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Immune cell therapy approaches targeting tumor microenvironment

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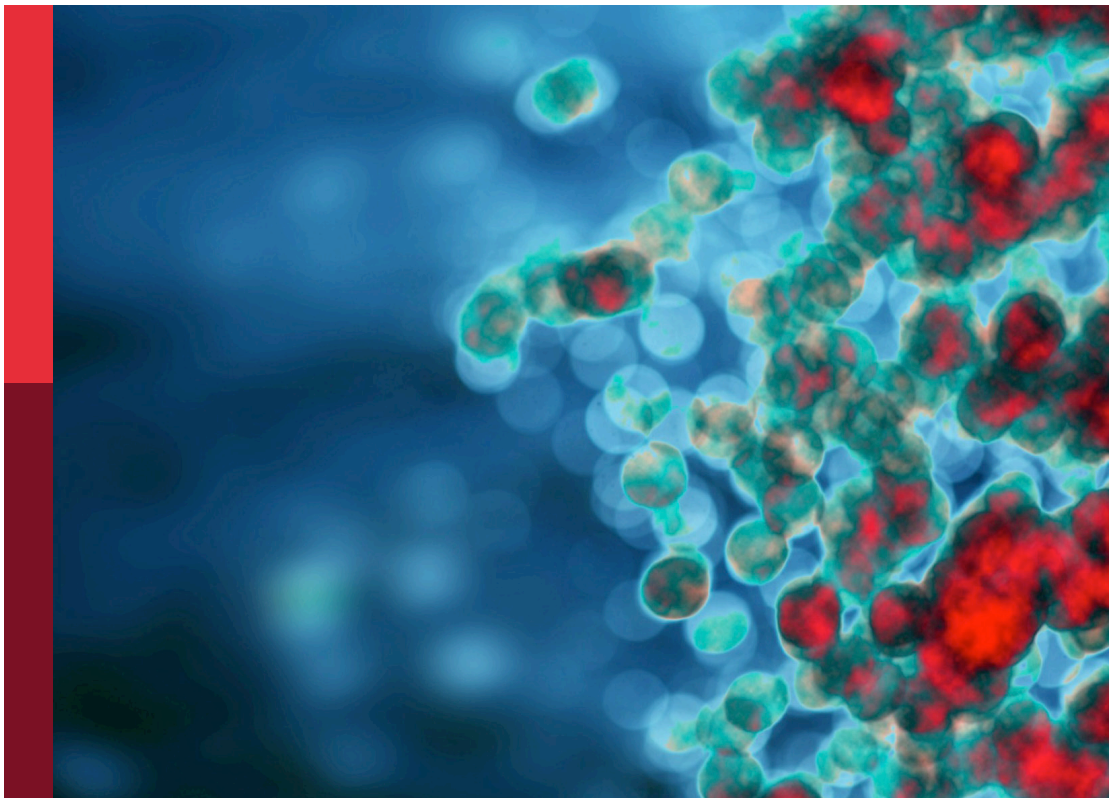
Published in
Frontiers in Immunology
Frontiers in Oncology



ISSN 1664-8714
ISBN 978-2-8325-6436-3
DOI 10.3389/978-2-8325-6436-3

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Immune cell therapy approaches targeting tumor microenvironment

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Citation

Thangaraj, J. L., Cari, L., Kalathil, S., Toubert, A., eds. (2025). *Immune cell therapy approaches targeting tumor microenvironment*. Lausanne: Frontiers Media SA. doi: 10.3389/978-2-8325-6436-3

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frontiers | Frontiers in Immunology

TYPE Editorial
PUBLISHED 28 May 2025
DOI 10.3389/fimmu.2025.1628689

OPEN ACCESS

EDITED AND REVIEWED BY
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RECEIVED 14 May 2025
ACCEPTED 15 May 2025
PUBLISHED 28 May 2025

CITATION
Patnaik SK and Kalathil SG (2025) Editorial: Immune cell therapy approaches targeting tumor microenvironment.
Front. Immunol. 16:1628689.
doi: 10.3389/fimmu.2025.1628689

Editorial: Immune cell therapy approaches targeting tumor microenvironment

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KEYWORDS

cancer, combination therapy, immunotherapy, tumor microenvironment, NK cells, immune checkpoint

Editorial on the Research Topic
Immune cell therapy approaches targeting tumor microenvironment

Introduction

Recent advances in immunotherapy have significantly improved clinical outcomes, particularly for cancers that were once considered untreatable or with poor prognosis. A growing body of evidence underscores the importance of both tumoral and non-tumoral components of the tumor microenvironment (TME) in driving cancer progression. The TME is a dynamic and complex ecosystem that includes not only cancer cells but also stromal cells such as immune cells, endothelial cells, and fibroblasts, as well as extracellular matrix components. These constituents can act jointly to create a pro-tumorigenic environment that facilitates tumor growth, metastasis, and therapeutic resistance, in particular, through immunosuppression, metabolic reprogramming, and angiogenesis. Interactions between cancer and other cells of the TME can also promote cancer cell heterogeneity, clonal evolution, and development of drug resistance mechanisms. Because of these reasons, the TME holds significant promise for improving cancer therapies. The genetic stability of the TME also makes it a compelling target for therapeutic interventions.

The articles in this Research Topic provide valuable insights that inform the development of innovative therapeutic strategies to target the TME in order to pave the way for improvised approaches for cell-based cancer immunotherapy. The findings of Puebla-Osorio et al. underscore the therapeutic potential of combining chimeric antigen receptor (CAR) T cell therapy with low-dose radiotherapy, particularly when tailored to specific tumor types and when the adoptively transferred effector T cells are precisely matched with radiation dosage. This innovative strategy has demonstrated promise across a range of preclinical models and holds significant potential for translation into clinical trials. Notably, it offers the unique advantage of eliciting therapeutic effects without incurring radiation-induced tissue damage. A potential role of ITGAL integrin in the immune landscape of non-small cell lung cancer is described by Zhang et al. Downregulation of ITGAL in tumors of this cancer as well as its association with poor prognosis indicate that ITGAL can be a valuable biomarker for predicting outcomes. The correlation between ITGAL expression and tumor

infiltration by $CD8^+$ T cells and macrophages implies that modulation of ITGAL expression or function can be a strategy to enhance anti-tumor immunity. The microbiome study of patients with acute myeloid leukemia by Zhong et al. underscores the growing recognition of the influence of intestinal microbiota on cancer. The study illustrates that antibiotics, besides controlling infections, can inadvertently affect the prognosis by altering key aspects of the immune biology and chemotherapy sensitivity. The work of Agostini et al. demonstrates that a nutritional medium rich in specific growth factors can enhance the migration of adipocyte stem cells. Such media may have therapeutic potential for cancer as well as regenerative applications.

Besides the above-mentioned research studies, the Research Topic includes a few reviews that bring us up to date on critical aspects of TME regarding immunotherapy. Li et al. review our understanding of homing of immune, stem, and circulating cancer cells to tumors. This work suggests that refining the homing ability of cells, especially in combination with other therapies, could be a strategy for cancer treatment. Chen et al. provide a comprehensive overview of the distinct cellular components of the ovarian cancer TME, elucidating their roles and associated pathways. Neutrophil extracellular traps (NETs), which are formed from DNA released by activated neutrophils, can impact cancer within the TME. Chen et al. provide a comprehensive overview of the latest research findings on the interaction between NETs and cancer, particularly in gynecologic tumors, serving as a valuable resource for future exploration in this field. The work of Qi et al. examines TME modulation by agonists of STING (stimulator of interferon genes) as a promising strategy to enhance immunotherapy efficacy. By targeting the immunosuppressive components of the TME, this approach aims to convert immunologically "cold" tumors into "hot" ones rendering them more susceptible to immune-mediated attack. Xu et al. highlight the intricate balance between different interleukins in the TME that influence tumor surveillance by NK cells. Targeting these cytokine pathways presents a promising strategy for enhancing NK cell-mediated tumor clearance and improving breast cancer outcomes. Engineering cytokines like IL-15, IL-12, and IL-18, along with the development of bispecific and trispecific antibodies, are likely to play a significant role in improving NK cell function within the TME. The immunosuppressive nature of TME in liver cancer remains a significant barrier to effective treatment. This immunosuppression arises from immune checkpoint molecules such as programmed death-1 (PD-1) and immunosuppressive cytokines that cause T cell exhaustion and inhibition. Immunotherapy, particularly with immune checkpoint inhibitors (ICIs), has shown great potential in improving outcomes for patients with advanced or unresectable hepatocellular carcinoma (HCC). The review by Hao et al. summarizes the latest clinical advancements in treatment strategies combining immunotherapy with targeted therapies for HCC treatment and discusses future directions and challenges in optimizing these regimens. Lastly, Dong et al. review current literature on the clinical benefit of combining ICIs with JAK inhibitors for enhancing anti-tumor immunity and overcoming resistance to immunotherapy in lung cancer and Hodgkin lymphoma.

Author contributions

SK: Writing – review & editing, Writing – original draft. SP: Writing – review & editing, Writing – original draft.

Funding

The author(s) declare that financial support was received for the research and/or publication of this article. This work was supported by National Institutes of Health, USA research grant R01CA255515-01A1.

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

frontiers | Frontiers in Immunology

TYPE Original Research
PUBLISHED 04 April 2024
DOI 10.3389/fimmu.2024.1382231

OPEN ACCESS

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RECEIVED 05 February 2024
ACCEPTED 21 March 2024
PUBLISHED 04 April 2024

CITATION
Zhang R, Zhu G, Li Z, Meng Z, Huang H, Ding C, Wang Y, Chen C, Li Y, Liu H and Chen J (2024) ITGAL expression in non-small-cell lung cancer tissue and its association with immune infiltrates. *Front. Immunol.* 15:1382231. doi: 10.3389/fimmu.2024.1382231

ITGAL expression in non-small-cell lung cancer tissue and its association with immune infiltrates

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Background: Integrin subunit alpha L (ITGAL) encodes an integrin component of LFA-1 and is a membrane receptor molecule widely expressed on leukocytes. It plays a key role in the interaction between white blood cells and other cells. There was a significant correlation between the expression of ITGAL and the tumor microenvironment in a number of cancers. However, experimental studies targeting ITGAL and immune cell infiltration in non-small-cell lung cancer (NSCLC) and the response to immune checkpoint inhibitor therapy are lacking.

Methods: Data were obtained from The Cancer Genome Atlas (TCGA), Gene Expression Omnibus (GEO), and Clinical Proteomic Tumor Analysis Consortium (CPTAC) databases to explore the relationship between ITGAL expression and prognosis, as well as the immune cell infiltration in patients with NSCLC. In addition, immunohistochemical staining for ITGAL and multiplex immunofluorescence (mIF) staining for ITGAL, CD20, CD68, CD4, and CD8 from tissue microarrays containing 118 tumor tissues and paired paracancerous tissues from patients with NSCLC were performed. The correlation between ITGAL expression and clinical factors, as well as the immunophenotypes of tumor-infiltrating immune cells, were also analyzed.

Results: In NSCLC tumor tissues, ITGAL was downregulated compared with matched paracancerous tissues, and low ITGAL expression was associated with a poor prognosis of NSCLC patients. Subsequently, immunohistochemistry results for tissue microarray showed that ITGAL expression was mainly elevated in tumor stroma and areas with highly infiltrated immune cells. ITGAL expression was higher in paracancerous tissues than tumor tissues. Furthermore, mIF results indicated that the patients with ITGAL-high expression tend had significantly higher CD8+ T cells, CD68+ macrophages, CD4+ T cells, and CD20+ B cells infiltration in their tumor tissues. Immunophenotypes were classified into three categories, that is deserted, excluded, and inflamed types, according to each kind of immune cell distribution in or around the cancer cell nest. MIF results showed that ITGAL expression level was correlated with the immunophenotypes. Furthermore, ITGAL expression was associated with the prognosis of NSCLC in

patients with immune checkpoint inhibitor therapy and the patients with high ITGAL expression tends have better outcomes.

Conclusions: ITGAL may be used as a biomarker for assessing the immune microenvironment in patients with NSCLC.

KEYWORDS

ITGAL, immune microenvironment, NSCLC, biomarker, immune cell

Introduction

Approximately 2.1 million new cases of lung cancer are diagnosed each year, and 1.8 million deaths are caused by this disease (1). Overall, lung cancer consists of two major groups: small-cell lung cancer (SCLC) and non-small-cell lung cancer (NSCLC), which account for about 15% and 85% of lung cancer cases, respectively. Of these, lung adenocarcinoma (LUAD) is the predominant subtype of NSCLC cases (2, 3). In spite of significant advances in diagnosis and treatment technology, NSCLC still has a very low overall cure rate and survival rate, especially when it is metastatic (4). In current cancer treatment, immunotherapy has been shown to be effective in treating a wide range of cancer types, including NSCLC (5, 6). However, not all NSCLC patients benefit from immunotherapy, which may be related to the tumor's immune microenvironment. In order to develop new immunotherapy targets for NSCLC, it is imperative to identify specific immune-related molecules.

ITGAL, also known as integrin αLβ2 (CD11a/CD18, CD11a), encodes an integrin component of LFA-1 and is a membrane receptor molecule widely expressed on leukocytes (7). ITGAL is an important molecule in the interaction between leukocytes and other cells (e.g., endothelial cells), and its main function is to participate in cell adhesion and migration, which are important for cellular immune responses, inflammatory responses, etc. (8–10). Recent studies have discovered that ITGAL is enriched in the tumor microenvironment, drawing much interest in oncology (11–13). ITGAL was reported to promote leukocyte migration and adhesion through an increase in the expression of Cx3cr1 and Ccl5, which in turn control leukocyte migration and mitogen production (14). Upregulation of ITGAL expression was strongly associated with immunomodulators, chemokines, and levels of CD8+ T cells, CD4+ T cells, B cells, monocytes, neutrophils, macrophages, T cells, natural killer (NK) cells, and myeloid dendritic cell infiltration in gastric adenocarcinomas (STAD). Further, some studies indicate that ITGAL might play a key role in cancer growth and transformation, making it a potentially useful target in the treatment of a wide variety of cancers (15–17). The main effect of resveratrol on NSCLC treatment may be related to immune signaling pathways, according to a mechanistic study of resveratrol in NSCLC (18). In NSCLC, the activated T cell (CD4 memory) pathway is mainly responsible for regulating the immune microenvironment, and ITGAL expression is significantly correlated with the immune microenvironment, according to some studies (19). In spite of this, the possible mechanisms of ITGAL on tumor development and immune interaction with NSCLC are still unknown, and experimental studies addressing the correlation between ITGAL and immune cell infiltration are limited.

We used immunohistochemical staining to diagnose ITGAL expression in 118 samples of NSCLC tissue and paraneoplastic tissue. Multiplex immunofluorescence staining (mIF) was also performed on the tissue microarrays to explore the relationship between ITGAL expression and infiltration of several immune cells, as well as the possible immunomodulatory role of ITGAL in NSCLC. We aim to uncover the critical role ITGAL plays in NSCLC, the possible connection between ITGAL and tumor-infiltrating immune cells, and the mechanism by which ITGAL may affect this process.

Materials and methods

NSCLC tissue samples and NSCLC tissue microchip

The Department of Lung Cancer Surgery of Tianjin Medical University General Hospital (TMU) collected lung cancer tissue and paraneoplastic lung tissue from five patients with NSCLC. Tissue microchips containing 118 pairs of primary lung cancer tissues and corresponding adjacent cancerous tissues were purchased from Saville Biotech (Wuhan, China). Each patient provided informed consent, and the study was approved by the Tianjin Medical University Ethics Committee.

Immunohistochemistry

The sections were first deparaffinized and hydrated, and antigen retrieval was achieved using a sodium citrate buffer before incubation with anti-human CD11a/integrin alpha L polyclonal antibody (dilution, 1:150) [(15574-1-AP), Proteintech] overnight at 4°C.

TBST was added three times to the slide, followed by the addition of rabbit anti-igG (Proteintech). The slide was incubated for 1 hour at room temperature after being washed three times in TBST. After washing the slides with phosphate-buffered saline (PBS), diaminobenzidine was used to expose them, and then hematoxylin was used to counterstain them. The immunohistochemistry (IHC) results were categorized as negative (-) and positive (+) according to the degree of staining, and each tissue was quantified according to the degree and extent of immunohistochemical staining using a 12-point counting method (0, 1, 2, and 3 according to the degree of staining, multiplied by the extent of staining, 1, 2, 3, and 4). In a blind review of the staining results, two pathologists without prior knowledge of clinicopathological data evaluated the results independently.

Multiplex immunofluorescence

Multiplex immunofluorescence (mIF) staining and primary evaluation were performed by Yucebio Technology (Shenzhen, China). Xylene was used to dewaxe the 5-millimeter slides produced from formalin-fixed, paraffin-embedded (FFPE) blocks. We then rehydrated the slides with decreasing concentrations of ethanol, then fixed them for 10 minutes with 10% neutral buffered formalin. Next, the slides were stained with markers of CD20 (E7B7T) XP® rabbit monoclonal antibody (mAb) (48750S, Cell Signaling Technology), recombinant anti-CD4 antibody [EPR6855, (ab133616), Abcam], anti-CD8 monoclonal antibody [4B11, (MA1-80231), Thermo Fisher Scientific], CD11a/integrin alpha L polyclonal antibody (15574-1-AP, Proteintech), anti-CD68 [KP1, (ZM-0060), ZSGB-Bio], anti-pan cytokeratin antibody [KRT/1877R, (ab234297), Abcam], followed by incubation with blocking proteins for 10 minutes. A secondary polymer antibody conjugated to horseradish peroxidase (HRP) was applied after blocking, followed by tyramide signal amplification (TSA). DAPI staining of the slides was carried out for 10 minutes, followed by imaging using Vectrapolaris (Akoya Biosciences).

Data source and bioinformation analysis

TCGA (https://portal.gdc.cancer.gov/ (accessed on 20 Nov 2023) and GEO (https://www.ncbi.nlm.nih.gov/geo/ (accessed on 20 Nov 2023) databases were used to obtain seven datasets: TCGA-LUAD contained 510 LUAD samples; TCGA-LUSC contained 498 LUSC samples; GSE116959 contained 57 tumor samples and 11 normal samples; GSE68571 contained 86 tumor samples and 10 normal samples; GSE30219 contained 59 tumor samples and 14 normal samples; GSE33532 contained 16 tumor samples and 20 normal samples and GSE93157 contained 35 non-small cell lung cancer samples, 5 head and necks quamous cellcarcinoma, and 25 melanoma samples. The mRNA expression and clinical correlation of ITGAL in LUAD and LUSC were analyzed using UALCAN (http://ualcan.path.uab.edu/ (accessed on 20 Nov 2023) website and TCGA database. ITGAL expression and its correlation with immune cells, immune scores, and immune infiltration were analyzed using the Sangerbox tool. We analyzed overall survival (OS) and progression-free survival (PFS) using Kaplan-Meier plotting websites.

Statistical analysis

GraphPad Prism 8 (GraphPad Software, La Jolla, CA) was used for statistical analyses. Data from the three groups of repeated experiments were presented as mean ± standard deviation and independent samples, t-tests were used to analyze the quantitative data. Tables used chi-square tests to detect differences between groups, and box plots used the unpaired Wilcoxon method to test for differences between groups. Survival analyses We calculated the optimal cut-off value for ITGAL using the R package maxstat and assessed prognostic differences between samples using the logrank test. $P < 0.05$ was considered a statistically significant difference.

Results

ITGAL expression was downregulated in primary NSCLC tissue

In order to determine whether human ITGAL plays an oncogenic role in NSCLC, the TCGA and GEO databases were used to examine expression levels of ITGAL in LUAD and LUSC. A significant difference was observed between tumor and paraneoplastic lung samples for ITGAL mRNA expression in TCGA-LUAD and TCGA-LUSC($P<0.001$) (Figures 1A, B). For further validation, we detected the expression of ITGAL in LUAD by GEO databases, GSE68517 and GSE116959, and in LUSC by databases GSE33532 and GSE30219. A significant reduction in ITGAL protein levels was observed in LUAD and LUSC compared to normal tissues (Figures 1C, D). In a subsequent study, we examined the expression of ITGAL mRNA in tumor and peritumor lung tissue samples from five patients with NSCLC. Tissues from tumors expressed less ITGAL than healthy tissues (Figure 1E).

Expression of ITGAL protein and correlation with clinical factors in lung cancer tissue

Tissue microarrays containing tissue from 118 patients with primary NSCLC and paired paracancerous tissues were used for ITGAL IHC staining and scoring. As shown in the table below, the tissue microarrays contained characteristics about patients with lung cancer (Table 1). The IHC results showed that ITGAL was mainly distributed in the plasma membrane and the cell membrane, with a patchy or nested distribution. In accordance with the median score of ITGAL, patients were divided into two groups: those with high ITGAL expression and those with low ITGAL expression (Figure 2A). The following table presents the results of the ITGAL analysis stratified by the median immunohistochemical

TABLE 1 Clinical characteristics of patients with lung cancer tissue microarrays.

Characteristics	Tumor (N=118)
Age	
<=61	59 (50%)
>61	59 (50%)
Gender	
Female	65 (55.08%)
Male	53 (44.91%)
Pathological diagnosis	
Other	10 (8.47%)
Adenocarcinoma	90 (76.27%)
Squamous carcinoma	18 (15.25%)
Stage	
I-II	91 (77.12%)
III-IV	27 (22.88%)

score into high and low expression for each clinical factor (Table 2). By analyzing the IHC results of each patient, we found that paraneoplastic tissues had significantly higher ITGAL expression compared to matched tumor tissues (Figures 2B, C). The expression of ITGAL was higher in tumor tissues from female patients than male patients, and in tumor tissues from early-stage patients than advanced-stage patients (Figures 2D, E). In order to further validate our findings, we examined ITGAL expression at various stages of NSCLC tumor progression and lymph node metastases using the TCGA database. The ITGAL expression of LUAD and LUSC at stratified clinical stages were lower than that of paracancer normal tissues (Figures 2F, H). Meantime, the ITGAL expression of LUAD and LUSC at stratified N stages were lower than that of paracancer normal tissues (Figures 2G, I).

Moreover, ITGAL was prominently highly expressed in the stroma area of the tumor tissues and on the membrane and cytoplasm of macrophages and lymphocytes aggregated in these areas (Figure 2J).

NSCLC patients with high expression of ITGAL had a better prognosis

KM survival curves were used to analyze the NSCLC prognosis based on ITGAL expression. In lung adenocarcinomas, patients with higher levels of ITGAL expression had a higher OS rate (HR=0.74, P=6.7e^{-03}) and PFS rate (HR=0.73, P=3.3e^{-07}). The prognosis was better when ITGAL expression was high (Figures 3A, B). Similarly, in lung squamous cell carcinoma, patients with higher expression of the ITGAL gene had longer OS time (HR=0.73, P=1.7e^{-03}) and PFS time (HR=0.55, P=3.7e^{-03}) (Figures 3C, D). A similar result was found in the TCGA database when analyzing OS among patients with LUAD. Those with high expression of ITGAL had a better prognosis (HR=0.61, P=9.3e^{-04}) (Figure 3E).

ITGAL expression was correlated with immune infiltrate patterns of NSCLC tumor tissue

With the help of mIF, we examined the expression of ITGAL in tissue microarrays and the distribution of tumor-infiltrating immune cells. Table 3 lists the labeled antibodies used and the cells and/or cellular components that were recognized. Figure 4 shows a representative diagram of mIF staining. In accordance with the immunohistochemical analysis, high levels of ITGAL were detected in tumor stroma (Figure 5A). At the same time, we found that areas of high ITGAL expression in NSCLC tumor tissues had different immune cell aggregates or high expression of immune cell markers (Figure 5B). Furthermore, we grouped the patients into ITGAL-high and ITGAL-low groups based on the median expression of ITGAL. As shown in Table 4, patients with ITGAL-high expression had significantly higher infiltration of CD8+ T cells, CD68+ macrophages, CD4+ T cells, and CD20+ B cells in their tumor tissues, while patients with ITGAL-low expression had significantly lower infiltration of CD8+ T cells, CD68+ macrophages, CD4+ T cells, and CD20+ B cells in their tumor tissues (Table 4).

Furthermore, immunophenotypes were classified into three categories according to each kind of immune cell distribution in or around the cancer cell nest: deserted, excluded, and inflamed types. ITGAL expression levels for individual immune cells were compared for each immunophenotype (Figure 5C). ITGAL expression level is correlated with immune cell immunophenotypes, according to the results. For example, the CD8, CD20, CD68, and CD4-positive exclusion group had the lowest degree of ITGAL-expressing cell infiltration in lung cancer tissues, whereas the inflamed group had the highest degree of ITGAL-expressing cell infiltration in lung cancer tissues. It is therefore possible that ITGAL expression is related to the infiltration of CD4+ and CD8+ T cells, macrophages, and B cells in the lung (Figures 5D-G). We examined the correlation of different immune cell surface markers with ITGAL expression using the TCGA database, and similarly, we found that the expression of CD4, CD68, CD20, CD8A, and GZMB was positively correlated with ITGAL expression (Figures 5H-L).

ITGAL expression was associated with the prognosis of NSCLC in patients on immune checkpoint inhibitor therapy

Programed death-1, programmed death-ligand 1 (PD-1, PD-L1), and cytotoxic T-lymphocyte antigen-4 (CTLA-4) are immune checkpoint molecules that protect tumor cells from immune attack. T lymphocytes can be restored to their cytotoxic abilities against tumor cells with the use of immune checkpoint inhibitors (ICIs). This treatment, however, is only effective in some patients. As the immunophenotypes of tumor-infiltrating lymphocytes are reported

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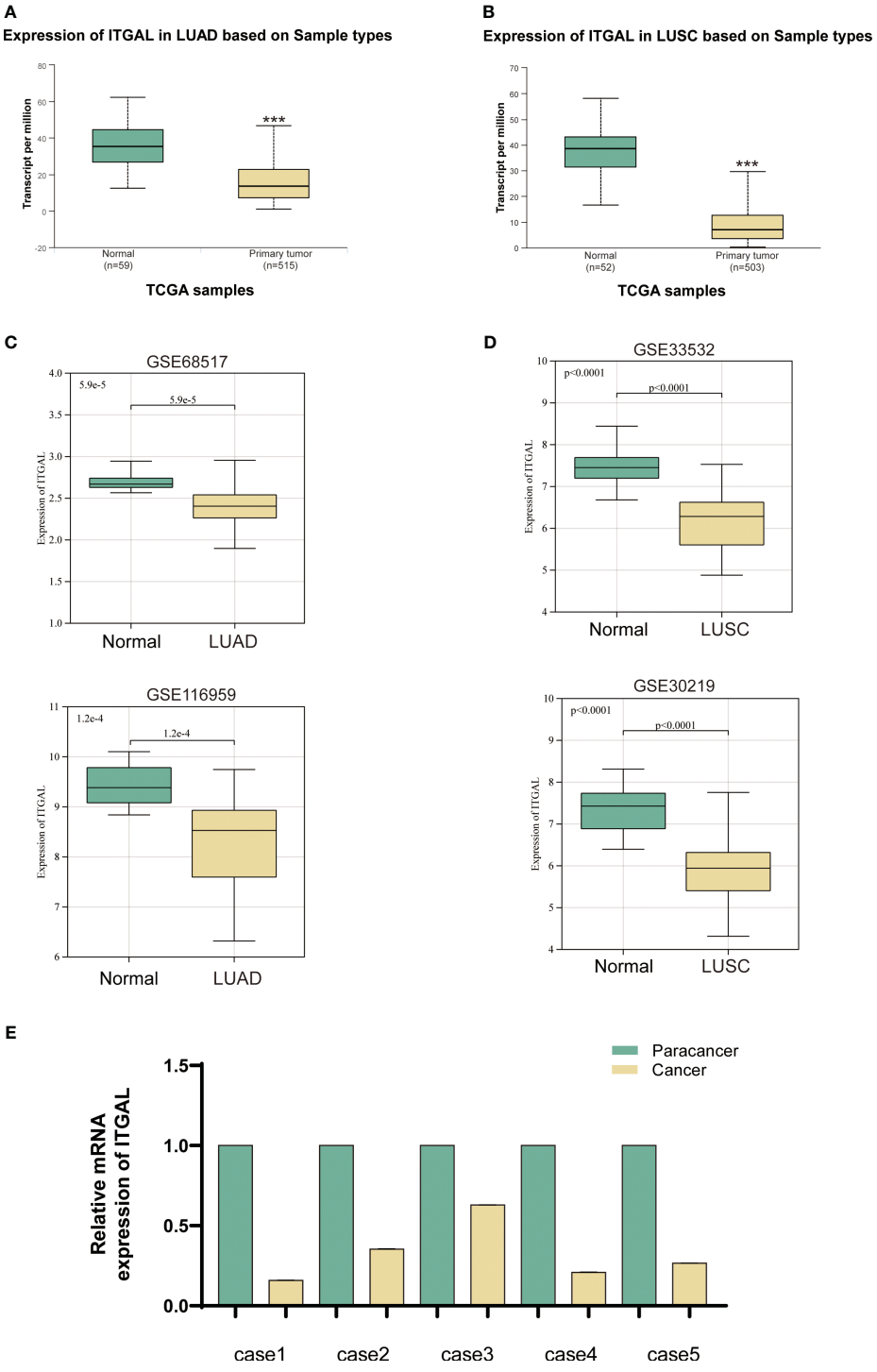

FIGURE 1
ITGAL expression was downregulated in primary NSCLC tissue. **(A, B)** Expression of ITGAL mRNA in LUAD and LUSC tissues in the TCGA database. **(C)** Verification of ITGAL expression in LUAD tumor tissues in the GEO database (GSE116959 and GSE68517). **(D)** Verification of ITGAL expression in LUSC tumor tissues in the GEO database (GSE33532 and GSE30219). **(E)** Detection of ITGAL mRNA levels in tissue samples from five patients with NSCLC. ***p<0.001.

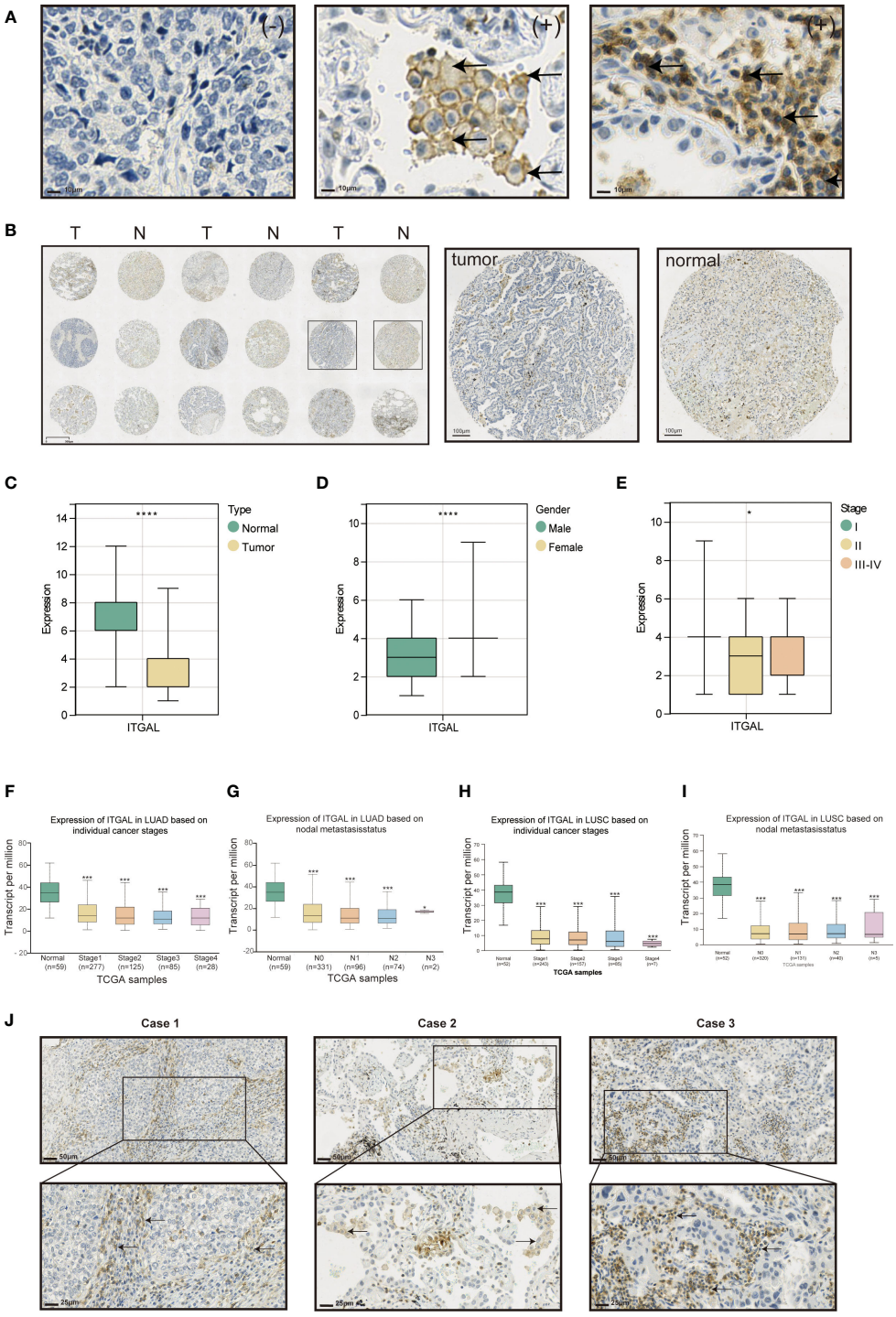

FIGURE 2
Expression of ITGAL protein and correlation with clinical factors in lung cancer tissue. **(A)** Immunohistochemical staining of ITGAL in lung cancer tissue microarrays. **(B)** Expression of ITGAL in tumor tissues and corresponding normal tissues in lung cancer tissue microarrays. **(C)** Immunohistochemical staining scores of ITGAL in lung cancer tissue microarrays stratified by **(D)** histology type, **(E)** gender, and **(F)** stage. **(F–I)** ITGAL expression levels in patients with LUAD and LUSC stratified by tumor stage and lymph node metastasis status in the TCGA database. **(J)** Expression of ITGAL in lung cancer tissue microarray tumor tissues. *p<0.05, ***p<0.001, ****p<0.0001.

10.3389/fimmu.2024.1382231

TABLE 2 Immunohistochemical results of ITGAL immunohistochemistry of lung cancer tissue microarrays and their relationship with clinic pathophysiological factors.

Characteristics	ITAGL		Total (N=118)
	low (N=82)	high (N=36)	
Pathological diagnosis			
Other	6 (60%)	4 (40%)	10
Adenocarcinoma	65 (71.43%)	26 (28.57%)	91
Squamous carcinoma	10 (58.82%)	7 (41.18%)	17
Age			
<=61	41 (69.49%)	18 (30.51%)	59
>61	40 (67.80%)	19 (32.20%)	59
Gender			
Female	44 (67.70%)	21 (32.30%)	65
Male	37 (69.81%)	16 (30.19%)	53
Stage			
I-II	62 (68.13%)	29 (31.87%)	91
III-IV	19 (70.37%)	8 (29.63%)	27

to be correlated with the response rate and prognosis of patients treated with ICIs and ITGAL expression is correlated with the immunophenotypes of tumor-infiltrating lymphocytes, we further investigated whether ITGAL expression correlated with the response and prognosis of patients who underwent ICI therapy. In this dataset, 22 patients had lung adenocarcinoma, 13 patients had lung squamous cell carcinoma, 5 patients had head and neck squamous cell carcinoma, and 25 patients had malignant melanoma. In Table 5, the detailed clinical characteristics are shown. The results of the prognostic analysis showed a better outcome for patients with immunotherapy and high ITGAL expression (z=-1.60, P=0.11) (Figure 6A). Furthermore, we grouped the patients into responders and non-responders and compared the ITGAL expression between those two groups. Results showed that ITGAL expression was higher in responders than non-responders, although this did not reach statistical significance since the non-responders contained only five cases. (Figure 6B).

Immune cell infiltration was linked to anomalous ITGAL expression in NSCLC

TIDE is a method for predicting whether tumors will respond to immunotherapy based on T-cell function. As part of the TIDE algorithm, cytotoxic T-cell analysis (CTL) is used to evaluate the

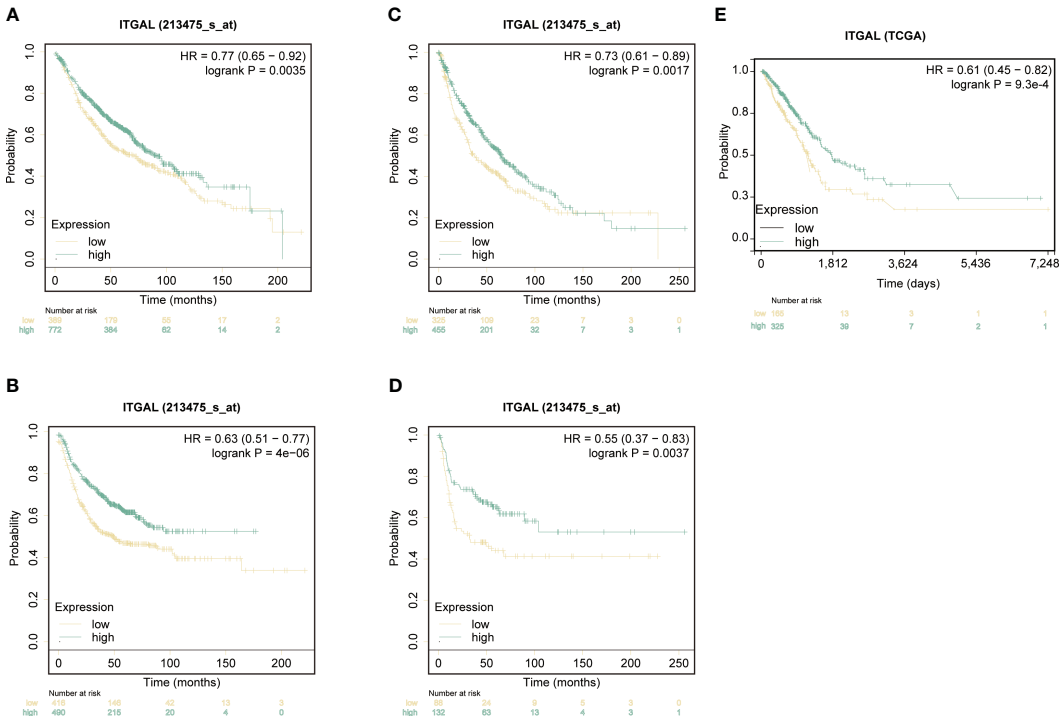

FIGURE 3
ITGAL expression correlated with prognosis in patients with non-small-cell lung cancer. **(A)** Kaplan-Meier survival curves for overall survival (OS) based on ITGAL expression levels in LUAD. **(B)** Kaplan-Meier survival curves for progression-free survival (PFS) based on ITGAL expression levels in LUAD. **(C)** Kaplan-Meier survival curves for OS based on ITGAL expression levels in LUSC. **(D)** Kaplan-Meier survival curves for PFS based on ITGAL expression levels in LUSC. **(E)** The effect of ITGAL on overall survival in LUAD was analyzed in the TCGA database.

10.3389/fimmu.2024.1382231

TABLE 3 Multicolor immunofluorescence labeling of antibodies and the cells they recognize.

Marker	Antibodies	Color	Cells/Components
1	CD20	Opal-520	B-cell
2	CD4	Opal-620	Helper T-cell
3	CD8	Opal-480	Cytotoxic T-cell
4	CD68	Opal-570	Macrophage
5	PanCK	Opal-780	Tumor epithelial cell
6	DAPI	DAPI	Nucleus

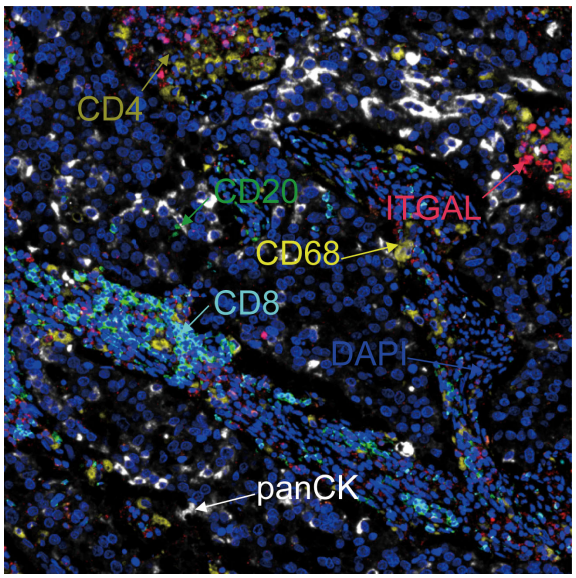

FIGURE 4
Representative diagram of mIF staining.

cytotoxicity of T-cells. There was a positive correlation between CTL expression and ITGAL (r=0.561, P=6.77e^{-05}) (Figure 6C). In order to further explore the relationship between ITGAL and CTL, we investigated the correlation between ITGAL and CTL in the TCGA database and found that ITGAL expression in LUAD and LUSC was positively correlated with CTL levels. (Figures 6D, E).

Among NSCLC patients, Spearman's analysis revealed a positive correlation between ITGAL expression and immune scores (estimated score, immune score, and interstitial score). (Figures 7A–C). To further elucidate the immune properties of ITGAL related to the tumor immune microenvironment, we evaluated genes involved in immune stimulation and found that 30 genes were positively associated with ITGAL in LUAD and LUSC (Figure 7D). According to the TCGA database, CD4+ T cells, CD8+ T cells, and macrophages from patients with NSCLC expressed ITGAL significantly more than CD8+ T cells (Figure 7E). ITGAL may be a potential prognostic marker of immunotherapy efficacy for NSCLC when combined with our multicolor immunofluorescence results.

Discussion

In this study, we performed ITGAL immunohistochemistry and multicolor immunofluorescence on tissue microarrays containing 118 patients with NSCLC to characterize the expression of ITGAL in NSCLC and whether there is a relationship between it and immune cell infiltration. We found that ITGAL was enriched in the mesenchyme in NSCLC tumor tissues, while ITGAL-expressing regions in tumor tissues were accompanied by immune cell aggregation and high expression of immune cell markers. However, ITGAL expression was lower in NSCLC than in normal tissues. This further suggests that ITGAL functions as a protective factor *in vivo* and has the potential to be a biomarker for assessing the immune microenvironment in NSCLC patients.

ITGAL is a cell adhesion molecule that is essential for the migration and adhesion of immune cells in inflammation and immune responses (20, 21). In current oncology studies, expression of the ITGAL gene has been associated with the progression and aggressiveness of renal (22), ovarian and colorectal cancers (23, 24). However, its expression and clinical relevance in NSCLC is unknown. We found that ITGAL expression was significantly down-regulated in NSCLC cells compared to normal lung epithelial cells by including analysis of the TCGA database, the GEO database, and our collection of tissue mRNA from NSCLC patients. Similarly, at the protein level, ITGAL showed different expression patterns under different circumstances. To begin with, we found that tumor tissues expressed less ITGAL protein than healthy tissues. It appears that ITGAL may function as a tumor suppressor in NSCLC. The second finding was that ITGAL expression was significantly higher in the tumor tissues of female patients than in the tumor tissues of male patients, suggesting that the tumor immune microenvironment differs between females and males. Finally, our results showed that the protein expression level of ITGAL was significantly higher in stage I tumors than in stage II or III–IV tumors, indicating that ITGAL was associated with the progression of lung cancer, including disease stage and degree of tumor differentiation.

In the tumor immune microenvironment, immune cell infiltration has been shown to play a role in cancer development (25). The integrin α-L chain encoded by the ITGAL gene is involved in regulating immune cell activity in the tumor microenvironment (7). In T-cell immunotherapy, ITGAL proteins may be involved in tumor recognition by immune cells as the starting point for cytotoxic T cells to generate immune synapses with cancer cells. In recent studies, ITGAL has been found to play a role in the generation of primary resistance to NSCLC immunotherapy (7, 26). In our study, we also found that ITGAL expression correlated not only with the number of multiple immune cells infiltrated but also with the mode of infiltration. Its main site of expression was the tumor stroma, while less expression was found on the surface of tumor cells. Therefore, ITGAL may play a greater role in regulating immune cell function in the tumor microenvironment (27). Gastric cancer has been found to express ITGAL significantly more than peritumor samples in several studies. A high ITGAL expression was significantly associated with the type of sample, the subgroup, the

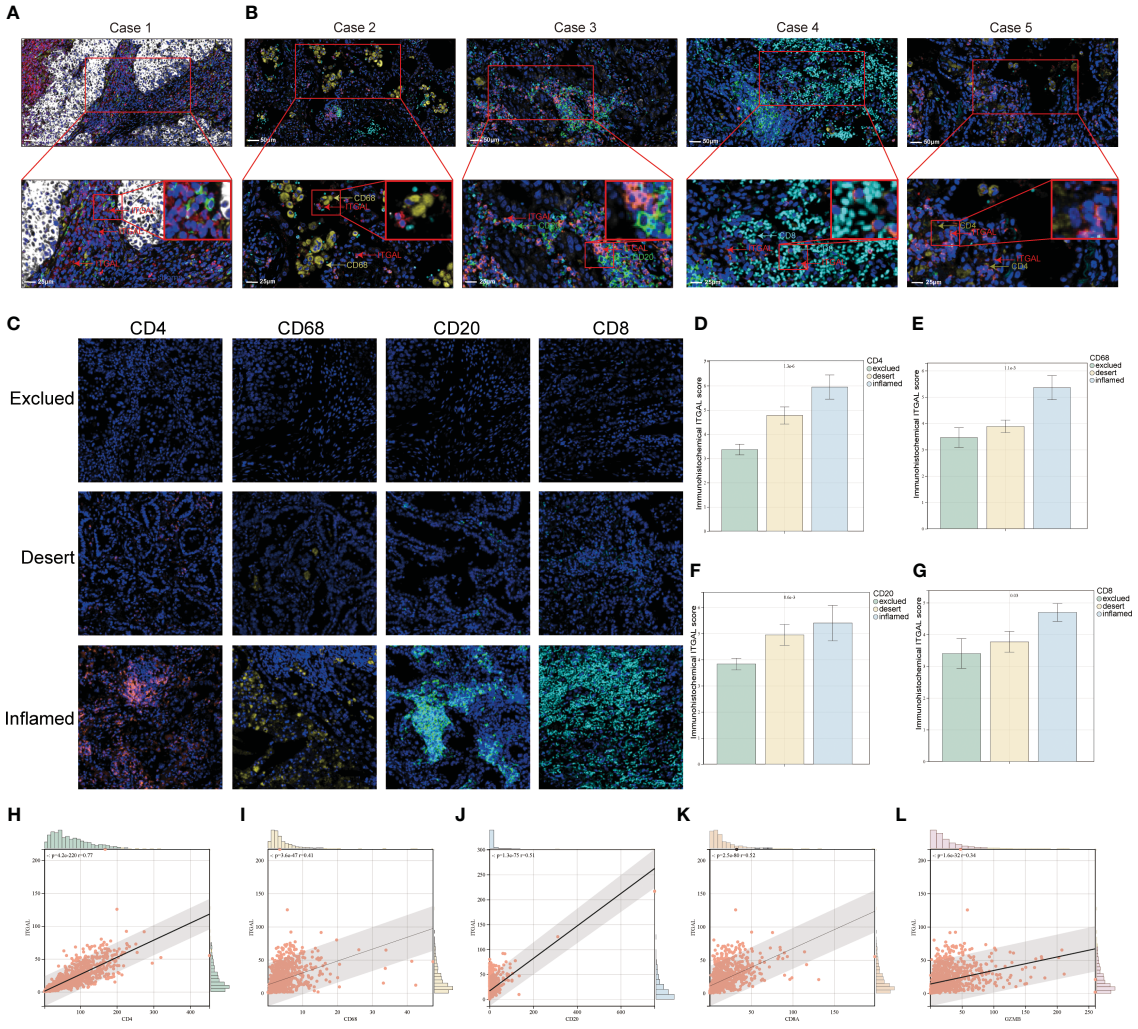

FIGURE 5
Relationship between ITGAL expression and pattern of immune infiltration in NSCLC tumor tissue. **(A, B)** Multicolor immunofluorescence assay to detect the location of ITGAL expression and immune cells in lung cancer tissue microarray tumor tissues. **(C)** Patients with lung cancer were categorized into inflamed, excluded, and deserted immunophenotypes according to the degree of infiltration of CD20, CD8, CD4, and CD68-positive cells. **(D–G)** Comparison of ITGAL-positive cell infiltration in patients with different immunophenotypes. **(H–L)** Relationship between different immune cell surface markers and ITGAL expression in the TCGA database.

tumor stage, the lymph node stage, and the low survival rate for gastric cancer (12, 19). In contrast, our study found that non-small-cell lung cancer expressed low levels of ITGAL, while controls expressed high levels. Some studies have reported that the upregulation of ITGAL expression was closely associated with the level of infiltration of immunomodulators, chemokines, CD8+, CD4+ T cells, B cells, monocytes, neutrophils, macrophages, T cell modulators, NK cells, and myeloid dendritic cells in gastric adenocarcinoma (STAD) (19). A multicolor immunofluorescence staining and score analysis were used to investigate the relationships between ITGAL and different types of immune cells. Immunofluorescence staining showed that CD4, CD68, CD2, and CD8 non-infiltrating groups had lower infiltration of ITGAL-expressing cells, whereas the highly infiltrating group had higher infiltration of ITGAL-expressing cells. This suggests that ITGAL may play a role in regulating immune cell infiltration, affecting the organism's tumor microenvironment and immune response, and may play a moderately important role in immune escape, leading to tumor immune alterations that result in a poor prognosis for patients with NSCLC. Further research is needed to determine the exact mechanism by which ITGAL affects immune infiltration in NSCLC.

In conclusion, this study was the first to demonstrate the role of ITGAL in promoting immune cell infiltration in NSCLC by immunohistochemistry and multiplex immunofluorescence and confirmed the important role of ITGAL in the immune microenvironment of NSCLC using public databases. The role of ITGAL and its ligands in the tumor microenvironment has been considered as a potential therapeutic target. Studies have been conducted to explore ways to inhibit the invasion and metastasis of tumor cells by interfering with ITGAL-ICAM interactions (28). It has been shown that the expression level of ITGAL is associated with the efficacy of ICI in patients with NSCLC (29), suggesting that ITGAL might be useful as a molecular marker of NSCLC and

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TABLE 4 Immunohistochemical results of ITGAL immunohistochemistry of lung cancer tissue microarrays and their relationship with staining results of various indicators in tumor tissues.

Characteristics	ITAGL		pvalue
	high (N=36)	low (N=82)	
CD8			0.030
Mean ± SD	10.42 ± 9.61	7.60 ± 9.38	
Median [min-max]	8.46 [0.15,47.86]	3.88 [0.09,45.30]	
CD20			<0.001
Mean ± SD	2.61 ± 4.57	1.36 ± 3.08	
Median [min-max]	0.90 [0.0e+0,21.75]	0.15 [0.0e+0,12.44]	
CD68			<0.001
Mean ± SD	6.13 ± 5.08	3.15 ± 3.05	
Median [min-max]	4.56 [0.08,19.13]	2.35 [0.11,14.86]	
CD4			<0.001
Mean ± SD	4.61 ± 5.05	1.31 ± 2.66	
Median [min-max]	2.88 [0.0e+0,21.82]	0.39 [0.0e+0,19.36]	

TABLE 5 Clinical characteristics of immunotherapy datasets.

Characteristics	Tumor (N=65)
Age	
<65	43 (66.15%)
≥65	22 (33.85%)
Gender	
Female	50 (76.92%)
Male	15 (23.08%)
Cancer Type	
LUAD	22 (33.85%)
HEADNECK	5 (7.69%)
MELANOMA	25 (38.46%)
LUSC	13 (20.00%)
Drug	
NIVOLUMAB	28 (43.08%)
PEMBROLIZUMAB	37 (56.92%)

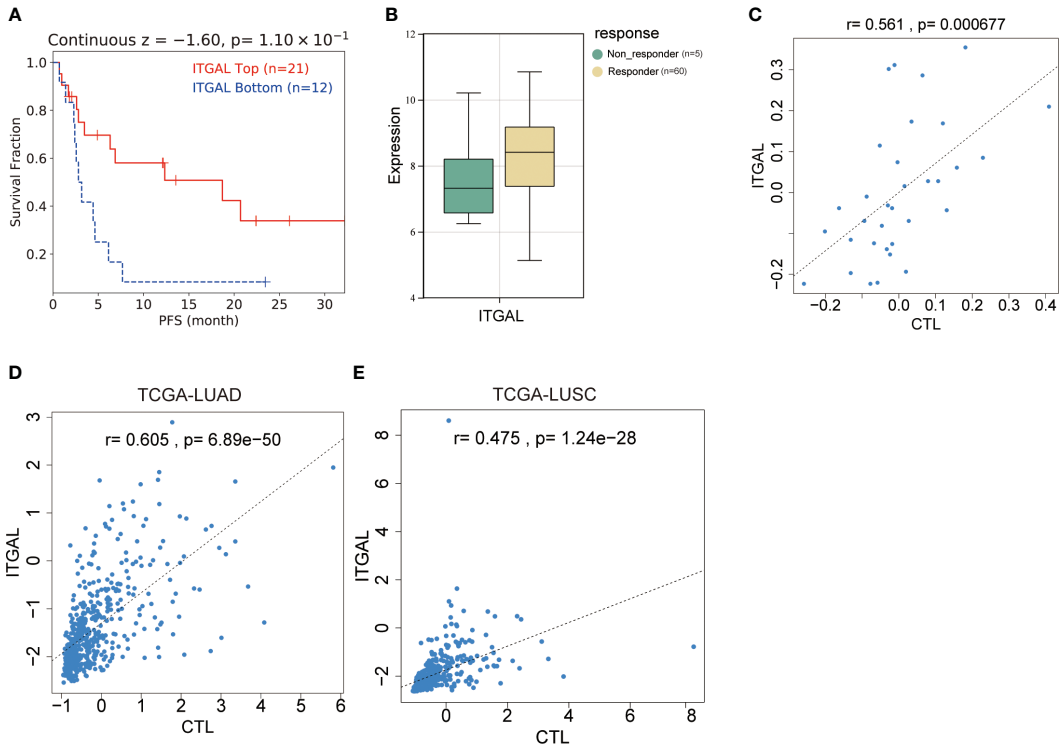

FIGURE 6
Relationship between ITGAL expression and prognosis of NSCLC in patients treated with immune checkpoint inhibitors. **(A)** Survival curve analysis of patients with lung adenocarcinoma receiving immunotherapy. **(B)** ITGAL expression in patients with lung adenocarcinoma who were responders and non-responders to immunotherapy. **(C)** Correlation analysis of ITGAL gene expression with TIDE score in patients with lung adenocarcinoma receiving immunotherapy. **(D)** Correlation analysis of ITGAL gene expression with the TIDE score in LUAD from the TCGA database. **(E)** Correlation analysis of ITGAL gene expression with the TIDE score in LUSC from the TCGA database.

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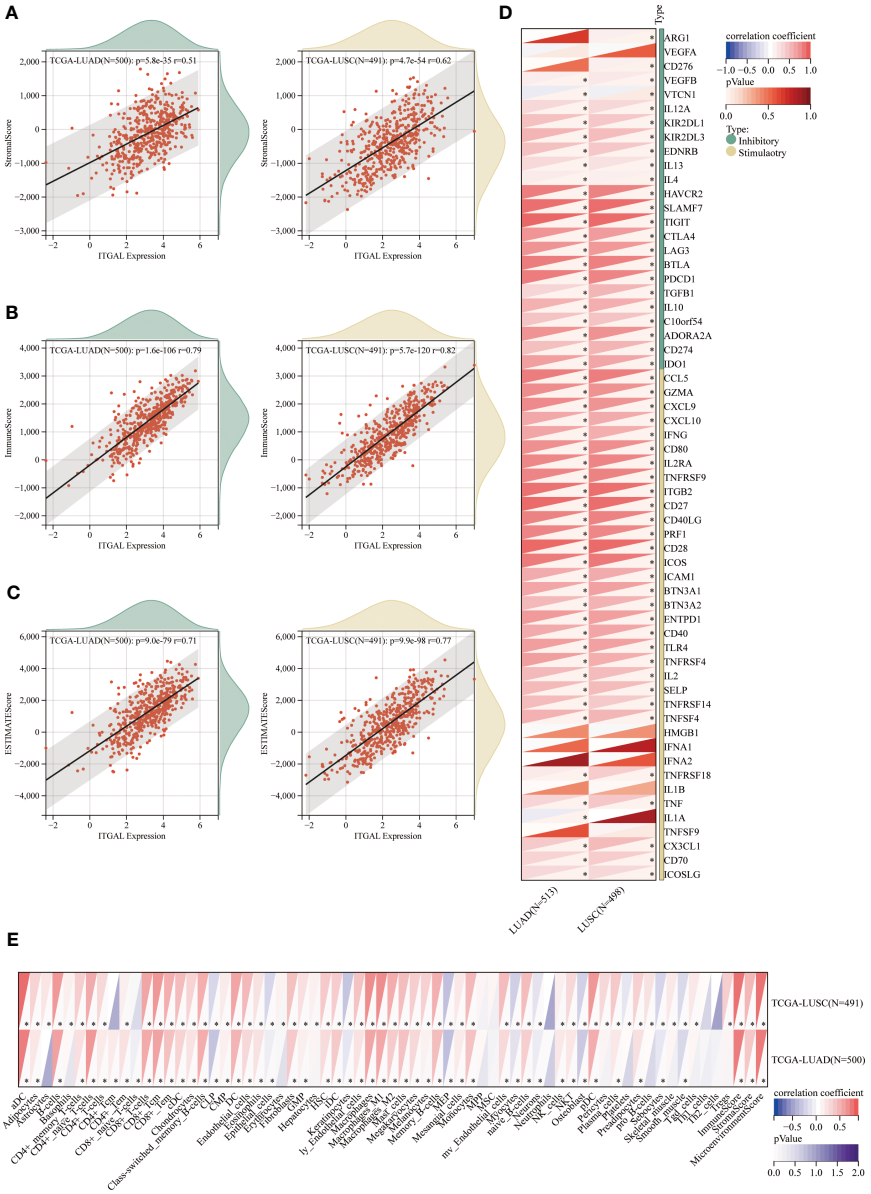

FIGURE 7
Relationship between immune cell infiltration and aberrant ITGAL expression in NSCLC detected by the TCGA database. **(A-C)** Relationship of ITGAL expression with estimated LUAD and LUSC scores, immunity scores, and stromal scores. **(D)** Relationship between ITGAL and immune checkpoint genes in LUAD and LUSC. **(E)** Differences in immune cell expression between high and low ITGAL groups in LUAD and LUSC. *p<0.05.

as a predictor of ICI efficacy. However, there are several limitations. First, our study only focused on the correlation between ITGAL and immune cell infiltration in NSCLC; the specific mechanism of ITGAL involvement in immune infiltration in NSCLC is unclear and requires further investigation. Second, bioinformatics and patient tissue samples have only been used to validate the relationship between ITGAL and immune cell infiltration. Considering the complexity of the tumor immune microenvironment, animal models may provide a better understanding of ITGAL's role in NSCLC immunity. The purpose of this study was to examine the relationship between ITGAL expression and immune infiltration and its impact on NSCLC patients' prognoses. This adds to our understanding of the critical role of ITGAL in human tumors, including NSCLC.

Conclusions

Our present study provided evidence that increased ITGAL expression promoted immune cell aggregation in NSCLC and may be used as a biomarker for immune infiltration. Furthermore, ITGAL expression may be used to predict the efficacy of ICI therapy in patients with NSCLC treated with ICIs. There are multiple possible roles for ITGAL in regulating immune cell

infiltration in NSCLC tissues, as revealed by our study; however, the molecular mechanisms of ITGAL in tumorigenesis and its clinical application prospects warrant further investigation.

Data availability statement

Publicly available datasets were analyzed in this study. This data can be found here: TCGA: TCGA-LUAD, TCGA-LUSC; GEO: GSE116959, GSE68517, GSE30219, GSE33523, GSE93157.

Ethics statement

The studies involving humans were approved by Tianjin Medical University Ethics Committee. 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

RZ: Data curation, Investigation, Methodology, Software, Visualization, Writing – original draft, Writing – review & editing. GZ: Data curation, Formal analysis, Software, Validation, Writing – review & editing. ZL: Data curation, Methodology, Software, Writing – review & editing. ZM: Formal analysis, Methodology, Writing – review & editing. HH: Software, Validation, Writing – review & editing. CD: Methodology, Software, Writing – review & editing. YW: Software, Validation, Writing – review & editing. CC: Methodology, Software, Validation, Writing – review & editing. YL: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Project administration, Resources, Supervision, Writing – review & editing. HL: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Supervision, Validation, Writing – review & editing.

Funding

The author(s) declare that financial support was received for the research, authorship, and/or publication of this article. This study was supported by the National Natural Science Foundation of China 82172569 (supported by Yongwen Li), 82072595 and 61973232 (supported by Jun Chen), Tianjin Medical Key Discipline (Specialty) Construction Project TJYXZDXK-061B (supported by Jun Chen), Tianjin Health Science and Technology Project TJWJ2022XK005 (supported by Jun Chen), 5ZC20179 (supported by Hongyu Liu) and Beijing Science and Technology Innovation Medical Development Fund grant KC2021-JX-0186-57 (funded by Jun Chen). Funding sources were not involved in study design, data collection and analysis, publication decision or manuscript writing.

Conflict of interest

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

The author(s) declared that they were an editorial board member of Frontiers, at the time of submission. This had no impact on the peer review process and the final decision.

Publisher's note

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frontiers | Frontiers in Immunology

TYPE Correction
PUBLISHED 26 April 2024
DOI 10.3389/fimmu.2024.1415814

OPEN ACCESS

APPROVED BY
Frontiers Editorial Office,
Frontiers Media SA, Switzerland

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RECEIVED 11 April 2024
ACCEPTED 17 April 2024
PUBLISHED 26 April 2024

CITATION
Zhang R, Zhu G, Li Z, Meng Z, Huang H, Ding C, Wang Y, Chen C, Li Y, Liu H and Chen J (2024) Corrigendum: ITGAL expression in non-small-cell lung cancer tissue and its association with immune infiltrates.
Front. Immunol. 15:1415814.
doi: 10.3389/fimmu.2024.1415814

Corrigendum: ITGAL expression in non-small-cell lung cancer tissue and its association with immune infiltrates

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KEYWORDS

ITGAL, immune microenvironment, NSCLC, biomarker, immune cell

A Corrigendum on

ITGAL expression in non-small-cell lung cancer tissue and its association with immune infiltrates

by Zhang R, Zhu G, Li Z, Meng Z, Huang H, Ding C, Wang Y, Chen C, Li Y, Liu H and Chen J (2024) *Front. Immunol.* 15:1382231. doi: 10.3389/fimmu.2024.1382231

In the published article, there was an error in the affiliation(s)for author Yongwen Li [3*]. Instead of "Yongwen Li [3*]", it should be "Yongwen Li [4*]".

Additionally, there was an error in Figures 1, 2 as published. There is an error in the typographical order of Figures 1, 2, the Figures 1, 2 are in reverse order. The corrected Figure 1 and its caption ITGAL expression was downregulated in primary NSCLC tissue appear below.

The corrected Figure 2 and its caption Expression of ITGAL protein and correlation with clinical factors in lung cancer tissue appear below.

There was also an error in the Funding statement. The funding statement does not clearly state Jun Chen's funding number: "The author(s) declare that financial support was received for the research, authorship, and/or publication of this article. The National Natural Science Foundation of China grants 82172569 was funded by Yongwen Li. The Tianjin Health Science and Technology Project 5ZC20179 was funded by Hongyu Liu. All other funds were funded by Jun Chen."

The correct Funding statement appears below.

FUNDING

"The author(s) declare that financial support was received for the research, authorship, and/or publication of this article. This study was supported by the National Natural Science Foundation of China 82172569 (supported by Yongwen Li), 82072595 and 61973232

(supported by Jun Chen), Tianjin Medical Key Discipline (Specialty) Construction Project TJYXZDXK-061B (supported by Jun Chen), Tianjin Health Science and Technology Project TJWJ2022XK005 (supported by Jun Chen), 5ZC20179 (supported by Hongyu Liu) and Beijing Science and Technology Innovation Medical Development Fund grant KC2021-JX-0186-57 (funded by Jun Chen). Funding sources were not involved in study design, data collection and analysis, publication decision or manuscript writing."

Finally, in the published article, there were some errors in the text. In the results section, due to the change in the order of Figures 1, 2, the references to the figures need to be changed accordingly.

In the results section, ITGAL expression was downregulated in primary NSCLC tissue and Expression of ITGAL protein. Correction has been made to references to figures in paragraphs. This sentence previously stated:

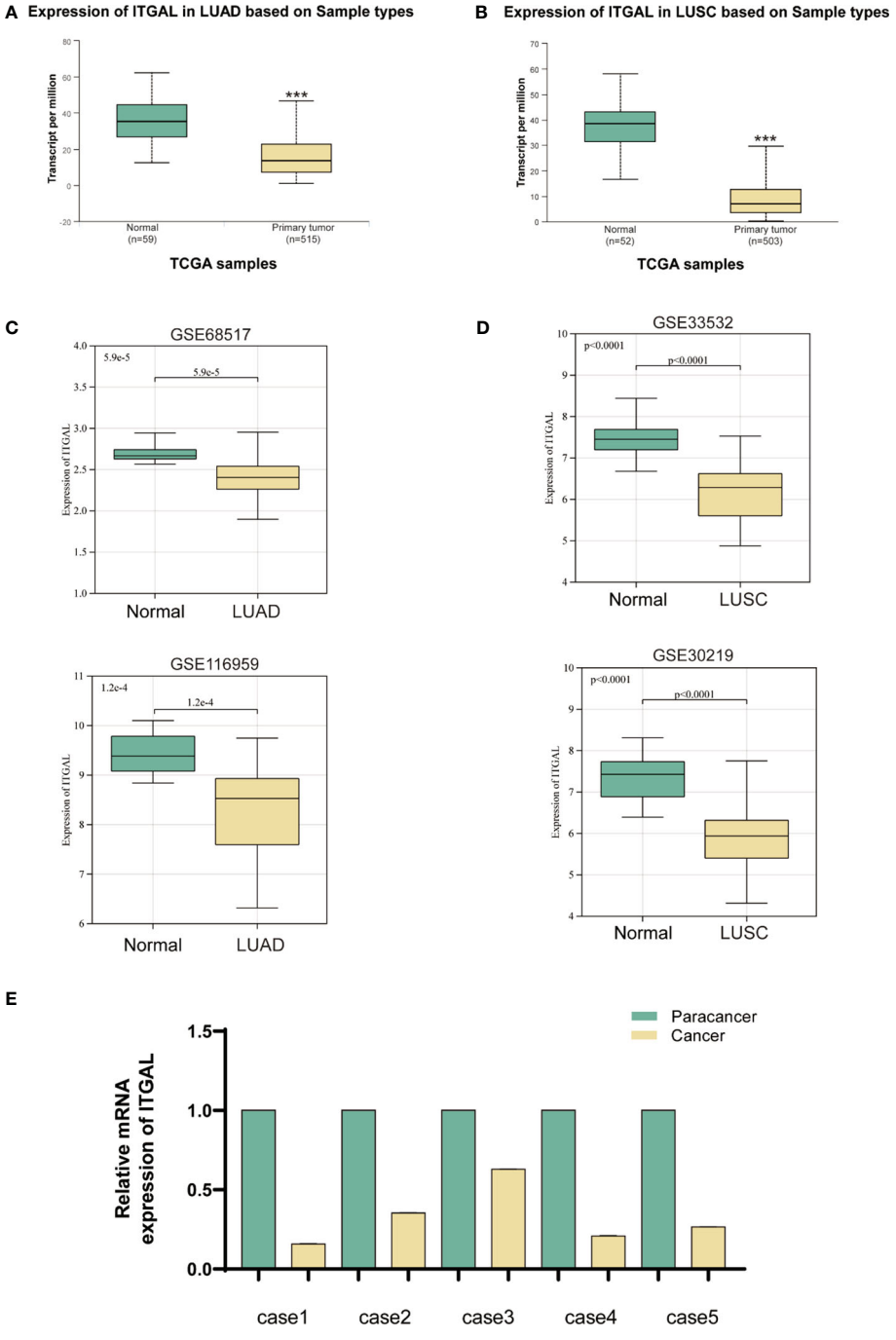

FIGURE 1

"In order to determine whether human ITGAL plays an oncogenic role in NSCLC, the TCGA and GEO databases were used to examine expression levels of ITGAL in LUAD and LUSC. A significant difference was observed between tumor and paraneoplastic lung samples for ITGAL mRNA expression in TCGA-LUAD and TCGA-LUSC(P<0.001) (Figures 2A, B). For further validation, we detected the expression of ITGAL in LUAD by GEO databases, GSE68517 and GSE116959, and in LUSC by databases GSE33532 and GSE30219. A significant reduction in ITGAL protein levels was observed in LUAD and LUSC compared to normal tissues (Figures 2C, D). In a subsequent study, we examined the expression of ITGAL mRNA in tumor and peritumor lung tissue samples from five patients with NSCLC. Tissues from tumors expressed less ITGAL than healthy tissues (Figure 2E)."

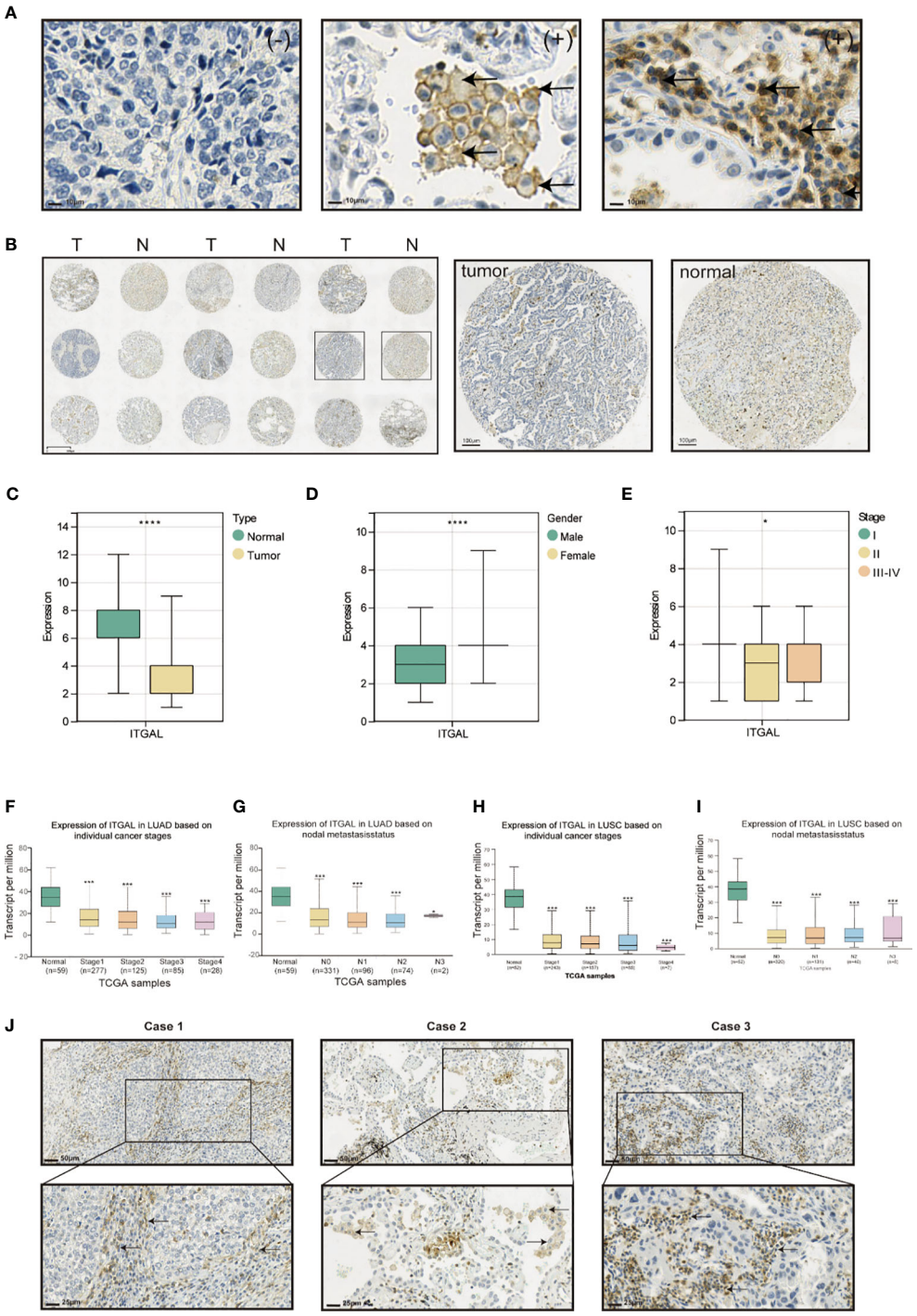

FIGURE 2

The corrected sentence appears below:

"In order to determine whether human ITGAL plays an oncogenic role in NSCLC, the TCGA and GEO databases were used to examine expression levels of ITGAL in LUAD and LUSC. A significant difference was observed between tumor and paraneoplastic lung samples for ITGAL mRNA expression in TCGA-LUAD and TCGA-LUSC($P<0.001$) (Figures 1A, B). For further validation, we detected the expression of ITGAL in LUAD by GEO databases, GSE68517 and GSE116959, and in LUSC by databases GSE33532 and GSE30219. A significant reduction in ITGAL protein levels was observed in LUAD and LUSC compared to normal tissues (Figures 1C, D). In a subsequent study, we examined the expression of ITGAL mRNA in tumor and peritumor lung tissue samples from five patients with NSCLC. Tissues from tumors expressed less ITGAL than healthy tissues (Figure 1E)."

In the results section, Correlation with clinical factors in lung cancer tissue. Correction has been made to references to figures in paragraphs. This sentence previously stated:

Tissue microarrays containing tissue from 118 patients with primary NSCLC and paired paracancerous tissues were used for ITGAL IHC staining and scoring. As shown in the table below, the tissue microarrays contained characteristics about patients with lung cancer (Table 1). The IHC results showed that ITGAL was mainly distributed in the plasma membrane and the cell membrane, with a patchy or nested distribution. In accordance with the median score of ITGAL, patients were divided into two groups: those with high ITGAL expression and those with low ITGAL expression (Figure 1A). The following table presents the results of the ITGAL analysis stratified by the median immunohistochemical score into high and low expression for each clinical factor (Table 2). By analyzing the IHC results of each patient, we found that paraneoplastic tissues had significantly higher ITGAL expression compared to matched tumor tissues (Figures 1B, C). The expression of ITGAL was higher in tumor tissues from female patients than male patients, and in tumor tissues from early-stage patients than advanced-stage patients (Figures 1D, E). In order to further validate our findings, we examined ITGAL expression at various stages of NSCLC tumor progression and lymph node metastases using the TCGA database. The ITGAL expression of LUAD and LUSC at stratified clinical stages were lower than that of paracancer normal tissues (Figures 1F, H). Meantime, the ITGAL expression of LUAD and LUSC at stratified N stages were lower than that of paracancer normal tissues (Figures 1G, I).

Moreover, ITGAL was prominently highly expressed in the stroma area of the tumor tissues and on the membrane and cytoplasm of macrophages and lymphocytes aggregated in these areas (Figure 1J).

