revealed the activation of various signaling pathways potentially involved in tumor progression, particularly the integrin-dependent signaling pathway and the TGF-β signaling pathway. Signaling pathways activated by the cytokine TGF-β regulate a large number of biological processes, such as cell division, migration, and differentiation, and their effects vary depending on the type of target cells and their microenvironment. One of the primary mechanisms of TGF-β action involves interaction with Smad proteins, leading to the regulation of numerous genes. On the other hand, TGFβ can activate the function of MAP kinases, specifically p38, through Smad-independent mechanisms. It is also noteworthy that transcriptomic sequencing revealed an increase in the expression of osteoprotegerin (OPG), also known as a member of the 11b superfamily of TNF receptors (TNFRSF11B). This receptor is a soluble protein whose main function is to inhibit TRAIL-induced apoptosis. For various solid tumors, an association of OPG content with tumor aggressiveness has been demonstrated (77–80). It has been shown that OPG secretion is mediated by the activation of two signaling cascades, namely p-38 and ERK1/2, which, in turn, are activated in response to the cytokine IL-1β produced by macrophages (81), which is consistent with our results.

These findings suggest that while M1 macrophages are typically considered anti-tumor, their activity can, under certain conditions, promote tumor progression by exerting selective pressure that favors the outgrowth of resistant and more aggressive clones. This paradox highlights the complex and context-dependent nature of macrophage–tumor interactions and underscores the need for nuanced approaches in cancer therapy that go beyond simple macrophage activation. Rather than broadly stimulate M1 functions, future strategies should aim to preserve their cytotoxic potential while minimizing the pro-tumoral effects of sustained inflammatory signaling. The specific signaling pathways responsible for this shift remain to be identified, but therapeutic targeting of downstream cytokine effects and modulation of the tumor microenvironment may help prevent the unintended promotion of tumor growth.

Conclusions

The role of the immune system in tumor progression is the subject of study in leading laboratories around the world. The development of oncoimmunology and immunotherapy for tumors has revolutionized the treatment of cancerous diseases. Macrophages, natural killers, and T-cells play a central role in the destruction of tumor cells. The nature of the interaction between the tumor and its microenvironment is multifaceted. On one hand, tumor cells can reprogram immune competent cells and suppress their anti-tumor activity, while on the other hand, tumor cells can develop resistance to the cytotoxic effects of macrophages and other immune competent cells.

As illustrated in Figure 1, M1 macrophages, despite their cytotoxic activity against tumor cells, can inadvertently drive tumor evolution by selecting for resistant cell populations. In a heterogeneous tumor microenvironment, M1 macrophages eliminate sensitive tumor cells, but their activity may leave behind and promote the expansion of resistant clones, resulting in a more aggressive tumor phenotype. This selection pressure ultimately leads to tumor relapse with enhanced resistance characteristics. In contrast, the absence of such selective pressure may preserve tumor cell sensitivity, as shown in the right panel of the figure. Here, the tumor retains a mixed population without the dominance of resistant phenotypes, underscoring the paradoxical role of cytotoxic macrophages in tumor progression.

In summary, recent years have provided compelling evidence for a new function of cytotoxic macrophages in tumors – namely, their ability to participate in the selection of more malignant tumor cells and to promote tumor progression. Current literature explains the minimal success of therapeutic strategies aimed at altering the phenotype of macrophages to cytotoxic. It is clear that there is a need to completely reconsider macrophage-mediated therapy strategies and adjust them, possibly by focusing on reducing the overall number of macrophages in malignant neoplasms.

The interaction between tumor cells and the immune system is complex and dynamic. As our understanding of this relationship deepens, it reveals that while immune cells are traditionally viewed as protectors against cancer, under certain conditions they can facilitate cancer adaptability and survival. This paradoxical behavior highlights the intricate balance of immune responses within the tumor microenvironment, where the same factors that are meant to fight the tumor can also end up supporting it. Thus, a nuanced approach is required in developing immune-based therapies, one that not only aims to activate immune responses but also precisely targets these responses to avoid unintended support of tumor growth and resistance. This ongoing research emphasizes the importance of developing targeted therapies that can selectively modulate the immune landscape of tumors, thereby enhancing the efficacy and specificity of cancer treatments.

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Advances in the study of TIM3 in myelodysplastic syndrome

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Myelodysplastic syndromes (MDS) are heterogeneous myeloid clonal disorders derived from hematopoietic stem cells. The incidence of MDS (1.51/100,000 in China, 4-5/100,000 in Europe and America) is higher than any subtype of leukemia. In recent years, the imbalance of immune regulation and tumor microenvironmental disorders have received increasing attention in the pathogenesis of MDS. T-cell immunoglobulin and mucin-domain containing protein 3 (TIM-3) is an important inhibitory immune checkpoint molecule, widely expressed in T cells, NK cells, and dendritic cells, monocytes/macrophages and other immune cells. Numerous studies have confirmed that TIM-3 is aberrantly expressed in a variety of solid and hematologic tumors and plays an important role in regulating tumor escape and immune depletion. In this paper, we focus on reviewing the relevant studies of TIM-3 in MDS and summarize the findings of our team in this field. We also discuss the potential application of TIM-3 in the diagnosis and treatment of MDS in conjunction with the latest clinical trials. Blocking TIM-3 has both 'tumor cell-targeted inhibition' and 'immune function remodeling' dual roles in MDS disease progression, which provides new therapeutic strategies and hope for MDS patients.

KEYWORDS

T-cell immunoglobulin mucin 3 (TIM-3), myelodysplastic syndrome (MDS), immune checkpoints, immune escape, targeted therapy

1 Introduction

Myelodysplastic syndromes (MDS) can present with ineffective hematopoiesis, peripheral blood cytopenia, abnormal bone marrow cell development, and a high risk of transformation into Acute Myeloid Leukemia (AML). Currently, there is no effective, safe, and easily accessible treatment for this disease.

In recent years, with the rise of immunotherapy, the use of immune checkpoint inhibitors in tumors has gradually become a hot research topic. It has been found that these molecules are expressed on immune cells and inhibit their activation, which ultimately leads to immune escape of tumor cells and accelerates tumor metastasis and spread. And immune checkpoint inhibitors can block the immune escape of tumor cells and restore the body's immune recognition and killing of tumors. Inhibitors of immune checkpoint

molecules such as PD-1/PD-L1 and CTLA-4 have achieved significant efficacy in a variety of solid tumors and have gradually expanded into the field of hematologic malignancies (1).

The pathogenesis of MDS has not been fully clarified, and the more accepted explanations are: molecular genetic variation of primitive hematopoietic stem cells, proliferation of abnormal precursor cells (2); imbalance of the body's immunosurveillance system, abnormal bone marrow microenvironment, and disorders of the immune microenvironment. The bone marrow microenvironment is mainly composed of cellular components (immune cells, vascular endothelial cells, osteoblasts and mesenchymal stromal cells, etc.) and bone marrow ecological niche (3). Under the regulation of these immune cells, e.g., MDSC cells and malignant clonal hematopoietic cells in the bone marrow of MDS patients are increased in number and secrete immunosuppressive factors, chemokines, and growth factors to reduce the proliferation of effector T cells and NK cells, the increased number of Treg cells leads to immunosuppression, and the aberrant activation of inflammatory signaling pathways by MSCs drives the development of MDS, etc., which suppresses normal immune responses and causes an Bone marrow inflammatory microenvironment, leading to immune escape of malignant clonal cells, impaired clearance, and ultimately promoting the occurrence and development of MDS/AML (4) (5). Bone marrow microenvironment and immunoinflammatory disorders as one of the key pathogenesis of MDS, so immunosuppressants may become an alternative treatment to demethylating drugs, however, there are still some patients who are insensitive to PD-1/PD-L1 or CTLA-4 monoclonal antibody, which requires us to continue to explore more potential targets in diseases such as MDS.T-cell immunoglobulin mucin 3 (TIM-3) is precisely in this context as a novel immune checkpoint that has received much attention.

TIM-3 is expressed in a variety of immune cells and tumor cells, and regulates immune responses and inflammatory pathways through interactions with its ligands (e.g. Gal-9, HMGB1, CEACAM1 and PtdSer). Numerous studies have shown that high TIM-3 expression is associated with poor prognosis in solid tumors and hematologic malignancies, and is closely linked to processes such as maintenance of tumor stem cells, remodeling of the tumor microenvironment, and immune depletion (6–8). In MDS, the mechanism of action and clinical value of TIM-3 is emerging. In this paper, we will comprehensively review the expression and function of TIM-3 in MDS, explore its immunoregulatory mechanism in the process of disease onset, development and transformation, and describe the current application value of TIM-3 in MDS treatment.

2 Structure and biological function of TIM-3

2.1 Overview of the TIM family

Members of the TIM family (T cell immunoglobulin and mucin domain family) include TIM-1 to TIM-8, of which TIM-1, TIM-3, and TIM-4 have been clearly identified and well-studied in humans (9). TIM-3 consists of three structural domains: the extracellular region containing the immunoglobulin variable domain (IgV), mucin region, and stalk region, while the transmembrane and intracellular regions are enriched with tyrosine residues for mediating the activation or inhibition of downstream signaling pathways (10). TIM-3 was initially identified in CD4+ helper T cells (Th1 cells) and CD8+ cytotoxic T lymphocytes (CTLs) and was regarded as a negative regulatory receptor. With further research, TIM-3 has also been widely demonstrated in innate immune cells such as dendritic cells, NK cells, monocytes/macrophages, mast cells, etc., and plays a key role in a variety of tumor and inflammatory environments (6–11).

2.2 Main ligands and signaling pathways

TIM-3/HMGB1 pathway: which can attenuate the innate immune activation by blocking dendritic cells from recognizing the nucleic acids originating from tumors, and which in turn suppresses tumor immune surveillance (15, 16); TIM-3/PtdSer pathway: helps to clear apoptotic cells and inhibit immune hyperactivation under normal conditions; in tumor or chronic inflammatory environments, it may be exploited by tumor cells to evade immunity (17).

The identified TIM-3 ligands include galactose lectin-9 (Gal-9), carcinoembryonic antigen-associated cell adhesion molecule 1 (CEACAM1), high mobility group protein 1 (HMGB1) and phosphatidylserine (PtdSer) (12–14). Binding of different ligands to TIM-3 triggers multiple downstream signaling pathways and is involved in the regulation of T cell tolerance, immune depletion, and antigen presentation by dendritic cells. For example: TIM-3/Gal-9 pathway: Binding to Gal-9 on the surface of T cells inhibits IFN-γ secretion and induces apoptosis in Th1 and Th17 cells (11) (15, 16); in the tumor microenvironment, this pathway plays an important role on myeloid-derived suppressor cells (MDSC) and depleted CD8+ T cells (17); TIM-3/CEACAM1 pathway: Our team found that TIM-3 interaction with CEACAM1 not only affects T cell tolerance, but also correlates with the NF-κB/NLRP3/Caspase-1 inflammatory axis, which is involved in inflammation and immune escape in the tumor microenvironment (18); TIM-3/HMGB1 pathway: It can impair innate immune activation by blocking the recognition of tumor-derived nucleic acids by dendritic cells, which in turn suppresses tumor immunosurveillance (19, 20); TIM-3/PtdSer pathway: It helps to clear apoptotic cells and inhibit immune hyperactivation under normal conditions; and may be exploited by tumor cells to evade immunity in tumor or chronic inflammatory environments (21) (Figure 1).

Currently, the TIM3-related signaling pathways in AML/MDS are mainly TIM-3/Gal-9 and TIM-3/CEACAM1, while the TIM3/HMBG1 and TIM-3/PtdSer pathways have been less well studied in AML and have not been studied in MDS.

3 Role of TIM-3 in hematologic malignancies

3.1 TIM-3 in AML and leukemia stem cells

In acute myeloid leukemia (AML), TIM-3 is an important surface marker on leukemia stem cells (LSC). Studies have shown

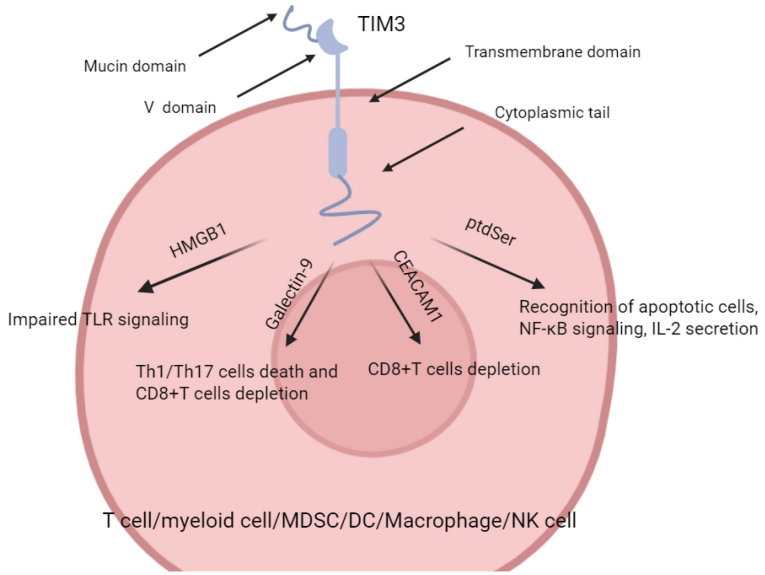

FIGURE 1
Models for TIm3–ligand (Gal-9 and CEACAM1) interactions. (Cited in: Nat Rev Immunol. 2020 Mar;20(3):173-185).

that TIM-3 is highly expressed on LSC but not or lowly expressed on normal hematopoietic stem cells (HSC), and that blocking TIM-3 not only slows down the leukemic progression in AML mice, but also reduces the number of leukemic stem cells transplanted into mice (22). Our team compared the TIM3 expression levels of HSCs in MDS patients, AML patients, and healthy volunteers, and found that the TIM3 expression levels of HSCs in high-risk MDS and AML patients were abnormally high, and TIM3+ HSCs exhibited aberrant differentiation, hyperproliferation, and reduced apoptosis (24). Thereafter, Japanese scholars further suggested that the interaction of TIM-3 with Gal-9 could promote LSC proliferation by activating signals such as NF-κB and β-catenin, and was closely associated with poor prognosis (23).

In addition, Vadim V Sumbayev's team found that TIM3 interacts with HMGB1 and induces the secretion of VEGF (angiogenic protein vascular endothelial growth factor), which promotes bone marrow angiogenesis, thereby alleviating hypoxic conditions induced by an increased number of LSC cells, which in turn supports the survival and proliferation of LSCs (25). PtdSer is considered to be one of the key one of the "eat-me signals" and promotes the uptake of apoptotic cells. Last year, Fredrik B Thorén et al. performed a genetic screen on the K562 leukemia cancer cell line and found that deletion of the TMEM30A gene leads to the accumulation of PtdSer on the outer side of the cell membrane, which binds to TIM3, which in turn inhibits NK cells leading to immune escape. The results of this phenomenon were consistent across a variety of leukemia cell lines and lymphoma cell lines, and targeted blockade of PtdSer or TIM3 reversed the occurrence of immune escape in TMEM30A-deficient tumor cells (26). In conclusion, the above findings further demonstrate that TIM3 plays an important role in AML pathogenesis and that combining multiple targets (e.g., TIM-3 with other leukemia-associated molecules) to inhibit LSC exhibits stronger anti-leukemic activity than a single target (27).

3.2 Progress of TIM-3 in MDS

3.2.1 Abnormal expression of TIM-3 in MDS hematopoietic stem/progenitor cells and osteoblasts

Recent studies have revealed that the immune checkpoint molecule TIM3 is aberrantly expressed in a variety of malignant hematologic diseases.Our team's TIM-3 assay of bone marrow hematopoietic stem cells (HSC) from MDS patients revealed that TIM-3-positive HSC with aberrant differentiation, hyperproliferation, and reduced apoptosis were strongly associated with higher conversion rates and shorter median survival in patients (24). In addition, in the bone marrow microenvironment of MDS patients, osteoblast activity is significantly reduced and TIM-3 is abnormally highly expressed in osteoblasts, and this high expression may further perturb the balance of the bone marrow ecological niche and promote disease progression (25).

Our team further found that despite the similar morphology of TIM3+ and TIM3- stem cells in MDS patients, TIM3+ stem cells had lower colony-forming ability and more pronounced karyotypic abnormalities, suggesting that they may represent early malignant clones (24). Meanwhile, myeloid-derived suppressor cell (MDSC) cells highly expressed TIM3, CEACAM1, and Gal-9, and inhibited apoptosis of TIM3+ stem cells through the TIM3/Gal-9 and TIM3/CEACAM1 pathways, whereas targeted blockade of the pathways reversed their anti-apoptotic effects (17, 18). We again confirmed by animal models that TIM3+ stem cells, especially in combination

with MDSC, showed enhanced expansion capacity *in vivo* but impaired differentiation potential, further supporting their malignant clonal properties and the pro-cancer role of MDSC (24).

The study by Toshio Asayama's team provides an important addition to the role of TIM3 in MDS progression. They demonstrated again that TIM3 expression on the surface of primitive cells and plasma levels of the TIM3 ligand, galactoselectin-9 (Gal-9), are dynamically elevated with the transformation of MDS to AML and are closely associated with primitive cell proliferation, disease progression, and prognosis (27). In addition, the team demonstrated that the bone marrow microenvironment induced the upregulation of TIM3 and Gal-9 expression, and thus they concluded that the TIM3-Gal-9 signaling axis may contribute to MDS disease progression and transformation to AML.

In conclusion, these findings collectively model the multiple roles of TIM3 in the pathogenesis of MDS, where TIM3 acts as a primitive cell-intrinsic regulator to promote malignant clonal proliferation and disrupts bone marrow microenvironmental homeostasis as well as promotes disease progression and accelerates leukemic transformation in conjunction with the ligand Gal-9. This provides a new rationale for the development of antitumor therapies targeting TIM3.

3.2.2 Role of TIM-3 in the immune microenvironment of MDS

In addition to its pro-proliferative and anti-apoptotic roles in MDS malignant clones, aberrant expression of TIM-3 in immune cells further exacerbates immune escape and disease progression in MDS. Our team analyzed in bone marrow samples from MDS patients by multicolor flow cytometry and found that compared to healthy controls, the proportion of the TIM3+ subpopulation of CD8+ T cells was significantly elevated in MDS patients, but the secretion of granzymes and perforin by this population of cells was decreased, along with the up-regulation of expression of apoptosis-sensitive marker CD95 (Fas), which suggests that there is functional exhaustion of TIM-3+ CD8+ T cells (28). In addition, PD-1 co-expression of TIM3+ CD8+ T cells was significantly elevated compared to controls, suggesting that TIM3 may synergize with other immune checkpoint molecules to jointly mediate T cell dysfunction (29). Subsequently, we have elucidated that TIM3 can regulate the formation of the immunosuppressive microenvironment in MDS through different ligand-dependent pathways: The TIM-3/Gal-9 signaling axis promotes myeloid-derived suppressor cell (MDSC) expansion and induces CD8+ T-cell functional depletion (17); whereas, TIM-3/CEACAM1 interaction, which in turn enhances the immunosuppressive capacity of MDSC, promotes secretion of inhibitory cytokines such as IL-10 and TGF-β, which ultimately exacerbates the bone marrow inflammatory microenvironment (18). Asayama et al. proposed on this basis that the TIM-3/Gal-9 signaling axis and imbalanced bone marrow microenvironment not only contribute to the pathogenesis of MDS, but also accelerates the transition of MDS to secondary AML (sAML) by inducing proliferation of progenitor cells and immune escape and thereby accelerating MDS) transformation (27).

In addition to CD8+ T cells, aberrant expression of TIM3 in the helper T cell (Th) subpopulation also affects immune homeostasis in MDS. Our team found that TIM-3 expression was significantly upregulated in Th1, Th17 and regulatory T cells (Treg) in MDS patients, and, of particular importance, TIM-3+ Treg cells exhibited dysfunction and their TGF-β secretion capacity was reduced (30), suggesting that TIM-3 may weaken the inhibitory capacity of Treg on effector T cells by altering its cytokine profile and while enhancing the overall immunosuppressive microenvironment and playing an important role in immune escape.

Recent studies have also revealed the critical role of TIM3 in intrinsic immune cells. In dendritic cells (DCs), TIM3 maintains the tolerogenic phenotype of DCs by inhibiting NLRP3 inflammatory vesicle activation. Knockout experiments confirmed that TIM3-deficient DC cells significantly enhanced the activation and expansion of CD8+ T cells and stem cell-like T cells (TSCM), and promoted anti-tumor immune responses (31). In addition, the expression of TIM3 in the monocyte-macrophage system also has a dual regulatory role: on the one hand, TIM3+ macrophages exhibit an M2-type polarization tendency, with impaired phagocytosis and antigen-presentation; on the other hand, TIM3 can promote the secretion of immune-suppressive cytokines through the regulation of the NF-κB signaling pathway, which further deteriorates the inflammatory microenvironment of MDS (32), allowing the tumor cells to evade immune surveillance and attack the organism, accelerating tumor progression and immune escape.The immunomodulatory role of TIM3 was further supported by the clinical study of Moiseev et al. who found that the proportion of TIM3-positive NK cells (CD56+TIM3+) was significantly increased in patients with MDS and, together with CD8+TIM3+T cells and CD4+TIM3+T cells, constituted the immune signature of poor prognosis. Multivariate analysis showed that patients with high TIM3-expressing immune cell populations had shorter progression-free survival (PFS) and worse prognosis (33).

In summary, these findings point to the conclusion that the aberrant expression of TIM-3 in MDS progenitor cells and immune cells builds a complex regulatory network: TIM3 directly inhibits the anti-tumor activity of T/NK cells, promotes the immunosuppressive function of MDSC and M2-type macrophages, and alters the immune-regulatory properties of DC cells and Treg cells. These effects form a "tumor-immune microenvironment" positive feedback loop that drives disease progression. Therefore, therapeutic strategies targeting TIM-3 (e.g., TIM3 monoclonal antibody or combined PD-1/CTLA-4 blockade) may not only directly inhibit tumor growth, but also reshape the immune microenvironment by lifting the suppression of DC cells by TIM3, enhancing the cross-presentation capacity of tumors, and activating CTL cells; reprogramming the polarization

of macrophages to enhance phagocytosis; and restoring the virulence and proliferative capacity of NK/T cells, thus reversing the suppression of the immune microenvironment, and improving the immunoregulatory properties of DC cells and Treg cells. Reversing the suppressed state of immune microenvironment, improving new direction for MDS immunotherapy. However, due to the great heterogeneity of MDS, the degree of immunosuppression and microenvironment of different patients are also different, and there may be differences in the efficacy of TIM3 inhibitors after application.

3.3 Significance of TIM-3 in other hematologic tumors

TIM3 also showed high expression levels in other hematological malignancies, and our team found that high expression of TIM-3 was present on myeloma cells of multiple myeloma (MM) patients and correlated with disease progression, and was also found to be closely related to the activation of the NF-κB signaling pathway; knockdown of TIM-3 significantly inhibited cell proliferation and induced apoptosis, and bortezomib had a synergistic NF-κB pathway inhibition, suggesting that TIM-3 could be a potential future therapeutic target for MM (34). It has been found that the expression of TOX, TOX2 protein and TIM3 is elevated in T-cell acute lymphoblastic leukemia (T-ALL), and that TOX and TOX2 proteins can directly induce the transcription and expression of TIM3, preventing apoptosis, whereas targeting TXO or TIM3 slows down the growth of tumors (35). A study found that with the disease progression of B-cell acute lymphoblastic leukemia (B-ALL), the expression of TIM-3 in T cells and its ligand galectin-9 were significantly upregulated in both primitive cells and MSCs, and the upregulation of the expression of TIM-3 and galectin-9 was negatively correlated with the disease prognosis, and the study also demonstrated that CAR19-TIM3- Fc T cells could promote the expansion of transduced and memory T cells *in vivo* and improve the antileukemic efficacy and durability of CAR19 T cells in B-ALL (36), however, the results of another study on TIM3 in B-ALL were contradictory, they also found that the expression of TIM3 was elevated in B-ALL CD34+CD19+B primitive cells, but TIM3+B primitive cell transplanted mice showed no significant difference in EFS and OS from TIM-B primitive cell transplanted mice (37). There is increasing evidence that TIM3 expression is elevated in CML stem cells, CD4+ and CD8+ T cells in both primary and relapsed patients with chronic myeloid leukemia (CML), inducing T-cell depletion, and that blocking TIM3 may improve the immune response generated by the discontinuation of TKI inhibitors and concurrently target leukemic stem cells, preventing the disease from relapsing (38, 39). In addition, the role of TIM-3 in chronic lymphocytic leukemia (CLL) and certain lymphomas has been gradually gaining attention, but more mechanistic and clinical studies are needed to clarify the specific role played by TIM3 and to confirm the clinical value of TIM3.

4 Novel therapeutic strategies and clinical progress related to TIM-3

4.1 Monoclonal antibody monotherapy and combined multi-target blockade programs

Based on the multiple mechanisms of TIM-3's role in tumor and immunity, a variety of monoclonal antibodies against TIM-3 have entered the preclinical and clinical research stage in the last decade, including blockade of TIM-3 alone and combined blockade with other immune checkpoints, such as PD-1/PD-L1, CTLA-4, LAG-3, TIGIT, etc. (40, 41). For myeloid tumors, the significance of TIM-3-targeted therapy is even more prominent: not only may it directly inhibit the proliferation of primitive/stem cells, but also partially restore the immune depletion of T cells or NK cells.

4.2 TIM-3 blockade in combination with demethylating drugs

In the treatment of MDS and AML, demethylating agents (HMAs) such as azacitidine (AZA) and decitabine (DAC) are widely used, but drug resistance and relapse still occur in most patients. Some investigators have tried to combine TIM-3 monoclonal antibody with HMA and found that it can enhance inhibition of tumor cells and improve the immune microenvironment. Several clinical trials are currently evaluating the efficacy of such combination regimens in MDS and AML.

Several clinical trials have been conducted in combination with demethylating agents. Sabatolimab (MBG453) is a humanized IgG4 anti-TIM-3 monoclonal antibody that specifically binds to TIM-3 and blocks its binding to ligands. STIMULUS-MDS1 (NCT03066648): enrolled patients with high/very high-risk MDS versus newly diagnosed patients with primary AML. The study showed an overall favorable safety profile for combination therapy in both the MDS and AML populations, with higher remission rates (both complete and partial) in the MDS group compared to the AML group, and some clinical benefit in some patients with adverse risk gene mutations (e.g., TP53). In another phase II trial (NCT04150029), 18 patients with AML were enrolled and given a three-drug combination of Sabatolimab + Venetoclax + Azacytidine, which was shown to be comparable in safety and tolerability to the two-drug combination of Venetoclax + Azacytidine. The preliminary results of these trials provide important evidence for the use of TIM-3 monoclonal antibody in MDS: in combination with demethylating drugs, it can enhance the response of high-risk MDS patients to a certain extent and is well tolerated, bringing new therapeutic hope for MDS patients.

4.3 TIM-3-CAR-T and bispecific CAR-T/CAR-NK

In the field of cell therapy, chimeric antigen receptor T cell (CAR-T) technology has been successfully applied to a variety of B-cell tumors. In recent years, TIM-3 has also been studied as a target for AML or MDS

cells and introduced into CAR-T cells to selectively kill leukemia cells with high TIM-3 expression (42). In addition, some teams have also explored bispecific CAR-T, such as targeting both TIM-3 and CD13, which demonstrated higher tumor clearance and relatively controllable toxicity to normal hematopoietic stem cells in AML mouse models (43). To further minimize the possible adverse effects of CAR-T such as severe cytokine release syndrome (CRS) and neurotoxicity, some investigators are trying to introduce CAR into NK cells (CAR-NK) (44). Preliminary results show that TIM-3-CAR-NK exhibits better anti-tumor activity in both *in vitro* and ex vivo experiments. Although such studies are still in the early exploratory stage, they offer new possibilities for personalized cell therapy for MDS and AML.

5 Latest clinical trial progress and challenges

With more clinical trials, the mechanism of action and efficacy of TIM-3 inhibitors in hematologic tumors have been further confirmed. However, the following challenges need to be noted:

5.1 Combined blockade with other immune checkpoints

The current clinical results regarding TIM3 monoclonal antibody monotherapy for MDS disease are not satisfactory, and TIM3 inhibitors need to be co-applied with other target drugs to achieve the expected results. According to the results of clinical trials of TIM3 in MDS/AML/CMML (NCT04878432, NCT04812548,NCT03066648,NCT03946670), the combination of TIM3 inhibitors with demethylating drugs (decitabine or azacitidine) and/or small-molecule targeted drugs or immune checkpoint inhibitors is better than monotherapy. better than single-agent application, partly because of biased results due to too few recruits, and partly because checkpoint molecules such as TIM-3, PD-1, CTLA-4, LAG-3, and TIGIT may play different roles at different stages and in different cell types, or there may be mutual compensation. It is sometimes difficult to achieve sustained clinical remission by blocking a pathway alone, and multi-agent combinations are not only efficacious but also safer for patients who are not suitable for intensive chemotherapy or after stem cell transplantation. However, more clinical studies are needed to overcome the clinical challenge of optimizing the timing, dosage and strategy of combination therapy, as well as assessing patient resistance and tolerability.

5.2 Evaluation of efficacy in patients with adverse risk gene mutations

Mutations such as TP53 are prevalent in high-risk MDS/AML and the prognosis is usually poor. Whether the trial data suggest that TIM-3 blockade may also have some efficacy in such patients requires further large-scale validation.

5.3 Immune-related toxicities and drug resistance

Similar to other immunotherapies, TIM-3 blockade may bring autoimmune or inflammatory side effects, such as over-immune activation and myelosuppression, etc. Moreover, new immune escape pathways may emerge in the tumor cells and the microenvironment, which may lead to secondary drug resistance.

5.4 Compared with other immune checkpoint-targeted drugs

there have been hundreds of clinical trials of immune checkpoint inhibitors for MDS currently under investigation, such as TIM3, PD-1/PD-L1, CD47, CTLA-4, Clever-1 inhibitors, etc. However, none of the clinical effects of single-agent therapy are satisfactory, and the core strategy of treatment is still combination of demethylating drugs, and the combination of drugs in the primary treatment of higher-risk MDS patients with ORR up to 60-80% (NCT03066648, NCT04623216, NCT03248479, NCT05428969). In comparison, TIM3 inhibitors have a slightly weaker ORR than PD-1 and Clever-1 inhibitors, but have a stronger overall safety profile, with no typical irAE occurring at present, and may be more suitable for MDS patients intolerant of PD-1 toxicity.TIM3 is uniquely advantageous in that it can target both T-cells and myeloid tumor cells, making it more suitable for patients with a highly suppressed immune microenvironment (e.g., high Treg infiltration).

In recent years, the development of tumor immunotherapy has provided new therapeutic ideas for malignant hematological diseases such as MDS, etc. TIM-3, as an important immune checkpoint molecule, is often highly expressed in myeloid and lymphoid tumor cells on the one hand, which promotes malignant proliferation and immune escape, and on the other hand, it can also be expressed on a wide range of immune cells (e.g., T cells, NK cells, DC cells, macrophages, etc.), which affects immune effects and inflammatory microenvironment. For MDS, the mechanism of action of TIM-3-targeted therapy may combine the advantages of "tumor cell inhibition" and "immune activation", but more large-scale phase III trials are needed to validate the survival benefit, and it may become an alternative option for PD-1-resistant or highly immunosuppressive MDS in the future. In the future, it may become an alternative choice for PD-1-resistant or highly immunosuppressive MDS.

6 Discussion

TIM-3, as an emerging inhibitory immune checkpoint molecule, plays an important role in the pathogenesis of myeloid malignant tumors, especially MDS and AML. At the tumor cell level, high expression of TIM-3 promotes the proliferation and anti-apoptosis of primitive cells and LSC; at the level of the immune

environment, TIM-3 induces the depletion of T cells and NK cells, and regulates inhibitory cell populations, such as MDSCs, dendritic cells, and macrophages, to fuel immune escape; at the level of the myeloid ecological niche, TIM-3 may be associated with the dysfunction of osteoclasts and stromal cells, and TIM-3 may be associated with osteoblast and stromal cell dysfunction at the bone marrow ecological level, and influence the progression of MDS through inflammatory signaling axes (e.g., NF-κB/NLRP3).

Immunotherapy, with its unique mechanism of "remodeling the body's immune system to recognize and kill tumors", is bringing new therapeutic hope for a variety of malignant hematological diseases. Monoclonal antibodies against TIM-3 (e.g. Sabatolimab) and their combination with other immune checkpoint inhibitors or HMA, BCL-2 inhibitors, etc. have achieved certain results in MDS/AML clinical trials, and some patients have achieved long-lasting remission, while cellular therapeutic strategies, such as CAR-T/CAR-NK, have provided a new way of thinking for refractory relapse cases. However, there are still many challenges: for example, the difference in efficacy of TIM3 inhibitors in different disease stages, and how to combine with other checkpoint inhibitors or chemotherapeutic agents in order to obtain the optimal synergistic effect.

Based on the current challenges, future MDS-related studies could further explore the association between TIM3 ligand expression and the degree of myeloid cell infiltration and efficacy. Meanwhile, we should pay more attention to patients with refractory relapsed or drug-resistant MDS, clarify the mechanism of drug resistance and the role of immune microenvironmental disorders, deepen the understanding of the pathogenesis and drug resistance mechanisms, and increase the number of clinical trials of TIM3 multidrug combination therapy for MDS, so as to provide more clinical basis for TIM3 combined with PD-1/PD-L1, Clever-1 and other inhibitors.TIM3 inhibitors in clinical application should pay attention to the combination of molecular typing, immune microenvironment inhibition stratification and dynamic monitoring, so as to develop a more precise and individualized immunotherapy program.

In summary, the study of TIM-3 provides new possibilities for the pathogenesis and clinical treatment of MDS. With the accumulation of evidence from more large-scale clinical trials, TIM-3 is expected to become a key molecule in the precision treatment and immunotherapy of MDS, and may play an indispensable role in improving the prognosis of MDS patients in the future.