The corrected sentence appears below:

Tissue microarrays containing tissue from 118 patients with primary NSCLC and paired paracancerous tissues were used for ITGAL IHC staining and scoring. As shown in the table below, the tissue microarrays contained characteristics about patients with lung cancer (Table 1). The IHC results showed that ITGAL was mainly distributed in the plasma membrane and the cell membrane, with a patchy or nested distribution. In accordance with the median score of ITGAL, patients were divided into two groups: those with high ITGAL expression and those with low ITGAL expression (Figure 2A). The following table presents the results of the ITGAL analysis stratified by the median immunohistochemical score into high and low expression for each clinical factor (Table 2). By analyzing the IHC results of each patient, we found that paraneoplastic tissues had significantly higher ITGAL expression compared to matched tumor tissues (Figures 2B, C). The expression of ITGAL was higher in tumor tissues from female patients than male patients, and in tumor tissues from early-stage patients than advanced-stage patients (Figures 2D, E). In order to further validate our findings, we examined ITGAL expression at various stages of NSCLC tumor progression and lymph node metastases using the TCGA database. The ITGAL expression of LUAD and LUSC at stratified clinical stages were lower than that of paracancer normal tissues (Figures 2F, H). Meantime, the ITGAL expression of LUAD and LUSC at stratified N stages were lower than that of paracancer normal tissues (Figures 2G, I).

Moreover, ITGAL was prominently highly expressed in the stroma area of the tumor tissues and on the membrane and cytoplasm of macrophages and lymphocytes aggregated in these areas (Figure 2J).

The authors apologize for these errors and state that they do not change the scientific conclusions of the article in any way. The original article has been updated.

Publisher's note

frontiers | Frontiers in Immunology

TYPE Original Research
PUBLISHED 14 May 2024
DOI 10.3389/fimmu.2024.1404228

OPEN ACCESS

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RECEIVED 20 March 2024
ACCEPTED 30 April 2024
PUBLISHED 14 May 2024

CITATION
Agostini F, Vicinanza C, Lombardi E, Da Ros F, Marangon M, Massarut S, Mazzucato M and Durante C (2024) *Ex vivo* expansion in a clinical grade medium, containing growth factors from human platelets, enhances migration capacity of adipose stem cells. *Front. Immunol.* 15:1404228. doi: 10.3389/fimmu.2024.1404228

Ex vivo expansion in a clinical grade medium, containing growth factors from human platelets, enhances migration capacity of adipose stem cells

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Introduction: Adipose tissue mesenchymal stem/stromal cells (ASC) can be used as advanced therapy medicinal product in regenerative and cancer medicine. We previously demonstrated Supernatant Rich in Growth Factors (SRGF) can replace fetal bovine serum (FBS) to expand ASC by a clinical grade compliant protocol. The therapeutic potential of ASC is based also on their homing capacity toward inflammatory/cancer sites: oriented cell migration is a fundamental process in this scenario. We investigated the impact of SRGF on ASC migration properties.

Methods: The motility/migration potential of ASC expanded in 5% SRGF was analyzed, in comparison to 10% FBS, by standard wound healing, bidimensional chemotaxis and transwell assays, and by millifluidic transwell tests. Mechanisms involved in the migration process were investigated by transient protein overexpression.

Results: In comparison to standard 10% FBS, supplementation of the cell culture medium with 5% SRGF, strongly increased migration properties of ASC along the chemotactic gradient and toward cancer cell derived soluble factors, both in static and millifluidic conditions. We showed that, independently from applied migratory stimulus, SRGF expanded ASC were characterized by far lower expression of α-smooth muscle actin (αSMA), a protein involved in the cell migration machinery. Overexpression of αSMA induced a significant and marked decrease in migration capacity of SRGF expanded ASC.

Discussion: In conclusion, 5% SRGF addition in the cell culture medium increases the migration potential of ASC, reasonably through appropriate downregulation of αSMA. Thus, SRGF could potentially improve the therapeutic impact of ASC, both as modulators of the immune microenviroment or as targeted drug delivery vehicles in oncology.

KEYWORDS

adipose tissue, mesenchymal stem/stromal cells, migration, chemiotaxis, millifluidic transwell assay, drug delivery, good manufacturing practice, supernatant rich in growth factors

1 Introduction

Mesenchymal stem/stromal cells (MSC) are characterized by the ability to sustain cell growth and homeostasis and by the property to balance inflammatory conditions in the microenvironment (1). Moreover, such cells can reach primary and metastatic cancer masses after intravenous infusions (2). In virtue of such homing properties, MSC can be exploited as a potential drug delivery vehicle for therapeutic purposes in cancer patients. Fabrication of an advanced therapeutic medical product (ATMP), based on MSC, requires an efficient cell expansion protocol, in compliance with good manufacturing practice (GMP) guidelines. As previously published (3), we described a GMP compatible protocol to efficiently expand adipose tissue derived MSC (ASC). The protocol was based on the introduction, in the cell culture medium, of a medium additive, that we defined as Supernatant Rich in Growth Factors (SRGF). Sterile SRGF was derived from human platelet concentrates, in which growth factors were released from thrombocytes by the addition of $CaCl_2$, as platelet activation trigger (4). Reliability and consistency of SRGF biologic functions were demonstrated in a previous work (5), in which we showed that such medium additive can be fully standardized pooling together at least 16 products derived from single donors. For such reasons, SRGF can be considered as compliant with GMP guidelines for ATMP fabrication. SRGF was shown to be more efficient than other similar medium additives, as e.g. platletet lysate, in promoting growth of MSC (6). ASC expanded in presence of SRGF were also shown to strongly interact, in microfluidic conditions, with selected types of cancer cells, suggesting a peculiar affinity of such ASC for target tumors (7). Nevertheless, the MSC homing process from circulating blood to cancer cells is not limited to direct cell-cell interaction but it's also characterized by oriented migration through a chemotactic gradient toward the cancer target (2). During cell migration, cells extend protrusions, shaped by actin, that adhere to the extracellular matrix through integrin clusters, defined as focal adhesions (8). Focal adhesions are highly multifaceted protein complexes in which few factors can play pivotal roles. Paxillin (PAX) is considered as a primary scaffold in the architecture of the focal adhesion protein complex (9). PAX was previuosly shown to be recruited at adhesion sites located in the forward part of a moving cell (10). Vinculin (VINC) and PAX provide a connection between actin and integrins: in particular, VINC is a mechano-sensing protein and its downregulation was linked to increased cell motility in bidimensional conditions (11). Both VINC and PAX play a crucial role in modulating the interaction with Focal Adhesion Kinase (FAK) (12, 13). FAK is a phosphorylation activated enzyme controlling formation and disassembly of focal adhesion (14). Previous publications showed α-smooth muscle actin (αSMA) is required for cell diapedesis (15), even though it was also shown to inhibit migration of selected primary cells (16). We previously showed (17) that FilaminA (FLNA) is increased in fast migrating cells, as MSC derived from the bone marrow of multiple myeloma patients. In this work, we aimed to investigate the impact of SRGF on the migration potential of expanded ASC. Thereafter, we also attempted to investigate molecular mechanisms explaining SRGF mediated effect on the migratory potential of expanded ASC.

2 Methods

2.1 Cell isolation and expansion

ASC were purified from stromal vascular fraction isolated from lipoaspirates obtained from female breast cancer patients that underwent mammary reconstruction by lipofilling after mastectomy. The study was in accordance with the Declaration of Helsinki (2004) and it was approved by Ethics Committee of CRO Aviano, National Cancer Institute, IRCCS (protocol number: CRO-2016–30). The study was conducted in accordance with the local legislation and institutional requirements. The participants provided their written informed consent to participate in this study. Expanded ASC were isolated from 5 patients in which liposuction was performed from the same anatomical area (thigh) for breast reconstruction in disease free conditions. Results included in this work were obtained using cells derived from at least 3 out of the 5 selected patients.

Stromal vascular fraction isolation and ASC expansion were performed as previously published (3). Briefly, a solution of collagenase, 0.15 U/ml final concentration (NB 6 Good Manufacturing Practice grade, SERVA Electrophoresis GmbH, Heidelberg, Germany) was added to the washed lipoaspirate. After 1 hour incubation at 37°C and collagenase neutralization by human albumin solution (Albital 200 g/l, Kedrion S.p.A., Lucca; Italy), the lower phase was recovered and centrifuged at 400 x g for 10 minutes at +4°C. The cell pellet was washed with a solution composed by 10% human albumin, 10% Anticoagulant Acid Citrate Dextrose Solution – A (ACD-A; Haemonetics Corporation, Braintree, MA; USA), 2 U/ml heparin (Epsodilave, HOSPIRA ITALIA S.r.l., Napoli; Italy) in Ringer Lactate solution (S.A.L.F. S.p.A. Laboratorio Farmacologico, Bergamo; Italy). Cell viability was controlled by Trypan blue dye exclusion test and cell identity was confirmed by flow citometry, exactly as previousy published (3). Stromal vascular fraction cells were frozen in autologous or AB serum containing 5% dimethylsulfoxide (CryoSure-DMSO, Li StarFish, MI; Italy). Thawed SVF cells were separately plated in standard T25 tissue culture flasks (BD Biosciences, Bedford, MA; US) with Minimum Essential Medium Eagle - Alpha Modification (α-MEM) (Lonza; Basel, Switzerland) with 100 IU/ml of Penicillin, 100 mg/ml of Streptomycin (both from Merck, Rahway, NJ; US) added with 10% vol/vol FBS or in parallel with 5% vol/vol SRGF. SRGF was used at 5% as we previously demonstrated that further increasing SRGF concentrations failed to confer relevant advantages in terms of ASC proliferation rate (18). Cells were cultured in clinical grade incubators (MCO-19AIC UV; Sanyo, Osaka, Japan) at 37°C, 5% CO_2 and 100% humidity. Non-adherent stromal vascular fraction cells were removed after 24 hours. Further ASC expansion was performed using 10% FBS or 5% SRGF α-MEM media. In this work, as indicated for specific experiments, SRGF was reduced at concentrations of 0.3; 0.6; 1.25; 2.5% vol/vol. Upon 80–90%

confluence, ASC were detached by trypsin-ethylenediaminetetraacetic acid (EDTA) (TrypLe Select 10X, Thermo Fisher Scientific, Waltham, MA; US) and subcultured again. ASC expanded in 5% SRGF and 10% FBS media fully detached from plastic surface, respectively, after 2 and 5 minutes exposure to trypsin-EDTA. Resuspended cells were seeded (at P1 and at each following cell passages) at 2.5–3.0 x 10^3 cells/cm^2.

Human hepatocellular carcinoma HepG2 and human fibrosarcoma HT1080 cell lines were expanded in Dulbecco's modified Eagle's medium (Lonza) with high glucose and 10% FBS. The human glioblastoma cell line T98G was maintained in Eagle's minimal essential medium (Lonza) supplemented with Earle's basic salt solution and 10% FBS. Cells were cultured in clinical grade incubators (MCO-19AIC UV; Sanyo, Osaka, Japan) at 37°C, 5% CO_2 and 100% humidity. All growth media were supplemented with 100 IU/ml of Penicillin, 100 mg/ml of Streptomycin (both from Merck). Conditioned medium (CM) from each cancer cell line was obtained exposing subconfluent cells to fresh medium containing the appropriate FBS concentration (as indicated in each experimental condition). After 24 hours, the supernatant was collected and centrifuged at 4500xg for 15 minutes. Aliquots were stored at -20°C until use.

2.2 Spheroid formation

To expand ASC as spheroids we seeded 2 x 10^4 ASC/well in low attachment 96 U-bottom well plates (Nunclon Sphera, Thermo Fisher Scientific) in the two different culture conditions, i.e. in presence of 5% SRGF or of 10% FBS. After cell aggregation in spheroids (24 hours), cells were transferred to 35 mm low attachment plates (35 mm Dish Nunclon Sphera, Thermo Fisher Scientific). After additional 24 hours incubation, spheroids were centrifuged for 5 minutes at 450 x g. To dissociate ASC from spheroids, the supernatant was discarded and trypsin-EDTA (1x) was added to spheroids. After 3 minutes incubation (37°C), an excess of complete medium was added to inhibit trypsin activity and cells were centrifuged at 450 x g. ASC expanded in 5% SRGF were dissociated twice with trypsin-EDTA, while 10% FBS were dissociated three times. Finally, the solution was passed through a strainer (40 µm cutoff), centrifuged at 450 x g and washed with PBS. The cell pellet was resuspended in the appropriate medium (i.e. in presence of 5% SRGF or of 10% FBS), and used for the functional assay.

2.3 Wound healing assay

ASC (1 x 10^4 cells) expanded in presence of 5% SRGF or of 10% FBS were seeded in each chamber of a Culture-Insert 2 Well (Ibidi, Gräfelfing; Germany) device, coated with bovine collagen type I (Thermo Fisher Scientific) at the concentration of 50 ng/µl (1 hour incubation at room temperature). After complete adhesion, the separating chamber was removed, allowing spontaneous cell movements toward the empty space. During the assay, α-MEM medium containing 1% FBS or 1% SRGF was applied to reduce as much as possible cell proliferation, without potentially affecting cell motility. After 6 and 24 hours from assay beginning, images were taken by inverted phase contrast microscope (Olympus CKX41, Olympus Italia Srl, Milano; Italy). Thresholded images were analyzed by ImageJ (19) and percent covered area in the middle channel were considered.

2.4 Bidimensional chemotaxis migration assay

To assess the impact of a chemotactic stimulus on ASC migration after expansion in presence of 5% SRGF or of 10% FBS, ASC were seeded at low concentration (1.000 total cells) in µ-slide chemotaxis devices (Ibidi). The device is composed of a central narrow channel in which cells are seeded. The channel is flanked on its left and right sides by a wing-shaped reservoir that, in virtue of its geometry, allows the onset of a chemotactic gradient. The central channel was coated with bovine collagen type I (Thermo Fisher Scientific) and after complete adhesion, cells were exposed to oriented gradients of chemotactic stumuli. Serum free α-MEM medium was injected into the left reservoir, while a medium containing only 2.5% FBS, or cancer cell CM containing 2.5% FBS, was introduced into the right reservoir. As technical negative control, in parallel assays, serum free medium (supplemented with antibiotics) was added in both right and left reservoirs. Cell migration was monitored by time course imaging of cells seeded in the middle channel (1 image/30 minutes). Environmental conditions during time lapse imaging were controlled maintaining temperature at 37°C. Manual tracking function of ImageJ was used to define trajectories of moving cells. Datasets of cell coordinates changing over time were analyzed by Chemotaxis and Migration tool (Ibidi). Forward Migration Index (FMI), as calculated by Chemotaxis and Migration tool, was considered as indicator of active cell migration, determined by the chemotactic stimulus. Mean FMI values scored in negative control experiments performed in each condition were closely approximate to 0 (data not shown).

2.5 Transwell assay in static conditions

2 x 10^4 ASC, expanded in presence of 5% SRGF (if not otherwise stated for setup experiments) or of 10% FBS were seeded in duplicate in transwell chambers (Transwell Permeable Support; Costar, WA, US), previously coated with bovine collagen type I (Thermo Fisher Scientific) at the concentration of 50 ng/µl (1 hour incubation at room temperature). Transwell chambers were equipped with polyester (PET) or polycarbonate (POLY) septa (both 8.0 micron cutoff pore size). To trigger cell migration, complete medium containing 5% FBS (if not otherwise stated) or cancer cell CM (with the same FBS concentration) were used as chemoattractants. Serum free medium was applied to the upper compartment. After 24 hours in cell culture incubators (Sanyo) at 37°C, 5% CO_2 and 100% humidity, migration was stopped fixing cells with 4% cold (+4°C) paraformaldehyde (Sigma-Merk, St Louis,

MO; US). ASC on both septum sides were stained with 4′,6-diamidino-2-phenylindole (DAPI) (Thermo Fisher Scientific) and visualized by a fluorescence microscope (Nikon, Tokyo, Japan) equipped 10x objective and with camera for image capture. Images, taken in the middle of the septum almost covered the whole area of migration septa. Pictures were then analyzed by Imagej and total ASC (nuclei of migrated and non-migrated cells) were counted by the "Analyze particles" plugin. Objects size threshold was set over 50 squared pixels to avoid inclusion of artifact dots in the final count. Thereafter, non-migrating ASC were scraped from the upper part of the septum by a cotton swab, and additional images of migrated cells were captured and analyzed as above. Transmigration index was estimated as percent ratio between migrated cells (post scrape) and total cells (before scrape) in each chamber.

2.6 Transwell assay in dynamic millifluidic conditions

To assess ASC migration potential in millifluidic conditions, we took advantage of the LifeBox2 device (Ivitech, LU, Italy), equipped with the peristaltic pump LiveFlow (Ivitech), compatible with the incubator environment. The LiveBox2 chamber is composed of 2 compartments, separated by a collagen coated (see above) PET septum. Resembling the architecture of static transwell chambers, the lower compartment was filled with chemoattractant media composed of complete medium added with 10% FBS or of cancer cell CM (with the same FBS concentration). Flow was not applied to the lower compartment closing tightly together inlet and outlet catheters. Otherwise, serum free medium flow (500 µl/minute, maximal flow rate) was applied to the upper compartment and ASC, previously expanded in presence of 5% SRGF or of 10% FBS, were injected into the close circuit through the reservoir. The final concentration of circulating cells was 2.4 x 10^4 ASC/ml. After 24 hours, the LiveBox2 chamber was disassembled taking advantage of the appropriate device to avoid disruption or perturbation of the adherent cell layer. The PET septum was carefully removed and cells were fixed by 4% cold (+4°C) paraformaldehyde (Sigma-Merk), stained with DAPI (Thermo Fisher Scientific) and analyzed as abovementioned for static assays. In order to analyze the major part of migration surface area, five pictures were taken in each septum before and after non-migrating cell removal by cotton swab. ASC transmigration index (%) was calculated by the same formula applied for static transwell assays.

2.7 Transient transfection

ASC were seeded in transwell chambers and a DNA vector (pRP-CMV-hACTA2; from Vectorbuilder; Chicago, IL, US), encoding for aSMA in fusion with the reporter green fluorescent protein (aSMA-GFP), was transfected using Lipofectamine Stem Transfection Reagent (Thermo Fisher Scientific) and following manufacturer's instructions. Lipofectamine was chosen as this reagent couples adequate transfection efficiency and reduced impact on cell viability. As control condition, cells were transfected with an empty vector (pRP-CMV-GFP; from Vectorbuilder) encoding for the only green fluorescent protein (GFP). A ratio of 1µg DNA to 2µl Lipofectamine was chosen to perform the transfection. To setup the appropriate protocol, 15 or 30 ng DNA/seeded cell were administered. Protein expression, was evaluated by western blot or immunofluorescence assays, 24 hours after transfection.

2.8 Western blot assay

Proteins were extracted from cells using a lysis buffer, composed by PBS + 0.5% NP40 non ionic detergent (Thermo Fisher Scientific) supplemented with protease and phosphatase inhibitors (Merck KGaA). Protein concentrations were measured using a BCA protein assay kit (Thermo Fisher Scientific) and a proper amount of cell lysate was loaded into a 4–20% protean TGX gel (Bio–Rad, Hercules, CA, USA). After electrophoresis, proteins were transferred to 0.2 µm PVDF membrane using a Trans–Blot Turbo device (BioRad, Hercules, CA, USA). Membranes were blocked for 1 hour with 5% milk proteins (Blotting Grade Blocker –BioRad, Hercules, USA) in Tris-buffered saline with 0.1% Tween 20 detergent (TBST). Primary antibodies were added at appropriate concentration in 5% milk in TBST overnight at 4°C. The following antibodies were used: anti FAK (1:1000, Abcam, Cambridge, UK, #ab76496); anti phospho FAK Y397 (1:1000, Abcam, #ab81298); anti αSMA (1:1000, Sigma-Merk, #A5228); anti VINC (1:3000, Abcam, #ab129002); Anti PAX (1:1000, Abcam, #ab32084); anti –FLNA (1:1000, Proteintech, Manchester, UK, #67133–Ig), anti –Phosphorylated FLNA S2152 (1:1000, ABclonal, Woburn, MA, USA #AP0783), and anti– glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (1:3000, Merkmillipore, Burlington, NJ, USA, #CB1001); Anti GFP (1:1000, Roche, Basel, Switzerland, #11814460001). After the incubation with proper secondary antibodies anti–mouse HRP (1:1000, GE Healthcare, Milan, Italy, #GENA931) or anti–rabbit HRP (1:1000, SouthernBiotech, Birmingham, AL, USA, #4030–05), the immunoreactive bands were visualized with Amersham ECL Prime Western Blotting Detection Reagent (Cytiva, Marlborough, MA, USA) and Chemidoc Imaging System (BioRad, Hercules, CA, US). Relative band quantification was performed by the ImageJ software.

2.9 Immunofluorescence assays

ASC were seeded on ethanol treated cover glasses and transfected as above mentioned. After 24 hours, cells were fixed with 4% cold paraformaldehyde (Sigma-Merk), permeabilized by 0.1% Triton X100 (Sigma-Merck), blocked with 2% BSA and incubated with Anti αSMA (1:250, Sigma-Merk, #A5228) and with Rhodamine Red™-X (RRX) AffiniPure™ F(ab')$_2$ Fragment Goat Anti-Mouse IgG (H+L) (1:300, Jackson Immunoresearch, PA, US) as secondary antibody. As control condition, fixed and permeabilized cells were stained with the only secondary antibody. After nuclei counterstaining by DAPI (Thermo Fisher Scientific), glass coverslides were mounted by ProLong Antifade

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mounting medium (Thermo Fisher Scientific). Cells were analyzed by fluorescent microscopy (Nikon).

2.10 Statistical analysis

In this work data were reported as mean ± S.E.M. Statistical differences between two mean values were analyzed by Student's T-test, as detailed in each figure legend and in results. Statistical mean differences between three or more groups were analyzed by ANOVA test, as detailed in each figure legend and in results. When appropriate, *post hoc* analysis was performed by Tukey's Honest Significant Difference (HSD) test. Correlation between two variables was analyzed by linear regression analysis.

3 Results

3.1 Enhanced chemoattractant-mediated planar migration of SRGF-ASC

In this work, we investigated the impact of the medium additive SRGF on migration potential of ASC. Thus, we expanded ASC in presence of 5% SRGF (SRGF-ASC) and, as control condition, in presence of 10% FBS (FBS-ASC). To analyze SRGF impact on ASC motility, in planar conditions and in absence of a chemotactic stimulus, we performed standard wound healing assays. Figure 1A shows a time course analysis of covered area changes in the artificial gap between cells: no significant differences in motility rate were shown between FBS-ASC and SRGF-ASC (Student's T-test for paired data between 6 and 24 hours migration mean results). Afterward, still in planar configuration, we exposed cells to a chemotactic gradient created by HT1080 CM and, as reference, by a fresh cell culture medium containing 2.5% FBS. The 2.5% FBS concentration was selected as ASC migration potential was sub-maximally stimulated in presence of 5% FBS as chemoattractant (data not shown). As displayed in Figure 1B, both FBS-ASC and SRGF-ASC actively migrated toward 2.5% FBS and toward HT1080 CM. Noteworthy, SRGF-ASC, but not FBS-ASC, showed significantly greater forward migration index toward HT1080 CM, when compared to the reference chemoattraction medium containing 2.5% FBS (p=0.008 Two-Way Factorial ANOVA for Independent Samples; *, p=0.03, *post hoc* analysis by Tukey's HSD test). Moreover, SRGF-ASC migration capacity toward HT1080 CM was significantly (§, p=0.02, *post hoc* analysis by Tukey's HSD test) higher when compared to FBS-ASC. In contrast, no differences in ASC forward migration index were identified when experiments were performed using complete cell culture medium, or HT1080 CM, both containing 10% FBS (data not shown).

3.2 Improved transmigration potential of SRGF-ASC: relationships with chemoattractant concentrations

We analyzed transmigration potential of expanded ASC by standard transwell assay. To setup the experimental conditions, we tested the impact on ASC migration taking advantage of different septa separating upper and lower transwell chamber. In such experiments, we used as chemoattractant a cell culture medium containing 10% FBS. As displayed in Figure 2A, both FBS-ASC and SRGF-ASC showed a comparable very high migration potential across POLY septa. On the other hand, when PET septa were used, SRGF-ASC transmigration potential toward the chemoattractive stimulus was markedly and significantly higher than FBS-ASC (p=0.04; Two-Way Factorial ANOVA for Independent Samples; p=0.03 *post hoc* analysis by Tukey's HSD test). For such reasons, we further investigated the impact of SRGF on ASC transmigration

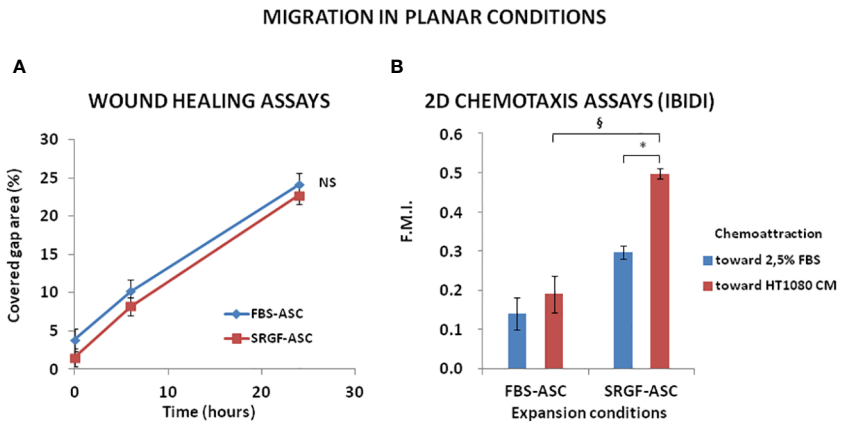

FIGURE 1
FBS-ASC and SRGF-ASC migration in planar conditions, with or without chemotactic stimulus. **(A)** shows, in a time course analysis, that, without chemotactic stimulus, there is not significant difference between FBS-ASC and SRGF-ASC motility rate. (Student's T-test for paired data between 6 and 24 hours migration mean results). NS, not significant. **(B)** shows FBS-ASC and SRGF-ASC actively migrated toward 2.5% FBS and toward the HT1080 CM (F.M.I., mean Forward Migration Index). SRGF-ASC, but not FBS-ASC, showed greater migration potential toward HT1080 CM, *vs* 2.5% FBS (p=0.008; Two-Way Factorial ANOVA for Independent Samples; *, p=0.03 *post hoc* analysis by Tukey's HSD test). Independently from chemoattraction type, SRGF-ASC showed a far greater migration capacity *vs* FBS-ASC (§, p=0.02, *post hoc* analysis by Tukey's HSD test).

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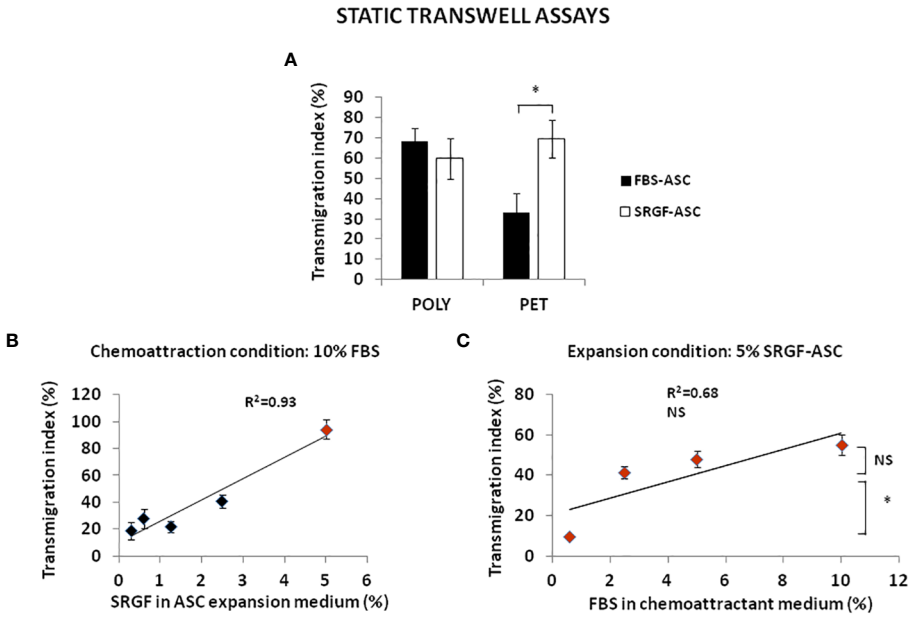

FIGURE 2
FBS-ASC and SRGF-ASC transmigration potential, analyzed by standard transwell assay in static conditions. **(A)** shows that both FBS-ASC and SRGF-ASC strongly migrated across polycarbonate (POLY) septa, without significant differences. When polyester (PET) septa were used, SRGF-ASC transmigration potential was markedly and significantly higher *vs* FBS-ASC. (p=0.04; Two-Way Factorial ANOVA for Independent Samples; *, p=0.03 *post hoc* analysis by Tukey's HSD test). **(B)** shows the linear relationship between SRGF concentrations in ASC expansion medium (5 days culture) and ASC transmigration potential, using 10% FBS as chemoattractant (Linear regression analysis: p=0.02; R^2 = 0.93). **(C)** demonstrates that FBS concentrations in the chemoattraction medium are not directly correlated to transmigration potential of 5% SRGF-ASC (Linear regression analysis: not significant; R^2 = 0.68). Differences between mean transmigration indexes measured when ASC migrated toward 2.5% FBS or 10% FBS were not significant. Otherwise, cell migration potential was significantly decreased when 0.6% FBS was used. (p=0.03 ANOVA for repeated measures; *, p=0.04 *post hoc* analysis by Tukey's HSD test). NS, not significant.

potential using PET septa only. We cultured, for at least 5 days, our ASC in cell culture media containing different concentrations (0.3; 0.6; 1.25; 2.5; 5% vol/vol) of SRGF as medium additive. As depicted in Figure 2B, using the same chemoattractant stimulus, i.e. a cell culture medium containing 10% FBS, migration potential of SRGF-ASC was shown to be significantly correlated to percent SRGF concentrations used for previous ASC expansion (Linear regression analysis: p=0.02; R^2 = 0.93). Analogous assays involving ASC expanded in presence of FBS were not feasible as decreasing FBS concentrations below 10% caused an unacceptable reduction of cell proliferation rate and/or viability (data not shown). Otherwise (Figure 2C), focusing on ASC expanded in presence of 5% SRGF, the FBS concentration in the chemoattraction medium (lower chamber), was not directly correlated to ASC migration potential (Linear regression analysis: not significant; R^2 = 0.68). In particular, a steep decrease in cell migration potential was observed when FBS was used as chemoattractant at concentrations below 2.5% (p=0.03 ANOVA for repeated measures; *p=0.04 *post hoc* analysis by Tukey's HSD test).

3.3 Increased transmigration of SRGF-ASC toward cancer derived chemoattraction: results in static and dynamic conditions

We investigated the impact of cancer cell derived chemotactic stimulus on transmigration of SRGF-ASC. Results summarized in Figure 3A were obtained using standard static transwell assays. Both FBS-ASC and SRGF-ASC showed a greater migration potential toward HT1080 CM, than in the direction of a reference medium that contained only 5% FBS (p=0.002; Two-Way Factorial ANOVA for Independent Samples; *, p=0.04 *post hoc* analysis by Tukey's HSD test). Interestingly, SRGF-ASC better migrated toward HT1080 CM, when compared to FBS-ASC (§, p=0.03 *post hoc* analysis by Tukey's HSD test). In turn, we analyzed SRGF-ASC migration potential toward T98G and HepG2 CM: as shown in Figure 3B, when compared to HT1080, SRGF-ASC equally migrated toward HepG2 and T98G derived chemotactic stimuli (NS, not significant, ANOVA for independent measures). Thus, in order to better define SRGF impact on ASC diapedesis potential, we took advantage of a challenging migration assay, as the millifluidic transwell chamber. In such experimental setting, the bottom chamber (i.e. below the PET septum) containing the chemotactic medium, was kept in static conditions. Otherwise, expanded ASC were allowed to flow through the upper chamber within a close circuit system, perfused by a peristaltic pump. As displayed in Figure 4A, we demonstrated that, in analogy with abovementioned results, migration rate of SRGF-ASC was not directly related to FBS concentrations in the lower chamber (Linear regression analysis: not significant; R^2 = 0.55) and that the fraction of migrating cells markedly decreased when FBS was reduced below 2.5% (p=0.008 ANOVA for repeated measures; *p=0.006 *post hoc* analysis by Tukey's HSD test). Millifluidic experimental conditions were

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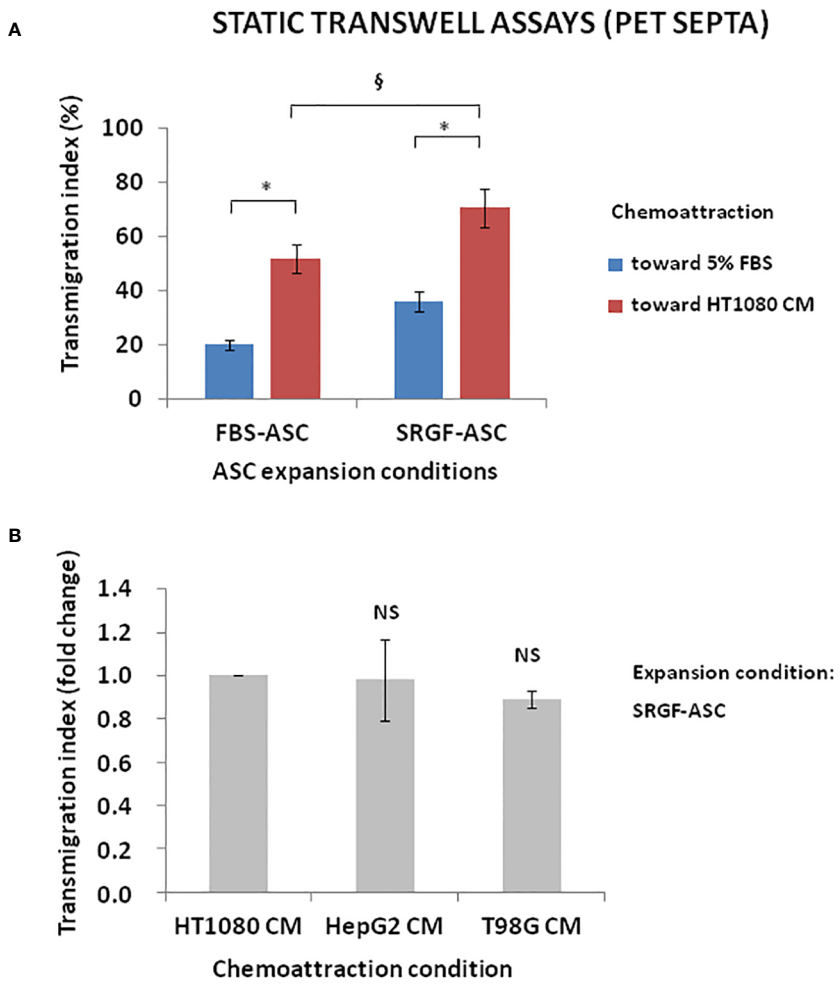

FIGURE 3
ASC transmigration in static conditions toward cancer cell CM. **(A)** shows that both FBS-ASC and SRGF-ASC were characterized by a greater migration potential toward HT1080 CM than toward 5% FBS (p=0.002; Two-Way Factorial ANOVA for Independent Samples; *, p=0.04 *post hoc* analysis by Tukey's HSD test). SRGF-ASC better migrated toward HT1080 CM when compared to FBS-ASC ([§], p=0.03 *post hoc* analysis by Tukey's HSD test). **(B)** shows that SRGF-ASC migration potential (fold change) toward T98G and HepG2 CM was not different, when compared to HT1080 (NS, ANOVA for independent measures). NS, not significant.

considered as most challenging for ASC adhesion and migration and, thus, CM and reference medium containing 10% FBS were adopted in the experimental setup. As reported in Figure 4B, both FBS- and SRGF-ASC displayed an higher migration potential, through the porous septum, toward HT1080 CM, when compared to the reference chemoattraction medium (p=0.008 Two-Way Factorial ANOVA for Independent Samples; *, p=0.04 *post hoc* analysis by Tukey's HSD test). Moreover, the migration potential of SRGF-ASC toward HT1080 CM was significantly higher, when compared to FBS-ASC ([§], p=0.03 *post hoc* analysis by Tukey's HSD test). To further complete the analysis of SRGF impact on ASC migration potential, FBS- and SRGF-ASC were expanded as floating spheroids, to be in turn dissociated into single cells (SCDS) and injected in the millifluidic tranwell system. Also in such conditions (Figure 4C), HT1080 CM increased migration potential of both FBS- and SRGF-ASC, when compared to the reference chemoattraction condition (p=0.01 Two-Way Factorial ANOVA for Independent Samples; *, p=0.04 *post hoc* analysis by Tukey's HSD test). Again, the migration capacity of SRGF-ASC toward HT-1080 medium was significantly higher, when compared to FBS-ASC (*, p=0.04 *post hoc* analysis by Tukey's HSD test).

3.4 Increased migration capacity of SRGF-ASC is mediated by αSMA downregulation

We attempted to elucidate potential mechanisms underlying the impact of SRGF on migration capacity of ASC. We analyzed, by western blot, expression level of selected proteins extracted from FBS- and SRGF-ASC cultured in transwell chambers, with or without a chemotactic stimulus (10% FBS medium). As expected, the chemotactic stimulus significantly increased (Figure 5), the availability of phosphorylated FAK (p=0.03 Two-Way Factorial ANOVA for Independent Samples; *, p=0.03 *post hoc* analysis by Tukey's HSD test), while total FAK expression levels were not differently regulated by chemoattraction, or expansion conditions.

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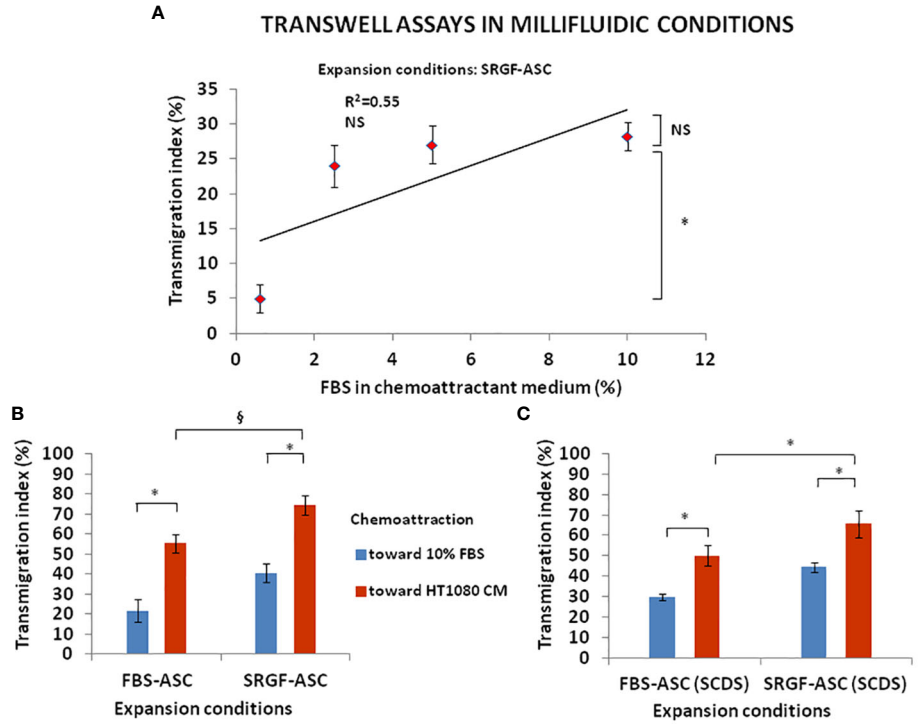

FIGURE 4
FBS-ASC and SRGF-ASC migration potential in millifluidic conditions. **(A)** shows that, in millifluidic conditions, migration rate of SRGF-ASC was not directly related to FBS chemoattractant concentrations (Linear regression analysis: not significant; R^2 = 0.55), and that migrating cells markedly decreased when FBS was reduced below 2.5% (p=0.008 ANOVA for repeated measures; *p=0.006 *post hoc* analysis by Tukey's HSD test). NS, not significant. **(B)**, shows that both FBS-ASC and SRGF-ASC better migrated toward HT1080 CM, *vs* the reference chemoattraction medium (p=0.008 Two-Way Factorial ANOVA for Independent Samples; *, p=0.04 *post hoc* analysis by Tukey's HSD test). The migration potential of SRGF-ASC toward HT1080 CM was significantly higher, *vs* FBS-ASC (§, p=0.03 *post hoc* analysis by Tukey's HSD test). **(C)** shows transmigration potential, in millifluidic tranwell system, of FBS-ASC and SRGF-ASC expanded as single cells dissociated from spheroids (SCDS). SRGF-ASC better migrated than FBS-ASC, both toward 10% FBS or toward HT1080 CM. HT1080 CM increased migration potential of both FBS-ASC and SRGF-ASC *vs* the reference chemoattraction condition (p=0.01 Two-Way Factorial ANOVA for Independent Samples; *, p=0.04 *post hoc* analysis by Tukey's HSD test). The migration capacity of SRGF-ASC toward HT-1080 medium was significantly higher, when compared to FBS-ASC (*, p=0.04 *post hoc* analysis by Tukey's HSD test).

Expression levels of VINC, phosphorylated FLNA, total FLNA, and PAX were not affected by the chemotaxis, and neither by ASC expansion conditions (Figure 5). Otherwise, in SRGF-ASC, αSMA availability was extremely lower than in FBS-ASC, independently from ASC exposure to a chemotactic stimulus (Figure 5) (p=0.01 Two-Way Factorial ANOVA for Independent Samples; §, p=0.005 *post hoc* analysis by Tukey's HSD test). To challenge the hypothesis that SRGF increases ASC reducing αSMA levels, we overexpressed GFP-αSMA in our SRGF-ASC. As shown in Figure 6A, optimal production of GFP-αSMA was achieved 24 hours after transfection of 30 pg of DNA vector per seeded cell. As demonstrated by immunofluorescence assay (Figure 6B), after GFP-αSMA transfection, SRGF-ASC cells expressed a markedly higher amount of intracellular αSMA, when compared to non transfected cells. Noteworthy, SRGF-ASC overexpressing GFP-αSMA recapitulated cell morphology of FBS-ASC (Figure 6B). Thus, 24 hours after DNA delivery to SRGF-ASC, we initiated a transwell assay lasting 24 additional hours. As demonstrated in Figure 6C, migration capacity of SRGF-ASC overexpressing GFP-αSMA was strongly reduced when compared to SRGF-ASC expressing the only GFP protein (p=0.009; Student's T-test for paired data).

4 Discussion

ASC can be used for cell therapy applications in several clinical settings in virtue or their homing potential toward inflammation sites and cancer masses (2). In fact, they can be potentially used as drug delivery vehicles (20) or as anti-inflammatory agents specifically favouring local tissue regeneration and microenvironment repair (21). Migration is one of the basic steps of the ASC homing process toward the target (22). Expansion of ASC is required to apply such cell product in clinical settings and it must be performed in compliance with GMP guidelines, preserving integrity of ASC biological features. SRGF was previously shown as a suitable ancillary product to expand ASC for potential applications in human cell therapy (3). Such growth factor mixture, in fact, is devoid of animal components and it can be considered as a standardized ancillary product, suitable for the GMP compliant and consistent expansion of ASC (5). SRGF can strongly increase ASC proliferation rate (3), but the impact of such compound on ASC migration was not previously assessed. Thus, this work was principally aimed to investigate the impact of SRGF on motility and migration properties of ASC after *ex vivo* expansion in GMP compatible conditions. We adopted 5% (vol/vol), as highest

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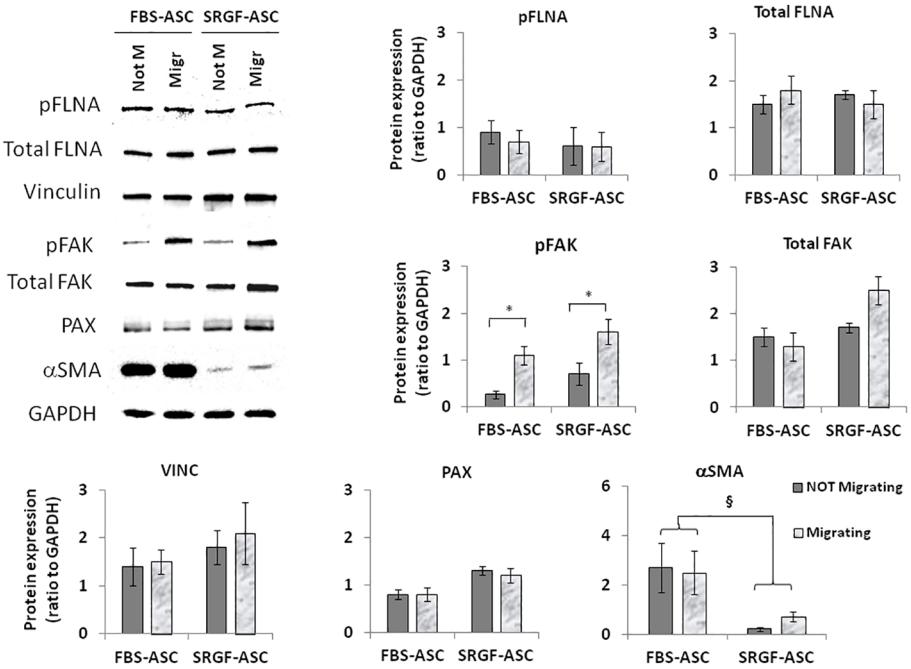

FIGURE 5
Representative images and quantification of western blot analysis performed on not-migrating (Not M) or migrating (Migr) FBS-ASC and SRGF-ASC. Migration was triggered using a 10% FBS medium. The chemotactic stimulus increased the availability of phosphorylated FAK (p=0.03 Two-Way Factorial ANOVA for Independent Samples; *, p=0.03 *post hoc* analysis by Tukey's HSD test), while total FAK expression levels were not differently regulated by chemoattraction, or expansion conditions. Expression levels of VINC, phosphorylated FLNA and PAX were not affected by chemotaxis or by ASC expansion conditions. Otherwise, in FBS-ASC, αSMA availability was extremely higher than in SRGF-ASC, independently from ASC exposure to a chemotactic stimulus (p=0.01 Two-Way Factorial ANOVA for Independent Samples; §, p=0.005 *post hoc* analysis by Tukey's HSD test).

SRGF concentration in cell culture, as we previously showed (3) that further increasing the concentration of such ancillary product didn't improve biological features of such cells. We here showed that, in absence of a chemotactic stimulus, SRGF failed to significantly affect cell motility, i.e. the rate of gap closure in wound healing assays. Previous publications investigated the impact of medium supplementation with an ancillary product defined as platelet lysate. This product shares similarities with SRGF and, in accordance with our results, authors (23) showed that the rate of wound healing was not significantly modified by platelet lysate addition to the cell culture medium. Nevertheless, in scratch assays performed with mesenchymal stem cells derived from rodents (24), platelet lysate supplementation to the cell culture medium was shown to improve gap closure rate. Interestingly, our results showed that ASC expansion in a 5% SRGF containing medium, improved cell migration properties toward a chemotactic gradient, both in planar 2D conditions, or in standard transwell assays. In the planar 2D setting, ASC migration potential was sub-maximally stimulated by 5% FBS as chemoattractant (data not shown), and this flattened differences between FBS- and SRGF-ASC. After preliminary assays, we empirically selected 2.5% FBS as chemoattractant concentration in planar 2D chemotaxis assay: in such conditions, differences between FBS- and SRGF-ASC were clearly evident. Interestingly, we found that SRGF-ASC were particularly attracted by different growth media in which previously expanded cancer cell lines released chemoattractive substances. In this simplified model, such condition resembles the process of ASC tumor specific homing. At our knowledge this is the first report directly showing that ASC expansion in presence of human growth factors derived from platelets can amplify cell migration capacity toward an artificial chemotactic stimulus or toward cancer cells. A previous work (25) showed that, after expansion in presence of platelet lysate, MSC derived from Wharton's jelly better migrated toward primary untransformed cells as fibroblasts. Only indirect evidence derived from microarray analysis previously showed that platelet lysate can downregulate transcription of migration related genes, when compared to FBS expanded ASC (26). Otherwise, in a previous work, only a statistical prediction model, considering cell dimension, proliferation rate and contact inhibition, suggested that ASC seeded at low density in presence of platelet lysate could display enhanced migration capacity (27). Otherwise, previous papers focused on platelet lysate as source of chemoattractive stimuli. In a recent work (28), MSC derived from humbilical cord and expanded in a medium containing 10% FBS better migrated, toward media containing platelet lysate at higher concentrations. The same work (28) also demonstrates that migration related genes were significantly upregulated. Similarly, migration of rat MSC, expanded in 10% FBS, was significantly stimulated by platelet lysate, as chemoattractant (29). Interestingly, our SRGF expanded ASC were equally and strongly affected by the chemoattraction exerted by each selected cancer cell line, as HT-1080 but also T98G and HepG2. In a previous work (7), we showed that, in microfluidic conditions, direct adhesion of SRGF-ASC on a monolayer of

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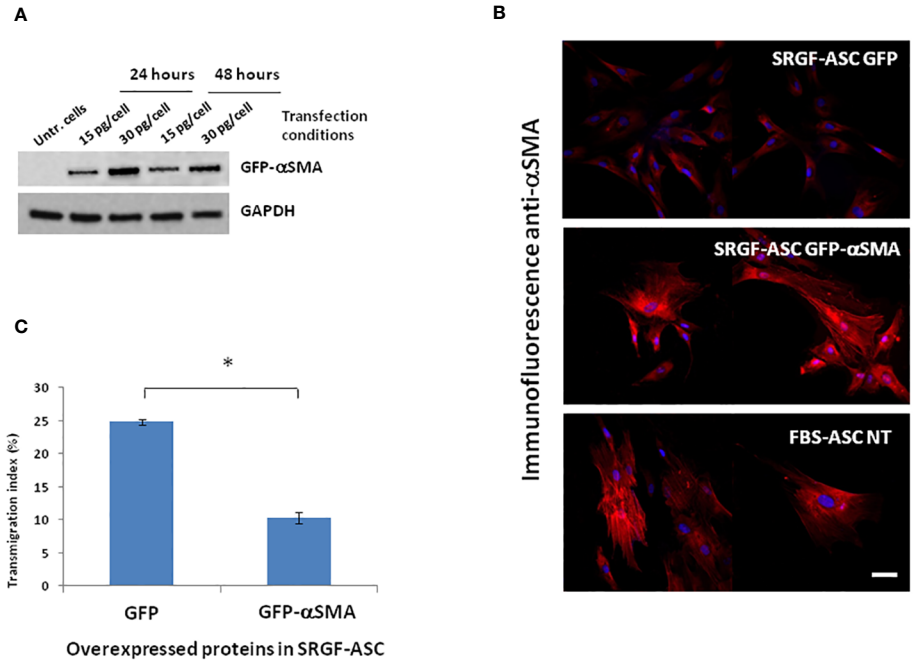

FIGURE 6
Morphology and migration potential of ASC overexpressing GFP-αSMA. **(A)** reports representative western blot showing that optimal production of GFP-αSMA was achieved 24 hours after transfecting 30 ng DNA per cell. **(B)** shows representative images of immunofluorescence assays perfomed using an anti αSMA primary antibody on fixed cells. Images of control cells stained with the only secondary rhodamin labeled antibody showed no signal (not reported). Top panel: SRGF-ASC transfected by a GFP encoding vector (control). Middle panel: SRGF-ASC overexpressing GFP-αSMA. Lower panel: non-transfected (NT) FBS-ASC. After GFP-αSMA transfection, SRGF-ASC cells recapitulated FBS-ASC morphology and expressed a markedly higher amount of intracellular αSMA, when compared to GFP transfected SRGF-ASC. **(C)** shows that migration capacity of SRGF-ASC overexpressing GFP-aSMA was strongly reduced *vs* SRGF-ASC overexpressing the only GFP protein. Only GFP or GFP-aSMA expressing cells were imaged by fluorescence microscopy and considered for transmigration index calculation (*, p=0.009; Student's T-test for paired data). White solid bar, 10 µm.

hepatocarcinoma cells was far weaker than on adherent HT-1080 and T98G. Such results are not in contrast with the present work as, we here investigated chemotaxis, but not direct cell-cell interaction, as ASC homing mechanism. Nevertheless, further investigations are required to better characterize the capacity of different hepatocarcinoma cell lines to attract ASC. As peculiar feature of this work, we also explored migration potential of our SRGF-ASC taking advantage of an *in vitro* millifluidic system, closely recapitulating microenvironmental and reologic conditions occurring *in vivo*. This system can evaluate migration properties considering also docking and firm adhesion of flowing cells onto the porous substrate. Even in this challenging conditions, SRGF-ASC demonstrated to be more prone to diapedesis than FBS-ASC, especially toward cancer cells. In these experiments FBS chemoattractant was set at 10% FBS, while in static experiments FBS was maintained at 5%. Noteworthy, we demonstrated that, both in static and dynamic settings, ASC migration is affected only when FBS chemoattractant concentration is below 5%. To perform such assays in millifluidic conditions, we chose the higher concentration (10%) of chemoattractant FBS, considering the biological complexity of the assay itself. Expanding ASC as floating spheroids in static conditions can improve anti-inflammatory, angiogenic, and tissue reparative/regenerative effects of mesenchymal stem cells (30), in turn potentially improving cell stemness. We aimed to assess if SRGF can improve ASC migration properties also when cells were expanded as spheroids, and we directly assessed this issue in the most challenging conditions of millifluidic transwell assays. We showed that, in comparison with ASC expanded as adherent single cells, ASC expansion in the condition of floating spheroid didn't confer additional advantage in terms of migration potential. Nevertheless, we confirmed that ASC derived from spheroids expanded in presence of SRGF were characterized by improved migration properties when compared to FBS expanded counterparts, especially toward cancer cells.

In order to elucidate a potential mechanism underlying the increased migration capacity of SRGF-ASC in presence of a chemoattractive gradient, we compared expression of selected candidate proteins involved in ASC migration machinery. We evidenced the upregulation of FAK phosphorylation after cell exposure to the migration stimulus. This effect was paralleled by only a minimal increase of total FAK content in migrating SRGF-ASC. Several previous publications demonstrated that cell exposure to a chemoattraction stimulus can induce FAK phosphorylation (31, 32): thus, the observed effect was considered as a demonstration that ASC actually responded to the applied chemotactic gradient. In the analysis of molecular mechanisms potentially explaining SRGF mediated impact on ASC migration capacity, ASC were exposed to the only chemotactic stimulus determined by 10% FBS, i.e. not by cancer cell CM. Such choice was taken to simplify our model,

considering the evidence that CM just increased migration properties of our SRGF cells, without differential effects on such cellular feature. In our results, ASC expansion in presence of SRGF failed to deregulate VINC and PAX expression. As above mentioned, expression of these two proteins is mandatory for cell migration, but they both appear to be not involved in the SRGF mediated increase of ASC migration potential. We previously showed that levels of phosphorylated FLNA are increased in highly migrating cells, as MSC derived from multiple myeloma patients (17). In present results, phoshorilated FLNA levels were not affected by SRGF or FBS addition to the cell culture medium or by the migration stimulus. Such evidence can be explained considering the different source of stem cells we here analyzed: multiple myeloma cells are, in fact, known to establish a cross-talk with surrounding MSC, in the bone marrow, that confers peculiar migration and proliferative properties to such stem cells (33, 34). Otherwise, our ASC were isolated form stromal vascular fraction derived from adipose tissue that was harvested from the thigh, i.e. distally form neoplastic lesions. Moreover, at the moment of liposuction, enrolled breast cancer patients were in a disease-free condition. Otherwise, independently from application of the chemotactic stimulus, αSMA expression levels were barely detectable in SRGF-ASC, while in FBS-ASC its expression was markedly higher. A previously published paper (35) confirms that MSC, expanded in presence of platelet derived growth factors, can be characterized by diminished occurrence of stress fibers, composed of actin filaments. Thus, we challenged the hypothesis that reduced αSMA expression could improve ASC migration. Artificial silencing of αSMA expression in FBS-ASC would not be technically feasible as transient transfection of such cells is poorly efficient (36). So, we overexpressed αSMA in our SRGF-ASC, and we showed that cells rapidly changed their morphology and stress fiber composition, resembling features of FBS-ASC. More importantly, we showed that migration capacity was impaired in SRGF-ASC cells transiently overexpressing αSMA: this strongly suggests that changes in αSMA availability could mediate the increase of migration potential demonstrated in SRGF-ASC. In the cell model of smooth muscle cells, selective upregulation of αSMA decreased cell proliferation and migration through Rac1 inhibition, even though stress fiber formation was not affected (16). An additional work showed that (37) αSMA inhibition in fibroblasts could increase migration rate of such cells, altering the architecture of focal adhesions and, thus, possibly immobilizing cells on the adhesion surface. Other works showed that migrating liver cells exhibited more intense expression of αSMA (38), thus suggesting that such protein is required for appropriate cell contractility. We may speculate that SRGF modulates αSMA synthesis at appropriate levels, correctly mediating cell adhesion, stiffness and contractility properties, in turn allowing efficient cell migration. This work has not fully elucidated the impact of SRGF on ASC motility and migration and especially on molecular mechanisms involved in this scenario. The panel of screened proteins involved in the migration machinery was limited and we can't exclude the contribution of other factors in the SRGF mediated regulation of ASC migration potential. Moreover, further evidences in animal models could additionally shed light on SRGF mediated effects on expanded ASC, better characterizing the capacity of such cells to reach target cancer and/or inflammation sites. Results enclosed in this work can only show that, expanding ASC in presence of SRGF, could provide a cell therapy product, compatible with GMP guidelines, characterized by improved cell migration capacity and, thus, by potential utility in cancer therapy. Additional steps are required to suggest a potential clinical application of these evidences. Mesenchymal stem cells are known to play a dual role on tumors (39), nevertheless. the influence of SRGF-ASC on target cancer cells was not investigated by the present study. Similarly, results enclosed in this work didn't assess whether SRGF-ASC can play a beneficial and appropriate function on immune system effector cells (40). Further experimental campaigns are required to confirm our observations and their potential implications on cancer and inflammatory diseases: nevertheless, we previously showed that SRGF expanded ASC can be genetically modified with elevate efficiency by the electroporation approach (36). Thus, we can conclude that appropriately engineered SRGF-ASC could potentially represent a suitable tool for targeted drug delivery against cancer growth and for the control of inflammatory diseases with reduced systemic impact on off-target sites.

Data availability statement

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

Ethics statement

The studies involving humans were approved by Ethics Committee of CRO Aviano, National Cancer Institute, IRCCS. 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

FA: Conceptualization, Data curation, Funding acquisition, Investigation, Methodology, Visualization, Writing – original draft. CV: Conceptualization, Data curation, Investigation, Methodology, Validation, Writing – original draft. EL: Formal analysis, Visualization, Writing – review & editing. FR: Formal analysis, Validation, Writing – review & editing. MiM: Formal analysis, Validation, Writing – review & editing. SM: Formal analysis, Resources, Writing – review & editing. MaM: Funding acquisition, Project administration, Writing – review & editing. CD: Funding acquisition, Project administration, Resources, Supervision, Validation, Writing – review & editing.

Funding

The author(s) declare financial support was received for the research, authorship, and/or publication of this article. The project

was supported by "Finchè ci siete voi ci sono anche io" Associazione ONLUS (grant # j31I17000440007), by a seed grant 5 x 1000 (grant #J35F21000650007) and by the Italian Ministry of Health-Ricerca Corrente.

Acknowledgments

We acknowledge the precious collaboration of the Breast cancer surgery unit of the CRO Aviano, National Cancer Institute, IRCCS, Aviano, Italy and of the Dipartimento Medicina Trasfusionale at CRO Aviano site. Moreover, we are grateful to Mrs Stefania Zanolin, for her precious technical support along the whole experimental campaign.

Conflict of interest

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

Publisher's note

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TYPE Review
PUBLISHED 05 June 2024
DOI 10.3389/fimmu.2024.1412328

OPEN ACCESS

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RECEIVED 04 April 2024
ACCEPTED 22 May 2024
PUBLISHED 05 June 2024

CITATION
Chen J, Yang L, Ma Y and Zhang Y (2024) Recent advances in understanding the immune microenvironment in ovarian cancer. *Front. Immunol.* 15:1412328. doi: 10.3389/fimmu.2024.1412328

Recent advances in understanding the immune microenvironment in ovarian cancer

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The occurrence of ovarian cancer (OC) is a major factor in women's mortality rates. Despite progress in medical treatments, like new drugs targeting homologous recombination deficiency, survival rates for OC patients are still not ideal. The tumor microenvironment (TME) includes cancer cells, fibroblasts linked to cancer (CAFs), immune-inflammatory cells, and the substances these cells secrete, along with non-cellular components in the extracellular matrix (ECM). First, the TME mainly plays a role in inhibiting tumor growth and protecting normal cell survival. As tumors progress, the TME gradually becomes a place to promote tumor cell progression. Immune cells in the TME have attracted much attention as targets for immunotherapy. Immune checkpoint inhibitor (ICI) therapy has the potential to regulate the TME, suppressing factors that facilitate tumor advancement, reactivating immune cells, managing tumor growth, and extending the survival of patients with advanced cancer. This review presents an outline of current studies on the distinct cellular elements within the OC TME, detailing their main functions and possible signaling pathways. Additionally, we examine immunotherapy rechallenge in OC, with a specific emphasis on the biological reasons behind resistance to ICIs.

KEYWORDS

ovarian cancer, tumor environment, immune cells, immune checkpoint inhibitor, immunotherapy

1 Introduction

Ovarian cancer (OC) is a prevalent form of gynecological cancer, recognized for its aggressive nature and tendency to metastasize (1). Its atypical presentation poses challenges for early detection and treatment, with approximately three-quarters of OC cases diagnosed at an advanced stage (2). OC includes various types, including epithelial tumors, sex cord stromal tumors, germ cell tumors, unclassified types, and metastatic secondary tumors, with epithelial ovarian cancer (EOC) accounting for over 95% of cases (3). EOC comprises diverse histological types, grades, and molecular profiles, primarily classified into type I and type II. Type I OC typically includes endometrioid carcinoma, low-grade serous carcinoma, clear cell carcinoma, and mucinous carcinoma, often arising from atypical proliferative (borderline) tumors (4). Type I OC is associated with mutations in genes such as K-Ras and PTEN, tends to present at early stages, exhibits slow growth, and carries a favorable prognosis. Conversely, type II OC originates from serous intraepithelial carcinoma of the fallopian tube, featuring high-grade serous carcinoma (HGSC), carcinosarcoma, and undifferentiated carcinoma subtypes (5). Research indicates that inflammation and endometriosis stemming from repeated ovarian cycles contribute to the development of type II OC. This type is frequently linked to mutations in the p53 and BRCA genes, HER2 overexpression, and is often diagnosed late, leading to a poor prognosis. It tends to be highly invasive and carries a high mortality rate (6). Currently, the primary approach to treating OC involves surgical resection alongside systemic radiotherapy and chemotherapy (7). In recent years, increased understanding of tumor immunity has led to the recognition of immunotherapy as a promising therapeutic option (8, 9). Despite the effectiveness of many treatments in managing OC, the disease still exhibits high rates of recurrence and low survival rates, underscoring the need for the development of new or enhanced therapeutic strategies.Tumor development is a complex process that unfolds in multiple stages. The tumor microenvironment (TME) plays a crucial role in facilitating the uncontrolled survival and growth of tumor cells, from initial carcinogenesis to fully developed cancer. Comprising various cell types and their driver molecules, such as immune cells, interstitial cells, endothelial cells, adipocytes, extracellular matrix, cytokines, and chemokines, the TME orchestrates diverse intracellular signaling pathways (10). Recent investigations underscore the significance of both the primary site TME and the microenvironment formed by distant metastasis in driving tumor proliferation, metastasis, invasion, drug resistance, and the preservation of tumor cell stemness (11). Immune checkpoints (ICs) are molecules expressed on immune cells that modulate immune system activation. Immune checkpoint inhibitors (ICIs) act by blocking the interaction between ICs and their ligands; thus, preventing T lymphocyte inactivation and exerting an anti-tumor effect.

In the past two decades, targeting the TME has become a key therapeutic strategy for solid tumors (12). The first successful pathway involved the programmed death 1 (PD-1)/programmed death ligand 1 (PD-L1) and cytotoxic T lymphocyte-associated protein 4 (CTLA-4) pathways, which are key mediators through which cancer cells evade antitumor T-cell-mediated cytotoxicity (13). Immune checkpoint inhibitors (ICIs) targeting PD-1/PD-L1 and CTLA-4 were the earliest developed, showing remarkable benefits in specific patients and revolutionizing the treatment landscape for numerous cancers (14). While these ICIs were also trialed in OC patients, phase III trials demonstrated that ICIs, either as monotherapy or combined with chemotherapy, did not yield statistically significant survival advantages (15, 16). The strong immunosuppressive environment and the number of participants involved in the OC TME are likely reasons for these disappointing results. Current data suggests that targeting the OC TME remains a challenging endeavor. However, within the context of the OC TME, concentrating solely on T cell activity and the PD-1/PD-L1 pathway might be overly restrictive. Achieving a broader characterization and a more thorough comprehension of the intricate interactions between OC tumor cells and their microenvironment is imperative to altering this trend.

This review summarized the related studies of different cell populations that combined the TME and immunotherapy challenge in OC.

2 Overview of the TME

The TME is the cellular milieu in which neoplastic cells or cancer stem cells reside. These include the blood vessels surrounding tumor cells, immune cells, other nontumor cells, the extracellular matrix, and signaling molecules (17, 18). The interactions between these components and tumor cells have a significant impact on tumor progression (19, 20). The TME consists of cancer cells, the extracellular matrix (ECM), cancer-associated fibroblasts (CAFs), a complex network of blood vessels, and diverse immune cells, including T-cells, B-cells, and cells linked to tumor progression (TACs). Cancer cells recruit and activate immune cells and stromal components, like lymphocytes, tumor-associated macrophages (TAMs), natural killer (NK) cells, dendritic cells (DCs), tumor-associated neutrophils (TANs), and myeloid-derived suppressor cells (MDSCs) (21–23), collectively establishing an anti-tumor inflammatory microenvironment during early tumor colonization or expansion; thus, impeding tumor growth (24, 25). However, prolonged exposure to tumor antigens and immune activation can deplete or alter effector cells, resulting in an immunosuppressive microenvironment that fosters tumor aggressiveness (26, 27). Given that the key cellular components sustaining this immunosuppressive microenvironment also exhibit anti-cancer properties during the initial tumor phases, they represent potential intervention targets. Figure 1 illustrates the cellular constituents within the TME.

CAFs are significant components of the tumor stroma and are primarily distributed in the perivascular or tumor peripheral fibrous mesenchyme (28, 29). CAFs produce cytokines, ECM components, and associated enzymes (30, 31). The ECM offers structural support to TME cells and plays a crucial role in cellular adhesion and infiltration (32, 33). ECM deposition leads to the creation of a dense fibrous mesenchyme enveloping the tumor, making the tumor tissue stiffer and more brittle than normal tissue. The buildup of ECM results in the formation of a dense fibrous mesenchyme encircling the tumor, increasing its stiffness and fragility compared to normal tissue. This forms a physical barrier hindering immune cell penetration and obstructing the efficient delivery of anti-cancer drugs to the TME. CAFs produce matrix

metalloproteinases that can modify the ECM and release chemokines, growth factors, and proangiogenic factors, thereby facilitating the malignant transformation of tumors (34–36). Due to the rapid growth of tumors and irregular blood flow patterns, tumors often face inadequate blood supply, leading to prolonged oxygen deprivation. Consequently, the tumor environment becomes acidic due to metabolic processes generating lactate and hydrogen ions. Vascular abnormalities and metabolic imbalances trigger signaling cascades, fostering the development of an immunosuppressive TME. This environment is infiltrated by diverse immune cells, including CD8+ or cytotoxic T lymphocytes (CTLs), crucial for tumor elimination. While CD8+ or cytotoxic T lymphocytes (CTLs) target tumor cells for destruction, regulatory T cells (Tregs) suppress the activity of effector T cells and promote immunosuppression within the TME (37–39). M1-type macrophages typically release Th1 cytokines, exerting pro-inflammatory and anti-tumor effects (40, 41). However, TAMs in the TME predominantly exhibit the M2 subtype and can induce angiogenesis and tumor invasion by secreting Th2 cytokines (42, 43). NK cells can eliminate target cells through the release of granzymes and perforin or by facilitating antibody-dependent cytotoxicity via their Fc receptors (44, 45). Nevertheless, the killing activity of T cells is hindered by the accumulation of TGF-β in the TME, and the antigen-presenting function of DCs is impaired by the hypoxic and inflammatory conditions of the TME (46, 47). Additionally, MDSCs, acting as negative immune regulators within the TME, suppress T-cell activation and the functions of various immune cells (48, 49).

3 The characteristics of the TME in OC

The participation of the TME is pivotal in the advancement and dissemination of OC. Substances released within the OC TME interact with tumor cells, facilitating their invasion and metastasis (50). Moreover, the TME holds potential as both a diagnostic biomarker and a target for therapeutic intervention in OC (51). Research has indicated that CAFs stimulate the elevation of pyruvate dehydrogenase kinase 1 (PDK1) expression in cancer cells through proteins secreted within the TME. PDK1 regulates metabolism and enhances cellular adhesion, migration, invasion, angiogenesis, and anchorage-independent growth of OC cells. This, in turn, leads to tumor invasion and migration (52). TAMs often infiltrate ascites from patients with advanced OC. The overexpression of TAMs has been linked to a negative prognosis for patients with tumors. Research findings suggest that TAMs boost tumor angiogenesis by secreting factors such as vascular endothelial growth factor (VEGF), tumor growth factor-β (TGF-β), matrix metalloproteinases, hypoxia-inducible factors, and adrenomedullin (53). TAMs also release growth factors that facilitate the growth and early metastasis of OC (54). Additionally, TAMs contribute to an immunosuppressive

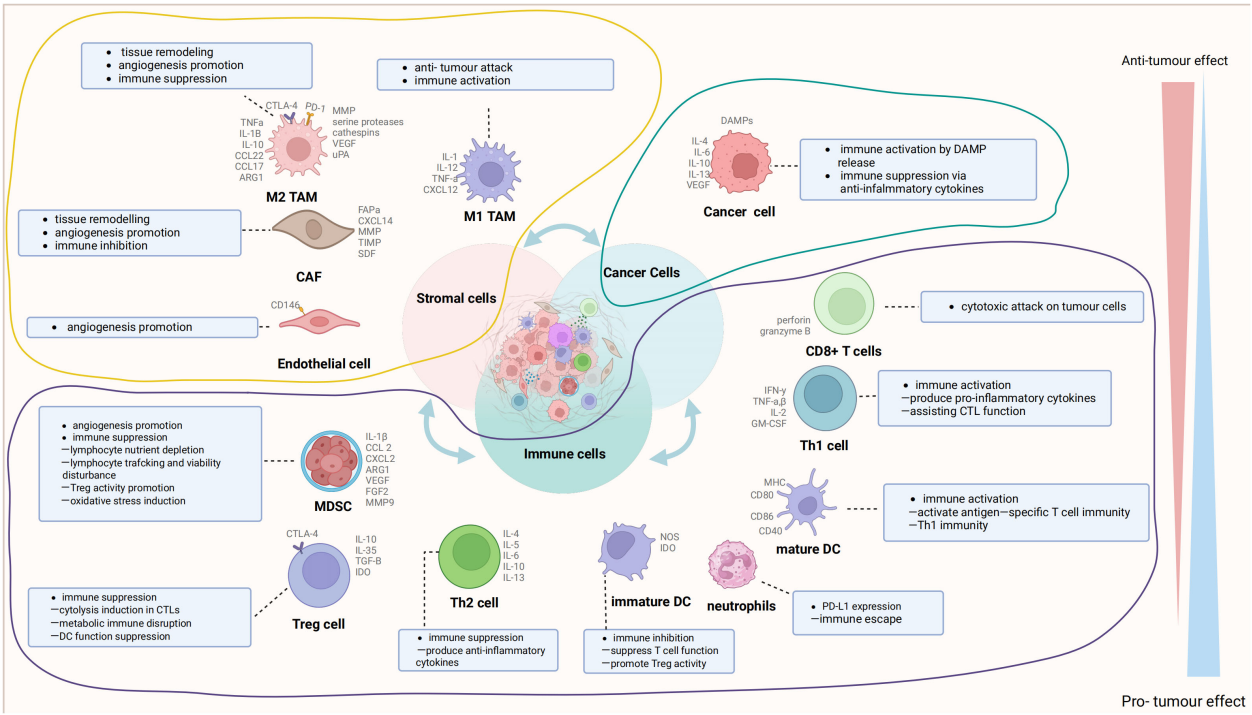

FIGURE 1
The cell com ponents in the tumor microenvironment (TME). The TME is mainly composed of cancer cells, immune cells, and stromal cells. These cells interact with receptors such as the molecules they secrete, histocompatibility complex (MHC) molecules, programmed cell death protein-1 (PD-1), and others to form an evolutionary microenvironment. The figure was generated by Biorender.com. TAMs, tumor-associated macrophages; DCs, dendritic cells; MDSCs, myeloid-derived suppressor cells; CAFs, cancer-associated fibroblasts.

microenvironment by releasing modulators that affect T-cells, thereby aiding tumor immune evasion (55). The interplay between the TME and tumor cells regulates the initiation, progression, and metastasis of OC. Some TME factors can influence how OC patients respond to treatment. Studies have shown that altering the TME can improve the efficacy of OC chemotherapy (56, 57). However, immune effector cells within the TME face inhibition not only from tumor cells but also from regulatory T cells (Tregs), immature DCs, MDSCs, and TAMs. This fosters an immunosuppressive microenvironment conducive to immune evasion (58, 59). The TIME can impact the development of OC cells. Both immune and targeted therapies against the TIME have shown promising results. Additionally, reversing the suppressive immune microenvironment in combination with antiangiogenic therapeutic regimens holds promise as a potential treatment for recurrent ovarian epithelial cancers (60, 61). The TIME could influence the development and progression of OC, which can ultimately affect patient survival.

4 The biological roles and functions of different immune cells in OC

4.1 TAMs

TAMs constitute a specific subset of macrophages crucial for shaping the TME. TAMs are commonly present in various tumor types and exhibit pro-tumorigenic activities, significantly influencing angiogenesis, metastasis, drug resistance, and anti-tumor immune suppression (62). Macrophages have the ability to polarize into two distinct activation states known as classical activation (M1 phenotype) and alternative activation (M2 phenotype) (63–65). M1 TAMs, also known as inflammatory macrophages, are stimulated by factors like IFN-γ, TNF-α, and lipopolysaccharide (LPS). They secrete pro-inflammatory cytokines such as TNF-α, IL-1β, IL-12, IL-18, and nitric oxide, exerting phagocytic and cytotoxic effects on target cells (66). Additionally, M1-like TAMs exhibit increased expression of MHC-II, CD68, CD80, and CD86 co-stimulatory markers. Conversely, M2-like TAMs, also known as macrophages with anti-inflammatory properties, are primarily induced by cytokines like IL-4, IL-13, CSF-1, IL-10, and TGF-β. M2-type TAMs produce anti-inflammatory mediators such as IL-10 and TGF-β, participate in type II immune responses, and promote tumor angiogenesis, proliferation, invasion, and metastasis. Phenotypically, M2-type TAMs express surface markers like CD206, CD163, and Arg1 (67). Parayatha et al. demonstrated the specific localization of TAMs within the peritoneal cavity of mice bearing homozygous ID8-VEGF ovarian carcinoma using nanoparticles composed of hyaluronic acid and encapsulating miR-125b (HA-PEI-miR-125b). This resulted in the repolarization of macrophages towards an immune-activated phenotype. Combining intraperitoneal injection of paclitaxel with HA-PEI-miR-125b nanoparticles augmented the anti-tumor efficacy of paclitaxel during late-stage disease progression. The study findings suggest that HA-PEI-miR-125b nanoparticles are well-tolerated and warrant further investigation in clinical trials. Bai et al. (68) observed a direct correlation between the expression level of CTHRC1 and the degree of endosomal infiltration by M2-like CD68+ CD163+ TAMs, accompanied by increased STAT6 phosphorylation in EOC. Furthermore, recombinant CTHRC1 protein (rCTHRC1) induced a dose-dependent M2-like macrophage phenotype, as evidenced by STAT6 signaling pathway activation. The conditioned cultures of Lenti-CTHRC1 EOC cells promoted macrophage M2 polarization, while CTHRC1 knockdown eliminated STAT6-mediated macrophage M2 polarization. This study suggested that CTHRC1 may have a significant impact on regulating macrophage M2 polarization in the ovarian TME, indicating its potential as a therapeutic target for antitumor immunity (69). Xia et al. revealed that Tim-4+ TAMs originate from embryonic sources and persist locally. They also observed that these Tim-4+ TAMs promote tumor growth *in vivo* and display increased oxidative phosphorylation. Additionally, Tim-4+ TAMs adapt to counter oxidative stress through mitosis. Depletion of Tim-4+ TAMs via ROS-induced apoptosis, resulting from genetic defects in the 200 kDa autophagy-related FAK family interacting protein, enhances T-cell immunity and suppresses ID8 tumor growth *in vivo*. Furthermore, the study noted similarities between human ovarian cancer-associated macrophages expressing complement receptor (CRIg) and murine TAMs expressing Tim-4 in terms of transcriptional profile, metabolism, and function. These findings suggest that targeting CRIg-positive (Tim-4-positive) TAMs could be a promising therapeutic approach for ovarian cancer patients with peritoneal metastases (70). Hoover et al. developed the IKFM transgenic mouse model to assess the impact of increased macrophage NF-κB activity on synthetic mouse models of TBR5 and ID8-Luc OC during two distinct timeframes: 1) established tumors; and 2) during tumor implantation and early tumor growth. Upon sacrifice, various parameters, including tumor weight, ascites volume, ascites supernatant and cells, and solid tumors were collected. Immunofluorescence staining and qPCR analyses were employed to investigate macrophage and T-cell populations in solid tumors and/or ascites. Additionally, ELISA was used to analyze soluble factors in ascites. Comparisons between the control and IKFM groups were performed using the two-tailed Mann–Whitney test (71). Ardighieri et al. discovered that immunoconverted HGSCs contain CXCL10-producing M1-type TAMs that closely resemble T cells. A subset of these M1-type TAMs also coexpressed TREM2. M1-polarized TAMs were almost undetectable in T-cell-poor clear cell carcinoma (CCC). Single-cell RNA sequencing confirmed the overexpression of antigen processing and gene expression programs by CXCL10+ IRF1+ STAT1+ M1-type TAMs within tumors. These results support the clinical significance of the CXCL10+ IRF1+ STAT1+ macrophage subset as a potential biomarker indicating the activation of T cells within tumors. This discovery offers a novel approach to identify patients who may have a greater likelihood of responding positively to immunotherapy targeting T cells or macrophages. In a study conducted by Le et al. (72), M2 macrophage supernatant was shown to have a modest enhancing effect on the proliferation, invasion, and migration abilities of A2780/DDP cells. However, this effect was counteracted in a manner that varied with the dose of TPL administered. Notably,

when TPL was combined with cisplatin (DDP), TPL significantly reduced the tumor burden and prolonged survival in mice through its ability to inhibit the polarization of M2 macrophages and downregulate the expression of CD31 and CD206. Additionally, the sequencing results revealed that DDP upregulated Akkermansia, while TPL upregulated Clostridium. Notably, the combined effect of DDP and TPL led to the decrease in Lactobacillus and Akkermansia abundance. These findings underscore the importance of M2 TAMs in the migratory capacity, invasiveness, and tolerance to DDP in EOC, as well as elucidate the mechanism by which TPL reverses M2 macrophage polarization (73). Long et al. uncovered a direct correlation between the malignancy level of ovarian cancer (OvCa) cells and the formation of OvCa-TAMs spheroids. They also identified that CCL18 induces macrophage colony-stimulating factor (M-CSF) transcription via zinc E box binding isozyme 1 (ZEB1) in OvCa cells, subsequently driving the polarization of M2-TAMs. Thus, a reciprocal interaction loop involving CCL18-ZEB1-M-CSF was elucidated between OvCa cells and TAMs within spheroids. The study proposes that the formation of OvCa-TAMs spheroids leads to an invasive phenotype of OvCa cells, constituting one of the specific feedback loops of CCL18-ZEB1-M-CSF. Inhibition of ZEB1 reduces OvCa-TAMs spheroids in ascites, impedes OC metastasis, and enhances the prognosis of OC patients (74). Wu et al. demonstrated that the administration of BETi resulted in a significant increase in programmed cell death among THP-1 monocytes and macrophages. Moreover, BETi selectively hindered the viability of CCR2+ macrophages while inducing their transition into a phenotype resembling M1 cells. RNA-seq analysis unveiled that BETi specifically targeted cytokines and chemokines associated with macrophage inflammation in ovarian cancer. The combined application of ABBV-075 (a BET inhibitor) and bevacizumab demonstrated enhanced efficacy in suppressing tumor growth, reducing infiltration by macrophages, and prolonging the lifespan of mice bearing tumors compared to that of the control groups or individual treatments. This study suggested that BETi plays some role in selectively targeting CCR2+ TAMs and improving the effectiveness of AVA in treating OC. Khan et al. uncovered that VSSP reduced peritoneal TAMs and prompted M1-like polarization of TAMs in an ID8 systemic model of EOC. Moreover, VSSP treatment mitigated the suppressive effects of peritoneal TAMs and granulocytes on CD8+ T cell responses to ex vivo stimuli. Additionally, ex vivo exposure to VSSP induced M1-like polarization of TAMs derived from patients with metastatic OC and differentially alleviated their suppressive phenotype. These findings indicate that VSSP reshapes myeloid responses, disrupting the inhibitory pathway and potentially enhancing the efficacy of VSSP administration in the TME to improve anti-tumor immunity (75). Werehene et al. found that increased expression of epithelial pGSN was linked to the apoptosis of M1 macrophages via augmented activation of caspase-3 and reduced production of iNOS and TNFα. Furthermore, independent prognostic analysis demonstrated that epithelial pGSN expression could forecast progression-free survival. These results suggest that pGSN regulates inflammation by modulating the abundance and function of various macrophage subtypes within the ovarian TME. Thus, targeting pGSN may offer a promising therapeutic avenue to overcome immune-mediated chemotherapy resistance in OVCA (76). Li et al. unveiled that a novel circular RNA, circITGB6, exhibited significant elevation in tumor tissues and sera of platinum-resistant OC patients. Mechanistic investigations revealed that circITGB6 directly interacts with IGF2BP2 and FGF9 mRNA, forming a circITGB6/IGF2BP2/FGF9 RNA-protein ternary complex in the cytoplasm. This complex stabilizes FGF9 mRNA and induces the polarization of TAMs toward the M2 phenotype. Furthermore, *in vivo* reversal of OC CDDP resistance was observed upon blocking circITGB6-induced M2 macrophage polarization using antisense oligonucleotides targeting circITGB6. These findings unveil a novel mechanism of platinum resistance in OC and suggest that circITGB6 could serve as a promising prognostic marker and therapeutic target for patients with this disease (62). Chen et al. revealed that myricetin inhibits the alternatively activated (M2) polarization of TAMs and reduces the secretion of tumorigenic factors by TAMs, thereby counteracting the pro-tumorigenic effect of TAMs on OC cells. Furthermore, cardamonin inhibited tumor growth and decreased the expression of CD163 and CD206 in xenografted nude mice. Additionally, STAT3 was found to be closely associated with mTOR activity. To conclude, these findings suggest that myricetin has the potential to inhibit the protumor function of TAMs by reducing M2 polarization through the inhibition of mTOR. Therefore, OC patients could benefit from the use of myricetin as a promising therapeutic intervention. Chen (77) et al. conducted a bioinformatics analysis to screen for DEGs associated with OC. They identified NEAT1 as a highly expressed gene in M2-derived extracellular vesicles (EVs) and OC cells cocultured with M2-derived EVs. NEAT1 was found to adsorb miR-101–3p, leading to increased expression of ZEB1 and PD-L1. Both *in vitro* and *in vivo* experiments illustrated that NEAT1, delivered via EVs derived from M2 cells, stimulated the proliferation of OC cells, triggered apoptosis in CD8+ T cells, and facilitated tumor growth. Research suggests that M2-derived EVs containing NEAT1 have a tumor-promoting effect on OC through the miR-101–3p/ZEB1/PD-L1 axis (78). Yin, Wang et al. found that TAMs expressing Siglec-9 were associated with an immunosuppressive microenvironment in tumors. Blocking Siglec-9 inhibited SHP-1, an inhibitory phosphatase, leading TAMs to display an anti-cancer phenotype. Combining Siglec-9 inhibition with anti-PD-1 antibody enhanced the cytotoxicity of CD8+ T cells in tissues with abundant Siglec-9+ TAMs. These findings suggest that the presence of Siglec-9+ TAMs may independently predict poor survival outcomes and serve as a potential biomarker for PD-1/programmed death ligand-1 immunotherapy in HGSC. Further exploration of targeting Siglec-9+ TAMs for therapy is warranted (79). Brauneck et al. observed that M2 macrophages in HGSOC frequently express TIGIT, CD226, TIM-3, and LAG-3 compared to HDs. Higher TIGIT expression correlated with increased tumor grades in HGSOC, suggesting prognostic significance. Blocking TIGIT reduced the frequency of M2 macrophages, and combining TIGIT and CD47 blockade enhanced phagocytosis of (OC cells by TAMs compared to CD47 blockade alone. These findings propose a combination approach of TIGIT and CD47 blockade to enhance anti-CD47

treatment efficacy. As reported by Brauneck (80), Le et al. discovered that supernatant from M2 macrophages promoted the growth, infiltration, and motility of A2780/DDP cells. However, the TPL reversed this effect in a dose-dependent manner. Moreover, the combination treatment of TPL and DDP significantly reduced the tumor burden by inhibiting the polarization of M2 macrophages. The experiment led to a longer lifespan for mice and a decrease in CD31 and CD206 levels. Sequencing showed that DDP increased Akkermansia, whereas TPL increased Clostridium. Moreover, DDP and TPL together decreased Lactobacillus and Akkermansia. These findings suggest that M2 TAMs, invasiveness, and tolerance to DDP in epithelial EOC are significantly affected by migratory capacity, and that TPL can revert M2 macrophage polarization (73). TAMs are summarized in Table 1.

4.2 CAFs

In comparison to normal fibroblasts (NFs), CAFs exhibit distinctions in morphology, function, and gene expression (81). CAFs not only foster local tumor growth but also facilitate distant metastasis through diverse mechanisms. Moreover, CAFs contribute to tumor immune evasion by secreting a variety of cytokines and chemokines that impact the recruitment and function of immune cells (35, 82). Tumor cells can evade immune surveillance by establishing an immunosuppressive microenvironment. According to Wu et al., the activation of extracellular signal-regulated kinase induced by collagen type XI alpha 1 (COL11A1) prompts the translocation of p65 to the nucleus, thereby activating TGF-β3. COL11A1 overexpression in cells promotes tumorigenesis and the formation of CAFs. TGF-β3 inhibits CAF activation, whereas TGF-β3 promotes CAF activation. COL11A1 and IGFBP2 expression is upregulated in human tumors with elevated levels of TGF-β3, which correlates with reduced survival rates. These findings suggest that targeting CAFs in ovarian tumors positive for COL11A1 could be effectively achieved through an anti-TGF-β3 treatment approach. This study was conducted by Wu (2020) and Kim et al. (year not provided). This study analyzed various CAFs isolated from OC tissues and compared their gene expression profiles. The expression profile revealed that GLIS (1 Glis family zinc finger) was among the genes whose expression increased in metastatic CAFs (mCAFs).

TABLE 1 Summary of TAMs.

Cell types	Phenotype
Inflammatory monocyte	CD14+,HLA-DR high,CD11c+,CD64+
M1 macrophage	HLA-DR+,CD68+,CD80+,CD86+
M2 macrophage	HLA-DR+,CD68+,CD163+,CD206+,CD200R
M-MDSC	CD11b+,CD33+,CD14+,HLA-DR low
G-MDSC	CD11b+,CD33+,CD15+,CD66b+,HLA-DR low

A significant increase in both gene mRNA and protein expression was observed. Reducing GLIS1 in mCAFs significantly decreased the migratory, invasive, and wound healing abilities of OC cells. Additionally, an animal study indicated that knocking down GLIS1 in CAFs reduced peritoneal metastasis. These results imply that CAFs' overexpression of GLIS1 enhances the migration and metastasis of OC cells, suggesting that targeting GLIS1 could be a promising therapeutic approach to inhibit OC metastasis (83). A study by Akinjiyan et al. found that DDR2 regulates the expression of POSTN in CAFs associated with OC. Furthermore, the presence of both DDR2- and POSTN-expressing CAFs resulted in a greater tumor load than the presence of CAFs lacking DDR2 and POSTN. Notably, coinjection of DDR2-deficient CAFs expressing POSTN with ovarian tumor cells led to a significant increase in tumor burden. These findings suggest that DDR2 regulates the expression of periosteal proliferative proteins through integrin B1 (integrin B1). There was a strong correlation between DDR2 expression in the tumor stroma and POSTN expression in the stromal cells of OC patients. Consequently, the regulation of OC metastasis through periosteal proliferative proteins is influenced by DDR2 expression in CAFs (84). Lin et al. reported that periostin (POSTN) enhanced integrin/ERK/NF-κB signaling through autocrine effects, resulting in polarization to M2 macrophages *in vitro*. Tumors overexpressing SKOV3 with POSTN contained more tumor-associated macrophages than did the controls. Similarly, the number of CAFs was increased in metastatic tumors derived from SKOV3 cells overexpressing POSTN. In terms of clinical relevance, POSTN expression correlated with late-stage disease and low overall patient survival. The results suggest that POSTN integrin NF κB-mediated signaling contributes to the enhancement of M2 macrophages and CAFs. This indicates that POSTN might serve as a valuable prognostic indicator and a potential target for therapeutic interventions. Akinjiyan et al. observed that the invasive capability of tumor cells decreased when exposed to CAF conditioned media (CM) lacking DDR2 or arginase-1. However, this invasive deficiency was not observed in cells consistently overexpressing arginase-1 (85). The presence of CM from DDR2-depleted CAFs with constitutive arginase-1 overexpression restored this invasion defect. Moreover, the supplementation of exogenous polyamines to CM derived from DDR2-depleted CAFs increased tumor cell invasion. DDR2-depleted CAFs exhibited reduced levels of SNAI1 protein in the arginase-1 promoter region. These findings illustrate how DDR2 regulates collagen production by modulating arginase-1 transcription, which is a crucial source of arginase activity and L-arginine metabolites in OC models (86). Studies indicate that CAFs can communicate with neighboring cells through exosomes, thereby participating in OC initiation and progression. Guo et al. treated CAFs with a microRNA-98–5p (miR-98–5p) inhibitor, isolated exosomes and cocultured them with OC cells. Finally, the impact of exosomal miR-98–5p on cisplatin resistance in OC cells was investigated. CDKC1A expression levels were greater in cisplatin-sensitive OC cell lines. CDKC1A expression was inhibited through the targeting action of miR-98–5p. Furthermore, CAF-derived exosomes containing miR-98–5p led to enhanced proliferation and cell cycle progression in OC cells. Additionally, exosome-mediated delivery of miR-98–5p

promoted cisplatin resistance and downregulated CDKN1A in nude mice. These experimental findings suggest that CAF-originated exosomes with increased miR-98–5p levels promote the emergence of cisplatin resistance by suppressing CDKN1A expression in nude mice (87). Cui et al. identified that miR-630 overexpression increased FAP and α-SMA levels in NFs, inducing their transformation into CAFs. miR-630 targets KLF6, and inhibiting miR-630 or enhancing KLF6 expression mitigated EV-induced CAF activation. EVs triggered the NF-κB pathway via the miR-630/KLF6 axis. The invasion and metastasis of OVCAR8 cells were enhanced by the CM from NFs that had been pretreated with EVs. However, the promotion by NFs was partially hindered when miR-630 in EVs was downregulated. These findings suggest that miR-630 is transported into NFs through EVs, leading to the activation of CAFs and facilitating OC invasion and metastasis by inhibiting KLF6 and activating the NF-κB pathway. Our study provides insight into the mechanism underlying OC invasion and metastasis within the TME (88). Sun et al. assessed the expression of secretory leukocyte protease inhibitor (SLPI) in OC cells, tissues, CAFs, and EVs, and examined the impact of exogenous SLPI on OC cells *in vitro*. The investigation revealed a significant increase in SLPI protein expression in a subset of CAFs characterized by high FAP levels and low α-SMA expression. This upregulation correlated with higher tumor grade and decreased overall survival (OS). Notably, SLPI proteins from CAFs could be encapsulated into EVs for targeted delivery to OC cells, activating the PI3K/AKT pathway. Additionally, a strong association was observed between elevated levels of encapsulated SLPI in plasma samples from OC patients and advanced tumor stage. These findings provide evidence for the oncogenic role of EV-encapsulated SLPI secreted by CAFs in driving TACs, suggesting its potential as a prognostic biomarker for OC (89). Mo et al. identified miR-141 as an exosomal miRNA that reprograms stromal fibroblasts into pro-inflammatory CAFs, promoting metastatic colonization. Mechanistically, miR-141 targets YAP1, a key effector of the Hippo pathway, and enhances the production of GROα by stromal fibroblasts. Stroma-specific knockout (cKO) of Yap1 in a mouse model results in a microenvironment enriched for GROα and promotes tumor colonization *in vivo*, but this effect is reversed by depletion of Cxcr1/2 in OvCa cells. The results highlight the relevance of YAP1/GROα in clinical samples and propose a potential therapeutic intervention to impede the formation of pre-metastatic niches and metastatic progression in OC (90). Han et al. isolated exosomes from primary omental NFs and CAFs obtained from OC patients and assessed their impact on metastasis. Among the down-regulated miRNAs by CAF-Exo, miR-29c-3p in OC tissues was associated with patient metastasis and survival. Elevating miR-29c-3p levels significantly reduced the metastasis-promoting effect of CAF-Exo by directly targeting matrix metalloproteinase 2 (MMP2). These findings provide evidence for the significant contribution of exosomes derived from omental CAFs to peritoneal metastasis in OC, which may be partly explained by the alleviation of low levels of miR-29c-3p-mediated inhibition on MMP2 expression (91). Sun et al. identified a specific overexpression of microRNA (miR)-296–3p in EVs derived from activated CAFs. The proliferation, migration, invasion, and drug resistance of OC cells were significantly increased by the upregulation of miR-296–3p in laboratory experiments. Tumor growth was also stimulated *in vivo*. Mechanistic investigations demonstrated that miR-296–3p facilitated OC progression through direct targeting of PTEN and SOCS6 genes and activation of AKT and STAT3 signaling pathways. Elevated levels of miR-296–3p within plasma-derived EVs were strongly associated with tumorigenesis and chemotherapy resistance in OC patients. These findings offer new evidence supporting the involvement of CAF-derived EVs carrying miR-296–3p in promoting OC progression and suggest the potential of miR-296–3p encapsulated within CAF-derived EVs as a diagnostic biomarker and therapeutic target for OC treatment (92). The transfer of DNA from OC cells to CAFs was facilitated by cisplatin, as discovered by Liu and colleagues. This process induces activation of the CGAS-STING-IFNB1 pathway in CAFs, resulting in the release of IFNB1. Consequently, the resistance of cancer cells to platinum-based drugs is augmented. High levels of STING expression in the tumor stroma have been correlated with poor prognosis, while inhibition of STING expression heightens susceptibility to OC. The association between the CGAS-STING pathway and platinum drug resistance in CAFs suggests that targeting STING could represent a promising approach for combination therapy in OC, offering potential opportunities for enhancing treatment outcomes (93). Jiang et al. obtained CAFs and NFs from ovarian tumors and healthy ovaries, respectively. Overexpression of miR-1290 in CAFs significantly increased viability, DNA synthesis, and cell invasion in OC cells, and altered the expression of epithelial-mesenchymal transition (EMT) markers in OC cells. Finally, overexpression of miR-1290 in CAFs increased tumor growth in a nude mouse xenograft tumor model. These findings suggest that the miRNA/mRNA axis in OC CAFs may regulate the proliferation and invasion of OC cells through the Akt/mTOR pathway (94).

Moreover, cytokines derived from CAFs also exert significant biological effects. Thongchot et al. assessed cancer cell migration using the Transwell migration assay and investigated the role of interleukin-8 (IL-8) in OC cell migration, along with its mechanistic connection to autophagy. Additionally, the pro-migratory impact of IL-8 was mitigated by pharmacologically inducing autophagy with rapamycin or metformin. Neutralizing anti-IL-8 antibodies counteracted the inhibitory effect of OVCAFs-CMMPs. The experimental results argue for the involvement of IL-8 released by CAFs in ovarian tumors. Autophagy in the ovarian TME is inhibited to promote cancer cell migration (95). Ji et al. uncovered that IL-8 secretion by CAFs can trigger the activation of normal ovarian fibroblasts (NFs) through diverse signaling pathways. Moreover, IL-8 was found to enhance the malignant growth of OC cells in animal models and increase their resistance to cisplatin (CDDP), evidenced by elevated IC50 values for OC cells. Further investigations revealed that IL-8 promotes cancer cell stemness induction via Notch3, with a positive correlation observed between elevated IL-8 levels in ascites and Notch3 expression in OC tissues. In essence, the activation of Notch3-mediated signal transduction through IL-8 secretion from CAFs and cancer cells significantly contributes to promoting stemness in human OC. These findings may offer a novel approach for treating

OC (96). Jin et al. reported that collapsin response mediator protein-2 (CRMP2) from CAFs is a key regulator mediating these cellular events in ovarian cancer (OvCA). *In vitro* investigations utilizing recombinant CRMP2 (r-CRMP2) demonstrated that this protein stimulates OvCA cell proliferation by activating the hypoxia-inducible factor (HIF)-1 alpha-glycolytic signaling pathway, invasion, and migration. Analysis of patient samples revealed abundant expression of CRMP2 in OvCA, strongly correlated with cancer metastasis and an unfavorable prognosis. Inhibition of CRMP2 in CAFs by neutralizing antibodies significantly ameliorated tumors in mice *in vivo*. Our findings provide new insight into TME-based OC treatment (97). Dai et al. reported that the migration of OC cells cocultured with CAFs was significantly enhanced. In addition, the density of CAFs in metastatic sections was greater than that in primary OC primary tumor sites. We found that co-culture of SKOV3 with recombinant human stromal-derived factor-1α (SDF-1α) significantly inhibits cisplatin-induced cytotoxicity and apoptosis in a dose- and time-dependent manner, with the CXCR4 antagonist AMD3100 blocking this effect. These results suggest CAFs may contribute to malignant OC metastasis by promoting tumor cell migration. In OC, CAFs' resistance to cytotoxic drugs may be mediated through SDF-1α/CXCR4 signaling (98). Hu et al. identified a subset of CAFs expressing INHBA as significant promoters of tumor growth and immune suppression in OC. Reanalyzing patient samples revealed metastatic tumors with high INHBA(+) CAF levels also had increased regulatory Tregs. Co-culturing human ovarian CAFs with T cells showed direct contact between INHBA(+) CAFs and T cells is crucial for promoting Treg differentiation. This involves activating autocrine PD-L1 expression in CAFs through SMAD2-dependent signaling triggered by INHBA/recombinant activin A, ultimately facilitating Treg differentiation. These findings highlight the therapeutic potential within the INHBA(+) subset for advanced OC treatment, especially considering its typically poor response to immunotherapy.

In summary, the above studies illustrate how CAFs contribute to cancer advancement through the secretion of growth factors, cytokines, and chemokines, as well as ECM degradation. Additionally, CAFs can release taste-promoting cytokines locally; thus, aiding in the spread of OC cells. Moreover, CAFs promote immune evasion by upregulating immune checkpoint ligands and immunosuppressive cytokines, hindering the infiltration of antitumor CD8+ T lymphocytes, and triggering antitumor responses by interacting with other immune cells. Increasing evidence suggests that CAFs mediate chemotherapy resistance in OC, which supports the role of CAFs as promising therapeutic targets for treating OC.

4.3 MDSCs

MDSCs constitute a highly diverse array of immune cells emerging in various physiological and pathological contexts, notably in inflammatory settings like cancer, infection, trauma, and autoimmune diseases (99). Primarily originating from the bone marrow, MDSCs include immature myeloid cells, including early granulocytes, monocytes, and other myeloid precursors, which typically mature into immune cells such as neutrophils, monocytes, and macrophages under normal conditions (100). However, in inflammation or the TME, the differentiation of MDSCs is disrupted by signaling molecules such as cytokines and growth factors, resulting in their accumulation. MDSCs suppress immune responses by inhibiting T-cell activation and proliferation, promoting regulatory Treg development and function, and modulating other immune cell activities (101). Their accumulation is a key mechanism enabling tumors to evade immune surveillance and fuel tumor growth. MDSCs directly support tumor cell survival and dissemination through immunosuppression and also contribute to angiogenesis and TME alterations (102). Furthermore, MDSCs indirectly facilitate tumor development by promoting tumor cell invasiveness and metastasis (103).

MDSCs consist of two main groups of cells, mononuclear MDSC (M-MDSC) and polymorphonuclear MDSC defined as CD11b+Ly6ChighLy6G- cells and CD11b+Ry6ClowLy6G- cells, respectively (104); therefore, individuals equivalent to M-MDSCs are defined as CD33+CD14+HLA-DR -/low CD15 - cells, while PMN-MDSCs are defined as CD13+CD14-CD15+ or CD33 +CD14-CD66b+ cells. Abundant M-MDSCs have been observed in the peripheral blood and ascites of OC patients, with their accumulation and inhibitory activity primarily attributed to ascites-derived IL-6 and IL-10, along with downstream STAT3 signals (105). Okta et al. found a correlation between elevated M-MDSC quantities in tumors and the progression to advanced stages and higher grades of EOC. They also noted differences in immunosuppressive patterns between EOC patients and healthy donors, with a significant increase in ARG/IDO/IL-10-expressing M-type and PMN-MDSCs in the blood of EOC patients. The accumulation of these subpopulations positively correlated with TGF-β and ARG1 levels in plasma and peritoneal fluid (PF). In OC patients, prolonged survival was significantly associated with reduced levels of circulating and intratumoral M-MDSCs, indicating their potential clinical significance (106). Horikawa et al. observed that treatment with anti-VEGF led to the up-regulation of granulocyte-monocyte colony-stimulating factor (GM-CSF), promoting the migration and differentiation of MDSCs while inhibiting the proliferation of CD8+ lymphocytes. Targeting GM-CSF improved therapy efficacy by reducing MDSC infiltration and increasing CD8+ lymphocytes. Additionally, enhanced expression of GM-CSF was found in bevacizumab-resistant patients, suggesting that GM-CSF plays a role in recruiting MDSCs to suppress tumor immunity induced by hypoxia caused by anti-VEGF therapy. Targeting GM-CSF could overcome resistance to this therapy for OC (107). In their study, Li et al. identified various genes that were differentially expressed in EOC cells. Notably, when EOC cells were cocultured with MDSCs, a significant increase in the expression level of colony-stimulating factor 2 (CSF2) was observed. Furthermore, successful depletion of CSF2 has been accomplished in these cells. Interestingly, the downregulation of CSF2 expression effectively counteracted the increase in EOC cell stemness induced by MDSCs. Additionally, inhibition of p-STAT3 also led to a significant reversal in the

promotion of EOC cell stemness caused by MDSCs. In addition, CSF2 expression levels correlated with EOC clinical stage. These findings suggest that MDSCs enhance EOC cell stemness by inducing the CSF2/p-STAT3 signaling pathway. Enhancing the effectiveness of conventional treatments could be achieved by focusing on MDSCs or CSF2 as viable targets (108). Okla et al. discovered that M-MDSCs exhibited greater PD-L1 expression than MO/MA in both blood and ascites samples. The expression of PD-L1 was notably elevated in ICs compared with that in TCs, although PD-L1+ TC levels were more prominent in endometrioid and mucinous tumors. Furthermore, there was a direct association between the levels of circulating sPD-L1 and the numbers of PD-L1+ M-MDSCs and PD-L1+MO/MA in the bloodstream. Neither PD-L1 nor sPD-L1 served as prognostic indicators for overall survival (OS). The experimental findings suggested that while PD-L1 may not predict OC outcomes, its upregulation indicated immune impairment without prognostic implications. Moreover, PD-L1+ myeloid cells in blood correlated positively with sPD-L1, suggesting sPD-L1 might serve as a non-invasive surrogate marker for immune surveillance of PD-L1+ myeloid cells in OC (109). McGray et al. proposed exploring other therapeutic combinations to enhance CD8+ T cell function using the primary/booster vaccine platform. They observed moderate tumor control enhancement with CD27 agonists or antibody-mediated granulocyte depletion post-vaccination, while adding anti-PD-1 therapies further improved treatment outcomes. These findings underscore the potential of immunotherapies with well-defined mechanisms of action as a basis for identifying combination approaches for treating OC (110). pi et al. reported that high expression levels of mTORC2 were associated with shorter survival in EOC patients, whereas mTORC1 was unrelated to patient prognosis. Azd2014 inhibits the mTOR signaling pathway in OC cells and suppresses cell proliferation. Azd2014 specifically reduces the migration and aggregation of MDSCs in the peritoneal tissue of EOC patients and the aggregation of MDSCs in EOC peritoneal tissue but not in the spleen. In addition, AZD2014 treatment after cisplatin chemotherapy delayed the recurrence of EOC. Our findings suggest that high mTORC2 expression in EOC portends a poor prognosis. Notably, in tumor-bearing mice, AZD2014 reduced MDSC accumulation and delayed tumor growth and recurrence. (Pi 34560229). Yang et al. reported that there was an increase in the proportion of MDSCs in the peripheral blood of obese mice. Additionally, IL-6 significantly enhanced the expression levels of S100A8 and S100A9 in MDSCs. Furthermore, the infiltration of MDSCs into OCs was directly related to the level of IL-6 expression. The levels of IL-6 observed in OC tissues were positively associated with the expression levels of S100A8 and S100A9. Finally, LMT28 can inhibit tumor growth by suppressing IL-6. Obesity promotes immune evasion and metastasis in OC by upregulating IL-6 and promoting the expression of the MDSC-associated immunosuppressive genes S100A8 and S100A9 (111). Chen et al. reported that Ankrd22 knockdown increased CCR2 expression in CD11b+ Ly6G+ Ly6Clow cells and the immunosuppressive activity of PMN- MDSCs. In a mouse model of tumor xenografts, CD11b+ Ly6G+ Ly6Clow cells organized biochemically from Ankrd22-/- mice significantly enhanced the proliferation of OC cells. RNA sequencing revealed a significant increase in the expression of Wdfy1 in these cells. Furthermore, a potential small molecule compound activating ANKRD22 was found to weaken the immunosuppressive activity of Ankrd22+/+ PMN-M MDSCs. These findings suggest ANKRD22 as a promising target for reversing the immunosuppressive effects of PMN-M DSCs (112). Wang et al. explored the influence of METTL3 on IL-1β secretion and inflammasome activation in the context of OC. The study observed increased OC cell growth in Mettl3-cKO mice, along with a transition in macrophage polarization from reduced M1 to increased M2 during OC progression. Additionally, Mettl3 deficiency in myeloid cells resulted in increased secretion of CCL2 and CXCL2 in peritoneal lavage fluid. Importantly, the deletion of Mettl3 amplified IL-1β secretion induced by viable ID8 cells. These insights shed light on how METTL3-mediated m6A methylation affects the immune response against OC (100).

4.4 DCs

DCs are vital to the human immune system, serving as essential mediators in capturing, processing, and presenting antigens, and acting as a crucial bridge between innate and adaptive immunity (113). These cells are distributed throughout multiple tissues, organs, the blood, and lymphatic systems. The primary function of DCs is to recognize and capture antigens, presenting them to T cells to initiate a T-cell-mediated immune response (114, 115). After capturing antigens, DCs migrate from peripheral tissues to lymph nodes, undergoing maturation and exhibiting elevated levels of major histocompatibility complex (MHC) molecules and costimulatory molecules. The critical role of DCs in initiating and regulating the immune response highlights their importance as a research target in immunotherapy. Modifying the activity and function of DCs may lead to new vaccine developments and therapeutic approaches to effectively combat cancer and other immune-related diseases (116). Additionally, DCs play a significant role in studying autoimmune diseases, infectious diseases, and transplant rejection. Gao et al. found that high expression of Growth differentiation factor-15 (GDF-15) was linked to the infiltration of immune DCs in immunoreactive high-grade plasmacytoid carcinoma. Moreover, GDF-15 inhibited the maturation of DCs. Overexpression of CD44 in DCs inhibited GDF-15 effects on DC synapse length and number. The inhibitory effect of GDF-15 on CD11c, CD83, and CD86 expression was attenuated in DCs overexpressing CD44, and this inhibitory effect was further enhanced in DCs knocked down for CD44, while CD44 overexpression suppressed the inhibitory effect of GDF-15 on DC migration. These findings suggest that GDF-15 may inhibit the function of CD44 in DCs by interacting with it, thereby promoting immune escape from OC (117). According to a study by Luo et al., it was discovered that the Th17-DC vaccine positively impacted the TME by increasing the presence of Th17 T cells and remodeling the bone marrow microenvironment. This resulted in an improved survival rate for mice compared to those treated with a cDC vaccine. While immune checkpoint blockade (ICB) showed limited effectiveness against OC, the administration of a Th17-inducing

dendritic cell (DC) vaccination sensitized OC cells to PD-1 ICB, effectively overcoming IL-10-induced resistance. The efficacy of the Th17-DC vaccine, either alone or in combination with ICB, was found to be mediated by CD4 T cells rather than CD8 T cells, highlighting the potential benefits of utilizing biologically relevant immunomodulators like the Th17-DC vaccine in OC therapy as a means to remodel the TME and enhance clinical response to ICB therapy (118).

4.5 NK cells

NK cells are important immune cells that mediate tumor immunosurveillance (119). The recognition and elimination of target cells by NK cells do not require prior exposure to pathogens, especially those infected with viruses and mutated tumor cells (120). Immunosurveillance relies heavily on the pivotal contribution of NK cells, responding rapidly and directly killing cells that do not express sufficient amounts of MHC I molecules (121, 122). This recognition mechanism allows NK cells to bypass the immune evasion strategies of certain pathogens and tumor cells. In addition to their direct killing function, NK cells can influence and regulate other immune cells by secreting cytokines to promote an immune response (119, 123). Meer et al. found that N-803 also enhances HPC-NK cell-mediated leukemia killing. Treatment of OC spheroids with HPC-NK cells and N-803 increased target killing. In immunodeficient mice harboring human OCs, the binding of N-803 to whole human immunoglobulin supported the persistence of HPC-NK cell N-803 binding to whole human immunoglobulin, preventing Fc-mediated HPC-NK cell depletion. In addition, the combination therapy reduced tumor growth. These results suggest that N-803 is a promising agent for enhancing the proliferation and function of HPC-NK cells both in laboratory settings and in animal models. Integrating N-803 into HPC-NK cell therapies could potentially improve the efficacy of cancer immunotherapy (124). Meer et al. noted that gemcitabine did not affect the characteristics or function of HPC-NK cells, though OC cells showed increased expression of NK cell activating ligands and death receptors. While pretreatment of OC cells with gemcitabine did not enhance HPC-NK cell function, the combination of HPC-NK cells and gemcitabine was effective in killing OC cells *in vitro*. Additionally, this combination therapy decreased tumor growth in OC mouse models. These findings support that the joint application of HPC-NK cells and gemcitabine enhances the destruction of OC cells both in the laboratory and *in vivo* environments. This supports further exploration of this treatment strategy in patients with recurrent OC (125). Using a murine experimental model of advanced EOC, Vloten et al. reported that Orf virus (OrfV) is a therapeutic agent. It was demonstrated in experiments with knockout mice that OrfV therapy requires classical type 1 dendritic cells (cDC1s). In addition, cDC1s control antitumor NK and T-cell responses, thereby mediating the antitumor efficacy of OrfV. Primary tumor resection is a commonly used treatment for human patients and is effectively combined with OrfV to achieve optimal therapeutic outcomes. achieves optimal therapeutic effects. Furthermore, cDC1s were associated with NK cells in human OC, and intratumoral NK cells were positively correlated with survival. These findings demonstrate the potential of OrfV as an NK-stimulating immunotherapy for the treatment of advanced OC (126). Fraser et al. discovered that the expression of genes related to cytotoxicity and signaling pathways decreased in NK cells isolated from the ascites of OC patients. Similarly, NK cells obtained from treated healthy donors also displayed downregulation of genes involved in cytotoxic pathways. These findings indicate that both ascites and CA125 impede the anti-tumor activity of NK cells by suppressing gene expression responsible for their activation and ability to kill cancerous cells at the transcriptional level. This study provides a deeper insight into how ascites inhibits NK cell function and suggests potential strategies for reactivating these immune cells as part of OC immunotherapy (127). Raja et al. discovered that Protein phosphatase 4 (PP4) inhibitors or agents that knock down PPP4C in combination with carboplatin triggered inflammatory signaling. Inhibiting PP4 results in reduced CD8 T-cell migration. Co-culturing NK-92 cells and OC cells via PPP4C or PPP4R3B suppression enhances NK cells' ability to eliminate OC cells. In an immunocompetent mouse model, stable knockdown of PP4C significantly restrains tumor growth. These findings propose that PP4 inhibitors can stimulate inflammatory signaling and enhance immune cell response efficacy. Hence, further investigation into PP4 inhibitors' use in combination with chemoimmunotherapy for treating OC is justified (128). Luo et al.'s study illustrated that expanded natural killer cells (eNK-EXO), loaded with cisplatin, sensitize drug-resistant OC cells to cisplatin's antiproliferative effects. Additionally, eNK-EXO can activate NK cells within the immunosuppressive TME, and researchers have explored the underlying mechanism involved. In conclusion, the inherent antitumor activity of eNK-EXO suggests their potential as therapeutic agents for OC. Furthermore, utilizing eNK-EXO as carriers for cisplatin can enhance the effectiveness of drugs against drug-resistant OC cells. Additionally, eNK-EXO has shown promise in reversing the immunosuppressive effects of NK cells. These findings present significant prospects for the clinical application of eNK-EXO in treating OC and pave the way for further investigations into its efficacy in other solid tumor treatments (129). Steitz et al. discovered that tumor-associated NK cells induced TRAIL-dependent apoptosis in mesothelial cells upon encountering activated T cells. Moreover, the upregulation of TRAIL expression in NK cells and the enhanced cytotoxicity to mesothelial cells were primarily driven by T cell-derived TNFa. Importantly, apoptotic mesothelial cells were observed in the peritoneal fluid of HGSC patients. Conversely, HGSC cells exhibited resistance to TRAIL, indicating a cell type-selective killing effect of NK cells. The findings support a synergistic role of T cells and NK cells in breaching the mesothelial cell barrier in HGSC patients (130).

4.6 T cells

T lymphocytes play a pivotal role in the body's immune response against tumors, capable of combatting both pathogens

and tumor cells (131) and identifying processed antigenic fragments through the T-cell receptor (TCR) (132). The main subsets of T cells include CD4+ T cells and CD8+ T cells. CD4+ T cells primarily regulate the immune response by releasing various cytokines to activate or suppress other immune cells. CD8+ T cells, also referred to as CTLs, possess the ability to directly eliminate virus-infected cells or tumor cells (133).

Chen et al. observed a notable rise in TIGIT expression among CD4+ Tregs. Furthermore, the application of anti-TIGIT therapy led to a decline in the CD4+ Tregs proportion, with no discernible effect on CD4+ and CD8+ T cells or NK cells. Additionally, TIGIT inhibition resulted in a decrease in the level of immunosuppression induced by CD4+ Tregs. Survival analysis indicated that anti-TIGIT treatment notably enhanced the survival rate of mice with OC. These findings suggest that TIGIT contributes to enhancing the response to CD4+ Tregs and mediating immunosuppression within the OC model. Consequently, inhibiting TIGIT could be a viable therapeutic approach for patients with OC (134). Silveira et al. discovered that P-MAPA treatment elevated the levels of TLR2 and TLR4 in OC while decreasing the count of regulatory Tregs. Furthermore, the interaction of P-MAPA with IL-12 notably augmented the population of CD4+ and CD8+ effector cells in draining lymph nodes. Concerning inflammatory mediators, P-MAPA raised the levels of the proinflammatory cytokine IL-17, whereas P-MAPA + IL-12 increased the levels of IL-1β. These findings suggest that P-MAPA upregulates TLR2 and TLR4 signaling while attenuating tumor immunosuppression. Furthermore, P-MAPA, in combination with IL-12, enhances antitumor immune responses, opening a new therapeutic avenue in the fight against OC (135). Werehene et al. observed that under chemosensitive conditions, the secretion of sEV-pGSN decreased. This led to an increase in IFNg release by T cells, resulting in decreased intracellular glutathione (GSH) production and increased susceptibility of chemotherapy-sensitive cells to cis-dichloroplatinum (CDDP)-induced programmed cell death. In cases of chemotherapy resistance, OC cells showed increased secretion of sEV-pGSN, inducing apoptosis in CD8þ T cells. Consequently, IFNg secretion decreased, leading to elevated GSH production. These findings suggest that sEV-pGSN plays a role in suppressing immune surveillance and regulating GSH production, contributing to the development of chemoresistance in OC (136). Desbois et al.'s research identified two distinct features of T-cell exclusion from tumors: 1) loss of antigen presentation by tumor cells; and 2) upregulation of TGFβ and stromal activation. Moreover, TGFβ significantly inhibits T-cell infiltration. *In vitro* experiments demonstrated that TGFβ reduces MHC-I expression in OC cells. Additionally, TGFβ stimulates fibroblasts and enhances ECM production, potentially forming a physical barrier to impede T cell entry. These findings propose that targeting TGFβ could be a promising strategy to overcome immune rejection by T cells and enhance the clinical efficacy of cancer immunotherapy (137). McCaw et al. reported that the class I HDAC inhibitor entinostat upregulated pathways and genes associated with the cytotoxic function of CD8+ T cells while downregulating the expression of myeloid-derived suppressor cell chemoattractant factors. The inhibitory potential of regulatory T cells in tumors and associated ascites was significantly diminished, leading to a reversal in the CD8-Treg ratio. These findings illustrate that class I HDAC inhibition fosters intratumoral CD8 T cell activation by disrupting the suppressive network in the EOC TME. Consequently, class I HDAC inhibition may render advanced EOC susceptible to immunotherapeutic modalities (138). Sima et al. uncovered that the absence of TG2 in mice resulted in an augmentation of cytotoxic responses of CD8+ T cells specific to tumor antigens present in ascites. Additionally, the depletion of CD8+ T cells hastened the accumulation of ascites in TG2-/- mice. CD8+ T cells obtained from tumor-bearing TG2-/- mice exhibited characteristics associated with effector T cells. Mechanistically, the absence of TG2 amplifies signals that facilitate the activation of T cells. Intraperitoneally growing cancer cells produced a stronger immune response to TG2 deletion. Furthermore, TG2 expression in the stroma but not in the tumor was indirectly correlated with the number of tumor-infiltrating lymphocytes. The results demonstrated that the TME in TG2-/- mice has a reduced tumor load, enhanced T-cell activation and effector function, and loss of immunosuppressive signals. This leads to the hypothesis that TG2 is an attenuator of antitumor T-cell immunity and a novel immunomodulatory target (139). Muthuswamy et al. reported high expression of CXCR6 on chemokine receptors in OC patients. The analysis revealed a connection between CXCR6 and increased CD103 levels, correlating with enhanced patient survival. Furthermore, CXCR6 acts as an exclusive marker for tumor-specific memory CD8+ T cells residing within the tumor rather than circulating in the bloodstream. Elimination of CXCR6 in these specific CD8+ T cells leads to reduced retention within tumor tissues, resulting in a weakened resident memory response and compromised control over OC. These results emphasize the vital role of CXCR6 in immune surveillance and OC management by promoting the retention of resident memory T cells within tumor tissue. Future studies should explore utilizing CXCR6 to improve resident memory responses against cancer (140). Tsuji et al. conducted a thorough analysis at the single-cell level and proposed a model in which CD103TCF1+ recirculating T cell precursors differentiate in response to tumor antigen recognition, underscoring the significant anti-tumor function of CD103+ TRM cells in OC (141). Kamat et al. identified elevated levels of CCL23 in both ascites and plasma samples from patients diagnosed with high-grade plasmacytoid OC (HGSC). These increased levels were associated with increased expression of exhaustion markers CTLA-4 and PD-1 on CD8+ T cells in tissues exhibiting higher CCL23 levels and macrophages. Through *in vitro* experiments, it was demonstrated that CCL23 prompts the upregulation of immune checkpoint proteins on CD8+ T cells by phosphorylating GSK3β. These results underscore the role of macrophage-derived CCL23 in shaping the immunosuppressive TME in OC, promoting a depleted T cell phenotype (142). Zhu et al. reported that OC tissues had greater expression of Rab8a, Hsp90a, and Il6 than neighboring normal tissues. IL-6 levels were correlated with the number of LC3+ EVs in ascites, and the percentage of HSP90α+ LC3+ EVs and the ROMA index of patients were positively correlated. In addition, LC3+ EVs induced elevated IL-6 production by CD4+ T cells, which was inhibited by anti-

HSP90α or anti-TLR2. These findings demonstrate the associations of LC3+ EV levels and the percentage of HSP90α+ LC3+ EVs with elevated IL-6 in the ascites of EOC patients. HSP90α on human EOC LC3+ EVs stimulates IL-6 production by CD4+ T cells via TLR2 (143). In their study, Zhang et al. detected a specific group of tissue-resident memory T cells (Trm) characterized by the presence of both TIM-3 and CXCL13 markers within EOC samples. Notably, compared with other patients, high-grade plasmacytoid EOC patients with TIM-3-positive Trm cells experienced significant improvements in OS. Moreover, the presence of CXCL13-positive CD8-positive T cells demonstrated a strong association with positive responses to anti-PD1 ICIs among patients, suggesting that combining PD-1 blockers with agents targeting TIM-3 could reactivate anticancer immunity against EOC (144). Another study conducted by Vlaming et al. revealed an interesting subset of exhausted CD8+ TNFRSF1B+ T cells linked to disease progression in OC patients. Their findings from both laboratory experiments and analyses of OC patients consistently supported the notion that increased expression levels of TNFRSF1B on activated CD8+ T cells corresponded to increased clinical malignancy levels and poorer prognoses. Furthermore, the inhibition of TNFRSF1B led to a notable modification of the immune microenvironment in an OC mouse model, resulting in suppressed tumor growth. These findings highlight the potential clinical significance of targeting TNFRSF1B for immunotherapy and enhance our understanding of the factors contributing to the limited success observed in OC immunotherapeutic approaches (145). Yakubovich et al. reported that cancer cells that migrated into/out of tumors possessed more mesenchymal stromal cells than those that exited and deserted tumors. Furthermore, high LGALS3 expression was associated with EMT *in vivo*. Significantly, CD8+ T cells displayed increased expression of LAG3, a marker of T cell exhaustion. These findings suggest that the EMT process in OC cells facilitates interaction between cancer cells and T cells through the LGALS3 - LAG3 pathway, potentially leading to a decrease in T cell presence within tumor infiltrates and thereby suppressing the immune response against tumors (146).

4.7 Tregs

Tregs, a subset of immune-suppressing cells, play a pivotal role in maintaining immune system equilibrium and preventing autoimmune diseases. They regulate the activity of other immune cells through both contact-dependent and cytokine-mediated mechanisms (147). Dysregulation of Tregs is associated with the onset of various conditions, including autoimmune diseases, allergic reactions, and immune evasion in tumors (148). When activated by their environment, Tregs can dampen the anti-tumor immune response by releasing inhibitory cytokines like IL-10, IL-35, and TGF-β; thus, fostering tumorigenesis (149, 150). Tregs are distinguished by the expression of CD25 and Foxp3, a key marker crucial for their suppressive function. Xu et al. reported that after activation of TLR8 signaling in CD4+ Tregs, the proliferation of naive CD4+ T cells was greater than that in controls. Moreover, glucose uptake and glycolysis in TLR8-activated CD4+ Treg cells were decreased. In addition, TLR8 signaling downregulates the mTOR pathway in CD4+ Tregs. Pretreatment of CD4+ Tregs with 2-deoxy-d-glucose (2-DG) and Schisandra chinensis also reduced the inhibition of Teff proliferation. There were no significant differences between CD4+ Tregs pretreated with 2-DG and those pretreated with pentaphosphatoflavone prior to TLR8 signaling activation and those treated with inhibitors alone, demonstrating that TLR8-mediated reversal of the inhibitory effect of CD4+ Tregs on the microenvironment of cocultured OC cells is causally related to glucose metabolism (147). Shan et al. explored the presence of HVEM in the peripheral blood of OC patients and analyzed the proportion of CD4+CD25+Foxp3 positive Tregs cells using flow cytometry. They also established OC cell lines with varying levels of HVEM expression. Moreover, it was discovered that overexpressing HVEM enhanced the production of IL-2 and TGF-β1 cytokines, activated STAT5, and increased Foxp3 expression, ultimately resulting in an increase in Treg positivity rate. These findings provide experimental evidence elucidating how HVEM expression in OC cells can upregulate Tregs through the STAT5/Foxp3 signaling pathway, offering insights into potential clinical strategies for treating OC (151).

5 The potential of antitumor therapy on the immune microenvironment of OV

5.1 Chemotherapy

Chemotherapy is a widely employed medical treatment for cancer, involving the use of one or more chemical agents to eradicate or curb the proliferation and division of cancer cells. These chemotherapeutic agents function by damaging the DNA of cancer cells, obstructing crucial phases of cell division, suppressing necessary hormones or signals for cell growth, and facilitating the programmed death of the cancer cells themselves. Given that cancer cells often divide more rapidly than normal cells, chemotherapeutic agents are particularly effective at targeting these swiftly multiplying cells, although they may also impact other rapidly dividing healthy cells in the body, such as those in hair follicles, blood, and the digestive tract, leading to various side effects (152). Chemotherapy is critical in treating OC, with systemic administration being typical, allowing the drugs to circulate through the bloodstream and target both the primary tumor and any microscopic metastatic foci elsewhere in the body (153). Treatment usually involves a combination of drugs, predominantly those containing platinum and paclitaxel, which impede tumor growth by various mechanisms that damage the DNA of cancer cells; thus, preventing their replication and division. Chemotherapy may serve as a primary treatment (neoadjuvant or postoperative adjuvant chemotherapy) to lessen tumor size and reduce recurrence risk, or as palliative care for advanced or recurrent OC to extend survival and enhance the quality of life. Despite chemotherapy's role in treating OC, its side effects and the impact on patient quality of life must be considered. Common side effects include nausea, vomiting, hair loss, fatigue,

low white blood cell count, and anemia. Thus, the selection and management of chemotherapy regimens should consider the tumor characteristics, overall patient condition, and patient preferences. The influence of TME on the therapeutic outcomes of chemotherapy has been thoroughly investigated. Immune cells within tumors can suppress growth by disrupting immune regulatory tumor cells, yet they may also foster tumor resistance to treatment by affecting tumor immunogenicity and selecting tumor clones that contribute to immune evasion (154). Furthermore, immune cells in the TME play a dual role in cancer development and metastasis. Cells such as Type 1 helper T cells (Th1), cytotoxic T lymphocytes (CTL), and natural killer cells (NK cells) contribute to an immune-stimulating environment. Conversely, the regulatory cells of the TME, including Type 2 helper T cells (Th2), TAMs, regulatory T cells (Tregs), and myeloid-derived suppressor cells (MDSCs), create an immunosuppressive environment and are linked with adverse outcomes (155). These cells either support tumor eradication or promote tumor escape by removing immunogenic tumor cells or modifying tumor immunogenicity (156). Additionally, chemokines and cytokines are significant components of the tumor immune microenvironment (TIME), playing a pivotal role in balancing oncogenic and anti-tumor immune responses. The intricate interactions between cancer cells and immune niches influence immunotherapy and various other anti-cancer treatments. With advancements in personalized medicine and targeted therapeutic approaches, integrating chemotherapy with other treatments like targeted therapy and immunotherapy offers more options and hope for OC patients. It also highlights that combining agents targeting TME and chemotherapeutics may overcome drug resistance and yield synergistic effects.

5.2 Radiotherapy

Radiotherapy is a medical procedure that employs high-energy radiation, such as X-rays, gamma rays, or proton beams, to address cancer and certain non-cancerous conditions. This method functions by damaging the DNA of cancer cells, inhibiting their ability to divide and multiply, and ultimately resulting in their death. Radiotherapy is typically localized to the tumor area, which minimizes harm to the adjacent healthy tissue. It can be applied as an independent treatment or combined with other therapeutic strategies, such as surgery, chemotherapy, or immunotherapy, to develop a comprehensive cancer treatment plan tailored to the specific objectives and modalities of treatment. The primary limitation of radiotherapy in treating OC lies in its general indication for localized conditions, whereas OC frequently affects multiple locations on the peritoneal surface and within the abdominal cavity. Furthermore, the tissues within the abdominal cavity are particularly prone to radiation-induced damage, which restricts the safe dosage of radiation that can be administered (157). Therefore, radiotherapy, such as palliative treatment against a single residual tumor or treatment for the recurrence of OC, is mainly used in specific cases of OC, especially if multiple lines of chemotherapy are used. Nevertheless, radiotherapy continues to be an option for selected OC patients. In advanced stages of OC, radiotherapy can alleviate pain, control bleeding, or manage other symptoms; thus, enhancing the quality of life for patients. Recent advancements in radiotherapy techniques, such as intensity-modulated radiotherapy (IMRT), stereotactic radiotherapy (SBRT), and proton therapy, have significantly enhanced the precision and safety of treatments. These improvements may broaden the application of radiotherapy in OC treatment in the future.

5.3 Immunotherapy

Immunotherapy has emerged as a significant treatment modality for tumors in recent years (158). Rather than directly targeting tumor cells, immunotherapy engages the body's immune system to recognize and eliminate tumors. This category includes ICIs, immunomodulators, cancer vaccines, and cellular therapies like CAR-T therapy. ICIs are particularly crucial as they enhance the immune response by blocking checkpoint proteins within the immune system, such as PD-1, PD-L1, and CTLA-4. These proteins typically maintain immune balance and prevent the immune system from attacking normal cells, but many cancer cells exploit these mechanisms to evade immune detection and destruction. By inhibiting these checkpoints, the suppression is removed, allowing the immune system to target cancer cells effectively.

The use of immunotherapy in managing OC is increasing, although its role is less defined compared to other cancers due to the immunosuppressive microenvironment of OC, which hampers the ability of immune cells to infiltrate and eliminate cancer cells. However, initial clinical trials and studies have indicated benefits for certain OC patients, particularly those who express PD-L1 or have a high mutational burden. Research is also ongoing in utilizing cancer vaccines and cellular therapies in OC treatment. These vaccines aim to prime the immune system to recognize and attack cancer cells bearing specific antigens. While still in the early stages for OC, immunotherapy has shown promise and offers an alternative for patients who respond poorly to conventional treatments like surgery and chemotherapy. As understanding of the immune microenvironment of OC deepens and immunotherapy strategies are optimized, it is anticipated that more effective treatment options will be provided and outcomes for OC patients will improve.

5.3.1 PD-1 and PD-L1 inhibitors

PD-1 is expressed on the surfaces of immune cells, such as T cells and B cells, with two ligands, PD-L1 (B7-H1, CD274) and PD-L2 (B7-DC, CD273) (159). PD-L1 is found on subsets of activated T cells, B cells, and macrophages, while PD-L2 is primarily on antigen-presenting cells (160). In physiological conditions, the PD-1/PD-L1 axis reduces inflammation-induced tissue damage and T-cell responses, playing a role in maintaining homeostasis to prevent autoimmune diseases (161). However, when activated T cells express PD-1 combined with tumor cells expressing PD-LI, the PD-1/PD-LI axis promotes immune escape from tumor cells through a variety of mechanisms (162). The PD-1/PD-LI axis induces T-cell tolerance by inhibiting TRC signaling (163). They

can also inhibit the proliferation of CD8+ T lymphocytes to promote the death of antigen-specific T cells and tumor-infiltrating T lymphocytes, thereby suppressing the antitumor immune response (164). In addition, the PD-1/PD-LI axis can inhibit the PI3K/ALK and RAS/MEK/ERK signaling pathways to hinder the normal proliferation cycle of T lymphocytes, and the PD-1/PD-LI axis can downregulate the phosphorylation of mTOR, AKT, and ERK2 to upregulate the expression of PTEN, which promotes the transformation of CD4+ T cells into Tregs and inhibits the activity of effector T cells (165). An overview of PD-1/PD-L1 interaction-mediated T-cell inhibition is displayed in Figure 2. The immune checkpoint inhibitors used for the treatment of ovarian cancer in randomized clinical trials are summarized in Table 2.

Fukumoto et al. found that the combination of HDAC6 inhibition and anti-PD-L1 ICB exhibited a synergistic effect in OCs with ARID1A inactivation. This therapeutic strategy notably suppressed the transcription of CD274, the gene responsible for PD-L1 encoding. In preclinical mouse models treated with ACY1215, an HDAC6 inhibitor, and anti-PD-L1 ICB, there was a significant reduction in tumor burden and an improvement in survival rates. These favorable outcomes were linked to the activation and increase of IFNγ-positive CD8 T cells. These findings suggest that targeting HDAC6 in conjunction with ICB could represent a promising approach for treating cancers with ARID1A mutations (166). Shang et al. observed that transcript at the distal tip (HOTTIP), interleukin-6 (IL-6), and PD-L1 were all highly expressed in OC tissues. There was a positive correlation between IL-6 and PD-L1 levels; furthermore, HOTTIP was found to enhance IL-6 expression by binding to c-jun. This interaction subsequently promoted PD-L1 expression and facilitated immune escape in neutrophils, while inhibiting T cell proliferation and tumor immunotherapy effectiveness. The study suggests that HOTTIP's role in increasing IL-6 secretion leads to enhanced PD-L1 expression in neutrophils, impairing T cell activity and promoting immune evasion by OC cells. Thus, targeting HOTTIP may offer a potential therapeutic strategy for OC (167). Cai et al. reported that PD-L1 was not significantly expressed in the TME of human OC. In contrast, B7-H3 was found to be highly expressed in both tumor cells and antigen-presenting cells within the tumor. This discrepancy highlights the complexity of immune responses in OC and suggests that other targets like B7-H3 may be crucial in developing effective therapies. Experiments conducted on mice with ID8 OvCa tumors revealed that B7-H3 expressed by tumor cells

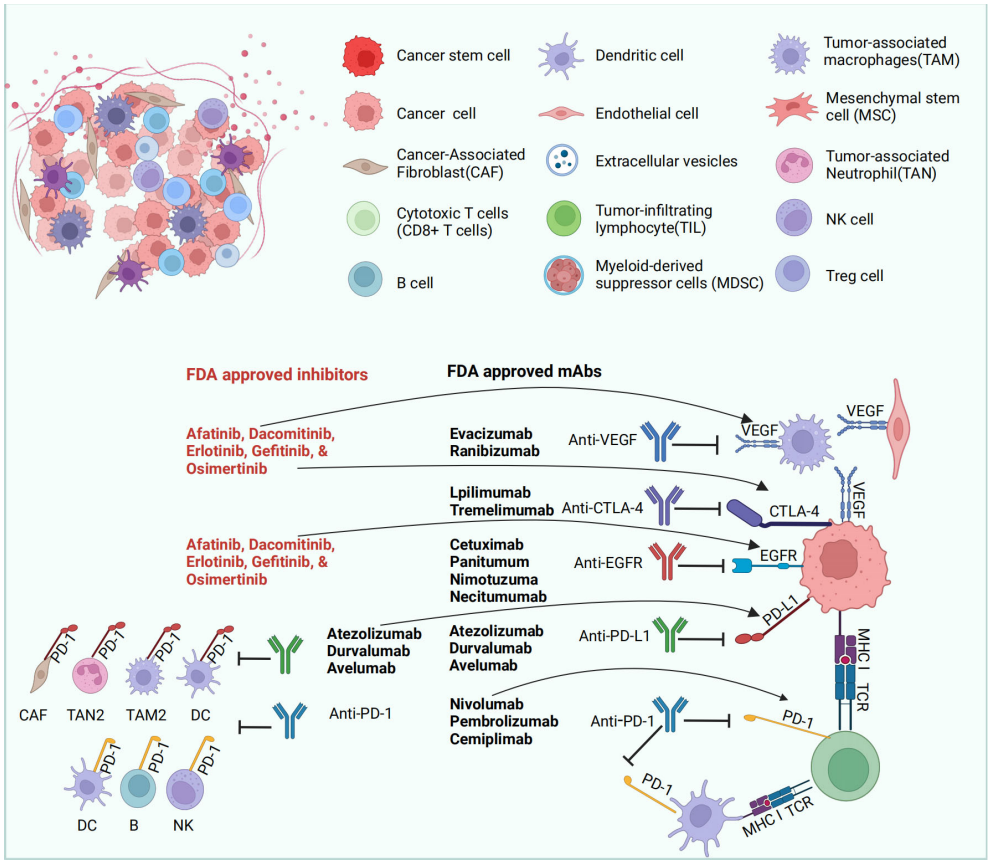

FIGURE 2
PD-1/PD-L1 interaction-mediated T-cell inhibition. Many mechanisms, such as genomic aberrations, oncogenic transcription factors and pathways, and post-translational regulation and transport, are involved in the regulation of PD-L1 expression. In addition, anti-PD-1/PD-L1 antibodies can block the activation of PD-1/PD-L1. APCs can absorb tumor antigens and regulate T-cell responses through interactions between the main MHC and TCR. APC can also regulate T-cell activity by regulating the interaction between PD-L1/PD-L2 and PD-1, as well as the interaction between B7 and CD28. The figure was generated by Biorender.com.

TABLE 2 Immune checkpoint inhibitors used related agents for ovarian cancer in randomized clinical trials.

Checkpoint inhibitor	Clinicaltrials.gov Identifier	Status	Agents	Combination agents
CTLA-4	NCT04678102	Recruiting	PHI-101	/
CTLA-4	NCT00094653	Completed	Ipilimumab	/
PD-1	NCT02674061	Completed	Pembrolizumab	Doxorubicin and Bevacizumab
	NCT02054806	Completed		
	NCT02865811	Completed		
PD-1	UMIN000005714	Unknown	Nivolumab	Ipilimumab
PD-L1	NCT01375842	Completed	Atezolizumab	Bevacizumab
PD-L1	NCT01772004	Completed	Avelumab	Axitinib, Docetaxel, and Doxolubicin
PD-L1	NCT02718417	Terminated	Avelumab	Carboplatin and Paclitaxel

strongly inhibited the immune response against tumors, while its expression on host cells did not have the same effect. Blocking B7-H3 has been found to prolong survival in mice with ID8 tumors, indicating that B7-H3 expressed by tumors may inhibit the function of CD8+ T cells. This suggests that B7-H3 could be a potential therapeutic target for patients who do not respond effectively to PD-L1/PD-1 inhibitors (168). Lampert et al. observed varied responses among some OC patients, where a lower overall response rate (ORR) and a higher disease control rate (PRþSD) were reported. Treatment was associated with enhanced expression of several factors, increased systemic production of IFNγ and TNFα, and an increase in tumor-infiltrating lymphocytes, indicative of an immunostimulatory environment. Notably, higher IFNγ levels were linked to improved PFS, whereas elevated levels of VEGFR3 were associated with reduced PFS. These observations suggest that the combination of PARP inhibition (PARPi) and anti-PD-L1 therapy has modest clinical activity in recurrent OC. The use of olaparib/duvalizumab demonstrated an immunomodulatory effect in patients, highlighting the necessity of blocking the VEGF receptor pathway to enhance the efficacy of this combination treatment (169). Zhang et al. reported that the use of abcetinib as monotherapy significantly increased CD8+ T-cell and B-cell infiltration in an ID8 mouse model of OC, indicating potential benefits of this approach in enhancing anti-tumor immunity. The analysis showed that abercetinib induced proinflammatory immune responses in the TME. Compared with control cells, abciximab-treated ID8 cells secreted more CXCL10 and CXCL13. The synergistic antitumor effect of abemaciclib and anti-PD-1 combination therapy depended on CD8+ T cells and B cells. The research argues that the combination of cyclin-dependent kinases 4 and 6 inhibitors (CDK4/6i) with anti-PD-1 antibodies enhances the effectiveness of anti-PD-1 therapies and holds significant potential for the treatment of OC characterized by poor immune infiltration (170). Wan et al. identified that bivalent antibodies activate T cells and natural killer (NK) cells, leading to NK cells transitioning from an inactive to a more active cytotoxic state. This transformation suggests a substantial role of NK cells in the immune response induced by ICB in HGSC, highlighting the involvement of previously uncharacterized CD8 T cells in mounting an immune response. The observed alterations were partially attributed to the downregulation of the bromodomain-containing protein BRD1 by double-stranded antibodies. These findings suggest that modifying the state of NK cells and T cell subsets could be essential for eliciting an effective anti-tumor immune response and propose that immunotherapies, such as BRD1 inhibitors, which can induce such changes, may enhance treatment efficacy against HGSC (171). Yang et al. discovered that the presence of CXC-chemokine ligand 13 (CXCL13) in culture stimulates the expansion and activation of CXCR5+ CD8+ T cells *in vivo*. These T cells were found to be closer to CXCL13 within tumors and showed a propensity to migrate towards it *in vitro*. Additionally, patients with higher levels of CD20+ B cells and the presence of CXCL13 exhibited improved survival rates. When used in conjunction with anti-PD-1 therapy, CXCL13 effectively hindered tumor growth by enhancing the influx of cytotoxic CD8+ T cells and promoting the maintenance of CXCR5+ CD8+ T-cells within tertiary lymphoid structures (TLS). These findings strongly advocate for targeting CXCL13 along with PD-1 blockade as a potential therapeutic strategy for treating high-grade serous carcinoma (HGSC) (172). Laumont et al. found that the presence of CD39, CD103, and PD-1 together identifies specific subsets of TILs, namely CD8þ T cells and CD4þ regulatory Tregs. The co-expression of these markers correlates with a reduced diversity in the T cell receptor (TCR) among triple-positive CD8þ TILs. Additionally, triple-positive CD8þ effector cells uniquely showed higher expression of TIGIT compared to CD4þ Tregs. This simultaneous expression signifies the involvement of these highly activated immune cells in cytolytic, humoral, and regulatory immune responses. Notably, triple-positive TILs have significant prognostic value and present promising targets for combination immunotherapy involving PD-

1 blockade as well as targeting both CD39 and TIGIT receptors (173). Seitz et al. found that overexpression of CXCL9 leads to T cell clustering, which results in delayed ascites formation and improved survival rates. This chemokine had effects similar to anti-PD-L1 therapy in an ICB-resistant mouse model, although the impacts were less pronounced in Brca2-deficient tumors. Moreover, the clear cell subtype, known for its high responsiveness to ICB among OC patients, exhibited a significantly greater prevalence of high CXCL9 tumors compared to other subtypes. These findings underscore the crucial role of CXCL9 in enhancing the efficacy of ICB treatments for preclinical OC and suggest that CXCL9 inducers could serve as both a viable predictive biomarker and a potent co-administration partner for improving ICB effectiveness in this cancer type (174). Dong et al. reported low expression of cullin 3 in OC (CUL3) and speckle-type POZ protein (SPOP). From a functional perspective, CUL3 degrades the PD-L1 protein by forming a complex with SPOP. By degrading the PD-L1 protein, CUL3 overexpression inhibited tumor formation, enhanced the chemosensitivity of mouse OC cells and reduced the malignant features and immune escape of OC cells. The findings indicate that the CUL3/SPOP complex facilitates PD-L1 degradation, thereby suppressing immune evasion and enhancing chemosensitivity in OC cells, presenting a promising therapeutic avenue for OC treatment (175). Lv-PD1-γδ T cells, developed by Wang et al., produced humanized anti-PD-1 antibodies. These cells amplified tumor cell proliferation and cytotoxicity, resulting in enhanced therapeutic efficacy and survival rates in mice harboring ovarian tumors. There was no potential tumorigenicity in immunocompromised NOD/SCID/γ-fasted mice. In addition, Lv-PD1- γδ T cells showed excellent tolerance and safety in humanized NOD/SCID/γ-fasted mice. The experimental results suggested that Lv-PD1-γδ T cells could attenuate or eliminate immunosuppression and maximize cytotoxicity by secreting anti-PD1 antibodies locally in the tumor and thus could serve as a promising anticancer "off-the-shelf" cell therapy (176).

5.3.2 CTLA-4 inhibitors

The surface of T cells expresses CTLA-4, which is a molecule that acts as an immune checkpoint with inhibitory functions. CTLA-4 regulates T-cell activation and maintains immune system self-tolerance, preventing the immune system from attacking normal tissues (161). CTLA-4 competitively inhibits immune activation signaling and reduces T-cell activity by binding to B7 molecules (CD80 and CD86) (177). By blocking the function of CTLA-4, CTLA-4 inhibitors can inhibit T-cell activation, thereby improving the capacity of the immune system to recognize and attack cancer cells (178, 179). Although CTLA-4 inhibitors have shown significant effects in boosting immune responses, they may also lead to overactivation of the immune system to attack normal tissues and cause immune-related side effects (180). With an improved comprehension of immune checkpoint pathways and the advancement of novel immunotherapeutic approaches, the anticipation is that CTLA-4 inhibitors and their combined therapies will offer more efficacious treatment alternatives for a broader spectrum of cancer patients.

Friese et al. used a CTLA-4 blocking antibody, which was added during initial tumor-inflating lymphocyte (TIL) culture, and found that CTLA-4 blockade favored the propagation of CD8+ TILs in ovarian tumor fragments. In addition, the addition of CTLA-4 blocking antibody at the initial stage of TIL culture produced more potent antitumor TILs than did standard TIL culture. This phenotype was retained during the rapid amplification phase. These findings suggest that targeting CTLA-4 in the intact TME of a tumor fragment enriches tumor-responsive TILs and thus improves the clinical outcome of TIL-based ACT in OC (181). An investigation conducted by Swiderska et al. focused on three proteins associated with the immune response, namely, PD-1, PD-L1, and CTLA-4. To assess their effectiveness, receiver operating characteristic (ROC) curves were generated, and the area under the curve (AUC) was calculated to determine the sensitivity and specificity of these parameters. Utilizing Cox regression models, both univariate and multivariate analyses were carried out during this research endeavor. The findings from this study strongly suggest that considering CTLA-4 as a prospective biomarker could prove valuable in diagnosing OC while highlighting that elevated concentrations of PD-L1 and PD-1 serve as unfavorable prognostic indicators for this particular form of cancer (182). Chen et al. performed survival analyses on a subset of patients who were followed up. The results showed that the genotype and allele distribution frequency of the rs5742909 C/T polymorphism in cytotoxic T-lymphocyte antigen-4 (CTLA-4) differed significantly between patients and controls. Compared with the CC genotype, the CT + TT genotype significantly reduced the risk of developing EOC. However, no significant correlation was detected between the rs231775 G/A and rs3087243 G/A polymorphisms and susceptibility to EOC. The results confirmed that in women, the CTLA-4 rs5742909 C/T polymorphism may decrease the genetic predisposition to EOC (183).

5.3.3 Tumor vaccines

Tumor vaccines are a new approach to immunotherapy and are considered a highly promising therapeutic modality in the field of tumor therapy. Tumor vaccines can induce body-specific antitumor immune responses by actively delivering tumor antigens to the body. Tumor vaccines can also make use of widely distributed T cells in the body to recognize and kill tumors (184). Tumor vaccines both improve the efficiency of tumor killing and overcome the drawbacks of conventional difficulties in completely removing tumors (161, 185). Currently, more clinical trials have been conducted in the field of tumor vaccines. However, most of the trials have small sample sizes, and a significant number of them have not achieved the expected results. Antigens, adjuvants, vectors, and the autoimmune status of the body play a dominant role in causing poor results in tumor vaccines. It is important to optimize the composition and timing of tumor vaccines with respect to these aspects to enhance the effectiveness of the vaccine. At the same time, the design of vaccines with individualized characteristics and preventive ability is highly important for improving tumor treatment and preventing tumor recurrence and metastasis.

Adams et al. demonstrated the potential of isolating ascites monocytes within the ID8 model and boosting their role as genuine antigen-presenting cells (APCs) by employing Toll-like receptor (TLR) 4 LPS, TLR9 CpG-oligonucleotide, and an interleukin-10 receptor (IL-10R) blocking antibody. Activated ascites monocytes efficiently curbed tumors and malignant ascites *in vivo*. Similarly, human ascites monocytes exhibited tumor-associated antigens (TAAs) under steady-state conditions. Notably, activated ascites mononuclear cells preserved their capacity to activate TALs even in the presence of ascites fluid. The findings suggest that ascites monocytes inherently harbor tumor antigens and can serve as potent antigen-presenting immune cells following a brief *in vivo* activation. This innovative ascites APC vaccine can be swiftly prepared, is straightforward, and cost-effective, making it an appealing option for OC treatment (186). Block et al. employed a Th17 induction protocol to generate DCs and loaded them with the HLA class II epitope of folate receptor alpha (FRα). Mature antigen-presenting DCs were subcutaneously injected. The majority of patients completing the respective interventions developed Th1, Th17, and FRα antibody responses post-vaccination. Prolonged relapse-free survival was associated with the presence of antibody-dependent cell-mediated cytotoxicity targeting FRα. Among the patients evaluable for efficacy, fewer than 40% remained relapse-free at the data cutoff. The findings indicate that vaccinating DCs with Th17-induced FRα loading is safe, induces antigen-specific immunity, and extends remission (187). Tanyi et al. improved vaccine-induced immune responses by combining ASA and low-dose IL-2 with the OCDC-Bev-Cy treatment. In the ID8 ovarian model, animals that received this treatment exhibited prolonged survival, elevated levels of perforin T cells within tumors and CD8+ T cells specific to neoantigens, and decreased expression of endothelial Fas ligand and intratumoral T-regulatory cells. These findings suggest that the ID8 model holds promise for the future development of OC trials (188). Nishida et al. observed a notable increase in the proportion of highly active tetrameric WT1-CTLs within CD8+ T lymphocytes (%tet-hi WT1-CTL) and a rise in WT1235-IgG levels post-vaccination. Furthermore, the elevated WT1235-IgG levels significantly prolonged progression-free survival. Unfavorable clinical outcomes associated with the WT1 vaccine included lower serum albumin levels, multiple tumor lesions, poor performance status, and excessive ascites. These results underscore that patients with refractory OC develop antigen-specific cellular and humoral immunity following WT1 vaccine administration. Both %tet-hi WT1-CTL and WT1235-IgG levels serve as prognostic markers for evaluating WT1 vaccine efficacy (189). Zhang et al. devised a fused cell membrane (FCM) nanovaccine, termed FCM-NPs. In addition, FCM-NPs exhibited the immunogenicity of tumor cells and the antigen-presenting ability of DCs and stimulated naïve T lymphocytes to produce large numbers of tumor-specific cytotoxic CD8+ T lymphocytes. FCM-NPs demonstrated robust immune activation both *in vitro* and *in vivo*. These findings highlight the potential of FCM-NPs to impede OC growth and hinder metastasis. FCM-NPs are poised to emerge as a novel tumor vaccine for OC treatment (190). As per Fucikova et al., although a higher tumor mutational burden (TMB) and significant CD8+ T-cell infiltration have been associated with improved outcomes in EOC patients undergoing conventional chemotherapy, this correlation does not apply to female patients undergoing DCVAC treatment. Conversely, positive responses to DCVAC were observed among individuals with below-average TMB levels and an excessive presence of CD8+ T-cells. These responses were accompanied by indications of enhanced effector function and specific tumor destruction within peripheral blood samples. In conclusion, our findings suggest that while heavily infiltrated "hot" EOC cases may benefit from chemotherapy, females diagnosed with less infiltrated "cold" EOC may require a dendritic cell-based vaccine as an alternative approach to elicit a clinically significant immune response against cancer (191). Zhao et al. conducted a study comparing the efficacy of co-formulated preparations of ICC and CPMV, either naturally bonded through CPMV-cell interactions or chemically coupled, with mixtures of PEGylated CPMV and ICC. The results revealed that blocking ICC interactions by PEGylation of CPMV did not significantly affect mice inoculated with the simple mixture of ICCs and (PEGylated) CPMV adjuvant. However, when co-formulated CPMV-ICCs were administered, approximately 70% of the mice survived, and 60% of the surviving mice successfully rejected tumor growth in a re-challenge experiment. These findings underscore the necessity of delivering cancer antigens and adjuvants together for effective OC vaccine development (192). Kos et al. reported a clinical therapeutic benefit in a small number of patients, including three partial responders (PR) and three patients with stable disease (SD) for more than six months. Clinical benefit was achieved in 1/3 of immunological responders and in less than 30% of evaluated patients. High levels of mRNA for various molecules linked to terminally differentiated T cells were expressed by immunological nonresponders in pretreatment peripheral blood mononuclear cell samples. These findings suggest that the combination of p53MVA and pembrolizumab immunotherapy shows promising efficacy against tumors in individuals with intact T-cell function in their peripheral blood. The identification of markers indicative of fully differentiated T cells prior to treatment could serve as a means to predict patients who are nonresponsive to p53MVA/pembrolizumab (193).