Author contributions

XG: Visualization, Writing – original draft, Writing – review & editing. SY: Writing – original draft. JT: Writing – original draft. YW: Writing – original draft. ZS: Writing – review & editing. RF: Writing – review & editing. LL: 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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Trends and hotspots in research related to tumor immune escape: bibliometric analysis and future perspectives

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Background: Tumor immune escape, a defining hallmark of malignant tumors, enables cancer cells to thrive within the host by evading detection and attack by the immune system. While immune checkpoint inhibitors, such as PD-1/PD-L1 antibodies, have delivered significant clinical advances, their effectiveness is tempered by modest response rates and a growing challenge of drug resistance. In this study, we aimed to explore the development process and trend of tumor immune escape, analyze the current hot spots, and predict the future research directions.

Methods: A bibliometric analysis was conducted in this study to retrieve and analyze 1839 publications from January 1, 2009 to February 14, 2025 related to tumor immune escape. Literature was obtained from Web of Science Core Collection (WoSCC) and data visualization and trend analysis were performed using VOSviewer, CiteSpace, Bibliometrix software package.

Results: The bibliometric analysis indicates that research on tumor immune escape has primarily focused on China, the United States, and European countries. China ranks first in research output and impact, with notable contributions from institutions like the Sun Yat-sen University System and the University of Texas System. The journal with the most publications is Frontiers in Immunology, while the most cited article globally is Jiang P's 2018 publication in Nature Medicine, titled "Signatures of T cell dysfunction and exclusion predict cancer immunotherapy response." Keyword co-occurrence and burst analysis indicate that the field has undergone a thematic evolution. Early research centered around classical immune checkpoint molecules and T cell exhaustion, while more recent trends have shifted toward the tumor microenvironment (TME), multi-target combination immunotherapies, and mechanisms of immune evasion involving metabolic reprogramming and the microbiome. The integration of artificial intelligence (AI) and machine learning (ML) in immunotherapy prediction and biomarker discovery has also gained momentum, highlighting a growing cross-disciplinary approach.

Conclusion: This bibliometric study provides a comprehensive overview of the intellectual landscape, research hotspots, and developmental trajectory of tumor immune escape research over the past 14 years. By mapping influential nation, authors, core journals, reference, and keyword bursts, this work not only summarizes major contributions in the field but also helps researchers better understand its evolution and emerging directions. Based on the observed

patterns, we propose three key areas that warrant further exploration: (1) advancing interdisciplinary research at the intersection of the microbiome, metabolism, and immune regulation; (2) integrating artificial intelligence and multi-omics data to enhance predictive modeling and therapeutic precision; and (3) combining multi-modal therapeutic strategies to overcome immune escape more effectively.

KEYWORDS

tumor immune escape, bibliometric analysis, tumors, tumor microenvironments, immune checkpoint inhibitors

1 Introduction

Cancer has become a globally prevalent and serious economic and social problem, with increasing incidence and high mortality rates (1). Although the traditional three main therapies (surgery, radiotherapy, and chemotherapy) remain the cornerstone of clinical treatment, their efficacy is limited by significant toxicities and patient response heterogeneity. Targeted therapies have achieved significant breakthroughs in treating specific malignancies by blocking key oncogenic signaling pathways, such as those involving the epidermal growth factor receptor, HER2, estrogen receptor, vascular endothelial growth factor recepto, and multikinase inhibitors (2). However, issues of acquired drug resistance and inadequate therapeutic efficacy remain unresolved, particularly in cancers with complex pathophysiologic mechanisms.

The advent of cancer immunotherapies has revolutionized treatment approaches, particularly with immune checkpoint inhibitors like anti-PD-1/PD-L1 antibodies marking a landmark advancement. This breakthrough, awarded the 2018 Nobel Prize in Physiology or Medicine, combats tumor immune evasion by enhancing T-cell-mediated antitumor immune responses (3). However, despite these advances, not all patients benefit clinically due to the dynamic complexity and spatial heterogeneity of TME (4). Recent studies have demonstrated that malignant cells employ various strategies to create immune-evasive microenvironments, including metabolic reprogramming, secretion of immunosuppressive factors, and epigenetic modulation of antigen-presentation mechanisms (5, 6).

Tumor immune escape is a phenomenon where tumor cells avoid immune system recognition and attack, enabling them to grow and metastasize. This is a key strategy for tumor survival and progression (7). The interaction between immunity and cancer in regulating tumor growth is considered a cancer hallmark. Anti-tumor immunity involves innate and adaptive immune responses that control cancer development and proliferation. Tumor immune escape poses a major obstacle to effective anticancer therapy (8). Many factors induce tumor immune escape, including low tumor cell immunogenicity, tumor-specific antibody recognition as self-antigens, tumor surface antigen regulation, tumor-induced immune privilege, and tumor-induced immunosuppression. Research mainly focuses on the latter factors. Cancer cells can evade the immune system by activating immune checkpoints, altering the surrounding microenvironment, causing antigen presentation and recognition abnormalities, and undergoing metabolic reprogramming to inhibit T-cell activity. This allows cancer cells to survive and proliferate within the host (9). This mechanism is significantly influenced by programmed death receptor 1/programmed death receptor-ligand 1 (PD-1/PD-L1), which regulates immune tolerance and escape within TME (7, 10–12). When the PD-1 receptor on activated T cells interacts with the PD-L1 receptor on cancer cells, it weakens cytotoxic T lymphocyte effects, helping malignant cells resist immune attacks and promoting immune escape (13).

In recent years, research into tumor immune escape mechanisms and their role in cancer progression has surged exponentially. Reviews have explored key issues in this field from molecular pathways and clinical interventions perspectives (4, 14). However, a systematic overview of the discipline's development and knowledge structure evolution is still lacking, as is a clear definition of research foci and potential blind spots. Bibliometrics, an effective tool for assessing discipline dynamics, can objectively identify core contributing countries, institutions, and scholars, and reveal landmark high-impact literature. It can also track historical changes in research hotspots, capture emerging frontier directions, and locate under - explored scientific issues (15). Such analyses have been successfully applied to TME (16), checkpoint inhibitor development (17) and other immunotherapy - related fields. Notably, while bibliometric studies on the PD-1/PD-L1 signaling axis or CAR-T cell therapies have been reported (18), there remains a lack of comprehensive and systematic analyses specifically focused on the field of tumor immune escape. Therefore, this study aims to comprehensively analyze the research landscape, evolutionary pathways, and future trends in tumor immune escape using a multidimensional bibliometric approach. This will provide a data - driven decision - making basis for optimizing immunotherapeutic strategies and basic research directions.

Abbreviations: TME, tumor microenvironment; AI, artificial intelligence; ML, machine learning; GBM, Glioblastoma.

2 Materials and methods

2.1 Data collection and sources

Bibliometric analysis offers a systematic framework for identifying developmental trends and research hotspots within a discipline over a defined period. The selection of an appropriate database is critical to ensuring data reliability and analytical rigor. Among available options, the Web of Science (WoS) stands out for its multidisciplinary coverage of high-impact scientific journals and robust citation indexing. Compared to Scopus and MEDLINE/PubMed, WoS provides more comprehensive information that is particularly well-suited for bibliometric analysis (17). In this study, we selected the Web of Science Core Collection as our primary data source, as it is widely recognized for its depth, accuracy, and authority in indexing peer-reviewed literature (19). Its extensive journal coverage ensures that the retrieved publications reflect contemporary research trajectories in immunology and oncology. This choice enables accurate, representative data extraction and supports a thorough exploration of valuable research insights.

2.2 Search strategy and criteria

A literature search was conducted on February 14, 2025, to retrieve original articles and reviews on tumor immune escape published between 2009 and February 14, 2025. To avoid temporal bias due to real-time database updates, the search was completed in a single day. The search strategy was as follows: ((((TS=("Immune Escape, Tumor")) OR TS=("Tumor Immune Escape") OR TS=("Tumor Immune Evasion") OR TS=("Evasions, Tumor Immune") OR TS=("Evasion, Tumor Immune") OR TS=("Immune Evasions, Tumor") OR TS=("Tumor Immune Evasions") OR TS=("Immune Evasion, Tumor")))). Only journal articles and reviews published in English were included in this analysis. Other publication types—such as letters, editorials, conference abstracts, meeting reports—and all non-English publications were excluded to ensure consistency and comparability of the bibliometric dataset. The eligible records were exported in plain-text format with the "Full Record and Cited References" option selected to enable comprehensive metadata extraction. The final dataset contained information on publication counts, citations, titles, authors, affiliations, countries, keywords, and journals. In total, 1,839 records met the inclusion criteria. The detailed screening process is presented in Figure 1.

2.3 Data analysis

For data processing and analysis, we used Microsoft Excel in combination with three specialized tools: Bibliometrix 4.3.3 (an R-based package), VOSviewer 1.6.20, and CiteSpace 6.4.R1.

VOSviewer, developed by van Eck and Waltman, generates bibliometric network visualizations using node-link diagrams. It visualizes collaboration patterns by clustering nodes chromatically, where node size represents publication volume and edge thickness indicates collaboration strength between entities (e.g., countries or institutions).

CiteSpace, created by Chaomei Chen, is a Java-based software for detecting research frontiers. It employs timeline mapping and citation burst detection, with keyword clustering to reveal thematic domains. Clustering reliability is validated when silhouette values

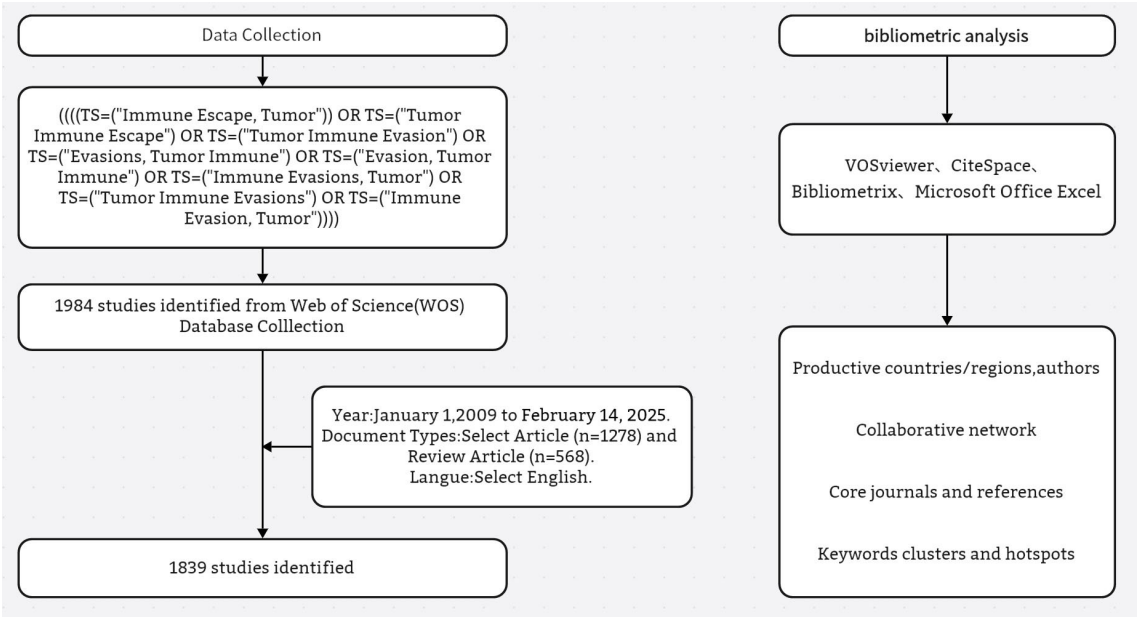


FIGURE 1
A process flowchart.

exceed 0.5 and modularity Q-values exceed 0.3, indicating strong internal consistency and significant structural separation.

Bibliometrix, an R-integrated package, enables statistical analysis of scholarly outputs including publication frequencies, citation metrics, and national contributions. Its algorithms support cross-comparison among journals and countries, contributing to a quantitative understanding of academic productivity.

3 Result

3.1 Trends in publications and citations

As per the formulated research strategy, 1,839 tumor immune escape - related publications were obtained from the WoSCC database between 2009 and 14 February 2025. Figure 2 presents the annual publication and citation counts for tumor immune escape research from 2009 to 14 February 2025.

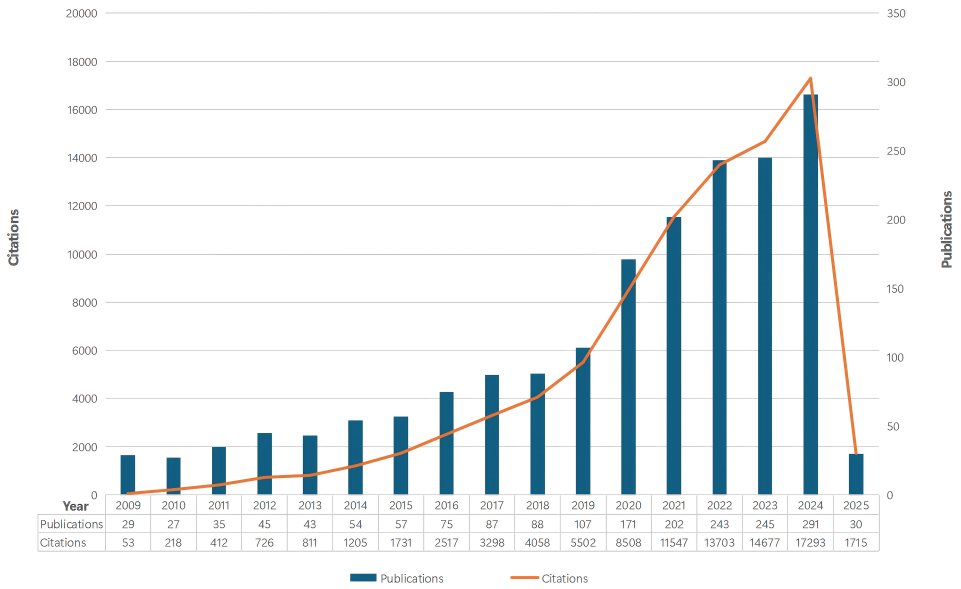
The steady publication increase from 2009 to 2018 shows great attention and interest in this field. The steeper growth curve from 2018 onwards indicates significant expansion, likely due to the 2018 Nobel Prize in Physiology or Medicine awarded to Professors James P. Allison and Tasuku Honjo for their work on the CTLA - 4 and PD - 1/PD - L1 pathways. The rising citation trend suggests ongoing research impact and the need for more prospective studies to highlight its global relevance.

3.2 National and institutional analyses

A total of 65 countries and 2,184 institutions participated in tumor immune escape research. Table 1 ranks the top ten countries by number of publications and total citations. China (n = 958) was the most productive country, accounting for 52.1 per cent of the total number of publications, followed by the United States (n = 281, 15.3 per cent), and Germany (n = 114, 6.2 per cent). The US and China have nearly identical total citations, 28,052 and 28,142 respectively, far surpassing other nations and highlighting their influence in this field. The UK has the highest average citations per publication at 131. Multiple country publication (Figure 3) refers to the proportion of publications in this field involving contributions from multiple countries and is used to assess international collaboration levels within a research area in a given country. China has the highest number of publications, but the proportion of multiple country publication with other countries is relatively low at 13.2%. However, France (43.2%) and the UK (69.6%) have a high proportion of multiple country publication, indicating their significant contributions to international cooperation.

A minimum threshold of 7 articles was set to filter out 30 countries meeting the criteria, as shown in Figures 4A, B. This reveals a wide - ranging network of international cooperation, with the US, China, and various European countries serving as key hubs. The United States led international collaboration with the highest total link strength at 303, underscoring its central role in the global tumor immune-escape network. China followed at 204, and Germany at 117, together highlighting these nations' pivotal contributions to cross-border research and knowledge exchange in the field. Notably, the closest collaboration exists between the US and China. Additionally, publication timelines were analyzed through a VOSviewer - based visual map of organizational collaboration overlays (Figure 4B). It is worth noting that China started publishing later than most other leading countries in this field.

Among the top 15 institutions ranked by publication count (Table 2), Sun Yat-sen University leads with 72 publications, followed by the Chinese Academy of Sciences with 69. This indicates Sun Yat-sen University has the greatest international



Year	2009	2010	2011	2012	2013	2014	2015	2016	2017	2018	2019	2020	2021	2022	2023	2024	2025
Publications	29	27	35	45	43	54	57	75	87	88	107	171	202	243	245	291	30
Citations	53	218	412	726	811	1205	1731	2517	3298	4058	5502	8508	11547	13703	14677	17293	1715

FIGURE 2
The number of annual papers and citations on tumor immune escape research has been steadily increasing from 2009 to 2025.

TABLE 1 Top 10 productive countries of publications on tumor immune escape.

Rank	Country	Articles n(%)	SCP	MCP	MCP %	Country	TC	AC
1	CHINA	958 (52.1%)	832	126	13.2	CHINA	28142	29.40
2	USA	281 (15.3%)	203	78	27.8	USA	28052	99.80
3	GERMANY	114 (6.2%)	83	31	27.2	GERMANY	6284	55.10
4	ITALY	79 (4.3%)	62	17	21.5	ITALY	3270	41.40
5	FRANCE	44 (2.4%)	25	19	43.2	UNITED KINGDOM	3013	131.00
6	JAPAN	38 (2.1%)	34	4	10.5	SPAIN	2154	107.70
7	NETHERLANDS	32 (1.7%)	21	11	34.4	NETHERLANDS	1842	57.60
8	KOREA	25 (1.4%)	21	4	16	FRANCE	1783	40.50
9	UNITED KINGDOM	23 (1.3%)	7	16	69.6	CANADA	1647	109.80
10	IRAN	20 (1.1%)	13	7	35	JAPAN	1631	42.90

NP, number of publications; SCP, single country publication; MCP, multiple country publication; TC, total citation; AC, average citations.

influence. These institutions are significant not only in publication quantity but also in impact. Notably, the University of Texas System, despite ranking eighth in publication count (45 publications), holds the top spot in betweenness centrality (0.11 centrality), suggesting its research is highly collaborative internationally and highly influential in tumor immune escape.

Figure 5A shows the top 15 institutions with citation outbreaks. Shandong First Medical University & Shandong Academy of Medical Sciences have recently experienced citation bursts, indicating significant potential in tumor immune escape research. In the co - occurrence graph (Figure 5B), node size represents co - occurrence frequency, and links show co - occurrence relationships. Nodes with purple rounded corners have high betweenness centrality (≥0.1), such as the University of Texas System and UTMD Anderson Cancer Center, which play key roles in connecting diverse research communities.

3.3 Analysis of journals

To identify active and influential journals in tumor immune escape, a visual analysis of published journals was done, uncovering 1,839 related publications in 472 academic journals. FRONTIERS IN IMMUNOLOGY had the most publications (126), followed by

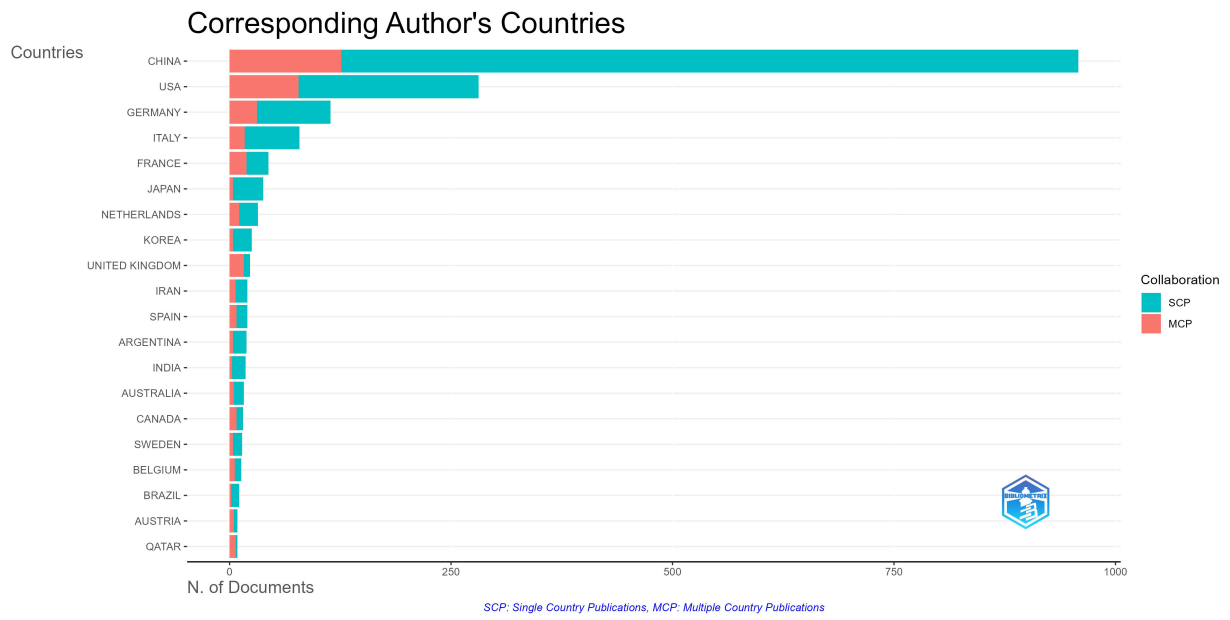


FIGURE 3
Top 20 most corresponding author's country in the tumor immune escape field.

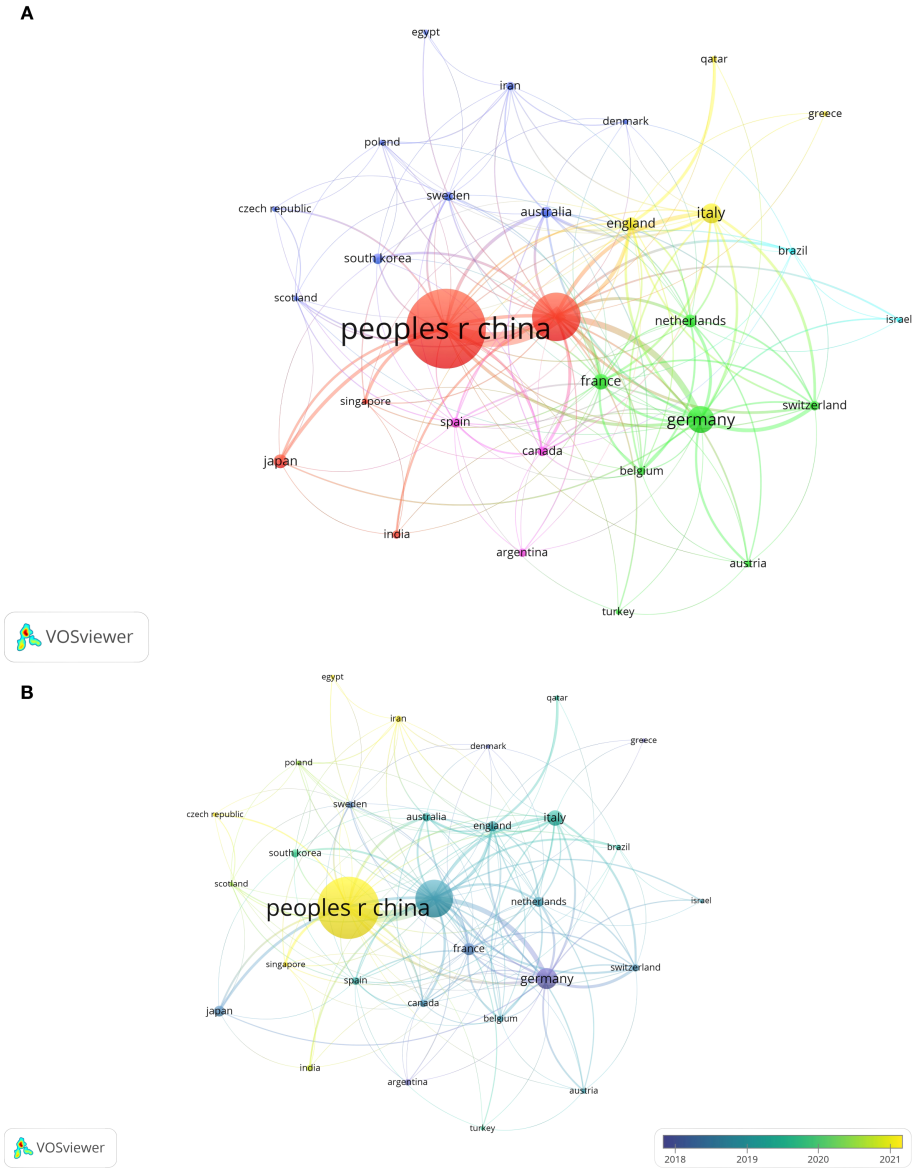


FIGURE 4
(A) Visual map of national/regional citation networks. Each circle/node's size shows the number of papers published. The connection strength between circles/nodes is indicated by the line thickness, with colors representing clusters of related objects in the network. Each circle/node stands for a separate country/region. **(B)** Visualisation map of the country/region citation overlay. Purple nodes are organisations that started research in this area earlier, while yellow nodes are those that began later.

CANCERS (54) and CANCER RESEARCH (31) (see Table 3). Notably, CANCER RESEARCH has the highest impact factor (12.5) and average citations (107) among the top 10 journals, underscoring its significant impact in tumor immunology.

Figure 6 Application of Bradford's Law showing core journals for tumor immune escape research.

The double figure overlay reveals a single citation pathway in numerous inter - field links between journals (Figure 7). Interestingly, publications on MOLECULAR, BIOLOGY, GENETICS are mainly cited by publications on MOLECULAR, BIOLOGY, IMMUNOLOGY and MEDICINE, MEDICAL, CLINICAL.

3.4 Author contributions and co-occurrence

In this study, 12,322 authors were involved in the study. Table 4 lists the top 10 most prominent authors in tumor immune escape research. Xuitao Cao leads the list with 11 articles and 1737 citations, followed by Kebin Liu with 10 articles and 558 citations. Notably, Li Yong has published fewer articles but has the second highest number of citations. His 2018 publication was the first article on tumor immune escape, marking him as a highly promising emerging figure in the field (Table 4).

TABLE 2 Top 15 core institutions in terms of publications.

Rank	Institution	NP	Centrality	Country
1	Sun Yat Sen University	72	0.09	China
2	Chinese Academy of Sciences	69	0.05	China
3	Central South University	55	0.04	China
4	Shanghai Jiao Tong University	55	0.02	China
5	Chinese Academy of Medical Sciences - Peking Union Medical College	54	0	China
6	Fudan University	48	0.02	China
7	Zhejiang University	46	0.09	China
8	University of Texas System	45	0.11	USA
9	Institut National de la Sante et de la Recherche Medicale (Inserm)	44	0.07	France
10	Huazhong University of Science & Technology	40	0.06	China
11	Helmholtz Association	38	0.05	Germany
12	Nanjing Medical University	36	0.01	China
13	University of California System	35	0.04	USA
14	Southern Medical University - China	34	0.01	China
15	Harvard University	33	0.01	USA

NP, number of publications.

Figure 8 illustrates the collaborative network of 45 authors who have published five or more articles. These authors are clustered into five distinct collaborative groups. While each group demonstrates strong internal collaboration, there is limited interaction between groups. This pattern indicates a relative lack of intergroup communication and suggests the need to strengthen inter-institutional and international collaboration within the field.

3.5 Citation and reference analyses

Table 5 resents the top 10 most cited articles on tumor immune escape. The first two articles, each with over 2,800 citations, lead significantly over the remaining entries, underscoring their substantial influence in the field.

The most cited article globally is 'Signatures of T cell dysfunction and exclusion predict cancer immunotherapy response' by Jiang P, published in Nature Medicine in 2018 with 3,151 citations. This article introduces TIDE, an alternative biomarker for predicting immune checkpoint blockade (ICB) response, offering novel ideas for immune checkpoint blockade prediction and laying the foundation for immunotherapy prognosis forecasting (20).

The second most cited article globally is Ansell SM's 'PD-1 blockade with nivolumab in relapsed or refractory Hodgkin's lymphoma', published in the New England Journal of Medicine in 2015. This study presents nivolumab as a new PD-1 blockade antibody and is the first to evaluate its efficacy and safety in relapsed or refractory Hodgkin's lymphoma, providing a crucial basis for subsequent clinical applications in combating tumor immune escape (21).

Figure 9A highlights 20 core publications that experienced significant citation bursts, underscoring their influence and cutting-edge contributions to the tumor immune escape field during the analyzed timeframe. Early foundational literature, such as the work by Rabinovich et al. (22), experienced a dramatic citation surge between 2009 and 2011. This article became a landmark in tumor immunology by synthesizing previously fragmented immune escape mechanisms into a unified conceptual framework. It identified key immunotherapeutic targets, addressed unmet needs in immunotherapy research, and catalyzed the translation of basic science into clinical practice. Building upon earlier conceptual frameworks, Hanahan D provided a comprehensive synthesis of the hallmarks of cancer, in which immune evasion was recognized as an emerging hallmark and the TME was emphasized as a critical component influencing tumor progression and therapeutic resistance (23). This conceptual integration laid important theoretical groundwork for subsequent research into the mechanisms of tumor immune escape.

Between 2013 and 2017, three citation burst references were identified, all of which were pivotal clinical trials (24–26). This period marked a significant turning point, as immune checkpoint inhibitors (ICIs) transitioned from preclinical exploration to clinical application. Among these, the landmark study by Hodi FS et al. (26) demonstrated that ipilimumab significantly improved overall survival in patients with advanced melanoma (median OS increased from 6.4 to 10.1 months), leading to its FDA approval in 2011 for metastatic melanoma. Ipilimumab thereby became the

FIGURE 5
This analysis focuses on research institutes related to tumor immune escape. **(A)** It visualises co - author and research institution collaborations in this field. **(B)** It presents a co - occurrence mapping of research institutions. Here, node size shows co - occurrence frequency, and links indicate co - occurrence relationships. Nodes with purple circles have high betweenness centrality (≥0.1).

first immune checkpoint inhibitor approved globally, inaugurating a new era in cancer immunotherapy. Four additional citation burst articles identified between 2015 and 2020 (21, 27–29) focused on PD-1 inhibitors, likely reflecting the momentum generated by the FDA approvals of nivolumab and pembrolizumab in 2014. These approvals marked a major breakthrough in immunotherapy and spurred a surge of clinical and translational research into immune checkpoint blockade strategies. In 2018, Jiang P and colleagues (20) developed TIDE (Tumor Immune Dysfunction and Exclusion), a computational framework designed to model the two major mechanisms of tumor immune escape and predict responses to ICI therapy. Beyond its direct predictive value, TIDE played a pioneering role in bridging AI and tumor immunology, setting the stage for subsequent applications of machine learning in decoding immune evasion. Notably, this study attracted widespread attention between 2021 and 2023 and remains the most cited article in the field to date, underscoring its foundational significance and groundbreaking impact. The article exhibiting the most intense citation burst was “Global Cancer Statistics 2020: GLOBOCAN Estimates of Incidence and Mortality Worldwide for 36 Cancers in 185 Countries” by Sung H et al. (30), published in 2021 in CA: A Cancer Journal for Clinicians (impact factor: 503.1). Utilizing GLOBOCAN 2020 data, this study provided a comprehensive overview of the global cancer burden, highlighting substantial

TABLE 3 Top 10 core journals.

Rank	Journal	h_index	NP	TC	AC	2024 JCI division	IF (2024)
1	FRONTIERS IN IMMUNOLOGY	36	126	4034	32	Q1	5.7
2	CANCERS	23	54	1508	27	Q2	4.5
3	CANCER RESEARCH	22	31	3321	107	Q1	12.5
4	ONCOIMMUNOLOGY	22	30	1484	49	Q1	6.5
5	FRONTIERS IN ONCOLOGY	19	54	1792	33	Q2	3.5
6	PLOS ONE	19	23	1893	82	Q1	2.9
7	CANCER IMMUNOLOGY RESEARCH	18	20	1267	63	Q1	8.9
8	JOURNAL FOR IMMUNOTHERAPY OF CANCER	18	33	1259	38	Q1	10.3
9	INTERNATIONAL JOURNAL OF MOLECULAR SCIENCES	16	34	781	22	Q2	4.9
10	JOURNAL OF IMMUNOLOGY	16	19	1981	104	Q2	3.6

h_index, Hirsch index; NP, number of publications; TC, total citation; AC, average citations; IF, impact factor.

regional differences in cancer incidence and mortality, and exploring the underlying epidemiological factors. The publication has since served as both an authoritative data source and an essential reference for global oncology research and clinical practice.

The use of co - citation cluster analysis offers an objective illustration of the knowledge structure within a research area. For a more detailed description of the co - cited reference groups, a network diagram was generated. The degree of association between articles

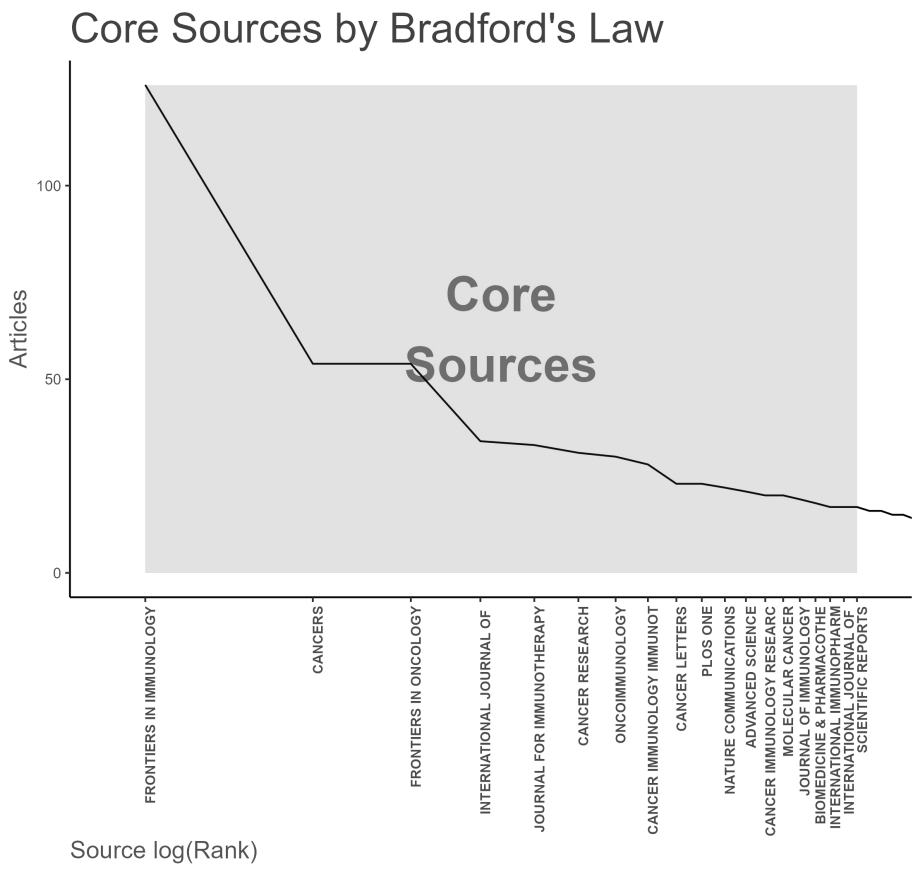


FIGURE 6
Analysis of academic journals related to tumor immune escape. Bradford's law in academic journals.