5.3.4 Chimeric antigen receptor-T cells

CAR-T-cell immunotherapy is one of the more established forms of cellular therapy and involves the artificial modification of T cells taken from the patient's body. T cells are artificially modified by the addition of chimeric antigen receptors (CARs), which can recognize tumor antigens, giving T cells the ability to recognize tumor cells. The modified T cells are then infused back into the body to achieve tumor recognition and killing (194). CAR-T-cell therapy was initially developed for certain types of hematological cancers (195) and has already achieved significant therapeutic results under these conditions. The successful use of CAR-T-cell therapy has stimulated widespread interest and research into its application in the treatment of solid tumors. The rapid generation and administration of CAR-T cells are shown in Figure 3, while the antigens used in CAR-T-cell therapy for ovarian cancer in randomized clinical trials are summarized in Table 3.

Garcia et al. reported that T cells expressing a specific chimeric antigen receptor (CAR) targeting the Müllerian inhibiting

substance type 2 receptor (MISIIR) demonstrated targeted responses when exposed to antigens under laboratory conditions, effectively eradicating tumors with overexpressed MISIIR receptors within living organisms. Furthermore, these MISIIR CAR-T cells recognized various types of ovarian and endometrial cancer cell lines and exhibited the ability to destroy tumor samples obtained from patients without harming normal primary human cells. These results demonstrate that MISIIR-targeted therapy for OC and other gynecological malignancies can be achieved using CAR technology. *In vitro* experiments conducted by Li et al. illustrated the potent capacity of chimeric antigen receptor T (CAR-T) cells to eliminate OVCAR-3 cells while releasing a plethora of cytokines. Moreover, therapeutic efficacy was observed, along with significantly prolonged survival time in OVCAR-3 tumor-bearing mice. The experimental findings revealed that the killing capability of dual CAR-T cells on OVCAR-3 cells mirrored that of single CAR-T cells *in vitro*. However, *in vivo*, the killing efficacy of dual CAR-T cells led to a noteworthy enhancement and substantial extension of the lifespan of mice harboring tumors. *In vivo*, PD1-anti-MUC16 CAR-T cells exhibited stronger antitumor activity than single CAR-T cells. The current experimental data may lead to clinical studies (196). Shu et al. designed a truncated CD47 CAR without intracellular signaling structural domains. The CD47 CAR facilitates binding to CD47+ cells; thus, increasing the possibility of eradicating TAG72+ cells via the TAG72 CAR. In addition, we can reduce damage to normal tissues by monopolymerizing CD47 CAR. These findings suggest that expressing both TAG-72 CAR and CD47-truncated monoclonal CAR on T cells may be an effective dual CAR-T-cell strategy for the treatment of OC and is also applicable to other adenocarcinomas (197). Schoutrop et al. discovered that administering CAR T cells expressing an MSLN CAR construct incorporating the CD28 structural domain (M28z) led to a significant extension in survival. Despite a modest response rate, MSLN-4–1BB (MBBz) CAR T cells achieved long-term remission. Intratumorally infiltrated M28z and MBBz CAR T cells exhibited upregulation of PD-1 and LAG3 in response to antigens, while the respective ligands were expressed by MSLNþ-positive tumor cells. This suggests that immunosuppressive pathways within the ovarian tumor microenvironment hinder the persistence of CAR T cells. These findings highlight the promise of MSLN-CAR T cells for OC treatment (198). Guo et al. focused on the development of 5T4 CAR-T cells, which are second-generation human CAR-T cells engineered to specifically target the 5T4 protein. These CAR-T cells not only secrete cytotoxic cytokines but also exhibit lysogenic cytotoxicity against tumor cells. Additionally, the adoptive transfer of 5T4 CAR-T cells significantly delayed tumor formation. These findings underscore the potential effectiveness and viability of employing 5T4 CAR-T cell immunotherapy for OC treatment and lay a robust theoretical groundwork for future clinical studies targeting 5T4 cells (199). Liang et al. devised an innovative strategy to enhance the efficacy of CAR-T cell therapy by designing a tandem CAR that targets two antigens, one of which exhibits secretory activity (IL-12). *In vitro* experiments demonstrated that compared with single-target CAR-T cells, tandem CAR-T cells efficiently killed antigen-positive OC (OV) cells and showed enhanced secretion of cytokines. More importantly, the tandem CAR-T cells prolonged the survival of mice by reducing tumor size and enhancing antitumor activity. The

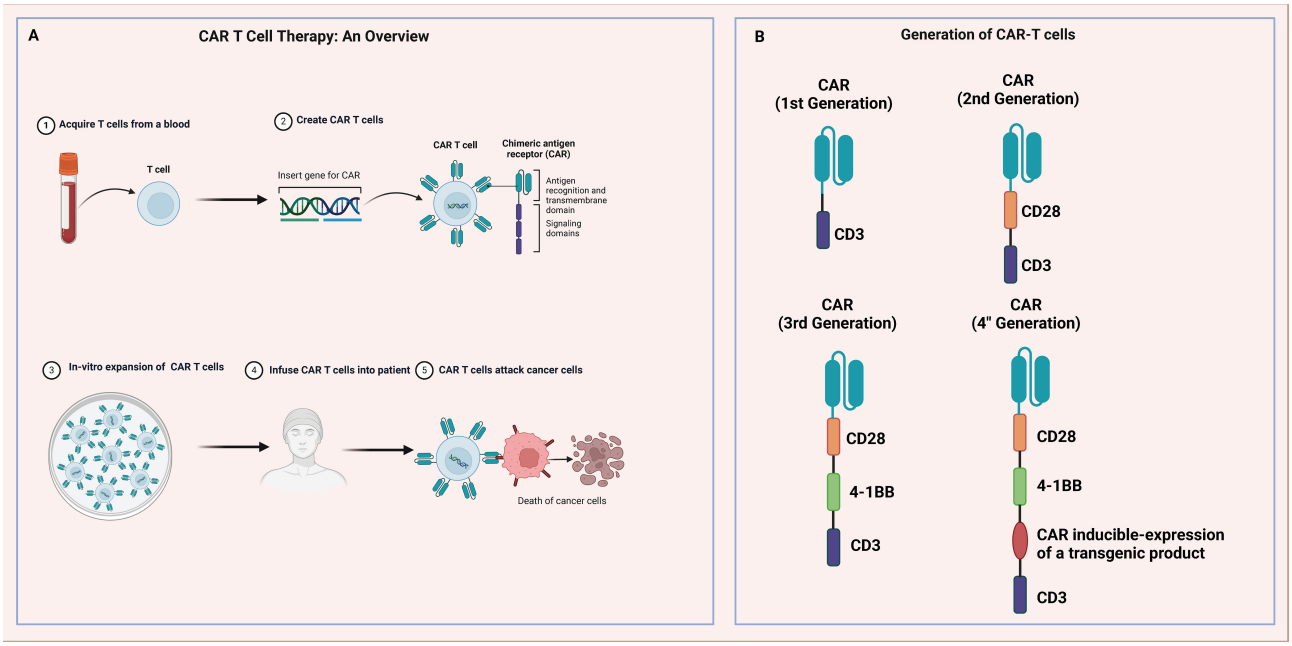

FIGURE 3
Generation of the brief and administration of CAR-T cells. The production and administration of CAR-T cells in cancer patients. **(A)** T cells are collected from patient blood through a single collection and genetically engineered to express CARs. After amplifying CAR-T cells *in vitro*, they are used in cancer patients. The reconstructed CAR-T cells are able to recognize their targets and kill tumor cells expressing that target. **(B)** Explanation of the basic structure of fourth-generation CAR-T cells. The figure was generated by Biorender.com.

TABLE 3 Antigen used in CAR-T-cell therapy for ovarian cancer in randomized clinical trials.

Molecular	Clinicaltrials.gov Identifier	Status	Agents	Primary objectives
Mesothelin	NCT03916679	Unknown	MSTL-CAR-T	The feasibility and safety of anti-MESO CAR-T cells in
	NCT03799913			treating patients with MESO-positive ovarian cancer.
	NCT04562298	Terminated	MSTL-CAR-T	Valuate the safety, tolerability, pharmacokinetics, and
				anti-tumor efficacy profiles of the LCAR-M23 CAR-T-cell therapy.
	NCT03814447	Unknown	MSTL-CAR-T	Determine the safety and feasibility of anti-MESO CAR-T
				cells therapy for Refractory-Relapsed Ovarian Cance
	NCT04503980	Unknown	MSTL-CAR-T	Valuate the safety and tolerability of autologous MSLN-CAR-T
				Cells secreting αPD1-MSLN-CAR T cells in patients with solid tumors.
	NCT05372692	Completed	MSTL-CAR-T	The effect of MSTL-CAR-T in patients with mesothelin-positive
				drug-resistant relapsed ovarian cancer.
	NCT01583686	Terminated	MSTL-CAR-T	Evaluate the safety of the administration of anti-mesothelin CAR engineered.
				peripheral blood lymphocytes in patients receiving a nonmyeloablative
				conditioning regimen, and aldesleukin. Determine if the administration
				anti-mesothelin CAR engineered peripheral blood lymphocytes and
				aldesleukin to patients following a nonmyeloablative but
				the lymphoid depleting preparative regimen will result in clinical
				tumor regression in patients with metastatic cancer.
	NCT03054298	Active, not recruiting	MSTL-CAR-T	Establish safety and feasibility of both intravenous administration and local
				delivery of lentiviral transduced huCART-meso cells with or
				without lymphodepletion.
	NCT02580747	Unknown	MSTL-CAR-T	Determine the safety and feasibility of the MESO CAR-T,
				determine the duration of *in vivo* survival of CART-meso cells.
	NCT05568680	Recruiting	MSTL-CAR-T	Assess the safety, feasibility, and potential activity of a single
				intravenous (IV) dose of SynKIR-110 administered to subjects with mesothelin-
				expressing advanced ovarian cancer, mesothelioma, and cholangiocarcinoma.
	NCT02159716	Completed	MSTL-CAR-T	Establish the safety and feasibility of intravenously administered lentiviral
				transduced CART-meso cells administered with and without cyclophosphamide
				in a 3 + 3 dose escalation design in patients with metastatic pancreatic cancer,
				serous epithelial ovarian cancer, or pleural mesothelioma.
	NCT03025256	Active, not recruiting	MSTL-CAR-T	To determine the safety and/or recommended dose of intrathecal (IT) nivolumab
				in combination with systemic nivolumab treatment in melanoma and
				lung cancer with leptomeningeal disease (LMD).
MUC16	NCT05239143	Recruiting	PD1-MUC16-CAR-T	To determine the safety, tolerability and response of P-MUC1C-ALLO1 in adult
				subjects with advanced or metastatic epithelial derived solid tumors.
MUC1	NCT04025216	Terminated	CART- TnMUC1	To determine the safety, tolerability, feasibility, and preliminary efficacy of

(Continued)

TABLE 3 Continued

Molecular	Clinicaltrials.gov Identifier	Status	Agents	Primary objectives
				CART-TnMUC1 cells engineered to express a CAR capable of recognizing
				the tumor antigen, TnMUC1 and activating the T cell.
TAG72	NCT05225363	Recruiting	TAG72-CAR T	To evaluate the safety and tolerability of TAG72-CAR T cells in
				participants with recurrent epithelial ovarian cancer (EOC).
				To determine the maximum tolerated dose (MTD). III. To identify the
				recommended phase 2 dose (RP2D).
FSHR	NCT05316129	Recruiting	FSHCER-CAR T	Evaluate the safety of treatment with FSHCER-CAR T, with or without
				conditioning chemotherapy in participants with recurrent or
				persistent ovarian, fallopian tube, or primary peritoneal cancer.

results demonstrated that IL-12-secreting tandem CAR-T cells can enhance immunotherapeutic efficacy by reducing tumor antigen escape and improving T-cell function, which may be a promising strategy for the treatment of OV and other solid tumors (200). Chen et al. illustrated the effectiveness of anti-MSLN CAR-T cells in treating OC by inducing programmed cell death in MSLN-positive tumor cell lines. This led to suppressed tumor growth and increased cytokine levels compared to control groups. Subsequently, an *in vivo* experiment targeted OC-derived xenografts, demonstrating the safety and efficacy of autologous anti-MSLN CAR-T cell therapy for patients with this condition. These findings reinforce the potential efficacy of employing an anti-MSLN CAR-T treatment strategy for OC and offer initial data for future clinical trials (201). Shen et al. developed a novel CAR-T-cell therapy that effectively targets TM4SF1 for OC treatment. *In vitro* experiments demonstrated that CAR-T cells can specifically eliminate TM4SF1-positive tumor cell lines, while *in vivo* studies revealed that these modified immune cells significantly inhibited SKOV3-derived tumor growth. Possible therapeutic options for the management of OC may involve targeting TM4SF1, which has encouraging potential. Furthermore, there is a possibility for the advancement of immunotherapy utilizing TM4SF1 as a basis (202). Schoutrop et al. evaluated the therapeutic efficacy of MSLN-CAR-T cells and the characteristics and number of different MSLN-CAR-T cells and found that M1xx CAR-T cells had greater antitumor potency and durability than conventional second-generation M28z and MBBz CAR-T cells. In addition, M1xx CAR-T cells exhibited improved *in vitro* generation capacity and were characterized by self-renewal genes. These findings indicate that MSLN-CAR T cells, which express a mutated CD3ζ strand containing only one immunoreceptor tyrosine-based activation motif (ITAM), demonstrate enhanced potential for treating OC. Employing CAR T cells with precisely calibrated activation potential may lead to improved clinical responses in solid tumors (203). Ranoa et al. conducted a comparative analysis on the efficacy of four Tn-dependent CARs with different affinities toward the Tn antigen. The 237 CAR and a mutant with significantly higher affinity, along with a CAR with lower affinity, effectively controlled advanced ID8Cosmc-KO tumors. Tumor regression was more pronounced with a single dose of intraperitoneal intravenous CAR. The most successful CARs were associated with the antigen possessing the highest affinity. Furthermore, less effective CARs exhibited tonic signaling, leading to cytokine expression independent of the antigen. These findings provide evidence for the potential use of affinity-enhanced CAR-T cells in treating advanced OC, where different patterns of inflammatory cytokine release were observed *in vitro* among successful CARs. Importantly, Tn-dependent CAR-T cells, which are considered highly effective, do not exhibit any toxicity to the immune system of tumor-bearing mice (204). Xu et al. conducted a thorough investigation into the therapeutic potential of PTK7 CAR-T cells against OC, both *in vitro* and *in vivo*. Their study unveiled significant PTK7 expression in OC tissues and cells, indicating its suitability as a target for CAR-T cell therapy. Through the TREM1/DAP12 signaling pathway, researchers observed robust cytotoxicity of PTK7-targeted CAR-T cells against OC cells expressing PTK7 in laboratory settings. Additionally, these engineered immune cells effectively eradicated tumors in animal models. These findings suggest that employing TREM1/DAP12-based PTK7 CAR-T cell therapy shows promise as an innovative approach for treating OC; however, further evaluation is necessary to determine its safety profile and clinical efficacy through rigorous clinical trials (205). Mun et al. developed Muc16-specific CAR T cells (4H11) capable of producing a bispecific T cell engager (BiTE), composed of a TCR mimetic antibody (ESK1). This BiTE selectively binds to the WT1-derived epitope RMFP, presented by the HLA-A2 molecule. Compared with 4H11 CAR-T cells alone, secreted ESK1 BiTE redirected additional T cells toward WT1 on tumor cells, resulting in improved anticancer activity against Muc16-overexpressing cancer cells. These observations were made in both laboratory experiments and a mouse model of tumors. This novel strategy of dual orthogonal cytotoxicity targets distinct surface and intracellular tumor-associated antigens, indicating potential for overcoming resistance to CAR-T cell therapies not only in EOC but also in other malignancies (206). Mondal et al. characterized an important cluster of positively charged residues known as PPCR within

domain 2 of the Fas protein structure. This cluster plays a crucial role in blocking apoptotic signaling triggered by mutated forms of either FasL or Fas, thereby affecting both tumor cells and T cells. Furthermore, our study sheds light on how Fasl interacts with its receptor, Fas, at the PPCR interface through various mechanisms. These findings suggest that employing death agonists could potentially offer an effective therapeutic avenue for targeting FAS and improving cancer immunotherapy outcomes, not only for OC but also for various solid tumors (207). Biotec et al. verified the high expression and consistency of the tumor-associated antigen FOLR1 in primary OC samples. Subsequently, a range of potential CAR T cells were engineered to target the identified markers, and their efficacy against OC cell lines was validated in laboratory and animal models. Ultimately, an automated manufacturing process for these candidate CAR T cells was developed through additional laboratory tests. These results underscore the potential of utilizing anti-FOLR1 CAR T cells as a therapeutic strategy for treating OC and other tumors expressing FOLR1 (208).

6 Discussion and prospects

OC is difficult to diagnose at an early stage, and its five-year survival rate is low. Henceforth, it is crucial to develop new methods for early screening of OC and new therapeutic strategies. As the role of the TME in OC metastasis has received increasing attention, understanding the interactions between immune cell molecules in the TME and tumor cells at primary and metastatic sites and elucidating the molecular mechanisms of the TIME and tumor cells in OC metastasis are urgently needed to provide guidance for the clinical development of new diagnostic markers and therapeutic targets for OC. Since most tumor cells are weakly immunogenic, it is difficult to induce specific immune responses against these antigens in the body. Immunotherapies, such as vaccines to stimulate the host antitumor immune response, the application of genetically engineered cytokines, the infusion of immune effector cells, etc., to assist surgery, radiotherapy, and chemotherapy, in the treatment of OC, have promising application prospects. The intricate interplay between cancer genomic alterations and the complex microenvironment poses challenges to the development of effective immunotherapies. Successful immunotherapy for OC hinges on stimulating antigen-presenting cells, mitigating the suppressive immune microenvironment, and enhancing effector T-cell activity. The modulation of cell-mediated immune responses entails both inhibitory and stimulatory signals. Immune checkpoint receptors play a crucial role in dampening T-cell activation to prevent hyperactivation. Figure 4 illustrates the constituents and therapeutic targets of the TME.

Certain types of tumors express immune checkpoints, allowing them to evade the immune system hence blocking these checkpoints is crucial in immunotherapy. Lysovirus therapy, a novel anti-tumor strategy, triggers an immune response against tumors by rapidly proliferating and ultimately killing tumor cells in response to lysovirus infection (209). While immune checkpoint inhibitors have shown promise in various tumors, their efficacy in OC therapy is relatively low. To enhance the anti-tumor response rate, several studies are combining them with chemotherapy or small molecule inhibitors as adjuvant therapy. However, applying CAR-T cell therapy to solid tumors faces challenges like effective penetration into tumor tissues, overcoming immunosuppressive factors, and minimizing damage to normal tissues. Despite these challenges, CAR-T cell therapy holds significant potential in cancer treatment. Ongoing research explores new target antigens, improved CAR designs, novel management strategies, and combination therapies to expand CAR-T therapy's application and efficacy. As research advances, CAR-T cell therapy is expected to offer hope to more cancer patients. Immunotherapy represents a paradigm shift in OC treatment, but effectively enhancing its efficacy remains a priority. A deeper understanding of the OC immune microenvironment and immune escape mechanisms could lead to novel therapeutic strategies. By using cutting-edge technologies, personalized immunotherapy can be implemented by considering tumor biology and the microenvironment. Therefore, investigating the integration of chemotherapy with immunotherapy or other strategies is essential to improve efficacy and mitigate immune escape in OC treatment.

Given its poor prognosis and high mortality rates, OC has presented significant challenges in treatment, especially in advanced stages where options are scarce. Immunotherapy holds potential for OC treatment, offering better survival outcomes, long-lasting responses, and manageable safety profiles in advanced cases. Nonetheless, current single-agent studies have not yielded substantial survival benefits. In addition to the immunosuppressive properties of OC, this may be due to the complexity of the OC microenvironment, which prevents the activity of immune cells and should, therefore, be targeted by the TME. Temporal factors play a crucial role in modulating the suppression of the immune response and tolerance, as well as influencing the quantity and functionality of TILs. Elucidating the interplay between OC cells and the surrounding mesenchymal environment is imperative for identifying effective therapeutic approaches and reliable prognostic biomarkers. All elements of the TME must be taken into account when devising new therapeutic approaches, particularly for "variable tumors" or "cold tumors." These strategies may involve initiating T-cell responses, utilizing vaccines and CAR-T cells targeting neoantigens in tumor cells, inducing immunogenic cell death in *in situ* vaccination strategies, and simultaneously targeting checkpoint inhibitors responsible for T-cell dysfunction or failure.

7 Conclusion and prospectives

In this study, we conducted a thorough analysis of the diverse cellular components within the TME and their functions in controlling OC progression and treatment responses. Furthermore, we offered a detailed evaluation of the obstacles linked to ICI-based therapies in OC. Altogether, advancing our understanding of the TME will facilitate the development of precise and effective treatment approaches for OC.

The tools currently employed to examine the TME include genomics to investigate gene expression features in high-grade serous ovarian cancer. Verhaak et al. identified four distinct

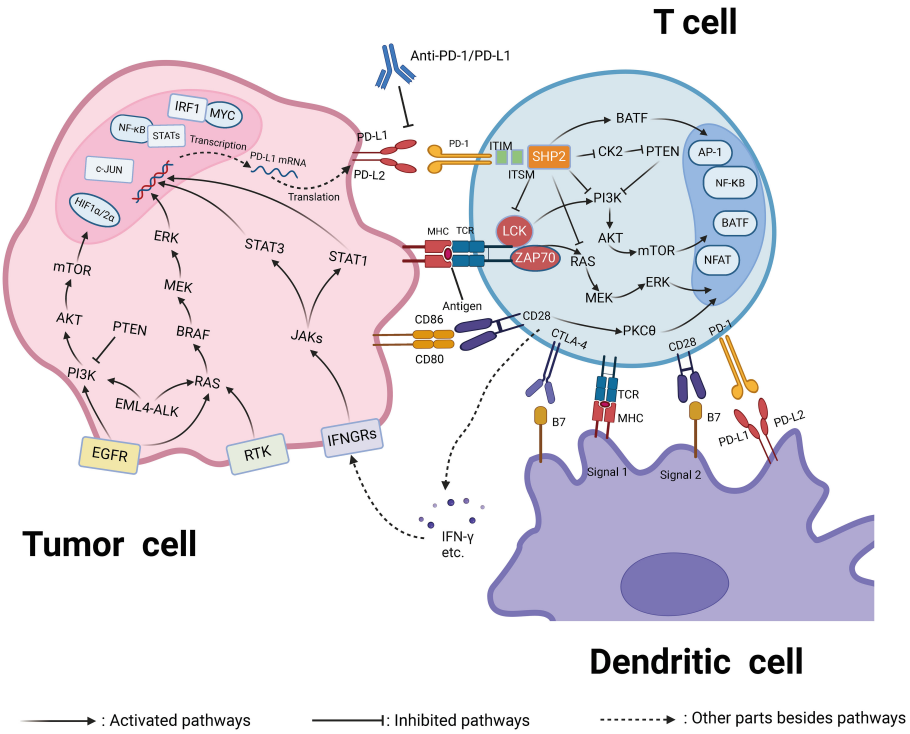

FIGURE 4
The components and therapeutic targets of the tumor microenvironment (TME). The TME is composed of various cells, including cancer stem cells, immune cells, and stromal cells. Treatment targets and intervention measures for the TME. Drugs approved by the US Food and Drug Administration for targeting VEGF, CTLA-4, EGFR, PD-L1, and PD-1.

genetic categories in a study on ovarian tumors, designated as differentiated, immune responsive, mesenchymal, and proliferative (210). It has been determined through IHC that T lymphocytes are increased in the immune-responsive group, while an increase in connective tissue proliferation associated with infiltrating stromal cells is noted in the mesenchymal group. The survival outcomes for patients in the immune-responsive group are the most favorable. Several malignant tissues exhibit more than one of these four gene clusters. These findings were corroborated using an independent dataset of 879 high-grade serous ovarian cancer expression profiles. Additional insights into survival outcomes and platinum resistance were gained using survival prediction models related to BRCA1/2 mutation status, postoperative residual disease, and disease staging (210). Therefore, the development of similar gene classification models might assist in selecting patients for targeted or immunotherapy, or in predicting patient prognosis. For instance, it has been observed that patients exhibiting mesenchymal traits may respond more effectively to therapies such as angiogenic inhibitors. Additional methods for studying the high-grade serous ovarian carcinoma TME include integrating proteomics with other genomic data (211), and employing multiple parameter analysis techniques (such as gene expression, matrix proteomics, cytokine and chemokine expression, ECM parameters, and biomechanical properties) on single biopsy samples to enhance understanding of the events occurring within the tumor tissue (212). Novel tools for investigating ovarian TME include the utilization of artificial microenvironments to monitor the progression of ovarian cancer (213). The TME is a complex and dynamic entity that may vary between primary disease and recurrence. In devising more effective treatments, it is crucial to consider existing immune suppression and emerging mechanisms of therapeutic resistance (214). Cancer treatment with immunotherapy has not achieved the same success as treatments for other types of cancer (215). Combining immunotherapy, such as PD-1 blockade, with other checkpoint inhibitory molecules like anti CTLA-4, anti TIM-3, anti LAG-3, PARP inhibitors, kinase inhibitors, chemotherapy drugs (216), dendritic cell vaccines, CAR T cell therapy (217), or other therapies have been validated to be successful measures to overcome multiple immunosuppressive mechanisms in TME. In fact, targeting complements in OC-TME is a new approach to developing effective immunotherapy. A randomized phase 2 clinical trial (NCT04919629) is currently underway, investigating the combination of a complement inhibitor, anti-PD1, and anti-VEGF for recurrent ovarian cancer patients. The results of this trial may guide future strategies involving complement inhibition in ovarian cancer TME alongside other treatments.

Author contributions

JC: Writing – review & editing, Writing – original draft. LY: Writing – original draft, Validation. YM: Writing – review &

editing, Visualization, Investigation. YZ: Writing – review & editing, Writing – original draft, Visualization, Validation.

Funding

The author(s) declare financial support was received for the research, authorship, and/or publication of this article. This study was supported by the Liaoning Provincial Natural Science Foundation (2023JH2/101700177 and 2023-BS-043).

Acknowledgments

This work was supported by the Liaoning Cancer Hospital & Institute (Shenyang), China Medical University (Shenyang) and Dalian University of Technology (Dalian).

Conflict of interest

The reviewer LZ declared a shared parent affiliation with the authors to the handling editor at the time of review.

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

Publisher's note

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Glossary

OC	ovarian cancer
EOC	epithelial ovarian cancer
TME	tumor microenvironment
ICs	immune checkpoints
ICIs	immune checkpoint inhibitors
ECM	extracellular matrix
CAFs	cancer-associated fibroblasts
TACs	tumor-associated cells
TAMs	tumor-associated macrophages
NK	natural killer
DCs	dendritic cells
TANs	tumor-associated neutrophils
MDSCs	myeloid-derived suppressor cells
CTLs	cytotoxic T lymphocytes
CTL	cytotoxic T lymphocytes
NK	natural killer
PDK1	pyruvate dehydrogenase kinase 1
VEGF	vascular endothelial growth factor
TGF-β	tumor growth factor-β
CTLA-4	cytotoxic T-lymphocyte antigen-4
IL-10R Ab	interleukin-10 receptor-blocking antibody
TLR	Toll-like receptor
LPS	lipopolysaccharide
rCTHRC1	recombinant CTHRC1 protein
CRIg	complement receptor
CCC	clear cell carcinoma
DDP	TPL+cisplatin
ZEB1	zinc E box binding isozyme 1
s mCAFs	metastatic CAFs
POSTN	periostin
CM	conditioned media
DDR2	Discoidin Domain Receptor 2
NFs	normal fibroblasts
EVs	extracellular vesicles
OS	overall survival
cKO	stroma-specific knockout
NFs	normal fibroblasts
EMT	epithelial-mesenchymal transition

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CDDP	cisplatin
CRMP2	Collapsin response mediator protein-2
SDF-1α	stromal derived factors-1α
TIME	tumor immune microenvironments
PF	peritoneal fluid
CSF2	colon-stimulating factor 2
PARPi	PARP inhibition
ORR	overall response rate
APCs	antigen-presenting cells
CXCL13	CXC- chemokine ligand 13
ICB	immune checkpoint blockade
TIL	tumor-inflating lymphocyte
TAA	tumor-associated antigen
TLR	Toll-like receptor
FCM	fused cell membrane
CAR	chimeric antigen receptor
CAR-T	chimeric antigen receptor T
RMF	RMFPNAPYL
ALPS	autoimmune lymphoproliferative syndrome

frontiers | Frontiers in Immunology

TYPE Review
PUBLISHED 05 August 2024
DOI 10.3389/fimmu.2024.1418965

OPEN ACCESS

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RECEIVED 17 April 2024
ACCEPTED 23 July 2024
PUBLISHED 05 August 2024

CITATION
Hao L, Li S, Ye F, Wang H, Zhong Y, Zhang X, Hu X and Huang X (2024) The current status and future of targeted-immune combination for hepatocellular carcinoma.
Front. Immunol. 15:1418965.
doi: 10.3389/fimmu.2024.1418965

The current status and future of targeted-immune combination for hepatocellular carcinoma

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Hepatocellular carcinoma (HCC) is one of the most common cancers and the third leading cause of death worldwide. surgery, transarterial chemoembolization (TACE), systemic therapy, local ablation therapy, radiotherapy, and targeted drug therapy with agents such as sorafenib. However, the tumor microenvironment of liver cancer has a strong immunosuppressive effect. Therefore, new treatments for liver cancer are still necessary. Immune checkpoint molecules, such as programmed death-1 (PD-1), programmed death-ligand 1 (PD-L1), and cytotoxic T lymphocyte antigen-4 (CTLA-4), along with high levels of immunosuppressive cytokines, induce T cell inhibition and are key mechanisms of immune escape in HCC. Recently, immunotherapy based on immune checkpoint inhibitors (ICIs) as monotherapy or in combination with tyrosine kinase inhibitors, anti-angiogenesis drugs, chemotherapy agents, and topical therapies has offered great promise in the treatment of liver cancer. In this review, we discuss the latest advances in ICIs combined with targeted drugs (targeted-immune combination) and other targeted-immune combination regimens for the treatment of patients with advanced HCC (aHCC) or unresectable HCC (uHCC), and provide an outlook on future prospects. The literature reviewed spans the last five years and includes studies identified using keywords such as “hepatocellular carcinoma,” “immune checkpoint inhibitors,” “targeted therapy,” “combination therapy,” and “immunotherapy”.

KEYWORDS

HCC, ICIS, targeted-immune combination, anti-PD-1, anti-PD-L1, CTLA-4

1 Introduction

Liver cancer is the sixth most common malignancy and the third leading cause of cancer-related death worldwide (1). Currently, hepatocellular carcinoma (HCC) accounts for about 75-85% of primary liver cancer, and is among the most common malignant tumors, posing a serious threat to public health (2). The Barcelona Clinic Liver Cancer (BCLC) system is the most commonly recommended staging system for HCC. Based on the underlying liver function, as assessed by the Child–Pugh score, and the performance status, HCC patients can be classified into BCLC stage 0, A, B, C and D (3). Most clinical practice guidelines recommend excision, ablation, and transplantation for patients with early HCC (BCLC 0, A). For patients with intermediate (BCLC B) and advanced (BCLC C) HCC, preferred treatments include transcatheter arterial chemoembolization (TACE), systemic therapy, local ablation therapy, radiotherapy and targeted drug therapy with sorafenib (Figure 1) (4–8). However, the treatment of advanced HCC (aHCC) remains inadequate. The tumor's propensity for invasion, metastasis and recurrence results in low overall survival (OS), high mortality and poor prognosis.

HCC is a chronic inflammatory cancer that expresses multiple antigens and mediates immune responses. In recent years, immunotherapy has shown beneficial results in HCC (9). Immune checkpoint inhibitors (ICIs) therapy, especially antibodies targeting the programmed death-1 (PD-1)/programmed death-ligand 1 (PD-L1) pathway, represents a major breakthrough in oncology drug development over the past decade. ICIs exerts anti-tumor effects by blocking the interaction between immune checkpoint proteins and their ligands to prevent T cell inactivation (10, 11). However, not all HCC patients respond to immunotherapy. Moreover, monotherapy has a lower objective response rate (ORR) and no significant improvement in OS (12, 13). Therefore, researchers are exploring more effective combination therapies for HCC. Recently, the combination of ICIs and antiangiogenic drugs has shown promising results (14). More studies are also exploring the use of different types of ICIs in combination with various targeted drugs. In this review, we provide the latest advances in the use of ICIs combined with targeted drugs (targeted-immune combination) and targeted-immune combination other regimens for the treatment of aHCC or unresectable HCC (uHCC). Additionally, we provide an outlook on future prospects and potential developments in this evolving therapeutic landscape.

2 Immune checkpoint inhibitors combined with targeted drugs

Currently, anti-PD-L1 includes atezolizumab and durvalumab and so on (15). Anti-PD-1 mainly includes nivolumab, pembrolizumab, sintilimab, camrelizumab, tislelizumab and so on (16). Anti-cytotoxic T lymphocyte antigen-4 (CTLA-4) includes tremelimumab and ipilimumab and so on (17). The clinical application of ICIs represents a revolutionary milestone in oncology, but ICIs has a low response rate. Increasingly, clinical studies are combining ICIs with other treatments to achieve better treatment results and improve patient survival (Figure 2) (18). Sorafenib was the first oral tyrosine kinase inhibitor (TKI) approved for the treatment of advanced HCC (19). Subsequently, other TKIs such as lenvatinib, regorafenib, cabozantinib, and vascular endothelial growth factor receptor (VEGFR) inhibitors like ramucirumab and VEGF inhibitors like bevacizumab have been approved as first- or second-line treatments (20–23). More recently,

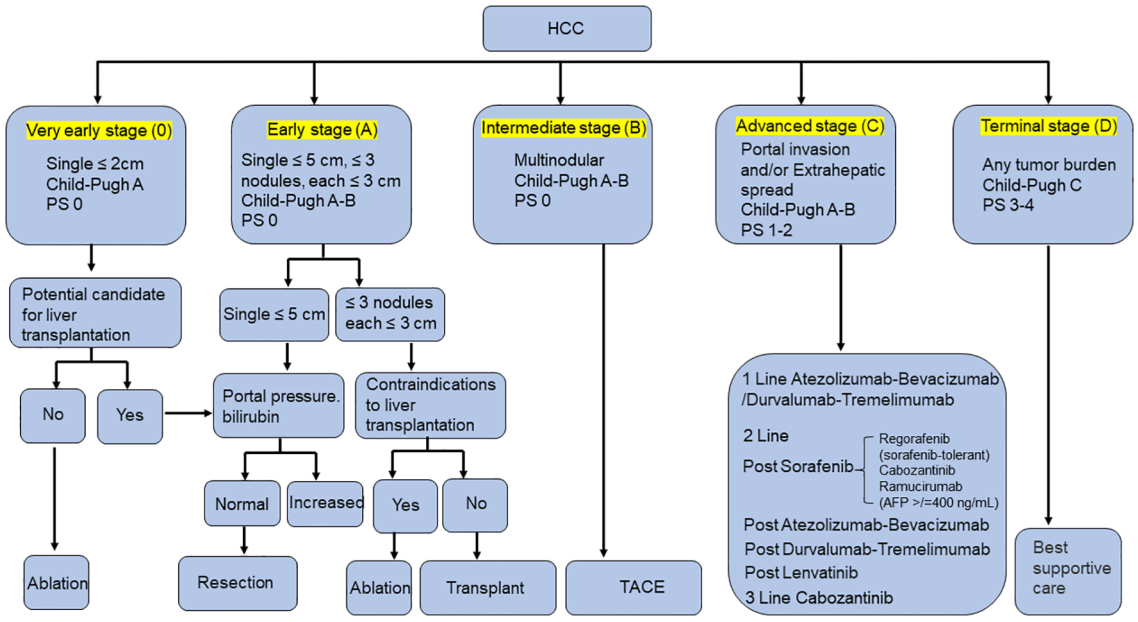

FIGURE 1
Overview of BCLC staging and treatment strategy in HCC.

10.3389/fimmu.2024.1418965

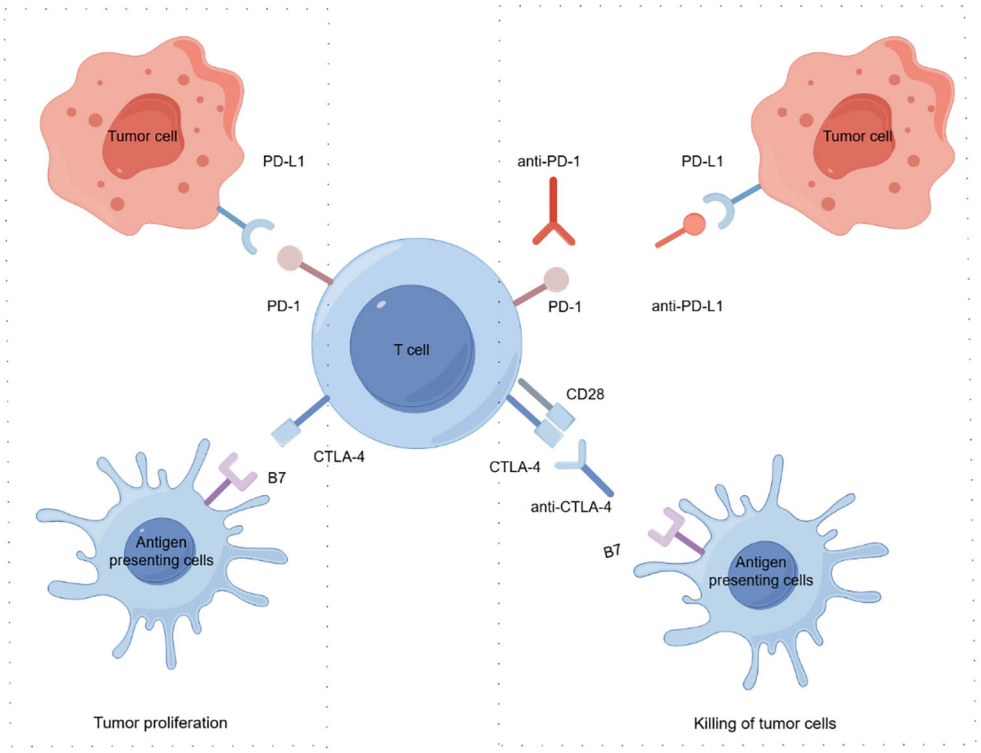

FIGURE 2
Mechanisms of tumor immune evasion and suppression of immune checkpoints following restoration of anti-tumor immunity Tumor cells evade immune surveillance by promoting immune checkpoint activation. Tumor cells express the immune checkpoint activator PD-L1 and produce antigens, which are captured by antigen presenting cells. These cells present antigens to cytotoxic $CD8^+$ T cells through the interaction of major histocompatibility complex (MHC) molecules and T cell receptor (TCR). T cell activation requires costimulatory signaling mediated by B7 and CD28 interactions. Inhibitory signals from CTLA-4 and PD-1 checkpoints inhibit T cell responses and promote tumor proliferation. ICIs, such as anti-PD-L1, anti-PD-1, and anti-CTLA-4, block immunosuppressive checkpoints (CTLA-4, PD-1, and PD-L1, respectively), thereby restoring anti-tumor immune responses. By Figdraw.

the combination of ICIs and VEGF inhibitors (atezolizumab plus bevacizumab) has been approved for the treatment of aHCC (19). Studies have demonstrated the efficacy and safety of anti-PD-1 combined anti-angiogenesis therapy in a real-world cohort of patients with uHCC (24). Additionally, anti-PD-1 combined with TKIs has proven to be an effective and safe strategy for patients with portal vein tumor thrombus (PVTT) (25). A Phase I/II study showed that BMS-986,205 combined with nivolumab showed a DCR of 50% and no incidence of grade 4-5 adverse events (AEs), suggesting that this combination offers manageable safety and lasting benefit in unresectable/metastatic HCC patients (26).

2.1 Anti-PD-L1 plus targeted drugs

2.1.1 Atezolizumab plus bevacizumab

The combinations of atezolizumab and bevacizumab are summarized in Table 1.

A Phase 1b study has shown that atezolizumab combined with bevacizumab is effective and has a tolerable safety profile in uHCC patients who have not previously received systemic therapy (27). The ORR (36% in group A) and disease control rate (DCR) (71% in group A). Treatment with Atezolizumab plus bevacizumab in group F resulted in clinically meaningful improvement in median progression-free survival (mPFS) by 2.2 months and a reduced risk of progression or death (27). In the phase 3 IMbrave050 study, recurrence-free survival improved in patients who received atezolizumab plus bevacizumab compared to those under active surveillance after HCC resection or ablation (28). The IMbrave150 trial demonstrated that atezolizumab plus bevacizumab significantly improved median overall survival (mOS) and mPFS in uHCC patients compared to sorafenib after a median follow-up of 8.6 months (29). 12 months after initial analysis of IMbrave150, atezolizumab plus bevacizumab maintained consistent safety and tolerability (30). After an additional 12 months of follow-up, this combination achieved a mOS of 19.2 months, mPFS of 6.9 months, and ORR of 30% compared to sorafenib (30). Atezolizumab plus bevacizumab showed good efficacy and safety in patients with uHCC and partially advanced liver cirrhosis in a real-world setting (40). Among 171 patients (BCLC stage A:B:C: D=5:68:96:2), this combination effective as both first-line and post line therapy (41). In a German study, the combination significantly improved rates of OS and PFS (31). In Taiwan, the combination provided a 37.5% response rate in patients with aHCC who received systemic therapy for the first time, with a mPFS of 8.6 months and a mOS of 24.9 months (32). Patients who achieved an objective tumor

TABLE 1 Clinical trials with atezolizumab and bevacizumab in HCC.

Combinations	Trial	patient Number	mOS	mPFS	ORR/DCR	3 or/and 4 AEs	Ref.
atezolizumab plus bevacizumab	phase 1b (GO30140)NCT02715531	A 104, F109	A:17.1 months; F:8.3 months	F:5.6 months	A:ORR:36%,DCR:71%	A:20%; F:8%	(27)
atezolizumab plus bevacizumab	phase III (IMbrave050) NCT04102098	668	NA	NA	NA	41%	(28)
atezolizumab plus bevacizumab	phase III (IMbrave150) NCT03434379	501	NA	6.8 months	ORR:28%	57%	(29)
atezolizumab plus bevacizumab	12 months after the primary analysis of IMbrave150	501	19.2 months	6.9 months	ORR:30%	43%	(30)
atezolizumab plus bevacizumab	German	100	20.3 months	6.3 months	ORR:36%	64%	(31)
atezolizumab plus bevacizumab	Taiwan	40	24.9 months	8.6 months	DCR:85%	3 AEs:67.5% or 4 AEs:50%	(32)
atezolizumab plus bevacizumab	Taiwan	35	22.2 months	5.2 months	ORR:23%,DCR:72%	9%	(33)
atezolizumab plus bevacizumab	Japan	52	NR	4.7 months	ORR:15.4%,DCR:57.7%	Any grade AEs:69%	(34)
atezolizumab plus bevacizumab	Korean	121	NR	6.5 months	ORR:24%,DCR:76%	10.70%	(35)
atezolizumab plus bevacizumab	older (age ≥ 65 years) and younger (age < 65 years)	191	older:14.9 months, younger:15.1 months	older:7.1 months, younger:5.5 months	ORR:older:27.6%, younger:20%;DCR: older:77.5%,younger:66.1%	older:20.7%;younger:20.0%	(36)
atezolizumab plus bevacizumab	elderly and non-elderly	317	3, 6, and 9 months: elderly:95.8%, 90.0%, 83.9%, non-elderly:96.2%, 89.5%,80.8%	3 and 6 months: elderly:76.6% and 50.3%; non-elderly:74.8% and 54.2%	ORR:elderly:30.5%,non-elderly:22.8%;DCR: elderly:83.9%, non-elderly:80.2%	≥3 AEs:elderly:39.2%; non-elderly:21%	(37)
atezolizumab plus bevacizumab	overweight (BMI ≥ 25) and non-overweight (BMI < 25)	191	overweight:15.1 months; non-overweight:14.9 months	overweight:7.1 months;non-overweight:6.1 months	ORR:overweight:27.2%; non-overweight:22.0%; DCR:overweight:74.1%; non-overweight:71.9%	≥3 AEs:overweight:19.2%; non-overweight:21.7%	(38)
atezolizumab plus bevacizumab	without PH and with PH	146	without PH:18.4 months; with PH:18.8 months	without PH:8.6 months; with PH:5.8 months	ORR:without PH:31.7%; with PH:26.8%	without PH:78%; with PH:79.9%	(39)

ICIs, immune checkpoints inhibitors; ORR, objective response rate; DCR, disease control rate; mPFS, median progression free survival; mOS, median overall survival; AEs, adverse events; NR, not reached; NA, not available.

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response had a 24-month OS rate of 81%, while those with stable disease had a 24-month OS rate of 57% (32). The most common adverse events were proteinuria and hypertension (32). The Taiwan-Tainan Medical Oncology Group H01 Trial, involving 35 patients reported an overall response rate of 51%, ORR of 23%, and DCR of 72% (33). The mPFS and mOS were 5.2 months and 22.2 months, respectively (33). In Japan, patients receive atezolizumab plus bevacizumab as first line (n=23), second line (n=16), third line (n=6), fourth line (n=3), fifth line (n=3), or sixth line (n=1) (34). According to Response Evaluation Criteria in Solid Tumors (RECIST), the ORR and DCR for all patients were 15.4% and 57.7%, respectively (34). Patients who received the combination as first-line treatment were significantly longer than those who received atezolizumab as late-stage treatment (34). In Korean patients with aHCC, atezolizumab plus bevacizumab showed efficacy and safety consistent with the Phase III trial results. The ORR of 121 patients was 24.0%, DCR was 76%, and the mPFS was 6.5 months (35). Studies have also shown that patients of different ages, particularly the elderly, benefit from atezolizumab and bevacizumab (36, 37). This combination has proven effective in patients with HCC who are overweight, have portal hypertension (PH), and have relatively good liver function (38, 39, 42–44). Early changes in HCC perfusion could predict the long-term therapeutic response of atezolizumab plus bevacizumab, facilitating personalized treatment for HCC patients (45).In case analysis, the study has found that a patient with unresectable aHCC with major portal vein tumor thrombus (Vp4 PVTT) cases responded significantly to atezolizumab plus bevacizumab (46). This combination showed a powerful anti-tumor effect in such cases (46). In another case, patient with hepatocellular and cholangiocarcinoma (CHC) and multiple lymph node metastases obtained PFS of 7.5 months after treatment with atezolizumab plus bevacizumab (47). There was also a case of unresectable multinodular HCC with a complete tumor response following atezolizumab/bevacizumab treatment, leading to liver transplantation due to liver failure. This therapy resulted in complete pathological remission of aHCC, but the safety of long-term treatment needed further evaluated (48). A 49-year-old woman with primary large HCC complicated with portal vein tumor thrombosis responded favorably to atezolizumab in combination with bevacizumab after disease progression through pembrolizumab and Lenvatinib (49). This suggests that HCC patients who are resistant to anti-PD-1 might benefit from anti-PD-L1, providing a potentially promising strategy for the treatment of HCC (49).

The combinations of ICIs, targeted drugs, and other treatments are summarized in Table 2.

2.1.2 Avelumab plus axitinib

A phase 1b study enrolled 22 Japanese patients who were treated with a combination of avelumab plus axitinib (50). The minimum follow-up time was 18 months. Grade 3 treatment-related adverse events (TRAEs) occurred in 16 patients (72.7%) (50). No grade 4 TRAEs or treatment-related deaths were reported (50).

2.1.3 Atezolizumab plus cabozantinib

The COSMIC 312 trial showed that atezolizumab plus cabozantinib had a PFS benefit compared to sorafenib in the first 372 randomized patients (90). However, there was no difference in OS in the interim analysis among the intention-to-treat population (90). In the most recent analysis, cabozantinib did not show an OS benefit compared to sorafenib in the intent to treat population (51). Nevertheless, subgroup analyses showed that potential benefits of cabozantinib in patients with hepatitis B etiology and baseline AFP of 400 ng/mL or higher. The PFS benefit of combination therapy was maintained with longer follow-up and in a larger group of intended treaters (51).

2.1.4 Atezolizumab plus bevacizumab plus lenvatinib

Although lenvatinib therapy did not provide a pseudo-combined immunotherapy effect after atezolizumab plus bevacizumab failure, it may still be comparable as a second-line treatment (91). Patients treated with atezolizumab and bevacizumab after lenvatinib treatment may experience rapid tumor growth and subsequent shrinkage (92). Lenvatinib has been effective and safe for treating aHCC patients who were previously treated with atezolizumab plus bevacizumab. It can effectively control anorexia, general fatigue and other adverse reactions without compromising its therapeutic effect (52). In a 68-year-old uHCC patient with adrenal metastases, lenvatinib was effective after atezolizumab plus bevacizumab treatment failure (93).

2.2 Anti-PD-1 plus targeted drugs

Anti-PD-1 improved OS and PFS in patients with aHCC (94). The efficacy of anti-PD-1 therapy, whether used alone or in combination with TKIs, varies depending on the metastatic site. Notably, a high response rate in vascular metastasis was associated with longer PFS (94). Anti-PD-1 might provide a synergistic benefit when used in conjunction with conventional therapy, potentially enhancing vascular responses in other organs (94).

2.2.1 Anti-PD-1 plus lenvatinib

The study also showed that the ORR (32.7%), DCR (80.0%), mPFS (10.6 months) and mOS (18.4 months) in combination with anti-PD-1 and lenvatinib were significantly higher than those in anti-PD-1 group (53). The simultaneous use of anti-PD-1 and lenvatinib could significantly improve the clinical outcome of aHCC (95). Different anti-PD-1 combined with lenvatinib have shown a good safety profile, guiding treatment options in patients with uHCC (96). Anti-PD-1 plus lenvatinib was a safe and effective conversion therapy for unresectable patients with aHCC (54). This combination was a promising new strategy for the treatment of HCC patients (97). Anti-PD-1 and lenvatinib therapy demonstrated an ORR of 45.0%, a PFS of 7.5 months and an OS of 22.9 months. These data suggested that Lenvatinib combined with nivolumab was a potential combination for aHCC (55). In first-line therapy for

TABLE 2 Clinical trials with ICIs and targeted drug and others treatments in HCC.

Combinations	Trial	patient Number	OS	PFS	ORR/DCR	3 or/and 4 AEs	Ref.
avelumab plus axitinib	Phase 1b(NCT03289533)	22	14.1 months	5.5 months	ORR:13.6%,DCR:68.2%	3 Aes:72.7%	(50)
atezolizumab plus cabozantinib	phase III(COSMIC-312)	837	16.5 months	6.9 months	NA	66%	(51)
atezolizumab plus bevacizumab plus lenvatinib	retrospective clinical study	25	10.5 months	6.0 months	ORR:25.0%,DCR:95.0%	30%	(52)
anti-PD-1 plus lenvatinib	retrospective clinical study	118	18.4 months	10.6 months	ORR:32.7%,DCR:80.0%	63.60%	(53)
anti-PD-1 plus lenvatinib	phase II	124	23.9 months	8.9 months	ORR:53.6%	42.90%	(54)
anti-PD-1 plus lenvatinib	real-world report	87	22.9 months	7.5 months	ORR:45.0%	42.50%	(55)
anti-PD-1 plus lenvatinib	phase III(NCT03713593)	1309	21.2 months	8.2 months	NA	62%	(56)
anti-PD-1 plus lenvatinib	retrospective clinical study	71	NA	9.3 months	ORR:34.1%,DCR:84.1%	NA	(57)
anti-PD-1 plus lenvatinib	retrospective clinical study	159	21.7 months	11.3 months	ORR:38.9%,DCR:92.6%	5.56%	(58)
anti-PD-1 plus lenvatinib	phase Ib	116	22 months	9.3 months	ORR:46%	64%	(59)
anti-PD-1 plus sorafenib	retrospective clinical study	93	19.23 months	8.63 months	ORR:21.4%,DCR:83.9%	32.10%	(60)
anti-PD-1 plus apatinib	phase Ib/II	28	13.2 months	3.7 months	ORR:10.7%	≥3 Aes:92.9%	(61)
anti-PD-1 plus rivoceranib	phase III(CARES-310)	842	22.1 months	5.6 months	ORR:25%	81%	(62)
anti-PD-1 and regorafenib	retrospective clinical study	17	NR	5.09 months	ORR:41.2%,DCR:64.7%	17.64%	(63)
Targeted-immune combination TACE	retrospective clinical study	139	14 months	10 months	ORR:38.7%,DCR:69.4%	3 Aes:3.2%	(64)
Targeted-immune combination TACE	retrospective clinical study	65	26.8 months	11.7 months	ORR:44.4%,DCR:93.3%	73.10%	(65)
Targeted-immune combination TACE	retrospective clinical study	168	29 months	16.2 months	ORR:76.7%,DCR:96.7%	≥3 Aes:30%	(66)
Targeted-immune combination TACE	retrospective clinical study	204	NR	24.1 months	ORR:70.4%,DCR:100.0%	35.30%	(67)
Targeted-immune combination TACE	retrospective clinical study	234	21.7 months	6.3 months	ORR:41.25%,DCR:86.25%	22.50%	(68)
Targeted-immune combination TACE	retrospective clinical study	84	26.7 months	8.2 months	ORR:86.96%,DCR:100%	≥3 Aes:56.53%	(69)
Targeted-immune combination TACE	retrospective clinical study	152	20.5 months	10.2 months	ORR:54.3%	≥3 Aes:43.6%	(70)
Targeted-immune combination TACE	retrospective clinical study	53	NA	8.5 months	ORR:54.9%,DCR:84.3%	≥3 Aes:32.1%	(71)
Targeted-immune combination TACE	retrospective clinical study	92	16.9 months	7.3 months	ORR:56.1%,DCR:85.4%	3 Aes:36.7%	(72)
Targeted-immune combination TACE	retrospective clinical study	41	21.7 months	14.5 months	ORR:68.3%	17.00%	(73)
Targeted-immune combination TACE	retrospective clinical study	169	10.9 months	19.6 months	ORR:66.7%,DCR:82.6%	14.80%	(74)
Targeted-immune combination TACE	retrospective clinical study	246	19.5 months	9.7 months	ORR:73%,DCR:89%	34.70%	(75)

(Continued)

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TABLE 2 Continued

Combinations	Trial	patient Number	OS	PFS	ORR/DCR	3 or/and 4 AEs	Ref.
Targeted-immune combination TACE	retrospective clinical study	87	24 months	9.7 months	ORR:52.4%,DCR:83.3%	19.00%	(76)
Targeted-immune combination HAIC	phase I(NCT04191889)	40	NR	10.38 months	ORR:77.1%,DCR:97.1%	≥3 Aes:74.3%	(77)
Targeted-immune combination HAIC	retrospective clinical study	405	18.0 months	10.0 months	DCR:83%	0.00%	(78)
Targeted-immune combination HAIC	retrospective clinical study	142	26.3 months	11.5 months	ORR:61.8%	89.80%	(79)
Targeted-immune combination HAIC	retrospective clinical study	248	17.7 months	10.9 month	ORR:59.5%	≥3 Aes:4.76%	(80)
Targeted-immune combination HAIC	retrospective clinical study	27	NR	10.6 months	ORR:63.0,DCR:92.6%	3 Aes:55.6%	(81)
Targeted-immune combination HAIC	retrospective clinical study	210	14.6 months	8.37 months	ORR:60.6%,DCR:84.8%	28.70%	(82)
Targeted-immune combination HAIC	retrospective clinical study	123	482 days	208 days	ORR:59%	33%	(83)
Targeted-immune combination radiotherapy	retrospective clinical study	33	9.8 months	8.0 months	ORR:76.6%	27%	(84)
Targeted-immune combination radiotherapy	retrospective clinical study	202	15.8 months	8.3 months	ORR:89.5%,DCR:94.7%	5.30%	(85)
Targeted-immune combination chemotherapy	retrospective clinical study	65	NR	10.6 months	ORR:67.3%	59.10%	(86)
Targeted-immune combination chemotherapy	retrospective clinical study	104	14.3 months	8.63 months	ORR:52.8%	41.50%	(87)
Targeted-immune combination chemotherapy	phase II(NCT04411706)	47	NA	9.0 months	ORR:50%,DCR:91.3%	28.30%	(88)
Dual immune checkpoint inhibitors combined with targeted drugs	phase I/II (CheckMate 040)	98	22.1	4.3 months	ORR:29%	74%	(89)

ICIs, immune checkpoints inhibitors; ORR, objective response rate; DCR, disease control rate; mPFS, median progression free survival; mOS, median overall survival; AEs, adverse events; NR, not reached; NA, not available.

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patients with aHCC, the LEAP-002 study showed that the pembrolizumab plus lenvatinib had an OS of 21.2 months and a PFS of 8.2 months (56). The most common grade 3-4 TRAEs were hypertension (56). Clinical data for pembrolizumab plus lenvatinib showed no unexpected adverse effects, showing positive responses and survival rates even in patients with high-risk tumors and Child-Pugh B status (57). In lenvatinib plus sintilimab group, the mOS was 21.7 months, and mPFS was 11.3 months (58). According to the mRECIST criteria, the ORR was 38.9%, and the DCR was 92.6% (58). ICIs plus lenvatinib provided significantly higher OS and PFS than lenvatinib (98). In addition, ICIs plus lenvatinib had significantly higher ORR (41.5%) and DCR (72.3% vs 46.7%) per RECIST v1.1 than lenvatinib (98). In a phase Ib single-arm study showed that lenvatinib plus pembrolizumab had a longer mPFS of 9.3 months (by mRECIST; 8.6 months by RECIST v1.1) per IIR, and mOS of 22.0 months (59). A 63-year-old male patient received combination immunotherapy with Lenvatinib and Pembrolizumab. He had a complete response (CR) nine months after treatment (99). Now, 22 months since initial treatment, there was no clinical evidence of disease progression. The current OS was 22 months (99).

2.2.2 Anti-PD-1 plus sorafenib

In hepatitis virus-associated HCC, both the mOS (19.23 months) and mPFS (8.63 months) were significantly improved in the TKIs (sorafenib or lenvatinib or regorafenib) plus ICIs (camrelizumab or sintilimab) group compared to the TKIs group (60). The DCR was also significantly higher in the TKI-ICIs group at 83.9% (60). Compared with anti-PD-1 alone, the combination of anti-PD-1 (nivolumab or pembrolizumab) and sorafenib showed better tumor control with an ORR of 22.4%, longer PFS (3.87 months) and OS (100). Importantly, there was no significant increase in grade 3 or 4 AEs, and a significant reduction in AFP levels was observed (100). Anti-PD-1 (nivolumab or pembrolizumab) therapy increases $CD4^+$ and $CD8^+$ T cell infiltration and provides vascular protection, which is beneficial for subsequent multi-kinase inhibitor therapy. In this sequence, sorafenib acts as an immune stimulator by promoting $CD8^+$ T cell infiltration (101). A 62-year-old man showed extensive tumor reduction after multiple treatments sintilimab combined with sorafenib. This suggested that the protocol was a promising therapeutic strategy for the treatment of HCC (102).

2.2.3 Anti-PD-1 plus cabozantinib

A 71-year-old metastatic HCC patient with RET amplification, high tumor mutation burden, and positive PD-L1 expression responded well to the combination of cabozantinib and nivolumab, achieving a PFS of over 25 months (103). Cabozantinib and nivolumab may be a good option for patients with aHCC, especially those with bone metastases (103). Studies have shown that TKIs (lenvatinib or apatinib) plus anti-PD-1 (nivolumab or pembrolizumab or sintilimab) is safe and effective in the treatment of uHCC (104). The mOS was 27.0 months and the 1-year OS rate was 83.6%. The mPFS was 15.0 months and the 1-year PFS rate was 77.0% (104).

2.2.4 Anti-PD-1 plus apatinib

In patients with advanced primary liver cancer (PLC), camrelizumab and apatinib achieved a manageable safety profile and good efficacy. The mPFS and mOS were 3.7 months and 13.2 months, respectively (61). A 250mg dose of apatinib is recommended as a combination therapy for further study of late-stage PLC therapy (61).

2.2.5 Anti-PD-1 plus rivoceranib

A Phase 3 study has shown that camrelizumab-rivoceranib met both primary endpoints, with an improvement of 6.9 months in mOS and 1.9 months in mPFS (per RECIST 1.1 by the BIRC) compared to the sorafenib group (62). The risk of death was reduced by 38% and the risk of progression or death by 48% (62). The combination therapy resulted in significantly higher response rates, longer lasting responses and higher DCR compared to the sorafenib group (62).

2.2.6 Anti-PD-1 plus regorafenib

Regorafenib combined with anti-PD-1 (camrelizumab or sintilimab) was safe and effective for treating aHCC, with a low incidence of severe AEs (63). Seventeen patients with BCLC-B and BCLC-C HCC were followed up for a median of 7.62 months (63). The ORR and DCR were 41.2% and 64.7%, respectively, and the mPFS was 5.09 months (63). In a refractory patient previously treated with sorafenib, progressive disease occurred during treatment with anti-PD-1 (nivolumab) and the anti-GITR (BMS-986156) in a Phase 1 clinical trial (105). Subsequently, a prolonged tumor response was achieved during third-line therapy with regorafenib according to RECIST v.1.1 criteria (105).

3 Targeted-immune combination local therapy

Based on targeted-immune combination, combined with local treatment means such as intervention and radiotherapy, the comprehensive treatment can improve the treatment efficiency of middle HCC and aHCC.

3.1 Targeted-immune combination TACE

The mOS of 14 months, mPFS of 10 months and ORR of 38.7% in the treatment of aHCC patients with TACE combined with atezolizumab and bevacizumab were significantly improved, with acceptable safety (64). This combination was effective reducing the early recurrence of HCC without severe complications (106). In a 74-year-old patient with HCC, the liver tumor achieved complete remission after TACE, but lung, bone, and lymph node metastases were observed (107). These metastases eventually decreased, showing partial response after continuous administration of atezolizumab plus bevacizumab (107). Compared to TACE combined with sorafenib, TACE combined with sorafenib and ICIs (camrelizumab or sintilimab) was a potentially safe and

effective treatment option for patients with aHCC who have previously received local regional therapy. These patients had higher DCR (82.8%), longer mPFS (6.9 months), and longer mOS (12.3 months) (108). TACE combined with lenvatinib plus anti-PD-1 (TACE-L-P) provided better treatment response and survival benefits, with manageable adverse events (65–70, 109). In 51 evaluable patients, the confirmed ORR was 54.9% and the DCR was 84.3% (71). The mPFS was 8.50 months (71). Grade ≥3 TRAEs was developed in 32.1% of patients (71). No new safety signals detected (71). TACE-L-P (camrelizumab or sintilimab) might have good anti-tumor activity in the treatment of uHCC. Toxicity was manageable, no unexpected safety signals (71). In HCC patients with portal vein tumor thrombus (PVTT), the DCR (80.00%), ORR (38.57%), mOS (23.5 months) and mPFS (7.5 months) of TACE-L-P (pembrolizumab or sintilimab) were significantly better than those of TACE+lenvatinib (110). The patients in TACE-L-P (sintilimab or tislelizumab or camrelizumab) group had prolonged mOS (16.9 months), longer mPFS (7.3 months) and higher ORR (56.1%) and DCR (85.4%) than those in TACE-L group (72). TACE-L-P (camrelizumab or tislelizumab or sintilimab) combined with Vp4 was effective and tolerated in treating uHCC, with a high tumor response rate and good prognosis (73). For HCC PVTT patients, compared with TACE combined with apatinib alone (TACE-A), TACE combined with apatinib and anti-PD-1 (TACE-A-P) significantly improved PFS, OS, and ORR, and the TRAEs was safe and controllable (111). TACE plus apatinib and TACE plus apatinib plus camrelizumab were feasible in patients with uHCC with a manageable safety profile. TACE plus apatinib plus camrelizumab showed additional benefits compared to TACE plus apatinib (112). The TACE plus donafenib plus toripalimab group showed higher ORR (66.7%) and DCR (82.6%), longer mPFS (10.9 months) and longer mOS (19.6 months) compared to the TACE plus sorafenib group (74). Patients treated with TACE combined with TKIs and ICIs (nivolumab or pembrolizumab or camrelizumab) had significantly longer OS than those treated with TKIs plus ICIs without TACE. Both groups tolerated severe AEs well, with no significant difference in incidence (75). Compared with TACE combined with molecularly targeted agents (sorafenib or lenvatinib or apatinib or regorafenib or bevacizumab), TACE combined with molecularly targeted agents plus ICIs (camrelizumab or sintilimab or pembrolizumab or tislelizumab or atezolizumab) improved the survival and tumor response of uHCC patients, and the toxicity is controllable (76). The mOS (24.00 months) and mPFS (9.70 months) were both significantly longer (76). HCC patients treated with TACE combined with molecular targeted agents (sorafenib or lenvatinib or apatinib or regorafenib) plus ICIs (camrelizumab), the formation of liquefaction necrosis increased (113). Larger tumor size and higher AFP levels were associated with more liquefaction necrosis in the tumor (113).

3.2 Targeted-immune combination HAIC

One study (NCT04191889) evaluated the benefit of camrelizumab and apatinib combined with HAIC-FOLFOX in patients with BCLC-C HCC (77). Thirty-five patients were enrolled. The ORR was 77.1% and the DCR was 97.1% (77). The mPFS was 10.38 months (77). The most common treatment-related AEs with grade ≥3 or above included reduced lymphocyte count (37.1%) and reduced neutrophil count (34.3%) (77). This combination showed encouraging results and manageable safety concerns (77). The HAIC plus anti-PD-1 group had a longer mOS of 18.0 months and a longer mPFS of 10.0 months, as well as a higher DCR (83%) and intrahepatic response (85%) (78). HAIC-FOLFOX plus lenvatinib plus anti-PD-1 (pembrolizumab or sintilimab or toripalimab or camrelizumab or tislelizumab) was an effective and safe treatment for HCC patients with PVTT. There were significant improvements in OS (26.3 months), PFS (11.5 months) and ORR (61.8%) (79). Pembrolizumab plus lenvatinib and HAIC prolonged median PFS (10.9 months) and OS (17.7 months) in newly treated uHCC patients with PD-L1 staining (80). The mOS was 43.6 months and post progression-free survival (PPS) was 35.6 months in anti-PD-1 plus lenvatinib plus HAIC group (114). Anti-PD-1 (camrelizumab or sintilimab ot toripalimab or nivolumab) combined with TKIs (lenvatinib or sorafenib or regorafenib or apatinib) and HAIC was safe and effective for aHCC. The ORR was 63.0%, the DCR was 92.6%, and the median PFS was 10.6 months. The most common grade 3 AEs were pain (7.4%) and elevated ALT (7.7%) (81). A meta-analysis has shown that HAIC based therapy improved the prognosis of patients with HAIC (115). Although HAIC combined with anti-PD-1/anti-PD-L1 (triple therapy) increased the incidence and severity of AEs, it produced higher ORR, longer PFS and OS compared to angiogenesis inhibitors plus anti-PD-1/anti-PD-L1 (115). Initial hepatic artery intervention plus anti-PD-1 and targeted therapy led to longer median PFS (8.37 months) and OS (up to 14.6 months) in BCLC-C HCC patients (82). Transarterial interventional therapy combined with TKIs (lenvatinib or sorafenib or apatinib) and anti-PD-1 (triplet regimen) produced excellent results and controllable AEs in patients with HCC and severe PVTT. Compared to double regimens, the triplet regimen resulted in longer median PFS (208 days) and OS (482 days) (83). Skeletal muscle index (SMI) combined with interventional therapy with ICIs (toripalimab or camrelizumab) and TKIs (lenvatinib) highlighted sarcopenia as an independent risk factor for OS in HCC patients treated with sorafenib or regorafenib, which could be of great help for personalized medical treatment of HCC patients (116). The meta-analysis suggested that triple therapy with TACE/HAIC, TKIs, and ICIs provided clinical benefit for uHCC in both short and long-term outcomes without an increase in severe AEs, though further validation is needed (117).

3.3 Targeted-immune combination radiotherapy

In comparison to the combination of ICIs (pembrolizumab or camrelizumab or sintilimab or atezolizumab) and antiangiogenic therapy (lenvatinib or sorafenib or donafenib or bevacizumab or apatinib), the inclusion of RT has improved DCR and survival outcomes in aHCC patients (118). The safety profile of this triple therapy was satisfactory (118). For HCC patients, transarterial

radioembolization (TARE) using Y-90 resin microspheres showed similar results to atezolizumab-bevacizumab (AB) (119). The mOS was 15.0 and 14.9 months for TARE and AB, respectively (119). The mPFS was 4.4 and 6.8 months for TARE and AB, respectively (119). ORR were 19.8% and 25% with TARE and AB, respectively (119). Atezolizumab plus bevacizumab combined with TARE improved OS and PFS outcomes compared to TARE alone (120). In a cohort of 30 patients with PLC and extrahepatic portal vein tumor thrombus (ePVTT), combining intensity-modulated radiotherapy (IMRT) with systemic atezolizumab systemic atezolizumab and bevacizumab yielded an ORR of 76.6%. The median OS was 9.8 months, and the median PFS was 8.0 months (84). Patients with aHCC treated with radiotherapy before and/or during nivolumab therapy had significantly higher PFS and OS, with generally acceptable toxicity profiles (121). In HCC patients, PVTT was more sensitive to radiotherapy (RT) than primary tumor (PT). Combining RT with anti-angiogenesis and ICIs in aHCC patients created surgical opportunities and may be promising for low-stage HCC patients with PVTT (122). Proton beam radiotherapy (PBT) combined with anti-PD-1/anti-PD-L1 was safe, with no accidental AEs. Concurrent therapy effectively treated aHCC through sustained local tumor necrosis and effective systemic tumor control (123). The mOS for the entire cohort was 12.9 months. In patients with advanced uHCC, immunotherapy with Y90-RE or nivolumab or atezolizumab/bevacizumab within 90 days appeared to be well tolerated, with a low incidence of severe AEs (124). Sequential ICIs (anti-PD-1: sintilimab or camrelizumab, ati-PD-L1: atezolizumab) plus bevacizumab plus bevacizumab therapy after radiotherapy for PVTT in patients with HCC was safe and feasible, potentially prolonging PFS (125). HCC patients treated with Y90+ICI had better ORR (89.5%) and DCR (94.7%) than those treated with Y90 plus TKI (85). The mPFS was 8.3 months and mOS was 15.8 months, patients had no significant combination therapy AEs attributed to radioembolization (85).

3.4 Targeted-immune combination chemotherapy

Atezolizumab plus bevacizumab combined with oxaliplatin (HAIC-FOLFOX) showed ORR of 67.3% based on mRECIST criteria and 44.2% based on RECIST 1.1 criteria (86). The mPFS of patients was 10.6 months (86). AEs were controllable, suggesting this combination may be a potential treatment option for aHCC (86). Anti-PD-1 (toripalimab) plus lenvatinib with Gemox chemotherapy as a first-line treatment option for advanced ICC (87). The mOS was 14.3 months and the mPFS was 8.63 months, and the median ORR was 52.8% (87). The incidence of grade 3 and 4 AEs was 41.5%, which was acceptable, tolerable and controllable (87). A single-arm Phase II clinical study met its pre-set primary endpoint, showing that sintilimab combined with apatinib plus capecitabine had a good safety profile and antitumor activity as a first-line treatment for uHCC (88). The ORR based on blinded independent image evaluation was 50.0% and the DCR was 91.3% (88).

3.5 Targeted-immune combination ablation

A 38-year-old male patient received prophylactic TACE after surgery (126). Three months after surgery, the patient developed multiple liver metastases (126). He underwent atezolizumab and bevacizumab combined with intratumor cryoablation (126). After treatment, the patient's tumor exhibited extensive necrosis, the disease has been effectively controlled (126).

4 Dual immune checkpoint inhibitors combined with targeted drugs

Tumor cells evade the immune system in several ways, so combining ICIs with different mechanisms of action could be an interesting therapeutic strategy (127). Inhibition of the B7-CTLA-4 pathway by anti-CTLA-4 play an anticancer role by increasing the level of activated $CD8^+$ T cells in the lymph nodes (128).

A meta-analysis showed that combining anti-PD-1 with anti-PD-L1 for uHCC improved OS, PFS, ORR, DCR, especially in patients with HBV infection and among Asian populations (129). While the incidence of any grade and grade 3-5 TRAEs was higher with combination therapy, the safety was manageable (129). Another Meta-analysis showed that anti-PD-1/anti-PD-L1 was superior to sorafenib and placebo in OS, PFS, ORR and DCR in uHCC patients, especially when anti-PD-L1 was combined with anti-VEGF (130). However, the incidence of AEs was slightly higher in patients treated with anti-PD-1/anti-PD-L1 (130). In cohort 6 of the CheckMate 040 study, the ORR for nivolumab and cabozantinib was 17%, and for the triplet therapy (nivolumab, ipilimumab, and cabozantinib) was 29% (89). The mPFS was 5.1 months and 4.3 months, and the mOS was 20.2 months and 22.1 months, respectively (89). The incidence of grade 3-4 TRAEs was 50% for the doublet and 74% for the triplet, with TRAEs leading to discontinuation in 11% and 23% of patients, respectively (89). Notreatment-related deaths occurred in either group (89). In a randomized Phase 1 trial, stereotactic body radiation therapy (SBRT) of nivolumab plus ipilimumab outperformed immunotherapy alone in patients with aHCC or uHCC (131). Adding 1mg/kg ipilimumab to the atezolizumab plus bevacizumab combination during induction was safe, showed acceptable toxicity and increased ORR and subsequently improved patient outcomes (132). For aHCC patients, the sequence of TKIs and ICIs treatment might not matter. Patients who are frail or have comorbidities that preclude them from tolerating the combination therapy (ICIs and TKIs/anti-VEGF) might benefit from continuous exposure to both drug classes (133).