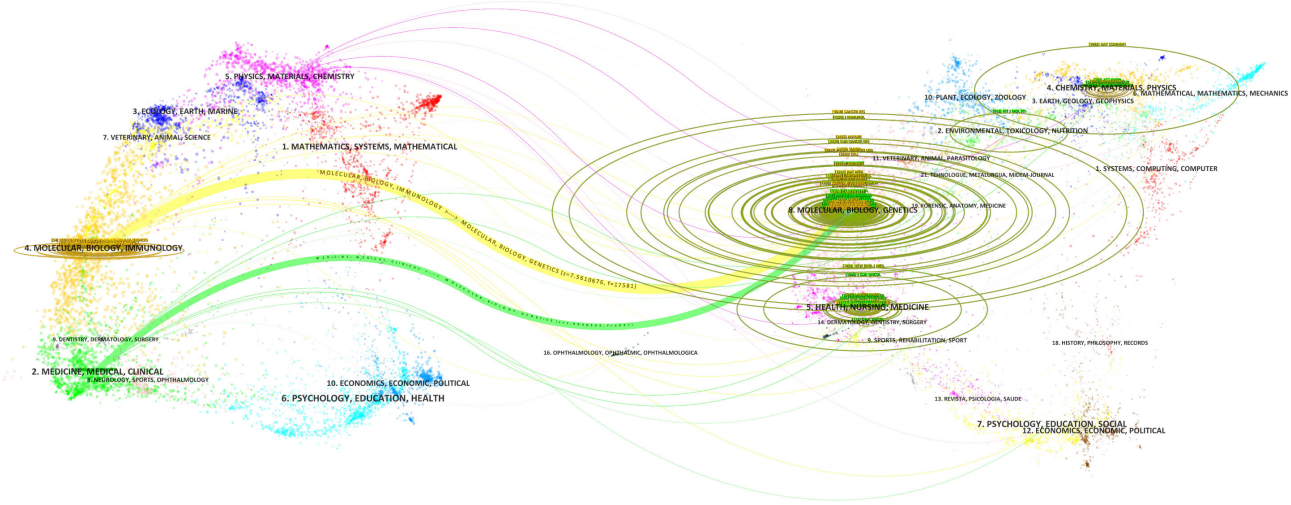


FIGURE 7
Double image overlay of journals in tumor immune escape research. This overlay visualises citation relationships between journals in this field. The lower graph shows citing journals on the left, cited journals on the right, with coloured lines indicating citation paths.

was categorized into 17 groups, which formed the basis for the clustering classification. The co - citation cluster analysis, as shown in the diagram, clearly reveals the knowledge structure of the research area. To fully describe the co - cited literature groups, a complex network diagram was constructed (as shown in Figure 9B). Research topics were classified into 17 categories based on co - citation relationships, forming a clear cluster structure. In this diagram, (1) metabolic reprogramming, being the largest cluster, indicates that metabolic reprogramming - related research holds a central position in the field and carries extensive academic influence.

Evidence of evolution over time among the study clusters is also apparent. For instance, the gradual evolution of (4) non-small cell lung cancer clusters into the emergence of (0) tumor-derived exosome, (3) tumor-associated macrophages and (1) metabolic reprogramming clusters. In the field of non-small cell lung cancer research, the transition from conventional to immunotherapeutic approaches has catalyzed the rapid evolution of tumor immunotherapy. Additionally, triple negative breast cancer is the most aggressive breast cancer type, having limited treatment choices and a poor prognosis (31). The figure shows that the triple negative breast cancer group gradually evolved into metabolic reprogramming and tumor-associated macrophage groups. This means immunotherapy breakthroughs bring hope to triple negative breast cancer clinical treatment.

The (7) PD-L1 cohort has gradually evolved into the (3) tumor-associated macrophages and (0) tumor-derived exosomes cohorts, reflecting the research process of tumor immune escape, which further promotes the exploration of the tumor microenvironment

TABLE 4 The ten most relevant authors and their works.

Rank	Author	h_index	NP	TC	PY_start
1	CAO XUETAO	9	11	1737	2009
2	LIU KEBIN	9	10	558	2016
3	RABINOVICH GABRIEL A.	9	11	590	2009
4	ELKORD EYAD	8	9	1049	2016
5	GUO WEI	8	10	680	2017
6	KOCH JOACHIM	8	8	403	2010
7	KOEHL ULRIKE	8	8	438	2010
8	LI WEI	8	9	342	2010
9	LI YONG	8	8	1609	2018
10	LU CHUNWAN	8	10	540	2016

NP, number of publications; TC, total citation; h_index, Hirsch index.

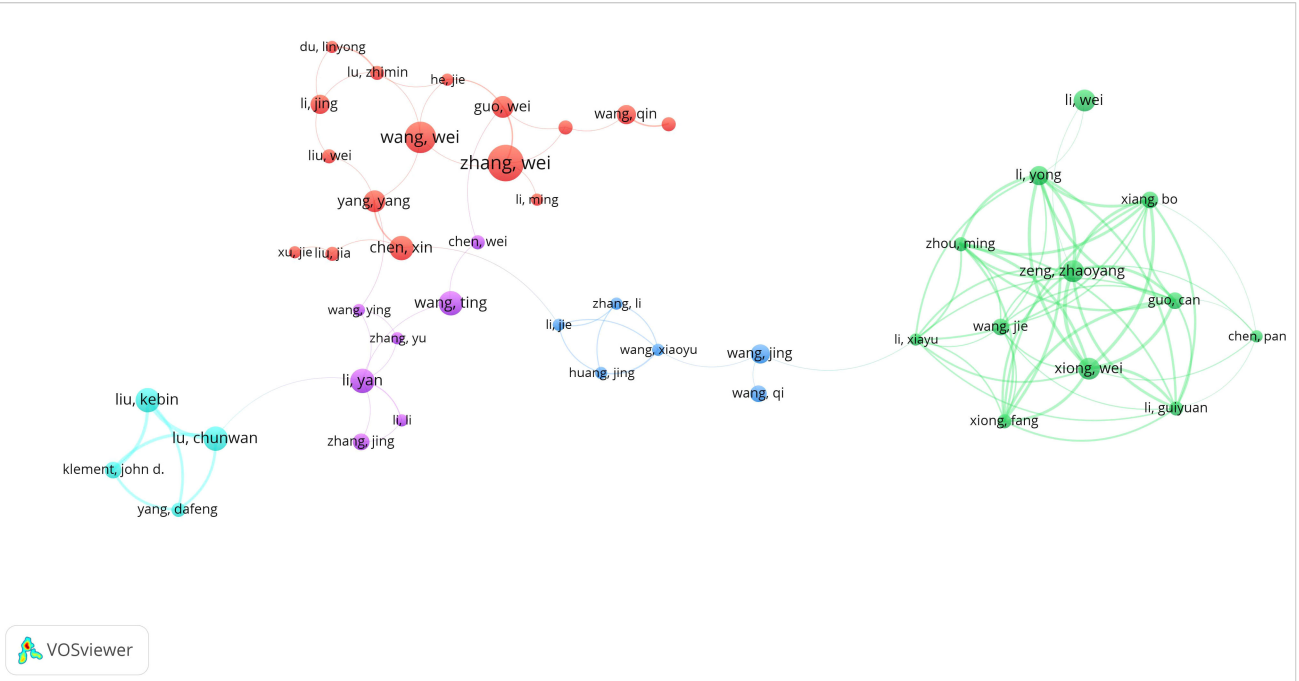


FIGURE 8
Authors' tumor immune escape study blood infection co-occurrence graph. 744 different colored nodes reflect authors in different clusters. Node size indicates co-occurrence frequency and links indicate co-occurrence relationships between authors.

TABLE 5 Top 10 core literatures.

Rank	Title	First author	Journal	Type	Year	Total citations
1	Signatures of T cell dysfunction and exclusion predict cancer immunotherapy response	Jiang P	Nature Medicine	Article	2018	3151
2	PD-1 blockade with nivolumab in relapsed or refractory Hodgkin's lymphoma	Ansell SM	New England Journal of Medicine	Article	2015	2834
3	Transforming Growth Factor-β Signaling in Immunity and Cancer	Batlle E	Immunity	Review	2019	1499
4	Targeting CXCL12 from FAP-expressing carcinoma-associated fibroblasts synergizes with anti–PD-L1 immunotherapy in pancreatic cancer	Feig C	Proceedings of The National Academy of Sciences of The United States of America	Article	2013	1481
5	LDHA-Associated Lactic Acid Production Blunts Tumor Immunosurveillance by T and NK Cells	Brand A	Cell Metabolism	Article	2016	1256
6	PD-1 and PD-L1 Checkpoint Signaling Inhibition for Cancer Immunotherapy: Mechanism, Combinations, and Clinical Outcome	Alsaab HO	Frontiers in Pharmacology	Review	2017	1237
7	Increased circulating myeloid-derived suppressor cells correlate with clinical cancer stage, metastatic tumor burden, and doxorubicin–cyclophosphamide chemotherapy	Diaz-Montero CM	Cancer Immunology, Immunotherapy	Article	2009	993
8	Role of the tumor microenvironment in PD-L1/PD-1-mediated tumor immune escape	Jiang XJ	Molecular Cancer	Review	2019	974
9	T-Cell Transfer Therapy Targeting Mutant KRAS in Cancer	Tran E	New England Journal of Medicine	Article	2016	966
10	Immune checkpoint blockade therapy for cancer: An overview of FDA-approved immune checkpoint inhibitors	Hargadon KM	International Immunopharmacology	Review	2018	872

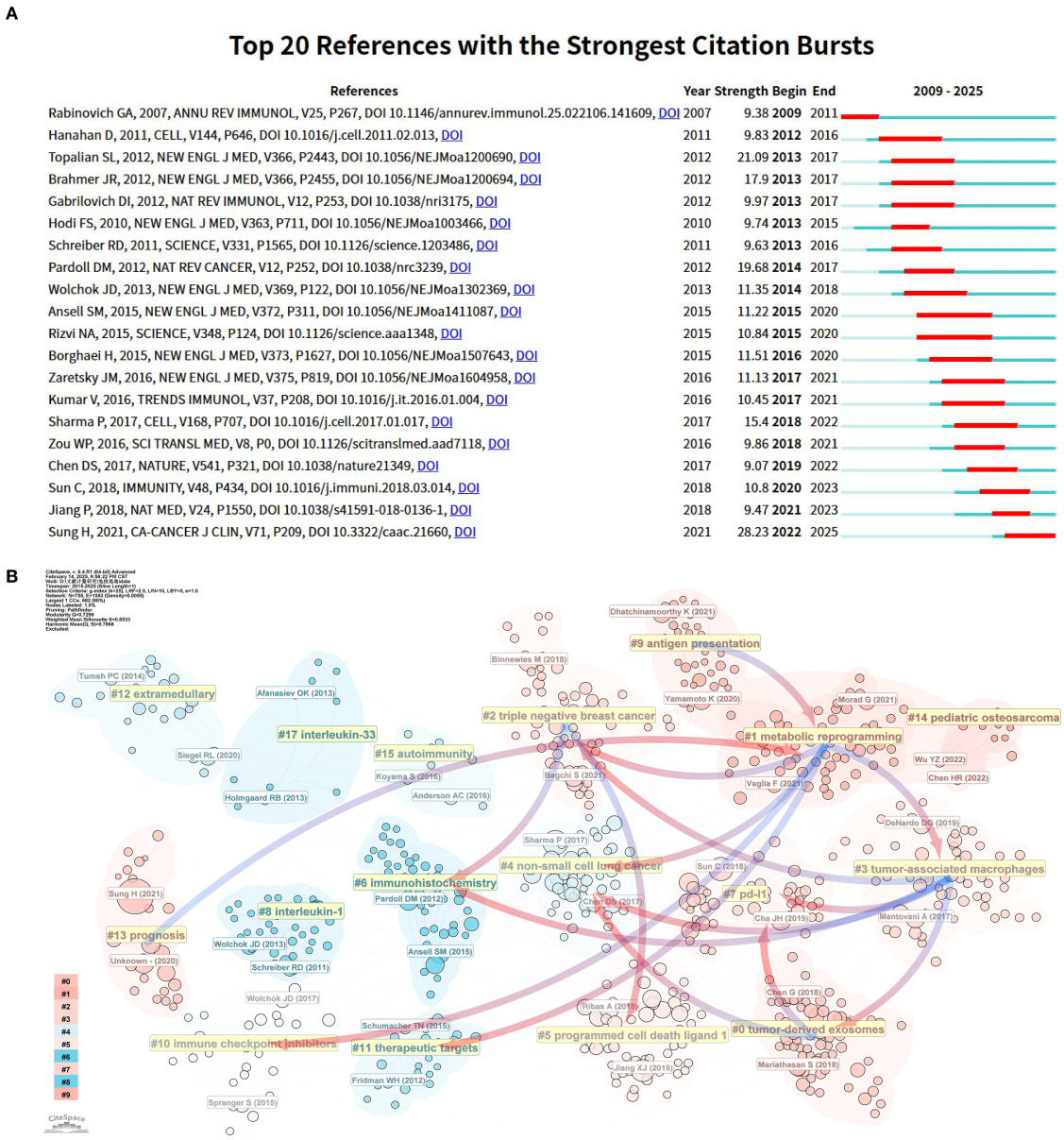


FIGURE 9
Analysis of references related to tumor immune escape. **(A)** The top 20 references with a significant increase in citation frequency. **(B)** Clustering of references according to similarity. Topics include #0 Tumorderived exosomes, #1 Metabolic reprogramming, #2 Triple-negative breast cancer, #3 Tumor-associated macrophages, #4 Non-small cell lung cancer, #5 PD-L1, #6 Immunohistochemistry, #7 PD-L1, #8 Interleukin-1 and so on. Linkage represents connections between different clusters, and the blue groups on the line evolve from the red ones.

due to the differentiated efficacy of PD-L1-targeted drugs. It reflects the in-depth exploration of the mechanism of tumor development and the continuous optimization of immunotherapy strategies in the field of tumor immunity.

Beyond the main evolutionary trends, several smaller groups indicate the ongoing development of specific research directions. Notably, (1) metabolic reprogramming cohorts have gradually evolved into (9) prognosis cohorts. This shift shows that as tumor metabolomics advances, researchers are paying more attention to predicting patient prognosis. Metabolic reprogramming is a key strategy for tumor cells to adapt to harsh microenvironments and maintain rapid proliferation and survival (32, 33). This metabolic alteration not only supports tumor growth but also closely correlates with tumor malignancy and poor patient prognosis (34), providing a solid theoretical basis for the evolution of metabolic reprogramming research towards prognosis. In clinical practice, accurately predicting patient prognosis is crucial for developing personalized treatment plans and improving survival rates.

3.6 Keywords co-occurrence analysis

Figures 10A, B highlight key themes in tumor immune escape research, including 'immunotherapy', 'pd-l1', 'cancer', 'expression',

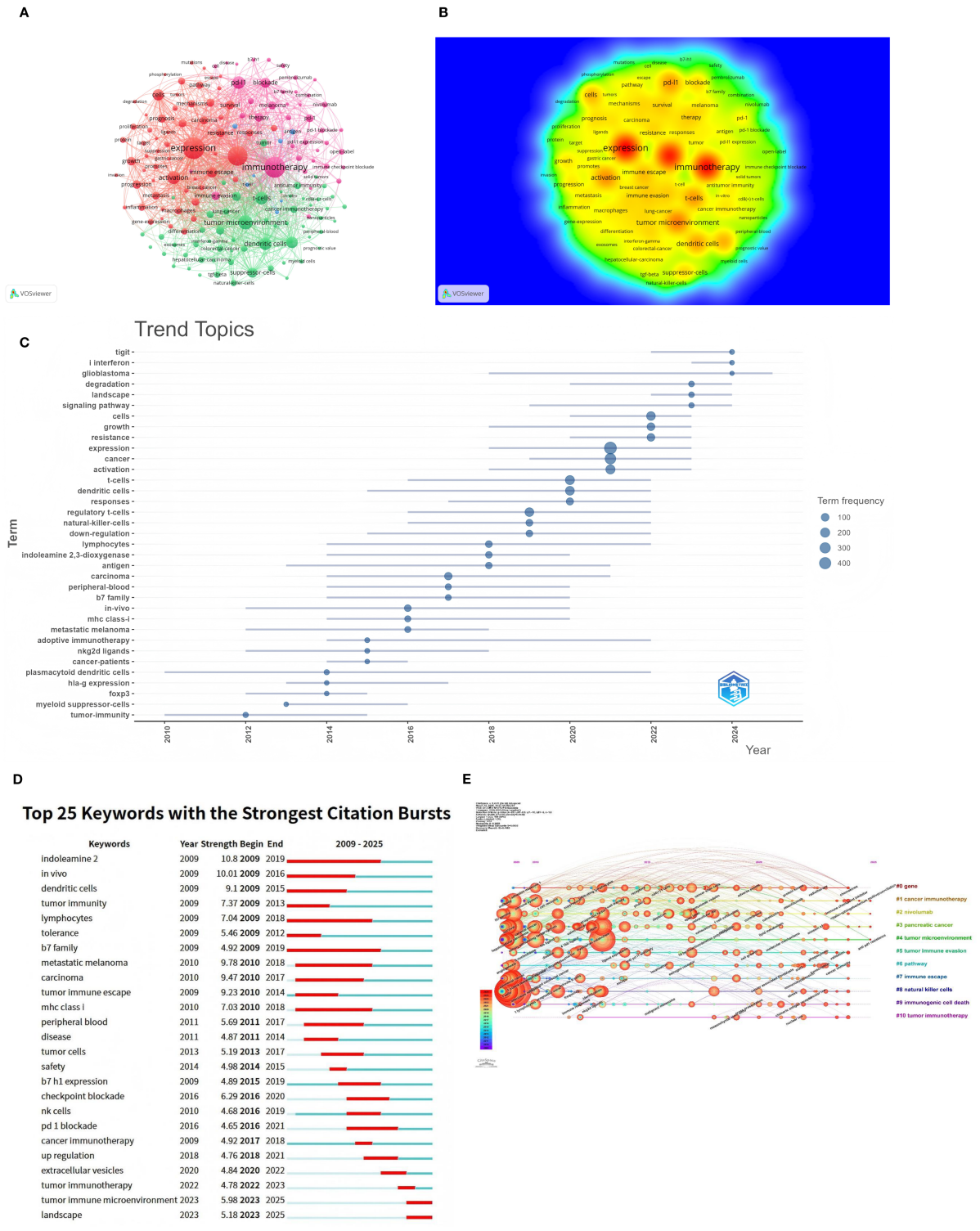


FIGURE 10
Keyword co-occurrence analysis in tumor immune escape studies. **(A)** Network visualisation for keyword co-occurrence analysis (n>5). Each node in the network represents a keyword, with the size of the node indicating the number of times the keyword occurs. Lines between nodes indicate co-occurrence between keywords; the larger the node size, the higher the frequency of the keyword. **(B)** Density visualisation of keyword co-occurrence analysis. This visualisation methodically illustrates the density and intensity of research themes within the designated field. Heat maps are utilised to accentuate areas of varying research intensity, with warmer colours denoting higher activity and stronger connections. **(C)** Trend themes from 2009 to 2025. The timeline illustrates the temporal progression of pivotal research themes within the field. The relative prominence of these research themes undergoes substantial fluctuations over the course of time, with larger nodes denoting elevated frequency and significance. **(D)** The 25 keywords with the strongest citation bursts are displayed. The blue line indicates the time axis, with the red segments denoting the start year, end year, and duration of each burst. **(E)** Timeline of keyword co-occurrence analysis. The timeline visualises the temporal evolution of key research topics in the field. The salience of each keyword undergoes a change over time, with larger and more concentrated nodes representing higher frequency and importance. The keywords are then organised into clusters on the right-hand side of the figure.

'tumor microenvironment', 'dendritic cells', and 't-cells'. These keywords are strongly interconnected, reflecting their central role in the research community. The density visualization reveals intense research activity around these themes, with warmer colors indicating areas of high interest.

Figures 10C, D depict the evolution of research topics in tumor immune evasion. Figure 10C analysis of theme word trends from 2009 to 2025 shows that "TIGIT," "type I interferon," and "glioblastoma" are the next few years' research Frontiers. Keyword burst analysis delineates three pivotal evolutionary stages in tumor immune escape research (Figure 10D). The pronounced bursts of "dendritic cells" and "lymphocytes" during 2009–2015 underscored foundational investigations into antigen presentation machinery and T-cell activation dynamics. Concurrently, sustained bursts of "indoleamine 2,3-dioxygenase" and "B7 family"(2009–2019) signaled the emergence of metabolic checkpoint pathways as critical regulators. The subsequent phase (2016–2021) witnessed transformative clinical advances, where bursts in "checkpoint blockade" and "PD-1 blockade correlated with therapeutic breakthroughs in immune checkpoint inhibitors, while renewed focus on "B7-H1 expression" (2015-2019) reflected its consolidation as a predictive biomarker. Current research has shifted toward tumor microenvironmental orchestration, exemplified by the burst in extracellular vesicles (2020–2022) highlighting exosome-mediated immune remodeling. Dominant ongoing bursts for tumor immune microenvironment and landscape (2023–2025) reveal accelerating adoption of spatial multi-omics and integrative biology frameworks to deconvolute immune evasion ecosystems. Notably, persistent attention to MHC class I (2010–2018) reflects enduring challenges in antigen presentation defects as core resistance mechanisms. Figure 10E categorizes keywords into 11 groups arranged chronologically, showing the most recent and prevalent keywords as 'Immune Checkpoint Inhibitors', 'Sphingobacterium multivorum', and 'Anti PD-1 Resistance'.

4 Discussion

4.1 Overall distribution

Research on tumor immune evasion has sustained growth from 2009 to 2024, with no sign of abating in 2024. This reflects growing interest and attention in the field (30). Immunotherapy has become a major strategy for cancer treatment. However, tumor immune escape remains a significant challenge to the efficacy of anticancer therapies. To understand the mechanism of tumor immune escape, many targeted approaches have been explored, and some drugs have been clinically applied and achieved better efficacy (35, 36). Globally, China, the United States, and European countries are major contributors to research on tumor immune evasion. These countries' research efforts have a clear advantage in addressing the global cancer threat. China leads in research output with 958 (52.1%) research papers published, showing the breadth and depth of its research and its great global influence. This is closely related to China's comprehensive cancer screening and registration system and the rising cancer mortality rate (37, 38). Meanwhile, the USA and Germany have published 281(15.3%) and 114(6.2%) publications respectively, showing that the USA and Europe also have a large influence in the field of tumor immune escape. China's cancer screening program, initiated in 1958, has significantly expanded its coverage over the past decade. This provides robust and credible primary data supporting research in the field of tumor immune escape (38). Presently, China faces a substantial cancer burden. According to the latest World Health Organization (WHO) estimates, the country accounts for 24% of global cancer incidence cases, with cancer mortality rates exceeding the global average (1, 39, 40). Consequently, the "Healthy China 2030" initiative designates cancer prevention and control as a strategic priority. Coupled with sustained investment in scientific research, these policies have enabled China to achieve significant progress in cancer prevention and treatment, while making substantial contributions to tumor immune escape research (41).

In the field of research institutions, China's top institutions excel in output and influence. All seven institutions with the highest number of relevant publications are in China. Sun Yat-sen University leads with 72 publications and a betweenness centrality of 0.09, showing its significant position and global collaborative contribution in tumor immune evasion research. Although the University of Texas System has fewer publications, its betweenness centrality of 0.11 reflects substantial contributions to global cooperation in this field. Notably, Shandong First Medical University & Shandong Academy of Medical Sciences has rapidly risen to become a prominent player in recent years. Despite strong domestic collaborations among Chinese institutions, international cooperation remains relatively limited, which may hinder overall progress in tumor immune evasion research. Therefore, strengthening international collaborations between research institutions is crucial for accelerating global research efforts and addressing the worldwide cancer challenge.

Bibliometric analysis shows the USA and France excel in international collaboration, especially in cross - national publications. The UK, despite fewer publications, maintains strong research networks with other countries, highlighting its role in advancing global tumor immune escape research. The United States not only demonstrates strong research output in the field of tumor immune escape research, but also maintains a high proportion of multinational collaborative publications. That edge traces back to decades of steady investment by the National Cancer Institute in fostering worldwide oncology partnerships (42). Additionally, the Cancer Genome Atlas (TCGA) (43) have established accessible genomic databases that have catalyzed the formation of multinational research consortia and promoted collaborative discoveries. These collaborations facilitate knowledge sharing and enhance international synergies, enabling effective responses to global cancer challenges. Notably, low - and middle - income countries, largely due to China's contributions, have significantly contributed to this research, helping overcome resource deficiencies and high cancer risks in these areas (44). Nevertheless, cross - border collaboration between research

institutions remains limited, which may impede research progress. Enhanced global research collaboration and resource sharing could facilitate tumor immune escape research in these regions and provide more diverse perspectives and data support in the global fight against cancer.

Among journals, Frontiers in Immunology publishes the most tumor immune escape studies, far exceeding other academic journals. While Cancer Research publishes fewer articles, it has the highest average citations and impact factor (12.5), underscoring its substantial influence in this field. Among authors, Cao XT from China is the most prolific and cited. He and his team summarize the role of tumor - associated macrophages (TAMs) in promoting tumor progression and drug resistance (45), explore antibody variable region engineering applications, and discuss future antibody engineering directions to enhance cancer therapy (46).

4.2 Evolution of research focus and translational impact

Our bibliometric analysis shows that tumor immune escape research has evolved from focusing on classical checkpoints like PD-1/PD-L1 to exploring more complex mechanisms such as T cell exclusion, antigen presentation loss, and TME dynamics. (Table 6) Recently, emerging hotspots include combination immunotherapies, AI-assisted predictive modeling, and the cross-talk between metabolic reprogramming and the microbiome—an area gaining notable traction.

These trends have clear translational value. Insights into immune evasion are driving the development of multi-target therapeutic strategies, particularly for tumors resistant to standard immunotherapies, such as glioblastoma (GBM) and MSS colorectal cancer. Advances in single-cell profiling, spatial transcriptomics, and AI tools are further enabling precision immune phenotyping and personalized treatment planning.

At the policy level, the increasing relevance of immune escape calls for integrating immune profiling into clinical workflows and national treatment guidelines. Promoting international collaboration and investment in emerging technologies will be key to accelerating progress and improving global cancer outcomes.

4.3 Research hotspots

Bibliometrics is crucial for processing and analyzing large-scale data to offer researchers insights into trends. Analyzing frequent keywords and subject terms can uncover changing trends and key themes, which are vital for understanding the field's evolution. Based on the above analysis, current major hotspots focus on areas like Immune Checkpoint Inhibitors, tumor immune microenvironment, and landscape. An in-depth analysis of these research hotspots can help better understand the progress of tumor

TABLE 6 Major molecular and cellular drivers of tumor immune evasion.

Category	Molecule/Cell	Definition	Mechanism of action
Immune Checkpoints	PD-L1	Transmembrane immunosuppressive protein	Binds PD-1 on T cells to inhibit activation and cytokine production
	CTLA-4	T-cell surface receptor	Competes with CD28 for B7 ligands on APCs, blocking co-stimulatory signals
Antigen Presentation	HLA-I	Major histocompatibility complex class I	Presents tumor antigens to CD8+ T cells; frequent allelic loss in tumors
	B2M	β2-microglobulin subunit	Essential for MHC-I complex stability; mutations cause antigen presentation failure
	TAP1/2	Transporter associated with antigen processing	Transports antigen peptides to ER for MHC-I loading; often downregulated in tumors
Suppressive Cytokines	TGF-β	Pleiotropic immunosuppressive cytokine	Induces Treg differentiation; blocks CD8+ T-cell proliferation; disrupts ribosomal P-stalk formation
	IL-10	Anti-inflammatory cytokine	Inhibits dendritic cell maturation; promotes M2 macrophage polarization; downregulates TAP1
Metabolic Regulators	IDO1	Tryptophan-catabolizing enzyme	Depletes tryptophan to induce T-cell anergy; generates kynurenine to activate Tregs
	CD73	Ecto-5'-nucleotidase	Converts AMP to adenosine, which binds A2AR on T cells to suppress activation
TME Suppressive Cells	Tregs	Regulatory T cells (CD4+CD25+FoxP3+)	Express CTLA-4 to deplete CD80/86 on DCs; secrete IL-10/TGF-β; directly kill CD8+ T cells via granzyme B
	M2 Macrophages	Alternatively activated macrophages	Secrete arginase-1 to deplete arginine (essential for T cells); produce VEGF for angiogenesis; express PD-L1
	CAFs	Cancer-associated fibroblasts	Secrete CXCL12 to block T-cell infiltration; produce TGF-β to induce T-cell exhaustion; create physical barriers

immune escape research and predict future developments in the context of current studies.

4.3.1 Current research hotspots

4.3.1.1 Novel immune checkpoint discovery and multi-target combination strategies

Antibodies targeting immune checkpoints such as the programmed death-1 (PD-1) receptor, its ligand PD-L1, or cytotoxic T lymphocyte-associated-4 (CTLA-4) have transformed the treatment of many tumor types. However, only a small percentage of patients produce a durable response. Consequently, researchers are actively exploring new immune checkpoints to target and combining therapies to achieve enhanced therapeutic efficacy.

TIGIT, a member of the poliovirus receptor (PVR)/nectin family, is expressed on T cells, NK cells, and Tregs (47, 48). It features an extracellular IgV domain, transmembrane region, and cytoplasmic ITIM/ITT motifs. By binding to CD155 (PVR) with high affinity, TIGIT competitively inhibits the co-stimulatory receptor DNAM-1 (CD226), thus suppressing the activation of T and NK cells (49, 50). The signaling pathway involved in this process, mediated by Grb2/SHIP1, results in the disruption of the MAPK and NF-κB pathways. In clinical trials, the anti-TIGIT antibody tiragolumab, when combined with the anti-PD-L1 antibody atezolizumab, demonstrated an improved overall response rate (ORR) of 37.3%, compared to 20.6% with monotherapy, in patients with PD-L1-high non-small cell lung cancer (NSCLC) (51).

LAG-3 (Lymphocyte Activation Gene 3), a member of the immunoglobulin (Ig) superfamily located on chromosome 12, is expressed on CD4+/CD8+ T cells, natural killer (NK) cells, regulatory T cells (Tregs), and plasmacytoid dendritic cells (52, 53). It inhibits T cell activation by binding with high affinity to MHC-II molecules on antigen-presenting or tumor cells, suppressing IL-2 and IFN-γ secretion. The intracellular domain of LAG-3 contains the S484, KIEELE, and EP motifs, which regulate cellular localization and TCR signaling (54, 55). In clinical trials, relatlimab (anti-LAG-3 monoclonal antibody) combined with nivolumab (anti-PD-1) achieved FDA approval for advanced melanoma (Phase III RELATIVITY-047 trial), demonstrating superior progression-free survival (PFS: 10.1 *vs.* 4.6 months) (56). Future multi-targeted combination therapies have great potential in the treatment of cancer.

4.3.1.2 NETs–TME interactions: a hotspot in tumor immunology

The tumor microenvironment (TME) is now regarded as crucial in cancer development, progression, and treatment (57). This heterogeneous system consists of a chemical TME (marked by acidic pH, hypoxia, and low nutrition), a cellular TME (including tumor cells, stromal cells, pericytes, endothelial cells, immune cells, and the extracellular matrix), and various signaling molecules like cytokines, chemokines, and growth factors within the microenvironment (58, 59). These components interact closely and consistently with tumor cells, thereby enabling the tumor to evade the immune system through different mechanisms (60).

Among the immune cells within the TME, neutrophils play a central role in all stages of cancer progression (61). Neutrophils contribute to tumor metastasis through the formation of extracellular traps (NETs), which protect tumors from effector T cell-mediated elimination (62). However, NETs may also have a dual role in the TME. In certain acute inflammatory conditions, NETs have been shown to inhibit melanoma cell migration and promote tumor lysis, suggesting that they can contribute to tumor elimination (63). Interestingly, depletion of the immune checkpoint receptor CD276 has been found to significantly reduce the expression of CXCL1, which ultimately diminishes neutrophil infiltration into tumors, thereby decreasing NET formation through the CXCL1-CXCR2 axis. This reduction in neutrophil-driven immune suppression can enhance NK cell infiltration, which may play a pivotal role in halting the progression of esophageal squamous cell carcinoma (64). Chronic stress has been shown to disrupt the normal circadian rhythm of neutrophils, leading to increased formation of neutrophil extracellular traps (NETs) via elevated glucocorticoid release. This alteration progressively shapes a TME that favors metastatic cancer progression (65).

Understanding the TME and the role of neutrophil extracellular traps (NETs) is essential for advancing cancer immunology. The TME serves not only as a physical and biochemical scaffold for tumor growth but also as a dynamic immunological hub that orchestrates immune evasion, metastatic potential, and therapeutic resistance. Moving forward, deeper investigation into the mechanistic crosstalk between NETs and the TME is critical. Unraveling these interactions will inform the development of novel immunotherapeutic strategies, including NET-targeted interventions, which may reshape the immunosuppressive landscape of solid tumors and improve clinical outcomes.

4.3.1.3 Type I interferon as a promising strategy to overcome ICB resistance

Despite the transformative success of immune checkpoint blockade therapies, their clinical efficacy remains highly heterogeneous across patient populations (66). A considerable proportion of patients exhibit primary resistance or develop adaptive resistance during treatment, often due to a highly immunosuppressive TME, insufficient tumor immunogenicity, or impaired effector immune responses (67). Type I interferons (IFN-I) have emerged as key modulators of antitumor immunity. IFN-I signaling coordinates a range of immune-regulatory processes, including dendritic cell (DC) maturation, $CD8^+$ T cell activation, macrophage polarization, and the induction of tumor cell senescence and apoptosis (68). Notably, IFN-I can also act directly on natural killer T (NKT) cells, enhancing their infiltration into the TME and further amplifying the immune response (69). A promising approach to overcoming ICB resistance involves combining checkpoint inhibitors with IFN-I–activating strategies, particularly in tumors characterized as immunologically "cold." (70).

Borui Tang et al. identified daurisoline (DS)—a bioactive alkaloid extracted from the rhizomes of the traditional Chinese medicinal herb Ban Yue Zi—as a potent inducer of IFN-I signaling. Mechanistically, DS stimulates IFN-I production via a TANK–TBK1–dependent pathway in tumor cells. The IFN-I released subsequently promotes NKT cell recruitment, enhancing antitumor immune activity (71). Importantly, their study demonstrated that combination therapy using DS with either anti–PD-1 antibodies or the STING agonist diABZI significantly remodeled the immune landscape of the TME. These findings suggest that DS-based combinations may serve as a viable strategy to overcome resistance in ICB-refractory tumors.

Similarly, Ruixuan Liu et al. engineered a bacterial strain, VNP-C-C, that co-expresses CCL2 and CXCL9, thereby facilitating immune cell mobilization and establishing a pro-inflammatory TME. This strategy induces immunogenic cell death (ICD) and activates the cGAS–STING pathway, resulting in elevated IFN-I production and a strengthened antitumor response (72). Interestingly, following VNP-C-C treatment, a marked upregulation of PD-1 expression on tumor-infiltrating T cells was observed—indicative of robust immune activation but also potential T cell exhaustion and immune escape. These findings highlight VNP-C-C as a potential priming agent for ICB-based combination immunotherapy.

In summary, these preclinical studies underscore the emerging role of type I interferon signaling as a central modulator of resistance to immune checkpoint therapies. While both DS and VNP-C-C have shown promising immunomodulatory effects in experimental models, neither has yet advanced to clinical trials. Nevertheless, the ability of IFN-I–targeted interventions to convert "cold" tumors into "hot" ones positions this axis as a compelling focus for future translational research and therapeutic development.

4.3.2 Future research hotspots

4.3.2.1 Cross-cutting studies of metabolic reprogramming and microbiome

The intersection of the microbiome and metabolic reprogramming has emerged as a prominent research focus in the field of tumor immune evasion, particularly in the context of colorectal cancer. The microbiome plays a pivotal role in shaping the TME by modulating inflammation and immune responses (73, 74). It influences cancer initiation, progression, metastasis, and immune escape mechanisms through both microbial actions and their metabolites (75, 76). Microbial metabolites such as short-chain fatty acids (SCFAs) and bile acids reprogram metabolic processes within the TME, either enhancing or inhibiting immune responses. For example, SCFAs, particularly butyrate, produced by beneficial bacteria like Bacteroides thetaiotaomicron, help maintain immune balance by promoting regulatory T cell (Treg) differentiation, which can suppress inflammation and support immune homeostasis (77). In contrast, dysbiosis—often linked to poor dietary habits—can favor pathogenic bacteria like Fusobacterium nucleatum, which promotes immune evasion by inducing M2 macrophage polarization, suppressing T cell responses, and enhancing inflammation, ultimately accelerating cancer progression (78). Furthermore, the metabolic competition within the TME between cancer cells, immune cells, and microbes adds another layer of complexity to immune evasion. Tumor cells reprogram their metabolism to favor glycolysis, thus depriving immune cells of essential nutrients like glucose and glutamine. This metabolic shift impairs T cell function and promotes immune suppression, facilitating tumor progression (75). The crosstalk between tumor metabolism, microbial metabolites, and immune responses underscores the potential for targeting these metabolic pathways to improve the efficacy of immunotherapies (79). In conclusion, the intersection of metabolic reprogramming and the microbiome offers a promising avenue for cancer research, particularly in tumor immune evasion. By understanding how microbial metabolites influence tumor metabolism and immune responses, this area could lead to new therapies that enhance immunotherapy effectiveness. Further exploration of this cross-cutting research will be key to developing personalized treatments that combine microbiome and metabolic strategies to overcome immune suppression.

4.3.2.2 Intelligent decoding of the tumor immune evasion landscape

As tumor immunology advances toward increasingly personalized and dynamic paradigms, the concept of an "immune evasion landscape" has emerged as a critical framework to describe the intricate, multidimensional interplay between tumors and the host immune system. Recent progress in high-throughput technologies—such as spatial transcriptomics (80), single-cell technologies (81, 82), and multi-omics integration (83)—has enabled unprecedented resolution in mapping this landscape. AI, empowered by access to high-dimensional biological datasets and breakthroughs in computational power and deep learning architectures, offers a transformative approach to decoding these complex interactions (84, 85). Several recent studies exemplify this trend. For instance, Hanqi Li et al. (86) integrated four histological dimensions to define three molecular subtypes of hepatocellular carcinoma (HCC), establishing an MSRS model validated through single-cell RNA sequencing, spatial transcriptomics, and functional assays. This model demonstrated robust prognostic capability and potential for guiding individualized therapy. In another study (87), researchers applied imaging mass cytometry and a graph-based AI model to compare non-small cell lung TMEs in people with and without HIV. Leveraging PageRank and diffusion maps, the model achieved 84.6% accuracy in classifying HIV-associated tumors and identified key immunosuppressive markers, such as PD-L2 on tumor-associated macrophages and CD25 on infiltrating T cells. Additionally, Liu et al. (88) developed a self-supervised learning (SSL) framework based on the Barlow Twins method to analyze over 1,600 H&E-stained colon cancer slides from TCGA-COAD and AVANT cohorts. Their model, trained without manual annotations, extracted latent features to define 47 histomorphological phenotype clusters (HPCs) that reflect immune infiltration, stromal disorganization, and tumor necrosis. The HPCs proved predictive of survival outcomes and treatment response, demonstrating how SSL can be leveraged for label-free,

interpretable profiling of the TME. In summary, these advances highlight the growing synergy between AI and tumor immunology. By enabling mechanistic, data-driven characterization of the immune evasion landscape, AI models are not only enhancing prognostic precision but also uncovering biologically meaningful therapeutic targets (89). Looking ahead, the integration of multi-modal data—including spatial, transcriptomic, proteomic, and morphological inputs—into unified AI frameworks will likely revolutionize our ability to anticipate tumor immune dynamics and design next-generation precision immunotherapies tailored to individual patients.

4.3.2.3 The future of glioblastoma: combination immunotherapy

GBM is the most common and aggressive form of primary brain tumor, characterized by a complex network of survival mechanisms that promote therapeutic resistance and immune evasion (90). Within its highly immunosuppressive TME, GBM stem cells—notorious for their intrinsic drug resistance—remain key contributors to treatment failure and disease recurrence (91). The blood–brain barrier further restricts the delivery of therapeutics, while tumor antigenic heterogeneity, limited neoantigen presentation, and T cell exclusion add layers of immune resistance (92). Thus, the most urgent challenge lies in designing integrated therapeutic strategies that concurrently target multiple immune escape mechanisms and reinforce the overall antitumor immune response (93).

The team led by Arrieta VA (94) used low-intensity pulsed ultrasound (LIPU) and intravenous microbubbles to open the blood-brain barrier and increase the concentration of liposomal doxorubicin and PD-1 blocking antibody. Additionally, it was found that when administered with LIPU/MB, doxorubicin's efficacy surpassed simple drug delivery; it significantly modulated the TME, potentially improving the presentation of tumor antigens to T cells, thereby enhancing the efficacy of T cell-based immunotherapy (including PD-1 blockade).

Luo F et al. (95) found that LRRC15 expression was elevated in GBM patients who did not respond to anti-PD-1 therapy. Therefore, they believe that targeting LRRC15 may provide a new strategy to enhance anti-PD-1 therapy and overcome immune therapy resistance in GBM.

In the preclinical model developed by the Xing YL team (96), it was found that BRAFi+MEKi can synergize with ICI by enhancing T cell activity and antigen presentation, thereby increasing the intrinsic sensitivity of tumors. However, the combination therapy has significant toxicity. Therefore, they propose incorporating galectin-3 inhibitors into treatment regimens for these gliomas as a promising strategy to improve treatment efficacy while controlling toxicity, thereby enhancing patients' overall quality of life.

In summary, while GBM remains highly resistant to current therapies, progress in blood–brain barrier-penetrating delivery systems, TME modulation, and biomarker-driven combinations has opened new avenues for immunotherapy. Future research should prioritize dissecting the immune evasion mechanisms—particularly those involving GBM stem cells, myeloid cells, and stromal factors—while advancing precision delivery technologies to enhance treatment efficacy. These efforts will be key to developing the next GBM of effective, personalized immunotherapeutic strategies for glioblastoma.

4.4 Limitations

This bibliometric analysis provides a comprehensive overview of tumor immune escape research, though several methodological limitations warrant acknowledgment. First, the keyword strategy, while designed for thematic specificity, may have inadvertently excluded conceptually related topics, leading to potential omissions in the broader immuno-oncology landscape. Second, exclusive reliance on the Web of Science Core Collection—though beneficial for standardization—may underrepresent applied or interdisciplinary studies more extensively indexed in databases such as PubMed or Scopus. Third, citation-based metrics are inherently time-sensitive, often disadvantaging recent publications and reflecting academic rather than translational impact. Fourth, the exclusion of non-English publications to ensure language consistency may introduce geographic bias, potentially overlooking contributions from non-English-speaking countries. Lastly, a certain degree of subjectivity is unavoidable in the interpretation and synthesis of bibliometric findings.

5 Conclusion and future perspectives

This bibliometric study provides a comprehensive overview of the intellectual landscape, research hotspots, and developmental trajectory of tumor immune escape research over the past 14 years. By mapping influential nation, authors, core journals, reference, and keyword bursts, this work not only summarizes major contributions in the field but also helps researchers better understand its evolution and emerging directions. Based on the observed patterns, we propose three key areas that warrant further exploration: (1) advancing interdisciplinary research at the intersection of the microbiome, metabolism, and immune regulation; (2) integrating artificial intelligence and multi-omics data to enhance predictive modeling and therapeutic precision; and (3) combining multi-modal therapeutic strategies to overcome immune escape more effectively.

Looking ahead, future research should emphasize translating mechanistic discoveries into clinically actionable strategies, particularly in identifying biomarkers that predict immune evasion and therapy resistance. Greater investment in large-scale, real-world immunotherapy data, along with the development of open-access, cross-platform analytical tools, will further support reproducibility and innovation. Moreover, fostering stronger international collaboration among researchers, institutions, and

countries will be vital to accelerating discovery in this field and promoting the global advancement of cancer immunotherapy.

Data availability statement

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

Author contributions

HZ: Formal Analysis, Funding acquisition, Methodology, Writing – original draft. YH: Data curation, Formal Analysis, Methodology, Software, Writing – original draft. XW: Formal Analysis, Investigation, Methodology, Validation, Writing – original draft. WX: Data curation, Formal Analysis, Investigation, Methodology, Software, Writing – original draft. YX: Resources, Supervision, Validation, Writing – review & editing, Funding acquisition.

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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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Cancer-derived exosomes: mediators of immune crosstalk and emerging targets for immunotherapy

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Exosomes, nanoscale extracellular vesicles secreted by various cell types, play pivotal roles in intercellular communication. In cancer, tumor-derived exosomes—referred to as cancer-derived exosomes (CDEs)—have emerged as critical regulators of immune evasion, tumor progression, and therapy resistance within the tumor microenvironment (TME). CDEs modulate immune cell function through the transfer of immunosuppressive proteins, cytokines, and non-coding RNAs, ultimately reprogramming immune surveillance mechanisms. This review provides an in-depth analysis of how CDEs influence major immune cell subsets—including T cells, B cells, NK cells, dendritic cells, macrophages, and myeloid-derived suppressor cells—thereby establishing an immunosuppressive TME. We also explore the potential of immune cell-derived exosomes (IDEs) as emerging immunotherapeutic tools capable of counteracting the suppressive effects of CDEs. Furthermore, we highlight exosome engineering strategies aimed at improving therapeutic cargo delivery, tumor targeting, and antitumor immune activation. Finally, we discuss how exosome profiling offers promise in liquid biopsy diagnostics and how integration with 3D tumor models and advanced bioengineering can accelerate the clinical translation of exosome-based cancer immunotherapies.

KEYWORDS

cancer-derived exosomes, immune-crosstalk, immune-modulation, immunotherapy, tumor microenvironment

Introduction

Exosomes, a subtype of extracellular vesicles ranging between 30 and 100 nm, play a crucial role in cell-to-cell communication by transporting proteins, lipids, and nucleic acids reflective of the state of the originating cell (1; 2, 3). Among their various physiological functions, cancer cell exosomes referred to as cancer-derived exosomes (CDEs) have

attracted growing interest for their involvement in tumor progression, immune evasion, and metastasis (4, 5). These vesicles interact intricately with immune cells, promoting immunosuppression in the tumor microenvironment and contributing to cancer hallmarks such as immune escape, largely through mechanisms such as exosomal PD-L1-mediated T cell inhibition (6).

Beyond their physiological role, exosomes have gained attention due to their clinical potential in cancer diagnostics, prognosis, and therapeutic monitoring. Their stability in bodily fluids and ability to carry tumor-specific biomarkers make them suitable candidates for liquid biopsies. Biomolecules such as exosomal PD-L1 and miRNAs have shown utility in predicting response to immune checkpoint inhibitors and tracking disease progression in cancers such as melanoma, breast, ovarian, and bladder cancer (7–10).

Recent findings also reveal that cancer therapies such as chemotherapy and radiation therapy can significantly alter the molecular composition and release of tumor-derived exosomes. These post-therapeutic changes can enhance tumor aggressiveness or signal treatment efficacy, depending on the context (11, 12). For example, chemotherapeutics such as paclitaxel and melphalan have been shown to increase exosome release *in vitro* (12, 13), while clinical samples from patients with leukemia and head and neck cancer show reduced exosomal proteins after treatment (14, 15). These discrepancies highlight the complex and context-dependent nature of exosome biology in the response to treatment.

To leverage the full therapeutic potential of exosomes, researchers are engineering immune and tumor-derived exosomes to deliver therapeutic agents such as siRNAs, chemotherapeutic drugs, and immune agonists (16). Various loading techniques, including electroporation, sonication, and surface conjugation, have improved cargo specificity and delivery efficiency (17). Engineered exosomes have been shown to cross biological barriers and target tumor sites with minimal toxicity (18, 19), but their clinical translation still faces hurdles such as standardization, targeting specificity, and large-scale production. This review explores the immunomodulatory functions of CDEs, their potential as biomarkers, and the engineering strategies aimed at overcoming current therapeutic limitations. To further assess the functional relevance and therapeutic impact of engineered exosomes, advanced 3D tumor models, such as spheroids, are emerging as valuable tools that more accurately recapitulate the tumor microenvironment compared to traditional 2D cultures.

Cancer hallmarks and tumor microenvironment

Cancer cells exploit intercellular communication similarly to healthy cells, but they use it to promote their growth by inhibiting cells that oppose them or activating regulators of cancer hallmarks. These hallmarks include immune evasion, sustained proliferation, metastasis, replicative immortality, angiogenesis, and apoptosis avoidance (20, 21). To survive harsh environments, cancer cells adopt "enabling characteristics" that maintain malignancy and create favorable conditions for tumor progression and metastasis (21). Within the tumor microenvironment (TME), cancer cells continuously maintain these hallmarks by releasing cancer-derived exosomes (CDEs), which regulate surrounding cells and adapt to the hostile TME. Therapeutic strategies can target CDE cargo production to disrupt hallmark maintenance or enhance immune cell function to counteract these cancer-promoting signals (Figure 1).

TME is a central hub where cancer hallmarks are enabled, providing favorable conditions for cancer cells while being hostile to normal host cells (21). It comprises cancer-associated fibroblasts (CAFs), immune and stromal cells, blood vessels, and extracellular vesicles (EVs), all of which coordinate to support metastasis and immune evasion through exosome-mediated signaling (21). Exosomes also facilitate tumor innervation via axonogenesis.

Tumor cells reprogram their metabolism toward glycolysis to fuel proliferation by upregulating the output of glucose transporters, and this promotes lactate production leading to the release of protons that acidify the TME and enhance exosomal cargo exchange (22). These exosomes carry factors like DLL4, TGF-β, and Tspan8 that promote angiogenesis and tumor progression (22). Additionally, fibroblasts are reprogrammed into CAFs, further supporting metastasis. Targeting the acidic conditions of the TME by navigating through anti-TME strategies aimed at increasing the pH may provide a therapeutic strategy by altering exosomal cargo profiles (22).

The building blocks of exosomes

Initially, EVs were described as fragments released by cells ubiquitously; however, it was only until the 1980s that exosomes were characterized as 'cellular waste units' which govern communication between cells (23). Subsequently, exosomes were stumbled upon in a study in 1983 where transferrin receptors (TfRs) migrated from the plasma membrane to mature reticulocytes, where they eventually reassembled into small vesicles within these cells (24). The discovery of exosomes marked a turning point in molecular biology as they revolutionized the previously held stance that they were solely for removing cellular garbage, to being the pioneers of cell-cell communication (25). In the past 20 years, exosomes have been progressively characterized and are gaining attention in therapeutics; however, as much as they have potential in therapeutics, their signaling nature is likened to that of a double-edged sword, as they also play a pathological role in diseases like cancer. Thus, understanding the physiological and pathological fate of exosomes requires a detailed exploration of their biogenesis.

Exosome biogenesis is triggered when cell cargo undergoes endocytosis within a cell, and the vesicle that buds into the plasma membrane is known as the early endosome (Figure 2) (26). At this stage, primary sorting takes place via the endosomal sorting complexes required for transport (ESCRT) and the fate of the cargo to be delivered is determined (26, 27). The main pathway of exosome biogenesis is the classic pathway that uses ESCRT complexes to release exosomes (27). ESCRTs are a group of

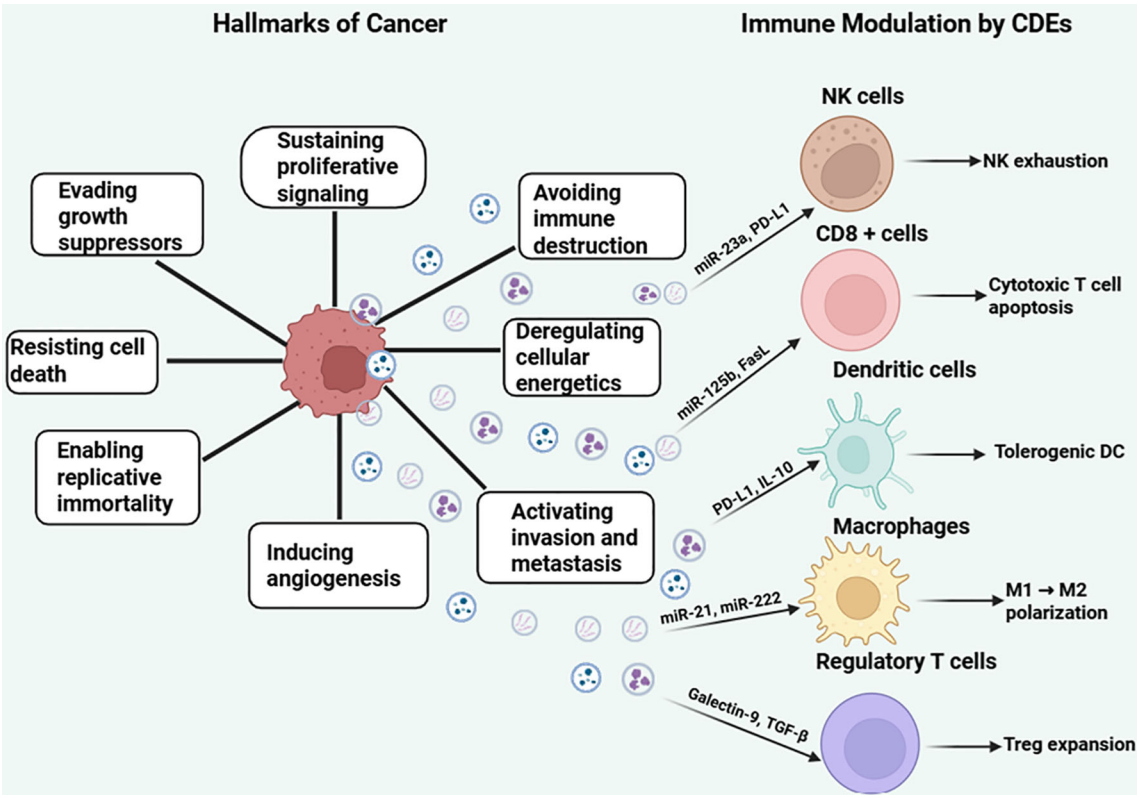

FIGURE 1
Hallmarks of cancer and immunomodulatory roles of cancer-derived exosomes (CDEs). The central cancer cell is surrounded by the eight classical hallmarks of cancer, including sustained proliferative signaling, evasion of growth suppressors, resistance to cell death, replicative immortality, induction of angiogenesis, activation of invasion and metastasis, deregulated cellular energetics, and avoidance of immune destruction. Cancer-derived exosomes (CDEs) are shown as vesicles released from the cancer cell, carrying immunosuppressive cargo such as miRNAs (e.g., miR-23a, miR-125b), proteins (PD-L1, Galectin-9, FasL), and cytokines (TGF-β, IL-10). These exosomes interact with key immune cells—natural killer (NK) cells, $CD8^+$ T cells, dendritic cells (DCs), macrophages, and regulatory T cells (Tregs)—to induce NK exhaustion, cytotoxic T cell apoptosis, Treg expansion, M1-to-M2 macrophage polarization, and tolerogenic DC phenotypes. The left panel illustrates the intrinsic hallmarks of cancer, while the right panel emphasizes the immunomodulatory effects of exosomal signaling on immune evasion, highlighting exosomes as mediators of tumor progression. Figure was designed using BioRender.com.

proteins that localize on the membrane of multivesicular bodies (MVBs) to organize cargo and release intraluminal vesicles (ILVs), which later form exosomes carrying cargo to their designated target cells (27). There are four different networks within ESCRT which are ESCRT-0, ESCRT-I, ESCRT-II, and ESCRT-III, all of which play distinct roles in the development of exosomes (28). An alternative pathway to exosome formation is the ESCRT independent pathway, and despite the different pathways, the exosomes that are released are alike in structure but vary in the cargo they carry (27).

Exosome biogenesis occurs alongside cargo packaging (Figure 2), with contents—proteins, lipids, and nucleic acids—reflecting the cell of origin (28). Key cargo includes RAB GTPases, ALIX, and TSG101, which are involved in membrane transport (28). RAB7, RAB11, RAB27, and RAB35 regulate exosome secretion by directing MVB trafficking and fusion with the plasma membrane (29). Tumor cells often upregulate RAB proteins to enhance exosome release, highlighting them as potential targets for cancer immunotherapy. Further research is needed on cancer-derived exosomal (CDE) RAB regulators.

In addition to regulation of exosome formation, exosome cargo also contains microRNA (miRNA) that regulate gene expression within recipient cells, and these are the highest population of RNA within exosomes (30). Exosomal miRNAs are very stable and are useful for studying exosomes (30). Under pathological conditions, tumor derived-exosome miRNAs have been found to promote lung cancer metastasis by silencing genes that down-regulate the epithelial mesenchymal transition (EMT) (31). In therapy, exosomal miRNAs are being used as tumor markers for the molecular diagnosis of tumors (32).

Where do these exosomes go?

To facilitate intercellular communication, the exosome absorption and secretion pathways can cross paths within a cell, but the nature in which these pathways intersect varies in complexity depending on the fate of exosome cargo (33). The mechanism by which cells absorb exosomes is classified into two,

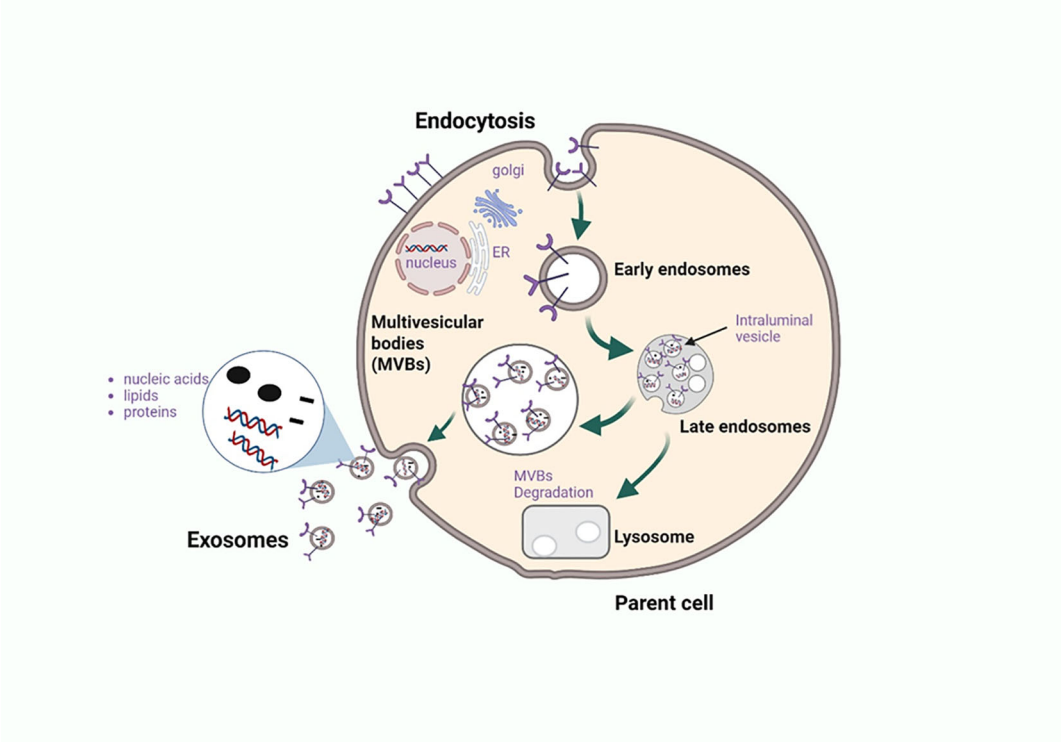

FIGURE 2
Biogenesis and exosome release from the parent cell. Exosomes are nanoscale extracellular vesicles formed through the endosomal trafficking pathway, beginning with the invagination of the plasma membrane to generate early endosomes. These early endosomes internalize diverse biomolecules such as proteins, nucleic acids, and lipids which are further sorted during maturation into multivesicular bodies (MVBs) or late endosomes. Within MVBs, inward budding of the limiting membrane generates intraluminal vesicles (ILVs) that are selectively loaded with cargo. MVBs can fuse with lysosomes for degradation, particularly when carrying damaged or incomplete cellular components, or merge with the plasma membrane to release ILVs as exosomes into the extracellular space. In the context of cancer, exosomes enriched with immunomodulatory proteins and nucleic acids act as critical mediators of immune crosstalk, promoting tumor progression, immune evasion, and systemic signaling. Elucidating the mechanisms of cargo sorting and release provides insight into novel therapeutic targets aimed at modulating exosome content or blocking their immunosuppressive functions. Figure was designed using BioRender.com.

one is non-specific and the other is specific uptake (34). All cells can utilize nonspecific mechanisms to absorb exosomes; however, specific uptake is necessary to allow the target cell to absorb all exosome contents relative to the host cell's specificity with respect to cargo sorting (34). Conservation of the signature of the host cell within the exosome through conserved tropism between host and target cells promotes exosome specificity via recognition motifs that can always be recognized on these target cells by exosomes (34). An example is neuroblastoma cells where exosomes only recognize cells positive for CD63 for cargo selection (34, 35).

Upon contact with the target cell, exosomes exert their function through direct fusion with the plasma membrane or internalization within the cell (34). Direct fusion occurs when transmembrane ligands on the exosome surface bind to receptors on the surface of target cells and these trigger a signaling cascade within the cells that exert functions that may be immunomodulatory or apoptotic in nature (34).). Internalization occurs when the primary function of the target cell is to engulf the exosome followed by the release of exosome contents into the cell (34). One of the ways in which internalization is achieved is through clathrin-mediated endocytosis where the vesicles are internalized and subsequently fused with endosomes (34). As cancer cells secrete exosomes aggressively to promote tumor microenvironment (TME) activities, they can also improve exosome uptake by overexpressing of transferrin which is an essential cargo during internalization through clathrin-mediated endocytosis (34). Here we can compare how cancer cells may up-regulate exosome secretion by enhancing RAB regulatory factors and they also enhance exosome uptake by target cells via transferrin overexpression to ensure the seamless transfer of CDE cargo.

Cancer-derived exosomes in cancer therapy

Cancer-derived exosomes (CDEs) are exosomes released by tumor cells in the TME and the primary way in which they regulate the TME is by altering the expression of immune cells (Figure 3) (36). Secondary mechanisms CDEs can employ in the TME include changing the way in which B cells, T cells, natural killer (NK) cells, and macrophages respond to the TME (36). CDEs have been studied extensively over the years, as they are key regulators of TME and may serve as potential biomarkers for

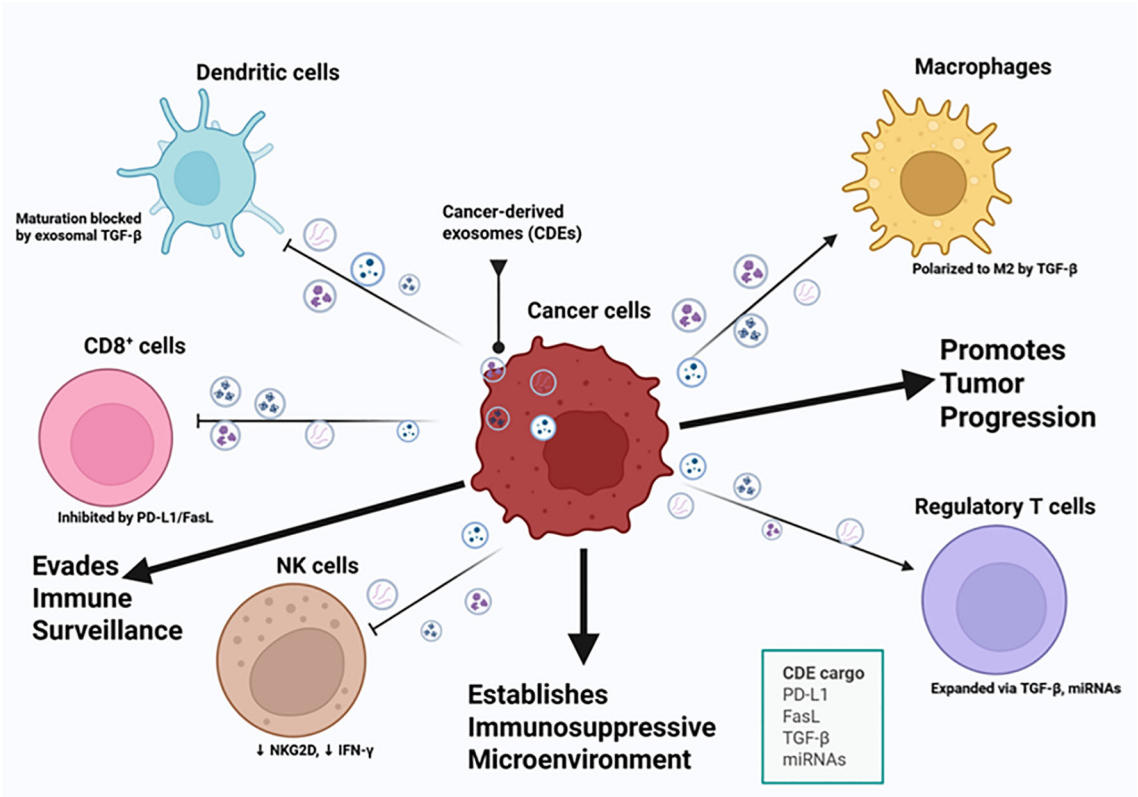

FIGURE 3
Cancer-derived exosomes mediate immune evasion and tumor progression. This illustration highlights key cancer hallmarks related to immune evasion and tumor progression, including the ability of cancer cells to avoid immune destruction, sustain proliferative signaling, induce angiogenesis, and activate invasion and metastasis. Cancer-derived exosomes (CDEs) carry immunosuppressive and oncogenic cargo, including PD-L1, FasL, TGF-β, and specific microRNAs (miRNAs), which modulate the function of key immune cell types within the tumor microenvironment. These exosomes inhibit CD8 + T cell activity through PD-L1 and FasL signaling, suppress natural killer (NK) cell cytotoxicity by downregulating NKG2D and IFN-γ, and block dendritic cell maturation via TGF-β. CDEs also promote the expansion of regulatory T cells and polarize macrophages toward an M2 phenotype, both contributing to an immunosuppressive microenvironment. This exosomal crosstalk effectively reprograms the immune microenvironment, allowing cancer cells to circumvent immune surveillance, establish an immunosuppressive niche, and promote tumor progression. Figure was designed using BioRender.com.

diagnosis (36). Apart from regulating immune cells in the TME, CDEs can reprogram stromal cells into cells that support the formation of premetastatic niches in surrounding tissues (2). Considering the dominant control CDEs have over immune cells, the rest of the review focusses on the mechanisms by which CDEs control immune cell activity, possible crosstalk with immune cell derived-exosomes and possible therapeutic targets that can be exploited in these signaling cascades.