5 Challenges in combination therapy for HCC

However, challenges remain, including drug resistance and AEs in combination therapy. First, ICIs in combination with targeted

drugs is unlikely to be cost-effective (134–137). Secondly, ICIs can encounter resistant (primary or acquired), which remains the leading cause of treatment failure (138). Drug resistance is complex and dynamic, as abnormal behavior at any step can lead to resistance. Therefore, developing new methods to reduce drug resistance is critical.

In addition, after ICIs treatment, an over-activated immune system can lose self-tolerance, leading to non-tumor auto-immune response, resulting in immune-related AEs (irAEs) (139). These effects are usually mild and manageable but can sometimes be life-threatening. Rash and itching are the most common clinical features (140). Other common adverse events include diarrhea and colitis, hepatotoxicity and elevated AST, elevated alkaline phosphatase and elevated ALT, thyroid dysfunction, lung, blood, and HBV reactivation (141–148). Therefore, necessary baseline assessment and screening should be performed before targeted-immune combination. For patients receiving immunotherapy, it is crucial to conduct routine medical history inquiry, manage underlying diseases, complete baseline screening, and adequate address underlying diseases or comorbidities before initiating immunotherapy. Baseline viral DNA screening and routine antiviral therapy for HBV patients.

6 Potential biomarkers of combination therapy

The combination of anti-PD-1/anti-PD-L1 plus anti-VEGF drugs may have significantly better clinical benefits (149, 150). However, not all HCC patients receiving combination therapy achieve the expected efficacy, and biomarkers are essential for predicting and evaluating treatment effect. A meta-analysis showed that atezolizumab in combination with bevacizumab was effective and well tolerated in treating aHCC (151). This combination demonstrated better tumor response rates in long-term, first-line, and low-dose therapy (151). Atezolizumab plus bevacizumab treatment could be expected to elicit an effective immune response in untreated uHCC patients (152–154). A low pretreatment neutrophil-to-lymphocyte ratio (NLR ≤ 2.22) might indicate longer OS (25.8 months) and PFS (14.0 months) for patients with uHCC treated with TACE plus TKIs (sorafenib or lenvatinib or apatinib) plus ICIs (camrelizumab) (155). Lenvatinib plus anti-PD-1 plays a unique immunomodulatory role by activating the immune pathway, reducing Treg cell infiltration, and inhibiting TGF-β pathway. Although these HCC do not respond to a single drug, they could benefit from the proposed combination drug (156). In mouse models, cabozantinib, especially when combined with anti-PD-1 therapy, induced neutrophil infiltration, reduced the immunosuppressive environment and enhanced antitumor activity compared with monotherapy. Patients with reduced active neutrophil phenotypes in their tumors (about 30% of cases) might benefit the most from this combination (157). Cryo-thermal ablation could transform HCC from a “cold” tumor to a “hot” tumor. This technique, combined with anti-PD-1 and anti-CTLA-4, might be a promising method for improving HCC prognosis (158). Anti-PD-1 therapy enhanced the anti-tumor immune response in liver cancer models. When used with sorafenib, this immunotherapy approach was effective only when simultaneously targeting the hypoxic and immunosuppressive microenvironment with drugs such as CXCR4 inhibitors (159). The study has shown that albumin-bilirubin (ALBI) grading and sorafenib treatment history are predictors of OS in HCC patients treated with lenvatinib. For patients with prior sorafenib experience, ICIs combined with lenvatinib achieved better OS than lenvatinib alone (160). Alpha-fetoprotein (AFP) is a potential alternative biomarker for atezolizumab plus bevacizumab in HCC (161). A 3-week AFP ratio of 1.4 or higher may predict refractory atezolizumab combined with bevacizumab (162). A reduction of ≥20% in AFP at 3 weeks was associated with longer OS and PFS, showing potential as a biomarker of response (163). AFP response was a predictor of disease control, PFS, and OS, making it useful for predicting treatment outcomes in uHCC patients receiving ICIs (or not receiving TKIs or local therapy) (164). HCC with different genes can be divided into hot tumors and cold tumors based on tumor infiltrating $CD8^+$ T cells in mice. Hot tumors respond to anti-PD-1 therapy, while cold tumors are more suitable for combination therapy with anti-PD-1 and sorafenib (165). Therefore, developing predictive biomarkers with high specificity and sensitivity is crucial to accurately identify HCC patients most likely to benefit from combination therapy.

7 Conclusions

More than 70% of HCC patients are diagnosed at intermediate to advanced stage (BCLC stage B, C, or D) and require systemic treatment. Traditional TKI drugs, such as sorafenib, lenvatinib, have provided some hope, but their clinical efficacy is still unsatisfactory (166). Consequently, new strategies are being developed. ICIs have ushered a new era in the treatment of aHCC. The combination of ICIs and anti-VEGFA, represented by anti-PD-1/PD-L1 and anti-CTLA-4, provides patients with up to 35% more ORRs and is better tolerated than other approved treatments (40, 167). The approval of atezolizumab combined with bevacizumab establishes a new benchmark for the treatment of advanced HCC, with a mOS duration of 20 months. This raises the question of whether patients who benefit from atezolizumab combined with bevacizumab might benefit from targeted or targeted combination with other treatments. The studies analyzed in this review provide some evidence that each targeted drug and ICIs has unique immunomodulatory effects, and that the target population benefiting from these treatments may differ significantly. In addition, we describe the relevance of etiological dependent mechanisms that may influence the outcome of ICIs and their combinations. Effectively utilizing the synergistic effect of different anti-tumor mechanisms will be the focus of future research and is expected to transform the current landscape of HCC treatment.

However, challenges remain, including drug resistance, predictive biomarkers of treatment effectiveness, and AEs in combination therapy. The potential causes of immune resistance to ICIs in HCC patients are complex and varied. These include the upregulation of immune checkpoints, impaired antigen recognition and presentation by immune cells, abnormal activation and proliferation of immunosuppressive cells, increased inhibitory cytokines, and the compromised proliferation and function of anti-tumor immune cells within the complex tumor microenvironment (TME) (168). Additionally, loss of tumor antigen expression, tumor heterogeneity, and dysbiosis of the gut microbiota are associated with ICI resistance (169). Combination therapy has become a primary treatment approach. Current combination therapies include ICIs with another ICI, ICIs with targeted therapy, chemotherapy, radiotherapy, traditional Chinese medicine, or modulation of the gut microbiota. The application of these treatments has not only improved the ORR of patients but also mitigated ICI resistance in HCC patients (170). In addition, ICIs lead to irAEs (140). Other common adverse events include diarrhea and colitis, hepatotoxicity and elevated AST, elevated alkaline phosphatase and elevated ALT, thyroid dysfunction, lung, blood, and HBV reactivation (141–148). Therefore, necessary baseline assessment and screening should be performed before targeted-immune combination.

At the same time, the treatment process should be closely monitored to detect and deal with adverse reactions promptly. Although ICIs are promising for HCC treatment, their ORR remains relatively low. The discovery and application of biomarkers for ICIs treatment effect will help clinicians effectively screen patients who benefit from ICIs treatment and make personalized treatment more precise. However, the biomarkers of ICIs beneficiaries of liver cancer are still in the exploratory stage or lack of strong evidence, and the combination of multiple biomarkers may be a new development trend. In the future, developing new immunosuppressants, exploring new therapeutic approaches, and discovering new prognostic biomarkers will be essential to achieving better therapeutic effects. More trials with larger sample sizes are needed to further validate the efficacy of ICIs and targeted-immune combination therapy for aHCC.

Current clinical studies show that a Phase I trial of nivolumab combined with cabozantinib as neoadjuvant therapy for three months resulted in 12 out of 15 patients successfully undergoing resection. Five patients achieving major pathological response. Several studies are exploring other preoperative combination regimens. Larger cohorts are needed to validate the role of ICIs in the adjuvant setting (171). Analysis of 58 specimens from patients who had residual tumor cells after preoperative TACE treatment revealed that TACE increased intratumoral inflammation and tumor antigen expression, thereby enhancing the efficacy of immunotherapy (172).

In addition, we should strengthen the study of immunotherapy for metastatic liver cancer, mixed liver cancer and NASH-associated liver cancer. HBV reactivation can occur in patients with HBV-associated HCC treated with ICIs. Routine monitoring of HBV DNA and effective prophylactic antiviral therapy are necessary before and during combination therapy. In addition, clinical trials of the immunotherapy combination regimen are ongoing, opening up numerous new possibilities for perioperative conversion therapy for advanced liver cancer. If preoperative and postoperative immunotherapy studies show positive results, perioperative survival for uHCC will improve, potentially making liver cancer clinically controllable If targeted-immune combination can transform initially unresectable patients with advanced liver cancer into resectable patients with survival benefits, then the treatment strategy and surgical indications will change, greatly improving survival outcomes.

However, current Chinese and foreign guidelines for the treatment of liver cancer exhibit several key differences (Table 3), reflecting distinct clinical practices, cultural preferences, and regional pharmaceutical approvals. Chinese guidelines, such as those from the Chinese Society of Clinical Oncology (CSCO), often recommend conventional therapies. This integration mirrors local clinical practices and cultural preferences. In contrast, foreign guidelines, including those from the American Association for the Study of Liver Diseases (AASLD) and the European Association for the Study of the Liver (EASL), primarily focus on evidence-based Western medical practices.

Additionally, Chinese guidelines may emphasize the use of specific biomarkers and locally approved drugs, such as apatinib. Foreign guidelines typically recommend a broader array of diagnostic tools and systemic therapies, including advanced imaging techniques and a wider range of targeted and immunotherapy options. These differences underscore the importance of tailoring treatment strategies to regional practices and patient populations to optimize outcomes.

Recent advancements in liver cancer treatment have seen significant milestones, particularly in the first-line treatment. The National Comprehensive Cancer Network (NCCN) has made a groundbreaking inclusion of the atezolizumab and bevacizumab combination in its guidelines, marking the first approval of an immunotherapy combination for first-line treatment of liver cancer. Furthermore, the inclusion of Nivolumab as a monotherapy in the NCCN guidelines for first-line treatment underscores the expanding role of immunotherapy (173). Conversely, the European Society for Medical Oncology (ESMO) does not recommend chemotherapy as a first-line treatment, whereas Chinese guidelines still place high importance on it (174).

In the context of second-line treatment, the positions of the three major targeted therapies, such as regorafenib, cabozantinib, and ramucirumab, remain strong. However, significant controversy exists regarding the use of Nivolumab and pembrolizumab as second-line treatments. While NCCN and CSCO guidelines affirm the “Nivolumab and pembrolizumab combination” for second-line therapy, the Pan-Asian ESMO guidelines exclude K drug, and ESMO guidelines do not recommend either (173). The NCCN guidelines uniquely highlight the dual immunotherapy combination of nivolumab and ipilimumab as the first approved immunotherapy combination for second-line treatment.

TABLE 3 Applicable conditions and guideline recommendations for different treatment methods in liver cancer.

Treatment Method	Applicable Conditions	Guideline Recommendations	Notes
Atezolizumab + Bevacizumab	First-line treatment, suitable for patients without severe bleeding risks	NCCN, AASLD, EASL	Increases ORR, provides conversion therapy opportunities for unresectable HCC
Nivolumab Monotherapy	First-line treatment, suitable for patients without severe liver dysfunction	NCCN	First inclusion in first-line treatment, immunotherapy monotherapy
Pembrolizumab	Second-line treatment, suitable for patients with prior treatment failure	NCCN, CSCO	Controversial as a second-line treatment
Nivolumab + Ipilimumab (O+Y)	Second-line treatment, suitable for patients with prior treatment failure	NCCN	Dual immunotherapy, first approved combination in second-line
Chemotherapy	First-line treatment, suitable for patients unable to receive immunotherapy or targeted therapy	Chinese guidelines	Not recommended by ESMO, highly recommended in China
Regorafenib	Second-line treatment, suitable for sorafenib-intolerant patients without severe adverse effects	NCCN, CSCO, ESMO	Targeted therapy, good tolerability
Cabozantinib	Second-line and third-line treatment, suitable for patients with disease progression after first-line therapy	NCCN, CSCO, ESMO	Multi-targeted kinase inhibitor
Ramucirumab	Second-line treatment, suitable for patients with AFP > 400 ng/mL and disease progression	NCCN, CSCO, ESMO	Specific inhibitor, high specificity
Camrelizumab	Second-line treatment, suitable for patients with advanced liver cancer and prior treatment failure	Chinese guidelines	Comparable efficacy to imported PD-1 inhibitors
Sorafenib	First/second-line treatment, suitable for patients unable to undergo surgery or local treatment	AASLD, EASL	Traditional targeted therapy, widely used
Lenvatinib	First/second-line treatment, suitable for patients with good liver function and without severe adverse effects	AASLD, EASL	Traditional targeted therapy, good tolerability
Durvalumab + Tremelimumab	First-line treatment, suitable for patients contraindicated for bevacizumab	AASLD	Dual immunotherapy, provides dual immune suppression
Surgical Resection	Suitable for BCLC 0-A stage patients, some BCLC B and C patients after multidisciplinary discussion	AASLD	Requires discussion in large liver centers
Liver Transplantation	Suitable for recurrent liver cancer patients meeting Milan criteria	AASLD, Chinese guidelines	Surgery preferred for recurrent liver cancer in China

Additionally, the domestic drug camrelizumab has shown comparable efficacy to imported PD-1 inhibitors in second-line treatment of aHCC (174).

The AASLD recommends the use of serum AFP combined with ultrasound for liver cancer screening. Previously, the AASLD limited surgical resection indications to BCLC stage 0-A but now acknowledges that some BCLC stage B and C patients may be eligible for surgery following multidisciplinary discussion at large liver centers, particularly for patients with BCLC stage B and Vp1-Vp2 type portal vein tumor thrombus. For the first time, the AASLD also recommends adjuvant therapy post-surgery, currently advocating for the T+A regimen (175).

For recurrent liver cancer, liver transplantation is preferred if the patient meets the Milan criteria. However, in China, surgical resection remains the first choice for patients with recurrent HCC who are still eligible for surgery. For advanced liver cancer or intermediate liver cancer unsuitable for TACE, the AASLD recommends the T+A regimen as the first choice. For patients with contraindications to bevacizumab, the STRIDE regimen (durvalumab combined with tremelimumab) is recommended. For those contraindicated to immunotherapy, sorafenib or lenvatinib is recommended. In second-line treatment, the first choices are sorafenib or lenvatinib, previously used as first-line therapies (175).

In European guidelines, the T+A regimen is recommended as the first choice for systemic treatment-naive patients, with sorafenib or lenvatinib as alternative first-line options. Cabozantinib, regorafenib (for sorafenib-tolerant patients), and ramucirumab (for patients with AFP > 400 ng/mL) are recommended as second-line therapies following sorafenib treatment (176).

For tumor response evaluation, RECIST 1.1 is the preferred standard for assessing the response to systemic therapy. Other evaluative standards, such as immune-related RECIST and mRECIST, require further validation through prospective studies (176). Overall, the treatment guidelines for liver cancer in different regions reflect their respective clinical practices and cultural

backgrounds, underscoring the importance of tailoring treatment strategies to regional circumstances and patient populations.

Author contributions

LH: Data curation, Formal analysis, Investigation, Methodology, Writing – original draft. SL: Data curation, Formal analysis, Funding acquisition, Writing – original draft. FY: Writing – original draft. HW: Writing – original draft. YZ: Writing – original draft. XZ: Writing – original draft. XYH: Formal analysis, Funding acquisition, Writing – original draft. XPH: Formal analysis, Investigation, Validation, Writing – original draft.

Funding

The author(s) declare financial support was received for the research, authorship, and/or publication of this article. The present study was financially supported by the National Natural Science Foundation of China (No. 81973840 and No. 81273748); Science and Technology Program of Hebei (223777156D); Clinical Medical School Graduate Research Innovation Practice Project (2023KCY06).

Acknowledgments

The authors acknowledge using Figdraw (https://www.figdraw.com/static/index.html#/) to create the schemata (Figure 2).

Conflict of interest

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

Publisher's note

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frontiers | Frontiers in Immunology

TYPE Review
PUBLISHED 24 September 2024
DOI 10.3389/fimmu.2024.1449441

OPEN ACCESS

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RECEIVED 15 June 2024
ACCEPTED 09 September 2024
PUBLISHED 24 September 2024

CITATION
Xu J, Gao H, Azhar MS, Xu H, Chen S, Li M, Ni X, Yan T, Zhou H, Long Q and Yi W (2024) Interleukin signaling in the regulation of natural killer cells biology in breast cancer. *Front. Immunol.* 15:1449441. doi: 10.3389/fimmu.2024.1449441

Interleukin signaling in the regulation of natural killer cells biology in breast cancer

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In the field of breast cancer treatment, the immunotherapy involving natural killer (NK) cells is increasingly highlighting its distinct potential and significance. Members of the interleukin (IL) family play pivotal regulatory roles in the growth, differentiation, survival, and apoptosis of NK cells, and are central to their anti-tumor activity. These cytokines enhance the ability of NK cells to recognize and eliminate tumor cells by binding to specific receptors and activating downstream signaling pathways. Furthermore, interleukins do not function in isolation; the synergistic or antagonistic interactions between different interleukins can drive NK cells toward various functional pathways, ultimately leading to diverse outcomes for breast cancer patients. This paper reviews the intricate relationship between NK cells and interleukins, particularly within the breast cancer tumor microenvironment. Additionally, we summarize the latest clinical studies and advancements in NK cell therapy for breast cancer, along with the potential applications of interleukin signaling in these therapies. In conclusion, this article underscores the critical role of NK cells and interleukin signaling in breast cancer treatment, providing valuable insights and a significant reference for future research and clinical practice.

KEYWORDS

breast cancer, interleukin, natural killer cell, immunology, immunotherapy, tumor microenvironment

Introduction

Innate immunity and adaptive immunity represent the core defensive mechanisms of the human immune system. Among these, natural killer (NK) cells—an integral subset of innate lymphoid cells (ILCs)—are crucial for addressing intracellular microbial threats and mediating tumor responses (1). Unlike cytotoxic T cells, NK cells do not necessitate antigen presentation

for their cytotoxic functions. This characteristic provides a complementary mechanism to the adaptive immune system (2).

The development, maturation, and cytotoxic functions of NK cells are intricately regulated by various factors, with interleukins (ILs) playing a pivotal role. As cytokines secreted by white blood cells, ILs are essential for mediating cell communication and regulating the immune system. Through their binding to specific receptors, ILs modulate immune cell activity, thereby influencing NK cell growth, differentiation, survival, and apoptosis (3). Each interleukin exhibits distinct biological functions and mechanisms of action within NK cells, highlighting their significant role in immune regulation.

Breast cancer has a profound impact on women's health globally, being the most prevalent cancer among women in terms of both incidence and mortality (4). Despite significant advancements in breast cancer treatment—such as chemotherapy, radiotherapy, targeted therapy, and surgery—that have markedly improved prognosis (5), a substantial number of patients continue to experience cancer recurrence. Immunotherapy, the latest advancement in treatment, has transformed breast cancer management. Immune checkpoint inhibitors that target CD8+ T cells, particularly PD-1/PD-L1 inhibitors, have significantly expanded and revitalized the tumor-specific T cell pool, leading to notable improvements in patient outcomes (6). However, many patients either do not respond to these therapies or develop acquired resistance. Furthermore, T-cell-based immunotherapies are associated with risks, including excessive T cell proliferation, which can lead to cytokine release syndrome or graft-versus-host disease. In severe instances, these conditions may result in life-threatening attacks by donor cells on the recipient's tissues (7). These challenges underscore the urgent need for the development of novel immunotherapies that target alternative effector cells.

The clinical success of immune checkpoint inhibitors that stimulate T cells has driven research into immunotherapy strategies targeting NK cells for breast cancer treatment (6, 8). NK cells possess unique attributes not shared by T cells; their ability to simultaneously activate and inhibit multiple receptors helps prevent damage to healthy cells. Furthermore, unlike T cells, NK cells do not undergo extensive proliferation upon activation, which diminishes the risk of cytokine release syndrome or graft-versus-host disease (9). Consequently, many studies have leveraged the tumor-killing capabilities and inherent advantages of NK cells to investigate novel immunotherapeutic approaches for breast cancer (2, 10, 11). In this review, we first summarize the impact of interleukins on NK cell development, maturation, and cytotoxic functions. We then explore the role of interleukins within the breast cancer tumor microenvironment, particularly regarding NK cell-mediated killing of breast cancer cells. Finally, we review the latest clinical studies and advancements in the use of NK cells for breast cancer treatment.

Development, maturation, and effector functions of human NK cells

NK cells, belonging to the innate lymphoid cell (ILC) family, constitute 5-20% of all circulating lymphocytes in humans (12, 13). Initially, it was believed that NK cells developed solely in the bone marrow (BM); however, recent studies have revealed that NK cell development also occurs in extramedullary sites, including secondary lymphoid tissues (SLT) such as the tonsils, spleen, lymph nodes, as well as in the liver and uterus (14). In the BM, NK cells originate from multipotent hematopoietic stem cells (HSCs). These HSCs undergo a series of differentiation steps to produce common lymphoid progenitors (CLPs), which further differentiate into common innate lymphoid cell progenitors (CILCPs) with a lineage commitment towards the ILC spectrum. Under the influence of specific transcription factors, CILCPs differentiate into natural killer progenitors (NKPs) with a commitment to the NK cell lineage. Subsequently, cytokines such as T-bet and Eomesodermin drive the maturation of NKPs into functional NK cells, including immature NK cells (iNK) and eventually mature NK cells (mNK) (15–17). Although CD56 expression is a marker of NK cell maturation, full maturation is characterized by a gradual downregulation of CD56. NK cells are classified into $CD56_{bright}$ (iNK) and $CD56_{dim}$ (mNK) subsets, with $CD56_{bright}$ NK cells primarily found in SLT and $CD56_{dim}$ NK cells predominantly circulating in the blood (18). Both subsets express activating receptors NKp46 and NKp80 and produce cytokines; however, only $CD56_{dim}$ NK cells exhibit cytolytic activity. Thus, the downregulation of CD56 during NK cell maturation is closely associated with the acquisition of antitumor cytotoxicity (19, 20). CD56bright NK cells are generally regarded as precursors to $CD56_{dim}$ NK cells, with differentiation marked by an increase in CD94/NKG2C and CD16 expression (21). Nevertheless, some studies suggest that $CD56_{dim}$ NK cells may arise directly from NKPs (22). Throughout the differentiation process from HSCs to NK cells, there is a progressive loss of stemness and changes in the expression of various molecules that define different stages of NK cell development and function. We summarize the developmental process and molecular changes in the differentiation of human HSCs into NK cells (Figure 1).

Interleukin signaling's impact on NK cells

The expression of cytokines and their respective receptors is crucial for NK cell development (23). Interleukins, a diverse class of cytokines, influence NK cells through various mechanisms. They often work in concert with other cytokines to modulate NK cell regulation, particularly during the differentiation of hematopoietic stem cells (HSCs). In the bone marrow, IL-3 and IL-7 play a role in conjunction with cytokines such as SCF/KL and Flt3L/Flk2 in regulating the differentiation of HSCs into common lymphoid progenitors (CLPs) (23). Additionally, Flt3L or SCF can synergize with IL-15 to significantly enhance NK cell proliferation (24).

Interleukins are crucial for the survival, proliferation, and activation of NK cells. Early research demonstrated that both low-dose continuous infusion and intermittent administration of IL-2 effectively expanded CD56+ NK cells in patients with metastatic cancer (25). Moreover, NK cells cultured with IL-2 or

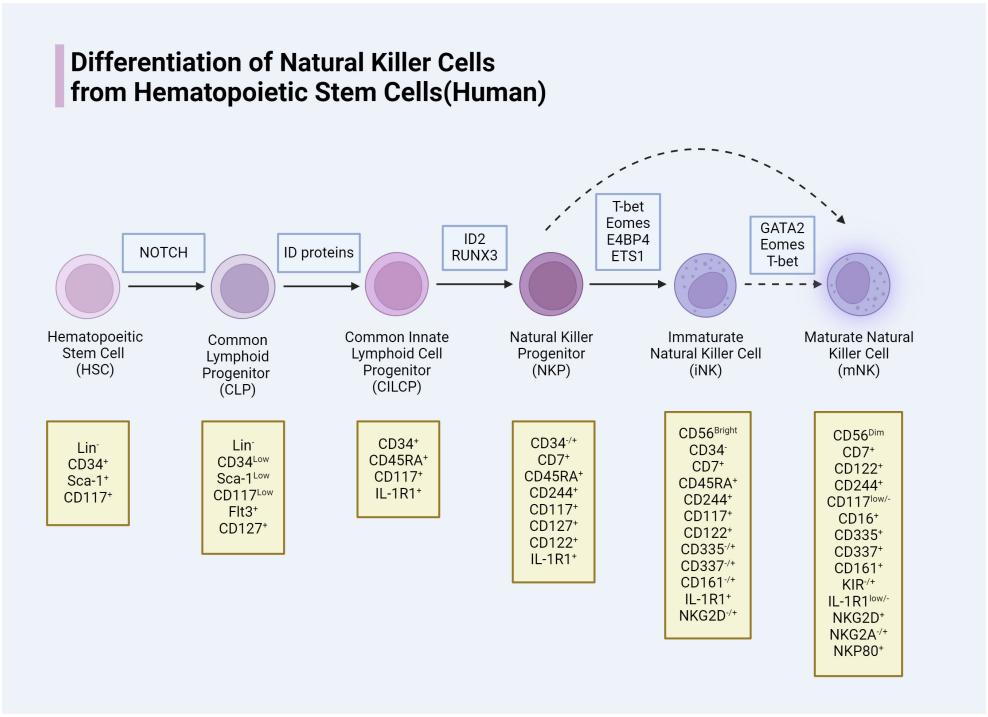

FIGURE 1
Human NK cells need specific transcription factor, in the process of development and different phases mean a loss of different molecular and expression.

IL-15 exhibit enhanced proliferation, activation, and increased sensitivity to therapeutic drugs (26). A study by Perez et al. confirmed that IL-21 significantly enhanced the cytotoxicity of CD56+ NK cells derived from cord blood/CD34+ cells (27). Additionally, the effects of interleukins on NK cells are often interdependent. Synergistic interactions between certain interleukins can amplify their impact on NK cells. Strengell et al. (28) found that the combined action of IL-21 with IL-15 or IL-18 enhances the production of IFN-γ in both NK cells and T cells, leading to increased cytotoxicity. Similarly, Wendt et al. observed that the combination of IL-2 and IL-21 resulted in more pronounced NK cell proliferation (29). Subsequent studies have identified various signaling cascade molecules downstream of key activation receptors (30).

Interleukin signaling plays a critical role in inducing the differentiation of NK cells. While NK cells generally differentiate from hematopoietic stem cells (HSCs), numerous studies have shown that bone marrow-derived CD34+ cells can be induced by cytokines to differentiate into NK cells *in vitro* (31). This differentiation process is heavily influenced by interleukin signaling. Ambrosini et al. demonstrated that IL-1β inhibits ILC3 while promoting the maturation of cord blood CD34+ precursor NK cells (32). Although IL-12 and IL-15 are also believed to be involved in this differentiation process, further studies are required to confirm their roles.

The common gamma chain (γc) signaling is indispensable for NK cell development, homeostasis, and function. For instance, IL-7 plays a pivotal role in the transformation of HSCs into CD122+ NK progenitors (NKPs), while IL-15 is crucial for NK cell lineage commitment and the maturation of CD122+ NKPs into mature NK cells (33). The γc chain (CD132) is a 40 kDa Type I transmembrane glycoprotein that acts as a shared subunit in the receptors for several interleukins, including IL-2, IL-4, IL-7, IL-9, IL-15, and IL-21 (34). These subunits pair with specific alpha chains to form complete cytokine receptors. Upon cytokine binding, the γc chain is instrumental in activating multiple signaling pathways, particularly the Janus kinase (JAK)/signal transducer and activator of transcription (STAT) pathways, which are crucial for regulating NK cell functions (35). Essential members of the γc family and the key downstream molecules of the JAK/STAT pathway are outlined for a comprehensive understanding (Figure 2).

The expression of some interleukin receptors represents different stages of NK cell development. Such as the expression of IL-7Rα (CD127) means that the formation of the CLP, the expression of IL-2Rβ (CD122) means that the formation of NKP, the expression of IL-1R1 is Pre-NK cells into end-stage, will grow into iNK mark. These close relationships constitute the basic architecture between NK cells and interleukin signaling, and interventions targeting the relevant mechanisms are expected to change the status quo of NK cell antitumor resistance.

Interleukins and the anti-tumor function of NK cells

The anti-tumor function of NK cells begins even before the tumor occurs. An 11-year-long study found that individuals with

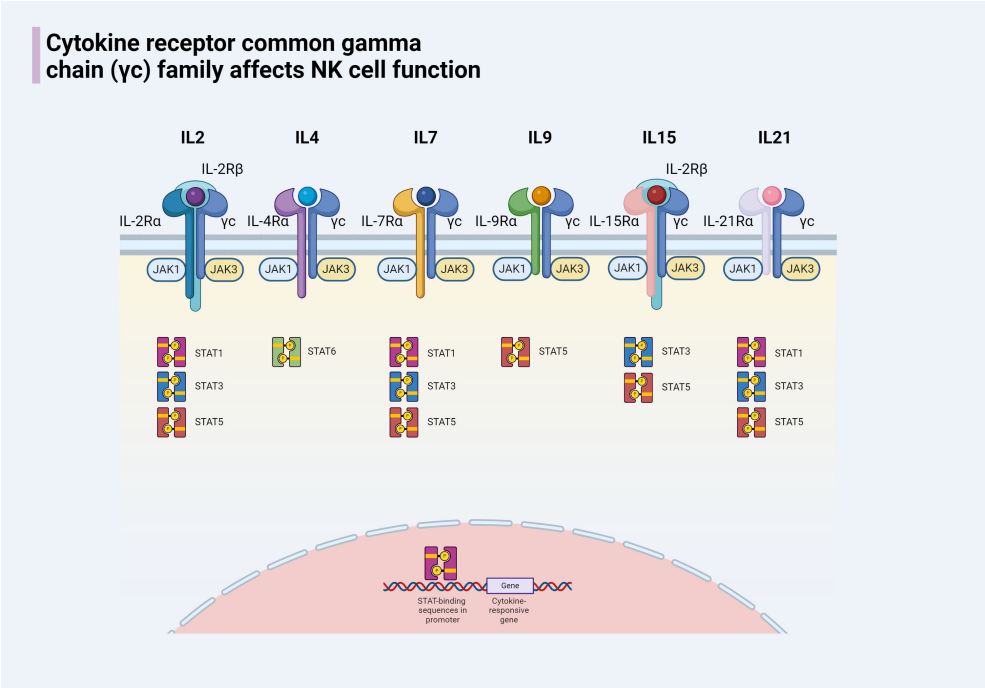

FIGURE 2
The cytokine receptor common γc chain family of cytokines includes IL-2, IL-4, IL-7, IL-9, IL-15, and IL-21. Members of this family share the common characteristic of having a γc chain and each has a unique α chain. Except for the receptors for IL-2 and IL-15, which have an additional IL-2Rβ chain, the receptors for other members are composed of two chains. When the receptors for members of the γc family on the surface of NK cells bind to their respective cytokines, the associated JAK enzymes are activated, leading to the phosphorylation of STAT proteins. The phosphorylated STATs form dimers and are transported to the nucleus, where they regulate gene expression, affecting the development of NK cells and modulating the immune response.

low cytotoxic reactivity of peripheral NK cells have a higher probability of developing cancer (36). Another study pointed out that patients with congenital NK cell deficiencies have an increased incidence of malignant tumors (37). If routine tumor monitoring fails, that is, when an individual develops cancer, NK cells can still continue to exert their anti-tumor functions.

The "recognition-connection-elimination" trilogy of NK cells

Recognition of tumor cells

The anti-tumor process of NK cells can be roughly summarized into a "recognition-connection-elimination" trilogy. NK cells can recognize tumor cells through various mechanisms. The first method is missing self recognition. Normal cells express major histocompatibility complex class I (MHC-I) molecules on their surface, which can send "self" signals to NK cells to prevent them from being attacked (38, 39). However, cancer cells often reduce or lose the expression of MHC-I, thus failing to deliver effective inhibitory signals to NK cells, allowing NK cells to recognize and attack these "self-lacking" cells (40). The second method is the balance of activating and inhibitory receptors on NK cells. As we all know, there are a large number of activating and inhibitory receptors on the surface of NK cells, and the function of NK cells depends on the balance of the activation degree of these receptors. Activating receptors can recognize specific molecules on the surface of cancer cells, such as stress-induced molecules or viral proteins. When the signal from the activating receptor exceeds the signal from the inhibitory receptor, NK cells are activated and attack the target cells (40). Some specific receptors on the surface of NK cells, including the immunoglobulin superfamily (such as KIR), C-type lectin family (such as NKG2 receptors), and natural cytotoxicity receptors (NCR) (41, 42), can recognize specific ligands on cancer cells, thus completing the recognition of cancer cells. In addition, the low-affinity IgG Fc receptor (such as CD16) on the surface of NK cells can also mediate the recognition of NK cells by binding to specific IgG antibodies attached to the surface of tumor cell (43). In addition to the above recognition methods, interleukin signals can also participate in the recognition of cancer cells by NK cells by enhancing/reducing the recognition ability of NK cells. These cytokines (such as IL-12, IL-15, and IL-18) are usually released by other immune cells in the tumor microenvironment and can enhance the recognition and killing ability of NK cells to cancer cells (44).

Formation of immunological synapse

After completing the recognition of tumor cells, an immunological synapse (IS) is formed between NK cells and them to complete the connection process (45, 46). The term IS originates

from the synapse in the nervous system and has similar characteristics of cell-to-cell contact and signal transmission (47). The confirmation of cancer cells enables IS to obtain activation signals, and the activation signals form and stabilize IS through the remodeling of the cytoskeleton, leading to the interaction between NK and target cells, thereby playing the role of immune checkpoints and completing the killing of cancer cells (48). Interleukins also play an important role in this process. The WAS protein can promote the branching of filamentous actin (F-actin) and is necessary for the aggregation of F-actin at the NK cell immunological synapse. However, WAS protein-deficient NK cells have been proven to recover function by activating the WASp homolog WAVE2 through IL-2 *in vitro* (49). In another study, IL-2 could restore the damage of IS that occurred in the treatment of leukemia by allogeneic transplantation of NK cells derived from umbilical cord blood (50).

Mechanisms of NK cell-mediated killing

The killing of cancer cells by NK cells occurs through two primary mechanisms: direct killing and indirect killing. In the direct killing mechanism, NK cells recognize cancer cells and deliver granzymes and perforin through the immunological synapse (IS). Perforin inserts into the plasma membrane of the target cell, creating pores through which granzymes enter. These granzymes then activate caspases, which promote a cascade involving IL-1β-converting enzyme (ICE) superfamily proteases (51), leading to the formation of apoptotic bodies and, ultimately, the apoptosis of cancer cells (52). ICE, or caspase-1, plays a crucial role in this process by cleaving the precursors of interleukins, such as pro-IL-1β and pro-IL-18, into their mature, biologically active forms, thereby activating their functions (53). Another direct killing mechanism involves the binding of the CD16 receptor on the surface of NK cells to the Fc region of immunoglobulins. This interaction induces phosphorylation within the immunoreceptor tyrosine-based activation motif (ITAM) domains of the high-affinity IgE receptor and CD3ζ, leading to the targeted killing of cells. This mechanism is known as antibody-dependent cellular cytotoxicity (ADCC) (54). Additionally, NK cells can induce apoptosis in target cells by expressing members of the TNF ligand superfamily, such as Fas ligand (FasL, CD95L) or TNFSF10 (TRAIL, CD253), thereby mediating a delayed killing effect (55, 56).

NK cells can also complete the killing of cancer cells by promoting other cells to secrete killing substances. When the receptors on the surface of NK cells react with tumor ligands, NK cells will release Th1-type cytokines, including FN-γ, TNF, and granulocyte/macrophage colony-stimulating factor (GM-CSF) (1), activate T cells, dendritic cells (DC), and so on to complete anti-tumor functions (57). We summarize the mechanisms and ways in which NK cells participate in tumor killing (Figure 3). In this process, the participation of interleukin signals is also indispensable. Some synergistic effects have produced interesting results, and some immune cells activated by NK cells can also feedback to NK cells. For example, Th1 cells activated by IFN-γ secreted by NK cells can

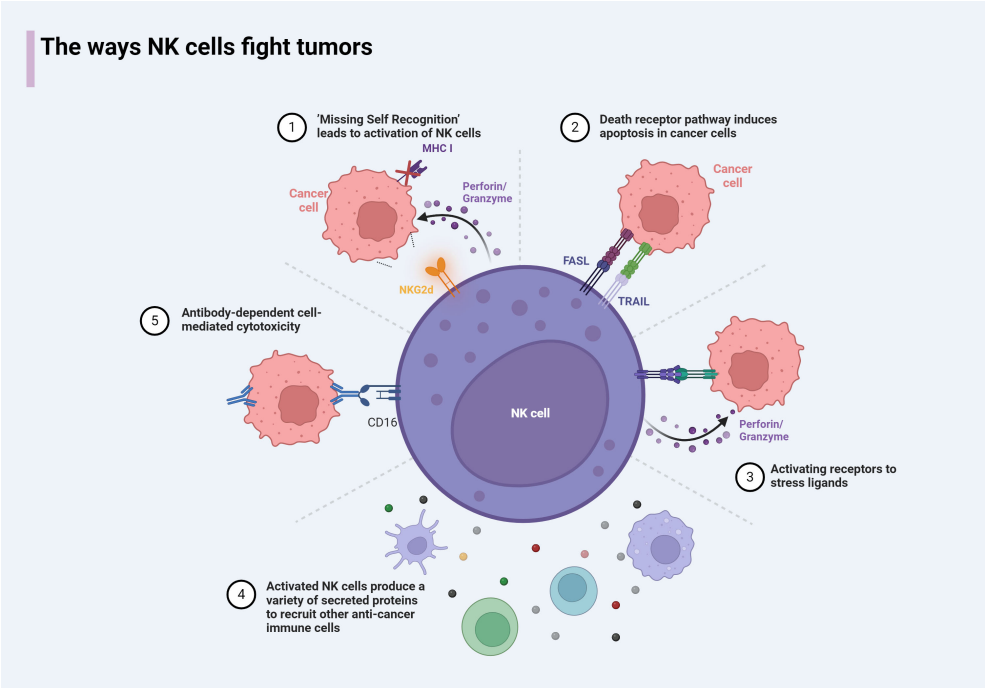

FIGURE 3
The antitumor mechanism of NK cells. 1) NK cells result in the release of intracellular perforin and granzyme due to loss of inhibition of sensing receptors or upregulation of stress ligands. 2) NK cells express death receptors, which can induce apoptosis in target cells expressing death receptor ligands. 3) Activates receptor that binds to stress ligand, leads to the release of perforin/granzyme by NK cells and completion of killing. 4) NK cells express cytokines that activate the recruitment of other immune cells to the tumor microenvironment. 5) The antibody-bound target activates NK cells through CD16 binding on the Fc portion of the antibody.

secrete IL-12 to further enhance the activity of NK cells (58). Type I IFN-α/IFN-β triggered by DCs stimulated by NK cells will cause the expression of IL-15Rα on DCs and the production of IL-15 from plasmacytoid DCs (59), which in turn induces the proliferation of NK cells (60). In addition, IL-15, IL-12, IL-23, IL-27, and IL-18 produced by DCs have also had a profound impact on the function of NK cells (61). These findings are crucial for the development of new cancer therapies.

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Breast cancer tumor microenvironment and interleukin signaling with NK cells

NK cells are known for their significant anti-cancer effects in hematological tumors, but infiltrating and influencing solid tumors, including breast cancer, presents a greater challenge. Nevertheless, current research still associates increased infiltration and activation of NK cells within solid tumors with improved overall survival across various cancers, including breast cancer (62). The infiltration of NK cells into tumors requires them to extravasate from blood vessels and navigate through the extracellular matrix and tumor stroma. While in circulation, NK cells interact with ligands on their surface, such as PSGL-1, via selectin family members like E-selectin, enabling them to roll along the endothelium of blood vessels. During this process, NK cells are activated by chemokines released within the tumor microenvironment, which guide them to tumor sites through the dynamic interaction between chemokine receptors and their corresponding ligands secreted in the tumor microenvironment (TME) (2). Additionally, cytokine signaling within the microenvironment where human NK cells reside significantly influences the type of effector functions they perform (63). However, once NK cells enter the TME, they often encounter negative regulation by immunosuppressive cells and molecules, leading to a functionally impaired state known as "immune exhaustion" (64). To counteract this, it is crucial to dissect the regulatory mechanisms governing the function of tumor-infiltrating NK cells. Moreover, not all tumor-infiltrating NK cells exert anti-cancer effects. For instance, research by Thacker et al. (65) identifies specific immature NK cells that actually promote the progression of triple-negative breast cancer. These diverse findings highlight the complex role of NK cells in the breast cancer microenvironment, with interleukin signaling playing a critical role in these processes. We outline the dual role of interleukin signaling in the breast cancer TME in Table 1.

Carcinostasis

Interleukins and NK cells interact in the immune response, jointly influencing the progression of breast cancer and the outcomes of treatment. Most interleukins can enhance the cytotoxic function of NK cells by activating and expanding them.

IL-2

IL-2 is a non-glycosylated protein composed of 133 amino acid residues, with a three-dimensional structure consisting of four α-helices that form a compact bundle. IL-2 exerts its biological effects by binding to its receptor complex, which is composed of three

TABLE 1 Interleukin families and their role in breast cancer.

Interleukin	Receptors	Function	Refs
IL-2	IL-2/IL-15Rβ–γc sIL-2Rα IL-2Rα–IL-2/IL-15Rβ–γc	Enhances *in vitro* expansion of NK cells for cytotoxicity against breast cancer cells and IFN-γ production	(66–68)
IL-15	IL-15–IL15Rα + IL-2/IL-15Rβ–γc	Enhances NK cell cytotoxicity against breast cancer cells	(68–71)
IL-21	IL-21R–γc	Enhances NK cell cytotoxicity against breast cancer cells	(72)
IL-8	CXCR1, CXCR2	Increases the sensitivity of breast cancer cells to NK cell-mediated lysis	(73)
IL-10	IL-10Rα–IL-10Rβ	Enhances NK cell activity, impedes breast cancer metastasis	(74)
IL-33	ST2 IL-1RAcP	Enhances NK cell activation and increases the tumor infiltrating NK cells Blocking IL33/ST2 and PD-L/PD-1 enhances the NK cells cytotoxicity of breast cancer cells	(75–77)
IL-30	IL-6Rα-gp130	Inhibits the production of IFNγ by NK cells	(78)
IL22	IL-22Rα1–IL-10Rβ IL-22Rα2	Increases CD155 expression in breast cancer cells, impairing NK cell function by promoting CD226 receptor internalization	(79)

distinct protein subunits: the α, β, and γ chains (80). Upon receptor binding, IL-2 activates signaling molecules such as Janus kinase (JAK) and signal transducer and activator of transcription (STAT). This activation not only promotes T cell proliferation and the generation of cytotoxic T lymphocytes but also enhances the cytotoxic activity of NK cells (81).

As early as 1993, researchers utilized IL-2 to treat peripheral blood lymphocytes (PBLs) from patients with stages I-III breast cancer (n = 41) *in vitro*. The activity of NK cells showed a clear dose-dependent increase and was significantly higher than the baseline NK cell activity observed in healthy controls (66). In the presence of trastuzumab and pertuzumab, IL-2-activated NK cells exhibited significantly higher cytotoxicity levels against SK-BR-3 cells compared to untreated cells (67). Similarly, NK cells stimulated by IL-21 demonstrated significantly increased cytotoxicity against the MCF-7, SKBR3, and T47D breast cancer cell lines (82). Moreover, IL-21 significantly enhanced the cytolytic activity and IFN-γ production of *ex vivo* expanded NK cells when targeting trastuzumab-coated breast cancer cells.

The stability and half-life of IL-2 in the body are relatively short, which limits its efficacy in clinical treatment. To address this limitation, researchers have developed Alb-IL2, a fusion protein composed of human serum albumin (Alb) and IL-2. The design of Alb-IL2 aims to enhance the *in vivo* stability and circulating half-life of IL-2, while reducing potential toxicity, thereby improving its efficacy and safety in clinical applications (83). Studies have demonstrated that the combination of intratumorally injected STING agonists, systemically administered Alb-IL2, and anti-PD-1 checkpoint blockade therapy (collectively referred to as CIP therapy) can stimulate both innate and adaptive anti-tumor immune responses in a triple-negative breast cancer model (83). The synergy between type I interferons activated by STING agonists and IL-2 enhances the expression of IFNAR-1 and CD25 on pulmonary NK cells through a positive feedback mechanism, promoting sustained NK cell activation. These activated NK cells continuously express granzymes, effectively combating and eliminating tumor metastasis.

HER2-positive breast cancer can be targeted by humanized anti-HER2/neu monoclonal antibodies (rhu4D5 or Herceptin). The specific lysis rates of IL-2-activated NK cells against rhu4D5-coated HER2/neu+ (MCF-7Her2/neu) and HER2/neu- (MDA-468) breast cancer cell lines were 35% and 3%, respectively ($p < 0.05$) (84). In the presence of IL-2, NK cells activated by rhu4D5-coated breast cancer cells also produce substantial amounts of IFN-γ, while surface activation markers CD25 and CD69 are upregulated. Therefore, the concurrent use of rhu4D5 monoclonal antibody and IL-2 treatment in patients with HER2/neu-positive breast cancer may yield superior therapeutic outcomes.

IL-15

IL-15 is a multifunctional cytokine composed of 162 amino acids, containing two disulfide bonds and two N-linked glycosylation sites (80). Structurally similar to the IL-2 gene, IL-15 belongs to the four-α-helix bundle cytokine family. IL-15 exerts its effects by binding to the IL-2Rβ/γc heterodimer, which it shares with IL-2, thereby activating the JAK/STAT pathway and promoting the activation and proliferation of immune cells. Various cells, including macrophages, monocytes, dendritic cells, epithelial cells, and fibroblasts, can produce IL-15 (85). This cytokine is particularly crucial for the proliferation and maintenance of NK cells. By binding to the IL-15 receptor on NK cells, IL-15 activates signaling pathways that promote NK cell proliferation and survival. Additionally, IL-15 regulates the terminal maturation of NK cells by modulating the expression of surface molecules such as CD122, which is essential for maintaining NK cell responsiveness to IL-15. IL-15 also enhances NK cell cytotoxicity by upregulating the expression of cytotoxic molecules such as perforin and IFN-γ and acts as a chemotactic factor, aiding in the migration and localization of NK cells. Research by Juliá et al. found that IL-15 enhances the efficacy of trastuzumab in treating triple-negative breast cancer (TNBC) by activating NK cells and dendritic cells (70). The study demonstrated that the combination of IL-15 and trastuzumab promotes the expression of CD25 and CD69 on NK cells and enhances the secretion of IFN-γ and TNF-α, thereby improving the anti-tumor response.

Combined use of recombinant IL-2 and recombinant IL-15 increases the number of NK cells while maintaining their purity (86). In addition, the combined use of IL-2, IL-15, and IL-18 has been shown to upregulate the expression of NK cell receptors, such as CD314, CD158a, and CD107a (69). NK cells stimulated by this cytokine combination secrete higher levels of TNF-α, IFN-γ, perforin (PRF1), and granzyme B, significantly enhancing their cytotoxic activity against breast cancer cells. Studies have demonstrated that IL-2 or IL-15 can restore and augment trastuzumab-triggered antibody-dependent cellular cytotoxicity (ADCC), thereby effectively enhancing the therapeutic efficacy of trastuzumab *in vivo* (68). The combination of trastuzumab with IL-15 and IL-2 has the potential to improve clinical outcomes in the treatment of triple-negative breast cancer. Furthermore, Roberti et al. demonstrated that IL-2 and IL-15 can enhance trastuzumab-mediated ADCC against breast cancer cells (87). This enhancement is primarily attributed to the ability of IL-2 and IL-15 to upregulate NK cell activation receptors, including CD16 and NKG2D, and to increase the production of IFN-γ.

NK cells release extracellular vesicles (EVs) that carry cytolytic proteins, indicating significant therapeutic potential. Notably, EVs derived from NK cells pre-treated with IL-12, IL-15, and IL-18 have demonstrated the ability to penetrate the core of tumor spheroids. This ability is linked to variations in the expression of NKG2D ligands MICA/B on the surface of tumor spheroids and can be inhibited by the application of anti-NKG2D antibodies (88). Building on this finding, researchers, including Zhu, have enhanced the therapeutic potential of NK-EVs in immunotherapy by pre-treating NK cells with IL-15 (71). They isolated NK-EVs from both untreated NK cells and those treated with IL-15 (NK-EVs^IL-15) from the culture medium of NK-92MI cells. These EVs were then purified using precise ultracentrifugation and density gradient centrifugation techniques. The study revealed that, compared to conventional NK-EVs, NK-EVs^IL-15 exhibited significantly enhanced cytolytic activity against human breast cancer cell lines *in vitro* and promoted the expression of molecules related to NK cell cytotoxicity. In a mouse tumor graft

model, NK-EVs^IL-15 significantly inhibited tumor growth without causing substantial toxicity to normal cells or mice, providing compelling evidence for the application of NK-EVs in cancer treatment.

It is worth noting that IL-15 has been shown to delay the formation of primary tumors and prevent or reduce metastasis in various mouse tumor models. For example, when breast tumor cells are intravenously injected into IL-15-deficient (IL-$15^{-/-}$), wild-type C57BL/6, IL-15 transgenic (TG), and IL-15/IL-15Rα-treated C57BL/6 mice, the metastasis rate is significantly higher in IL-$15^{-/-}$ mice, while mice treated with IL-15 TG or IL-15 exhibit almost no metastasis (89). Interestingly, when NK cells are depleted from the control C57BL/6 mice, the number of metastatic foci formed is considerably lower than in IL-$15^{-/-}$ mice, suggesting that in the absence of IL-15, other immune cell types may contribute to metastasis. Further investigation by the authors revealed that the lack of IL-15 in IL-$15^{-/-}$ mice may lead to the polarization of CD4^+ T cells towards a Th2 phenotype, promoting the formation of M2 macrophages, which are believed to contribute to metastasis. This study suggests that while IL-15's effect on NK cells is crucial, IL-15 also influences other immune cells, which can indirectly contribute to breast cancer metastasis.

Cytokine-inducible SH2-containing protein (CISH) is a key negative regulator of the IL-15 signaling pathway (90). Studies have shown that CISH interacts with JAK1, inhibiting its activity and thereby controlling NK cell responses to IL-15. This regulation is crucial for maintaining NK cell homeostasis and function. Notably, mice deficient in CISH exhibit resistance to breast cancer metastasis, indicating that CISH acts as a potent checkpoint in NK cell-mediated tumor immunity. To further elucidate the mechanisms by which CISH regulates NK cell activity, Bernard and colleagues employed a novel conditional mouse model ($Cish^{fl/fl}Ncr1^{iCre}$) (91). Their results suggest that the absence of CISH lowers the activation threshold of NK cells within the IL-15 cytokine signaling pathway. In $Cish^{fl/fl}Ncr1^{iCre}$ NK cells, IL-15 stimulation leads to increased expression of IFN-γ and CD107a, thereby inhibiting the metastasis of breast cancer cells.

Early studies demonstrated that recombinant single-chain IL-15 (rhIL-15), produced by E. coli, exhibits significant antitumor activity (91). To further enhance the clinical potential of IL-15, Stravokefalou et al. developed an IL-15/IL-15Rα complex (hetIL-15) to improve its stability and biological activity (92). Compared to rhIL-15, hetIL-15 has a longer half-life and demonstrates superior therapeutic efficacy. The therapeutic effect of hetIL-15, in combination with chemotherapy and surgery, has been investigated in a metastatic triple-negative breast cancer 4T1 mouse model, showing promising results.

Klopotowska et al. found that IL-15 can enhance the tolerance of NK cells to oxidative stress; however, this effect on antioxidant defense is short-lived (93). Additionally, recent studies have indicated that prolonged use of IL-15 may lead to NK cell functional exhaustion (94). These findings highlight the need for further research to determine the optimal use of IL-15 in order to maximize the persistence and expansion of infused NK cells while minimizing the risk of functional exhaustion.

IL-21

IL-21 is a member of the IL-10 cytokine family, composed of four α-helices. Unlike some other cytokines that may exist as dimers or multimers, IL-21 typically functions as a monomer (95). Upon binding to its receptor, IL-21 requires a common gamma chain (also known as a common receptor subunit, such as IL-2Rγ or CD132) and a specific alpha chain (such as IL-21R). IL-21 plays a crucial role in regulating immune responses, not only activating NK cells and cytotoxic T lymphocytes but also promoting the differentiation of helper T cells (Th cells) into the Th1 subset (96). Although IL-21 has demonstrated significant therapeutic efficacy in the treatment of various solid tumors, including breast cancer, it faces challenges in clinical application, such as limited response rates and a short half-life. Combining IL-21 with other therapies, particularly PD-1/PD-L1 inhibitors, may further enhance the anti-tumor activity of T cells (97). A recent study explored the combination of apolipoprotein A1-modified doxorubicin liposomes (ApoA1-lip/Dox) with long-acting IL-21 for cancer treatment (72). This combined therapeutic approach not only significantly increased the number of tumor-infiltrating lymphocytes but also enhanced the cytotoxicity of CD8+ T cells and NK cells. The joint application of ApoA1-lip/Dox and IL-21 enhanced the anti-tumor effect while effectively reducing the toxicity associated with ApoA1-lip/Dox, offering a promising new strategy for treating challenging tumors such as triple-negative breast cancer. This approach could potentially improve therapeutic outcomes while reducing adverse reactions, providing patients with a safer and more effective treatment option.

While IL-21 holds considerable therapeutic potential, its use may also induce side effects, such as enhanced inflammatory responses, necessitating careful monitoring and management. The application of IL-21 in breast cancer treatment is still under investigation, and more clinical data are needed to confirm its efficacy and safety. With the rapid advancements in immunotherapy, IL-21 is poised to become an important component in the treatment of breast cancer.

IL-8

IL-8 is a chemokine with multiple biological functions that has attracted extensive attention in the field of cancer research in recent years. IL-8 primarily exerts its effects by binding to the CXCR1 and CXCR2 receptors (98). The expression of IL-8 is upregulated in a variety of solid tumors, including TNBC, and it promotes the infiltration of immunosuppressive cells into the tumor microenvironment, angiogenesis, epithelial-mesenchymal transition, and other processes by activating various signaling pathways, such as JAK/STAT and PI3K/Akt (99). These actions further contribute to tumor growth, invasion, metastasis, and drug resistance (100, 101). Studies have indicated that serum IL-8 levels are positively correlated with tumor burden in patients and negatively correlated with the therapeutic effects of immune checkpoint inhibitors. Particularly in patients treated with PD-1/PD-L1 antibodies, higher circulating IL-8 levels are associated with shorter survival times and poorer clinical outcomes. Given the role of IL-8 in tumor development, inhibitors targeting IL-8 are being developed and studied. For instance, HuMax-IL8 is a monoclonal antibody against IL-8 that has demonstrated

antitumor activity in preclinical models of low claudin triple-negative breast cancer (73). HuMax-IL8 can not only reduce the mesenchymal characteristics of cancer cells but also decrease the frequency of polymorphonuclear myeloid-derived suppressor cells found at tumor sites. Moreover, HuMax-IL8 increases the sensitivity of breast cancer cells to lysis mediated by immune effector NK cells and antigen-specific T cells *in vitro*. These research advancements suggest that IL-8 plays a significant role in the occurrence, development, metastasis, and immune suppression of tumors, and it may serve as a potential biomarker for predicting therapeutic outcomes and patient prognosis. At the same time, therapeutic strategies targeting IL-8 could offer new options for cancer treatment. Future research is needed to further validate the clinical efficacy of IL-8 inhibitors and to explore the optimal combination with other treatment modalities.

IL-10

IL-10 is a low molecular weight cytokine belonging to the four α-helix bundle cytokine family, composed of 162 amino acids. This includes a 48-amino acid signal peptide sequence and a 114-amino acid mature protein portion (102). IL-10 contains two intramolecular disulfide bonds that are crucial for maintaining its three-dimensional structural stability and biological function. Additionally, the IL-10 molecule has two N-linked glycosylation sites, where glycosylation modifications may influence its solubility, half-life, and biological activity (103). When IL-10 binds to its receptor, it first requires high-affinity binding through the IL-10Rα chain, followed by the formation of a high-affinity trimeric receptor complex involving the IL-2Rβ chain and the common gamma chain (γc), which are also shared with IL-2. Upon receptor binding, IL-10 activates signaling pathways such as JAK/STAT, thereby regulating the activity of immune cells. IL-10 is primarily produced by immune cells, including monocytes and macrophages, but it can also be expressed by certain epithelial cells and tumor cells. Studies have shown that IL-10 functions as an effective anti-metastatic agent, particularly in immunocompromised hosts. This anti-metastatic effect appears to be relatively independent of T cell function but is dependent on NK cell activity. Conversely, the inhibitory effect of IL-10 on tumor formation is T cell-dependent (74).

Carcinogenesis

IL-1β

IL-1β is an important inflammatory cytokine that plays a key role in the regulation of immune and inflammatory responses (104). IL-1β promotes the maturation of cord blood CD34+ precursor NK cells (32). In TME, IL-1β is a major cytokine for tumor progression (105). Recent studies have confirmed that IL-1β-induced hypoxia-inducible lipid droplet-associated (HILPDA) mediates the pre-metastatic stage of lung-resident mesenchymal cells (MCs) in breast cancer accumulate neutral lipids. These lipid-laden MCs transport their lipids to tumor cells and NK cells via exosome-like vesicles, leading to enhanced tumor cell survival and proliferation as well as NK cells dysfunction. Furthermore, blockade of IL-1β alone effectively improved the efficacy of immunotherapy using NK cells in attenuating lung metastases (106).

IL-22

DNAX accessory molecule-1 (DNAM-1, also known as CD226) is a co-stimulatory adhesion molecule expressed by NK cells and has a crucial role in tumor immunosurveillance (107). NK-92 cells stimulated by anti-CD226 antibody (sNK-92) are more cytotoxic to TNBC cells compared to NK-92 cells (108). As a ligand for DNAM-1, CD155, is expressed on many cell types, including transformed or infected cells, and directly affects NK cell function (109). In breast cancer, for example, high cellular CD155 expression has been associated with aggressiveness and poorer prognosis 31176775. Many kinds of interleukins act on DNAM-1 and further affect NK cell function, such as IL-15/IL-18/IL-27 (110). However, certain interleukins in the tumor microenvironment also inhibit the function of DNAM-1 on NK cells, thus helping tumors to evade immune surveillance.IL-22 acts through the IL-22 receptor (IL-22R) and is associated with a poorer prognosis, higher disease stage, and more rapid tumor progression (79, 111). Briukhovetska and colleagues found that IL-22 produced by T cells can increase the expression of CD155 in breast cancer cells, which in turn disrupts NK cell function by promoting the internalization of the activating receptor CD226. This ultimately leads to the promotion of lung metastasis in breast cancer (79).

IL-30

IL-30, also known as IL-27p28 or IL-27A, signals by recruiting gp130 (CD130) to form a homodimer, then uses IL6Rα (CD126) to transmit signals. Among the different molecular subtypes of breast cancer, IL-30 expression is particularly high in triple-negative and HER2-positive subtypes, which may be related to the aggressiveness and poor prognosis of these subtypes. IL-30 can promote the proliferation and invasive and metastatic capabilities of breast cancer cells. Moreover, IL-30 can also provide nourishment for breast cancer stem cells through an autocrine loop of CXCL10 and IL-23, and shape the immune environment and host outcomes, further indicating that IL-30 may play a key role in the maintenance of breast cancer stem cells and the immune escape of tumors (112). Sorrentino et al. found that the lack of endogenous IL-30 triggers the production of IFNγ by T cells and NK cells, thus hindering the progression of triple-negative breast cancer and improving survival (78). However, the specific mechanisms of action of IL-30 in breast cancer and its clinical significance still require further research. Understanding the role of IL-30 in breast cancer may help develop new treatment strategies, especially for aggressive subtypes of breast cancer that currently lack effective treatment methods.

Two-edged sword

IL-6

IL-6 has generally been studied as a derivative of NK cells. In breast cancer patients, tumor-infiltrating NK cell-derived IL-6 is

associated with tumors with higher MHC-I expression. In wild-type and IL-6 KO mouse models, inhibition of the IL-6/signal transducer and activator of transcription (STAT3) axis attenuated the suppression of T-cell responses, resulting in reduced tumor growth and metastatic spread (113). Another study showed that anti-IL-6 receptor (IL-6R) ameliorated helper T-cells, cytotoxic T-cells, and NK cells in the lymphatic system cells and reduced Tregs in relapsed and metastatic TNBC. Not only that, the combination of IL-6R and PD-L1 immunotherapy diminished TNBC cell stemness and M2 macrophage activity to a greater extent than monotherapy and showed better survival outcomes and the lowest postoperative recurrence and metastasis rates (114). However, IL-6 is likewise thought to inhibit breast cancer development. A study by Jin et al. demonstrated that NK cells in the TME inhibited the invasiveness of MDA-MB-231 cells, a human TNBC cell line, through IL-6-mediated inhibition of uPA. Cytokine array analysis revealed elevated levels of interleukin IL-10, IL-6, IL-8, C-C motif ligand (CCL)5, and CCL2 in the conditioned medium of co-cultured cells (115). The synergistic effect between these molecules still deserves further exploration.

IL-32

It was in IL-2-activated NK cells that IL-32 was originally discovered (116). However, in breast cancer, IL-32-induced vascular endothelial growth factor (VEGF) increases the migratory and invasive capacity of breast cancer and decreases its apoptosis via STAT3 activation (117). Interestingly, it also induces apoptosis and enhances the sensitivity of NK cells and cytotoxic T cells in other cancer types (116). Therefore, we have included it in this review despite the fact that it has not been studied to affect breast cancer after direct action on NK cells.

IL-33

IL-33, a member of the IL-1 cytokine family, has a unique fibrinogen-like domain and was identified in 2005 as a ligand for ST2 (also known as IL1RL1) (118). ST2 exists in two forms: a functional long form, ST2L, and a decoy receptor short form, sST2. When IL-33 binds to its receptor ST2, it activates multiple intracellular signaling pathways, including NF-κB and MAPK, which are crucial for the activation, proliferation, and cytokine production of immune cells (119). Despite containing a nuclear localization signal (NLS), IL-33 can also be released as a secretory protein through a non-canonical secretion pathway. IL-33 is primarily expressed by epithelial cells, endothelial cells, and immune cells and can be induced by various stimuli such as injury and inflammation. Recent research has highlighted the role of IL-33 in promoting Th2 immune responses, regulating allergic reactions, and tissue repair (120).

However, the role of IL-33 in tumorigenesis remains controversial. Some studies have indicated that IL-33 can effectively inhibit the development of lung metastases in mouse models of breast cancer. For example, in the 4T1 breast cancer model, IL-33 exerts antitumor effects by promoting macrophages to produce TNF-α and enhancing the activation and specific recruitment of NK cells to the tumor site. The activation of NK cells and the production of CCL5 induced by IL-33 contribute to the recruitment of NK cells in the tumor microenvironment, leading to a potent tumor rejection response (75). Gao et al. found that overexpression of IL-33 in 4T1 cells strongly inhibits tumor growth (121). IL-33 increases the number of tumor-infiltrating NK cells and CD8(+) T cells and their production of IFNγ. The antitumor effect of IL-33 requires the participation of both NK cells and CD8(+) T cells. In contrast, other studies suggest that the IL-33/ST2 axis may accelerate tumor progression and metastasis by promoting tumor angiogenesis and the accumulation of immunosuppressive cells. In breast cancer models, activation of the IL-33/ST2 pathway is associated with accelerated tumor growth and lung and liver metastasis. In tumor-bearing mice, IL-33 treatment reduced the cytotoxicity of NK cells, while the cytotoxicity of CD8(+) T cells did not change significantly, and the removal of CD8(+) T cells had no effect on the progression of breast tumors (76).

Further research has found that in ST2-deficient mice, the inhibition of breast cancer progression and metastasis is associated with enhanced NK cell cytotoxic activity and increased systemic Th1/Th17 cytokines (76). Specifically, there is an increase in the proportion of activated CD27high CD11bhigh NK cells, CD69+ NK cells, and KLRG- NK cells in tumor-bearing ST2-/- mice, as well as enhanced *ex vivo* killing activity of splenocytes, NK cells, and CD8+ T cells. Compared with wild-type mice, there is a significant increase in the number of NK cells expressing IFN-γ in ST2-/- mice, which may be related to an enhanced antitumor immune response.

Additionally, the IL-33/ST2 axis may interact with other immune regulatory pathways, such as the PD-L/PD-1 axis. In a recent study, the authors induced 4T1 breast cancer in BALB/C wild-type and ST2 knockout mice and treated the mice with anti-PD-1 and anti-IL-33. They found that simultaneous blockade of IL-33/ST2 and PD-L/PD-1 delayed tumor onset and slowed tumor growth (77). In ST2 knockout mice treated with anti-PD-1, enhanced cytotoxicity of NK cells against 4T1 tumor cells was associated with overexpression of miRNA-150 and miRNA-155, upregulation of NF-κB and STAT3, increased activation markers, and reduced immune suppressive markers. NK cells in ST2 knockout mice treated with anti-PD-1 tended to proliferate more and were less sensitive to apoptosis. In the spleens and primary tumors of ST2 knockout mice treated with anti-PD-1, the accumulation of immunosuppressive myeloid-derived suppressor cells and regulatory T cells was significantly impaired. Blocking the IL-33/ST2 axis may be a potential tumor treatment strategy. For example, using antibodies against IL-33 or ST2 can inhibit tumor growth and the accumulation of immunosuppressive cells.

Existing research results present differing views on the role of IL-33 in the TME, revealing its complex and multifaceted mechanisms of action. The effects of IL-33 may be influenced by various factors, including the specific TME, immune cells in the tissue, the stage of tumor development, and the type of tumor cells. Future research has the potential to uncover the specific role of IL-33 in tumor immune responses and explore how to develop effective cancer treatment strategies by modulating the IL-33/ST2 axis. In particular, the activation or inhibition of the IL-33/ST2 axis may produce very different immune responses in different tumor

microenvironments, which needs to be specially considered when designing targeted therapies. With a deeper understanding of the function of IL-33, we can look forward to new breakthroughs in the field of cancer treatment, especially for tumor types that are not sensitive to traditional treatments.

Recombinant and engineered interleukins boost function of NK cell to fight breast cancer

Interleukins have demonstrated tremendous potential in the field of breast cancer treatment, particularly with the application of IL-12 family cytokines. However, the full activation of the immune system and the poor stability of interleukins in the body may lead to significant side effects. Therefore, current research trends are focusing on the modification of interleukins or the combination of chemotherapy with immunotherapy, aiming to reduce their toxicity and enhance therapeutic efficacy.

In this context, researchers have designed an innovative nanosystem that utilizes a charge-reversal polydimethylaminoethyl methacrylate (PMet) platform to co-deliver doxorubicin (DOX) and plasmids encoding IL-12. This strategy aims to enhance the treatment effect on metastatic breast cancer through the combined application of chemotherapy and gene therapy. In a 4T1 breast cancer lung metastasis mouse model, this nanosystem can enhance NK cells and tumor-infiltrating cytotoxic T lymphocytes, regulate the polarization from tumor M2 macrophages to activated anti-tumor M1 macrophages, and simultaneously reduce immunosuppressive regulatory T (Treg) cells, increasing the expression of cytokines IL-12, IFN-γ, and TNF-α, showing better anti-tumor and anti-metastatic activity (122).

Furthermore, preoperative intratumoral injection of biodegradable poly(lactic acid) microspheres (PLAM) loaded with cytokines can stimulate a long-term and systemic tumor-specific immune response. This method utilizes the immune potential of autologous tumors to exert anti-tumor effects with minimal toxicity (123). IL-12, mainly produced by macrophages and dendritic cells, is a heterologous cytokine. IL-12 receptors are expressed on activated CD4+ and CD8+ T cells and CD56+ NK cells; thus, it does not induce the proliferation of resting peripheral T cells or NK cells but has a direct proliferative effect on pre-activated T cells and NK cells. In the Balb/c mouse 4T1 breast cancer model, PLAM injected alone with IL-12 after spontaneous metastasis showed anti-tumor effects. *In vivo* lymphocyte depletion studies confirmed that its anti-tumor effects are mainly mediated by NK cells, but this may enhance the immune suppression of T cells. The combination of IL-12 and TNF-α loaded PLAM as a new adjuvant immunotherapy can enhance the activity of tumor-specific CD8+ T cells. Intratumoral cytokine therapy shows great potential in clinical and immunological aspects, but to produce a lasting tumor-specific T cell response, rather than an NK cell response or a more detrimental immune suppression effect, precise cytokine combinations and sustained delivery methods are needed.

In a recent study, nanoparticles based on the low-temperature expansion effect of acid-sensitive materials mPEG-Dlinkm-PDLLA and Pluronic F127 were used to co-deliver hydrophobic chemotherapeutic drug PTX and the biologic macromolecule IL-12 (124). The nanoparticles enriched in the tumor site significantly inhibited the growth and metastasis of breast cancer cells 4T1 and extended the overall survival time of tumor-bearing mice. The combination of PTX and IL-12 activated T lymphocytes and NK cells to release IFN-γ, selectively inhibited regulatory T cells, induced tumor-associated macrophages to differentiate into the M1 type, thereby improving the tumor immunosuppressive microenvironment.

Gene therapy can well control the location and quantity of interleukins to maximize the improvement of NK cell expansion and function. Zhao et al. injected mice inoculated with 4T1 mouse breast cancer cells with plasmids encoding IL-15 and everolimus and found that both IL-15 gene therapy and everolimus significantly reduced tumor size (125). IL-15 gene therapy increased the proportion of CD4+ T cells and NK cells but had no effect on CD8+ T cells. Everolimus reduced the number of CD8+ T cells but had no effect on CD4+ T cells and NK cells compared to the control group. Although effective individually, no synergistic effect was observed with the combined treatment of everolimus and IL-15 gene therapy.

IL-10 is considered an immunosuppressive cytokine, which is related to various inhibitory and regulatory cell populations in the tumor microenvironment, including tolerogenic dendritic cells, regulatory CD4+ T cells, B10 cells, M2 macrophages, and myeloid-derived suppressor cells. Specific single nucleotide polymorphisms have been found to drive overexpression of the IL-10 gene, thereby promoting the progression of various malignant tumors. Multiple clinical studies have shown that upregulation of IL-10 levels is strongly correlated with tumor staging and poor prognosis in breast cancer patients (126). Shen et al. designed an IL-10 protein trap through genetic engineering methods and delivered it to the tumor microenvironment through lipid-arginine-DNA (LPD) nanoparticles to reduce IL-10 levels and activate dendritic cells, thereby enhancing the anti-tumor immune response (127). In the 4T1 triple-negative breast cancer model, IL-10 trap gene therapy alone could significantly inhibit tumor growth and improve the host's survival time. The local and transient expression of the IL-10 trap gene can change the tumor microenvironment, reduce the accumulation of immunosuppressive cells, and promote the tumor-killing activity of cytotoxic T cells and NK cells. The potential of IL-10 as a therapeutic target is emphasized because it plays a key role in regulating the immunosuppressive microenvironment of breast cancer and other cancers.

Recombinant adenovirus-associated vector serotype 2 (rAAV2) is a viral vector widely used in gene therapy and gene function research. Yu et al. (128) constructed rAAV2-hIL15, which carries human interleukin 15 (hIL15) delivered to the body for long-term gene expression. The results showed that rAAV2-hIL15 can significantly delay the onset of breast cancer, inhibit tumor growth, and extend the lifespan of tumor-bearing mice. Moreover, rAAV2-hIL15 can express a large amount of IL15 protein, ultimately activating the cytotoxic activity of NK cells. This gene therapy strategy may be used to increase the local expression level of IL-15, thereby enhancing the activity of NK cells, and is expected to become a new potential therapeutic tool for immunotherapy of breast cancer.

Clinical trials

In breast cancer treatment, several clinical trials have been conducted to explore the efficacy and safety of using NK cells or interleukins to influence NK cell activity. Burns et al. conducted a phase I/II clinical trial to evaluate the safety, immune activation effects, and potential efficacy of IL-2-activated NK cell infusion or IL-2 priming therapy in autologous transplantation for lymphoma and breast cancer (129). The results of this study indicated that IL-2-activated NK cell/IL-2 priming therapy could be safely administered, enhancing the cytolytic function of fresh peripheral blood mononuclear cells and increasing cytokine levels. However, with this dosage and IL-2 dosing regimen, no improvement in patient disease outcomes was observed. This study provided preliminary evidence for the potential therapeutic use of IL-2-activated NK cells post-autologous transplantation, although their clinical efficacy did not meet expectations, valuable information for future research and treatment strategies development. To enhance the therapeutic efficacy of IL-2-activated NK cells, exploring strategies such as increasing the number of NK cell infusions, using high-purity NK cells or their subsets, combining with tumor-reactive monoclonal antibodies, combination therapy with other cytokines like IL-12, or enhancing treatment efficacy through NK cell inhibitory receptor blockade may be necessary.

In a phase I clinical trial on Trastuzumab combined with IL-2 in HER2-positive metastatic breast cancer, it was found that Trastuzumab combined with IL-2 was a well-tolerated outpatient treatment regimen, resulting in NK cell expansion and enhanced *in vitro* targeted killing of HER2-expressing cells (130). This provided preliminary clinical trial results for the combination therapy of HER2-positive metastatic breast cancer, emphasizing the safety and potential clinical benefits of this treatment regimen. Recchia et al. conducted a clinical study on maintenance immunotherapy using IL-2 and 13-cis-retinoic acid (RA) in patients with metastatic breast cancer after chemotherapy (131). The study found that maintenance immunotherapy was well tolerated in patients with metastatic breast cancer and improved lymphocyte and NK cell counts and CD4+/CD8+ ratio, delaying disease recurrence. However, randomized trials are needed in the future to further validate these findings.

In a phase II clinical study evaluating allogeneic NK cell therapy in recurrent solid tumor patients (mainly ovarian cancer and breast cancer patients), lymphodepletion pre-treatment was administered to 20 patients, followed by infusion of NK cells from haploidentical donors and low-dose IL-2 to promote NK cell expansion. The infusion of allogeneic NK cells was associated with transient chimerism and may be limited by reconstructed recipient Treg cells (132). This study also mentioned some unforeseen serious adverse events, such as tumor lysis syndrome (TLS) and passenger lymphocyte syndrome (PLS), adding to the understanding of potential consequences of allogeneic NK cell therapy.

Recent phase I clinical trials have explored various administration methods of recombinant human IL-15 (rhIL-15), including intravenous infusion monotherapy, subcutaneous injection, and continuous intravenous infusion (133–135). Although these treatment methods significantly increase the total number of circulating NK cells, patients may experience adverse reactions related to recombinant cytokine administration.

These clinical studies will greatly impact the field of breast cancer treatment. We have listed relevant clinical studies in Table 2. It is worth noting that although interleukins theoretically have the potential to enhance immune responses and anti-tumor activity, their clinical application still faces challenges, including how to optimally activate the immune system, avoid excessive immune-related toxicity, and predict and select patient populations most likely to benefit from treatment.