Building on this understanding of CDE–mediated immunosuppression, it is crucial to examine the roles of the various immune cells within the TME. Immune cells including regulatory T cells (Tregs), B cells, myeloid-derived suppressor cells (MDSCs), macrophages, dendritic cells, natural killer (NK) cells, and monocytes serve as both targets and mediators of exosome-driven signaling, shaping antitumor immunity or, conversely, contributing to immune evasion. Understanding how these immune cells interact with exosomal cargo provides a foundation for developing strategies that harness immune-derived exosomes (IDEs) to restore immune surveillance and enhance cancer immunotherapy.

T cells

T cells, key players in the adaptive immune response, originate in the bone marrow as pro-T cells and mature in the thymus, where they become capable of protecting the host from infections and cancer (37). Immature T cells initially lack a T cell receptor (TCR) and gain antigen specificity through VDJ recombination during maturation, committing to a single antigen for their lifespan as naïve T cells (37). CD4+ T cells, known as helper cells, coordinate immune responses primarily through cytokine release and play a critical antitumor role despite limited cytotoxicity (38, 39). On the contrary, CD8+ T cells are highly cytotoxic and can induce apoptosis in cells presenting antigens recognized by their TCRs (39).

One study showed that CDEs were found to decrease IFN-γ, a critical cytokine in immune responses, in CD4+ and CD8+ T cells, as well as a decrease in Tregs that regulate immune responses by maintaining self-tolerance and exaggeration of immune responses (Figure 3) (40, 41). Another study showed that under an immune competence state, PDL-2 from CDEs are manipulated in a PD-1-

mediated mechanism which serves to damage the integrity of T cells by upregulating Tregs and downregulating tumor-infiltrating T cells (TIL-Ts) (42).

When we focus on the study by (41), the effect of CDEs on IFN-γ and Tregs is independent of each other, however, they conjointly decrease the immune response with the TME. As Tregs naturally controls exaggerated immune responses, it does not necessarily mean that immune responses stay upregulated when Tregs is depleted as Tregs is mostly active when immune responses stay abnormally consistent above a certain threshold. This may indicate that CDEs within this context prioritize depleting IFN-γ which is more critical for immune response efforts in the TME. This may also suggest that the decrease of Tregs in the presence of CDEs is dependent on the type of cancer cells the study was using, TME conditions etc. which plays a role in the way CDEs dictate the pro-tumorigenic conditions in the TME. As opposed to the Liu et al. (43) study where CDEs were shown to directly increase Tregs to downregulate the immune response. Here we can observe that in the study (40; Hussain and Malik, 2022), the decrease in Tregs is not directly associated with cancer progression, however, in another study (42), an increase in Tregs is the major factor associated with cancer progression. This contrast in studies highlights the versatility of CDEs in their ability to manipulate a variety of immune cells and should be considered when studying their effect on T cells. Overall, these studies show how exosomes within the TME further cancer progression by promoting an immunosuppressive environment by downregulating CD4+ and CD8+ T cell function but also highlight the need for more research into counteractive measures against T cell manipulation.

B cells

B cells support adaptive immunity alongside T cells through antigen-specific mechanisms (44). Although cancer research has traditionally focused on T cells, recent studies highlight the importance of tumor-infiltrating B cells (TIL-Bs) in enhancing T cell responses (44). TIL-Bs contribute to antitumor activity through the presentation of specialized antigens and interactions with T and NK cells, helping to transform the tumor microenvironment (TME) into a hostile space for cancer cells (45). Although the influence of tumoral exosomes on TIL-Bs remains underexplored, emerging research continues to define their role. Additionally, B cells produce antigen-specific antibodies, which generate memory cells for rapid secondary responses, and assist in directing NK and myeloid cell cytotoxicity toward tumors (44).

A group of B cells known as regulatory B cells (Bregs) has been found to support tumor immunosuppression, however, the mechanism by which they inhibit antitumor immunity in TME is still unknown (46). In a colorectal cancer (CRC) study, CDEs were shown to enhance Bregs activity by carrying long noncoding RNA (lncRNA) in their cargo (46). The lncRNA in question is known as HOTAIR, where cancer-derived HOTAIRs differentiated B cells into a regulatory phenotype associated with programmed death ligand 1 (PD-L1), these PD-L1+ B cells then inhibit the cytotoxic activity of CD8 + T cells promoting an immunosuppressive TME (46). More recently, a study done on exosomes from a murine CRC cell line shows that these CDEs prevent B cell proliferation and survival, moreover, they polarize B cells into the regulatory B cell phenotype that contributes overall to the decreased immune response toward cancer (47). The effect of CDEs from the murine CRC cell line crossed into T cell territory as they were also involved in altering the activity of CD8+ T cells (48). The results found in CRC cells show the extent to which CDEs will promote an immunosuppressive environment where they polarize immune cells into phenotypes which promote TME. These studies also highlight the need for more research on preventing immune cell polarization into phenotypes favorable for cancer progression.

Macrophages

Macrophages play a vital role in both innate and adaptive immunity, forming the first line of defense before full immune activation (49). Their phenotype is shaped by cytokine signals: lipopolysaccharides induce the pro-inflammatory M1 type, while IL-4/IL-13 promote the anti-inflammatory M2 type (49). Among the M2 subtypes, tumor-associated macrophages (TAMs) are the most notable. Activated by A2 adenosine receptor agonists and TLR ligands, TAMs support tumor proliferation within the tumor microenvironment (TME) (M. 50). Due to its abundance and tumor-promoting role, reprogramming TAMs from the M2 to the anti-tumor M1 phenotype is a promising immunotherapeutic strategy (51). In particular, this M1/M2 polarization mirrors how B regulatory cells (Bregs) are driven into immunosuppressive phenotypes by cancer-derived exosomes (CDEs), a recurring mechanism through which CDEs manipulate various immune cells, including macrophages.

Studies have shown that CDEs in breast cancer promote macrophage M2 polarization by delivering circ-0001142, which is a circular RNA (circRNA) recently found to be highly expressed in breast cancer cells, subsequently interfering with autophagy and increasing tumor proliferation (52, 53). A defining signature of the formation of the premetastatic niche, necessary for metastasis, is the entry of immunosuppressive macrophages where CDEs polarize macrophages into the M2 phenotype distinguished by enhanced expression of PD-L1 and promoting tumor metastasis (Figure 3) (5). A study by Theodoraki et al. (54) shows that exosomes derived from HNSCC cells are involved in macrophage polarization into the M2 phenotype and are accompanied by increased levels of CXCL4. As recent studies continue to suggest the influence of CDEs on macrophage polarization into pro-tumorigenic phenotypes, this is a significant gap in CDE research, as more studies need to be done to counteract this mechanism and promote M1 phenotypes necessary for an anti-tumorigenic initiative.

Another study has shown that CDEs in cervical cancer delivered the TIE2 protein, involved in vascular quiescence and angiogenesis, to macrophages that promoted angiogenesis in TME (55, 56). CDEs have also been found to deliver miRNAs to macrophages in an intrahepatic cholangiocarcinoma study, such as miR-183-5p, which

polarizes macrophages into the PD-L1 + phenotype, which similarly to PD-L1+ B cells, inhibits the cytotoxicity of CD8+ T cells promoting an immunosuppressive environment (46, 57). A combined initiative of B cell and macrophage immunotherapy initiative has the potential to prevent polarization into immunosuppressive phenotypes, and this is more effective than individual immune cell immunotherapies.

Dendritic cells

Dendritic cells (DCs), key antigen-presenting cells in conjunction with macrophages and B cells, bridge innate and adaptive immunity (58). They exist in immature and mature forms. Immature DCs, found on mucosal surfaces, express low MHC levels but are antigen processing and migratory. Mature DCs have reduced antigen processing but enhanced migration (58). Using pathogen recognition receptors (PRRs), DCs detect PAMPs or DAMPs, internalize antigens, and present them via MHC to T cells (58). Beyond pathogens, DCs also process tumor antigens. Reduced DC levels in cancer suggest tumor-driven suppression of DC function within the tumor microenvironment (59).

Studies have shown that CDEs promote immunosuppressive TME by suppressing DC maturation and activity (Figure 3) (42). With the loss of DC function, tumor antigens cannot be processed and presented to T cells that contribute to cancer cell proliferation (42). DC differentiation is directly related to MDSC expression levels to the extent that loss of function of MDSC directly affects DC maturation (42). CDEs inhibit DC differentiation by interfering with myeloid cells, and employ molecules such as prostaglandin E2 (PGE2), TGF-β and heat shock proteins (42). CDEs derived from prostate cancer were found to prevent DC differentiation leading to accumulation of their MDSC precursors known to be involved in suppressing the immune response (60). Another study shows that exosomes acquired from the cerebrospinal fluid (CSF) of glioblastoma multiforme (GBM), one of the most aggressive and common brain tumors, contained Galectin-9, which is a molecule involved in preventing DC cell maturation (42, 61).

As DCs are crucial for antigen presentation, CDEs ensure their inactivity, lowering the frequency of immune responses in the TME. Until this point, it is evident that cancer immunotherapy should not only be directed towards only a subset of immune cells and rather all immune cells as CDEs employ a variety of mechanisms to promote immunosuppressive TMEs. Upregulating a subset of immune cells in the TME during cancer immunotherapy does not necessarily solve the problem, as CDEs focus their efforts on down-regulating a different subset of immune cells and this highlights the complexities of developing a therapeutic strategy to counteract CDEs. Like the suggestion of a combined B-cell and Macrophage immunotherapy, there should also be a combined DC and MDSC immunotherapy approach, as there is a correlation between DC differentiation and MDSCs which has the potential to produce greater therapeutic effects in cancer immunotherapy.

Myeloid-derived suppressor cells

Myeloid-derived suppressor cells (MDSCs) inhibit both innate and adaptive immunity and are heterogeneous in transcriptional activity and differentiation states (62, 63). Under pathological conditions such as cancer, MDSCs resemble neutrophils or monocytes but deviate from their normal immune functions to promote tumor progression (64). Like Tregs, MDSCs regulate immune responses, but their suppressive functions are amplified in cancer and chronic inflammation (63). Pro-inflammatory cytokines such as PGE2 and TGF-β hinder DC maturation and promote MDSC differentiation, contributing to immune evasion (42). CDEs also alter DC development and increase MDSC accumulation, leading to localized immunosuppression in the TME (60). Targeting DC differentiation may offer a strategy to reduce MDSC-mediated suppression and restore immune competence.

When we shift the focus to MDSCs-derived exosomes derived in the TME, it was observed that these exosomes promoted the development of castration-resistant prostate cancer by upregulating the S100A9/circM1D1/miR-506-3p axis (65). S100A9 is a calcium binding protein that is said to have implications in cancer associated with inflammation, circM1D1 expression is highly upregulated in prostate cancer cells treated with MDSC exosomes and miR-506-3p was found to be an inhibitor of CRC progression through EZH2-targeted mechanisms (65–67). MDSC exosomes in this study were associated with faster progression, migration, and invasion of prostate cancer cells (65). In a concurrent experiment, they observed that circM1D1 downregulated MDSC exosome-mediated prostate cancer progression, and S100A9 from MDSC exosomal cargo was able to convert circM1D1 expression to sponge miR-506-3p, masking its antitumoral effects and effectively promoting prostate cancer cell progression (65). This demonstrates that the promotion of tumor progression in the MDSC context can occur in two ways, which are through CDE mediated mechanisms and through MDSC exosomal mechanisms. Immunotherapy would have to be targeted at the regulators of each pathway such as HSP70 or the S100A9/circM1D1/miR-506-3p axis, however, targeting CDEs may produce more promising results, as they inhibit the activity of MDSCs before they even reach a stage of producing pro-tumorigenic exosomes.

Studies done on CDEs of renal cancer have shown that MDSC-mediated immunosuppression in TME is achieved through antigen-specific mechanisms and is highly dependent on the presence of HSP70 as a regulatory factor (68). These findings have potential in the therapeutic landscape by actively blocking MDSC activity or preventing the expression of HSP70 (42, 68). Furthermore, a study carried out on highly metastatic colorectal cancer cells shows that CDEs contain lncRNA MIR181A1HG which promotes liver metastasis through MDSC recruitment and is also a key player in extracellular matrix remodeling (69). As the primary mechanism used by CDEs for cancer proliferation is through MDSC recruitment, more strategies aimed at halting CDE-mediated MDSC recruitment must be studied to bridge this research gap as

the only strategy available to date is targeting DC differentiation which is still in development.

Natural killer cells

Natural killer (NK) cells are innate lymphocytes involved in antitumor and antiviral responses (70). Their activation depends on signals from activating or inhibitory receptors, allowing them to distinguish self from nonself through recognition of MHC I (70, 71). Once activated, NK cells kill compromised cells by releasing cytotoxic granules that induce apoptosis (70). However, in cancer, a subset called dysfunctional NK cells fails to eliminate malignant cells due to the immunosuppressive tumor microenvironment (TME) (72). The TME alters NK function by disrupting activating signals, enhancing inhibitory pathways, and interfering with metabolism. Restoring NK activity by targeting these disruptions is a key focus of cancer immunotherapy.

A study was conducted in CDEs from samples of hepatocellular carcinoma (HCC) adjacent to NK cell function where qRT-PCR was used to identify circular ubiquitin similar to PHD and ring finger domain 1 RNA (circUHRF1) in HCC CDEs (73). circUHRF1 in the HCC CDEs cargo was found to promote immunosuppression in the TME by contributing NK cell dysfunction in HCC (73). The mechanism by which circUHRF1 acts is by promoting TIM-3 expression, which is involved in T cell exhaustion during cancer, and downregulates miR-449C-5p, which is a gene silencer for the Tim-3 gene (73, 74). circUHRF1 is not only involved in TME regulation, as it also presents a challenge in cancer immunotherapy by resisting anti-PD1 therapy (73). So far, CDEs have portrayed a variety of mechanisms to counteract immune cell function, showing that they are the focal point of immunosuppressive efforts by tumor cells in the TME.

A study carried out on CDEs derived from oral cancer (OC) cell lines shows an elevation of TGF-β via mass spectrometry analysis of protein cargo of these exosomes (75). In OC studies, TGF-β is involved in inhibiting NK function in OC samples (42, 75). The enrichment of TGF-β coincides with the inhibition of key NK cell receptors such as NKG2D and NKp30, however, the hypothesis suggests that the deeper lying mechanisms need to be studied (75). It is evident that targeting TGF-β in cancer immunotherapy has the potential to restore the function of DCs and NK cells within the TME. Flow cytometry analysis of OC CDEs together with NK cells further revealed the gradual decrease over a week in killer cell lectin-like receptor k1 (KLR-K1) and the natural cytotoxicity triggering receptor 3 (NCR-3) (75). KLR-K1 is a critical receptor in immune cells that promotes an antitumor effect against cancer, while NCR-3 is responsible for NK cell identification as well as destruction of target cells (76, 77). The study on OC-derived CDEs reveals that CDEs gradually suppress natural killer (NK) cell function rather than causing immediate inhibition. This temporary lag phase presents a potential window for therapeutic intervention to prevent NK cell suppression in cancer immunotherapy.

Monocytes

Monocytes, derived from the bone marrow, are key components of the innate immune system (78). In cancer, they act as critical regulators, capable of both pro- and anti-tumorigenic functions (79). They typically accumulate early during tumor development and metastasis. While monocytes can induce tumor cell apoptosis through cytokine release and phagocytosis, this has mainly been observed *in vitro*, with *in vivo* relevance still unclear (78, 79). Monocytes can differentiate into tumor-associated macrophages (TAMs) or suppress T cell activity, aiding tumor immune evasion. Their dysfunction in cancer highlights the need for therapies that target monocyte-driven tumor progression.

CDEs in colorectal cancer have been shown to interfere with monocyte differentiation into macrophages, limiting tumor antigen presentation to the immune system (80). When monocytes merge their membranes with CDEs, this alters their phenotype into a phenotype that does not express the human leukocyte antigen-DR (HLA-DR), its costimulatory molecule, and only expresses a surface marker CD14 (81). This is significant as the altered monocyte phenotype forms an integral mediator in tumor immunosuppression in the TME and other mechanisms CDEs employ to interrupt monocyte differentiation include disrupting the STAT3 signaling cascade and promoting the formation of reactive oxygen species (ROS) (82). The disruption of monocyte differentiation by CDEs builds on previous discussions about macrophages, where CDEs primarily downregulate immune cells that induce a domino effect on the function of adjacent immune cells targeted toward the TME region. This means that therapeutic efforts can be directed at the source of the domino effect rather than only a single immune cell to ensure that all immune cells are effective against cancer cells.

To better illustrate their role in shaping the tumor immune microenvironment, Table 1 summarizes the major cargos carried by CDEs and their downstream effects on immune targets involved in tumor immunosuppression.

These examples underscore how CDE cargos actively remodel the immune landscape, setting the stage for therapeutic strategies aimed at disrupting exosome-mediated immunosuppression. In contrast, immune-derived exosomes (IDEs), such as those secreted by dendritic cells or activated T cells, can be engineered to carry immunostimulatory molecules, tumor antigens, or checkpoint inhibitors to activate the immune system against cancer (86). These therapeutic IDEs offer the potential to reverse the immunosuppressive tumor microenvironment, enhance antigen presentation, and stimulate robust adaptive immune responses.

Therapeutic potential of immune-derived exosomes

Strategies targeting crosstalk between CDEs and immune cells aim to reverse immunosuppression within the TME. Exosomes are

TABLE 1 Summary of CDE cargos and their downstream effects on immune targets in tumor immunosuppression.

CDE cargo	Immune target	Mechanism of action	Effect	Reference
PDL-2	T cells	PD-1 mediated; Tregs upregulated and TIL-Ts downregulated	Damages integrity of T cells	(42)
lncRNA (HOTAIR)	B cells	Promotes the polarization of B cells into PDL-1 mediated Bregs phenotype	Diminishes cytotoxic activity of CD8+ T cells	(46)
circRNA (circ-0001142)	Macrophages	Polarize Macrophages into M2 phenotype	Interferes with autophagy and promotes tumor proliferation	(52, 53)
CXCL4	Macrophages	Polarize Macrophages into M2 phenotype	Promotes tumor proliferation	(54)
TIE2	Macrophages	Active in the presence of VEGF-A and Angiopoietin in TME	Promotes angiogenesis in the TME	(55, 56, 83)
miR-183-5p	Macrophages	Polarizes macrophages into PDL-1+ phenotype; Transported by M2 TAM regulated Akt/NF-KP pathway	Inhibits cytotoxic activity of CD8+ T cells; Accelerates cancer progression	(46, 57, 84)
TGF-β, PGE2 and heat shock proteins	Dendritic cells	Interfere with myeloid cells which downregulate DC differentiation	Leads to loss of DC driven tumor suppression	(60).
Galectin 9	Dendritic cells	Prevents DC cell maturation via Gal-9/Tim-3 signaling	Leads to loss of DC driven tumor suppression	(42, 61, 85)
circUHRF1	Natural Killer cells	Inhibits miR-449C-5p which is responsible for silencing Tim-3 gene. Upregulation of Tim-3 disrupts NK activity	Loss of NK cell contributes to tumor immunosuppression	(73, 74)
lncRNA MIR181A1HG	Myeloid Derived Suppressor cells	Promotes upregulation of MDSC	Metastasis, ECM remodeling and tumor immunosuppression	(69)

promising as diagnostic biomarkers, drug delivery vehicles, and therapeutic targets in cancer immunotherapy (87). While CDEs often promote immunosuppression, IDEs, such as those secreted by dendritic cells or activated T cells, can be harnessed as therapeutic agents (86). IDEs can be engineered to deliver tumor antigens or immunostimulatory molecules, activating adaptive immunity and counteracting the immunosuppressive effects of CDEs. Given that the immune balance is shaped by this exosomal interplay, increasing the function of the IDEs could restore immunocompetence and counteract the hallmarks of cancer. Figure 4 illustrates the mechanisms by which IDEs exert therapeutic effects. IDEs, secreted by immune cells such as dendritic cells, macrophages, $CD8^+$ T cells, and NK cells, deliver pro-apoptotic miRNAs, antitumor drugs, and therapeutic proteins to cancer cells, inducing apoptosis, inhibiting proliferation, and triggering cytotoxicity with minimal systemic toxicity (88).

B cell-derived exosomes

B cell-derived exosomes (BDEs) are released by B cells and have been found to carry an MHC-II molecule conjugated with a peptide (pMHC-II) (89). This pMHC-II is only released by BDEs upon B cell activation so that helper T cells can initiate their immune response to that antigen (89). BDEs have potential as therapeutic drug carriers when it was shown that they can carry miR-155 in mouse models (89). In the context of cancer regulation, plasma cell-derived exosomes which are derivatives of B cells regulate tumor proliferation by carrying miR-330-3p which downregulates TPX2; a critical gene involved in sustaining melanoma cell proliferation (89). Protocols surrounding down-regulation of TPX2 through BDEs have not been fully optimized and need to be validated, however, they show great potential to inhibit the development of melanoma (89). In another study, BDEs were treated with zinc oxide nanocrystals (ZnNCs) and these promoted cytotoxicity against Burkitt lymphoma (90). These BDEs were further modified by adding an anti-CD20 monoclonal antibody to promote lymphoma cell specificity (90). However, BDEs have great potential in cancer immunotherapy, because of the limited number of studies they have not been fully characterized in this context (90). Modifications of BDEs show great promise regardless, as observed with results obtained from Burkitt lymphoma studies (90).

T cell-derived exosomes

T cell-derived exosomes (TDEs) are released by T cells and characterized according to the functions of parent T cells such as cytotoxic effects, regulation of antibody release by B cells, specificity against antigens and mediating cytokine release (91). TDEs regulate immune responses by coordinating the activity of other immune cells in mediating APCs (91). Considering that T cells are divided into CD8 +, CD4 + and Tregs, each subset releases their own exosomes which have their own distinct functions (91). Multiple studies have shown that CD8 + TDEs control information transfer between immune cells and tumor cells (91). These CD8 + TDEs promote T cell cytotoxicity which subsequently destroys tumor cells (91). A study has shown that CD8 + TDEs have increased programmed cell death- 1 (PD-1) expression which promotes toxicity by binding to PD-L1 and downregulating PD-L1 induced suppression of cytotoxic T cells (91). In addition to mediating information exchange between tumor cells and immune cells, CD8+ TDEs are also involved in halting tumor progression (91).

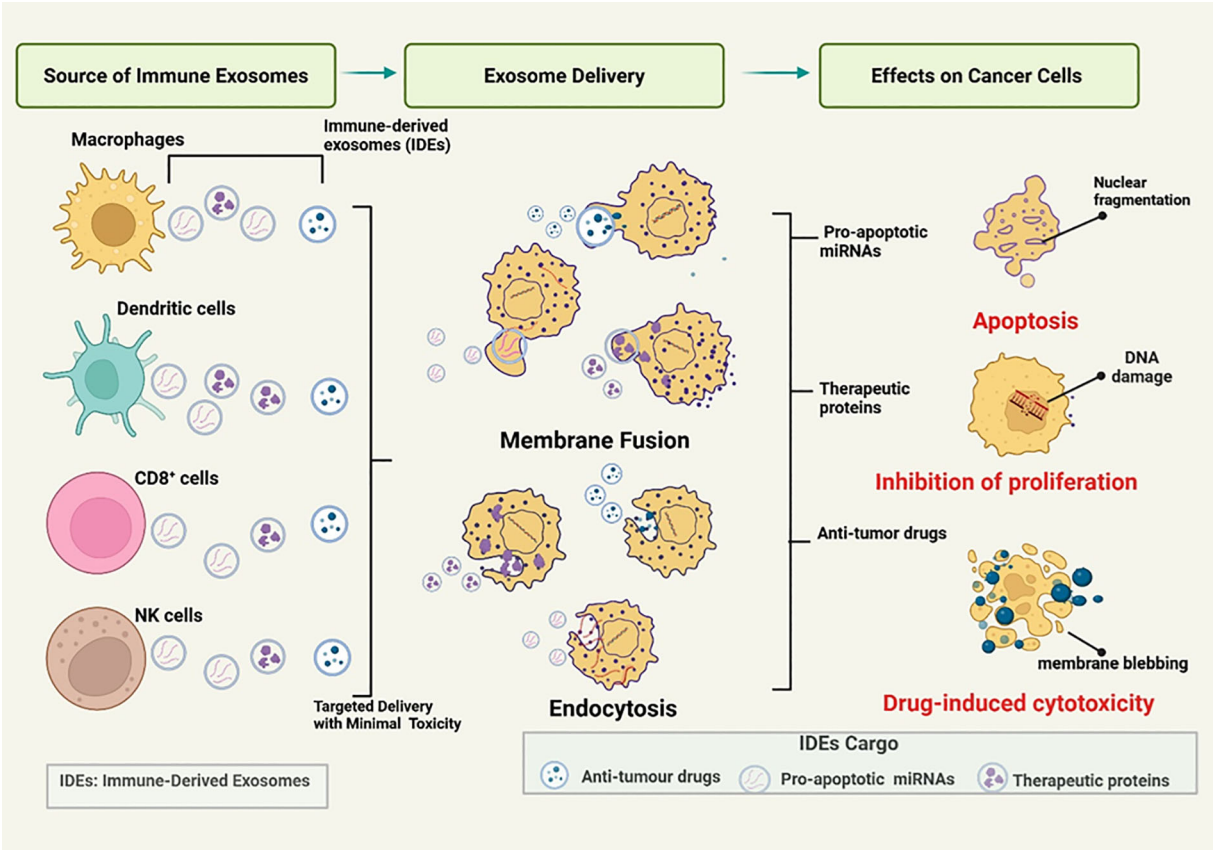

FIGURE 4
Mechanisms by which immune-derived exosomes (IDEs) mediate therapeutic effects. Immune cells - including macrophages, dendritic cells, CD8 + T cells, and natural killer (NK) cells - release immune-derived exosomes (IDEs) loaded with therapeutic cargo such as pro-apoptotic microRNAs, antitumor drugs, and therapeutic proteins. These IDEs are taken up by cancer cells through membrane fusion or endocytosis, enabling targeted delivery with minimal systemic toxicity. Upon delivery, the cargo induces distinct anticancer effects: (1) apoptosis, driven by pro-apoptotic miRNAs and characterized by nuclear fragmentation; (2) inhibition of proliferation, mediated by therapeutic proteins that cause DNA damage and cell cycle arrest; and (3) drug-induced cytotoxicity, where antitumor drugs trigger membrane blebbing and cell death. Collectively, IDEs represent a multifunctional platform that integrates immune surveillance with targeted therapeutic action against cancer cells. Figure was designed using BioRender.com.

Another study has shown that CD8 + TDEs without CD45RO carry miR-765 which is involved in inhibiting estrogen-driven development of uterine corpus endometrial cancer (UCEC) (91). Another way that CD8 + TDEs can down-regulate tumor proliferation is by depleting supporting mesenchymal tumor stromal cells (MTSCs) (91). CD8 + TDEs are not only involved in antitumor responses and can also be protumor, making therapeutic avenues around TDEs more complex (91). Exosomes from spent CD8 + T cells disrupt the production of crucial antitumorigenic cytokines such as IFN-γ, IL-2 and this causes CD8 + T cells to lose their cytotoxic abilities in antitumorigenic responses (91).

CD4 + TDEs promote antitumor responses by mediating crosstalk between CD4 + T cells and other important immune cells such as macrophages, NK cells, and CD8 + T cells (91). CD4 + TDEs carry miR-25-3p, miR-155-5p, miR-215-5p and miR-375 which promote CD8+ T cell-mediated antitumorigenic responses (91). CD4 + TDEs initiate these antitumor responses without provoking Tregs immune regulation (91). Tregs on the other hand, contrary to their other T cell counterparts, are more involved in immunosuppressive activity and are usually more pronounced in the TME (91). In a HNSCC study, patients received a variety of chemotherapeutic drugs such as cetuximab and ipilimumab, and Tregs-derived exosome expression was monitored (91). It was found that Tregs-derived exosome expression was increasing from its standard levels, indicating that Tregs-derived exosomes may serve as biomarkers in HNSCC (91). It can therefore be understood that up-regulation of factors that promote T cell derived exosome secretion can be promising for cancer immunotherapy, which negates g the effects of CDEs in the TME. It is evident that under pro-tumorigenic conditions, the balance needs to be shifted in favor of T cell derived exosome secretion to activated T cells which had their functions impaired by CDEs.

Macrophage-derived exosomes

Previously we mentioned that CDEs employ mechanisms to convert anti-tumorigenic macrophages M1 like into the more aggressive pro-tumorigenic M2 like phenotype known as TAMs and this can be manipulated in a therapeutic context in the reverse to promote more M1 like phenotypes through macrophage-derived exosomes (MDEs) (92). The first strategy to promote M1 phenotypes is to target and prevent TAM formation, and this can be done using a variety of mechanisms (92). The first mechanism is to block macrophage recruitment for pro-tumorigenic purposes, and this is done using inhibitors such as vascular endothelial growth factor (VEGF) or colony stimulating factor (Figure 4) (92). The second mechanism is by reducing the number of TAMs in the TME and many studies have used liposomal chondrates that reduce the vasculature in this region, preventing adequate blood supply to the TAMs (92). The third mechanism is to condition TAMs to a more favorable M1-like phenotype, and this can be achieved using cytokines such as IL-12 or M2 inhibitors such as miR-125b (92). Another mechanism involves the inhibition of the CD47-SIRPα pathway for advanced macrophage cell phagocytosis (92). Considering that CD47 is a marker that is highly expressed in cancer cells and interacts with SIRPα to prevent their own phagocytosis, this pathway can be inhibited through anti-CD47 or anti-SIRPα therapy leading to more phagocytosis of cancer cells (92).

In the case of MDEs, these can be engineered into the M1 like phenotype as they inherit their characteristic traits from macrophages and may serve as anticancer drug vehicles (92). These MDEs were modified with aminoethyl anisamide (AA), which binds to the α receptors in lung cancer and plays a role in stopping pulmonary metastasis of nonsmall cell lung cancer (92). A study was carried out in macrophage-derived M1 exosomes where these exosomes were polarized into the M1 phenotype with the aid of M1 enhancers such as NF-KB p50 siRNA, which silences the antiapoptotic activity of NF-KB-P50 in cancer cells, and miR-511-3p (93, 94). The surface of these M1 MDEs was also lined with IL4R-Pep1 so that they can bind to the IL4R receptors of TAMs (94). It was found that these TAMs took up these M1 MDE binding peptides and downregulated essential M2 macrophage genes that ultimately promoted the expression of M1 markers while downregulating M2 markers (94). Modifying these M1 MDEs contributed to stopping tumor growth, preventing the expression of key M2 cytokines while concurrently promoting the expression of M1 cytokines (94). M2 reprogramming using M1 MDEs is a promising strategy in cancer immunotherapy, as the global decrease of TAMs in the TME means that certain cancer hallmarks cannot be sustained as the immune response is in favor of immunocompetence rather than immunosuppression. Halting the activity of TAMs may indicate that other immune cells will follow suit with M1 macrophages considering the proximity of their crosstalk and more studies need to be done to ensure the maintenance of the M1 phenotype in cancer immunotherapy.

Natural killer cell-derived exosomes

NK cell-derived exosomes (NKDEs) are derivatives of NK cells and can perform signature NK cell functions according to the signal from NK activation or NK inhibitory receptors (95). When NK cells are stimulated to kill cancer cells, NK cells release NKDEs that perform antitumorigenic activities by releasing cytotoxic molecules such as perforin, granzymes, and miRNAs (Figure 4) (95, 96). NKDEs show great potential as enforcers of immune modulation and cancer immunotherapy due to their intrinsically latent antitumor influence (96). Therefore, it can therefore be assumed that NKDEs activity is silenced under pro-tumorigenic conditions, as parent NK cells have little function under these conditions (95). However, since this is a two-way road in terms of cancer immunotherapy against CDEs, studies have found ways to use NKDEs to deliver therapeutic drugs against cancer and the activation of NK-activated responses to promote cytotoxicity.

A recent study of triple negative breast cancer exploited the cargo carrying ability of NKDEs to determine whether they could deliver Sorafenib, an antitumor drug, to these cancer cells (97). The study wanted to compare Sorafenib administration with NKDEs versus without NKDEs and it was found the administration of Sorafenib with NKDEs significantly increased the cytotoxicity towards triple negative breast cancer spheroids (*in vitro* tumor mimics), highlighting the promising potential of NKDEs in cancer immunotherapy (97, 98). In a study conducted on NKDEs loaded with oxaliplatin, NKDEs were confirmed to have benefits such as inherent inhibition of tumor growth and their ability to enhance the antineoplastic activity of oxaliplatin in CRC therapy (99). Recent studies around NKDE cancer immunotherapy focus on increasing the apoptosis inducing ability of NKDEs, as they are more potent than other techniques. The delivery of chemotherapeutic drugs such as sorafenib using NKDEs may have increased specificity for tumor cells and reduce side effects of chemotherapy, making it a promising avenue for cancer immunotherapy.

Dendritic cell-derived exosomes

Dendritic cell-derived exosomes (DDEs) are vesicles released by DCs and possess the phenotypic characteristics of DCs which include the MHC complex, costimulatory components, and other surface markers required for communication with other immune cells (100). DDEs have more potential in tumor rejection using immune cells than traditional DC immunotherapy methods (100). DDE immunotherapy is more effective than DCs as they can maintain DC immunostimulatory characteristics without degrading and the stability of their membranes provides increased frozen storage for up to 6 months (100). DDEs possess both types of MHC molecules: MHC-I and MHC-II; and they can stimulate both helper T cell activity as well as cytotoxic T cell activity (100). The most abundant proteins in DDEs are the EGF factor 8 (MFG-E8) milk fat globule, which increases target cell exosome uptake (100).

What separates DDEs from exosomes from other immune cells is their enhanced antigen-presenting abilities, however, DCs produce greater T-cell responses (100). Some mechanisms by DDEs that stimulate antigen presentation to T cells include binding of APCs and they transfer their MHC/peptide complex to the APC, removing the need for any antigen processing (100). Another mechanism involves DDE-mediated tumor manipulation in adenocarcinoma cells that reactivate primed T cells and produce an IFN-γ mediated T cell response (100). The ability of DDEs to weaponize tumors to promote immunocompetence indicates that DDEs show great promise in cancer immunotherapy by coordinating T-cell responses against cancer cells.

A recent study produced a nano vaccine platform using DDEs and patient-specific neoantigens for personalized cancer immunotherapies (J. 49). The nano vaccine was designed for efficient cargo loading and increased cargo transportation times to lymph nodes which led to antigen specific B and T cell responses that had beneficial biosafety as well as biocompatibility (J. 49). The use of this nano vaccine system was found to significantly oppose tumor proliferation, had longer survival times, slowed down tumor incidence and eradicated lung metastasis in certain cancer models (49). The introduction of personalized DDE nano vaccine platforms provides a significant advantage in cancer immunotherapy as this eliminates the reliance on cell-based immunotherapy which is less efficient and has lower biocompatibility. In a study done by Safaei et al. (101), exosomes derived from triple negative breast cancer cells (TNBCC) could induce immunogenicity and this meant that they could improve DC vaccine immunotherapy for cancer patients.

These personalized nano vaccine systems provide a powerful avenue in DDE based immunotherapy to effectively deliver molecules which coordinate T cell responses against cancer cells as they overcome the barrier of biosafety and biocompatibility, which were major issues in DC based immunotherapy. This immunotherapy combined with the immunotherapy of other immune cell derived exosomes may pave the way for chemotherapy free cancer treatments which are mostly non-invasive.

Despite their promise, the clinical efficacy of IDE-based therapies remains limited in solid tumors compared to hematologic malignancies. This challenge arises from the hypoxic and immunosuppressive TME, which impairs T cell activity, remodels the extracellular matrix and vasculature, and drives immune suppression through Tregs, MDSCs, and TAMs (87). Addressing these barriers will be essential for unlocking the full therapeutic potential of immune-derived exosomes. In the following section, we provide a comparative overview of CDEs and IDEs, highlighting their contrasting roles in tumor progression and immune activation.

Dual faces of exosomes in cancer: drivers of immunosuppression and agents of immunotherapy

Exosomes serve as critical mediators of intercellular communication within the tumor microenvironment, exerting dual functions by either suppressing or stimulating immune responses, and offering opportunities for therapeutic engineering (Figure 5). CDEs carry immunosuppressive and oncogenic cargo such as PD-L1, FasL, TGF-β, and specific microRNAs, which suppress $CD8^+$ T cell cytotoxicity, impair natural killer (NK) cell activity, and block dendritic cell maturation (81). In addition, they encourage the expansion of regulatory T cells and direct macrophages towards an M2 phenotype, thus strengthening an immunosuppressive TME that supports tumor growth, angiogenesis, invasion, and metastasis (57). This capacity of CDEs to alter immune cell function underscores their pivotal role in tumor immune evasion.

In contrast, IDEs secreted by NK cells, $CD8^+$ T cells, dendritic cells, and macrophages offer an immunostimulatory counterbalance. These vesicles are enriched with cytotoxic proteins such as perforin and granzymes, cytokines like IFN-γ, and pro-apoptotic microRNAs that restore immune surveillance and trigger cancer cell death (88). By leveraging these properties, bioengineered exosomes can be tailored to transport tumor antigens, checkpoint inhibitors, or therapeutic drugs, facilitating accurate delivery and reducing systemic toxicity (102). By shifting the emphasis from CDE-driven immunosuppression to IDE-mediated immune activation, therapeutic exosome engineering has the potential to transcend the shortcomings of existing immunotherapies for solid tumors and pave the way for a novel era of precision cancer treatments.

Thus, understanding and harnessing the opposing functions of CDEs and IDEs provides a strategic avenue for developing next-generation exosome-based therapies that precisely modulate the tumor-immune interface. Exosome-based strategies demonstrate how leveraging the immune system can be effective against cancer. Similarly, other immunotherapy methods, including immune checkpoint inhibitors, CAR-T cell therapy, and cancer vaccines, seek to restore or boost antitumor immunity. Each of these approaches operates through unique mechanisms and comes with its own set of benefits and obstacles.

Other cutting edge cancer immunotherapies

Immune checkpoint inhibitors (ICIs) are one of the latest cancer immunotherapies which steer away from the conventional chemotherapy treatments and are being used to treat a variety of solid and liquid tumors (103). ICIs primarily act on T cells by removing any form of suppression of T cell activity from cancer cells, and this increases the cytotoxicity and antitumorigenic potential of T cells as well as other immune cells (103). The most prominent immune checkpoint pathway that cancer cells exploit to evade the immune system is the PD-1/PD-L1 pathway and ICIs, by blocking PD-1 or PD-L1 to prevent immune evasion (104). The goal of anti-PD1 or anti-PDL1 treatment is to activate cytotoxic T cells within the TME by forming a blockade between the immunosuppressive PD-1/PD-L1 ligand receptor complex (104). Immune checkpoint immunotherapy should be combined with

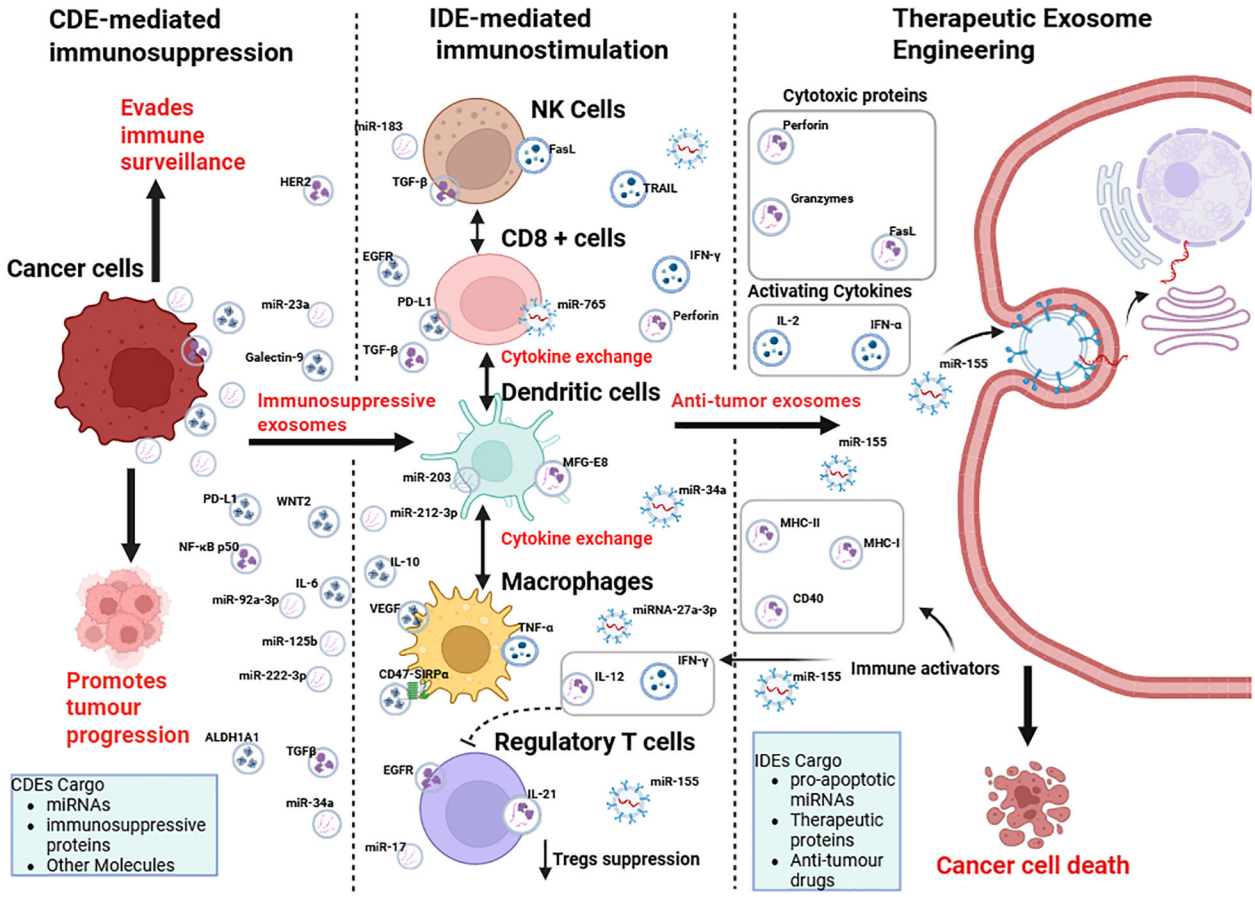

FIGURE 5
Crosstalk between cancer cells and the immune system via cancer-derived exosomes (CDEs) and immune cell-derived exosomes (IDEs), and their potential for therapeutic exosome engineering. Cancer cells release immunosuppressive exosomes (CDEs) containing miRNAs, immunosuppressive proteins, and other molecules, which promote immune evasion and tumor progression by modulating NK cells, $CD8^+$ T cells, dendritic cells, macrophages, and regulatory T cells. In contrast, immune cell-derived exosomes (IDEs) carry pro-apoptotic miRNAs, cytokines, and cytotoxic proteins that stimulate anti-tumor immunity. Therapeutic exosome engineering aims to exploit IDE cargo (e.g., perforin, granzymes, IL-2, IFN-α, miR-155) to deliver immune activators and anti-tumor drugs, ultimately inducing cancer cell death. Figure was designed using BioRender.com.

engineered immune cell-derived exosomes to ensure global activation of cytotoxic T cells and NK cells to combat cancer. The combination of a variety of cancer immunotherapies may increase the specificity against a variety of cancer types, however, these treatment options may be costly, which is a challenge.

Chimeric antigen receptor T cell (CAR-T cells) therapy is an exciting avenue in cancer immunotherapy which has been successful in a variety of hematological malignancies (105). CAR-T cell therapy weaponizes T cells to bind tumors with overexpressed surface antigens (105). The T cells are modified with CAR which increases the specificity of T cells towards tumor surface antigens (105). Despite the FDA approval of six CAR-T cell therapies, there are still ongoing clinical trials on other diseases, and as with other cutting edge cancer immunotherapies, they present some dangerous side effects such as immune effector cell associated neurotoxicity syndrome (ICANS) (105). Considering that the goal of CAR-T therapy is essentially to arm T cells with the firepower to destroy cancer cells, these can be combined with loading of TDEs with cargo that increases T cell cytotoxicity.

In addition to established immunotherapies, recent studies indicate that cancer exosomes undergo notable transformations after treatment, affecting immune reactions and resistance to therapy (106). Grasping these post-treatment changes in exosomes is essential for enhancing immunotherapy results and addressing tactics for tumor evasion.

Post-therapeutic modulation of cancer exosomes: implications for immunity and resistance

Emerging evidence suggests that cancer treatments, including chemotherapy and radiotherapy, can profoundly reshape the composition and function of tumor-derived exosomes. These post-therapeutic changes influence immune responses, contribute to therapy resistance, and impact clinical outcomes. For instance, chemotherapeutic agents such as carboplatin, paclitaxel, and

irinotecan have been shown to markedly increase exosome release from HepG2 hepatocellular carcinoma cells, as measured by acetylcholinesterase activity assays (13). Exosome production in CAG human cells increased significantly 16 hours after treatment with melphalan, bortezomib, and carfilzomib, as measured by nanoparticle tracking analysis (11). Similarly, after paclitaxel treatment, an increase in exosome release was observed compared to untreated cells in MDA-MB-231 breast cancer cells (12). However, contradictions emerged when comparing these *in vitro* studies with ex vivo studies. A notable decrease in exosomal protein levels was reported in patients with acute myeloid leukemia (AML) after chemotherapy (14). Similarly, exosomal protein levels decreased in patients with head and neck cancer after oncological treatment (15). These discrepancies may be attributed to differences in exosome clearance, tumor burden, systemic immune responses, and technical variability between controlled *in vitro* conditions and the complex physiological environment represented in ex vivo patient samples.

Furthermore, after radiation therapy, exosomes derived from breast cancer cells (MCF7, SKBR3, and MDA-MB-231) irradiated with 2 Gy exhibited altered molecular profiles compared to controlled group without radiation (107). While these changes did not influence cell viability or radioresistance, irradiated exosomes increased migratory and invasive potential, in part through β-catenin downregulation—and were more readily internalized by endothelial cells, contributing to reduced expression of CD31 and vascular disruption. Pszczółkowska (2022) (108) reported a dose-dependent decrease in exosome concentration in both PC3 and DU145 prostate cancer cell lines after alpha radiation, although the reduction was not statistically significant. Furthermore, more radio-resistant DU145 cells secreted fewer exosomes than radio-sensitive PC3 cells. In addition, exosomes released by irradiated head and neck cancer cells induced DNA damage and replication stress in naïve recipient cells, evidenced by increased γH2A.X foci and activation of ATM/ATR kinases (109). These effects, which occur even before full exosome internalization, suggest a receptor-mediated bystander mechanism driven by radiation-altered exosomal signaling.

P-gp and other key ATP-binding cassette transporters linked to multidrug resistance are frequently present on exosome membranes (110). Exosomes can transfer P-gp from resistant to sensitive tumor cells, promoting drug resistance (111). In addition to ABC transporters such as P-gp, exosomes from resistant cancer cells also carry detoxifying enzymes such as glutathione S-transferases (GSTs), which neutralize reactive oxygen species and toxic metabolites generated by chemotherapy, thus reducing treatment efficacy (112). Furthermore, tumor-derived exosomes may also carry PD-L1, which can inhibit T cell activation and contribute to immune evasion by suppressing the antitumor immune response (113). In another study by Theodoraki et al. (9), exosomal PD-L1 was reported to be the earliest indicator of failure in treatment in patients with Head and neck cancer. These studies highlight the potential of exosome profiling as a noninvasive biomarker for predicting therapeutic response and guiding early intervention to prevent recurrence.

Cancer-derived exosomes: biomarkers of immune status and tools for diagnosis, therapy monitoring, and treatment

Exosomes have emerged as promising non-invasive biomarkers because of their stability in body fluids and their molecular cargo reflective of the tumor microenvironment. Their diagnostic, prognostic, and predictive potential is particularly evident in immunotherapy, where PD-L1+ exosomes have shown utility as biomarkers for immune checkpoint inhibitor (ICI) response and resistance (10). In the KEYNOTE-028 trial, patients with advanced solid tumors were selected based on 1% PD-L1 expression in tumor or stromal cells (114). While a phase I/II study in urothelial carcinoma showed that patients with 25% PD-L1 expression in tumor or immune cells had higher response rates to durvalumab (115). In another study, circulating exosomal PD-L1 was reported to serve as a predictive biomarker of pembrolizumab response in patients with melanoma (113). Elevated levels of PD-L1 before treatment were associated with T cell exhaustion and reduced therapeutic benefit, while an increase during treatment was associated with T cell reinvigoration and enhanced antitumor immunity. These findings support the use of PD-L1 levels, including exosomal PD-L1, to stratify patients likely to benefit from ICIs, and further highlight its potential as a non-invasive blood-based marker for monitoring and predicting therapeutic outcomes during anti-PD-1 therapy. Importantly, exosomal PD-L1 also complements existing diagnostic tools. Unlike tissue-based PD-L1 immunohistochemistry, which is limited by intratumoral heterogeneity and insufficient biopsy samples (116), circulating exosomal PD-L1 offers a repeatable, minimally invasive alternative that captures dynamic changes during therapy. This position as a valuable adjunct to conventional assays, particularly in patients where tissue availability or sampling frequency is a challenge.

These insights into exosomal PD-L1 not only reinforce its prognostic and predictive utility, but also exemplify the broader clinical relevance of liquid biopsy approaches, which offer a non-invasive means to dynamically monitor tumor evolution and therapeutic response. For example, in breast cancer, exosomal miR-1246 was reported to distinguish patients from healthy individuals using a gold nanoflare probe, which demonstrated high sensitivity and single-molecule specificity at relatively low cost compared to conventional qRT-PCR, while also offering faster turnaround times (7). ELISA assays, such as those used for protein markers like PD-L1 (106), are cost-effective but have limited multiplexing capacity. In contrast, nanosensor-based approaches for miRNAs provide higher sensitivity and adaptability, making them promising for clinical use where precision, scalability, and

affordability are essential. In colorectal cancer, Lui et al. (117) showed that CRC-secreted exosomal miR-1246 is internalized by hepatic stellate cells (HSCs), leading to their activation through the INSIG1/SREBP2/cholesterol metabolism axis, which reprograms the tumor microenvironment and promotes liver metastasis. Importantly, these findings suggest that exosomal miR-1246 could serve as a non-invasive biomarker for predicting colorectal cancer liver metastases. Similarly, in lung cancer, Huang and Qu (118) demonstrated that serum exosomal miR-1246 was significantly upregulated in non-small cell lung cancer (NSCLC) patients, correlated with lymph node metastasis and TNM stage, and acted as an independent prognostic factor for poor survival. ROC analysis confirmed its strong diagnostic performance, while dynamic changes in its levels reflected treatment response and recurrence. Together, these findings underscore the versatility of exosomal miR-1246 as a diagnostic and prognostic biomarker across multiple solid tumors, including breast, colorectal, and lung cancers. Furthermore, exosomal miR-105, miR-21, and miR-222 have shown promise as predictive markers for neoadjuvant chemotherapy and in the diagnosis of breast cancer (8). Furthermore, high levels of the exosomal protein CD82 have been associated with metastasis, likely reflecting its redistribution from tissues to exosomes during tumor progression (119). miR-210-3p, miR-5100, and miR-193a-3p were identified as novel biomarkers of lung cancer progression (120). In ovarian cancer, exosomal miR-200b and miR-200c have been reported to be associated with poorer overall survival, with their expression levels showing a significant correlation with CA-125 (Cancer Antigen 125) levels (121). Although miRNAs have been the main focus, long exosomal RNAs such as lncRNAs offer greater potential for tracking somatic mutations and gene expression changes. Exosomal lncRNA PCAT-1, detected in urine, has been proposed as an independent prognostic biomarker to assess relapse-free survival in patients with nonmuscle-invasive bladder cancer (122), further underscoring the potential of exosomes as liquid biopsies in cancer prognosis. Beyond bladder cancer, similar strategies are being explored in other solid tumors: for instance, exosomal lncRNA HOTAIR has been linked to poor prognosis and metastasis in breast cancer (123), while exosomal lncRNA MALAT1 has been shown to promote chemoresistance and predict outcomes in ovarian cancer (124). These findings highlight the broader applicability of exosomal lncRNAs as minimally invasive biomarkers for early detection, treatment monitoring, and therapeutic stratification across multiple cancer types.

While most ongoing exosome-based clinical trials focus on their diagnostic and prognostic potential, cancer-derived exosomes are increasingly recognized for their immunomodulatory roles, influencing antitumor immunity and opening new avenues for cancer therapy. Exosomes have garnered interest as therapeutic delivery vehicles due to their endogenous origin, low immunogenicity, ability to cross the blood–brain barrier, high target specificity and excellent biocompatibility. Nanosomes, an exosome–gold nanoparticle delivery system, were developed to deliver doxorubicin for lung cancer therapy (125). The study demonstrated an efficient intracellular distribution of doxorubicin and enhanced therapeutic efficacy in H1299 and A549 nonsmall cell lung cancer cells, highlighting the potential of exosome-engineered platforms for targeted cancer treatment. Similarly, glioblastoma and brain endothelial cell exosomes were loaded with paclitaxel and doxorubicin to facilitate transport across the blood-brain barrier to brain tumors in a zebrafish model (18). In another study, engineered exosomes (iExoSTINGa) were used to deliver the cyclic GMP-AMP small molecule STING agonist, resulting in enhanced antitumor immunity and suppression of subcutaneous tumor growth of B16F10 (19). Additionally, exosomes isolated from peripheral blood were successfully loaded with MAPK1 siRNA and used to deliver the siRNA into monocytes and lymphocytes, leading to targeted gene silencing (126).

Together, these studies underscore the versatility of cancer-derived exosomes as diagnostic tools and therapeutic platforms, further confirming their emerging role in precision oncology and immune modulation.

Engineering and isolation of exosomes for cancer therapy

Building on their natural capacity for intercellular communication, IDEs are now being engineered using a variety of physical, chemical, and biological techniques to enhance their specificity, cargo capacity, and therapeutic efficacy.

Exosome engineering strategies

These engineering strategies are critical to translating exosomes into clinically viable platforms. Various methods such as electroporation, sonication, transfection, and surface conjugation are used to load exosomes with therapeutic molecules, including nucleic acids, proteins, and drugs (17). Table 2 summarizes the most commonly used methods, their mechanisms, and representative examples from the current literature.

The compiled studies demonstrate the versatility of engineered exosomes as targeted delivery vehicles in cancer therapy, utilizing various types of cargo such as siRNAs, chemotherapeutic prodrugs, and immune agonists. Various engineering methods, such as electroporation, sonication, and incubation enable efficient loading and targeting of exosomes derived from mesenchymal stem cells, macrophages, and other cell types. These approaches collectively enhance therapeutic efficacy through mechanisms including gene silencing, immune activation, pH-responsive drug release, and improved tumor targeting, showing promise across multiple cancer types including pancreatic, melanoma, cervical, bladder, and glioblastoma.

Exosome isolation methods

Exosome isolation is a critical step that ensures purity and functional integrity before downstream applications. Commonly

TABLE 2 Exosome engineering and loading methods in cancer immunotherapy and targeted therapy.

Engineering methods	Cargo type	Cell type	Mechanism	Cancer type	Reference
Electroporation and vortexing	Galectin-9 siRNA, DOGEM (prodrug of gemcitabine), Indocyanine Green (ICG)	Bone marrow-derived mesenchymal stem cells (BM-MSCs)	pH-responsive release; synergistic chemotherapy, immunotherapy (T-cell activation), and phototherapy; galectin-9 silencing	Pancreatic cancer	48
Sonication + chemical modification	siRNA (KRASG12D)	Macrophages	Gene silencing of oncogenic KRAS to inhibit tumor growth	Pancreatic cancer	127
Exogenous incubation	STING agonist (cGAMP)	T cells	Activation of STING pathway to stimulate innate and adaptive antitumor immunity	Melanoma	19
Electroporation & folate decoration	Survivin siRNA	HEK293T-derived exosomes	Tumor targeting via folate, surviving knockdown, apoptosis	Cervical cancer	128
Incubation (drug diffusion)	Paclitaxel (PTX), Doxorubicin	Macrophages	Trans-BBB drug delivery to kill brain tumor cells	Glioblastoma	18

used methods include ultracentrifugation, size-exclusion chromatography, and immunoaffinity capture, each with distinct advantages and limitations. Table 3 provides an overview of these isolation strategies, emphasizing their mechanisms and the applications they have been investigated in.

Ultracentrifugation is known as the gold standard when it comes to exosomes isolation strategies (134). Differential ultracentrifugation and density-gradient approaches (including isopycnic and moving-zone methods) are the primary ultracentrifugation techniques traditionally employed for exosome isolation. Differential ultracentrifugation also known as simple ultracentrifugation or the pelleting method is the most widely used approach for exosome isolation, accounting for nearly half of reported studies (45.7%) (129). Its principle is straightforward: by applying increasing centrifugal forces, extracellular components in a fluid sample are sequentially separated according to their size, density, and shape. This method is favored for its ease of use, minimal technical expertise requirements, and suitability for processing large sample volumes without the need for complex pre-treatment (135). Despite this, extracellular fluids exhibit significant heterogeneity, and differential ultracentrifugation frequently results in the co-precipitation of microvesicles with non-vesicular entities like protein aggregates and lipoproteins (136). Consequently, this can result in low purity, potentially affecting subsequent applications (137). For example, Paolini and colleagues showed that exosomes isolated by this method exhibited poor and inconsistent biological activity compared to more purified samples (138). To improve exosome isolation, researchers have developed new centrifugation methods, among which density-gradient centrifugation is widely used to separate particles by density (131).

Isopycnic density-gradient centrifugation entails setting up a tube with layers of a biocompatible medium with varying densities, such as iodixanol or sucrose, arranged from highest density at the bottom to lowest at the top (139). The sample is carefully placed atop this gradient and subjected to extended ultracentrifugation (e.g., 100,000 × g for 16 hours). During this process, extracellular components like exosomes, apoptotic bodies, and protein

TABLE 3 Exosome isolation methods in cancer immunotherapy and targeted therapy.

Isolation methods	Cargo type	Cell type	Mechanism	Application	Reference
Differential Ultracentrifugation	Untreated exosomes (native cargo)	Plasma, urine, cell culture supernatant	Sequential centrifugation at increasing speeds to remove cells, debris, and larger vesicles; final pelleting of exosomes at high speed (100,000×g)	Widely used standard method; biomarker studies; therapeutic applications	129, 130
Density- gradient ultracentrifugation	Untreated exosomes (native cargo)	Plasma, serum, cell culture supernatant	Separation of vesicles based on buoyant density using sucrose or iodixanol gradients; improved purity compared to differential UC	Functional and proteomic studies; cancer biomarker discovery	130, 131
Size-Exclusion Chromatography (SEC)	Untreated exosomes (native cargo)	Plasma, cell culture supernatant	Separation based on vesicle size through porous matrix; preserves vesicle integrity and function	Functional studies, therapeutic applications, biomarker analysis	132
Immunoaffinity Capture (IAC)	CD16 marker	Plasma	Antibody binding to highly enriched exosome surface proteins enables selective isolation	Linking exosome origin to immunoregulatory function; biomarker discovery; HNSCC, other cancers	133

aggregates move through the gradient until reaching their isopycnic position, where their buoyant density matches that of the medium surrounding them. Although density-gradient centrifugation is widely regarded as the most effective approach for obtaining highly pure exosomes for downstream applications, it cannot distinguish extracellular vesicles of similar buoyant density but different sizes from exosomes (e.g., microvesicles) (140). To address the challenges of isopycnic centrifugation, moving-zone (rate-zonal) density-gradient centrifugation enables separation of particles by both size and density (141). This method allows for the isolation of vesicles with similar densities but varying sizes, such as exosomes, large microvesicles, and viruses. In this technique, the gradient medium is less dense than any component in the sample, and the centrifugation duration must be meticulously managed to avoid all particles sedimenting at the bottom. To reduce exosome loss, a dense cushion is frequently placed at the tube's base to maintain vesicles within the gradient while allowing denser particles to sediment.

Size-exclusion chromatography (SEC) is a size-based separation technique that isolates extracellular vesicles, including exosomes, by passing a biological sample through a column packed with a porous matrix (142). Larger vesicles are excluded from the pores and elute first, while smaller particles enter the pores and elute later, allowing gentle separation with minimal impact on vesicle structure and function. Within just a decade, several commercial SEC kits specifically designed for exosome isolation have been developed, including qEV (iZON) and PURE-EVs (Hansa Biomed). iZON has developed an automated exosome isolation system (qEV Automatic Fraction Collector) built on the SEC platform, incorporating weight-dependent fractionation and sample collection (143). This system enables fast, precise, and scalable exosome isolation, while reducing hands-on time and variability. SEC preserves the natural structure and biological activity of exosomes through passive gravity flow, avoiding the high shear forces and structural damage associated with ultracentrifugation (144). It enables rapid, simple, and reproducible isolation from small sample volumes without extensive pre-treatment, while physiological buffers maintain vesicle integrity. Compared to ultracentrifugation, SEC allows selection of defined vesicle subpopulations, minimizes sample loss, and achieves high yield, making it particularly suitable for functional and therapeutic studies (145).

Immunoaffinity capture leverages the specific binding between antibodies and proteins or receptors that are highly enriched on the surface of exosomes, allowing selective isolation from complex biological fluids (146). Common exosome markers include transmembrane proteins such as CD9, CD63, CD81, CD82, Rab5, Alix, and annexins, as well as other components like lysosome-associated membrane protein-2B, heat shock proteins, platelet-derived growth factor receptors, and lipid-related proteins (147–152). This approach underlies several commercial exosome isolation products, including the Exosome Isolation and Analysis Kit (Abcam), Exosome-Human CD63 Isolation Reagent (Thermo Fisher), and Exosome Isolation Kit CD81/CD63 (Miltenyi Biotec), providing high specificity while preserving vesicle functionality. Notably, immunoaffinity capture of CD3(+) (T cell-derived) and CD3(−) (tumor-derived) plasma exosomes from HNSCC patients showed that tumor-derived exosomes induce stronger T cell suppression, demonstrating the method's ability to link exosome origin to immunoregulatory function and disease progression (153).

Challenges and future directions

Despite promising preclinical results, several challenges hinder the clinical translation of exosome-based therapies. Standardization of exosome isolation and loading methods remains difficult, leading to variability in yield and cargo encapsulation efficiency (16). For instance, even though gradient ultracentrifugation can purify exosomes with minimal contamination, its processing volume is limited, requires expensive equipment, and demands highly trained personnel (154). Additionally, prolonged exposure to ultracentrifugal force can damage exosome structure and function, compromising downstream applications such as functional studies and drug development (155). Additionally, SEC's key challenge is that exosome preparations often display a broader size distribution, particularly at the lower end, indicating contamination with similarly sized particles such as protein aggregates and lipoproteins (156). To address this, combined strategies such as SEC with ultrafiltration or ultracentrifugation have been employed, resulting in higher-purity exosomes while preserving their functional integrity. Furthermore, the immunoaffinity capture approach is highly specific and preserves exosome function, but it is limited to exosomes that express the target antigen on a large proportion of vesicles (146), and it can be costly, difficult to scale, and may miss subpopulations lacking the selected marker.

Challenges in exosome engineering include low cargo loading efficiency, instability or premature leakage of cargo, population heterogeneity, altered biological function, limited scalability and reproducibility, potential safety and immunogenicity concerns, and regulatory or manufacturing barriers that hinder clinical translation (157). In response to restricted loading capacity, active cargo loading techniques have emerged, but these can lead to exosome aggregation, membrane damage, and necessitate rigorous purification (158). Endogenous loading consists of directly inserting therapeutic cargo into exosomes via the donor cell. This can be accomplished by either incubating the parent cells with the cargo or using gene editing to enhance the expression of target molecules for later encapsulation (159).

In addition to optimizing cargo loading, challenges such as target specificity and off-target effects must be addressed to prevent unintended immune responses or toxicity. One potential strategy is using autologous tumor cells as the source of exosome production, which can reduce neutralization by the patient's immune system and enhance therapeutic efficacy (17). While allogeneic engineered IDEs carry a higher risk of immunogenicity, autologous IDEs are generally better tolerated; however, both may still cause off-target effects on healthy cells, highlighting the need for precise targeting, rigorous safety evaluation, and careful design of therapeutic cargo (160).

Phase I clinical trials have initiated investigations into the potential of utilizing exosome-based therapies for cancer treatment. One specific study (NCT01550523) assessed glioma cell–derived exosomes that were engineered to carry an antisense molecule against the insulin-like growth factor I receptor (IGF1R), demonstrating both the feasibility and safety of exosome-based therapeutic delivery (161). In a separate trial (NCT01159288), exosomes derived from autologous dendritic cells (DEX) were used as a therapeutic vaccine for patients with metastatic melanoma, indicating safety and tolerability, yet lacking strong responses from $CD4^+$ or $CD8^+$ T cells. This underscores the necessity for further exploration into how exosome-mediated antigen presentation can be optimized (162).

Current and completed early-phase studies investigate a variety of therapeutic approaches, such as mesenchymal stromal cell-derived exosomes loaded with KRAS^G12D siRNA for treating metastatic pancreatic cancer (NCT03608631; 163), plant-derived exosomes used to transport curcumin for colon cancer therapy (NCT01294072; 164), exosomes sourced from autologous ascites combined with GM-CSF for colorectal cancer (165), and dendritic cell-derived exosomes evaluated as a maintenance immunotherapy following initial chemotherapy in non-small-cell lung cancer (166). Collectively, these studies highlight the diverse array of exosome-based strategies presently under clinical evaluation (Table 4).

Building on these early-phase studies, additional trials have demonstrated the feasibility and initial safety of engineered exosomes for targeted cancer therapy. However, translating these promising results into widespread clinical use is limited by challenges in large-scale manufacturing and quality control of clinical-grade exosomes (17). Standardized, GMP-compliant protocols are lacking, and scaling up while maintaining exosome purity, functionality, and batch-to-batch consistency remains a major bottleneck. Strategies such as automated bioreactor systems, advanced purification technologies, and the development of synthetic exosome mimetics are being explored to improve scalability, reproducibility, and safety for therapeutic applications (167).

Preliminary outcomes indicate that engineered exosomes are generally well-tolerated and capable of delivering therapeutic cargo, but immune responses and clinical efficacy have been variable. These findings underscore the need for optimized dosing strategies, improved targeting, and enhanced exosome engineering in future trial design to maximize therapeutic benefit, while advanced 3D ex vivo models and rigorous *in vivo* studies remain essential to fully evaluate pharmacokinetics, biodistribution, long-term safety, and therapeutic efficacy.