Conclusion and outlook

NK cell-related therapies for breast cancer hold promise in altering the current landscape of breast cancer immunotherapy. A plethora of cytokines, including interleukins, can be utilized to broadly enhance the cytotoxicity and cytokine response of NK cells, and are currently undergoing clinical testing. These clinical trials leverage the unique characteristics of NK cells against breast cancer, adeptly executing tumor killing across various mechanisms. In addition to traditional approaches such as engineering NK cells, such as CAR-NK, or utilizing gene editing techniques to knock out inhibitory receptors on NK cells or inhibit immune checkpoints, modification of cytokines will also have profound effects on NK cell function. Previous modifications often targeted antibodies, such as developing bispecific or trispecific antibodies capable of simultaneously activating NK cells and binding to tumor antigens, enhancing ADCC effects, among others. Current research indicates that modifications can also be applied to cytokines, including interleukins. Utilizing engineered fusion complexes of interleukins and their receptors, encapsulated within extracellular vesicles, has been shown to retard tumor growth,

TABLE 2 Clinical trial of NK Cell-Based therapy and the use of interleukins for the treatment of breast cancer and assessment of NK Cell status.

Treatment modalities	Phase	NCT	Status
Activation of primary NK cells	I/II	NCT03634501	UNKNOWN
Combination of allogeneic NK cells and targeted therapy	I	NCT05385705	RECRUITING
Combination of cryosurgery and NK cells immunotherapy	I/II	NCT02844335	COMPLETED
Combination of CAR-NK and targeted therapy	I	NCT05069935	TERMINATED
IL-7(observe the function of NK cells)	II	NCT01368107	COMPLETED
CAR-NK	I	NCT04106167	TERMINATED
Allogeneic NK cells as monotherapy and in combination with monoclonal antibody	I	NCT03319459	COMPLETED
Trastuzumab and IL-2(observe the function of NK cells)	II	NCT00006228	COMPLETED
Injection of rIL-2-activated NK cells	II	NCT00855452	COMPLETED

increase tumor infiltration of NK cells and CD8+ T cells, elevate expression of IFN-γ, cytotoxic granule components, and anti-apoptotic BCL-2 (136). These studies hold strong promise for application and have entered clinical research stages.

However, there are still many limitations to NK cell-related therapies for breast cancer, and there is much room for developing novel approaches to further enhance the NK cell immunotherapy platform. Improving NK cell penetration and targeting in solid tumors, enhancing their activation, cytolytic capabilities, and survival rates after encountering the harsh immune-suppressive tumor microenvironment are the most critical goals. Additionally, efforts should be directed towards mobilizing exhausted tumor-infiltrating NK cells. These studies will profoundly change the landscape of breast cancer treatment, and we eagerly anticipate the arrival of this day.

Author contributions

JX: Writing – original draft, Writing – review & editing. HG: Writing – review & editing. MA: Writing – review & editing. HX: Writing – review & editing. SC: Writing – review & editing. ML: Writing – review & editing. XN: Writing – review & editing. TY: Writing – review & editing. HZ: Conceptualization, Data curation, Investigation, Methodology, Software, Supervision, Writing – review & editing. QL: Formal analysis, Project administration, Validation, Writing – review & editing. WY: Funding acquisition, Resources, Visualization, Writing – review & editing.

Funding

The author(s) declare financial support was received for the research, authorship, and/or publication of this article. This study was funded by the Natural Science Foundation of the Hunan Province of China (Grant No. 2022JJ70143, 2022JJ70122); Wu Jieping Medical Foundation Clinical Research Special Grant Fund (Grant No. 320.6750.2023-18-17); Xinrui Tumor Support Therapy Research Project(Grant No. cphcf-2023-022); The China Postdoctoral Science Foundation (Grant No. 2023M733955).

Conflict of interest

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

Publisher's note

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frontiers | Frontiers in Immunology

TYPE Opinion
PUBLISHED 04 October 2024
DOI 10.3389/fimmu.2024.1459777

OPEN ACCESS

EDITED BY
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RECEIVED 04 July 2024
ACCEPTED 17 September 2024
PUBLISHED 04 October 2024

CITATION
Dong S and Ma Z (2024) Combination of JAK inhibitor and immune checkpoint inhibitor in clinical trials: a breakthrough.
Front. Immunol. 15:1459777.
doi: 10.3389/fimmu.2024.1459777

Combination of JAK inhibitor and immune checkpoint inhibitor in clinical trials: a breakthrough

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KEYWORDS

JAK inhibitor, immune checkpoint inhibitor, combination, immunotherapy, clinical trial

Introduction

Recently, two back-to-back clinical trials research studies published in *Science*. Zak et al. and Mathew et al. reported that combining these therapeutic approaches led to improved clinical responses compared to immune checkpoint inhibition alone in patients with relapsed or refractory Hodgkin lymphoma and metastatic non-small cell lung cancer (NSCLC), respectively (1, 2).

The landscape of cancer treatment, particularly immunotherapy, has evolved dramatically over the past few decades. Immune checkpoint inhibitors (ICIs), which increase the ability of the immune system to attack cancer cells, are a promising revolution (3). However, not all patients respond to ICIs, leading to the search for combination therapies that can enhance their efficacy (4). A promising approach is the combination of Janus kinase (JAK) inhibitors (JAKi) and ICIs. These studies explored the potential of this combination therapy for the treatment of various cancers. Before these two studies, this combination of therapies in cancer treatment was rarely undertaken. This is because the strategy appears to be paradoxical. Why can JAKi (which suppress immune cell activation and proliferation) increase the immune-mediated elimination of cancer cells?

JAKi are small molecules that block the activity of one or more members of the JAK family of enzymes. These enzymes are critical for the signaling of various cytokines and growth factors involved in hematopoiesis (5), immune function (6), and inflammation (7, 8), neuropathology (9). These drugs modulate the immune response by inhibiting JAKs, making them effective for treating autoimmune diseases. JAKi include Ruxolitinib, Itacitinib, and tofacitinib, each with varying specificities for JAK1, JAK2, JAK3, and TYK2 (10).

ICIs, such as nivolumab (anti-PD-1), pembrolizumab (anti-PD-1), and ipilimumab (anti-CTLA-4), block the inhibitory pathways that limit T-cell activation. These pathways include PD-1/PD-L1 and CTLA-4, which are often exploited by tumors to evade the immune system. By blocking these checkpoints, ICIs enhance T-cell responses against cancer cells, leading to improved antitumor activity (11).

In general, JAKi decrease T-cell activity in most autoimmune diseases. However, it is beneficial for patients with high T-cell activation in a tumor environment with conventional cognition. Therefore, only a few groups have attempted to combine

immunotherapy with inflammatory inhibitors for cancer therapy. It is a new mechanism that these two *Science* papers show that JAKi can improve antitumor responses, implying that a new rationale will be found.

Here, the authors hypothesized that JAKi can be combined with ICIs. This combination is based on complementary mechanisms. While ICIs boost the immune system's ability to recognize and attack cancer cells, JAKi modulate the immune environment to reduce factors that limit this response rather than directly targeting immune cells. 1). Enhancing T-Cell Activity: JAKi can help overcome the immunosuppressive environment within tumors by reducing the activity of myeloid-derived suppressor cells (MDSCs) and regulatory T-cells (Tregs). This enhances the activation and proliferation of cytotoxic T-cells, which are crucial for effective antitumor responses. 2). Reducing Immune Resistance: Chronic inflammation and persistent interferon signaling within the tumor microenvironment can lead to T-cell exhaustion. JAKi can reset chronic signaling, reducing T-cell exhaustion and improving the efficacy of ICIs.

Zak et al. explored the use of Ruxolitinib, a JAK1/2 inhibitor, in combination with nivolumab (anti-PD1) in mouse models of chronic lymphocytic choriomeningitis virus (LCMV) infection, a model for chronic viral infections and cancer. The study found that Ruxolitinib administration resulted in increased numbers of antigen-specific $CD8^+$ T-cells and reduced the viral load. Importantly, the combination of Ruxolitinib and nivolumab led to significant reductions in tumor growth compared to either treatment alone. A phase 1 trial conducted by Zak et al. assessed the efficacy of Ruxolitinib in combination with nivolumab in 19 patients with relapsed or refractory Hodgkin lymphoma who had previously received ICIs. The trial showed an impressive overall survival rate of 87% at two years, compared to 23.8% with ICIs therapy alone. Patients receiving combination therapy exhibited decreased numbers of myeloid progenitors and MDSCs, increased cytokine-producing $CD8^+$ T-cells, and improved antitumor immune responses.

Similarly, Mathew et al. investigated the combination of Itacitinib, a JAK1 inhibitor, and pembrolizumab (anti-PD1) in a mouse model of NSCLC. Combination therapy significantly improved survival and reduced tumor burden, with treated mice showing increased T-cell infiltration and reduced expression of exhaustion markers. They conducted a phase 2 clinical trial combining Itacitinib with pembrolizumab in 21 treatment-naïve patients with NSCLC. The trial demonstrated a median progression-free survival of nearly two years, significantly longer than the 6.5 to 10.3 months observed with pembrolizumab alone in other trials. Combination therapy led to a proliferative burst of $CD8^+$ T-cells and reduced T-cell exhaustion.

The mechanisms underlying the enhanced efficacy of the combination of JAK inhibitors with ICIs are multifaceted and involve several key processes: 1). Reversing T-Cell Exhaustion; 2). Modulating the Tumor Microenvironment; and 3). Enhancing Antigen Presentation.

However, combination therapy remains a challenge. 1). Safety and Toxicity, as combining two potent immune-modulating therapies raises concerns regarding increased toxicity and adverse effects. Careful monitoring and management of adverse effects are essential to ensure patient safety. 2). Patient Selection, that is, identifying patients who are most likely to benefit from combination therapy is critical. Biomarkers that predict response to JAKi and ICIs can help treat individual patients. 3). Optimal Dosing and Timing, as determining the optimal dosing and timing of JAK inhibitors in combination with ICIs is crucial for achieving the best clinical outcomes. 4). Resistance Mechanisms, as understanding the mechanisms of resistance to combination therapy is essential for developing strategies to overcome this resistance. Studies exploring the molecular pathways involved in drug resistance may facilitate the development of next-generation therapies.

In summary, the combination of JAKi and ICIs is a promising strategy for enhancing antitumor immunity and overcoming resistance to immunotherapy. Preclinical and clinical studies have demonstrated the potential of this approach in treating relapsed or refractory Hodgkin lymphoma and NSCLC, showing improved survival and tumor control compared to ICI therapy alone (1, 2). The mechanistic insights gained from these studies highlight the importance of reversing T-cell exhaustion, modulating the tumor microenvironment, and enhancing antigen presentation to achieve effective antitumor responses. However, challenges related to safety, patient selection, dosing, and resistance mechanisms must be addressed to fully realize the potential of this combination therapy. It is a very exciting insight and positive result to support combining anti-inflammatory inhibitors and immunotherapy, not restricted to the JAKi with anti-PD1/PD-L1 combination.

Discussion and prospective

Although these clinical trials provide promising preliminary data, it must be acknowledged that these studies have limitations in terms of sample size and study design. For example, the study by Zak et al. included only 19 patients, which may limit the generality of the results. Moreover, the lack of long-term follow-up data precludes the assessment of the durability of the therapeutic effects and potential long-term side effects, which should be further investigated in subsequent studies.

When discussing the safety of these clinical trials, it is crucial to analyze the immune-related adverse events associated with combination therapy. For instance, patients may experience severe immune-mediated side effects during treatment, such as aggressive autoimmune diseases and some else emerged inflammatory responses. Several serious immune-related adverse events were reported in the study by Zak et al., which warrants special attention in patient management to ensure safety.

While JAK inhibitors are typically considered to suppress immune cell activation, in specific tumor microenvironments, these drugs can selectively reduce the activity of suppressive immune cells such as myeloid-derived suppressor cells (MDSCs) and regulatory T cells (Tregs), thereby enhancing the activity of cytotoxic T cells. Thus, the role of JAK inhibitors is not merely to suppress immune responses, but to finely tune the tumor microenvironment to promote antitumor immunity. It may be a new strategy for combination (anti-inflammation drugs and anti-tumor growth drugs), such as TNF inhibitor, ACE (Angiotensin-converting enzyme) inhibitor, COX-2 inhibitor.

Biomarkers play a critical role in guiding patient selection and predicting the response to combination therapy with JAKi and ICIs. Current research is exploring various potential biomarkers, such as cytokine profiles within the tumor microenvironment, PD-L1 expression levels (12), and the extent of T-cell infiltration (13), all of which may be used to predict patient response to combination therapy. Future studies need to further elucidate the predictive value of these biomarkers to achieve more personalized cancer treatment.

Although these early clinical trials provide encouraging results, future research should focus on applying this combination therapy to a large range of cancer types. Additionally, exploring the combination of this approach with other emerging therapies, such as cancer vaccines and cellular therapies, could further enhance therapeutic outcomes. As we gain a deeper understanding of the mechanisms underlying the JAKi and ICI combination therapy, the development of next-generation therapies that are more targeted and have lower toxicity will also become an important direction for future research.

Author contributions

SD: Funding acquisition, Resources, Writing – review & editing. ZM: Writing – review & editing, Writing – original draft.

Funding

The author(s) declare financial support was received for the research, authorship, and/or publication of this article. This work was supported by the Natural Science Foundation of Colleges of Jiangsu Province, China (22KJB210001) and the Youth Foundation of the Natural Science Foundation of Jiangsu Province (BK20220704).

Conflict of interest

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

Publisher's note

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frontiers | Frontiers in Oncology

TYPE Original Research
PUBLISHED 09 October 2024
DOI 10.3389/fonc.2024.1407143

OPEN ACCESS

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RECEIVED 26 March 2024
ACCEPTED 04 September 2024
PUBLISHED 09 October 2024

CITATION
Puebla-Osorio N, Fowlkes NW, Barsoumian HB, Xega K, Srivastava G, Kettlun-Leyton C, Nizzero S, Voss T, Riad TS, Wong C, Huang A, Hu Y, Mitchell J, Kim M, Rafiq Z, He K, Sezen D, Hsu E, Masrorpour F, Maleki A, Leuschner C, Cortez MA, Oertle P, Loparic M, Plodinec M, Markman JL and Welsh JW (2024) Enhanced tumor control and survival in preclinical models with adoptive cell therapy preceded by low-dose radiotherapy.
Front. Oncol. 14:1407143.
doi: 10.3389/fonc.2024.1407143

Enhanced tumor control and survival in preclinical models with adoptive cell therapy preceded by low-dose radiotherapy

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Introduction: Effective infiltration of chimeric antigen receptor T (CAR-T) cells into solid tumors is critical for achieving a robust antitumor response and improving therapeutic outcomes. While CAR-T cell therapies have succeeded in hematologic malignancies, their efficacy in solid tumors remains limited due to poor tumor penetration and an immunosuppressive tumor microenvironment. This study aimed to evaluate the potential of low-dose radiotherapy (LDRT) administered before T-cell therapy to enhance the antitumor effect by promoting CAR-T cell infiltration. We hypothesized that combining LDRT with T-cell therapy would improve tumor control and survival compared to either treatment alone.

Methods: We investigated this hypothesis using two NSG mouse models bearing GSU or CAPAN-2 solid tumors. The mice were treated with engineered CAR-T cells targeting guanyl cyclase-C (GCC) or mesothelin as monotherapy or in combination with LDRT. Additionally, we extended this approach to a C57BL/6 mouse model implanted with MC38-gp100+ cells, followed by adoptive transfer of pmel+ T cells before and after LDRT. Tumor growth and survival outcomes were monitored in all models. Furthermore, we employed atomic force microscopy (AFM) in a small cohort to assess the effects of radiotherapy on tumor stiffness and plasticity, exploring the role of tumor nanomechanics as a potential biomarker for treatment efficacy.

Results: Our results demonstrated enhanced tumor control and prolonged survival in mice treated with LDRT followed by T-cell therapy across all

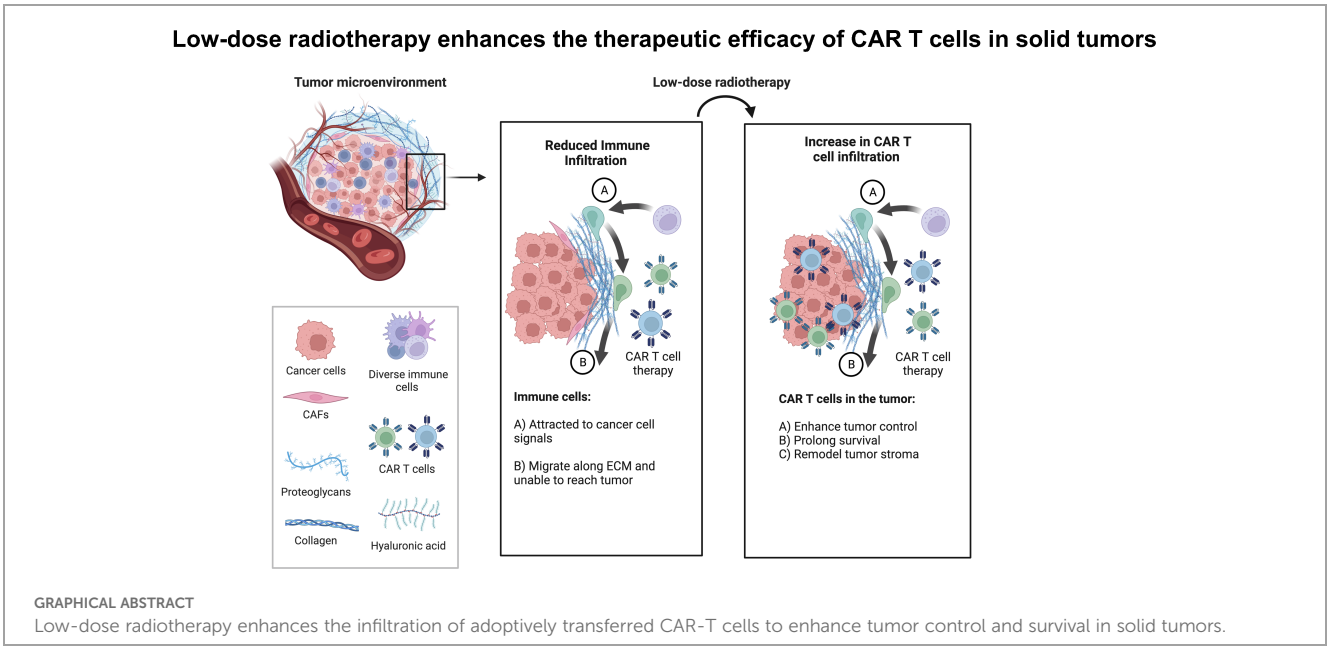
10.3389/fonc.2024.1407143

models. The combination of LDRT with CAR-T or pmel+ T-cell therapy led to superior tumor suppression and survival compared to monotherapy, highlighting the synergistic impact of the combined approach. Additionally, AFM analysis revealed significant changes in tumor stiffness and plasticity in response to LDRT, suggesting that the nanomechanical properties of the tumor may be predictive of therapeutic response.

Discussion: The findings of this study highlight the transformative potential of incorporating LDRT as a precursor to adoptive T-cell therapy in solid tumors. By promoting CAR-T and pmel+ T-cell infiltration into the tumor microenvironment, LDRT enhanced tumor control and improved survival outcomes, offering a promising strategy to overcome the challenges associated with CAR-T therapy in solid tumors. Additionally, the changes in tumor nanomechanics observed through AFM suggest that tumor stiffness and plasticity could be biomarkers for predicting treatment outcomes. These results support further investigation into the clinical application of this combined approach to improve the efficacy of cell-based therapies in patients with solid tumors.

KEYWORDS

NSG, NOD-SCID-IL2R gamma mice, LDRT, low-dose radiotherapy, RT, radiotherapy, CAR-T cells, chimeric antigen receptor T cells, solid tumors



GRAPHICAL ABSTRACT
Low-dose radiotherapy enhances the infiltration of adoptively transferred CAR-T cells to enhance tumor control and survival in solid tumors.

Introduction

Chimeric antigen receptor T cell (CAR-T) therapy has been less effective in treating solid tumors compared to hematologic malignancies, partly attributed to the immunosuppressive signals within the tumor microenvironment (TME). Components like stroma, cytokines, chemokines, checkpoint proteins, and metabolites hinder the function and persistence of adoptively transferred immune cells (1–3). Tumor-associated macrophages, regulatory T cells (Tregs), and myeloid-derived suppressor cells (MDSC) release cancer-associated cytokines, such as TGF-β and

Abbreviations: NSG, NOD-SCID-IL2Rgamma mice; RT, low-dose radiotherapy; RT, radiotherapy; CAR-T cells, chimeric antigen receptor T cells.

IL-10, further suppressing immune responses (4–7). Collagen overproduction by cancer-associated fibroblasts (CAFs) also forms a rigid stromal barrier around tumors, shielding them from immune surveillance (8–11) Different radiotherapy approaches have been explored to address TME challenges. Hypofractionated radiotherapy (HFRT) applied to the primary tumor induces an abscopal effect in distant metastasis (12) HFRT applied to the primary tumor followed by low-dose radiotherapy (LDRT) to the secondary lesion has shown promise in reducing TGF-β and enhancing intratumoral immune cell infiltration (6, 13, 14). However, the ideal doses of non-ablative radiotherapy to achieve the maximum therapeutic response, particularly when preceding cell therapy, still need to be determined (14–16). Studies have shown radiation therapy (RT) can synergize with checkpoint inhibitors by modulating the stroma to enhance T-cell infiltration (13, 17–23). Our team discovered that applying LDRT directly to tumors modulates the tumor stroma and microenvironment without causing damage to normal tissues (6, 13). De Selm et al. found that a single dose of 2 Gy sensitizes tumor cells to immune rejection by CAR-T cells. The study, conducted in a pancreatic adenocarcinoma model, demonstrated that antigen-positive and antigen-negative tumor cells become susceptible to CAR therapy when exposed to this radiation dose. Their findings provide promising insights into the successful application of CAR therapy for heterogeneous solid tumors, especially when coupled with local radiation as a conditioning regimen, a common component of standard tumor care (24).

LDRT demonstrates significant potential in augmenting the effectiveness of adoptive CAR-T cell therapy against solid tumors by reshaping the TME and bolstering immune cell infiltration. Our study employs a novel strategy using T-cell therapy for solid tumors post LDRT, capitalizing on its beneficial impact on the TME. Through this integrated radioimmunotherapy approach, we aim to redefine the efficacy of cancer treatment.

We evaluated the impact of administering LDRT before T-cell therapy on tumor growth control and survival across three mouse models. NSG mice were implanted with GSU or CAPAN-2 cell lines, followed by LDRT before CAR-T cell infusion. Similarly, C57BL/6 mice were injected with MC38-gp100+ murine colon adenocarcinoma cells and treated with pmel+ T cells before or after LDRT administration. Our results demonstrate that the sequential application of LDRT before T-cell therapy significantly improves tumor control and extends survival compared to control groups across all experimental models.

Also, we evaluated the efficacy of tumor growth control in a bilateral tumor model, where only the primary tumor received LDRT before CAR-T cell infusion. Remarkably, tumors subjected to LDRT before CAR-T cell infusion exhibited significantly superior tumor control compared to unirradiated tumors within the same mouse.

Although we refer to the administration of LDRT with CAR-T cells as combined therapy throughout this manuscript, LDRT was consistently administered before CAR-T cell infusion in all our experiments to mitigate potential harm to the infused cells. The only exception was observed in the pmel experiment, where one group received adoptively transferred gp100-sensitized pmel cells before LDRT, as previously described.

In summary, our innovative approach of integrating LDRT before T-cell therapy demonstrates promising results in enhancing the infiltration of adoptively transferred T cells and improving treatment outcomes for patients with solid tumors while minimizing additional toxicity.

Materials and methods

Mouse models

We used 8–12-week-old NSG (NOD-SCID IL2Rgamma) male mice, and 8–12-week-old C57BL/6 mice purchased from the Department of Experimental Radiation Oncology at The University of Texas MD Anderson Cancer Center; all mice were housed in the Experimental Radiation Oncology animal facility at MDA. All mouse studies included a minimum of 5 mice per group and were conducted under guidelines from the Institutional Animal Care and Use Committee. The NSG mice were implanted subcutaneously in the right hind leg (0.5×10^6 cells/mouse) with either GSU cells (Creative Bioarray cat #CSC-C6317J) or CAPAN-2 (2×10^6 cells/mouse) cells (American Type Culture Collection, HTB-80). To create a bilateral tumor model in NSG mice, we first implanted the primary tumor on the right hind leg (0.5×10^6 cells/mouse) on day zero, followed by a secondary implantation on the left hind leg (0.5×10^6 cells/mouse) five days later. The C57BL/6 mice were implanted similarly with (0.5×10^6 cells/mouse) MC38 murine colon adenocarcinoma cells expressing mouse gp100 [a gift from Dr. Patrick Hwu]. Procedures for the LDRT and T cell therapies are described below.

Radiotherapy

Radiation was delivered at total doses of 2 Gy, 4 Gy, 12 Gy, 24 Gy, or 36 Gy as follows: 1 fraction of 2 Gy; 1 fraction of 4 Gy; 2 fractions of 1 Gy each; 3 fractions of 4 Gy each; 3 fractions of 8 Gy each; or 3 fractions of 12 Gy each. We also used 4 fractions of 1Gy in later studies, and for the pmel study, we used 4 fractions of 1.4 Gy (total 5.6 Gy). The latter dose was chosen from a clinical radiation dose delivered to patients with metastatic disease (25). Radiation was delivered to the implanted tumors with a custom-built Cs-137 unit, with the rest of the mouse body shielded to avoid off-target effects.

In vitro irradiation of naïve T cells

We exposed freshly collected and sorted human CD3+ naive T cells to two fractions of 1Gy radiation in triplicate, with a 24-hour interval between exposures, *in vitro* using the X-RAD320 irradiator (Precision X-Ray Irradiation; Madison, CT, USA.) After exposure, we collected cells at 24, 48, and 72 hours to extract RNA for Nanostring analysis. We utilized the nSolver software to analyze the irradiation-exposed cells compared to their controls. Using the normalized dataset, we performed fold induction analysis

to assess the impact of irradiation exposure on various genes, including those involved in TCR signaling and DNA repair pathways.

Adoptive cell therapy

For adoptive cell therapy, we used second-generation CAR-T cells against GSU cells expressing guanyl cyclase-C (GCC) or against CAPAN-2 cells expressing mesothelin as follows: A leading signal domain was linked to the codon-optimized single heavy and light chain fragments recognizing GCC or mesothelin, followed by a spacer tethered to the transmembrane domain of CD28 (anti-GCC CAR) or CD8 (anti-mesothelin CAR). The construct was finalized with the intracellular domains of CD28 (anti-GCC CAR) -or 4-1BB (anti-mesothelin CAR)- and CD3. Mice were administered intravenously with 1×10^6, or 2.5×10^6 anti-GCC CAR-T cells (for the GSU model) or 1×10^5 anti-mesothelin CAR-T cells (for the CAPAN-2 model) 24h after the last dose of radiation. As a control, untransduced (UTD) T cells from the same donor were administered. Cells were maintained in liquid nitrogen until the day of the infusion when they were thawed at 37°C, their viability was determined, and 100 µL aliquots in PBS were prepared for injection. For the third mouse model, $5x10^6$ $pmel^+$ T cells were adoptively transferred into C57BL/6 mice implanted with $gp100^+$ MC38 cells at 24 h after the final dose of RT.

Tumor control and mouse survival

After implantation, tumors were left to grow to ~7 mm in diameter (~170 mm^3), which was reached by days 8 or 10 depending on the tumor model utilized; this size was ideal for testing various LDRT doses and schedules. Tumors were measured with high-precision calipers twice weekly, and the volumes calculated from tumor length and width measurements as described by Tomayco and Reynolds (26). Mouse survival was recorded over the experimental periods, and the survival curves were calculated with the Kaplan-Meier method (GraphPad). Tumor specimens were obtained when they reached the permissible size or at the end of the experiment; after volumes were measured, tumors were divided in half for assessment of tumor-infiltrating lymphocytes and creation of tissue microarrays (TMAs) as described below.

Statistical analysis

The differences in tumor growth among groups in each experiment were calculated using one-way ANOVA and Tukey's multiple comparison tests ($p< 0.05$). In addition, to factor in the unexpected loss of tumor measurements due to the tumor burden and death of affected mice, we implemented a one-way ANOVA mixed-effects analysis and Tukey's multiple comparison tests (p-value < 0.05). The differences in survival among groups were calculated using Chi-square analysis and Log-rank (Mantel-Cox) test for curve survival comparison ($p<0.05$).

Isolation and flow cytometry of tumor-infiltrating lymphocytes

Half of each tumor specimen was subjected to single-cell dissociation followed by gradient centrifugation with Lymphoprep (1.077 g/mL, StemCell Technologies). Blood samples were collected via tail vein in live animals and by heart puncture in sacrificed animals and preserved in heparin (10U/mL blood). The isolated cells were stained for flow cytometry with the following antibodies, all from BioLegend: anti human CD4 (PE/Dazzle-594; cat #357412, clone A161A1), CD8 (PerCP/Cyanine 5.5; cat #344710, clone SK1), CD45 (Brilliant Violet 650; cat #304044, clone HI30), CD3 (Brilliant Violet 711; cat #317328, clone OKT3), CD69 (allophycocyanin [APC]; cat #310910, clone FN50), CD279 (PD1) (PE/Cyanine7; cat #3621616, clone A17188B), CD197 (CCR7) (Brilliant Violet 605; cat #353224, clone G043H7), CD45RA (Brilliant Violet 785; cat #304140, clone HI100), and anti-mouse CD16/32 (cat #101302, clone 93). Doublets were removed by plotting forward-scatter height (FSC-H) vs. forward-scatter area (FSC-A); live single-cell populations were selected by plotting side scatter-area (SSC-A) vs. a Live/Dead marker, followed by identification of the lymphocyte population. $CD4^+$ and $CD8^+$ T cell subpopulations were then identified from the total $CD3^+$ fraction. Tumor-infiltrating lymphocytes were subjected to flow cytometry with a Cytek Aurora cytometer. The gating strategy is described in online Supplementary Figure 1. In addition, when required, we generated t-SNE maps to display T-cell populations using Cytofkit (27), an R-based interface available in Bioconductor (https://www.bioconductor.org/packages/3.5/bioc/html/cytofkit.html).

Tissue processing and immunostaining

Portions of tumor specimens were fixed in 10% neutral buffered formalin followed by paraffin-embedding, tissue sectioning (4 µm), and staining with hematoxylin and eosin (H&E). The H&E-stained slides were inspected visually and two 3-mm cores were selected from each tumor block for inclusion in a TMA. The TMA block was sliced in 4-µm sections and stained with a Leica Bond RX auto stainer and Opal 7-color multiplex kit (Akoya Biosciences) to detect human CD4, CD8, PD1, Ki67, granzyme B, and PanCK, with DAPI used for counterstaining. Multiplex IF-stained slides were imaged with a Leica Versa 8 fluorescent digital slide scanner. Slides were viewed by using Imagescope and HALO software. Biomarkers were quantified with a Leica cellular immunofluorescence algorithm. Quantitative data were exported in Excel and graphed and analyzed with GraphPad Prism version 9. One-way analysis of variance was used for statistical analyses.

Tissue nanomechanics

The nanomechanical characterization utilizing atomic force microscopy (AFM) was conducted using the ARTIDIS research device, which is an advanced AFM-based technique that enables

precise and detailed characterization of the mechanical properties of biological samples at the nanoscale, facilitating a deeper understanding of cellular and tissue mechanics in various biomedical applications. Biopsies obtained from mice tumors were securely mounted on standard TPP 9.2 cm^2 dishes from TPP (Switzerland) using a 2-component epoxy resin glue. We prepared 2-5 samples for measurements from mice receiving LDRT only, CAR-T only group, or LDRT before CAR-T cell therapy. The ARTIDIS chip holder pre-equipped with triangular DNP-S10 D probes from Bruker AFM Probes (USA) was utilized for nanomechanical measurements. These probes possess specific properties, including a nominal spring constant of 0.06 N m−1, a cantilever length of 205 μm, a tip height of 6 μm, and a tip radius of 20 nm. The spring constant in the air was determined through a fully automated calibration process employing the thermal tune method described by Sader et al. (28) Deflection sensitivity calibration was performed by acquiring force curves at the bottom of TPP 9.2 cm^2 dishes filled with degassed and sterile Custodiol solution. The ARTIDIS device integrated optical microscopes from Leica (Germany) and Navitar (USA), allowing for imaging and precise positioning control of both the cantilever and the sample.

To determine the elastic modulus of the samples, the AFM was operated in force-volume mode. This involved capturing 20 x 20 force-displacement curves arranged in square arrays (force maps), each map covering an area of 20 x 20 μm^2. This process resulted in 400 force curves per map. We acquired force maps for each sample at a minimum of 20 equidistant spots across the sample. During the acquisition of force-displacement curves, indentations ranging between 0.2 and 3 μm were sampled, applying a defined maximum force load (29, 30) of 1.8 nN and an indentation speed of 16 μm s−1.

Subsequently, the AFM data were analyzed using the ARTIDIS analysis software. The recorded curves were transformed into force vs. tip-sample distance curves. For each sample, all the force maps were evaluated to assess the quality of the force curves. We excluded maps with quality rates below 75% from further analysis. We considered signal-to-noise ratio, baseline tilt, and curve twist factors in selecting suitable maps for analysis (31). We evaluated 17 nanomechanical parameters between mice treated with radiation only, CAR-T cells only, and those treated with LDRT plus CAR-T cell therapy, with varying cell quantities (1, 2.5, and 5 million cells per mouse). The backward elastic modulus was determined using the contact area and slope, employing the Oliver and Pharr model (32), as previously established in the literature (30, 33). The elastic modulus of each force curve was spatially plotted in 2D to generate a stiffness map for the different samples. A Gaussian distribution was derived from the collected stiffness values across the sample groups. The force maps were further processed for visualization purposes using Gwyddion software (34).

Results

Determining radiotherapy dose for use with cell therapy

To effectively control tumor growth, adoptively transferred T cells must reach and infiltrate beyond the tumor stroma. Multiple factors affect this event, including the tumor microenvironment, its localization, and growth status at the time of treatment. Our study aimed to determine the best radiation dose to enhance tumor control before CAR-T cell therapy. Using an experimental model with human gastric cancer in NSG mice, we simplified the process by implanting tumors only in one leg. This allowed for targeted radiation and more straightforward tumor measurement. We found that injecting 1 million GSU cells per mouse created tumors reaching a suitable size (~170 mm^3) within eight days, ideal for testing different radiation doses and schedules (online Supplementary Figure S2A). This model setup enabled us to explore the optimal radiation approach for enhancing CAR-T cell effectiveness against tumor growth. For the GSU experiments, mice from each experimental group received focal irradiation on day 8 post-implantation, followed by the infusion of a single intravenous dose of 2 x 10^6 CAR-T cells recognizing guanyl cyclase-C (GCC) expressed on the GSU tumor cells 24 h after receiving their last dose of radiotherapy (Figure 1A). Mice receiving untransduced (UTD) naïve T cells or CAR-T cells alone succumbed to the tumor burden by day 18 and 35 after implantation, respectively (Figures 1B, C). Also, mice receiving 1Gy x 2F or 2 Gy x 1F prior to CAR-T cells had a marginal effect in controlling tumor growth, succumbing to the tumor burden on day 35 (Figures 1B, C). Although mice that received 12Gy x 3F or 8Gy x 3F followed by CAR-T cell therapy had an efficient control of tumor growth (Figure 1B); such regimens led to skin ulceration at the irradiation site that prompted early euthanasia on day 32. Notably, LDRT doses of 12 Gy (4-Gy x 3 fractions) or 4 Gy (4Gy x 1 fraction) administered before anti-GCC CAR-T cells, controlled tumor growth (Figure 1B) without adverse reactions at the irradiation site and prolonged survival until the termination of the experiment on day 54 after implantation (Figure 1C) (*** $p<0.0001$, [4Gy x 1 fraction+ CAR-T cells or 4Gy x 3 fractions+ CAR-T cells vs. CAR-T cells or the remaining combinations]; Long-rank Mantel-Cox test). Moreover, twice-weekly measurements of body weight revealed no weight loss related to CAR-T-cell infusion in any group (Figure 1D). To minimize potential RT-related side effects, we opted to use 4Gy x 1 fraction or 1Gy x 4 fractions in subsequent experiments, in part because the latter regime resembles the dose and schedule used to treat metastatic disease in the clinic (1.4Gy x 4 fractions) (25). Further testing is warranted to explore the potential therapeutic effects of administering three fractions of 4Gy (totaling 12 Gy) before CAR-T cell therapy. This intervention could regulate tumor growth and enhance survival in different mouse tumor models. In summary, combining CAR-T cell therapy with LDRT rather than high-dose radiotherapy makes therapeutic sense due to LDRT's low side effects, and its role in modulating the tumor stroma, as demonstrated by our recent findings (13, 25).

Radiotherapy optimizes the use of cell therapy

LDRT enhances the anti-tumor immune response (13, 25) and is gaining traction as an important approach to improve clinical outcomes. Recent data show that efficacy from autologous CAR-T cells or tumor-infiltrating lymphocyte therapies (35) depend on the

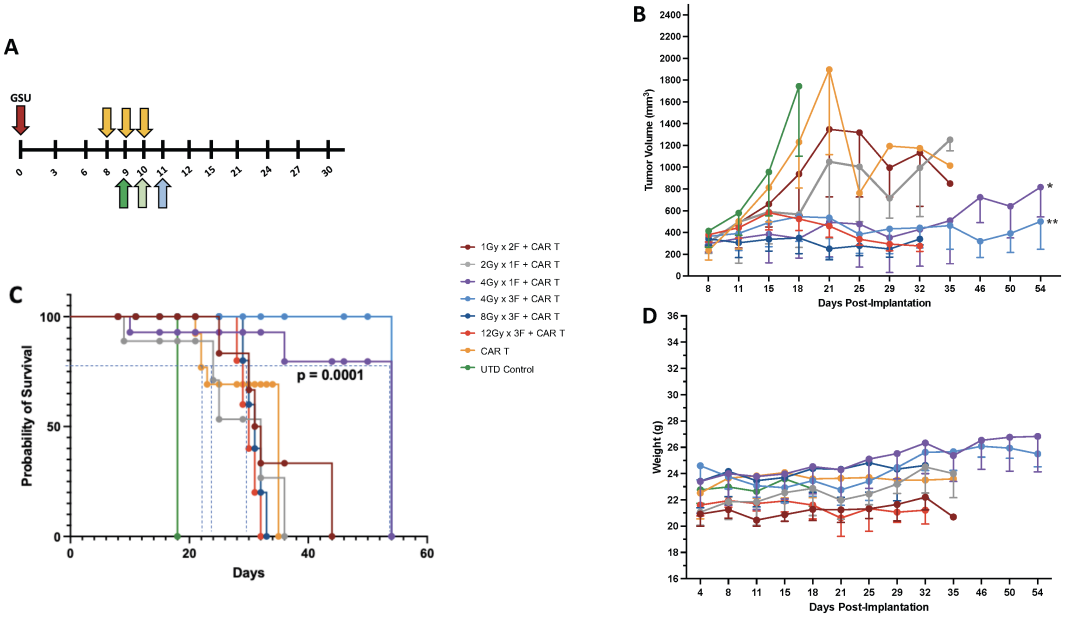


FIGURE 1
Establishing the best radiation dose and schedule for combining with CAR-T cell therapy. **(A)** We used the GSU tumor model to determine the proper radiation dosage combined with cell therapy; cell therapy was administered 24h after the last fraction of radiotherapy (each radiation fraction is indicated by a yellow arrow). If radiation was given in one fraction on day 8 (2 Gy or 4Gy), the infusion was administered on day 9 (dark green); if the radiation included two doses (1Gy in 2 fractions) on days 8 and 9, the mice received the infusion 24h after the last fraction on day 10 (light green). If the mice received 3 fractions on days 8, 9, and 10 (4, 8, or 12Gy in 3 fractions), the T cell infusion was administered 24h after the last fraction on day 11 (light blue). Two regimens (1 fraction of 4 Gy [purple lines] or 3 fractions of 4 Gy each [light blue lines]) in combination with CAR-T cell therapy led to superior tumor control **(B)** and **(C)** survival and **(D)** had little to no effect on body weight. Compared to the CAR-T-only group, the groups that received 4Gy x 1F (purple) or 4Gy x 3F (light blue) showed statistically better tumor control (*p<0.004 [4Gy x 1F + CAR-T cells vs. CAR-T cells] and **p<0.001 [4Gy x 3F +CAR-T cells vs. CAR-T cells]; one-way ANOVA and Tukey's multiple comparison tests; n=5.), and survival (*** p<0.0001, [4Gyx 1 fraction + CAR-T cells or 4Gy x 3 fractions + CAR-T cells vs. CAR-Ts or the remaining combinations]; Long-rank Mantel-Cox test). The two highest-dose schedules [3 fractions of 8 Gy each (dark blue) and 3 fractions of 12 Gy each (44, 45)] led to skin ulceration at the irradiation site.

fitness of the T cells used for their manufacturing or expansion; this fitness is inherent to each patient and is an absolute determinant of desirable therapeutic outcomes (36, 37). Because CAR-T cells alone often have high anti-tumor efficacy in xenograft models, we intentionally selected CAR-T cell resistant tumor models and/or suboptimal CAR-T numbers that have low to medium efficacy as a monotherapy to assess the extent of RT-induced enhancement in tumor control and survival. To determine the optimal number of CAR-T cells to be used with our chosen LDRT schedules (1Gy x 4 fractions or 4Gy x 1 fraction), we followed the schema depicted in Figure 2A. In this study, we conducted experiments using NSG mice implanted with GSU cells. On day 8, RT was administered, followed by the infusion of different doses of anti-GCC CAR-T cells ($1x10^6$ or 2.5×10^6) 24 hours after the final LDRT dose (day 9 or day 12; Figure 2A, indicated by dark green or light green arrows).

Our findings demonstrated that combining LDRT with any of the CAR-T cell doses resulted in significantly better tumor control than CAR-T cells alone, untreated T cells, or four fractions of 1Gy radiation therapy alone (Figures 2B, C).

Notably, 4Gy x 1 fraction LDRT followed by $1x10^6$ CAR-T cells showed significantly superior tumor control compared to all CAR-T doses alone (4Gy x 1 fraction + $1x10^6$ CART vs. $1x10^6$, or 2.5×10^6 CAR-T alone; p<0.0001, and 0.0004; n=5 mice per group). Moreover, the use of 4Gy x 1 fraction LDRT preceding any CAR-T dose ($1x10^6$, or 2.5×10^6) was significantly more effective than using 1Gy x 4 fractions LDRT followed by 2.5×10^6 CAR-T cells (4Gy x 1 fraction + $1x10^6$ CAR-T vs. 1Gy x 4 fractions + 2.5×10^6 CAR-T; p<0.002; n=5 mice per group).

In addition to tumor control, the combination treatments led to 100% survival up to day 60 (Figure 2D). We observed a period of robust tumor control from day 26 to day 47 (21 days) in mice receiving $1x10^6$ CAR-T cells after the administration of 1Gy x 4 fractions or 4Gy x 1 fraction and from day 26 to day 44 (18 days) in mice receiving 2.5×10^6 CAR-T cells after 1Gy x 4 fractions LDRT (Figures 2B, C, indicated by the dotted box).

Comparatively, 100% of mice receiving UTD succumbed to the tumor burden by day 23, and mice receiving CAR-T cells alone died by day 47 ($1x10^6$ CAR-T cells) and 54 (2.5×10^6 CAR-T cells) (Figure 2D). Mice treated with LDRT only (1Gy x 4 fractions or 4Gy x 1 fraction) also exhibited no control of tumor growth and succumbed to the tumor burden at day 44 and 47, respectively) (Figure 2D). Interestingly, one mouse that received 1Gy x 4 fractions before $1x10^6$ CAR-T cells demonstrated tumor growth suppression throughout the experiment (online Supplementary Figure 3D). Detailed tumor records for individual mice can be found in online Supplementary Figures 3A–I.

Based on these findings, LDRT can potentially enhance the efficacy of anti-GCC CAR-T cell therapy in this experimental model.

Next, to assess whether CAR-T cell therapy after LDRT was also effective in a different tumor model, we implemented a similar

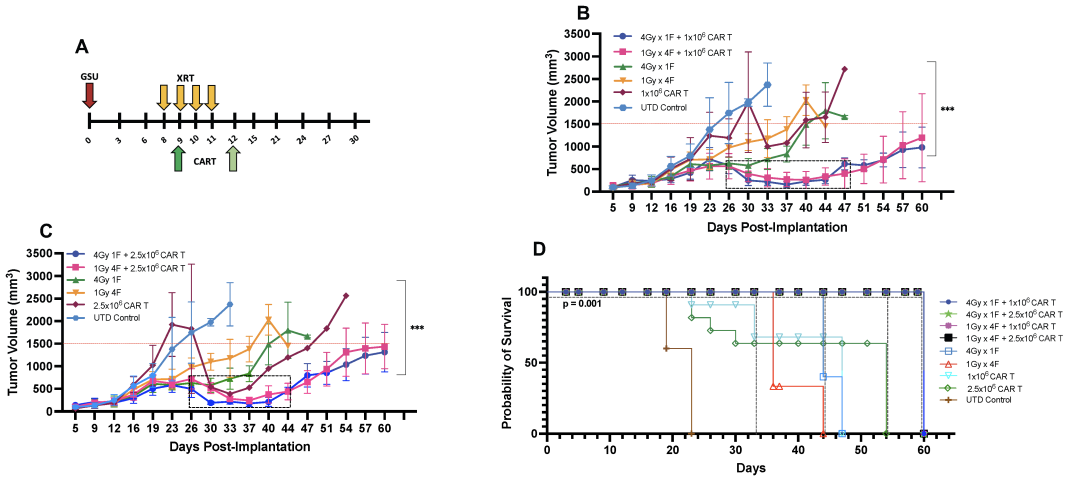


FIGURE 2
Determining the ideal CAR-T cell dose combined with radiation for mice bearing subcutaneous GSU gastric carcinoma tumors. **(A)** In an efficacy study using the GSU tumor model, if the radiation was given in one fraction (each radiation fraction is indicated by a yellow arrow) on day 8 (4Gy) post-tumor implantation, the infusion was administered on day 9 (dark green); if the radiation included four doses (1Gy in 4 fractions) on days 8, 9, 10, and 11, the mice received the infusion 24h after the last fraction on day 12 (light green). In a single experiment, we included two doses of CAR-T cells combined with two dose regimens of radiotherapy. **(B, C)** Mice receiving 1×10^6 anti-GCC CAR-T cells combined with either 1Gy x 4 fractions or 4Gy x 1 fraction showed superior tumor control (B; days 26–47) relative to the mice given the same radiation doses with 2.5×10^6 cells (C; days 26–44). **(D)** Mice that received dual therapies (4Gy x 1 fraction, 1Gy x 4 fractions plus 1 or 2.5×10^6 CAR-T cells; black, pink, green, and blue) showed extended survival relative to the other experimental treatments ($p<0.001$, [4Gy x 1 fraction, 1Gy x 4 fractions plus 1 or 2.5×10^6s CAR-T cells vs. monotherapies]; Long-rank Mantel-Cox test). (n= 5 mice per group).

therapeutic approach in NSG mice implanted with the mesothelin-positive human CAPAN-2 cell line. We first determined that by subcutaneously implanting $2x10^6$ CAPAN-2 cells per mouse, the tumor grew to an optimal size for radiotherapy of ~7 mm in diameter on day 10 post-implantation (online Supplementary Figure S2A). The CAPAN-2 cell line grew slower than the GSU cell line *in vivo* (online Supplementary Figure S2A). Because the CAPAN-2 cells were previously determined to be more sensitive to CAR-T treatment than the GSU model, we tested fewer CAR-T cells per mouse than in the GSU experiments, i.e., 0.5×10^5, 1×10^5, or 2×10^5. We found that 0.5×10^5 or 2×10^5 anti-mesothelin CAR-T cells led to low or maximum antitumor response, respectively, (online Supplementary Figure S2B), and 1×10^5 CAR-T cells per mouse had intermediate killing efficacy. The use of the latter dose made therapeutic sense as we sought to assess whether a moderate number of CAR-T cells combined with LDRT enhanced the overall response. We implanted all grouped NSG mice with 2×10^6 CAPAN-2 cells in the right hind leg and recorded the tumor growth twice weekly. The mice-bearing tumors were irradiated with the following doses of radiotherapy: 1Gy x 4 fractions, 4Gy x 1 fraction, 2 Gy x 1 fraction, and 2 Gy x 2 fractions. All radiotherapy doses preceded the infusion of 1×10^5 anti-mesothelin CAR-T cells, in which the CAR-T cells were administered 24 hours after the final radiation dose according to Figure 3A.

The results indicate that neither the groups treated with CAR-T cell nor radiotherapy alone controlled tumor growth as effectively as the combined therapies. In the CAPAN-2 tumor model, treatment with 1Gy x 4 fractions before CAR-T cell therapy showed marginal enhancement of control of tumor growth relative to CAR-T cells or radiation alone, although 60% of these mice survived 75 days after implantation (Figures 3B, F1). Notably, the use of 4Gy x 1 fraction prior anti-mesothelin CAR-T cell therapy led to the most effective tumor control (Figure 3C) and a 100% survival rate (Figure 3F1) at day 75 [i.e., size of tumors ~ 350 mm^3 after 4Gy x 1 fraction + CAR-T vs. ~ 1150 mm^3 after CAR-T only ($p<0.005$) or ~1650 mm^3 after 4Gy x 1 fraction only ($p<0.0003$)]. Also, 2 Gy x 2 fractions combined with anti-mesothelin CAR-T cells also significantly improved tumor control (Figure 3D) and survival (Figure 3F2) relative to LDRT alone [$p<0.0001$]) and CAR-T therapy alone ($p<0.0001$). In addition, 2 Gy x 1 fraction + CAR-T cells (Figure 3E; values for individual tumor records are shown in online Supplementary Figure S4) was marginally, but not significantly better than 1Gy x 4 fractions + CAR-T cells (tumor volumes of ~800 mm^3 vs. ~900 mm^3 at Day 75). These findings confirmed that LDRT plus anti-mesothelin CAR-T cell therapy was effective for controlling tumor growth and extending survival in NSG mice.

To test whether radiotherapy combined with cell therapy leads to similar outcomes in non-immunosuppressed mice, we used LDRT with adoptive cell transfer (ACT) of pmel-sensitized T cells in two separate pilot experiments. Before their infusion, the pmel splenocytes were activated with anti CD3/CD28 beads and maintained in IL-2 and IL-15- supplemented culture for 96 h. We implanted $5x10^5$ MC38-gp100$^+$ tumor cells on the right hind leg of C57BL/6mice and allowed the tumors to develop until they reached ~7 mm diameter before irradiating them with 1.4Gy x 4 fractions (Day 9 post-implantation). To test whether radiotherapy after the cell infusion affected the outcome, we adoptively transferred each mouse with $5x10^6$ pmel-sensitized T cells 24 h before initiating radiotherapy (Day 8 post-implantation). Also, we adoptively transferred pmel T cells 24 h after the last administered radiotherapy fraction (Day 13 post-implantation) (online

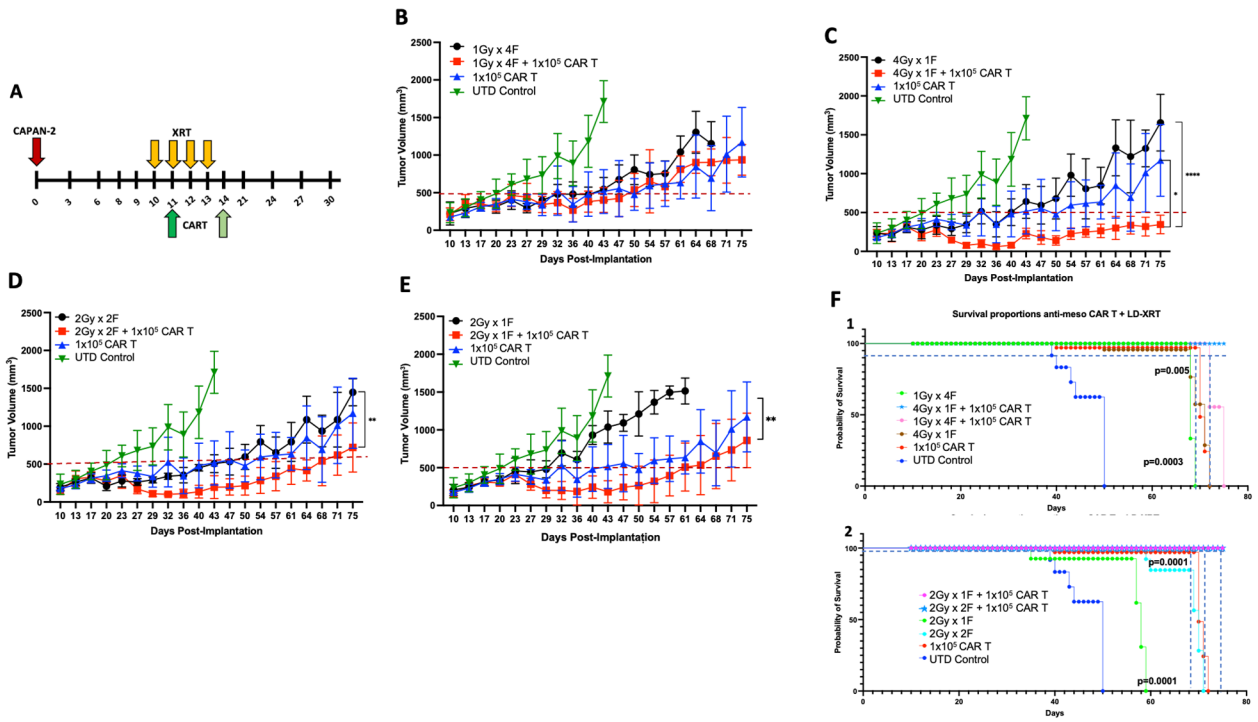


FIGURE 3
CAR-T cell therapy combined with radiotherapy in mice bearing subcutaneous CAPAN-2 tumors. **(A)** In the efficacy studies using the CAPAN-2 tumor model the radiation was given in one fraction on day 10 (4Gy) post-tumor implantation when tumors reached ~7mm in diameter and the infusion was administered on day 11 (dark green); if the radiation included four doses (1Gy x 4 fractions) on days 10, 11, 12, and 13; the mice received the infusion 24h after the last fraction on day 14 (light green). In a single experiment, we determined the efficacy of multiple radiation doses combined with a single dose of CAR-T cell therapy. In all four radiation dose groups in this experiment **(B–E)**, mice treated with dual therapy showed superior tumor control compared with mice receiving monotherapies. Mice treated with 1Gy x 4 fractions plus CAR-T cell therapy showed marginally better control of tumor growth relative to radiation or CAR-T cells alone **(B)**. Notably, the use of 4Gy x 1 fraction combined with CAR-T cell therapy resulted in the most significant tumor control **(C)** and a 100% survival rate **(F1)** at day 75 [i.e., compared to mice receiving CAR-T cells ($p<0.005$) or 4Gy x 1 fraction of LDRT ($p<0.0003$)]. Also, relative to LDRT alone ($p<0.0001$) and CAR-T therapy alone ($p<0.0001$), 2 Gy x 2 fractions combined with anti-mesothelin CAR-T cells also significantly improved tumor control **(D)** and survival **(F2)**. In addition, 2 Gy x 1 fraction + CAR-T cells **(E)** was marginally, but not significantly better than 1Gy x 4 fractions + CAR-T cells. (n=5 mice per group).

Supplementary Figure S5A). Our results indicate that mice receiving pmel-sensitized T cells 24 h before LDRT or 24h after the last LDRT dose showed better tumor control than mice receiving cell therapy only (day 30 post-tumor implantation, ACT 24h before LDRT vs. ACT, $p=0.0003$; ACT 24h after LDRT vs. ACT, $p=0.0001$) (Online Supplementary Figures S5B, C). These results are in line with the findings in the tumor xenograft models described here. Thus, combining the adoptive cell transfer of effector T cells with radiotherapy results in better tumor control.

Low-dose radiotherapy enhances the infiltration of adoptively transferred T cells

The efficacy of anti-tumor cell therapy requires robust intratumoral T-cell infiltration and sustained and active effector function. However, tumors generate multiple barriers within the TME that impair the infiltration of T cells. Tumor-infiltrating T cells suffer numerous changes affecting their anti-tumor activity. Using radiotherapy in low doses helps modulate the TME by increasing the infiltration of infused anti-tumor T cells and promoting beneficial changes, including the reduction of T-regs and TGF-β, and enhancing the proliferation of M1 macrophages, among others (13, 25).

Indeed, our results in the current study imply that LDRT enhanced the infiltration of infused CAR-T cells in the GSU model. To assess the persistence of infused anti-GCC CAR-T cells, we measured their presence in mice's bloodstream and tumor microenvironment (TME). We collected blood samples at two points: upon death from tumor burden (typically occurring earlier in mice receiving only CAR-T cells or untransduced donor T cells [UTD cells]) and at the experiment's end for mice that survived after combination therapy with CAR-T cells and LDRT. Thus, samples were collected at different time points for each group: on days 20-33 for the UTD mice; days 24-37 for the 5×10^6 CAR-T cell group; days 24-44 for the 1×10^6 CAR-T cell group; or days 24-54 for the 2.5×10^6 CAR-T cell group. We also collected samples at the end of the experiment (on day 60) from mice receiving dual therapy with 1Gy x 4 fractions or 4Gy x 1 fraction and infused with 1×10^6, 2.5×10^6, or 5×10^6 CAR-T cells. Mice receiving the dual therapy (4Gy x 1 or 1Gy x 4 fractions with 1×10^6, 2.5×10^6, or 5×10^6 CAR-T cells) showed significant increases in the intratumoral infiltration of CAR-T cells (human $CD3^+$ cells; Figures 4A, B; Supplementary Figures S6A, S7) relative to mice receiving anti-GCC CAR-T cells only (4Gy x 1

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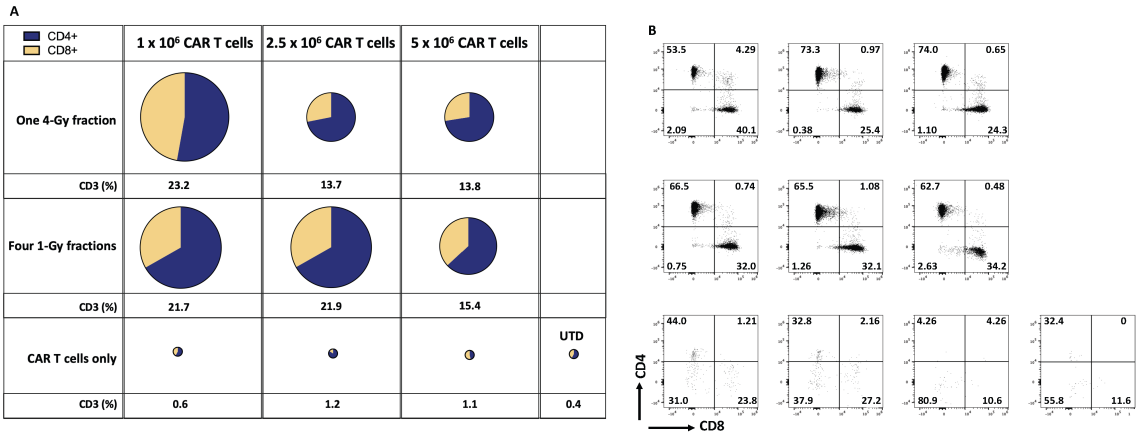

FIGURE 4
Radiation therapy enhances the infiltration of adoptively transferred anti-GCC CAR-T cells into the GSU gastric carcinoma tumor microenvironment. The average (n=3) of absolute numbers of $CD3^+$ CAR-T cells were determined in tumor samples from the indicated experimental groups. **(A)** The relative sizes of the pies represent the amount of $CD3^+$ cells, and the two colors indicate the relative proportions of $CD4^+$ cells (blue) and $CD8^+$ cells (yellow). Mice receiving dual therapy (4Gy x 1 or 1Gy x 4 fractions with $1x10^6$, or 2.5×10^6 CAR-T cells) showed significant increases in the intratumoral infiltration of CAR-T cells relative to mice receiving CAR-T cells only (4Gy x 1 fraction + $1x10^6$ CAR-T vs, 1 and 2.5×10^6 CAR-T cells. $p<0.01$, 0.004, and 0.04, respectively), and 1Gy x 4 fractions $^+$ 10^6 CAR-T cells showed a higher percentage of $CD3^+$ cells than 1 and 2.5×10^6 CAR-T cells alone (1Gy x 4 fractions + $1x10^6$ CAR-T vs, 1, 2.5×10^6 CAR-T cells; $p<0.02$, 0.01, respectively.) **(B)** Starting from the upper leftmost sample, examples of CD4 and CD8 distributions by flow cytometry, corresponding to the same treatment group as in panel **(A)**; samples from mice receiving CAR-T or UTD cells only showed a smaller number of tumor-infiltrating T cells. Tumor-infiltrating T cells were collected when mice reached the maximum permissible tumor volume and succumbed to the tumor burden (mostly in mice receiving monotherapy). However, mice that received the dual therapy were terminated at day 60, and samples collected. The gating strategy is described in online Supplementary Figure 1. (n=5 mice per group).

fraction + $1x10^6$ CAR-T cells vs 1, and 2.5 CAR-T cells. $p<0.01$, 0.004, and 0.04, respectively), and 1Gy x 4 fractions + $1x10^6$ CAR-T cells showed higher percentage of $CD3^+$ cells than 1 and 2.5×10^6 CAR-T cells alone (1Gy x 4 fractions + $1x10^6$ CAR-T vs 1, 2.5×10^6 CAR-T cells; $p<0.02$, 0.01, respectively.) Thus, regardless of the number of cells infused, the intratumoral infiltration of adoptively transferred CAR-T cells was consistently higher in mice receiving dual therapy. (Figure 4; Supplementary Figures S6A, S7).

To summarize, we found that infusing the fewest CAR-T cells ($1x10^6$ per mouse) after LDRT (4Gy x 1 or 1Gy x 4 fractions) led to better tumor control, extended survival, and better infiltration of CAR-T cells into tumors than using 2.5×10^6 CAR-T cells after LDRT. To confirm our findings, we repeated this experiment by using 4Gy x 1 or 1Gy x 4 fractions combined with 1×10^6 anti-GCC CAR-T cells and measured chimeric human $CD3^+$ cells in the blood. Our findings validated the previous experiment showing that the dual therapy led to better tumor control and better intratumor infiltration of the infused CAR-T cells. Specifically, administering 1Gy x 4 fractions or 4Gy x 1 fraction combined with 1×10^6 CAR-T cells led to higher percentages of chimeric human $CD3^+$ T cells in the blood (analyzed on day 60) than groups treated with UTD cells or CAR-T cells only analyzed on day 30 in the blood (endpoint; online Supplementary Figures 6, 7). Further, the administration of dual therapy (1Gy x 4 or 4Gy x 1 before 1×10^6 anti-GCC CAR-T cells resulted in higher percentages of tumor-infiltrating CAR-T cells than receipt of UTD cells only or CAR-T cells only. Notably, these results also show that although $CD4^+$ cells dominate the $CD4^+/CD8^+$ cell ratio in the blood and the tumor, the percentage of $CD8^+$ T cells in the tumor increases, which indicates a potential expansion of these cells due to their engagement with the targeted tumor proteins.

We also assessed the intratumoral infiltration of CAR-T cells by using multiplex immunohistochemical staining in a cross-section of the GSU tumors used for flow cytometry analyses described above (Figure 5). In that experiment, mice receiving 1Gy x 4 fractions followed by CAR-T cells showed higher intratumoral infiltration of CAR-T cells and increased $CD4^+$ and $CD8^+$ subpopulations relative to mice receiving 4Gy x 1 fraction prior CAR-T cells or CAR-T cells only, as well as an increase in the proliferation ($Ki67^+$) of $CD4^+$ and $CD8^+$ T cells and an increase in granzyme B production (Figures 5A–C). Moreover, treatment with CAR-T cells only led to more $CD4^+$ cells being retained outside the tumor (Figure 5C) than treatment with combination therapy. In short, even though statistical significance was not reached (probably because of the heterogeneity and low numbers of evaluated samples), administering 1Gy x 4 fractions seems to favor the increased intratumoral infiltration of total CAR-T cells and the effector molecule granzyme B, as well as increasing the $CD4^+$ and $CD8^+$ T cells relative to CAR-T cells only or 4Gy x 1 fraction followed by CAR-T cell therapy (Figure 5). Also, we performed quantitative analysis of tumor-infiltrating human CD3 + T cells by flow cytometry, showing that mice treated with both radiotherapy (one 4-Gy or four 1-Gy fractions) and CAR-T therapy (n=10) have a significantly higher percentage of tumor-infiltrating T cells compared to those treated with CAR-T therapy alone or untreated (UTD) control mice (p-value < 0.0001, mixed-effect analysis with multiple comparisons) (Figure 5D).

We repeated the above experiments with the CAPAN-2 model. The CAPAN-2-tumor-bearing mice showed comparable levels of infiltration of the anti-mesothelin CAR-T cells among all treatment groups: percentages of $CD3^+$ cells were 43% for CAR-T cells only; 45% for CAR-T cells + 2 Gy x 1 fraction; 48% for CAR-T cells + 2 Gy

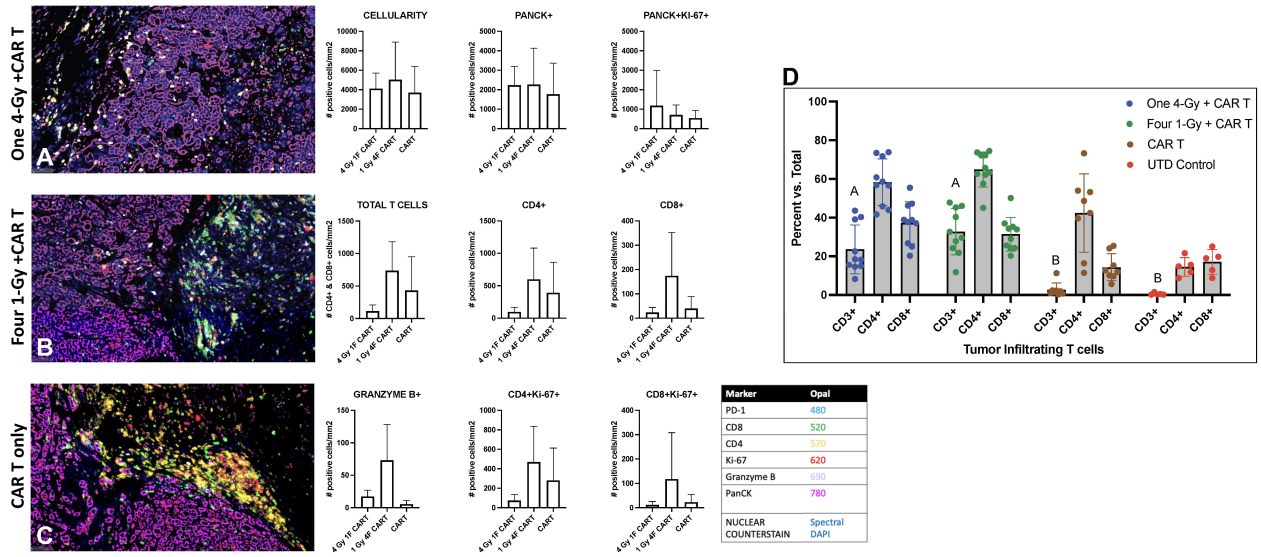

FIGURE 5
Radiation therapy enhances the infiltration of adoptively transferred anti-GCC CAR-T cells into GSU gastric carcinoma tumors in NSG mice. Mice given 1Gy x 4 fractions each **(B)** showed enhanced infiltration of CAR-T cells (total T cell, $CD4^+$, and $CD8^+$), increased proliferation ($Ki67^+$) of CD4 and CD8 cells, and increased numbers of granzyme B^+ cells in the tumor microenvironment relative to mice receiving 4Gy x 1 fraction **(A)** or CAR-T cells only **(C)**. Images captured at 20x. (n=5 mice per group). **(D)** Quantitative analysis of tumor-infiltrating human CD3+ T cells shows that mice treated with a combination of radiotherapy either one 4-Gy fraction or four 1-Gy fractions, and CAR-T therapy (n=10) exhibit a significantly higher percent of tumor-infiltrating T cells compared to those treated with CAR-T therapy alone or untreated (UTD) control mice (columns with different letter are statistically different, p-value < 0.0001, mixed-effect model analysis with multiple comparisons).

x 2 fractions; 38% for CAR-T cells + 1Gy x 4 fractions; and 32% for CAR-T cells + 4Gy x 1 fraction (online Supplementary Figure 8). The t-SNE maps [Cytofkit (27)] show the distribution of CD4 (cluster 10, green) and CD8 cells (cluster 7, yellow.) Although treatment with CAR-T cells + 4Gy x 1 fraction had relatively fewer tumor-infiltrating CAR-T cells (38%), those mice also had the best tumor control and survival compared with the other groups (Figure 3C).

Next, we used a bilateral tumor model to understand whether the dual therapy was sufficient to control tumor growth in non-irradiated tumors. Our results showed that using 1Gy x 4 fractions or 4Gy x 1 fraction before anti-GCC CAR-T cell therapy exhibited significantly better control of tumor growth in the primary tumor than mice receiving CAR-T cell therapy alone ($p<0.02$, Figure 6A). Also, mice receiving 1Gy x 4 fractions combined with CAR-T cells showed significantly better control in the primary tumor ($p<0.009$) than 4Gy x 1 fraction plus CAR-T cell therapy (Figure 6A). As expected, mice that received the dual therapies showed significantly better survival ($p<0.0001$) than mice receiving CAR-T cell therapy or radiotherapy alone (Figure 6C). All mice (n=5) receiving UTDs succumbed to the tumor burden by day 29. Four mice from the CAR-T group were eliminated by day 30 due to tumor burden, and only one mouse survived to day 39. We observed no significant differences in the control of the secondary non-irradiated tumor (Figure 6B) across treatment groups. Next, we analyzed the phenotype of CAR-T cells collected from primary and secondary tumors at the endpoint (Figure 6, primary and secondary; online Supplementary Figure 9 shows the corresponding heatmaps to the t-SNE maps generated by Cytofkit (27).) Except for the untreated (UTD) control group, all groups exhibited representative populations in the primary tumors, including $CD4^+PD\text{-}1^+$ (cluster -C23), terminally differentiated $CD4^+$ T cells ($CD4^+CD69^+CD45RA^+CCR7$-; C28), and activated $CD8^+$ cells ($CD69^+PD\text{-}1^+$; C26). Furthermore, in the secondary tumors, differences in the expression of CD4 (C28) and CD8 T cells (C34, C36) were not observed among all groups except for the UTD group.

The outcomes of this bilateral tumor experiment affirm the pivotal role of local low-dose radiotherapy in achieving enhanced tumor control when combined with CAR-T cell therapy. Notably, using LDRT proves a safe approach, as it does not compromise the populations of adoptively transferred CAR-T cells. We inferred this from the efficacy of combined therapy in controlling tumor growth, particularly in models where radiotherapy is not directly administered to adoptively transferred CAR-T cells. Nevertheless, in a smaller experiment, we aimed to determine the impact of applying LDRT directly to naïve T cells on their immune function and repair capabilities. Our investigation focused on assessing the effects of low-dose radiation therapy (LDRT) on human CD3+ naive T cells. Following LDRT exposure, we collected cells for RNA extraction and Nanostring analysis at specific times. Our analysis revealed differentially expressed genes associated with T cell receptor (TCR) signaling and DNA repair pathways through fold induction analysis. The gene expression data unveiled that LDRT induced moderate changes in pathways crucial for T cell function, with upregulation of genes such as TP53, COPS6, and FANCD2, indicating potential modulation of cell cycle progression and DNA repair mechanisms (online Supplementary Figure 6B). Interestingly, LDRT exposure also triggered the upregulation of genes within the PI3K/AKT/mTOR pathway, potentially promoting cell survival and proliferation. Additionally, modest upregulation of T cell activation and co-stimulation markers (CD3D, CD27, CD28) suggests partial T cell activation. The observed expression changes in immune

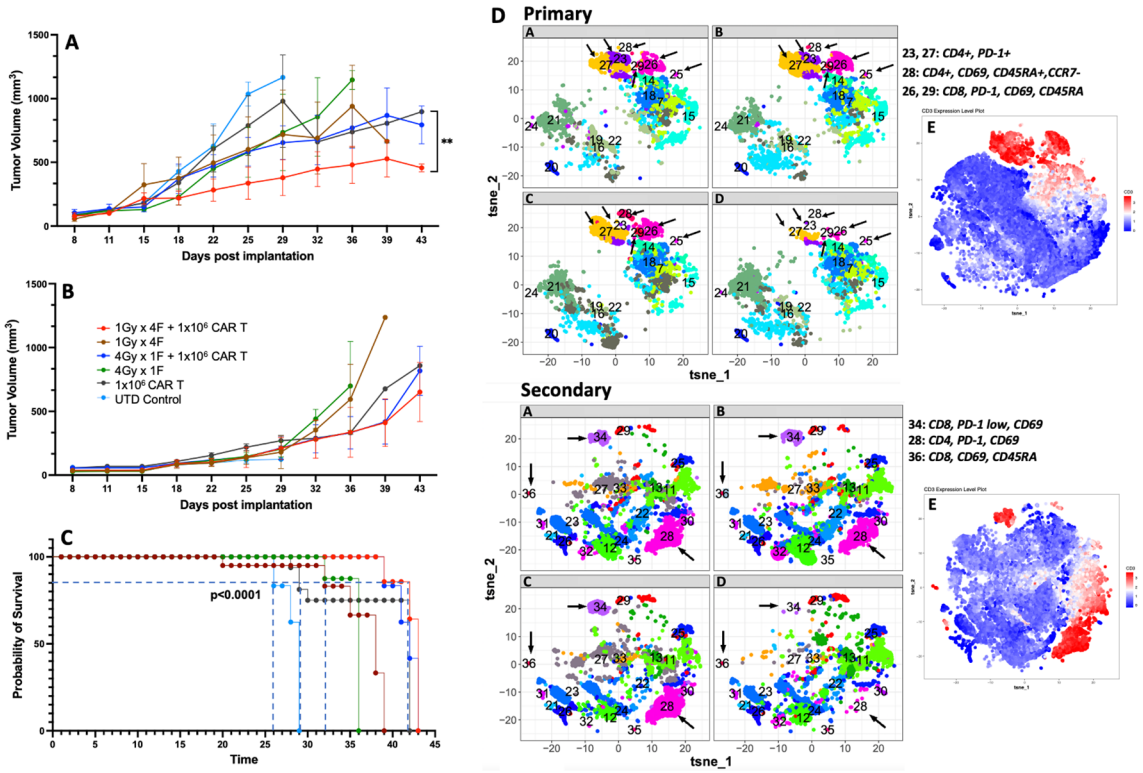

FIGURE 6
1Gy x 4 fractions (1Gy x 4F) or 4Gy x 1 fraction (4Gy x 1F) combined with anti-GCC CAR-T cell therapy exhibited significantly better tumor growth control and survival than CAR-T therapy alone in a bilateral tumor model. **(A)** Primary tumor growth comparison shows a significant difference ($p<0.02$) between mice receiving 1Gy x 4 fractions vs. mice receiving CAR-T cells only. Four mice from the CAR-T group were euthanized by day 30 due to tumor burden and only one mouse survived to the end of the experiment. Also, mice receiving 1Gy x 4 fractions combined with CAR-T cells showed significantly better control in the primary tumor ($p<0.009$) than 4Gy x 1 fraction + CAR-T cell therapy. Although no significant differences were observed in the control of the secondary tumor **(B)**, the dual therapy 1Gy x 4 fractions combined with CAR-T cell therapy showed enhanced control of the primary tumor and a significant survival rate (**(C)**, $p<0.0001$) than mice receiving CAR-T cell therapy or radiation therapy alone. **(D)** We also analyzed the CAR-T phenotype on cells collected at the time of their death (n=3). With exception to the UTD control, all groups had representative populations in the primary tumors including $CD4^+PD\text{-}1^+$ (C23), terminally differentiated $CD4^+$ T cells ($CD4^+CD69^+$, $CD45RA^+$, $CCR7^-$; C28), and activated $CD8^+$cells, ($CD69^+$, $PD\text{-}1^+$; C26). Also, in the secondary tumors, except for the UTD group, we did not observe changes in the expression of CD4 (C28) or CD8 (C34, C36) between the groups. E) The red clusters in both primary and secondary tumors, represent the distribution of the $CD3^+$ populations. (n= 5 mice per group). The t-SNE maps were generated using the R-based software interface Cytofkit (https://www.bioconductor.org/packages/3.5/bioc/html/cytofkit.html).

checkpoint molecules (CTLA4, LAG3, TIGIT) and cytokines (IL2, IL10, IL4, TNF) underscore a dynamic immune response elicited by LDRT. Notably, the upregulation of CD8A, CD8B, and NFKB1 indicates enhanced cytotoxic activity in irradiated T cells (online Supplementary Figure 6C). While these findings offer an initial understanding of LDRT's effects on normal donor non-activated T cells, further *in vivo* studies are imperative and currently underway to assess its impact on the function of adoptively transferred T cells. This study lays the groundwork for further exploration of LDRT's influence on T cell-based cancer immunotherapies.

A distinctive tissue nanomechanical signature in tumors treated with low-dose radiotherapy combined with CAR-T cells

To understand the effect of the combined low-dose radiotherapy (1Gy x 4 fractions) and CAR-T cell therapy on the stiffness and plasticity of the tumor stroma at the nanoscale level, we employed atomic force microscopy (AFM). Using AFM allowed us to quantitatively measure the mechanical properties of the cancer cells and obtain a unique nanomechanical signature associated with therapeutic efficacy (30, 31, 33). Our findings suggest that the combined use of the plasticity index and the stiffness or modulus backward approach, employed to derive mechanical properties like elastic modulus from force-distance curves acquired during indentation experiments, effectively delineates the impact of the combined therapy on the tumor stroma. This method reverses the indentation process to precisely ascertain the sample's elastic properties, providing unique insights into the effects of the treatment. Samples from mice receiving CAR-T cell infusion combined with LDRT demonstrated heterogeneously stiffer maps with higher plasticity index values, which refers to the measure of the tissue's ability to undergo plastic deformation when subjected to mechanical forces. In contrast, lower plasticity index values indicate that a tissue is more rigid and less likely to deform when subjected to mechanical forces (30, 31). In comparison, samples from mice treated solely with LDRT or CAR-T cell therapy exhibited

heterogeneously softer maps with lower plasticity index values (Figure 7A). The receiving operating characteristic (ROC) (38) curve analysis resulted in a 92% sensitivity, 100% specificity, and an accuracy of 95% (Figure 7B), defining the tissue mechanical phenotype in the combined therapy as plasticity high/stiffness heterogeneous (PlasHi StiffHet). Conversely, the signature associated with LDRT treatment alone was labeled as plasticity low/stiffness low (PlasLo StiffLo.).

Figure 7A presents a detailed histogram illustrating stiffness and plasticity index measurements for representative samples. Samples treated solely with LDRT or CAR-T cells displayed a distinct soft peak below 2 kPa, indicative of soft and aggressive cancer cells. Additionally, three out of five samples in each treatment group exhibited a stiffer cell signature within the 2-5 kPa range.

These samples displayed a soft matrix, with maximum stiffness values ranging from 1.5 to 4 kPa. A noticeable shift in the signature was observed when comparing these measurements to those obtained from samples treated with LDRT before CAR-T cells. In samples subjected to the combined treatment, the soft peak diminished in four out of five samples and completely disappeared in one sample, suggesting a reduced presence of soft, aggressive cells. The second cellular peak exhibited increased stiffness and more significant heterogeneity, indicating a potential signature of activated CAR-T cells warranting further investigation. Furthermore, a stiffer matrix peak emerged around 10 kPa, accompanied by an overall stiffening of the matrix, extending to 30 kPa.

Most analyzed samples displayed a single heterogeneous peak in the plasticity domain, which notably shifted towards higher plasticity in samples treated with the combined therapy. Specifically, among samples treated solely with LDRT, all five displayed a dominant peak around 0.6-0.65, with two out of five samples also presenting a second peak around 0.8. In contrast, in samples treated with CAR-T cells after LDRT, four out of five exhibited a peak between 0.7 and 0.8, with two also displaying a peak around 0.6. These findings highlight the potential of the ARTIDIS signature as a biomarker for effective CAR-T cell therapy combined with LDRT in tumor tissues.

In summary, employing AFM to explore the nanoscale effects of LDRT preceding CAR-T cell therapy on tumor stroma, a distinctive tissue nanomechanical signature emerged. This signature, labeled "PlasHi StiffHet," was characterized by heterogeneously stiffer maps with higher plasticity index values. In contrast, samples treated solely with LDRT or CAR-T cells exhibited a different signature labeled as "PlasLo StiffLo" with softer maps and lower plasticity index values. The analysis revealed a significant shift in the plasticity domain, suggesting that the identified ARTIDIS signature could be a biomarker for effective CAR-T cell infiltration in tumor tissues. The study showcased the potential of nanomechanical profiling to elucidate the mechanical phenotypes associated with therapeutic efficacy in combined cancer treatments.

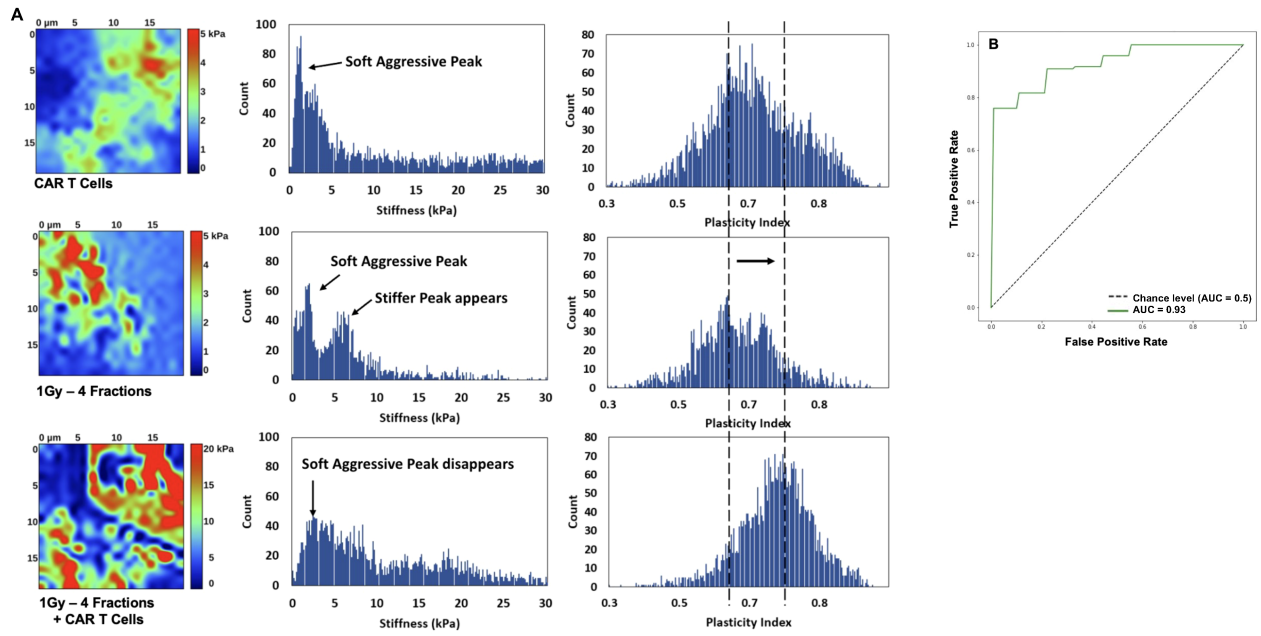

FIGURE 7
The distinctive signature of CAR-T cell therapy combined with LDRT is revealed through the combined analysis of plasticity index and stiffness. Mice receiving the combined therapy exhibited maps with varying stiffness and heightened plasticity index values and a reduction of aggressive soft cells. Additionally, there is an increase in stiffness of the second cellular peak and the emergence of a stiffer matrix peak around 10 kPa, resulting in an overall stiffening of the matrix **(A)**. Most samples exhibit a single peak in the plasticity domain, shifting towards higher plasticity with combined CAR-T cell and LDRT treatment. In contrast, mice treated solely with LDRT exhibit maps with heterogeneous softness and a low plasticity index. The ROC analysis yields significant results, with a 92% sensitivity, 100% specificity, and 95% accuracy, defining the tissue mechanical phenotype in the combined therapy as "PlasHi StiffHet." In contrast, either monotherapy, RT, or CAR-T cells is categorized as "PlasLo StiffLo" **(B)**. Samples treated solely with low-dose radiation or CAR-T cells manifest a soft peak below 2 kPa, corresponding to aggressive cancer cells. Some samples also display a stiffer cell signature in the 2-5 kPa range. (n=5 mice per group).

Discussion

Although immunodeficient mice are not a perfect model for testing the effectiveness of LDRT combined with cell therapy, they are nevertheless useful for testing therapeutic interventions against solid tumors. It is important to note that NSG models only partially translate into the clinic due to their limitations, such as lacking a functional immune system. However, despite these limitations, they remain valuable for preliminary testing, provide critical insights into therapeutic interventions, and help guide further research, making them an essential tool in the early stages of therapy development. We demonstrated that using LDRT combined with CAR-T cell therapy enhanced the control of tumor growth in NSG mice implanted with GSU or CAPAN-2 cells. Our approach included testing LDRT in several doses and schedules, delivered directly to implanted tumors, followed by a single intravenous injection of CAR-T cells recognizing either GCC or mesothelin 24 hours after the final LDRT fraction was delivered. Mice that received LDRT followed by CAR-T cell therapy survived longer and demonstrated significantly better tumor control compared to mice receiving monotherapy (i.e., CAR-T cells only, radiation LDRT only or untreated (UTD) T cells). We further chose to minimize the risk of RT-related side effects (e.g., skin ulceration) by focusing on a single dose of 4Gy or a low-dose of 1Gy x 4 fractions. We introduced using 4Gy divided into four fractions (1Gy x 4F) to emulate current dosing to treat metastatic disease in the clinic (25).

We also sought the ideal number of CAR-T cells for use as a single injection with the smallest possible effective LDRT dose. For the GSU model, we tested two doses of anti-GCC CAR-T cells (1×10^6, 2.5×10^6 cells/mouse) with two LDRT schedules (1Gy x 4 fractions and 4Gy x 1 fraction); for the CAPAN-2 model, we tested four LDRT schedules (1Gy x 4 fractions, 4Gy x 1 fraction, 2 Gy x 1 fraction, and 2 Gy x 2 fractions) and one dose of anti-mesothelin CAR-T cells (1×10^5cells per mouse). In this model, combining anti-mesothelin CAR-T cells with 1Gy x 4 fractions or 4Gy x 1 fraction both significantly enhanced tumor control and prolonged survival for up to 75 days after tumor-cell implantation. Although the mice treated with CAR-T cells exhibited similar survival rates in the pancreatic model, their tumor control was less effective compared to the combination therapy. Additionally, we substantiated the efficacy of dual therapy in immunocompetent mice. In these experiments, C57BL/6 mice were implanted with MC38-gp100+ colon adenocarcinoma cells. We administered LDRT at 1Gy x 4 fractions and infused pmel-sensitized T cells, either before or after the radiation treatment. Our findings mimicked those in the immunodeficient NSG mouse models, in that mice treated with adoptively transferred pmel-sensitized T cells combined with LDRT enhanced the control of tumor growth as compared to mice treated with cell therapy alone. This effect is likely due to the role of LDRT in modulating both the tumor stroma and the tumor microenvironment as evidenced previously (6, 13). We used the pmel model to demonstrate that combining low-dose radiation therapy (LDRT) with cell therapy is more effective than cell therapy alone. Although we did not design the experiment to show survival, which is essential for understanding treatment impact, this was due to the lack of mouse-generated CAR-T cells to replicate our experiments using human CAR-T cells. Recognizing the need for complete characterization, including survival outcomes, we are now developing a humanized mouse model. This model will better emulate human CAR-T cell specificity and allow for a comprehensive evaluation of the combined therapy's benefits.

Our findings unequivocally emphasize that the meticulous process of combining low-dose radiation (LDRT) with cell therapy demands a nuanced and tailored approach, eschewing a "one-size-fits-all" paradigm. The critical factor lies in carefully titrating doses of both radiation and CAR-T cells for optimal efficacy. For instance, administering 1Gy x 4 fractions combined with CAR-T cell therapy in the GSU model moderately controlled tumor growth and extended survival. However, this approach yielded different results in the CAPAN-2 model, where the most favorable response was observed with a single 4-Gy fraction coupled with 1×10^5 anti-mesothelin CAR-T cells, maintaining tumor volumes below 500 mm^3 and ensuring mouse survival for up to 75 days post-implantation.

It is crucial to stress that the choice of CAR-T cell dose is equally pivotal and must be carefully considered regarding both transduction efficiency and the cells' effectiveness in killing targeted tumor cells. Invariably, compared with mice receiving CAR or untransduced T cells, those treated with combinations of CAR-T cells and LDRT exhibited similar levels of tumor infiltration by CAR-T cells, as assessed by total CD3+ cells gated on the SSC-A vs. FSC-A lymphocyte fraction.

Although tumor-infiltrating CAR-T cells were not collected simultaneously across treatment groups in the GSU tumor model, a time course study would accurately elucidate the extent of intratumor infiltration of CAR-T cells. Our results showed that mice receiving CAR-T cell monotherapy had significantly lower numbers of tumor-infiltrating CAR-T cells than those receiving dual therapy (CAR-T cells combined with RT), as assessed by gradient isolation of tumor-infiltrating T cells. This disparity is likely due to differences in survival, as mice receiving CAR-T cell therapy died at least a month earlier than those receiving the dual therapy.

Our results underscore the importance of personalized dosing strategies for radiation and CAR-T cell therapies to maximize therapeutic outcomes in different tumor models.

CD4 T cells are known to arrive earlier than CD8 T cells at the site of infection or tissue damage. This earlier arrival is crucial for orchestrating the subsequent immune response, as CD4 T cells help recruit and activate other immune cells, including CD8 T cells. This temporal sequence ensures a robust and coordinated immune attack against the pathogen or damaged tissue.

The results of our study align with this understanding. Our findings using multiplex immunohistochemical staining in the GSU model confirmed that mice treated with 1Gy x 4 fractions of radiotherapy showed an overall increase in total infiltrating T cells, including CD4+ and CD8+ T cells, compared to mice given only CAR-T cells or CAR-T cells with 4Gy x 1 fraction. Although these findings were not statistically significant, they are consistent with previous reports and clinical data indicating that LDRT (1.4Gy x 4 fractions) preferentially enhance the intratumor infiltration of

CD4+ T and NK cells (6, 7, 13, 39). This observation reinforces the role of CD4 T cells in the early stages of immune infiltration and their potential impact on the overall immune response within the tumor microenvironment.

Moreover, by using a bilateral tumor model, we demonstrated that mice treated with either 4Gy x 1 fraction or 1Gy x 4 fractions with CAR-T cells led to significantly improved control of the primary (irradiated) tumor than mice with CAR-T cells alone. However, no significant differences in the control of the secondary tumor were observed. Our findings also revealed that the survival rate of mice treated with the combined therapy was significantly higher than those treated with either CAR-T or radiotherapy alone. This model emphasized the importance of administering low-dose radiotherapy locally, in conjunction with CAR-T cell therapy, to achieve superior tumor control. Also, our results indicate moderate changes induced by the direct exposure of normal donor non-activated T cells to two fractions of 1Gy in pathways crucial for T-cell function and the upregulation of genes promoting cell survival and proliferation. The observed upregulation of genes such as TP53, COPS6, and FANCD2 suggests potential modulation of cell cycle progression and DNA repair mechanisms in response to LDRT exposure, aligning with previous studies on the impact of radiation therapy on DNA damage repair pathways (40). Moreover, the modest upregulation of T cell activation and co-stimulation markers (CD3D, CD27, CD28), alongside dynamic changes in immune checkpoint molecules (CTLA4, LAG3, TIGIT) and cytokines (IL2, IL10, IL4, TNF), highlights the intricate interplay between LDRT and the immune response. Notably, the enhanced cytotoxic activity (CD8A, CD8B, and NFKB1) observed in irradiated T cells suggests a potential mechanism contributing to the improved tumor control observed in our study. These findings reinforce the importance of integrating low-dose radiotherapy with CAR-T cell therapy to optimize treatment outcomes.

We utilized atomic force microscopy (AFM) to investigate the impact of CAR-T cell therapy combined with LDRT on tumor stiffness and plasticity. Our findings reveal a distinctive characterization of the tumor stroma when subjected to the combined therapy. Mice receiving LDRT and CAR-T cell therapy exhibit stiffer maps and higher plasticity index values. Conversely, mice treated solely with LDRT display softer maps and lower plasticity index values.

The ROC analysis achieved notable results with a 92% sensitivity, 100 specificities, and accuracy of 95%, categorizing the mechanical phenotype of CAR-T cells as "PlasHi StiffHet." Samples treated exclusively with LDRT or CAR-T cells exhibit a soft peak associated with aggressive cancer cells. In contrast, samples treated with the combined therapy show a reduced presence of these cells and increased stiffness. The plasticity peak shifts towards higher values in samples treated with CAR-T cells and RT, indicating effective tumor control.

Our results on the nanomechanical changes in the tumor stroma underscore the importance of mechano surveillance by immune cells, including T cells. This concept, which refers to the ability of immune cells to sense and respond to the mechanical properties of their environment (41–43), plays a crucial role in their activation, motility, and overall function.

Our study's observed nanomechanical signatures likely reflect the dynamic interactions between CAR-T cells and the tumor microenvironment. T cells' ability to sense and adapt to the stiffness and plasticity of their surroundings is fundamental to their efficacy in targeting cancer cells. The heterogeneously stiffer and more plastic tumor stroma in the combined therapy group may facilitate better infiltration and activation of CAR-T cells, enhancing their therapeutic potential.

Understanding immune cells' mechanosensitive behaviors can provide deeper insights into their role in cancer therapy and help refine strategies for improving treatment outcomes. As we continue to explore these mechanical interactions, we aim to use this knowledge to develop more effective and targeted immunotherapies.

Finally, our findings underscore the therapeutic potential of combining cell therapy with LDRT, provided that it is tailored to the specific tumor type, and the adoptively transferred effector T cells are carefully matched with the radiation dosage. This innovative approach holds promise across a spectrum of preclinical models and has the potential for clinical trials, offering the unique advantage of inducing therapeutic effects without causing radiation-related tissue damage. Combining CAR-T cell therapy with RT, validated through successful preclinical studies, fuels high expectations for its forthcoming success in the clinic.

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 Institutional Animal Care and Use Committee. The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

NP-O: Data curation, Formal analysis, Methodology, Resources, Supervision, Writing – original draft, Writing – review & editing. NF: Formal analysis, Writing – review & editing. HB: Methodology, Supervision, Writing – review & editing. KX: Methodology, Validation, Writing – review & editing. GS: Formal analysis, Methodology, Writing – review & editing. CK-L: Methodology, Writing – review & editing. SN: Formal analysis, Writing – review & editing. TV: Methodology, Writing – review & editing. TR: Data curation, Methodology, Writing – review & editing. CW: Data curation, Supervision, Writing – review & editing. AH: Methodology, Writing – review & editing. YH: Methodology, Supervision, Writing –

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Funding

The author(s) declare financial support was received for the research, authorship, and/or publication of this article. This research project was funded by Takeda. The nanomechanical study was funded by ARTIDIS, AG.

Conflict of interest

JWW: Accuray (SAB, consulting), Alpine Immune Science (SAB, consulting, equity), Boehringer Ingelheim (SAB, consulting), Checkmate Pharmaceuticals (SAB, consulting, equity), China Medical Tribune (SAB, consulting), Genentech (SAB, consulting), GI Innovation (SAB, consulting), Kezar Life Sciences (consulting, SAB), Legion Healthcare Partners (SAB, consulting), Life Science Dynamic Limited (SAB, consulting), McKesson Corporation (SAB, consulting), Molecular Match (equity), Nanorobotix (SAB, consulting), OligoImmune (founder), Roche (SAB, consulting), Roche Molecular Systems (SAB, consulting), Nanobiotix (Research grant, Travel expenses, SAB), BMS (Research grant), Merck (Research grant), Varian (Research grant, Travel expenses, clinical sponsored research), Reflexion (Research grant, Travel expenses, Stock options, SAB), Hotspot Therapeutics (Research grant), Gilead (Research grant), Novocure (SAB), Oncoresponse (SAB, Stock options), Astra Zeneca (consultant, Research grant), Bayer Healthcare (Research grant), Kiromic (Research grant), Alkermes (Research grant, SAB), Artidis (Research grant), Sciclone (Research grant), Takeda (Research grant), Pebble Life Science (Research grant). HB and NP-O report consulting support from Research Guided Cancer Treatment Consults. Authors KX, CW and JM were employed by Takeda Development Centers Americas Inc. Authors GS, SN, PO, ML and MP were employed by ARTIDIS AG.

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

The author(s) declared that they were an editorial board member of Frontiers, at the time of submission. This had no impact on the peer review process and the final decision.

The authors declare that this study received funding from Takeda and was involved in the study design. The authors declare that ARTIDIS funded the tissue nanomechanical analysis from the study funded entirely by Takeda. ARTIDIS personnel was involved in the collection, analysis, interpretation, and writing of the tissue nanomechanical section 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/fonc.2024.1407143/full#supplementary-material

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frontiers | Frontiers in Immunology

TYPE Review
PUBLISHED 01 November 2024
DOI 10.3389/fimmu.2024.1421889

OPEN ACCESS

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RECEIVED 23 April 2024
ACCEPTED 30 September 2024
PUBLISHED 01 November 2024

CITATION
Chen H, Zhou Y, Tang Y, Lan J, Lin C, Chen Q and Kuang H (2024) Neutrophil extracellular traps in tumor progression of gynecologic cancers.
Front. Immunol. 15:1421889.
doi: 10.3389/fimmu.2024.1421889

Neutrophil extracellular traps in tumor progression of gynecologic cancers

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This article delves into the intricate interplay between tumors, particularly gynecologic malignancies, and neutrophil extracellular traps (NETs). The relationship between tumors, specifically gynecologic malignancies, and NETs is a multifaceted and pivotal area of study. Neutrophils, pivotal components of the immune system, are tasked with combating foreign invaders. NETs, intricate structures released by neutrophils, play a vital role in combating systemic infections but also play a role in non-infectious conditions such as inflammation, autoimmune diseases, and cancer. Cancer cells have the ability to attract neutrophils, creating tumor-associated neutrophils, which then stimulate the release of NETs into the tumor microenvironment. The impact of NETs within the tumor microenvironment is profound and intricate. They play a significant role in influencing cancer development and metastasis, as well as modulating tumor immune responses. Through the release of proteases and pro-inflammatory cytokines, NETs directly alter the behavior of tumor cells, increasing invasiveness and metastatic potential. Additionally, NETs can trigger epithelial-mesenchymal transition in tumor cells, a process associated with increased invasion and metastasis. The interaction between tumors and NETs is particularly critical in gynecologic malignancies such as ovarian, cervical, and endometrial cancer. Understanding the mechanisms through which NETs operate in these tumors can offer valuable insights for the development of targeted therapeutic interventions. Researchers are actively working towards harnessing this interaction to impede tumor progression and metastasis, opening up new avenues for future treatment modalities. As our understanding of the interplay between tumors and NETs deepens, it is anticipated that novel treatment strategies will emerge, potentially leading to improved outcomes for patients with gynecologic malignancies. This article provides a comprehensive overview of the latest research findings on the interaction between NETs and cancer, particularly in gynecologic tumors, serving as a valuable resource for future exploration in this field.

KEYWORDS

tumor immune microenvironment, neutrophils, neutrophil extracellular traps, metastasis and recurrence, gynecologic cancers, new treatment strategy

1 Introduction

Cancer is a kind of complex systemic disease, which is the result of a variety of factors (1, 2). Its formation and development are not only related to the random variation of the body's parenchymal cell genome, but also the disorder of the host immune microenvironment and the decline of immune surveillance ability play an important role in this process (1–3). The interaction between tumor cells and immune cells is also an important factor affecting tumor progression (1, 4, 5). The tumor immune microenvironment (TIME) is influenced by different chemokines and cytokines and is orchestrated by immune and tumor cells interacting (5–7). It is essential to the development, spread, invasion, and metastasis of malignancies. Immune cells invading tumors have the ability to either stimulate or prevent tumorigenesis. However, in the occurrence and development of tumor, which is a chronic inflammation, neutrophils also become an important force leading to the formation and progression of tumor, which is an "incurable wound" (8–10).

Tumor cells are a type of abnormal cells with immunogenicity (11, 12). In a state of maximum immunological vigilance, the immune system is able to promptly identify and eradicate aberrant transformed cells, thus preventing the occurrence of tumors (12). The microenvironment's inflammatory mediators have the ability to gather in additional neutrophils and contribute to the microenvironment (13). The hallmarks of inflammation that are linked to cancer are neutrophil recruitment and activation (14, 15). Tumor-associated neutrophils(TANs) are neutrophils that settle down or infiltrate the tumor microenvironment (15). T lymphocytes play an important role in the first stage of tumor immune editing "immune surveillance" (16). Interferon (IFN)-g-secreting cytotoxic CD8+ T lymphocytes (CTLs) are a major constituent of the TIME and constitute a uniform population of cytotoxic cells. CTLs promote anti-tumor responses and enhance prognosis for patients (17). During the aging process, naive T cells decrease, memory and effector T cells increase, the diversity and sensitivity of T cell receptors decrease, and the immune surveillance ability is weakened (17). Regulatory T cells (Tregs) foster tumor cells and evade the immune system by attenuating immunological responses. Th cells develop distinct populations with varying functions, including Th1, Th2, and Th17 cells (18). Th1 cells stimulate CTLs, influence anti-tumor immunity, and are linked to better prognoses. Conversely, Th2 cells limit Th1 responses, support humoral immunity, and are linked to a worse prognosis. Th17 cells show heterogeneity in human cancer, expressing diverse cytokines, transcriptional factors, and activated markers, which affects patient prognosis (18). Combined with increased exposure to external tumorigenic factors, the genomic instability of cells increases. Tumor cells with low-immunogenic cannot be eliminated and are in a dormant state, which are temporarily in the state of "immune equilibrium". When a certain stress event occurs and becomes the trigger, inflammation may break out in the body, and the immunosuppressive microenvironment created and manufactured by typical immune cells can promote tumor progression, thus entering the third stage of immune editing, "immune escape" (19, 20).

The initial line of defense against different types of infections and malignancies is the neutrophil (21). They primarily function as innate effector cells and account for over 70% of human circulating leukocytes. Neutrophils, the immune cell subtype with the greatest number of cells, are key players in acute infection and tissue damage (22, 23). They additionally prove the most beneficial for wound healing, which is due to their ability to degranulate, phagocytose, and form neutrophil extracellular traps (NETs) (1, 10).

In particular, the structure of NETs —"flying frame", produced by neutrophils has also been confirmed to be the material basis of inflammatory response and coagulation system (Figure 1). NETs were first identified as a nucleic acid-based structure highlighted in bacterial immunity in 2004 (24). Activated neutrophil-ejected net-like structures are formed out of decondensed extracellular chromatin filaments embellished with granular proteins, involving histones, which are frequently citrullinated, neutrophil elastase (NE), cathepsin G, myeloperoxidase (MPO), and matrix metalloproteinases 9 (MMP9) (25–28). In fact, according to recent discoveries, NETs have become essential for the emergence and progression of malignancies (26–29). The formation of pre-metastatic niches, the resurgence of dormant metastases, and the possibility for direct tumor growth via related proteases like NE and MMP9 via the proteolytic restructuring of laminin are all reliant on them (30–32). In order to promote the adherence, invasion, and migration of circulating tumor cells, NETs may additionally attach to them and serve as an adhesion substrate (28, 30, 32–35).

Insufficient information is known concerning the mutual interaction of NETs with invading immune cells in the TIME of gynecologic cancers, compared to the direct impact on cancer cells (29, 31). It is also not widely recognized how NETs, that encompass both innate and adaptive immunity, interact with TIME of gynecologic cancers. A more thorough understanding of the connection between the TIME and NETs might encourage more potent tactics. With the objective to gain insight into the synergies between NETs and TIME on progression of tumors, this review will bring together the body of knowledge currently accessible in this field.

2 The ways in which NETs form

Suicidal NETosis is and vital NET formation are the two major types of NET development (36, 37).

2.1 Suicidal NETosis

The onset of suicidal NETosis is initiated by the activation of neutrophils through various triggers, such as immunological complexes, specific autoantibodies, cholesterol or calcium salt crystals, or phorbol-12-myristate-13-acetate (PMA) (36, 38–40). This activation induces an elevation in cytosolic Ca2+ levels, which in turn activates the NADPH oxidase (NOX) complex, resulting in the generation of reactive oxygen species (ROS) via Raf/MEK/ERK signaling (41, 42). Subsequently, NOX and ROS complexes

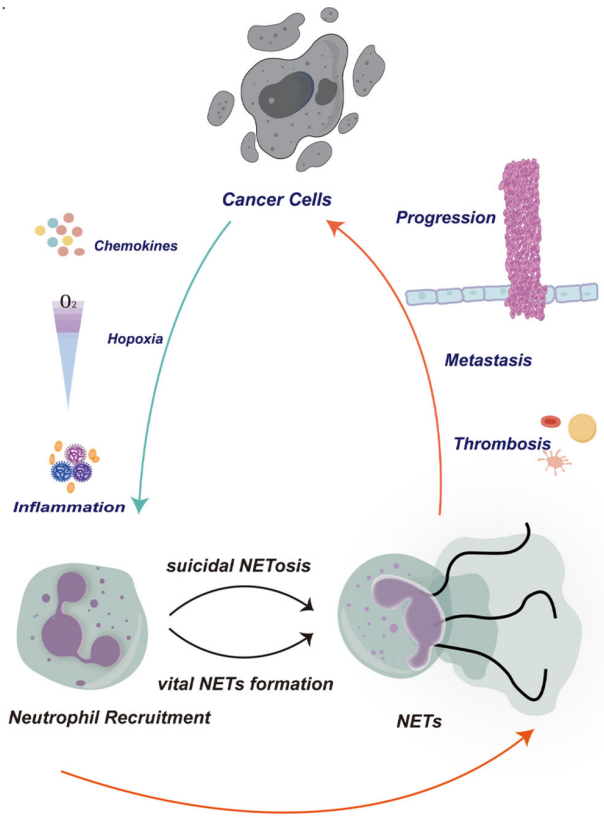

FIGURE 1
In the tumor microenvironment, cancer cells can release signaling molecules such as chemokines and cytokines to recruit neutrophils to the surrounding tissue of the tumor. These signaling molecules can attract neutrophils to migrate towards the tumor through various pathways. These neutrophils are activated and release neutrophil extracellular traps (NETs). The release of NETs can promote the migration and invasion of tumor cells, while also inducing the formation of blood clots to support the translocation of tumor cells. This phenomenon plays an important role in the tumor microenvironment, accelerating the progression of tumors by altering the tumor microenvironment, regulating immune responses, and influencing angiogenesis mechanisms. Therefore, the interaction between cancer cells, neutrophils, and NETs plays a crucial role in the progression and metastasis of tumors. A deeper understanding of this cooperative relationship can help us better comprehend the complexity of the tumor microenvironment and develop new therapeutic strategies.

stimulate protein-arginine deiminase 4 (PAD4) to modify histones, facilitating the movement of NE and MPO from neutrophil granules to the nucleus, thereby causing chromatin decondensation. The decondensed chromatin is eventually released during cellular lysis, combining with granular and cytosolic proteins in the cytoplasm. Suicidal NETosis is characterized by the demise of a cell through membrane dissolution, a process that may span several hours to complete (41, 43, 44).

2.2 Vital NETs

Shortly after neutrophil activation, the formation of vital NETs occurs independently of cell death and without membrane rupture. Stimuli such as lipopolysaccharide (LPS) from gram-negative bacteria or S. aureus initiate this process through complement receptors (CR) and toll-like receptors (TLRs) (45, 46). The NOX complex and reactive oxygen species (ROS) are not required for the generation of vital NETs. The release of nuclear DNA during the crucial NET formation process is associated with three specific morphological changes: nuclear decondensation, disruption of the nuclear envelope, and expansion of the nuclear envelope leading to vesicle release (29, 36, 44–46). Infectious diseases tend to encourage a higher frequency of vital NET formation compared to non-infectious diseases, as evidenced by the continued survival of neutrophils with their capacity for antimicrobial activities such as phagocytosis, chemotaxis, and bacterial killing (46–48).

2.3 Mitochondrial DNA NETs

In addition to the NETs formed by the release of nuclear DNA, research has identified NETs composed of mitochondrial DNA. This indicates that cells can utilize both nuclear and mitochondrial DNA when responding to infection or damage (49–51). Research has demonstrated that, after pretreatment with granulocyte/macrophage colony-stimulating factor (GM-CSF), neutrophils can generate NETs upon short-term stimulation with Toll-like receptor 4 (TLR4) or complement factor 5a (C5a) receptors. These NETs, produced by cells, contain mitochondrial DNA but are devoid of nuclear DNA. Additionally, inhibition of reactive oxygen species

(ROS) through pharmacological or genetic approaches reveals that NETs formation relies on ROS. Neutrophils activated by GM-CSF and C5a show higher survival rates compared to resting neutrophils, which do not produce NETs. Overall, mitochondrial DNA release and NET formation by neutrophils do not necessitate cell death or restrict cell lifespan (52). Another study investigated the role of mitochondrial DNA (mtDNA) in the formation of NETs following trauma and orthopedic surgery. The findings revealed that these NETs were solely composed of mtDNA, lacking nuclear DNA. They emerged as a response to injury and continued to exist post-surgery. These observations suggest that mtDNA-based NETs could act as an indicator of heightened immune activation and may impact the timing of surgical interventions to mitigate inflammatory complications (49).

2.4 Tumor cells cause NETs development

The detection of NETs in tumor tissues has been revealed in multiple instances (27–29, 31, 32, 34, 44). Electron microscopy demonstrated that neutrophils were destroyed and did not show signs of DNA-containing vesicles emerging from intact neutrophils, which is consistent with the co-cultivation of cancer cells with neutrophils leading to NETs formation within 3 hours (4, 34). All of these results pointed to the induction of suicidal rather than vital NETs production by cancer cells.

3 Mechanisms of NETs in cancer development and progression

In addition to promoting tumor formation by exacerbating chronic inflammation, neutrophils may also have a direct carcinogenic potential (10, 24). Recent studies have shown that in certain circumstances, neutrophils can directly participate in the formation and development of tumors (Figure 2). For example, neutrophils could directly promote tumor growth by releasing factors that stimulate tumor growth, promoting angiogenesis, and suppressing immune responses. Additionally, neutrophils may interact with tumor cells to promote tumor cell migration and invasion, thereby facilitating tumor spread and metastasis (10, 24, 25, 27, 28). Therefore, neutrophils may play a more complex role in tumor formation and development, beyond the mechanisms involving chronic inflammation. These new findings provide important clues for better understanding the interactions between different cell types in the tumor microenvironment and offer new research directions for future cancer treatment and prevention. Recently, in several chemical carcinogenesis mouse models,

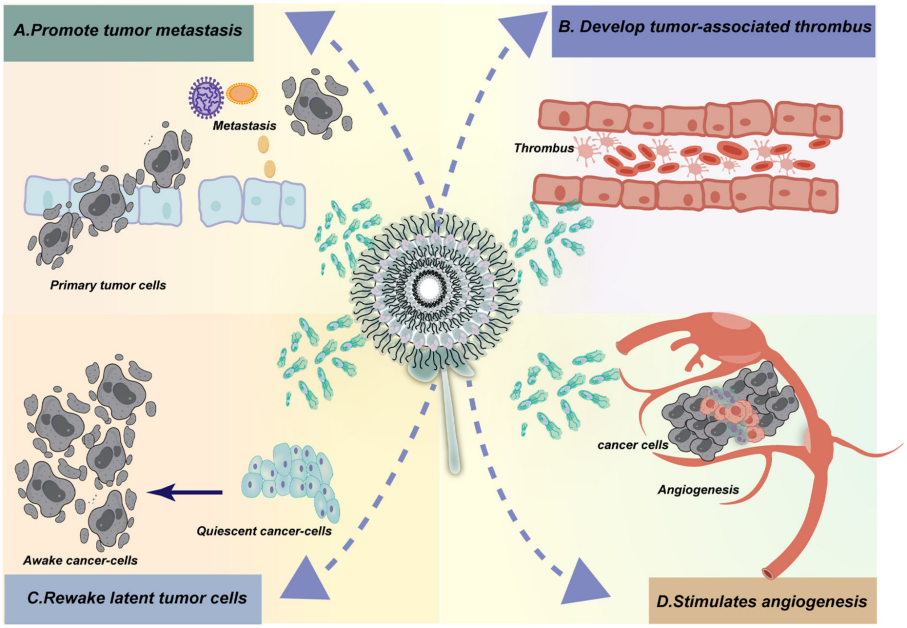

FIGURE 2
Neutrophil extracellular traps (NETs) have been implicated in cancer development and progression through various mechanisms. The activation of NETs by tumor cells has been shown to enhance the invasion and migration of tumors, thereby facilitating metastasis. Additionally, NETs released following surgery or under stress conditions have been implicated in stimulating tumor recurrence and metastasis by creating an inflammatory environment that supports cancer cell survival and proliferation. Furthermore, NETs aid in the survival of circulating tumor cells (CTCs) by promoting their adhesion and invasion into surrounding tissues. In the context of thrombosis, NETs composed of tissue proteases, cytokines, and DNA have been found to contribute to the development of venous and arterial thrombosis. Studies have shown that cancer patients have an increased risk of thrombosis, which can be attributed to the promotion of NET formation by tumor cells and platelets. Moreover, the release of NETs by neutrophils in different types of cancer has been associated with an elevated risk of thrombosis. Overall, NETs play a complex role in cancer development and progression, influencing various aspects of tumor biology including growth, angiogenesis, immune evasion, inflammation, and metastasis. Targeting NETs or their associated pathways may therefore represent a novel therapeutic strategy for cancer treatment.

neutrophils have been shown to be crucial for tumor formation (29–32). In the past decade, the emergence of single-cell technologies has revealed significant heterogeneity in the state of neutrophils within tumors. It is now recognized that myeloid-derived cells, including neutrophils, are highly plastic cells in cancer, akin to the diverse reservoir of monocytes/macrophages. Some of this heterogeneity may be related to differences in the ability of neutrophils to migrate or form NETs, as previously described in different stages of the neutrophil lifecycle (21).

3.1 NETs promote tumor metastasis

3.1.1 NETs can be activated by tumor cells which facilitates invasion and migration of tumors

The invasion and migration of tumors are enhanced by NETs (44, 53, 54). Certain breast cancer cells, such as those with significant G-CSF secretion abilities, have been shown *in vitro* to be able to activate neutrophils to make NETs, which can then be seen to promote the invasion and migration of the tumor cells and reverse their course. Additionally, pancreatic cancer cell lines produced by inducing NETs have improved invasion and migratory capabilities (55).

Polymorphonuclear myeloid-derived suppressor cells (PMN-MDSCs) play a crucial role in the tumor microenvironment, characterized by immune suppression that significantly reduces anti-tumor immune responses. PMN-MDSCs inhibit T cell immune responses, promote angiogenic factors, enhance the stem cell potential of tumor cells, and protect circulating tumor cells from host immune system attacks. This protective role aids in tumor metastasis and progression while conferring resistance to cancer immunotherapy (56–58). The C5a component of the complement system has emerged as a crucial immunoregulatory factor, capable of modulating various immune responses and influencing the activity of PMN-MDSCs (59). The findings from the study highlight a significant role for complement C5a in cancer progression, specifically in enhancing tumor growth and metastasis through the modulation of PMN-MDSCs. Recent research showed that by stimulating these immune cells, C5a aids the invasive capabilities of cancer cells via NETs, with the NETosis process being dependent on the high mobility group box 1 protein (HMGB1) produced by tumors. The study also identified that C5a promotes the expression of HMGB1 receptors, TLR4 and RAGE, on the surface of PMN-MDSCs, which could further facilitate tumor survival and immunity evasion. The experiments conducted in mouse models of lung metastasis showed that inhibiting C5a, its receptor, or the NETosis process resulted in reductions in circulating tumor cells and overall metastatic burden, suggesting potential therapeutic avenues. Moreover, clinical relevance was supported by findings that PMN-MDSCs from lung cancer patients exhibited increased migration and NETosis in response to C5a, with elevated levels of myeloperoxidase (MPO)-DNA complexes—a hallmark of NETosis—correlating with C5a levels in these patients. This research underscored the dual role of PMN-MDSCs in both immune suppression and facilitating tumor spread through NET formation, suggesting that targeting the C5a pathway could represent a promising strategy for developing innovative cancer therapies aimed at enhancing anti-tumor immunity and reducing metastasis (60).

3.1.2 Recurrence and metastasis of tumors can be stimulated by NETs resulting from surgery and under stress

The release of NETs can stimulate tumor recurrence and metastasis by fostering an inflammatory environment that promotes the survival and proliferation of cancer cells (61). In a recent research, there remained the possibility of recurrence for limited-stage breast cancer after surgery, and the window of opportunity for recurrence was nearly always between six and twelve-months following surgery (62, 63). Researchers hypothesized that this phenomenon could create circumstances for the survival and spread of tumor cells by influencing patients' stress responses and wound healing processes during surgery (61). A worse prognosis and increased risk of tumor recurrence were associated with patients with colorectal liver metastases who experience NETs surge following surgical removal of all malignancies (64, 65). Additionally, in mice models, it has been demonstrated that NETs could stimulate peritoneal metastases (66, 67). Moreover, there had been a greater probability of abdominal metastasis or recurrence following surgery for patients with colorectal cancer who had an increase in low-density neutrophils (LNDs) during the perioperative phase that were able to spontaneously produce NETs. While a number of tumor cells were captured and shielded by the newly generated NETs, the LDNs in patients' perioperative stress states prevented killer T cells from killing (66).

3.1.3 NETs aid in the survival of CTCs

As part of their medical treatment, the majority of individuals with cancer undergo surgery no fewer than once (68). Circulating tumor cells have the capability to enter the bloodstream and disseminate to distant sites, facilitated by surgical manipulation of the primary tumor. This phenomenon has been linked to potential advancements in tumor progression and the spread of metastases (69, 70). Researches have shown that surgical incisions can unintentionally promote the growth and spread of tumor (70). Postoperative infection represents a considerable concern, with a notable incidence rate, presenting a significant complication for cancer patients (71). This infection could result from a compromised immune system linked to the cancer itself, including complications such as intestinal obstruction or pneumonia. Alternatively, it could also be triggered by immunosuppression induced by standard cancer treatments such as chemotherapy and surgery (72). These circumstances markedly elevate the risk of postoperative infection. Research has indicated that up to 40% of cases suffered from postoperative infections (73). Unfavorable oncological outcomes have been associated with significant postoperative infections (74). Specifically, surgical procedures have been identified as triggering the release of NETs. These NETs possess the capacity to interact with circulating tumor cells, thereby potentially augmenting their capacity for adhesion and invasion into adjacent

tissues (75, 76). Chronic stress has been linked to persistent inflammation and weakened immune responses, both of which are known to promote the progression and metastasis of tumors. The induction of NETs due to stress could promote the migration and infiltration of cancer cells, thereby enhancing their spread to distant organs in the body (61). Jonathan Cools-Lartigue et al. have identified that NETs in the microvasculature of lung cancer cells produce DNA nets. These nets consist of chromosome-specific DNA and protein complexes. When released, DNA nets from NETs can interact with cancer cells, potentially facilitating tumor migration and invasion to distant sites in the body. Emphasizing the production and role of NETs shows potential in mitigating the progression and metastasis of lung cancer. Continued investigation via research and clinical trials has the potential to broaden therapeutic avenues for lung cancer patients, affirming NETs as a feasible target for intervention. This research may pave the way for novel treatment modalities focused on addressing NETs (77).

3.2 NETs help to develop tumor-associated thrombus

Research has demonstrated that NETs generated by neutrophils have been demonstrated to enhance the development of thrombosis in animal models. These NETs, which are made of tissue proteases, cytokines, and DNA, have the potential to contribute to venous and arterial thrombosis (29). Mantovani and colleagues were the first to report on the role of NETs in human thrombus formation. They found NETs components in venous and arterial thrombi retrieved from patients, suggesting that NETs may play a critical role in the early stages of thrombus formation (78). When platelets and red blood cells coagulate, the DNA skeleton that NETs produce not only acts as a reaction support point for them, but it also binds to tissue factor, the initiator of the exogenous coagulation pathway that originates from NETs. This increases the coagulation reaction's efficiency and promotes thrombosis (78). Studies revealed that compared to the general population, individuals with cancer have a greater likelihood of developing venous and arterial thrombosis. This increased risk is attributed to various factors, with one important factor being that cancer itself promotes the formation of NETs through multiple mechanisms, thereby facilitating thrombus formation. Tumor cells can directly promote the formation of NETs. Additionally, platelets in cancer patients play a key role in promoting NETs formation. Certain cytokines, such as granulocyte-macrophage colony-stimulating factor (GM-CSF) and IL-17, have also been found to increase NETs production, thus promoting thrombosis (79–81). Research has shown that co-culturing human pancreatic cell lines with neutrophils can increase the release of NETs by neutrophils, and can promote the occurrence of thrombus formation. This finding further supports the important role of NETs released outside neutrophils in promoting the process of thrombus formation (82). Another study observed that neutrophils isolated from patients with gastric cancer exhibited enhanced NETs formation ability compared to healthy controls (80). This suggests that gastric cancer could promote the process of neutrophils releasing NETs through certain mechanisms, potentially increasing the risk of thrombosis. NETs are also present in the blood clots of cancer patients. NETs released from neutrophils have been found in different types of cancer, and these NET components can play a role in both arterial and venous thrombosis. Increased formation of NETs may contribute to an increased risk of thrombosis (83). Additionally, studies have shown a positive correlation between circulating H3Cit (the ionized form of histone H3) and coagulation activation markers such as thrombin-antithrombin complexes. This positive correlation suggests that NETs released by neutrophils can promote activation of the coagulation system, thereby increasing the risk of thrombosis (83). The MPO-DNA complex is made up of DNA, the enzyme granule protein myeloperoxidase (MPO), and NETs, which are released by neutrophils. Studies have shown that patients with myeloproliferative neoplasms with thrombosis have higher levels of MPO-DNA complexes, which may reflect an increased formation of NETs in these patients (84). Research has shown that levels of circulating NETs (including components such as H3Cit) are increased in cancer patients, which may promote activation of the coagulation system, making these patients more prone to venous thromboembolism (85). Therefore, elevated plasma levels of H3Cit may serve as a potential biomarker for assessing the risk of venous thromboembolism in cancer patients. Denisa D Wagner and colleagues' research showed that in tumor-bearing mice, neutrophils enhanced the formation of NETs *in vitro* compared to control mice similar to cancer patients. This finding suggests that tumors may promote the generation of NETs by affecting the function of neutrophils, potentially increasing the risk of thrombosis. The researchers further observed elevated levels of H3Cit and extracellular DNA in the plasma in mouse models carrying tumors, leading to the occurrence of DNA-rich pulmonary thrombi *in vivo* (86). Another study showed that levels of NETs biomarkers in the plasma and levels of NETs in arterial and venous thrombi were increased in mice carrying 4T1 tumors. Comparing to control mice, mice with tumors formed larger thrombi. Interestingly, DNase I was effective in reducing arterial thrombus formation in both tumor-bearing and control mice, but only had a reducing effect on venous thrombus formation in tumor-bearing mice. This suggests that DNase I may have different regulatory effects on different types of thrombus formation (81). In a mouse model study of pancreatic cancer, it was found that IL-17 can promote the formation of NETs released by neutrophils (87). This suggests that IL-17 may promote the occurrence of this inflammatory reaction in pancreatic cancer patients, which may in turn affect the occurrence of thrombosis. Another study observed increased levels of circulating neutrophils and plasma NET biomarkers in mice carrying human pancreatic tumors. At the same time, increased level of H3Cit was found in venous thrombi (88). These results suggest that pancreatic cancer could increase the risk of thrombosis by promoting the formation of NETs released by neutrophils. Jak2V617F mice are a commonly used model for myeloproliferative neoplasms, which simulate patients with the Jak2V617F mutation. Studies have shown that compared to control mice, these Jak2V617F mice exhibit increased levels of NETs formation. This indicates that these mice have more NETs released into the surrounding environment. Furthermore, it

has been observed that these Jak2V617F mice display spontaneous pulmonary thrombosis, which may be attributed to the increased risk of thrombosis due to elevated NETs. In this model, treatment strategies targeting NETs have shown potential efficacy. Specifically, treatment with DNase I, deficiency of PAD4, and the JAK inhibitor Ruxolitinib have been found to reduce the occurrence of venous thrombosis in these Jak2V617F mice. DNase I degrades the DNA portion of NETs, PAD4 deficiency affects the formation of NETs, and the JAK inhibitor Ruxolitinib can decrease the risk of thrombosis by inhibiting the JAK-STAT signaling pathway to reduce inflammation in the body (89). Therefore, treatment strategies targeting NETs, such as the use of DNase I, PAD4 deficiency, or JAK inhibitors, may have potential benefits in reducing thrombosis in myeloproliferative neoplasm models, providing new insights for future clinical therapy.

3.3 NETs promote tumor growth by reawakening latent tumor cells

The majority of cancer patients ultimately die from the metastasis of distant tissues, rather than the primary tumor. The spread of cancer cells is a key process in cancer progression. Dormant cancer cells can remain in a quiescent state for extended periods of time after disseminating to other tissues, sometimes even for decades. These dormant cancer cells are sometimes described as 'seeds' asleep, and they are clinically undetectable (90, 91). When these dormant cancer cells are reactivated and awaken, they can lead to the recurrence of metastatic cancer. This phenomenon has been observed in many solid tumors, such as breast cancer, lung cancer, and so on (92–94). Although the understanding of the mechanisms and factors influencing cancer cell dormancy is limited, researchers are actively working to unravel this mystery in order to develop more effective cancer treatment strategies. Dormant cancer cells refer to a state in which cancer cells stop proliferating and spreading under certain conditions, entering a dormant state (90). These dormant cancer cells can survive in the body for a long time, waiting to be reawakened and start growing and spreading again at the 'right moment', leading to cancer recurrence and metastasis. In the early stages of cancer, slow-cycling cancer cells may spread early in the body and form tiny seeds in secondary organs, where they stay in specific 'niches' or 'seeding sites' awaiting the proper stimuli to reactivate them (90–93). The presence and reawakening of these dormant cancer cells are important factors leading to cancer recurrence and metastasis (93, 95, 96). The exact mechanisms underlying the awakening, re-initiation of proliferation, and metastasis of slow-cycling cells that have been overlooked by the immune system are still largely unknown.

Neutrophils play a crucial role in tumor development, especially in the relationship between inflammation and tumor progression (97). Neutrophils are part of the immune system and are extensively involved in regulating inflammation and infection (53). Their activities in the tumor microenvironment can impact tumor growth, spread, and metastasis (98). Neutrophils can influence tumor immune editing by forming NETs, affecting the interaction between the immune system and cancer cells, thus promoting the growth, invasion, and metastasis of cancer cells, leading to tumor deterioration and recurrence (4). Studies have found that NETs can awaken dormant cancer cells, causing these cancer cells to resume growth and spread, and thus may be one of the reasons for tumor recurrence and metastasis (4, 53, 99, 100). Inflammation plays an important role in the development of cancer, especially in the transition between cancer cell dormancy and metastasis (101). Studies have shown that in breast cancer survivors, elevated C-reactive protein levels are associated with a lower disease-free survival rate (102), indicating the impact of inflammation on cancer recurrence and metastasis. The long-term recurrence of breast cancer post-surgery highlights the presence of dormant cells in the body that can re-emerge under conducive conditions to form tumors. Studies have shown that during inflammation, NETs can trigger dormant breast cancer cells to exit their quiescent state and start proliferating (103). Additionally, research conducted by Arelaki et al. has demonstrated that NETs can promote the growth of colorectal cancer cells (104).

Albrengues and others proposed the seed and soil hypothesis in their research, referring to the tendency of tumor cells (seed) to migrate to specific organs in the local microenvironment that are favorable (soil). Among the many components in the tumor microenvironment, neutrophils and their products play a crucial role in tumor cell development, immune evasion, and metastasis. This suggests that neutrophils play a key role in regulating inflammatory responses in the tumor microenvironment and promoting the progression and metastasis of tumors. This research found that during the process of lung inflammation induced by lipopolysaccharide (LPS) or tobacco smoke, NETs are necessary to awaken dormant cancer cells and cause mouse metastasis. These NETs can concentrate neutrophil elastase (NE) and MMP 9 on their substrate-bound adhesive proteins, allowing for continuous cleavage to generate sites that trigger cancer cell awakening. These findings suggest that NETs may play an important regulatory role between lung inflammation and cancer metastasis. In the *in vitro* experiments of this study, researchers found that specific types of basement membrane proteins such as laminins-111, -211, -411, and -511 are crucial extracellular matrix (ECM) proteins that lead to the induction of cancer cell awakening by NETs. These proteins may play a significant role in promoting cancer cell awakening and metastasis. This research finding provides important references and insights for further exploration of the mechanisms of cancer cell dormancy and awakening as well as the development of related therapeutic strategies. The study further confirmed the role of NET-remodeled laminins in the process of lung inflammation, activating integrin $\alpha 3\beta 1$ activation sites, initiating the FAK/ERK/MLCK/YAP signaling pathway inside cancer cells, leading dormant cancer cells to reawaken. Based on this discovery, researchers developed inhibitory antibodies targeting NET-remodeled laminins, which could prevent the awakening of dormant cancer cells under conditions of lung inflammation. The findings of this study provide important references for the future development of therapeutic strategies targeting cancer cell dormancy and awakening mechanisms, and may offer new treatment options for preventing cancer recurrence (105).

3.4 NETs stimulate angiogenesis

Pathological angiogenesis is a common phenomenon in the process of inflammation and tumor growth. Both inflammation and tumors can trigger inflammatory reactions, leading to local tissue hypoxia and nutrient deficiency (106). The growth and metabolism of normal cells and tissues require an adequate supply of oxygen and nutrients, and tumor cells are no exception. Hypoxia, cellular metabolic demand, and nutrient deficiency stimulate angiogenesis. The blood supply needed to sustain tumor growth is achieved through a process called angiogenesis. Tumor cells produce signaling molecules, such as vascular endothelial growth factor (VEGF), to promote the growth and twisting of surrounding blood vessels, increasing their density and allowing them to obtain more oxygen and nutrients. This process is known as angiogenesis, and tumors ensure they have sufficient blood supply by promoting angiogenesis, allowing them to continue growing and spreading. Angiogenesis plays a crucial role in tumor growth and metastasis (12, 107, 108).

Angiogenesis is a complex and precise process that requires the coordinated interaction of multiple cells and molecules. Endothelial cells (BECs) play a crucial role in this process. This process is activated by angiogenic factors such as vascular endothelial growth factor (VEGF), angiopoietins (ANGPT), platelet-derived growth factor (PDGF), basic fibroblast growth factor (bFGF), epidermal growth factor (EGF), and CXCL 8/IL-8. Additionally, there are also anti-angiogenic factors that can inhibit angiogenesis. These pro-angiogenic factors and anti-angiogenic factors interact and regulate each other to maintain a dynamic balance in the process of angiogenesis. In pathological conditions such as tumors and inflammation, this balance may be disrupted, leading to abnormal angiogenesis (109–111). Under normal circumstances, the expression level of ANGPT 2 is low, but in areas of inflammation and in tumors, its expression increases significantly. Studies have shown that in cancer, the levels of ANGPT 2 rise, which may promote angiogenesis, tumor growth, and metastasis. Weak ANGPT 1-TIE 2 signaling could lead to inactivation of the AKT signaling pathway, thereby activating Foxo 1 and promoting the expression of ANGPT 2. In this scenario, ANGPT 2 promotes phosphorylation of TIE 2 to compensate for inadequate activation of TIE 2 induced by ANGPT 1, leading to increased endothelial cell chemotaxis and tube formation (112, 113). This suggests that in pathological conditions, ANGPT 2 may play a crucial role in regulating angiogenesis and endothelial cell function.

In addition to its traditional functions of killing bacteria and regulating inflammation, neutrophils also have the ability to produce and release a wide range of pro-angiogenic factors. Among them, VEGF-A is considered one of the most effective pro-angiogenic molecules, and it is present in human neutrophils and plays an important role. VEGF-A can be released through various signaling pathways, including in response to stimuli such as N-formyl-methionyl-leucyl-phenylalanine (fMLF), tumor necrosis factor-alpha (TNF-α), lipopolysaccharide (LPS), granulocyte colony-stimulating factor (G-CSF), and phorbol myristate acetate (PMA). These stimuli can activate human neutrophils, leading to the release of VEGF-A, and subsequently participate in the processes of inflammation and tumor angiogenesis (114–116). Aldabbous et al.'s research demonstrated the direct role of NETs in promoting angiogenesis both *in vitro* and *in vivo*. Their findings suggest that NETs could enhance endothelial cell proliferation and tube formation, thereby facilitating the process of blood vessel formation. Researchers applied multiple immunohistochemical staining to analyze the spatial distribution of NETs and microvessels in patient tissue samples. They established a subcutaneous tumor model in mice to observe the impact of NETs on tumor growth and used immunohistochemical staining to observe changes in tumor microvessel density (76). In the study, multiple immunohistochemical staining techniques were used to analyze the spatial distribution of NETs and microvessels in patient tissue samples. Establishing a subcutaneous tumor model in mice can be used to observe the effect of NETs on tumor growth, and immunohistochemical staining can be used to observe and analyze changes in microvessel density in tumor tissue. By labeling proteins related to blood vessels or labeling vascular endothelial cells to quantify the number and density of vessels, researchers found that NETs promote neovascularization and play a role in the progression of gastric cancer. Furthermore, the study found that blocking NETs associated with reduced microvessel density significantly inhibited tumor growth in a subcutaneous tumor model in mice. Tumor volume and mass in the inhibited group decreased by 61.3% and 77.9%, respectively, compared to the control group. Inhibiting the activity of NETs can effectively suppress tumor growth and development. These results suggest a potential role for NETs in tumor growth and angiogenesis, providing new insights for the development of novel cancer therapeutic strategies targeting NETs (117). Matrix metalloproteinases (MMPs) play an important role in the process of angiogenesis. MMP-9 has a promoting effect in angiogenesis. MMP-9 can promote the release of vascular endothelial growth factor (VEGF) from the extracellular matrix, increasing its activity and accelerating the process of angiogenesis. It also regulates the interaction between VEGF and its receptors, further regulating the progression of angiogenesis. The addition of neutrophils to pancreatic cancer cells can increase the budding rate by more than 2.5 times, indicating that MMP-9 may promote endothelial cell migration. After treatment with bevacizumab (a VEGF inhibitor) and doxycycline (a drug that can effectively inhibit angiogenesis like an MMP-9 inhibitor) for 14 days, the tumor volume in pancreatic cancer mice significantly decreased. Additionally, the average blood vessel density in pancreatic cancer mice also significantly decreased (118). Therefore, targeting the tumor vasculature system may be an effective strategy for treating tumors, especially those that are insensitive to traditional chemotherapy and radiation therapy. Studying the characteristics and mechanisms of the tumor vasculature system can provide crucial insights for developing more effective drugs targeting the tumor vasculature system. Further research on the role of MMPs, NETs, and their interactions in modulating angiogenesis and tumor growth could provide valuable insights for developing novel therapeutic strategies targeting the tumor vasculature system.

NETs release is mediated through the activation of Tie 2. Angiopoietin Tie2 receptor is an important member of the

angiopoietin family and plays a crucial role in regulating angiogenesis and inflammatory responses. The Angiopoietin Tie2 receptor is a dual-function receptor that can bind to two different ligands: angiopoietin-1 (Ang1) and angiopoietin-2 (Ang2). By binding primarily to its ligand Ang1, the Angiopoietin Tie2 receptor promotes vascular stability and repair, suppresses endothelial cell inflammatory responses, and regulates vascular wall permeability to maintain normal vascular function. In contrast, angiopoietin-2 regulates angiogenesis and inflammatory responses by interfering with Ang1/Tie2 signaling. The role of Ang2 is complex, as it may promote angiogenesis and inflammatory responses in some situations while inhibiting angiogenesis and increasing vascular permeability in others. The expression of Tie2 receptor in neutrophils may also influence the function and activity of neutrophils. Induction of angiopoietin Ang1 and Ang2 may trigger neutrophil production and release of platelet activating factor, regulate the expression of the β2 integrin complex (CD11/CD18), increase neutrophil chemotaxis, and other inflammatory activities. These actions may contribute to the regulation and response of neutrophils in inflammatory and immune reactions (119).

The irregular structure and leaky nature of tumor vasculature make it a vulnerable target for treatment. It is particularly suitable for destruction mediated by NETs. Studies have shown that NETs may promote tumor invasion and metastasis by affecting endothelial cell-cell contacts and increasing permeability. NETs can affect endothelial cell-cell contacts and increase permeability, including in the context of metastasis. Researchers found complement system activation in melanoma patients and mouse melanoma samples, highlighting tumor endothelium as the starting point of complement cascade. Complement-derived C5a promotes neutrophil recruitment, leading to neutrophil activation and release of NETs. Positioned near the vessel wall, NETs open the endothelial barrier, enhancing vascular leakage, promoting melanoma cell invasion and systemic dissemination. Further investigation revealed that neutrophil depletion in animals lacking C6 or deficiency in membrane attack complexes (MAC) formation can protect the vascular endothelium, preventing melanoma cell intravasation. Thus, inhibiting MAC-mediated neutrophil activation may be an effective strategy to eliminate melanoma hematogenous dissemination (120). Therefore, targeting tumor vascular disruption mediated by NETs may be an effective therapeutic strategy. By blocking the formation or activity of NETs, their impact on the tumor vascular system can be reduced, thereby decreasing the growth and spread of tumors. This therapeutic approach may provide a new avenue for treatment, especially for tumor types that are resistant to conventional treatment methods. Thus, research and development of therapies targeting NETs-mediated tumor vascular disruption may have significant clinical significance in the field of cancer treatment.

3.5 NETs in Regulating T cells in tumor microenvironment

Neutrophils, essential to the immune system, act as the initial responders to infections, especially bacterial ones (24, 25, 28). Researchers have demonstrated various interactions between neutrophils and T lymphocytes, along with their associated products. Notably, NETs can impact T lymphocytes through mechanisms such as direct contact, cytokine release, and modifications to the local microenvironment. Within the tumor microenvironment, these interactions can profoundly influence tumor progression and the effectiveness of immunotherapy. Consequently, it is essential to gain a comprehensive understanding of how NETs regulate T cells and how T cells influence NET formation to develop more effective immunotherapeutic approaches (14, 25, 48).

The findings from the study by Kati Tillack et al. have provided a compelling insight into the complex roles that neutrophils play beyond their traditional functions in pathogen elimination. By identifying the interaction between NETs and T cells, the research underscored a novel mechanism through which the innate and adaptive arms of the immune system communicate and cooperate. One key finding from the study was that NETs can reduce the activation threshold for T cells, allowing these adaptive immune cells to respond more effectively even in the presence of suboptimal stimuli. This suggested that NETs can enhance immune protection during infections by making T cells more responsive. The research emphasized the importance of the physical interaction between NETs and T cells for this priming effect, with signaling through the T cell receptor (TCR) being crucial for a robust immune response. Interestingly, the study also discovered that Toll-like receptor 9 (TLR9) does not contribute to this priming process, indicating that NETs influence T cell activation through other, as yet unidentified, signaling pathways. This opens new research avenues to explore alternative mechanisms by which NETs affect T cell function. The study enriches our understanding of the interplay between innate and adaptive immunity, highlighting the multifaceted role of neutrophils. It underscores the need for further investigation into the mechanisms through which NETs affect immune responses and opens up promising avenues for developing novel therapeutic interventions (121).

In a recent study, researchers proposed that NETs interact directly with infiltrating T cells, contributing to the establishment of an immunosuppressive tumor microenvironment. To facilitate the development of a NET-rich tumor microenvironment, researchers conducted liver ischemia/reperfusion (I/R) experiments in a cancer metastasis model and also injected NETs directly into subcutaneous tumors. The findings indicated that within this NET-rich tumor microenvironment, the majority of $CD4^+$ and $CD8^+$ tumor-infiltrating lymphocytes expressed a variety of inhibitory receptors and exhibited signs of functional and metabolic exhaustion. The application of DNase to target NETs in mice resulted in decreased tumor growth, reduced NET formation, and increased levels of functional T cells. *In vitro* experiments demonstrated that NETs contain the immunosuppressive ligand PD-L1, which contributes to T cell exhaustion and functional impairment. The use of PD-L1 knockout NETs or co-culturing NETs with PD-1 knockout T cells effectively negated this effect. Furthermore, elevated levels of soluble PD-L1 and myeloperoxidase-DNA (NETs markers) were observed in the serum of patients who had undergone resection surgery for colorectal liver metastasis. Neutrophils isolated from these patients' post-surgery were found to form NETs, leading to exhaustion and

functional impairment of $CD4^+$ and $CD8^+$ T cells. Following this, the researchers administered a PD-L1-blocking antibody during liver I/R. After a single dose of anti-PD-L1 was given during surgery, tumor shrinkage was noted three weeks later, accompanied by an increase in functional T cells within the tumor microenvironment. These results indicated that NETs can suppress T cell responses through metabolic and functional exhaustion, thereby facilitating tumor growth. Additionally, the deployment of DNase or targeting PD-L1 in NETs during surgery both contributed to reduced tumor growth, presenting a promising approach for enhancing immune competence in the tumor microenvironment (122).

4 NETs in gynecological cancers

4.1 NETs in cervical cancer

With almost 270,000 fatalities annually, cervical cancer ranks as the third most frequent malignancy worldwide. Even with the great improvements in surgery, chemoradiotherapy, and immunotherapy, approximately 40% of patients will eventually die from a recurrence following treatment with the intention of curing their illness (123, 124). Based on the anatomical extent of the tumor, the Union for International Cancer Control (UICC), tumor, nodes, metastasis (TNM) and the Federation International of Gynecology and Obstetrics (FIGO) classification system are used for prognostication and treatment recommendations in cervical cancer (125, 126). However, the clinical outcome varies significantly among patients with the same tumor stage (123, 126). In recent years, there has been a shift in the understanding of cancer development and progression from a focus solely on the tumor itself to a more holistic view that includes the entire microenvironment in which the tumor exists. The tumor microenvironment encompasses various factors such as immune cells, blood vessels, and extracellular matrix components, all of which play critical roles in supporting tumor growth and invasion (98, 127). It is now known that the biological behavior of invasion, metastasis, and tumorigenesis is caused by a disturbance of the dynamic equilibrium between the immunological components of the host and the tumor cells. Unbalance and ongoing interplay between malignant tumor cells and distinct stroma and immune cell subsets of the surrounding immunological microenvironment result in tumor progression (128). In contrast to the current histopathological techniques used for staging different cancers, such as lung cancer (129) and breast cancer (130), plenty immune-mediated statistics studies have found that the type, density, and location of immune cells in the tumor microenvironment serve as superior predictors of patient survival (98, 131, 132).

Researchers detected the formation of NETs in the tumor nests and stroma of 126 patients with cervical cancer using multiplex quantitative immunofluorescence technology, and discovered that: higher densities of stromal PMNs and NETs were correlated with poor outcome in a retrospective cohort of patients with cervical cancer, and high stromal NETs density was an independent prognostic factor for RFS. A closer examination of its relationship to the clinicopathological features of individuals with cervical cancer revealed that: the elevated density of stromal $CD66b^+$ cells was substantially correlated with the clinical stage (p=0.006). No correlation was observed between lymph node involvement, histology, or pathologic grade and stromal $CD66b^+$ cells. The results of the study indicate a positive correlation between the advancement of cervical cancer and a high density of neutrophils in the stromal tumor tissue. No statistically significant distinction was found between patients at different stages who had high or low concentrations of stromal $CD66b^+$ cells. The following conclusions were drawn from the investigation of the relationship between patient survival without recurrence and the concentration of NETs in tumor communities and tumor stroma. To further elucidate the effect of neutrophils affecting patient outcome, the researchers expanded this observation to include cervical cancer in Staging I–IV. The researchers found that the stromal tissue contained the majority of neutrophils. It was noted that neutrophils were primarily found in the stromal tissue. The association between short RFS and an elevated stromal $CD66b^+$ neutrophil density was shown to be significant, as opposed to a high tumor nest $CD66b^+$ neutrophil density. A favorable RFS was connected with low stromal $CD66b^+$ neutrophil density in univariate analysis, but in multivariate analysis, this component did not function as an independent predictor (133). Further researches are necessary to determine the significance of NETs activity associated with tumor boundary movement, as suggested by this finding, as demonstrated in Table 1. Using TNM staging rather of FIGO staging resulted in a deficiency of certain clinical prognostic variables. A further limitation of the study was the small number of participants, which could account for the lack of statistically significant differences in the clinical phase of subset evaluation of high- and low-density NETs. Further researches are necessary to elucidate the underlying process.

4.2 NETs in endometrial cancer

As for endometrial cancer, as demonstrated in Table 1, fluorescence microscopy was used by TV Abakumova et al. to investigate neutrophils and their capacity to create extracellular traps in the peripheral blood of 123 patients diagnosed with stage IA primary endometrial cancer. Enhancement of both aerobic and anaerobic cytotoxicity and phagocytosis, as well as a decrease in net activity, have all been demonstrated to occur as the relative quantity of neutrophils increases. Observations of altered neutrophil secretory activity included elevated MMP-1 levels, most likely from higher reactive oxygen species generation, lowered IL-2 levels, which is an inducer of cytotoxic action, and a marked rise in G-CSF levels. Patients with stage IA carcinoma of the endometrium have neutrophils that have changed in morphology and lost granules. They detected alterations in neutrophil secretory activity, marked by elevated MMP-1 levels—likely resulting from heightened the amount of reactive oxygen species production—decreased IL-2 levels, a trigger of cytotoxic activity, and a marked rise in G-CSF levels. Granule loss and a change in form are features of the neutrophil structure among individuals with stage IA endometrial cancer (134).

Using antibodies targeted against citH3, the team of investigators carried out IHC and IF analyses of EC tissue and

endometrial tissue from healthy individuals as controls for the purpose of assessing the presence of NETosis signatures in EC. They identified that leukocytes infiltrates that were positive for the NETosis biomarker identification citH3 were present in most tumor tissue samples. The general trend from grade G1 to grade G3 was upward. Nevertheless, there was no correlation found between the serum levels of citH3 and its tissue staining. This might be because the processes by which cfDNA is cleared in tissue differ from those in serum, or it could be because blood and tissue samples are collected at separate times. It has been discovered that endometrial cancer patients can be distinguished from individuals who are healthy using serum levels of cfDNA and cfmtDNA. In endometrial cancer samples, serum citH3 levels were significantly higher. In EC serum, there was a positive link between citH3 and cfDNA concentration, while in healthy individuals' serum, there was a negative correlation between cfmtDNA and citH3. Through the use of serum cfDNA and cfmtDNA in combination with serum citH3 concentration, these novel non-invasive indicators of NETosis in endometrial cancer can now be used for prognostic and diagnostic purposes as well. Further research is required to determine the prognostic significance of the combined indicators in different tumor types (135).

4.3 NETs in ovarian cancer

Of all the gynecological malignant tumors, epithelial ovarian cancer, especially HGSOC, has the greatest fatality rate (136, 137). While platinum-based the first-line chemotherapy medicines have demonstrated notable anti-tumor efficacy in numerous solid tumors, the absence of precise and accurate diagnostic tools and recurrence biomarkers has hindered the improvement of the five-year survival rate for ovarian cancer over the last ten years (138). Due to the fact that most patients get diagnosed at advanced stages, when they have widespread metastases throughout the peritoneal cavity, this is one of its most significant disadvantages (136–138). Accordingly, most OC tumor spread follows peritoneal fluid dynamics, while it can also propagate via systemic or lymphatic channels. The significance of deepening our comprehension of the constituent parts and mechanisms of peritoneal fluid is underscored by its distinctive manner of metastasis, which renders it the most representative biological fluid in the OC tumor milieu (139, 140). Furthermore, recurrence and resistance to chemotherapy are the primary reasons of death from ovarian cancer (141). A pressing issue that needs to be resolved is the research on the molecular mechanisms of metastasis, recurrence, and treatment resistance. In cancer research, NETs have garnered a lot of interest, while OC research is still in its early stages. Current research on the connection between NETs and OC was included in this review (Figure 3).

In an attempt to gain further insight into the possible significance of NETs markers in OC diagnosis and/or prognosis, recently published studies has inquired into whether they can serve as prognostic markers. The study by Singel et al. examined the levels of NE (markers of released neutrophil granule products occurring in NETosis, degranulation, and neutrophil death) and mtDNA (molecular patterns associated with mitochondrial damage released by tumor cells during necrosis) in ovarian cancer ascites. There was a non-linear association between ascites NE levels and PFS, with patients with the highest levels having a significantly shorter median PFS and a greater risk of disease progression within 12 months following initial surgery. It's noteworthy to note that mtDNA has been identified as a NETosis activation stimulant. When the trial was limited to a period of 12 months following surgery, which is clinically characterized as chemotherapy-refractory illness, survival analysis showed a significant positive relationship between mtDNA and NE levels and shorter progression-free survival. Moreover, it was shown *in vitro* that ascites can draw neutrophils and cause NETosis, indicating that mtDNA and other elements found in this bodily fluid may stimulate neutrophil reactions and encourage metastases (142). Thus, as demonstrated in Table 1, it was suggested that these pathways could function as possible indicators of prognosis and/or targets for treatment. Subsequent investigation revealed that a poor prognosis was linked to the ascites mtDNA level (but not the gDNA level because platelets are enucleated). This indicates that the mechanism of neutrophil cross-activation and NETosis in EOC ascites may be related to platelet-derived mtDNA and microparticles. Furthermore, the investigators noticed significant alterations in the capacity of enzyme inhibitors such as protease and DNAse I to stop platelet activation in ascites supernatants, indicating the presence of several mechanisms for platelet activation in the tumor microenvironment. As a result, they postulated that these pathways could serve as prognostic markers and/or therapeutic target.

In contrast to the earlier research conducted by Muqaku et al. (143) employed a comparable methodology to investigate the function of neutrophils in either stimulating or impeding tumor cell activity inside the tumor-associated microenvironment of high-grade serous ovarian cancer. NET-related molecules (NE and MPO) and local inflammatory markers (calprotectin heterodimers, including S100A8 and S100A9, which are also thought to be cytoplasmic markers of NETs) were found to be more abundant in ascites samples from patients with non-miliary metastases than in ascites samples from patients with miliary metastases. Several apparent disparities can be explained by the models suggested by the current data. Three layers make up the model: (1) hypoxic cellular stress initiates the development of NETs; (2) NETosis establishes distinctive microscopic features linked to certain biomarker profiles; and (3) NETosis modulates the adaptive immune system to enhance overall survival (142, 143).