The future of exosome-based immunotherapy lies at the intersection of mechanistic insight, bioengineering, and clinical translation. While CDEs promote tumor progression by suppressing immune surveillance, this same pathway can be harnessed by engineering immune cell–derived exosomes to deliver tumor antigens, siRNAs, or checkpoint inhibitors that stimulate antitumor immunity. Combination strategies, such as pairing dendritic cell–derived exosomes with cytotoxic T cell activation, may offer more durable and systemic effects, though multiplexed immunotherapies must also address the complexity of the TME and ensure affordability at scale.

To support translation, advanced 3D ex vivo models (e.g., tumor–immune organoids) will be critical for testing efficacy, biodistribution, and safety under physiologically relevant conditions. Equally important is the standardization of isolation and engineering workflows, improving loading efficiency, cargo stability, and reproducibility for clinical-grade production. Emerging technologies, including AI, machine learning, and multi-omics, could accelerate this process by identifying predictive biomarkers, optimizing therapeutic payloads, and enabling personalized exosome therapies.

In conclusion, clinical success will depend on overcoming barriers in engineering, large-scale manufacturing, and regulatory standardization, while leveraging new models and computational tools to shift the TME balance toward immune activation and cancer control.

TABLE 4 Current and finalized clinical trials concerning exosome-derived treatments in oncology.

NCT ID	Phase	Approach/cargo	Indication	Status/highlights	Reference
NCT01550523	I	Glioma exosomes carrying IGF1R antisense	Glioma	Feasibility and safety demonstrated	161
NCT01159288	I-II	Autologous DC-exosomes (DEX) vaccine	Metastatic melanoma/NSCLC	Tolerable; modest T cell activation	162
NCT03608631	I	MSC-derived exosomes with KRAS^G12D siRNA	Metastatic pancreatic cancer	Evaluating dose and safety	163
NCT01294072	I	Plant-derived exosomes delivering curcumin	Colon cancer	Safety/tolerability under investigation	164
—	I	Autologous ascites exosomes + GM-CSF	Colorectal cancer	Completed Phase I; showed antigen delivery	165
NCT01159288 (Phase II extension)	II	MHC class I & II–restricted antigens	NSCLC	Modest clinical benefit; median OS 15 mo; primary endpoint not met	166

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Multimodal cell-cell communication driving CD8$^+$ T cell dysfunction and immune evasion

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Effective anti-tumor immunity critically depends on functional CD8$^+$ T cells, yet in almost all solid tumors, these cells become dysfunctional, exhausted, or spatially excluded. This breakdown of immune surveillance arises not only from cell-intrinsic T cell exhaustion but also from multimodal communication among tumor, stromal, and immune cells within the tumor microenvironment (TME). This communication is mediated not only through direct receptor-ligand interactions but also through a suite of indirect mechanisms, such as metabolic competition, secretion of immunosuppressive metabolites and cytokines, extracellular vesicle exchange, and even mitochondrial transfer via tunneling nanotubes or membrane transfer through T cell trogocytosis. Together, these suppressive interactions impair CD8$^+$ T cell metabolism, effector function, and persistence, thereby enabling tumor immune evasion. In this review, we summarize current understanding of how multimodal cell-cell communication, including immune checkpoints, metabolic reprogramming, and stromal crosstalk, cooperatively drive CD8$^+$ T cell dysfunction. We also highlight emerging therapeutic strategies aimed at rewiring these suppressive networks, with emphasis on translational potential. A deeper understanding of the spatial, molecular, and metabolic context of CD8$^+$ T cell suppression offers new avenues to enhance the efficacy of cancer immunotherapies.

KEYWORDS

tumor microenvironment, CD8$^+$ T cell, multimodal cell-cell communication, suppression, dysfunction

1 Introduction

CD8$^+$ cytotoxic T lymphocytes (CTLs) are central mediators of anti-tumor immunity, capable of directly eliminating malignant cells through perforin-granzyme release and Fas-FasL signaling (1, 2). Their activation requires tumor antigens presentation by dendritic cells (DCs), co-stimulation signals (e.g., CD28-B7), and pro-inflammation cytokines [e.g., interleukin (IL)-12, interferon-gamma (IFN-γ)], leading to clonal expansion and cytotoxic

effector functions acquisition (3). Upon antigen-specific activation, CTLs proliferate and differentiate into two major subsets: effector $CD8^+$ T cells, characterized by high expression of granzyme, perforin, and IFN-γ, which eliminate target tumor cells; and memory $CD8^+$ T cells that possess self-renewal and multilineage differentiation capacities, providing a cellular reservoir for long-term immune surveillance (4, 5).

Under chronic antigen exposure, however, CTLs gradually lose effector function and upregulate inhibitory receptors such as programmed cell death protein 1 (PD-1) and cytotoxic T-lymphocyte associated protein 4 (CTLA-4), this dysfunctional state is termed T cell exhaustion (3). This trajectory of $CD8^+$ T cell differentiation and dysfunction proceeds through successive stages: naïve T cells → activated T cells → stem-like progenitor of exhausted T cells (Tpex) → effector-like or intermediate exhausted T cells → terminal exhausted T cells (6, 7). TME provides spatial niches that critically shape this progression (8). Tertiary lymphoid structures (TLS) and perivascular regions, enriched with DCs, maintain $TCF1^+$ Tpex cells, which preserve responsiveness to immune checkpoint blockade (ICB) (9–12). In contrast, tumor margins are enriched with $CD103^+$ tissue-resident memory T cells (Trm) associated with favorable patient prognosis, while the immunosuppressive and hypoxic tumor core drives T cells towards terminal exhaustion, reinforced by persistent antigen exposure (13–15).

This dysfunctional state is further exacerbated by immunosuppressive factors in the TME, including tumor-associated macrophages (TAMs, e.g., $IRF8^+$) (16) and inhibitory cytokine networks (17), ultimately impairing antitumor immunity. Preclinical and clinical studies consistently demonstrate that in solid tumors, $CD8^+$ T cells become functionally exhausted and metabolically impaired due to persistent antigen exposure and immunosuppressive mechanisms within the TME (18, 19). These mechanisms include direct inhibition by tumor and stromal cells, as well as indirect suppression via metabolic competition and soluble mediators, collectively impairing $CD8^+$ T cell function and antitumor immunity (8, 20, 21).

A critical axis of immune evasion involves direct cell-to-cell interactions that drive $CD8^+$ T cell dysfunction. Tumor cells exploit a repertoire of inhibitory ligands [e.g., PD-L1, B7 homolog 3 (B7-H3), and human leukocyte antigen (HLA)-E)] to engage checkpoint receptors [PD-1, Lymphocyte-activation gene 3 (LAG-3), Natural killer group 2 member A (NKG2A)] on T cells, thereby blunting TCR signaling and cytotoxicity activity. Immune cells such as regulatory T cells (Tregs) and myeloid-derived suppressor cells (MDSCs) further suppress CTLs through mechanisms including CTLA-4-mediated blockade of co-stimulation and PD-L1 expression. Cancer-associated fibroblasts (CAFs) reinforce this suppression both by expressing ligands such as PD-L1 and carcinoembryonic antigen-related cell adhesion molecule 1 (Ceacam-1), and by physically restricting $CD8^+$ T cells infiltration into tumor niches. Together, these interactions highlight the complexity of contact-dependent immunosuppression and underscore the limitations of current checkpoint blockade therapies.

Beyond direct contact, the TME imposes indirect suppression through metabolic hijacking, stromal crosstalk, and biochemical perturbations. Tumor cells aggressively outcompete T cells for essential nutrients including glucose and arginine, while releasing immunosuppressive metabolites such as lactate, adenosine and kynurenine. Extracellular vesicles, tunneling nanotubes and T cell trogocytosis further exacerbate suppression by transferring inhibitory cargos, such as dysfunctional mitochondria, inhibitory miRNAs, or even membrane fragments, to T cells. Meanwhile, cytokines (e.g., TGF-β) and ions (e.g., Mg^{2+}, ammonia) disrupt T cell metabolism, signaling and epigenetic programming. Stromal components such as CAFs and MDSCs amplify these effects by remodeling the extracellular matrix, secreting suppressive cytokines, and inducing hypoxia. Collectively, these processes create a hostile metabolic and structural niche that sustains T cell dysfunction.

These multimodal pathways act synergistically to impair $CD8^+$ T cell cytotoxicity and persistence, and spatial access into tumors, ultimately enabling immune evasion. Overcoming this coordinated suppression remains a major challenge in current cancer immunotherapy. In this review, we summarize recent advances in understanding the mechanisms of multimodal cell-cell communication, including immune checkpoint signaling, metabolic interference, and stromal crosstalk, that collectively drives $CD8^+$ T cell dysfunction (Figure 1). We further discuss emerging therapeutic strategies designed to disrupt these suppressive networks and restore anti-tumor immunity, with particular attention to combinatorial approaches with translational potential. A precise understanding of the spatial and molecular dynamics of $CD8^+$ T cell suppression will be pivotal for overcoming resistance to current immunotherapies.

2 Direct cell-to-cell interactions suppressing $CD8^+$ T cell function

The direct interaction between $CD8^+$ T cells and other cells in TME, including tumor cells, other immune cells and CAFs, is crucial for shaping anti-tumor immune responses. Direct contact through receptor ligand engagement and immunological synapses regulates $CD8^+$ T cell activation, effector function, and exhaustion. While stimulatory signals enhance cytotoxicity, some interaction induced inhibitory pathways blunt TCR signaling, cytokine production, and proliferation. This section reviews how tumor cells, immune cells, and CAFs suppress $CD8^+$ T cells function through surface expressed inhibitory molecules and checkpoint receptor-ligand interactions (Figure 2).

2.1 Tumor cell-to-$CD8^+$ T cell interactions

Tumor cells directly inhibit infiltrating $CD8^+$ T cells by engaging multiple inhibitory ligands. The PD-1-PD-L1 axis remains a dominant pathway: IFN-γ produced by activated T cells induces PD-L1 expression on tumor cells (22), which in turn

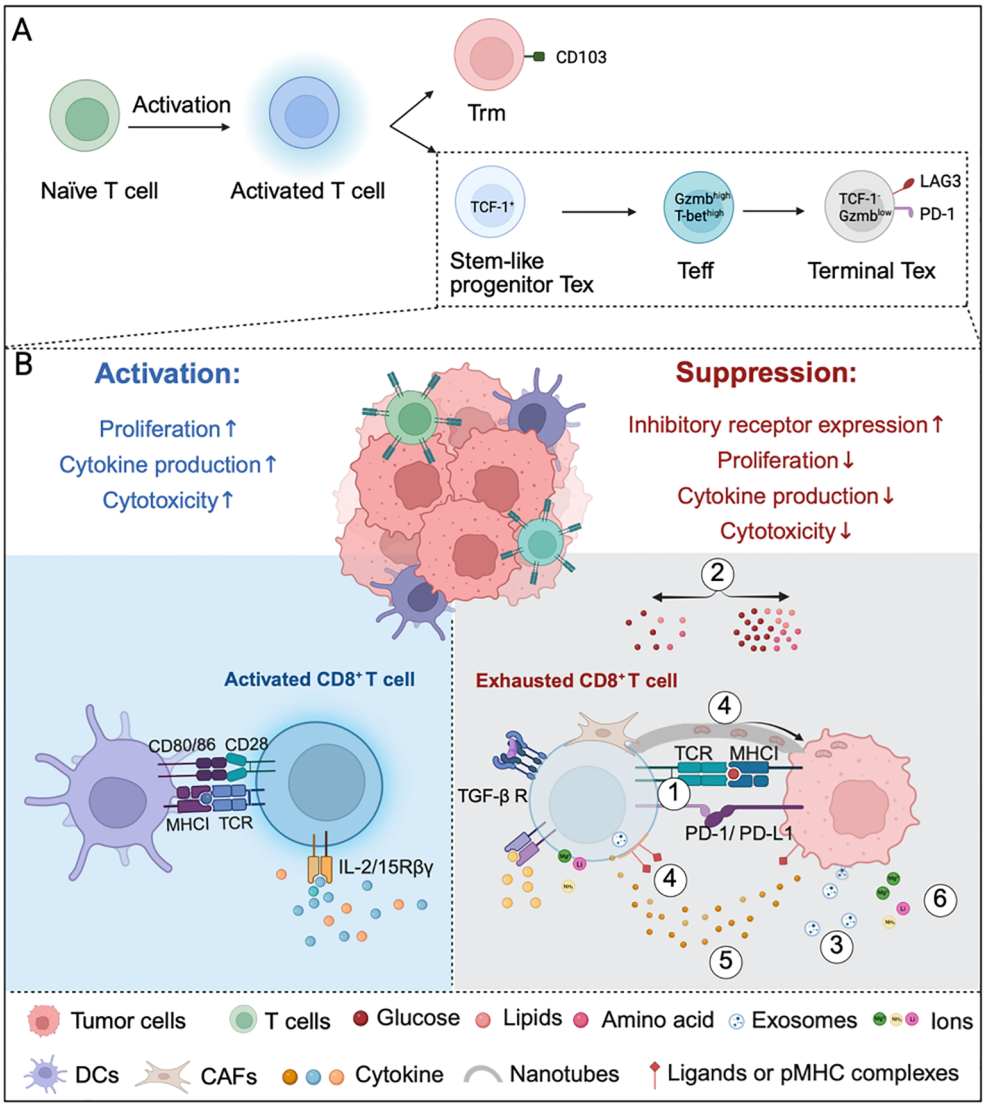

FIGURE 1
Multifaceted regulation of CD8$^+$ T cell function within the TME. **(A)** Differentiation and exhaustion of CD8$^+$ T cells under chronic antigen stimulation. Effector-like or intermediate exhausted T cells (Teff); Exhausted T cells (Tex); Tissue-resident memory T cells (Trm). **(B)** The TME exerts dual effects on CD8$^+$ T cells: it can promote T cell activation and effector functions, while simultaneously driving exhaustion and dysfunction. Left panel (Activation): Dendritic cells prime CD8$^+$ T cells through integrated signals, which collectively enhance T cell proliferation, migration, differentiation, cytokine production, and cytotoxic capacity. Right panel (Suppression): Tumor cells suppress CD8$^+$ T cell function through multiple mechanisms: (1) immunosuppressive ligand-receptor interactions [programmed cell death ligand 1 (PD-L1)-PD-1, transforming growth factor β (TGF-β)-TGF β receptor (TGF-βR)]; (2) nutrient competition (glucose, lipids, and amino acids); (3) tumor-derived exosomes; (4) intercellular material transfer via nanotubes and trogocytosis; (5) cytokines; and (6) release of immunosuppressive cytokines or metabolites ($Mg2^+$, lithium, and ammonia). These inhibitor cues collectively drive upregulation of checkpoint receptors, diminished proliferation, and self-renewal capacity, reduced cytokine production, and impaired cytotoxicity, ultimately driving CD8$^+$ T cells toward exhaustion. Image created with bioRender.com, with permission. Created in BioRender. Zhou, P. (2025) https://BioRender.com/e66x2mi.

binds PD-1 on CD8$^+$ T cells, delivering potent inhibitory signals that attenuate TCR signaling (e.g., reduced ZAP70 phosphorylation), cytokine secretion (e.g., IFN-γ), and cytotoxic activity, ultimately driving CD8$^+$ T cells into a dysfunctional state. Similarly, B7 ligands B7-1 (CD80) and B7-2 (CD86) on tumor cells engage CTLA-4 on activated CD8$^+$ T cells, outcompeting CD28 and thereby blocking co-stimulatory signals required for T cell activation, leading to CD8$^+$ T cell anergy.

Beyond these classical checkpoints, emerging ligand-receptor pathways are increasingly recognized. B7x-B7-H4, a member of the B7 family broadly expressed across tumors, which binds unidentified inhibitory receptor on activated, but not resting CD8$^+$ T cells (23–25). B7-H4 inhibits CD8$^+$ T cell responses at an early stage primarily by arresting cell cycle progression, suppressing TCR signaling, and reducing IL-2 production (26). Liver and lymph node sinusoidal endothelial cell C-type lectin (LSECtin), expressed in the liver and on multiple tumor types (e.g., melanoma), suppresses anti-tumor immunity by binding LAG-3 on CD8$^+$ T cells, where its KIEELE motif has been identified as structurally and functionally essential for LAG-3's inhibitory capacity. LAG-3

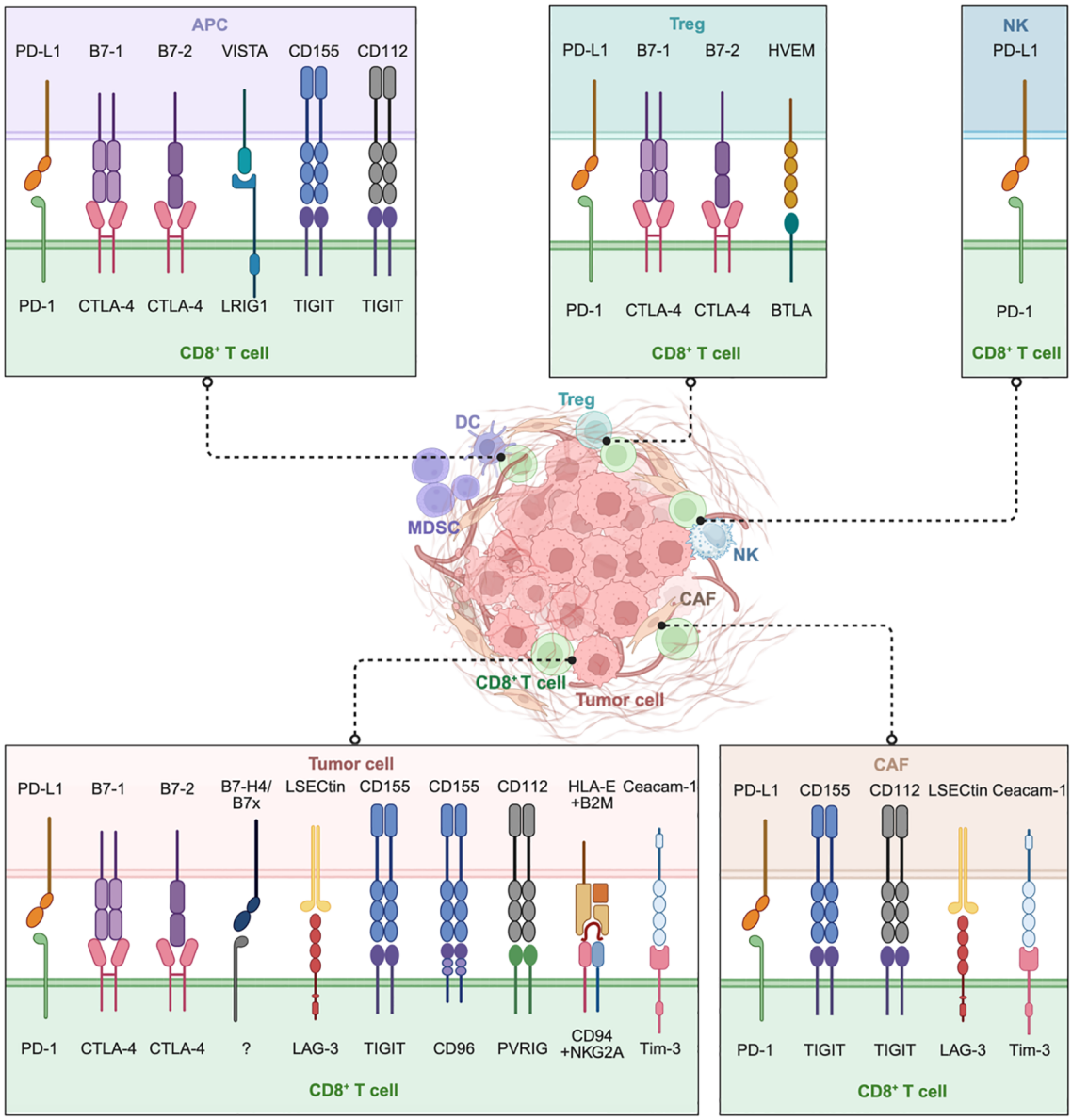

FIGURE 2
Direct cell-to-cell contact plays a critical role in the suppression of $CD8^+$ T cells within the TME. Tumor cells inhibit CTLs by engaging inhibitory ligands with corresponding receptors, while multiple immune cells, including antigen-presenting cells (APCs), regulatory T cells (Tregs), NK cells (NKs), and specialized $CD8^+$ Tregs, further suppress $CD8^+$ T cells through checkpoint molecules like PD-1, CTLA-4, and VISTA. Cancer-associated fibroblasts (CAFs) uniquely contribute by engaging in direct inhibitory signaling (such as PD-L1-PD-1, Ceacam-1-Tim-3) and by imposing physical barriers that restrict T cell infiltration. Collectively, this intricate intercellular communication network drives $CD8^+$ T cell dysfunction and exhaustion. Targeting these specific interactions, particularly beyond PD-1 and CTLA-4 (e.g., LAG-3, TIGIT, VISTA, PVRIG, CD96, NKG2A) and disrupting CAF-mediated suppression represent promising approaches to great reinvigorate $CD8^+$ T cell anti-tumor responses. Image created with bioRender.com, with permission. Created in BioRender. Zhou, P. (2025) https://BioRender.com/kjhqxg8.

signaling inhibits effector T cell function by associating with CD3, where co-engagement suppresses proliferation, IFN-γ secretion, and calcium mobilization (27, 28).

T cell immunoglobulin and ITIM domain (TIGIT), an Ig superfamily member specifically expressed in immune cells, binds CD155 on tumor cells, directly inhibiting effector $CD8^+$ T cell function (27). CD96, which also binds CD155, antagonizes the activating receptor CD226. Although CD96-mediated intracellular signaling remains incompletely characterized, its cytoplasmic ITIM domain suggests inhibitory potential (29). Notably, $CD155^{hi}$ lung adenocarcinoma (LUAD) cells dramatically reduce IFN-γ production in $CD8^+$ T cells, thereby suppressing antitumor immunity (30). Poliovirus receptor-related protein 2 (PVRL2), also known as CD112, expressed by tumor cells and tumor-associated myeloid cells, binds the late-induced inhibitory receptor PVRIG (CD112R) on activated $CD8^+$ T cells. The

PVRL2-PVRIG axis, mediated by PVRIG's ITIM domain, diminishes IL-12 receptor expression, suppresses cytotoxicity, and promotes $CD8^+$ T cell exhaustion (31, 32).

Additional interactions further reinforce this suppressive network. HLA-E-Qa-1^b complexes, presenting specific peptides processed by endoplasmic reticulum aminopeptidase 1-2 (ERAP1-2), engage the inhibitory natural killer cell group 2 member A (NKG2A)-CD94 heterodimer on a subset of $CD8^+$ tumor-infiltrating lymphocytes (TILs), leading to suppression of TCR signaling and consequent impairment of cytotoxic effector function (33, 34). Ceacam-1-Tim-3 interactions have also been implicated, although current support comes primarily from clinical evidence rather than experimental validation (35).

Collectively, these inhibitory dyads converge to restrain $CD8^+$ T cell cytotoxicity and persistence, underscoring the importance of multi-targeted checkpoint blockade.

2.2 Immune cell-to-$CD8^+$ T cell interactions

Multiple immune cell populations within the TME suppress $CD8^+$ T cell function through direct contact. Antigen-presenting cells (APCs), including DCs and macrophages, inhibit $CD8^+$ T cells through classic immune evasion pathways, like PD-L1-PD-1 axis (36–39). APCs also express VISTA (V-domain immunoglobulin suppressor of T cell activation), functioning as a ligand for immunoglobulin-like domains 1 (LRIG1) on $CD8^+$ T cells in a "trans" configuration, contributing to T cell inhibition and quiescence (40–42). Furthermore, constitutive expression of CD80-CD86 on APCs allows binding of CTLA-4 on activated $CD8^+$ T cells (43, 44). CTLA-4 not only transmits intrinsic inhibitory signals but also, on Tregs, mediates the trans-endocytosis and degradation of CD80-CD86 from the APC surface, thereby limiting co-stimulation for other T cells (43, 45). Follicular dendritic cells (FDCs) express CD112 and CD155, which engage TIGIT on TILs, promoting a dysfunctional state characterized by high co-expression of PD-1, and diminished production of IFN-γ, tumor necrosis factor-alpha (TNF-α), and IL-2 (46). Natural Killer (NK) cells upregulate PD-L1 upon tumor recognition and IL-18 stimulation, generating PD-$L1^{hi}$ NK cells that directly suppress $CD8^+$ T cell proliferation in PD-L1-PD-1-dependent manner (47). $CD45RA^-$ $CCR7^-$ (C-C motif chemokine receptor 7) Tregs exhibit upregulated CD80/CD86 expression alongside reduced HLA-DR, enabling potent suppression of $CD8^+$ T cell function through dual mechanisms: IL-10 secretion and cell-contact-dependent inhibition mediated by CD80/CD86-CTLA-4 interaction, as evidenced by diminished IFN-γ, granzyme B production, and proliferation (48). Herpes virus entry mediator (HVEM, also TNFRSF14), a member of the TNF receptor superfamily expressed by both immune and non-immune cells that is frequently upregulated in malignancies, engages B and T lymphocyte attenuator (BTLA) on T cells to trigger co-inhibitory signaling, thereby suppressing TCR-mediated activation and impairing cytotoxic effector function (49, 50). Intriguingly, $CD8^+$ T cells themselves may acquire suppression function. For example, a subset of $CD8^+$ T cells, identified in humans as $CD8^+HLA\text{-}DR^+$ T cells, can adopt regulatory functions, further constraining effector responses (51). LRIG1, expressed on $CD8^+$ T cells, interact with VISTA in cis or trans to suppresses anti-tumor immunity by inducing quiescence in $CD8^+$ T cells and limiting the development of effector T cells from progenitor and memory-like cells (40). In summary, the effectiveness of $CD8^+$ T cells in controlling tumors are significantly limited by an inhibitory interaction established immunosuppressive network in the TME.

2.3 CAFs-to-$CD8^+$ T cell interactions

CAFs suppress $CD8^+$ T cell function through both checkpoint signaling and structural modulation of the TME. CAFs frequently express PD-L1 (52), reciprocally upregulated through crosstalk with tumor cells via contact or soluble factors, which directly binds PD-1 on $CD8^+$ T cells and correlates with poor prognosis in cancers like esophageal carcinoma. Like tumor cells and FDCs, CAFs also express CD155 and CD112, engaging TIGIT on TILs. $TIGIT^+$ $PD\text{-}1^+$ T cells exhibit reduced IFN-γ, TNF-α, and IL-2 production and impaired cytotoxicity, marking dysfunctional $CD8^+$ T effector memory cells (T_{EM}) cells. Dual blockade of TIGIT and PD-1 reverses this exhaustion, restoring antitumor responses (46). In hepatic tissues, LSECtin on hepatic CAFs engages LAG-3 on $CD8^+$ T cells via the KIEELE motif, recruiting inhibitory signals through CD3 to suppress proliferation, IFN-γ, and calcium flux, dampening antitumor immunity (27). Moreover, CAFs express other immunosuppressive ligands: Ceacam-1 binds TIM-3 on $CD8^+$ T cells, reinforcing exhaustion (27, 53). Beyond checkpoint ligands, CAFs remodel the extracellular matrix, restrict $CD8^+$ T cells infiltration, and secret cytokines and exosomes that further impair function.

Through these diverse roles, CAFs act as key regulators of immune exclusion and resistance to immunotherapy. Targeting CAFs-$CD8^+$ T cells interactions represents a promising strategy for successful cancer immunotherapies combination with checkpoint blockade.

2.4 Therapeutic strategies targeting direct cell-cell interactions

Immune checkpoints such as PD-1 and CTLA-4 are critical regulators of immune tolerance, preventing excessive immune activation. Tumors exploit this mechanism through ligand overexpression (e.g., PD-L1) to suppress T-cell function and facilitate immune escape. ICB therapies targeting PD-1-PD-L1, CTLA-4, and LAG-3 have significantly improved survival in multiple cancers (54, 55). However, complete response rates remain limited (56), largely due to tumor heterogeneity and the complexity of the immunosuppressive in TME, underscoring the need for stratified and context-specific immunotherapy approaches (15).

The functional state of $CD8^+$ T cells, which serve as the core effector cells in antitumor immunity, is not shaped by a single signal

but instead by integrated crosstalk with diverse cell populations in the TME (57, 58). Accordingly, immunotherapy strategies are shifting from a T cell-centric focus toward approaches that modulate the cellular interactions within the TME to promote effective antitumor immunity. The central therapeutic goal is to enhance T cell recognition and effector function while simultaneously blocking tumor immune evasion pathways.

Immune checkpoint inhibitors (ICIs) represent the most direct strategy (49). Anti–PD-1-PD-L1 antibodies restore effector functions of $CD8^+$ T cells (such as cytokine secretion and cytotoxicity) by disrupting PD-L1-PD-1 inhibitory axis (49). Beyond classical ICIs, novel checkpoints such as TIGIT have been identified (59, 60). While anti-TIGIT monotherapy or combination therapy with anti–PD-1 has shown potential in some clinical trials, these approached remain insufficient to fully reinvigorate $CD8^+$ T cells, particularly in patients with advanced or high tumor burden (61, 62). To enhance TIGIT-targeted immunotherapy, combination regimens are being developed, including anti–CTLA-4 or anti-vascular endothelial growth factor (VEGF) agents in triple blockade (e.g., TIGIT + PD-1-PD-L1 + CTLA-4 or + VEGF), or combinations with chemotherapy (59). In addition, multiple bispecific and trispecific antibodies have also entered clinical development, showing preliminary potential in overcoming resistance.

Beyond checkpoint inhibition, targeting interactions between $CD8^+$ T cells and other immune cells offers additional therapeutic avenues (57). For example, anti-CTLA-4 antibodies (e.g., ipilimumab) function in part by depleting intertumoral Tregs, thereby relieving suppression on $CD8^+$ T cells (63). Additionally, combination therapy with doxorubicin and IL-12 has been shown to shift receptor signaling in tumor infiltrating $CD8^+$ T cells toward immunostimulatory pathways while reducing Treg infiltration, thus enhancing local effector activity (64).

CAFs present another major challenge to restrict $CD8^+$ T cell infiltration and function by constructing both physical and biochemical barriers (65). Overcoming CAF-mediated immunosuppression is thus critical for restoring $CD8^+$ T cell-mediated antitumor activity (66). In triple-negative breast cancer (TNBC), CAFs are particularly important therapeutic targets. Huo et al. engineered a CAF-targeted nanosystem co-loaded with a TGF-β inhibitor (LY3200882) and PD-L1 siRNA. Upon matrix metalloproteinase-2 (MMP2)-responsive release, LY3200882 preferentially modulates CAF activity, reducing extracellular matrix deposition and enhancing T-cell infiltration. Simultaneously, PD-L1 siRNA downregulates PD-L1 expression in both tumor cells and CAFs. This dual-action strategy effectively reverses CAF-driven immunosuppression, remodels the TME, and suppresses TNBC progression (67).

3 Indirect suppression via TME

The TME exerts profound indirect suppression on $CD8^+$ T cell responses, orchestrating a complex network of metabolic, biochemical and structural barriers that shape anti-tumor immunity. Mounting evidence indicates that tumors co-opt multifaceted pathways, including metabolic reprogramming, cytokine induction, receptor modulation, and immune checkpoint activation, to systemically impair $CD8^+$ T cell effector function, thereby fostering tumor progression. These immunosuppressive circuits are increasingly recognized as critical drivers of tumor immune evasion, positioning them as attractive therapeutic targets for restoring anti-tumor immunity. This section focuses on indirect TME-driven suppression, delineating how tumor cells and stromal elements orchestrate $CD8^+$ T cell suppression through metabolic competition (e.g., nutrient deprivation), intercellular communication (eg. exosomes, tunneling nanotubes, or trogocytosis), and microenvironmental perturbations (eg. cytokine networks, ionic imbalances, or ammonia accumulation) (Table 1). Collectively, these mechanisms establish an immunosuppressive niche that subverts $CD8^+$ T cell surveillance and therapeutic efficacy.

3.1 Tumor-$CD8^+$ T cell nutrient competition

The availability of nutrients within the TME has emerged as a pivotal determinant of $CD8^+$ T cell function. Compelling evidence indicates that enhanced nutrient uptake, glycolytic flux, and oxidative metabolism collectively potentiate $CD8^+$ T cell proliferation and effector differentiation within tumors. This metabolic adaptation is essential for sustaining anti-tumor responses. Nevertheless, the TME frequently imposes profound metabolic constraints, including nutrient deprivation and lipid accumulation, that directly impair $CD8^+$ T cell effector responses and immune surveillance. Strategies to overcome these barriers show therapeutic promise.

3.1.1 Glucose

Glucose metabolism plays a pivotal role in the TME, impacting both tumor progression and the functional capabilities of TILs (Figure 3). Tumor cells exploit the Warburg effect, consuming glucose and releasing lactate, which drives extracellular acidosis, hypoxia, disordered vasculature, and dense extracellular matrix within the TME (69, 73, 79, 114, 115). This nutrient competition restricts glucose availability to TILs, resulting in mitochondrial dysfunction and altered lipid metabolism, ultimately hindering T cell effector function and persistence. To sustain growth, tumor cells upregulate glucose transporters such as GLUT1 and GLUT3, and avidly consuming glucose and glutamine to promote T cell exhaustion and immune evasion (73, 74). In renal cell carcinoma, elevated tumor glycolysis corelates with reduced effector $CD8^+$ T cells (75). Nutrient deprivation triggers AMP-activated protein kinase (AMPK) activation, while suppressing mTOR thereby disrupting T cell differentiation (116). Moreover, dysregulation of glucose metabolism through pathways such as PI3K/AKT/mTOR signaling further impacts T cell activation, Ca^{2+} signaling, and O-GlcNAcylation, all of which are essential for T cell effector function (76, 77, 117).

TABLE 1 Indirect regulation of CD8$^+$ T cell dysfunction and exhaustion by the TME.

Classification of indirect suppression		Mechanisms and conclusions
Tumor-CD8$^+$ T cell nutrient competition	Glucose	In the tumor microenvironment, cancer cells or myeloid cells (68) outcompete CD8$^+$ T cells for glucose via the Warburg effect (69), leading to lactate accumulation (70, 71), acidosis, and metabolic stress (72), by upregulate glucose transporters GLUT1 and GLUT3 (73, 74), or elevated glucose metabolism (75). This nutrient deprivation impairs T cell mitochondrial function, mTOR signaling (76, 77), and effector responses, while promoting exhaustion markers (PD-1, LAG-3) and epigenetic dysfunction (72, 78–80). Targeting this metabolic competition may enhance immunotherapy efficacy (81).
	Lipids	Very-long-chain acyl-CoA dehydrogenase (VLCAD) (82), long-chain fatty acids (LCFAs) (83), arachidonic acid (82), lipid droplet (84), prostaglandin E2 (85), or PCSK9–63 impair CD8$^+$ T cell activity.
	Amino Acid	Depletion of arginine (86–88), alanine (89), glutamine (89), tryptophan (90), or accumulation of adenosine (91, 92), L-ornithine (90) suppress T cell activation, proliferation, and cytokine production.
Exosomes		Exosomes inhibit the function of CD8$^+$ T cells and enhance their apoptosis by delivering immunosuppressive molecules (e.g., cytokines (93, 94), regulatory miRNAs (93, 95, 96), and metabolic modulators (97)) or transmitting signals via direct contact (93, 94).
Nanotubes and Trogocytosis		Transfer of mitochondria (98), nutrients depletion (99) or "self-inhibition" (100) through acquisition of inhibitory ligands or "antigen loss" (101, 102), collectively rewire T cell metabolism and blunt antigen recognition, thereby hindering CD8$^+$ T cell function.
Cytokines		Inhibitory cytokines predominantly impair CD8$^+$ T cell proliferation and effector function, including TGF-β (103), IL-2 (104), IL-6 (105), IL-18R (106), IL-27 (107), or IL-10 (108, 109) and IL-35 (110).
Ions and Metabolites		Dysregulated Mg^{2+} (111), Lithium (112) and ammonia (113) levels interfere with T cell function and mechanisms.

Emerging evidence challenges the notion that immune dysfunction arises solely from tumor-driven nutrient deprivation. Reinfeld et al. demonstrated that myeloid cells, rather than T cells or tumor cells, exhibit the highest glucose uptake, while tumor cells preferentially rely on glutamine metabolism (68). These distinct metabolic programs are governed by intrinsic cellular programming mechanisms including differential mTORC1 activity and metabolic gene expression, rather than extracellular nutrient competition (68). Moreover, inhibiting glutamine metabolism was further shown to enhance glucose uptake across multiple cell types, suggesting a feedback mechanism between glucose and glutamine utilization. These findings emphasizes that immune metabolic dysfunction in the TME is shaped not only by nutrient deprivation but also by cell type-specific cellular metabolic programming, providing novel directions for metabolism-based therapeutic strategies.

Beyond nutrient depletion, additional metabolic barriers, including lactate accumulation, acidic pH, hypoxia, and elevated ROS, further contribute to T cell dysfunction by reprogramming metabolism and upregulating immune checkpoint expression (72). Notably, PD-L1 blockade has been shown to enhance T cell infiltration and metabolic fitness in glycolysis-low tumors (78). Conversely, inhibition of lactate dehydrogenase (LDHA) impairs CD8$^+$ T cell migration, proliferation, and effector functions (70), while blockade of OGR1 in melanoma restores CD8$^+$ T cell cytotoxic activity (71).

Together, glucose dysregulation in the TME not only hinders T cell effector functions but also increases the immune checkpoint expression and exhaustion, constituting a key mechanism of tumor immune evasion. These insights underscore the therapeutic potential of reprograming glucose metabolism by enhancing T cell glycolytic capacity, restraining tumor glycolysis, or targeting glutamine-glucose metabolic crosstalk, to overcome metabolic barriers and enhance immunotherapeutic efficacy (81).

3.1.2 Lipids

The interplay between lipids and CD8$^+$ T cell dysfunction within the TME has attracted growing interest, revealing complex mechanisms by which lipid accumulation and metabolism shape anti-tumor immunity. Lipid metabolism dichotomizes into opposing immunomodulatory pathways within the TME: one suppresses CD8$^+$ T cell effector function (118–120), while the other sustains or enhances CD8$^+$ T cell activation (121). This section highlights the specific immunosuppressive lipids present in the TME and delineate the mechanisms by which they impair CD8$^+$ T cell activity (Figure 3). For example, intrapancreatic CD8$^+$ T cells exhibit downregulation of very-long-chain acyl-CoA dehydrogenase (VLCAD), exacerbating the accumulation of lipotoxic long-chain fatty acids (LCFAs) and VLCFAs (82). Metabolic reprogramming through enforced VLCAD expression enhanced intratumorally T cell survival and persistence in a pancreatic ductal adenocarcinoma (PDA) mouse model, overcoming a major immunotherapy hurdle (82). LCFAs such as palmitate impede CD8$^+$ T cell proliferation and effector cytokine production (83). Among unsaturated fatty acids, oleic acid and linoleic acid exert divergent effects on tumor progression: linoleic acid reprograms tumor-infiltrating CD8$^+$ T cells from an exhausted phenotype towards a memory-like state, potentiating their effector function (122). Arachidonic acid induces ferroptosis in tumor cells but may concurrently trigger ferroptosis in tumor-infiltrating CD8$^+$ T cells (82). The TME induces lipid droplet accumulation in dysfunctional CD8$^+$ TILs through acetyl-CoA carboxylase-mediated metabolic reprogramming (84). Prostaglandin E2 impairs IL-2 sensing in human CD8$^+$ T cells, promoting oxidative stress and ferroptosis (85). Cholesterol and its derivatives critically modulate CD8$^+$ T cell function in context-dependent manner: cholesterol enhances TCR signaling, yet tumor cells derived PCSK9 dysregulates CD8$^+$ T cell cholesterol metabolism, thereby

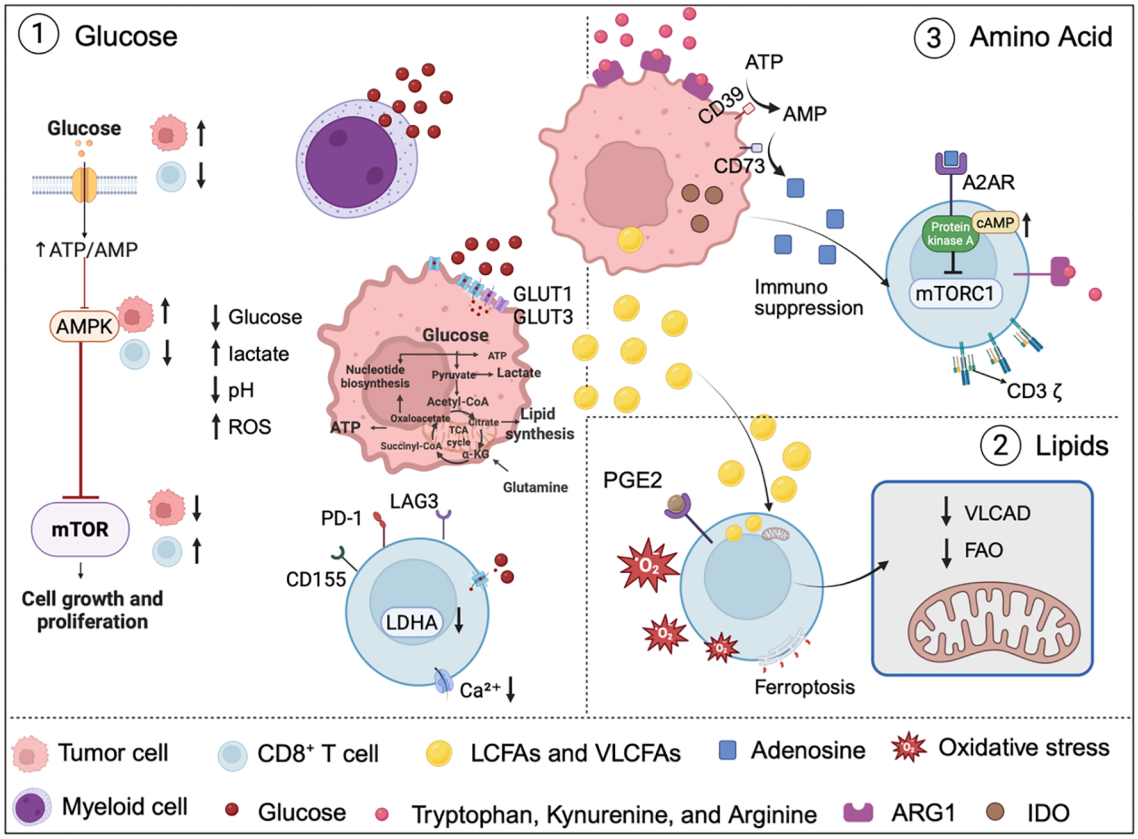

FIGURE 3
Metabolic Reprogramming in the TME Driving CD8$^+$ T Cell Dysfunction. Glucose, lipid, and amino acid metabolism in the TME collectively impair CD8$^+$ T cell function through nutrient competition, metabolite accumulation, and inhibitory signaling (1). Tumor cells and myeloid cells mediated glucose uptake and lactate accumulation suppress glycoses and mTOR activity in T cells. (2) Accumulation of long-chain fatty acids (LCFAs) and lipid abnormalities within T cells causes lipotoxicity and mitochondrial dysfunction. (3) Amino acid depletion by enzymes such as arginase 1 (ARG1) and IDO disrupts TCR signaling and generates immunosuppressive metabolites such as adenosine. These metabolic pathways collectively drive T cell dysfunction and represent potential therapeutic targets. Image created with bioRender.com, with permission. Created in BioRender. Zhou, P. (2025) https://BioRender.com/8h0fjul.

suppressing TCR signaling (123), while the oxysterol 27-hydroxycholesterol facilitates metastasis, an effect potently suppressed by CYP27A1 inhibition (124). Notably, in pancreatic tumors, CD8$^+$ T cell accumulation of LCFAs impairs mitochondrial function and fatty acid catabolism, recapitulating the proliferative and cytokine defects observed upon *in vitro* palmitate treatment (82). Rather than serving as an energy source, these accumulated lipids impair mitochondrial function and induce transcriptional reprogramming of lipid metabolism pathways, ultimately hampering CD8$^+$ T cell metabolic fitness and anti-tumor activity (82).

3.1.3 Amino acid

The TME orchestrates a complex metabolic interplay where amino acid availability profoundly impacts the functionality of CD8$^+$ T cells through diverse mechanisms (Figure 3). Amino acids serve as critical substrates for various cellular processes such as protein synthesis, epigenetic modifications (e.g., SAM-dependent methylation), and energy metabolism, making them highly contested resources between tumor cells and T cells. For example, in activated T cells, extracellular alanine is preferentially utilized for protein synthesis rather than catabolism. Arginine catabolism by arginase 1 (ARG1) and inducible nitric oxide synthase (iNOS) impairs TCR function by downregulating the CD3ξ chain expression (86). Moreover, ARG1-containing extracellular vesicles can traffic to draining lymph nodes, where their uptake by dendritic cells suppresses antigen-specific T-cell proliferation, as demonstrated in ovarian carcinoma models (87, 88). Adenosine further compromises T cell function and metabolic fitness through the A2AR/PKA/mTORC1 pathway, dampening both peripheral and tumor-infiltrating CD8$^+$ T cells (91, 92). Alanine deprivation delays the activation of naive and memory T cells (125), although it has limited effects on T cell effector function. In contrast, glutamine deprivation restricts metabolic flexibility, while SLC7A11, a multi-pass transmembrane protein, driven cysteine depletion promotes oxidative stress (89). L-ornithine has been shown to suppress T cell functionality, as observed in murine models of chronic viral infection where altered expression of hepatic urea cycle enzymes results in L-ornithine accumulation, leading to the inhibition of virus-specific CD8$^+$ T cell responses (126). Similarly, tryptophan

depletion triggers GCN2-mediated stress responses that suppress mTOR signaling, further restricting T cell activity (90).

Collectively, these metabolic perturbations disrupt T cell activation, proliferation, and the production of effector molecules, thereby contributing to immunotherapy resistance. Targeting this metabolic axis offers novel therapeutic strategies, such as inhibiting ARG1 or GLS in combination with immune checkpoint blockade, may restore amino acid homeostasis and reinvigorate antitumor immunity. Such strategies highlight a promising frontier that integrates metabolic and immunological intervention to overcome treatment resistance.

3.2 Exosomes

In various cancers, exosomes derived from tumor cells or stromal cells carry molecular cargo that induces dysfunction or exhaustion of $CD8^+$ T cells, thereby facilitating tumor progression and resistance to immunotherapy. Exosomes suppress $CD8^+$ T cell function and promote their apoptosis through two primary mechanisms: (1) delivery of immunosuppressive molecules and (2) ligand-receptor interactions that trigger contact-dependent signaling.

In the first route, exosomes transport inhibitory factors including cytokines [e.g., TGF-β (93), IL-8 (94)], regulatory miRNAs [e.g., microRNAs (93, 95) and circRNA (96)], and metabolic modulators (97) (e.g., lactate dehydrogenase LDHA and lactate). These cargos collectively impair T cell activation, disrupt inflammatory signaling pathways (eg. STAT1-IFN-γ) and compromise glycolytic metabolism. In the second route, exosome surface ligands, including PD-L1 (127) and FasL, engage corresponding receptors on $CD8^+$ T cells, driving exhaustion or apoptosis. Together, these coordinated immunosuppressive actions establish exosomes as critical mediators of T cell dysfunction in cancer, while also presenting potential therapeutic targets for enhancing immunotherapies. Recent studies demonstrate the breadth of this regulation. For example, Fan Xu et al. showed that IL-8 in exosomes derived from prostate cancer cells hyperactivates peroxisome proliferators-activated receptors (PPARα) in recipient $CD8^+$ T cells, which downregulates GLUT1 and hexokinase 2 to reduce glucose utilization while upregulating Carnitine O-palmitoyltransferase 1 and peroxisomal acyl-coenzyme A oxidase 1 to enhance fatty acid catabolism, ultimately exacerbating $CD8^+$ T cell starvation and promoting cellular exhaustion (94). Non-small cell lung cancer (NSCLC) cells release circUSP7 via exosome secretion, which upregulates SHP2 expression by sponging miR-934, thereby inhibiting $CD8^+$ T cell secretion of IFN-γ, TNF-α, granzyme B, and perforin and ultimately suppressing $CD8^+$ T cell function (128). Another example is the exosome circCCAR1, which is taken up by $CD8^+$ T cells and induces $CD8^+$ T cell dysfunction by stabilizing PD-1 protein (96). Collectively, these studies delineate a complex network whereby tumor and stromal cell-derived exosomes carry diverse molecular cargos, including circRNAs, cytokines and proteins, that induce $CD8^+$ T cell dysfunction, in addition offering novel opportunities for therapeutic targets.

3.3 Nanotubes and trogocytosis

The contribution of nanotubes and trogocytosis in regulating $CD8^+$ T cell function within the TME has become an emerging area, particularly regarding intercellular mitochondrial transfer and its consequences on T cell efficacy. Mitochondrial dysfunction in $CD8^+$ T cells represents a fundamental driver of T cell exhaustion in tumor contexts, making these intercellular communication mechanisms highly relevant to tumor immune evasion.

Current evidence reveals that nanotube-mediated mitochondrial transfer exhibits dual functionality. On one hand, nanotubes can restore T cell metabolic activity by delivering functional mitochondria; on the other hand, tumor cells often exploit this progress to transfer dysfunctional mitochondria containing mutations or oxidative damage, thereby promoting T cell failure. The principal inhibitory mechanisms of nanotubes toward $CD8^+$ T cells encompass metabolic subversion through mitochondrial hijacking (98) and nutrient deprivation (99). Using multimodal imaging and metabolic profiling, Tanmoy Saha et al. demonstrated that cancer cells hijack mitochondria from immune cells via tunneling nanotubes, simultaneously depleting immune cell function while metabolically empowering tumor cells (129). In contrast, Jeremy G. Baldwin et al. showed that bone marrow stromal cells transfer healthy mitochondria to $CD8^+$ T cells through intercellular nanotubes, thereby restoring $CD8^+$ T cell function and promoting anti-tumor responses (98). Together, these findings highlight the complex, context-dependent role of nanotubes in immune regulation and underscore their potential as therapeutic targets in cancer immunotherapy.

Trogocytosis, the direct transfer of membrane fragments and regulatory molecules during cell-cell contact, also play a crucial role on T cell function. In the TME, $CD8^+$ T cells that acquire inhibitory molecules from APCs or tumor cells can undergo suppression of cytokine production and proliferation through reverse signaling (45). Mechanistically, trogocytosis in $CD8^+$ T cells, where they acquire inhibitory ligands or pMHC complexes, can promote immune evasion, leading to T cell exhaustion mainly through “self-inhibition” (100) and “antigen loss” (101). For example, Lu et al. demonstrated that activation of trogocytosis in intratumoral CTL through the ATF3-CH25H axis dampened the anti-tumor immune response (100). Notably, $CD8^+$ T cells engage in cell-to-cell material exchange by obtaining pMHC from APCs or tumor cells in a TCR-dependent manner, may themselves become targets for killing by neighboring $CD8^+$ T cells (101, 102). While trogocytosis may prolong antigen receptor engagement and transiently enhance activation, sustained or excessive trogocytosis promote exhaustion (130). From a translational perspective, engineering CAR-T cells to resistant trogocytosis or to avoid the acquisition of inhibitory signals could improve their persistence and therapeutic efficacy in tumors (100, 131).

3.4 Cytokines

Multiple studies have elucidated the pivotal roles of cytokine signaling and the inhibitory receptor upregulation in driving $CD8^+$ T cell dysfunction within the TME. Cytokines impair $CD8^+$ T cell proliferation, cytotoxicity (e.g., granzyme B and perforin expression), and effector functions by inducing exhaustion, metabolic inhibition, and apoptosis. For example, TGF-β and IL-2 suppress $CD8^+$ T cell proliferation and cytotoxic activity (103, 104). Mechanistically, TGF-β reduces CXCR3 expression by binding to the CXCR3 promoter through Smad2, thereby diminishing $CD8^+$ T cell responsiveness to CXCL10. Ablation of the TGF-β receptor I (ALK5) restores CXCR3 expression, enhances T cell infiltration and cytotoxicity, and promotes tumor regression, these effects are partially reversed by CXCR3 blockade. Furthermore, chronic TGF-β1 signaling orchestrates terminal dysfunction of $CD8^+$ T cells through stable epigenetic reprogramming (17). Rebalancing TGF-β1-BMP signaling, for instance with BMP4 agonist SB4, preserves effector-memory programs, reduces exhaustion marker expression, enhances anti-tumor responses, and synergizes with ICB by restoring T cells responsive state.

IL-2 plays pivotal roles in regulating $CD8^+$ T cell proliferation, effector function, exhaustion, memory formation, and metabolic adaptability (132). Recent findings underscore the context-dependent effects of IL-2: while elevated IL-2 transiently enhance the proliferation and effector functions of $CD25^{hi}$ $CD8^+$ T cells, they also accelerate exhaustion (133–135). In chronic stimulatory settings such as tumor microenvironments, sustained IL-2 signaling drives $CD8^+$ T cell exhaustion through STAT5-mediated tryptophan hydroxylase 1 upregulation, generating 5-hydroxytryptophan that promotes inhibitory receptor expression and suppress effector function, revealing a conserved metabolic-epigenetic axis of T cell dysfunction in both mouse and human systems (104). Clinically, high-dose interleukin-2 (HD IL-2) has been employed for the treatment of advanced melanoma and renal cell carcinoma (136, 137), whereas low-dose recombinant human IL-2 selectively modulates the abundance of regulatory T (T_{reg}) cells, follicular helper T (T_{FH}) cells and IL-17-producing helper T (TH_{17}) cells (138). Through these effects, IL-2 promotes the development and survival of T_{reg} cells while inhibiting the differentiation of T_{FH} and TH_{17} subsets, thereby reshaping the immune milieu. Currently, multiple IL-2-based products are under clinical and pre-clinical investigation, requiring evaluation of their effects to reprogram dysfunctional state of anti-tumor $CD8^+$ T cells. Modulation of $CD8^+$ T cell exhaustion programs by IL-2 to promote the generation of effector cells with stem-like properties provides the immunological rationale for the combination therapy of IL-2 with PD-1 blockade (136, 139). Furthermore, engineered IL-2 partial agonists have been shown to preserve the stem-like properties and mitochondrial fitness of $CD8^+$ T cells, thereby enhancing anti-tumor immunity (140). In parallel, IL-6-STAT3 signaling, activated by STK31, also promotes $CD8^+$ T cell exhaustion in tumors (105), while IL-18 released in the TME through inflammasome activation drives T-cell exhaustion via IL2-STAT5 and AKT-mTOR signaling downstream of IL-18R (106).

Cytokine pathways also intersect with inhibitory receptor regulation. IL-27 upregulates PD-1 expression via STAT1 signaling yet paradoxically sustains $CD8^+$ T cell activity and synergizes with PD-1- PD-L1 blockade (107). Although IL-10 is classically categorized as immunosuppressive through its ability to induce inhibition, recent work suggests that IL-10 alleviates T cell exhaustion by promoting oxidative phosphorylation (OXPHOS) in $PD\text{-}1^+$ $TIM\text{-}3^+$ $CD8^+$ T cells. An IL-10-Fc fusion protein acts through IL-10 receptors on T cells to specifically enhance OXPHOS, proliferation and cytotoxicity in this subset, thereby reversing exhaustion and enhancing anti-tumor response (108, 109). Conversely, Treg-derived IL-10 and IL-35 cooperatively upregulate the expression of multiple inhibitory receptors and drive BLIMP1-dependent exhaustion of tumor infiltration $CD8^+$ T cells, further impeding antitumor immunity (110).

Collectively, these findings underscore the central role of cytokine-mediated signaling networks and inhibitory receptor upregulation in orchestrating $CD8^+$ T cell dysfunction within TME, emphasizing the therapeutic potential of targeting these pathways to reinvigorate anti-tumor immunity.

3.5 Ions and metabolites ($Mg2^+$, Lithium and Ammonia)

The immune function of $CD8^+$ T cells is profoundly affected by various ions and metabolites that modulate signaling and metabolic fitness. Magnesium (Mg^{2+}) functions as a critical second messenger that regulates $CD8^+$ T cell activity through metabolic circuits that sustain effector functions. Deficiency of intracellular free Mg^{2+} impairs NKG2D receptor expression on both NK cells and $CD8^+$ T cells, thereby compromising cytotoxic responses against pathogens such as Epstein-Barr virus (111). Lithium, widely used in psychiatric treatment, also exerts immunomodulatory effects on $CD8^+$ T cells. Mechanistically, cytoplasmic lactate promotes lysosomal proton influx, meanwhile lithium prevents lysosomal acidification by inhibiting vacuolar ATPase, thereby restoring diacylglycerol-PKCθ signaling to recruit monocarboxylate transporter 1 to mitochondria. This enabled lactic acid transport into mitochondria for $CD8^+$ T-cell energy production (112). Ammonia functions as a potent immunosuppressive metabolite within the TME. Elevated ammonia levels reprogram T cell metabolism, leading to exhaustion and proliferation arrest (113). Mechanistically, ammonia accumulation increases lysosomal pH, impairs lysosomal ammonia trapping capacity. This causes ammonia reflux into mitochondria, triggering mitochondrial damage and subsequent cell death (141). Collectively, these findings highlight distinct roles for ions and metabolites in shaping $CD8^+$ T anti-tumor immunity.

Indirect suppression in the TME operates through tightly interconnected metabolic, vesicular, structural, and cytokine

mediated pathways. These circuits converge $CD8^+$ T cells to impair metabolism, signaling, and effector function, driving exhaustion and immune escape. Understanding and therapeutically targeting these mechanisms will be essential for restoring durable anti-tumor immunity.

3.6 Integrative strategies to restore T cell function

The progress of immunotherapy has been driven by advances in immune checkpoint research, leading to the clinical approval of adoptive T cell therapy (142, 143). However, CAR-T cell therapies show limited efficacy in many solid tumors and are often linked to immune-related adverse events (144–146). Studies have shown that impaired mitochondrial quality in TILs reduces cytokine secretion and increases the expression of co-inhibitory receptors, while tertiary lymphoid structures in several cancers characterized by chronic inflammatory signaling (147). Moreover, the TME frequently lacks the pro-inflammatory cues or innate immune activation required for optimal T cell priming and expansion, thereby constraining therapeutic efficacy.

To overcome these barriers, emerging strategies aim to synergize innate immune activation with pro-inflammatory stimuli, extending therapeutic benefit beyond checkpoint inhibition, including nutritional interventions (148), oncolytic viruses (149), cGAS-STING agonists (150, 151), cytokine therapy (152), mitochondrial function modulation (153), and vaccine development (149, 154). Addressing metabolic dysregulation, such as lactic acid accumulation in TME (155–157), is particularly critical for maintaining T-cell stemness, emphasizing the importance of mitochondrial fitness in adoptive transfer approaches. Although IL-2 monotherapy showed early promise in metastatic renal cell carcinoma (RCC) and melanoma, its clinical utility was limited by toxicity and Treg activation, prompting a shift toward combination regimens (158, 159). Similarly, pharmacological activation of K^+ channels, such as with riluzole, a non-specific activator of the KCa3.1 channel, enhances cisplatin uptake in colorectal cancer patients with cisplatin resistance (160).

Improving the efficacy of ICIs requires addressing secondary inhibitory barriers in the TME, including immune-suppressive metabolite accumulation (113), nutrient competition (68), ion imbalances (e.g., high potassium environment), hypoxia, and acidosis-related metabolic hindrances (161, 162). Overcoming these multifactorial constraints is essential for fully unleashing the cytotoxic potential of T cells. Preclinical studies demonstrate that multi-targeted approaches can enhance antitumor efficacy, such as M7824, a bifunctional fusion protein simultaneously targeting PD-L1 and TGF-β (163). In addition, innovative platforms such as nanotube- and exosome-based drug delivery systems (164) and CRISPR-Cas9-based genetic engineering (165) are expanding therapeutic possibilities in personalized gene therapy.

4 Discussion

Effective antitumor immunity critically depends on functional $CD8^+$ T cells, whose suppression within the TME constitutes a major immune escape mechanism. This suppression occurs through two major routes: (1) Direct cell-to-cell interactions, including tumor cell-$CD8^+$ T cell contact (e.g., PD-L1-PD-1), inhibitory signals from CAFs, and immune cell crosstalk (e.g., DC-macrophage-T cell interactions); and (2) Indirect TME-driven mechanisms, such as metabolic competition (nutrient deprivation), intercellular communication (exosomes, tunneling nanotubes, T cell trogocytosis), and microenvironmental perturbations involving immunosuppressive cytokine networks (TGF-β), ionic imbalances (e.g., Mg^{2+} deficiency), and metabolite accumulation (e.g., ammonia).

Within this suppressive networks, $CD8^+$ T cell function is progressively impaired by diverse suppressive cues. Recent studies highlight that tumors directly suppress $CD8^+$ T cells via inhibitory ligand-receptor interactions, most prominently through the PD-1-PD-L1 axis and the CTLA-4-B7-1 (CD80)-B7-2 (CD86) pathway (56, 86, 166, 167). Additionally, APCs and CAFs suppress $CD8^+$ T cell function by engaging CTLA-4 on activated $CD8^+$ T cells, thereby constraining the availability of co-stimulatory signals. ICB therapies targeting PD-1-PD-L1, CTLA-4, and LAG-3 have improved survival in multiple cancers (54, 55). However, complete and durable responses remain limited, largely due to tumor heterogeneity, compensatory pathways, and the multifaceted suppressive networks in the TME (56, 168, 169). These limitations underscore the need for complementary or combinatorial strategies that extend beyond classical checkpoint inhibition. A2AR antagonists counteract adenosine-mediated immunosuppression in the TME, thereby restoring T cell-mediated tumor killing (170, 171). Currently, several A2AR antagonists (e.g., AZD4635, CPI-444, AB928) have advanced into Phase II clinical development for indications including prostate cancer and NSCLC (172). Notably, although these candidates vary in developmental stage and tumor type, they demonstrate synergistic effects when combined with PD-1/PD-L1 inhibitors, exhibiting superior antitumor activity compared to either agent alone (171). These findings highlight the potential of targeting metabolic pathways and nutrient competition presents promising avenue to enhance effector responses (169, 173, 174).

Despite these advances, our understanding of how direct and indirect communication networks suppress $CD8^+$ T cells in TME remain incomplete. A key challenge lies in decoding these interactions at sufficient resolution, cutting-edge platforms such as spatial resolved transcriptomics, single-cell CRISPR screening (175), nanotherapeutics (176, 177) are now being leveraged to dissect TME-T cell interaction at cellular and molecular levels. Likewise, clinical strategies like CAR-T cell therapy (178, 179) and bispecific antibodies (180) provided translational opportunities for targeting these networks. In particularly, extracellular vesicle-mediated signaling

(e.g., exosomes and tunneling nanotubes) represents an underexplored mechanism of tumor-driven immune evasion and a potential strategy of novel therapeutic targets.

Importantly, the functional state of $CD8^+$ T cells is tightly dictated by their local microenvironment niche, which is defined by spatial position and communicative interactions with neighboring cells. Ligand-receptor pairs are emerging as critical determinants of these intercellular communication (181, 182). Advances in single-cell and spatial multi-omics allow the dissection of these networks at both cellular and molecularly levels (183). In the parallel, advanced computational framworks enable the systematic analysis of immune infiltration, inference of cell phenotypes, spatial mapping of cellular interactions, and discovery of novel cell-cell communication events, with tools such as CellTalker, PyMINEr, CCCExplorer, SoptSC, NicheNet, CellPhoneDB, CellChat, and CSOmap (184–186).

Another major clinical challenge is the early prediction of immunotherapy efficacy (187). Platforms such as the gel-liquid interface co-culture model have recapitulated human immunity and tumor microenvironment interactions and identified circulating tumor-reactive T cells as biomarkers of treatment response in lung cancers (188). Integration of such ex vivo systems with omics and computational pipelines may accelerate biomarkers discovery.

Therapeutic strategies is increasingly focused on multi-target synergistic interventions (54). Dual-blockade strategies, such as combined PD-1-PD-L1 and TIGIT blockade (189), and tri-blockade regimes, integrating epigenetic modulators (e.g., HDAC inhibitors) with anti-angiogenic agents and PD-1 antibodies, have shown promise in refractory solid tumors by simultaneously remodeling the TME and restoring T cell function (190). Beyond blockade, and emerging therapeutic approach aims to sustain long-term T-cell function by preventing over-activation. An Fc-attenuated LAG-3-TCR bispecific antibody has been engineered to suppress T cell activity independently of MHC-II, demonstrating therapeutic potential in autoimmune models and offering a new avenue for sustaining T-cell function in cancer immunotherapy (191).

Collectively, the intrinsic cellular composition of the TME, coupled with pervasive immune evasion and multifaceted crosstalk, highlights the need for integrative therapeutic strategies that simultaneously target direct inhibitory interactions, metabolic competition, and intercellular communication.

Author contributions

LC: Writing – original draft, Writing – review & editing. QH: Writing – original draft, Writing – review & editing. PZ: Writing – review & editing, Writing – original draft.

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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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Glossary

TME	Tumor microenvironment
CTLs	Cytotoxic T lymphocytes
DCs	Dendritic cells
IL-12	interleukin-12
IFN-γ	Interferon-gamma
PD-1	Programmed cell death protein 1
CTLA-4	Cytotoxic T-lymphocyte associated protein 4
Tmp	Memory precursor T cells
Tpex	Progenitor of exhausted T cells
TLS	Tertiary lymphoid structures
TCF1	T-cell factor 1
ICB	Immune checkpoint blockade
Trm	Tissue-resident memory T cells
TAMs	Tumor-associated macrophages
TGF-βR	Transforming growth factor β receptor
B7-H3	B7 homolog 3
HLA	Human leukocyte antigen
LAG-3	Lymphocyte-activation gene 3
NKG2A	Natural killer group 2 member A
Tregs	Regulatory T cells
MDSCs	Myeloid-derived suppressor cells
CAFs	Cancer-associated fibroblasts
Ceacam-1	Carcinoembryonic antigen-related cell adhesion molecule 1
ZAP70	Zeta-chain-associated protein kinase 70
LSECtin	Liver and lymph node sinusoidal endothelial cell C-type lectin
LUAD	Lung adenocarcinoma
PVRL2	Poliovirus receptor-related protein 2
ERAP1-2	Endoplasmic reticulum aminopeptidase 1-2
NKG2A	Natural killer cell group 2 member A
TILs	Tumor-infiltrating lymphocytes
APCs	Antigen-presenting cells
VISTA	V-domain immunoglobulin suppressor of T cell activation
LRIG1	Ligand for immunoglobulin-like domains 1
FDCs	Follicular dendritic cells
TNF-α	Tumor necrosis factor-alpha
NK	Natural Killer
CCR7	C-C motif chemokine receptor 7
HVEM	Herpes virus entry mediator
BTLA	B and T lymphocyte attenuator
ICIs	Immune checkpoint inhibitors
VEGF	Vascular endothelial growth factor
TNBC	Triple-negative breast cancer
MMP2	Matrix metalloproteinase-2
GLUT1	Glucose transporters glucose transporter 1
AMPK	AMP-activated protein kinase
LDHA	Lactate dehydrogenase
VLCAD	Very-long-chain acyl-CoA dehydrogenase
LCFAs	Long-chain fatty acids
PDA	Pancreatic ductal adenocarcinoma
ARG1	Arginine catabolism by arginase 1
PPARα	peroxisome proliferators-activated receptors
NSCLC	Non-small cell lung cancer
iNOS	Inducible nitric oxide synthase
STAT3	Signal Transducer and Activator of Transcription 3
Mg^{2+}	Magnesium
OXPHOS	Oxidative phosphorylation
scTCR-seq	Single-cell T cell receptor sequencing.

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