Extraperitoneal spreading of ovarian cancer is characterized by a significant affinity for the omentum and has frequently been explained as a passive process that is driven by the mechanical movement of peritoneal fluid. Nevertheless, recent research on different kinds of tumors has demonstrated that metastatic tropism is a deliberate process whereby the tumor collaborates with different host cells to create favorable conditions at the intended site before colonization. Researchers have found that NETs indicators are present in the omentum of mice with ovarian tumors and people with early-stage ovarian cancer, and that these markers appear to be colonized at the site. However, in

TABLE 1 Neutrophil extracellular traps (NETs) in gynecological cancer and areas for further research.

Cancer Type	Technology Used	Clinicopathological Features	Key Findings	Further Research Needs	References
Cervical Cancer	Multiplex quantitative immunofluorescence	Elevated density of stromal $CD66b^+$ cells correlated with clinical stage;	Higher densities of stromal PMNs and NETs correlated with poor outcomes;	Investigate the activity of NETs in relation to the movement of tumor boundaries;	(133)
		No correlation found between stromal $CD66b^+$ cells and lymph node involvement, histology, or pathologic grade.	High stromal NETs density was an independent prognostic factor for recurrence-free survival.	Address limitations of TNM staging vs. FIGO staging;	
				Larger sample sizes needed to discern clinical prognostic differences;	
				Further research required to elucidate underlying processes.	
Endometrial Cancer	Fluorescence microscopy	Stage IA primary endometrial cancer; Peripheral blood analysis of 123 patients.	Neutrophils in patients showed altered morphology and loss of granules; Increased MMP-1 levels due to heightened reactive oxygen species production; Decreased IL-2 levels, reducing cytotoxic activity induction; Marked rise in G-CSF levels, affecting neutrophil function.	Clarification of how altered neutrophil function impacts disease progression; Longitudinal studies to assess changes in neutrophil behavior over time; Exploration of therapeutic implications based on neutrophil alterations.	(134)
	Antibodies targeted against citH3; IHC (Immunohistochemistry); IF (Immunofluorescence) analyses; Serum cfDNA and cfmtDNA analysis.	Endometrial cancer tissue samples; Healthy endometrial tissue as controls.	Presence of NETosis biomarker citH3 in most tumor tissue samples; Positive link between citH3 and cfDNA concentration in EC serum; Negative correlation between cfmtDNA and citH3 in healthy serum.	Determine prognostic significance in different tumor types; Mechanisms of NETosis in EC; Clinical Utility of NETosis Biomarkers; Impact of Treatment on NETosis.	(135)
Ovarian Cancer	Examination of ascites for NE levels, mitochondrial DNA, and their impact on NETosis.	Study focused on ovarian cancer ascites, investigating associations with progression-free survival and chemotherapy-refractory illness.	High NE levels correlated with shorter median PFS and higher risk of progression;	Explore mechanisms of neutrophil cross-activation in ovarian cancer ascites;	(142)
			mtDNA in ascites linked to poor prognosis and chemotherapy resistance;	Investigate therapeutic strategies targeting NETosis pathways;	
			In vitro evidence of ascites stimulating NETosis and neutrophil activation.	Validate mtDNA and NE levels as prognostic markers in larger cohorts;	
				Understand impact of ascites on tumor microenvironment and metastasis.	
	Examination of NETs in omental tissue;	Early-stage ovarian cancer patients; nonmetastatic ovarian cancer;	NETs within the omentum as a response to early-stage ovarian tumors;	Mechanisms of neutrophil recruitment; Long-term effects of NETs inhibition;	(67)
	Animal models (mice) and human patient samples used for research.	Neutrophil recruitment to omentum; omental metastases.	NETs presence in omental tissue of both humans and animal models;	Clinical trials to validate NETs inhibitors' efficacy in humans.	
			NETs inhibitors reduce omental metastases in animal models.		

(Continued)

TABLE 1 Continued

Cancer Type	Technology Used	Clinicopathological Features	Key Findings	Further Research Needs	References
	Quantification of H3Cit-DNA, dsDNA, and CA 125 plasma levels in peripheral blood samples	199 patients undergoing primary surgery for adnexal tumors	Patients with borderline or malignant ovarian tumors did not show higher levels of H3Cit-DNA or dsDNA plasma compared to benign tumor patients.	Investigate other potential biomarkers or combinations of biomarkers for more accurate diagnosis and prognosis of ovarian tumors.	(144)
			Borderline and ovarian cancer groups exhibited higher CA-125 levels.	Explore the mechanisms behind CA-125 elevation in different stages and subtypes of ovarian tumors.	
			CA-125 levels did not significantly affect the survival analysis of malignant ovarian tumors.	Conduct longitudinal studies to validate these findings and assess their clinical utility in guiding treatment decisions and monitoring disease progression.	

healthy women and animals without tumors, these markers are generally absent.

It is shown that the creation of NETs in the omentum prior to metastasis is an early response of neutrophils to ovarian tumors in the abdominal cavity by Lee, W., et al's findings, which imply that early ovarian tumors could release inflammatory signals to recruit neutrophils to the omentum and trigger NETs secretion (67). Interestingly, they found NETs in the omentum of patients with

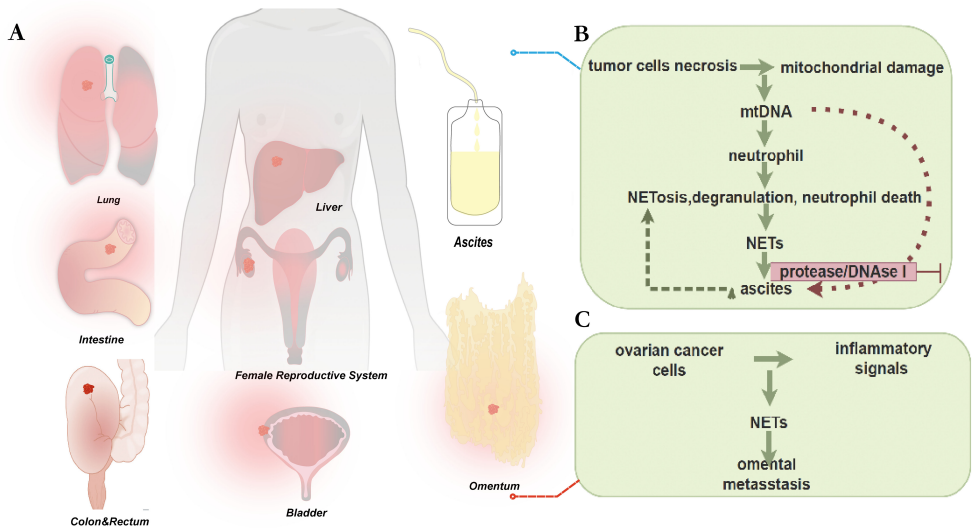

FIGURE 3
(A) Common routes of metastasis in epithelial ovarian cancer include several ways described as follows. Direct invasion of adjacent organs is a common route, especially in advanced stages of the disease. In addition, implantation metastasis in the peritoneum and within the abdominal cavity is also a common route, which can lead to the formation of peritoneal carcinomatosis. Lymphatic spread often occurs in the pelvic and abdominal lymph node regions. Hematogenous spread may also carry cancer cells to other organs, such as the liver, lungs, or other organs. Understanding these routes of metastasis is important for the diagnosis and treatment of epithelial ovarian cancer. In cancer research, NETs have attracted a lot of attention, while research on ovarian cancer is still in its early stages. **(B)** Detect levels of NE (a marker of neutrophil extracellular trap (NET) release, occurring in processes such as NETosis, degranulation, and neutrophil death) and mtDNA (a molecular pattern associated with mitochondrial damage, released by tumor cells during necrosis) in ascites from ovarian cancer patients. Levels of mtDNA and NE are significantly positively correlated with shorter PFS. Ascites can attract neutrophils and induce NETosis, potentially stimulating neutrophil responses and promoting metastasis. Ascitic mtDNA levels are associated with poor prognosis, potentially related to platelet-derived mtDNA and microparticles. There are multiple platelet activation pathways in the tumor microenvironment, which may serve as prognostic markers and/or therapeutic targets. **(C)** The peritoneal spread of ovarian cancer is found to be a deliberate process, in which cancer cells cooperate with host cells to create favorable conditions in predetermined locations. Research has shown that neutrophils respond to early ovarian tumors by producing NETs in the omentum, potentially manufacturing NETs in the omentum before metastasis. Ovarian cancer cells adhere to NET-positive neutrophils and omental metastasis, but tumor growth is significantly reduced in mice lacking NET formation. These findings suggest that intervening in NET formation may prevent omental metastasis and could help in understanding ovarian cancer treatment and related complications.

nonmetastatic early-stage ovarian cancer and in animals with ovarian cancers. Notably, NETs were found in the omentum of both patients with nonmetastatic early OC and mice with ovarian malignancies. Based on these data, it is possible that neutrophils responding early to intra-abdominal ovarian malignancies create NETs in the omental niche prior to metastases. Notably, ovarian cancer cells attached to NET-positive neutrophils and omental metastases but not primary tumor growth were markedly reduced in neutrophil-specific Padi $4^{-/-}$ mice defective in NETs formation but with normal white blood cell numbers and neutrophil chemotaxis. Omental metastases were dramatically decreased by NETs inhibitor therapy. When considered collectively, these findings raise the possibility that neutrophil inflow into the omentum may be a need for the development of premetastatic OC foci and imply that omental metastasis is prevented by interfering with NETs formation (67). Insights into ovarian cancer treatment and associated comorbidities may be gained from additional research into this remarkable host defense system.

Neutrophils with a variety of protumorigenic and antineoplastic characteristics are activated by stimuli within the tumor microenvironment. Through the quantification of H3Cit-DNA, dsDNA, and CA 125 plasma levels in peripheral blood samples taken from 199 patients undergoing primary surgery for adnexal tumors, researchers discovered that patients with borderline or malignant ovarian tumors did not have higher levels of H3Cit-DNA or dsDNA plasma than patients with benign tumors. The borderline and ovarian cancer groups had higher CA-125 levels, however the researchers concluded that this did not affect the survival analysis of malignant ovarian tumors (144).

5 Future prospects: the emergence of new technologies and devices in tumor treatment

The preceding discourse highlighted the potential of cancer surgery to inadvertently trigger the release of circulating tumor cells, thereby fostering tumor expansion and metastasis. Postoperative infections, a frequent complication, hold significant implications for oncological prognosis, particularly when infections escalate in severity. Neutrophils have been identified as key facilitators of cancer progression through the elaboration of NETs, which have been shown to interact with circulating tumor cells resulting in enhanced tumor cell adhesion and invasiveness. Consequently, these interactions have been linked to a less favorable prognosis for affected individuals. In light of recent advances in surgical treatment concepts and ongoing technological progress, a multitude of minimally invasive treatment modalities have surfaced. Presently, a host of ailments can be addressed through minimally invasive or non-invasive surgical interventions. These cutting-edge techniques employ thermal or cryogenic energy to induce necrosis within specified tissues, encompassing methodologies like focused ultrasound ablation, microwave ablation, radiofrequency ablation, and cryoablation (145–147).

5.1 High-intensity focused ultrasound

High-Intensity Focused Ultrasound (HIFU) is an advanced non-invasive thermal ablation modality that harnesses the power of high-energy ultrasound waves to precisely alter the architecture of targeted tissues, thereby enabling localized ablation therapy. By delivering focused ultrasound energy externally, HIFU triggers thermal necrosis within the target area. Moreover, when integrated with Magnetic Resonance Imaging (MR-HIFU), this technology allows for precise measurement and monitoring of temperature variations within the target tissue and its adjacent structures. This real-time feedback enables clinicians to make necessary adjustments during the treatment procedure (148, 149). Currently, FDA-approved HIFU applications encompass a wide range of clinical indications, including treatment of uterine fibroids, neurological disorders, as well as tumors affecting the prostate, breast, liver, and pancreas, among other anatomical sites (150). A comprehensive analysis was conducted on 153 cases of residual/recurrent cervical cancer within the previously irradiated region who received HIFU treatment. The investigation unveiled that following HIFU treatment, the objective response rate was 23.5% with a disease control rate of 93.5%. Notably, the median progression-free survival (mPFS) and median overall survival (mOS) were recorded at 17.0 months and 24.5 months, respectively. Moreover, it was observed that patients with lesions ≥1.40 cm prior to HIFU treatment and a post-treatment shrinkage rate ≥30% exhibited improved mPFS and mOS. Similarly, patients with post-treatment lesions ≤1.00 cm displayed increased mPFS, suggesting that HIFU possesses the ability to effectively enhance local control rates and extend patient survival (151). The findings indicate that HIFU treatment could emerge as the favored choice for individuals grappling with residual disease or recurrence post-radiotherapy for cervical cancer. Furthermore, all treatment-related adverse events were confined to mild complications such as skin burns, abdominal pain, and discharge. A recent investigation analyzed the outcomes of eight patients with recurrent ovarian cancer or metastatic pelvic tumors who underwent treatment. The study revealed that the pain relief rate among patients reached 60%, and there was an observable improvement in their short-term quality of life. The primary side effect of HIFU treatment was localized pain, with all patients reporting pain scores below 4 following the procedure. Additionally, all pain subsided within the first day after HIFU treatment. Notably, the study did not identify any instances of severe complications such as skin burns, intestinal perforation, or nerve damage. These findings suggest that HIFU may present a promising therapeutic approach for recurrent ovarian cancer, as well as metastatic pelvic tumors stemming from cervical cancer, endometrial cancer, and rectal cancer (152).

It is imperative to conduct additional research on the impact of high-intensity focused ultrasound on neutrophils and NETs. The necessity for further investigation lies in validating the potential effects of high-intensity focused ultrasound treatment on neutrophil function and the release of NETs, as well as understanding how these impacts may influence tumor growth and patient outcomes.

By delving deeper into the relationship between high-intensity focused ultrasound treatment and the immune response, a comprehensive understanding of the therapy's effects on the tumor microenvironment can be achieved, offering valuable insights to guide clinical practice and refine cancer treatment strategies. Future research endeavors should consider the intricate mechanisms of interaction between high-intensity focused ultrasound treatment and the immune system to effectively address challenges in cancer treatment.

5.2 Histotripsy

In the realm of clinical practice, the conventional method of focused ultrasound ablation primarily entails the induction of coagulative necrosis in tumor cells within the specific target region through thermal mechanisms. Nevertheless, the efficacy of this approach may be impacted by the vascular supply to the tumor, thus increasing the risk of incomplete necrosis. Additionally, prolonged exposure to thermal energy poses a potential threat of harm to surrounding healthy tissues. In order to achieve successful tumor ablation without compromising normal tissue integrity, investigators are delving into the possibilities of leveraging the non-thermal effects of focused ultrasound in disease treatment.

Histotripsy is an innovative non-invasive technology that utilizes focused ultrasound, similar to HIFU, yet with a fundamentally distinct mechanism which primarily involves mechanical effects at the cellular level for tissue destruction. This cutting-edge approach is rooted in the principles of non-thermal injury and non-ionizing radiation, employing a non-invasive, focused ultrasound methodology. By utilizing a low duty cycle (<1%) and short duration (microseconds to milliseconds) pulse ultrasound, this technique minimizes heat deposition while harnessing high peak negative pressure (>10 MPa) to induce cavitation bubbles within the target area, effectively pulverizing tissue into subcellular structures (153, 154). Furthermore, real-time ultrasound imaging serves as a valuable tool for guiding and monitoring the tissue destruction process. Histotripsy, in contrast to various other minimally invasive techniques, offers the distinct advantage of being non-invasive. This advanced technology is distinguished by its ability to precisely target and fragment solid tissues, such as tumors, effectively transforming them into acellular homogenate. The resulting fragments are subsequently absorbed by the body within a relatively short period of 1-2 months, leaving behind minimal scars (153, 155, 156).

To date, Phase III human clinical trials have been carried out to evaluate the safety and viability of Histotripsy in patients. Notably, a trial conducted in Barcelona (NCT03741088) involved the treatment of 11 liver tumor patients using the Vortx Rx device, with no reported adverse events. Following a two-month observation period, the average tumor shrinkage rate was found to be 71.8%, suggesting promising initial safety and effectiveness of the treatment in hepatic histotripsy (157). Since 2021, the United States and Europe have commenced two Phase I clinical trials exploring the efficacy of Histotripsy technology in the ablation treatment of primary and metastatic liver lesions. The outcomes of these trials have shown great promise, capturing the interest of the Food and Drug Administration in the United States. As a result, the FDA has fast-tracked the approval process for this cutting-edge technology. Histotripsy has demonstrated the ability to elicit strong innate and adaptive immune responses in animal models of melanoma and liver cancer. Evidence indicates that histotripsy leads to a significantly higher level of immune cell infiltration compared to radiation therapy or radiofrequency ablation. Additionally, histotripsy has been shown to provoke robust systemic anti-tumor immune responses and abscopal effects. Notably, histological examination of flank tumors has revealed a marked reduction in lung metastases compared to control groups. While the exact mechanisms are not yet fully understood, the overall therapeutic effects of histotripsy extend beyond local tumor ablation and may provide additional benefits while improving clinical outcomes for cancer patients. Further research is necessary to comprehensively elucidate its impact on formation of new tumor nodules. Despite substantial progress in the technical, preclinical, and clinical realms of histotripsy, significant future work remains in technology development, preclinical studies, and human research before histotripsy can become a widely adopted clinical treatment modality (158).

Future studies should place a strong emphasis on examining the effects of HIFU and histotripsy on the immune response in the tumor microenvironment, with a specific focus on neutrophil activity and the release of NETs. A deep understanding of the interactions between these therapies and the immune system is essential for improving treatment approaches, maximizing efficacy, and reducing risks. Progress in this area depends on conducting thorough research to uncover the mechanisms by which these treatments impact the tumor microenvironment and immune responses. This will establish a robust foundation for future developments in personalized medicine and immunotherapy.

5.3 NETs as potential cancer therapy targets

In the realm of cancer therapy, the exploration of NETs as potential targets presents an intriguing avenue for enhancing the immune response against tumors and potentially improving the effectiveness of current treatment modalities. This focus on disrupting NETs formation or function within the context of cancer aims to shift the delicate immune balance towards a more robust anti-cancer response. Researchers are delving into the intricacies of NETs to unlock their therapeutic potential within the cancer treatment. Unfortunately, current clinical trials have not yet defined the optimal treatment strategies for targeting NETs (159). Researchers are investigating the use of drugs to inhibit the formation of NETs. Some of these drugs can reduce NETs generation by targeting key components, such as DNA or specific proteins. Most experiments and clinical studies targeting NETs have focused on diseases other than cancer, such as autoimmune diseases and respiratory conditions or their complications (25). In autoimmune diseases like systemic lupus erythematosus, DNase has been shown to play a crucial role in degrading NETs. This mechanism allows DNase to not only reduce the number of NETs but also improve the clinical

symptoms of these conditions (160). Therefore, considering that DNase can disrupt the structure of NETs, these structures could be an ideal target for DNase therapy, potentially reducing their tumor-promoting effects. In a mouse model of breast cancer, researchers applied DNase treatment. The results indicated that DNase treatment significantly reduced tumor burden, suggesting that it may inhibit tumor growth by decreasing the quantity and function of NETs (161). In a mouse model of metastatic lung cancer, systemic administration of DNase also led to a reduction in experimental metastasis (99). Although DNase performs well in experimental models, its clinical application faces many challenges. Further researches are needed to determine how to combine DNase with existing cancer treatments, such as chemotherapy and immune checkpoint inhibitors, to maximize its therapeutic effects. In another study, researchers investigated the formation of NETs induced by Bacillus Calmette-Guérin (BCG) stimulation. The results showed that BCG-induced NETs possess cytotoxic properties, capable of inducing apoptosis and cell cycle arrest, while inhibiting the migration of bladder tumor cells. Additionally, NETs play a role *in vivo* by facilitating the recruitment of T cells and monocyte-macrophages, as well as causing tissue damage, which helps prevent tumor (162). In summary, NETs can exhibit different effects under various pathological conditions, potentially promoting or inhibiting tumor growth, which complicates targeted therapy. Another important consideration is the study of biomarkers associated with NETs to determine which patients might benefit the most from targeted NETs therapy. Currently known NETs formation biomarkers, such as H3Cit and MPO-DNA, may hold prognostic value for patients with cancer (99, 163). Understanding the mechanisms that regulate neutrophil and NETs behavior in the TME creates opportunities for therapeutic interventions to reshape immune responses against tumors. By targeting pathways and factors related to neutrophil-mediated immune suppression, it may improve the effectiveness of current anti-cancer treatments and pave the way for new therapies.

6 Conclusions

In conclusion, the interaction between NETs and TIME plays a critical role in the progression of gynecologic cancers. Neutrophils, as a key component of the innate immune system, have been shown to directly impact tumor formation and development beyond their role in chronic inflammation. The formation of NETs can promote tumor metastasis by enhancing invasion, migration, and creating an inflammatory environment that supports tumor cell survival and proliferation. Furthermore, NETs released under stress during surgical procedures have been linked to tumor recurrence and metastasis, highlighting the complex interplay between the immune system and cancer progression.

Understanding the mechanisms by which NETs influence the TIME in gynecologic cancers is crucial for developing targeted therapies that can modulate the immune response to tumors. Further research into the specific interactions between NETs, immune cells, and tumor cells may provide insights into novel treatment strategies that can improve outcomes for patients with gynecologic cancers. By unraveling the complex dynamics of NETs in the tumor microenvironment, we can pave the way for more effective and personalized approaches to cancer therapy in the future.

Author contributions

HC: Writing – original draft, Writing – review & editing. YZ: Writing – original draft. YT: Writing – original draft. JL: Writing – original draft. CL: Writing – original draft. QC: Writing – review & editing. HK: Writing – review & editing.

Funding

The author(s) declare financial support was received for the research, authorship, and/or publication of this article. This work supported by the Natural Science Foundation of Fujian Province, China (Grant No. 2022J05310).

Conflict of interest

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

Publisher's note

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frontiers | Frontiers in Immunology

TYPE Original Research
PUBLISHED 14 November 2024
DOI 10.3389/fimmu.2024.1486592

OPEN ACCESS

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RECEIVED 26 August 2024
ACCEPTED 21 October 2024
PUBLISHED 14 November 2024

CITATION
Zhong F-L, He J-J, Bai K-H, Shao R-N, Wu G-Y, Tian X-P, Wang D-W, Dai Y-J and Chen S-L (2024) Tigecycline-induced coagulation gene prognostic prediction model and intestinal flora signature in AML. *Front. Immunol.* 15:1486592. doi: 10.3389/fimmu.2024.1486592

Tigecycline-induced coagulation gene prognostic prediction model and intestinal flora signature in AML

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Infection is among the most common causes of death in patients with acute myeloid leukemia (AML) after chemotherapy. The anti-tumor effect of the intestinal microbiota in patients with AML is increasingly being recognized. Tigecycline, a broad-spectrum antibiotics, plays a vital role in the anti-infection treatment of AML patients with neutropenia and accompanying infections. Previously, this group reported that long-term use of tigecycline caused coagulation dysfunction in patients with hematological malignancies, increasing the risk of casualties. RNA sequencing was performed on CHO cells before and after tigecycline treatment. Further, the combined analysis of AML prognostic differentially expressed genes revealed 13 genes affected by tigecycline and closely related to AML prognosis. These genes were used for modeling analysis, and the results showed that the prepared model significantly improved the prognostic prediction efficiency for AML patients. The model also explored the correlation between prognosis score and immune cells infiltrating tumors and immune therapy targets. Moreover, 16S sequencing was performed on fecal samples from AML patients before and after tigecycline treatment. The results revealed that tigecycline significantly altered the distribution of intestinal microbiota in AML patients - These changes in microbiota are related to chemotherapy resistance. This study emphasizes the importance of intestinal microbiota in AML prognosis. Thus, the findings of this study show that the long-term use of antibiotics can not only cause dysbiosis of the intestinal microbiota but also indirectly affect the sensitivity of chemotherapy drugs, affecting the prognosis of AML patients.

KEYWORDS

tigecycline, intestinal microbiota, 16S sequencing, prognosis model, AML

Introduction

World Health Organization International Cancer Research Agency (IRAC) reported 19.29 million new cancer cases worldwide in 2020. Of these, 4.57 million new cancers were reported in China, accounting for about 23.7% of the total cases reported. Although chemotherapy can effectively extend the survival of patients with cancer (1), the medication scheme, dosage, and individual differences during chemotherapy cause varying degrees of bone marrow suppression in patients. Because chemotherapy plays a vital role in tumor treatment, the bone marrow suppression problem cannot be ignored (2, 3). According to relevant statistics, 80% of the patients will experience bone marrow suppression during tumor radiotherapy and chemotherapy, reducing neutral granulocytes, platelets, and red blood cells, leading to infection, bleeding, anemia, and other phenomena (4, 5). The decrease in neutral granulocytes is most prominent during bone marrow suppression. It decreases immunity and develops symptoms such as severe infection, fever, and fatigue. Infection is the most common and worst complication in elderly AL patients after chemotherapy and one of the causes of death (6).

Tigecycline can be quickly and widely distributed in the body to treat adult complex skin and soft tissue infections (CSSSIS) and adult complex abdominal infections (CIAIS) (7, 8). Tigecycline can induce apoptosis of leukemia (9). Inhibitory drugs combined with autophagy can further increase tigecycline's specific anti-leukemia effect and reverse CML resistance (10). Moreover, tigecycline can inhibit cell vitality, block cell cycles, and induce cell autophagy to treat multiple myeloma (11). Although various adverse reactions are reported on the diameter application, coagulation dysfunction is a rare side effect (12). The coagulation dysfunctions are related to multiple factors when treating immunosuppressive AL patients (13).

Abnormal severe infection and coagulation function are common causes of death in patients with leukemia. In this study, a novel prognostic model was constructed by genes regulated by tigecycline in patients with AML. Further, the tumor immune microenvironment and the intestinal flora changes induced by tigecycline were also investigated.

Materials and methods

Patient samples

Eleven patients with hematological malignancies diagnosed at the Sun Yat-sen University Cancer Center between 2021 and 2022 were enrolled in this study. All participants provided written informed consent per the regulations of the Institutional Review Boards of the Hospitals in agreement with the Declaration of Helsinki.

RNA-sequencing

The feature that CHO cells could be cultured in suspension and adherent at the same time made them be one of the important cells for platelet function research, and had been widely used in platelet function research in the world (14, 15). The CHO cells treated with tigecycline (0 mg/mL and 0.2 mg/mL) for 48 h were used for RNA sequencing, following previously reported methods (13). The raw data and proceed data were uploaded to NCBI (GSE198830). Here, the "edgeR" package was used to identify the differentially expressed genes (DEGs) between the CHO cell group treated with 0 mg/ml tigecycline and 0.2 mg/ml tigecycline. The following criteria were used: (a) fold change > 2 ($Log_2FC>1$ or <-1); (b) false discovery rate (FDR) < 0.05; and (c) gene expression levels > 1.

Quantitative real-time PCR

The expression of CRGs were measured by qRT-PCR. We followed the manufacturer's guidelines using the ESscience assay (QP002) and employed the $2-\Delta\Delta Ct$ formula to examine the relative expression levels. Primers are listed in Supplementary Table S1.

16S rRNA gene sequencing

Eleven patients with hematological malignancies were recruited; their feces specimens were collected before and on the third day using tigecycline for microorganisms 16s sequencing. The community DNA fragments were sequenced via the Illumina platform using paired-end sequencing. Both DADA2 and Vsearch were used to sequence denoising. Greengenes database (Release 13.8, http://greengenes.secondgenome.com/) was used to annotate species taxonomy and construct the phylogenetic tree. Next, multiple advanced bioinformatics methods were applied to analyze data, such as species composition analysis, alpha diversity analysis, beta diversity analysis, species difference and marker species analysis, and association network analysis. The raw data and proceed data were uploaded to NCBI (No: PRJNA967642).

Data acquisition

Gene expression information and the corresponding survival time data of patients with hematological malignancies were retrieved from public AML databases, including The-Cancer-Genome-Atlas (TCGA) and Gene-Expression-Omnibus (GEO: GSE37642, GSE71014, and GSE106291). Log_2 transformation was performed to normalize the expression profiles (Supplementary Figure S1).

Tigecycline-related prognostic signature model

The TCGA cohort was used as a training cohort, and other GEO databases were used as the validation cohorts (16). Overall survival (OS)-related DEGs of tigecycline were screened via Venn diagram analysis (17). The prognostic tigecycline signature was constructed using the LASSO regression analysis based on 10-fold cross-

validation penalized maximum likelihood estimators. The minimum criterion was used to choose the optimal penalty parameter (λ) values with a repetition frequency 1000. The CRG risk score (RS) was calculated for each AML patient using the following formula: RS = (0.041 * SEMA3C expression level) + (0.04 * SRC expression level) + (0.015 * PYCARD expression level) + (0.012 * MFHAS1 expression level) + (0.011 * ST6GALNAC4 expression level) + (0.0005 * HMGA1 expression level) - (0.038 * PLD1 expression level). Patients were further assigned to high-risk or low-risk sets based on the median of the RS. Kaplan-Meier and time-dependent receptor operating characteristic (ROC) curves were used to assess the predictability of the tigecycline signature. The PRC curve was analyzed by PRROC package in R.

Immune -infiltrating analysis

The relationship between the tigecycline signature and immune cells involved in the tumor microenvironment was evaluated using the Tumor Immune Estimation Resource (TIMER; cistrome.shinyapps.io/timer). The Pearson correlation analysis was done for an in-depth analysis of the relationship between Tregs and tigecycline signature. Also, the current critical immune checkpoints (ICKs), including PDCD1, CD274, TIGIT, CTLA4, LAG3, and IDO1, were examined to indirectly speculate the treatment response of immune checkpoint inhibitors in AML cells.

Statistical analysis

SPSS statistical software was used for normalization analysis. Mean ± standard deviation was used to present the measured data, and t-test or corrected t-test was used to analyze the significance of tigecycline signature and other variances. The χ^2 test was used to compare the categorical data. Continuous variables were subjected to analysis employing either the parametric Student's t-test or the non-parametric Wilcoxon rank-sum test, depending on the normality and homogeneity of variance assumptions. The log-rank test was employed to assess differences in OS distributions between two cohorts. For the evaluation of diagnostic accuracy, the time-dependent ROC analysis package was implemented to construct ROC curves and ascertain the AUC metric. Prognostic factors were identified via univariate and multivariate Cox proportional hazards regression analyses, facilitating the estimation of independent prognostic indicators (Supplementary Texts S1, S2). $P < 0.05$ indicated a statistical difference, and $P > 0.05$ showed no statistical difference.

Result

Prognostic significance of tigecycline-related genes in AML

RNA sequencing of CHO cells was conducted before and after tigecycline (0.2 mg/ml) treatment. The differentially expressed genes between these two groups were identified using the "edgeR" package (Supplementary Figures S2A-C). We first identified the gene expression differences in CHO cells before and after treatment with tigecycline through RNA sequencing. The results showed that the expression of most genes was downregulated after treatment with tigecycline (Figure 1A). We further validated using AML cell lines and found that the changes in these genes in AML cell lines treated with tigecycline were highly consistent with the changes in CHO cells (Supplementary Figure S9). Subsequently, these hamster derived genes were compared with human derived genes. Then, the TCGA database was used to analyze the prognostic significance and expression differences of the transformed human genes. Thirteen different genes were regulated by tigecycline, which was also closely related to AML prognosis (Figure 1B). Of these, ten genes (*EVL, FHL2, HMGA1, HMGA2, MFHAS1, MINK1, PXN, SEMA3C, SRC*, and *ST6GALN AC4*) showed lower expression in AML cells, and the remaining three genes (EXT2, PLD1, and PYCARD) showed elevated expression in AML cells compared with that in normal cells (Figure 1C; Supplementary Figures S2D, E). The survival results showed that elevated expression of these genes (except *EXT2* and *PLD1*) was related to poorer prognosis (Figure 1D).

Next, the TCGA dataset was selected as the training set to examine the prognostic significance of these 13 genes in AML. Kaplan–Meier analysis indicated that high-risk patients had a poorer prognosis than the low-risk patients in the TCGA dataset (Figure 1E). Further, time-dependent ROC curves were used to validate the survival prediction model. The specificity and sensitivity of AUC in AML was > 0.7 even at six years and > 0.8 at 5 years (Figure 1F). Further, GSE37642-96, GSE71014, and GSE106291 databases were used as validation sets to validate the prognostic model. These results were consistent with the results of the training set. The AUCs at five years were a minimum of 0.5, showing an upward trend over time (Figures 1G, H). In addition, the PRC value analysis results were consistent with the ROC analysis results (Supplementary Figure S3).

TRGs risk for clinical characteristics and prognosis in AML

Next, a nomogram with standard clinical variables and the TRGs risk score was created to expand the clinical applicability in AML (Figure 2A). The score of each patient was calculated by combining each prognostic criterion. These results indicated that patients with higher total scores had poorer prognosis. The nomogram predicted that OS was highly consistent with the observed OS of the ideal model and predicted the 1-, 3- and 5-year survival time (Figure 2B). Specifically, the AUC for 1-year OS in the merged score group was 0.822 [95% CI: 74.98 – 89.42], in the risk score group was 0.776 [95% CI: 68.87 – 86.24], in age was 0.699 [95% CI: 61.51 – 78.22], and in cytogenetic risk was 0.628 [95% CI: 54.12 – 71.52]. Also, the AUC for 3-year survival in the merged score group was 0.813 [95% CI: 71.19 – 91.40], in the risk score group was 0.751 [95% CI: 65.06 – 85.22], in age was 0.688 [95% CI: 59.17 – 78.41] and in the cytogenetic risk was 0.658 [95% CI: 54.56 – 77.03]. Further, the AUC for 5 years OS in the merged score group

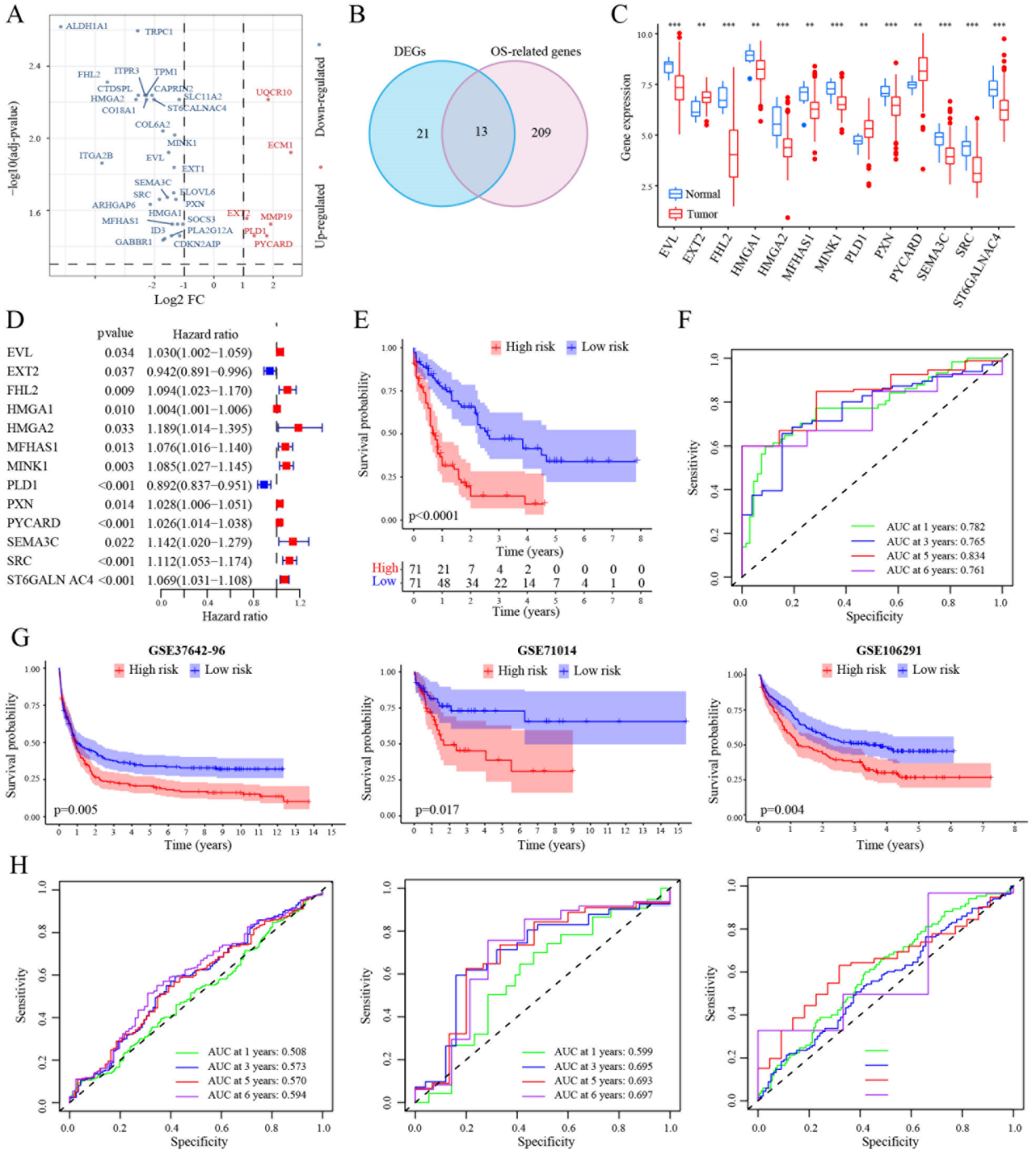

FIGURE 1
Construction of a prognostic signature in the TCGA-LAML cohort. **(A)** Volcano plot exhibiting 34 DEGs in GSE1159 cohort. **(B)** Venn diagram to identify 13 overlapping prognostic DEGs. **(C)** Expression signatures of 13 prognostic DEGs in GSE1159 cohort. **(D)** Forest plots showing the results of the univariate Cox analysis between gene expression and OS in TCGA-LAML cohort. **(E)** Kaplan-Meier curve of 7 MRG profile in the TCGA-LAML cohort. **(F)** Time-dependent ROC analysis of 7 MRG profile in the TCGA-LAML cohort. **(G, H)** Kaplan-Meier curve and time-dependent ROC analysis of 7 MRG profile in the GSE37642-96 (n=417), GSE71014 (n=104) and GSE106291 (n=250) cohorts, respectively. **, p < 0.01; ***, p < 0.001.

was 0.902 [95% CI: 81.51 – 98.96], in the risk score group was 0.801 [95% CI: 67.42 – 92.71], in age was 0.762 [95% CI: 70.66 – 81.82], and in the cytogenetic risk was 0.741 [95% CI: 57.85 – 90.36]. These results demonstrated that subjoining the risk score significantly improved overall survival prediction efficiency (Figures 2C-E).

Immune therapeutic targets of TRGs in AML

Recent studies have substantiated the immense growth in tumor immunotherapy (12). Thus, the relationship between TRGs risk and infiltrated tumor microenvironment in AML was investigated. The

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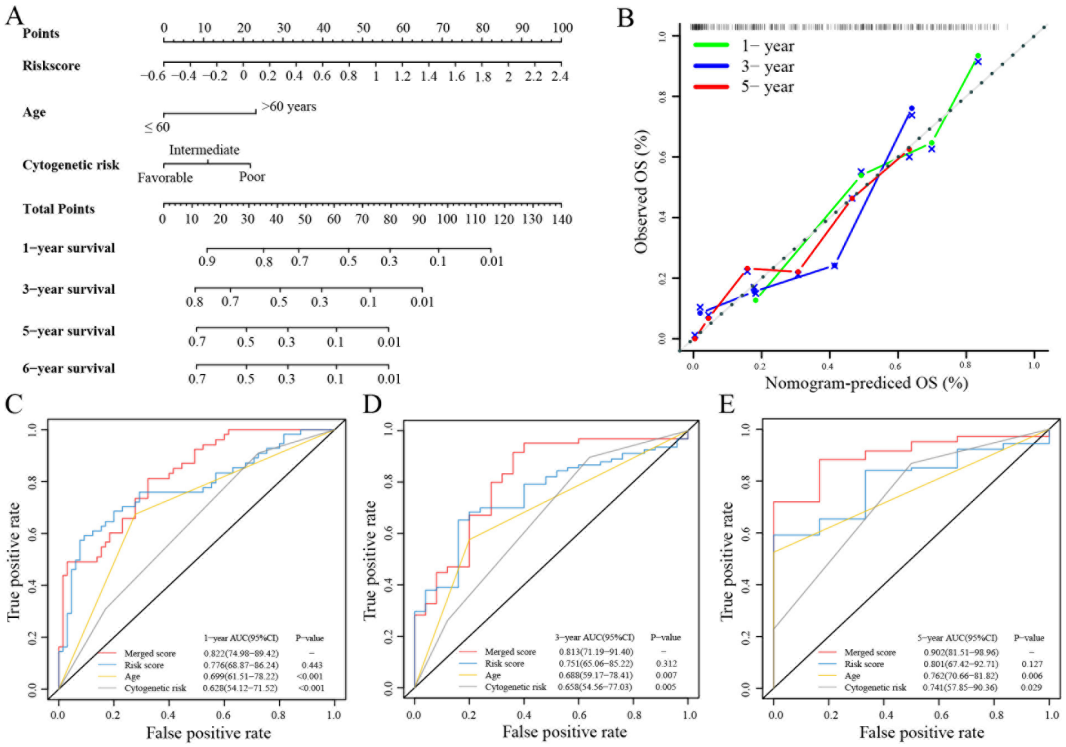

FIGURE 2
Building and validation of the nomogram to predict the OS of AML patients in the TCGA-LAML cohort. **(A)** Nomogram was built based on age, cytogenetic risk and risk score in the TCGA-LAML cohort. **(B)** Calibration plot of the nomogram. **(C–E)** Time-dependent receiver operating characteristic (ROC) curves of nomograms were compared based on 1-, 3- and 5-year OS of the TCGA-LAML cohort.

results showed that there was a significant difference in the immune scores between the high-risk group and the low-risk group in the (i) Treg, T cells gamma delta, Monocytes, and Mast cells resting in the TCGA-LAML database; immune scores of B cells memory, Treg, and Monocytes in the GSE71014 database; immune scores of B cells naïve, T cells follicular helper, T cells gamma delta, Monocytes, and Mast cells resting in the GSE37642-96 database and immune scores of Plasma cells, T cells CD4 memory activated, T cells gamma delta, NK cells resting, Monocytes, Macrophages M0, Mast cells resting, and Eosinophils in the GSE106291 database. Monocytes were the common cells in all four databases. Also, Treg cells were closely associated with TRGs risk in these databases (Figure 3A). Next, the relation between the TRGs risk score and common immunosuppressive marker molecules in AML was investigated. The results showed that the expression of four immunosuppressive markers (IL-10, FOXP3, TGFB1, IL-6, except FAP) was upregulated in the high TRGs risk group compared with that in the low TRGs risk group (Figure 3B). Also, the correlation between the TRGs risk score and common immunotherapy targets, such as PDCD1, TIGHT, IDO1, CD274, CTLA4, and LAG3, was investigated. Consistent with our predictions, except TIGIT ($p = 0.083$), the other targets PDCD1 ($p = 0.00069$), IDO1 ($p = 0.007$), CD274 ($p = 0.01$), CTLA4 ($p = 9.7e\text{-}08$), and LAG3 ($p = 2.7e\text{-}05$) showed elevated expression in the high TRGs risk group (Figure 3C). Therefore, combining chemotherapy and current immunotherapy-related drugs could improve the prognosis of patients in the high TRGs risk group.

Intestinal flora 16S sequencing

The change in the intestinal flora can affect the prognosis of patients with leukemia (18–20). Tigecycline, a broad-spectrum antibiotic, has the potential to induce intestinal flora disorders. Thus, 16S rRNA sequencing was done to investigate the microbial community changes regulated by tigecycline. The classified composition of microorganisms and different classification units were used in the two groups before and after applying tigecycline (Supplementary Figure S4). Ggtree was used to display the position of ASV/OTU in the evolutionary tree and the mutual evolution distance. Their composition and abundance, classification, and other information were visualized via hot charts and column diagrams (Supplementary Figure S5). The results of the alpha diversity index suggested that there was no apparent difference between the two groups (Supplementary Figure S6).

In contrast, the beta diversity analysis indicated the presence of significant differences between the two groups of microbial communities (Supplementary Figure S7). Next, ASV/OTU abundance tables were used to make petal diagrams for community analysis to further explore the differences between the species and to study which species were common and which were unique. After tigecycline treatment, a significant change was observed in the community composition. Only 326 species in the two groups were common (Figure 4A). Also, a significant difference in the trend of species composition of hot diagrams was observed (Figure 4B). Further, LEFSE was used to explore the search for

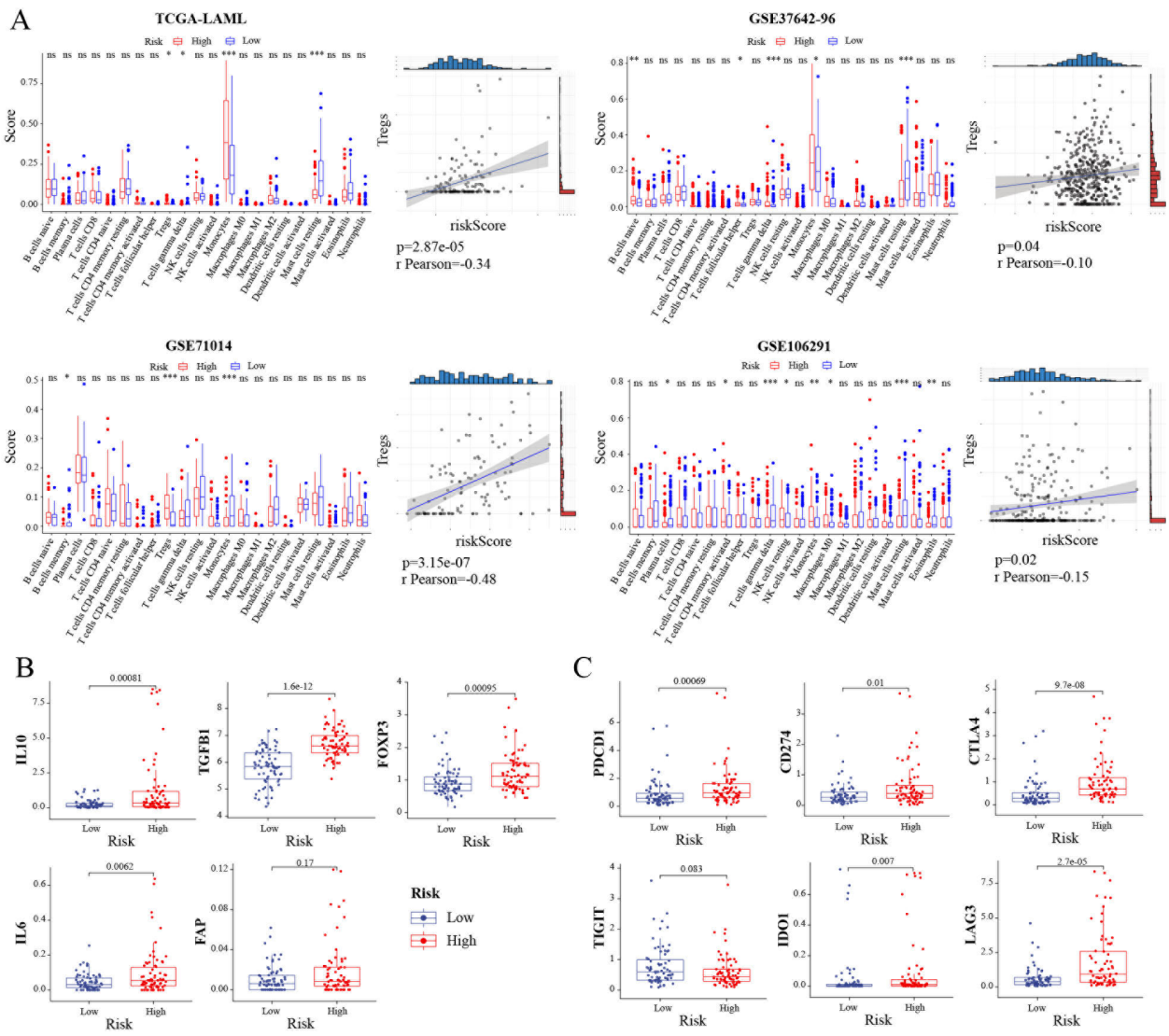

FIGURE 3
Landscape of tumor immune microenvironment between the high- and low-risk groups. **(A)** The scores of 22 immune cells and the correlation between riskscore and Tregs in the TCGA-LAML, GSE37642-96, GSE71014 and GSE106291 cohorts, respectively. **(B)** Investigations of tumor microenvironment immunosuppressive cytokines and markers. Expression level of IL10, TGFB1, FOXP3, IL6, and FAP in TCGA-LAML cohort. **(C)** Expression level of immune checkpoints in TCGA-LAML cohort. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

stable different species between the two groups and to identify the consistency of the microbiology group that showed similar performance in the two groups. There were significant differences in the presence of p_Acidobacteria, c_Thermoleophilia, c_Acidobacteria_6, o_iii1_15, and o_Pseudomonadales (Figure 4C; Supplementary Table S2). A classic and efficient machine learning algorithm (Random forest analysis) deepened the complex non-linear dependencies between samples and groups. The critical indicators of the top 5 were ASV_5376, ASV_7262, ASV_2328, ASV_3777, and ASV_6119, respectively (Figure 4D). Next, a specific microorganized community was identified in time and space via associated analysis, and a related network was constructed to determine the relationship between microorganisms. The network showed that these three modules were intertwined and closely connected. Also, the Zi and PI values showed that the network nodes (ASV/OTU) were divided into 4 parts. Most phylum (Firmicutes, Proteobacteria, Bacterodetes, and Actinobacteria) landed on the part of the Peripherals (Figure 4E).

For microbial ecology research, the functional potential of the flora was investigated. The analysis of metabolic pathways suggested that the primary functions included biosynthesis of amino acid and nucleoside and nucleotide, degradation of carbohydrate and carboxylate, fermentation, and glycolysis (Supplementary Figure S8). The layered sample metabolic pathway abundance tables were also used to analyze the pathway species. The results showed significant differences in stratification between the two groups and even in different samples (Figure 4F).

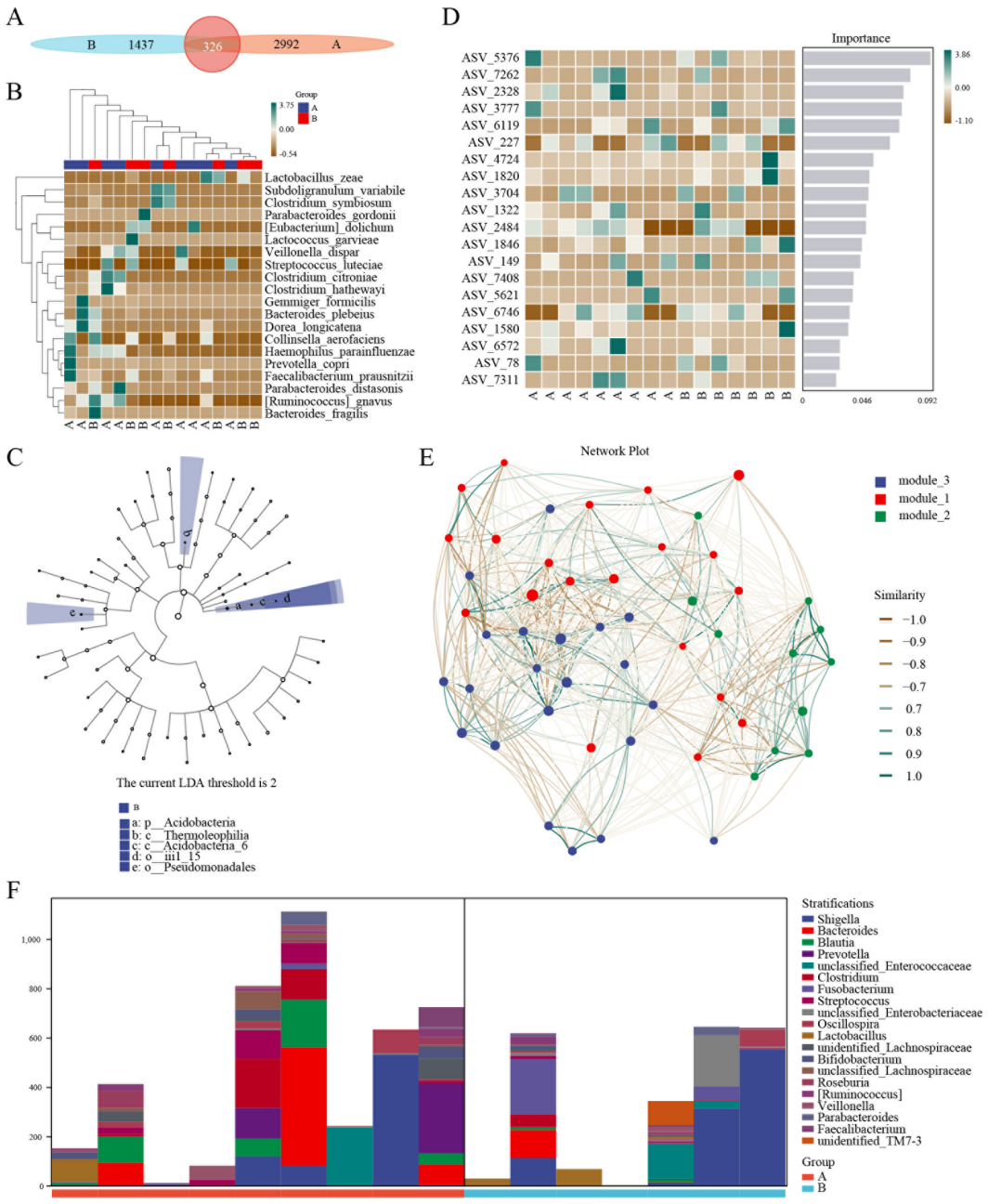

FIGURE 4
16S sequencing of AML patients treated with tigecycline. **(A)** ASV/OTU abundance analysis of patients before or after tigecycline treatment. **(B)** Hot diagrams of species composition in the trend of species. **(C)** LEFSE analysis showed the consistency of the microbiology group. **(D)** Hot diagrams of complex non-linearly dependencies between samples and groups. **(E)** The time and space microorganized community analysis between microorganisms. **(F)** The metabolic pathway analysis of stratification between the two groups.

Discussion

Tigecycline has a broad antibacterial spectrum with intense activity for Gram-positive and Gram-negative bacteria (21, 22). It is widely used to treat complex infections in patients with AML (23). The most common adverse reactions are discomfort in the digestive system, including fatigue, nausea, vomiting, diarrhea, and abdominal pain. The symptoms are mostly mild to moderate and disappear after the drug is discontinued (24). With the increase in clinical applications of tigecycline, there are increasing reports of abnormal coagulation function in non-tumor patients (25). This group has previously reported that using tigecycline to control complicated infections in tumor patients can cause apparent coagulation function abnormalities, especially in patients with AML (13, 26). However, the mechanism causing coagulation dysfunction was not clear.

Tigecycline is believed to inhibit vitamin K synthesis, affect coagulation factor synthesis, or inhibit the IL-6 mechanism to induce coagulation disorders (26, 27). Early studies in CHO cells revealed that tigecycline affected the changes in the internal genetic expression of the cells and affected the adhesion function of platelets. According to the genetic construction risk model based on the genes regulated by tigecycline and through analysis and verification of multiple databases, this study revealed that the prognosis of patients with high-risk group characteristics was significantly poorer.

The role of platelet function-related genes in tumor prognosis has drawn significant attention (28). Patients with triple-negative breast cancer TNBC could be divided into three subtypes according to platelet-related gene expression and variation levels (29). Many platelet-related genes in pancreatic ductal adenocarcinoma (PDAC) were significantly enriched in CTCs (30). The expression of HLA-E molecules on the tumor cell surface was upregulated to escape the immune surveillance of NK cells. Moreover, prognostic models of ovarian cancer patients have been established using tumor-domesticated platelets (TEP), which are used for early cancer screening and prognostic prediction (31, 32). Earlier, the authors of this study reported for the first time that tigecycline affected the procoagulant function by regulating the changes of hematopoietic related genes. In this manuscript, the authors report the first diagnosis model of platelet-related genes based on the regulation of tigecycline. Further, the prognostic prediction efficiency of this model was superior to the existing traditional methods in AML patients. Moreover, the combination of this model with conventional models can further improve the accuracy of AML prediction.

The immune microenvironment and microecological changes in the intestine are closely related to the tumor (33). There are several bacteria in the intestinal tract, and maintaining this homeostasis requires the participation of Treg on the one hand and immune cells to resist bacterial invasion on the other hand. Intestinal flora was reported to regulate the Th17/Treg cell differentiation in a mouse model of experimental autoimmune prostatitis by short-chain fatty acid propionic acid in various tumor types (34). TLR4 plays a sexual role in mice colitis by promoting the colonization of Akk bacteria in the intestine and upregulating the RORγt+ Treg cell-mediated immunosuppressive response (35). RORγt+ cells induce intestinal flora-specific differentiation of Treg cells (36). In this study, the application of tigecycline in patients with AML significantly affected the proportion and function of Treg cells in the body, which in turn caused changes in the intestinal flora and the prognosis of patients.

While there is no direct evidence at present to establish a connection between these microbial shifts and patient prognosis, a growing number of studies indicate that changes in the gut microbiome of individuals with AML are a contributing factor to their prognostic outcomes. AML patients receive multiple antibiotic treatments during induction chemotherapy, causing significant damage to the gut microbiota (37). In fact, our results indicated that tigecycline could also kill too many bacterial species in the intestinal microbiota, leading to alterations in bacterial diversity. For long-term treatment, reduced "good" microbiota could be troublesome such as increased "bad bacteria" infection potential and noncoding RNA dysfunctions linked to mitochondrial dysfunctions and AML. In light of this, we recommend exercising caution rather than imposing a ban on the use of tigecycline in patients experiencing bone marrow suppression. Moreover, we are committed to pursuing additional research to elucidate the intricate relationship between these elements. After chemotherapy and multiple antibiotic treatments, if the gut microbiota of AML patients wants to return to the initial antibiotic treatment state, it is necessary to reduce antibiotic pressure earlier or undergo fecal microbiota transplantation (Figure 5). Due to the

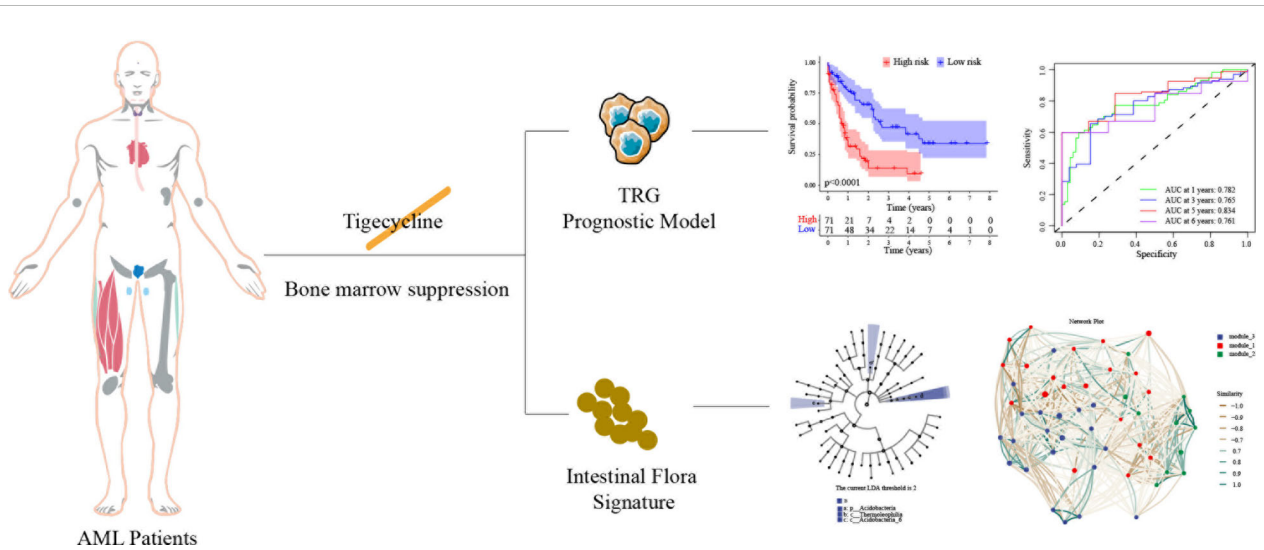

FIGURE 5
A conclusive hypothesis of the influence of tigecycline in AML patients.

stringent regulatory constraints on advanced antimicrobials such as tigecycline, rapid acquisition of additional samples is not feasible. Consequently, we are contemplating experimental protocols to assess the implications of our research findings across various neoplastic entities and patient prognoses. Our objective is to conduct a controlled clinical trial to substantiate the hypotheses advanced in further study.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/Supplementary Material.

Ethics statement

The studies involving humans were approved by State Key Laboratory of Oncology in South China, Guangdong Provincial Clinical Research Center for Cancer, Sun Yat-sen University Cancer Center. The studies were conducted in accordance with the local legislation and institutional requirements. Written informed consent for participation in this study was provided by the participants' legal guardians/next of kin.

Author contributions

F-LZ: Data curation, Methodology, Writing – original draft. J-JH: Investigation, Methodology, Validation, Writing – review & editing. K-HB: Data curation, Formal analysis, Software, Writing – original draft. R-NS: Investigation, Methodology, Validation, Writing – original draft. G-YW: Formal Analysis, Methodology, Validation, Writing – original draft. X-PT: Formal analysis, Methodology, Resources, Writing – original draft. D-WW: Resources, Supervision, Writing – review & editing. Y-JD: Funding acquisition, Project administration, Supervision, Writing – review & editing. S-LC: Writing – review & editing.

Funding

The author(s) declare financial support was received for the research, authorship, and/or publication of this article. This work was supported by National Natural Science Foundation of China (Project #: 82201739 and 82000144), Shenzhen Science and Technology Program#: JCYJ20220530160214031, the General Program for Clinical Research at Peking University Shenzhen Hospital (No. LCYJ2021037), the Research Foundation of Peking University Shenzhen Hospital (No. JCYJ2021007) and Guangzhou Basic and Applied Basic Research Foundation (202201011458), Shenzhen Biomedical Industry Major Public Service Platform and Core Technology Research Special Support Plan (No. XMHT20220104048).

Conflict of interest

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

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2024.1486592/full#supplementary-material

SUPPLEMENTARY FIGURE 1
A flowchart of data processing.

SUPPLEMENTARY FIGURE 2
(A) The volcano plot analysis of DEGs in GSE1159 cohort. **(B)** Heatmap of 34 DEGs in GSE1159 cohort. **(C)** The circos plot depicting the location on chromosomes of 13 prognostic DEGs. **(D)** The CNV frequency of 13 prognostic DEGs in TCGA-LAML cohort. **(E)** Genetic alterations of the 13 prognostic DEGs in CCLE, obtained from the cBioportal for Cancer Genomics (http://www.cbioportal.org/).

SUPPLEMENTARY FIGURE 3
PRC curve analysis of AML databases.

SUPPLEMENTARY FIGURE 4
The classification composition of microorganisms and different classification units in the groups before and after the application of tigecycline.

SUPPLEMENTARY FIGURE 5
Phylogenetic tree plot analysis of microorganisms in the groups before and after the application of tigecycline.

SUPPLEMENTARY FIGURE 6
Alpha diversity index analysis of microorganisms in the groups before and after the application of tigecycline.

SUPPLEMENTARY FIGURE 7
Hierachical clustering analysis of microorganisms in the groups before and after the application of tigecycline.

SUPPLEMENTARY FIGURE 8
Function analysis of microorganisms in the groups before and after the application of tigecycline.

SUPPLEMENTARY FIGURE 9
Validation of CRGs genes expression in AML cell line THP cells treated with tigecycline.

SUPPLEMENTARY TABLE 1
The primers of CRGs by qPCR.

SUPPLEMENTARY TABLE 2
The list of LEFSE analysis in the microbiology group.

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frontiers | Frontiers in Immunology

TYPE Opinion
PUBLISHED 06 December 2024
DOI 10.3389/fimmu.2024.1488345

OPEN ACCESS

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RECEIVED 29 August 2024
ACCEPTED 21 November 2024
PUBLISHED 06 December 2024

CITATION
Qi X, Cheng C, Zhang D, Yu Z and Meng X (2024) Exploring the synergy between tumor microenvironment modulation and STING agonists in cancer immunotherapy. *Front. Immunol.* 15:1488345. doi: 10.3389/fimmu.2024.1488345

Exploring the synergy between tumor microenvironment modulation and STING agonists in cancer immunotherapy

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KEYWORDS

tumor microenvironment (TME), STING agonists, cancer immunotherapy, immune suppression, macrophage polarization

Introduction

Cancer immunotherapy has revolutionized the treatment of various malignancies, particularly with the advent of immune checkpoint inhibitors and CAR-T cell therapies (1–3). These approaches have yielded impressive outcomes in a subset of patients, yet many still fail to achieve durable responses (4). One of the key reasons for this disparity in treatment outcomes is the presence of an immunosuppressive tumor microenvironment (TME), which plays a crucial role in limiting the effectiveness of immune-based therapies (5, 6). The TME comprises a complex network of cellular and molecular components, including tumor-associated macrophages (TAMs), regulatory T cells (Tregs), and myeloid-derived suppressor cells (MDSCs), all of which contribute to immune evasion and tumor progression (7–9).

The STING (stimulator of interferon genes) pathway has emerged as a promising target for cancer immunotherapy due to its ability to bridge innate and adaptive immune responses (10, 11). Upon activation by cytosolic DNA, the STING pathway triggers the production of type I interferons and other pro-inflammatory cytokines, leading to the activation of dendritic cells (DCs) and subsequent priming of T cells (12). This process is crucial for initiating a robust anti-tumor immune response. However, despite the potential of STING agonists to stimulate powerful immune responses, their efficacy in clinical settings has been limited, primarily due to the immunosuppressive nature of the TME, which can dampen the immune activation initiated by STING (13). This TME comprises various cellular components, including TAMs, regulatory Tregs, and MDSCs, which together contribute to a hostile immune environment that inhibits effective anti-tumor responses.

TAMs often adopt an M2-like phenotype within the TME, characterized by anti-inflammatory and tissue-remodeling activities that promote tumor growth and suppress effective immune responses (14). Recent studies have shown that activation of the STING pathway can lead to a shift in TAM polarization from M2 to M1, enhancing the secretion of pro-inflammatory cytokines such as TNF-α and IL-12, which are crucial for T cell activation and anti-tumor immunity. Tregs play a dual role in maintaining immune homeostasis but can hinder effective anti-tumor immunity by inhibiting cytotoxic T cell functions. Targeting Tregs through STING agonists may lead to a decrease in their suppressive effects, allowing for a more robust T cell response against tumor cells (15).

MDSCs represent a significant barrier to successful immunotherapy due to their ability to produce reactive oxygen species (ROS) and other immunosuppressive factors that inhibit T cell activation. Emerging evidence suggests that STING agonists may reduce MDSC levels or impair their function, thereby alleviating the suppression of T cell activity within the TME (16, 17). The extracellular matrix (ECM) and the physical characteristics of the TME, such as hypoxia and acidosis, also contribute to immune suppression. STING activation can enhance the remodeling of the ECM, thereby facilitating better immune cell infiltration and improving the therapeutic efficacy of STING agonists (18). Given these challenges, there is a growing interest in exploring synergistic combination strategies that not only modulate the TME but also enhance the overall effectiveness of STING agonists (19, 20). For instance, targeting specific components of the TME that contribute to immune suppression, such as TAMs, regulatory Tregs, and MDSCs, can create a more favorable environment for STING-mediated immune activation (21, 22). Recent studies have demonstrated that combining STING agonists with therapies like checkpoint inhibitors or bispecific antibodies leads to enhanced T cell responses and improved tumor regression. This synergistic approach has shown great promise not only in improving the efficacy of STING agonists but also in overcoming resistance mechanisms associated with current immunotherapies. By leveraging multiple therapeutic modalities, researchers aim to achieve more durable and effective anti-tumor responses, ultimately leading to better patient outcomes (23).

This article will delve into the characteristics of the TME, the role of the STING pathway in tumor immunotherapy, and how combining TME modulation with STING agonists can lead to more effective cancer treatments. This article uniquely contributes to the field by systematically evaluating the synergistic potential of STING agonists combined with TME-modulating therapies, which is often overlooked in current literature. Moreover, it emphasizes the critical need for personalized therapeutic strategies that consider the distinct characteristics of individual tumor microenvironments, thereby optimizing treatment efficacy. Additionally, the manuscript outlines future research directions that aim to elucidate the specific mechanisms by which STING pathway activation interacts with various TME components, paving the way for innovative clinical applications. Unlike previous studies that primarily focus on isolated therapeutic interventions, this manuscript provides a comprehensive overview of how the combination of STING agonists with diverse TME-targeting strategies can significantly optimize the immune response and improve patient outcomes.

Characteristics and challenges of the tumor microenvironment

TME is a complex and dynamic entity that plays a critical role in tumor progression and the response to cancer therapies (24, 25). It consists of various cellular components, including cancer cells, immune cells, fibroblasts, endothelial cells, and ECM components (26). Among the immune cells, TAMs, regulatory Tregs, and MDSCs are key players that contribute to the immunosuppressive nature of the TME (7–9).

TAMs often adopt an M2-like phenotype within the TME, characterized by anti-inflammatory and tissue-remodeling activities that promote tumor growth and suppress effective immune responses (27). These cells secrete cytokines such as IL-10 and TGF-β, which inhibit the activation and proliferation of cytotoxic T cells and natural killer (NK) cells, thereby fostering an environment that protects the tumor from immune attack (28). Tregs are another crucial component of the TME, functioning to maintain immune tolerance and prevent autoimmunity. However, in the context of cancer, Tregs suppress anti-tumor immunity by inhibiting the activity of effector T cells and secreting immunosuppressive cytokines like IL-10 and TGF-β. This contributes to the immune escape of cancer cells, allowing them to proliferate unchecked (29).

MDSCs are a heterogeneous population of immature myeloid cells that expand during cancer and other chronic inflammatory conditions. Within the TME, MDSCs suppress T cell function through the production of ROS, nitric oxide (NO), and arginase, further contributing to the suppression of anti-tumor immune responses (30). The immunosuppressive characteristics of the TME present significant challenges for effective cancer immunotherapy. The TME not only inhibits the function of immune effector cells but also creates physical barriers, such as dense ECM, that impede the infiltration of immune cells and therapeutic agents into the tumor. Moreover, the hypoxic and acidic conditions commonly found in the TME further exacerbate immune suppression and promote resistance to therapy (31).

In addition to the previously discussed immune cells such as TAMs, Tregs, and MDSCs within the TME, other cellular and non-cellular components also play significant roles. Endothelial cells, which line tumor blood vessels, are essential for tumor growth by supplying nutrients and oxygen (32). However, they also overexpress adhesion molecules and secrete chemokines, attracting immunosuppressive cells like Tregs and MDSCs, thus suppressing anti - tumor immune cells. Their abnormal vessel structure impairs drug delivery and favors tumor survival and metastasis (33). Stroma cells, especially fibroblasts, secrete ECM components, creating a fibrotic barrier that restricts immune cell infiltration (34, 35). They also secrete factors affecting tumor and immune cells' behavior, and understanding their crosstalk is key for devising strategies with STING agonists. Tumor cells, as the root of the problem, downregulate MHC expression, secrete

immunosuppressive factors like TGF - β and IL - 10, and undergo alterations for immune evasion and resistance to therapies (36). A comprehensive understanding of these TME components and their interactions is crucial for developing effective combination therapies, particularly those integrating STING agonists, to improve cancer immunotherapy outcomes.

Addressing these challenges requires innovative strategies that can modulate the TME to restore immune function and enhance the efficacy of cancer treatments. By targeting key components like TAMs, Tregs, and MDSCs, it may be possible to reprogram the TME from an immunosuppressive state to one that supports robust anti-tumor immunity, thereby improving the outcomes of immunotherapy.

Role of the STING pathway in tumor immunotherapy

The STING pathway is a crucial component of the innate immune system, playing a pivotal role in detecting cytosolic DNA, which often originates from viral infections or damaged tumor cells. Upon recognition of cytosolic DNA, the cyclic GMP-AMP synthase (cGAS) enzyme produces cyclic GMP-AMP (cGAMP), a second messenger that directly activates the STING protein (37). Once activated, STING translocates from the endoplasmic reticulum to the Golgi apparatus, where it triggers a signaling cascade leading to the phosphorylation of interferon regulatory factor 3 (IRF3) and the subsequent production of type I interferons (IFNs) and other pro-inflammatory cytokines (38).

Type I IFNs, such as IFN-α and IFN-β, are critical for bridging the innate and adaptive immune responses. They activate DCs, enhance antigen presentation, and promote the priming and activation of cytotoxic T lymphocytes (CTLs), which are essential for targeting and destroying tumor cells (39). This makes the STING pathway an attractive target for cancer immunotherapy, as it can initiate a robust immune response capable of overcoming the immunosuppressive TME. Preclinical studies have demonstrated that STING agonists can induce potent anti-tumor immunity by enhancing the infiltration and activation of effector immune cells within tumors. These agonists have shown the ability to convert "cold" tumors—those with low immune cell infiltration—into "hot" tumors that are more responsive to immunotherapy. In addition to promoting immune cell infiltration, STING activation can also lead to the direct induction of cell death in certain tumor types, further contributing to tumor control (40).

However, despite these promising effects, the clinical translation of STING agonists has encountered challenges. The immunosuppressive nature of the TME can dampen the immune response initiated by STING activation, limiting the therapeutic efficacy of STING agonists when used as monotherapy (41). Furthermore, the systemic administration of STING agonists carries the risk of inducing excessive inflammation, leading to potential toxicity (42). To overcome these challenges, there is increasing interest in combining STING agonists with other therapeutic strategies, such as immune checkpoint inhibitors or agents that modulate the TME (43). Such combination therapies aim to enhance the immune-stimulating effects of STING agonists while mitigating the suppressive influences of the TME, thereby maximizing the therapeutic potential of STING pathway activation in cancer immunotherapy.

Synergistic effects of tumor microenvironment modulation and STING agonists

TME plays a crucial role in determining the success or failure of cancer immunotherapies. As a highly immunosuppressive milieu, the TME inhibits the effective activation and function of immune cells, including those recruited by therapeutic interventions. This suppression poses a significant challenge to the efficacy of STING agonists, which rely on robust immune activation to exert their anti-tumor effects. Therefore, combining STING agonists with strategies that modulate the TME has emerged as a promising approach to enhance therapeutic outcomes (44, 45).

One of the primary strategies for modulating the TME is targeting TAMs, which often adopt an M2-like phenotype within tumors, characterized by immunosuppressive and pro-tumoral activities (46). Reprogramming TAMs from an M2 phenotype to a pro-inflammatory M1 phenotype can significantly enhance the immune-stimulating effects of STING agonists. M1-like TAMs produce pro-inflammatory cytokines such as TNF-α and IL-12, which support the activation of T cells and other effector immune cells. This shift in macrophage polarization can reduce the immunosuppressive burden of the TME, making it more permissive to the immune activation induced by STING agonists (47).

In addition to targeting TAMs, modulating the activity of regulatory Tregs within the TME is another promising approach. Tregs suppress the activity of cytotoxic T cells and other effector immune cells, thus contributing to immune evasion by tumors (29). By reducing the number or suppressive function of Tregs, the anti-tumor immune response can be enhanced. Combining Treg depletion strategies with STING agonists could lead to a more robust activation of the immune system, promoting a stronger and more sustained anti-tumor response (48).

MDSCs represent another key target within the TME. MDSCs inhibit T cell function through the production of ROS and NO, among other mechanisms (49). Reducing MDSC levels or blocking their suppressive activities can alleviate one of the major barriers to effective immunotherapy. When combined with STING agonists, MDSC-targeting strategies can further enhance immune activation by removing a significant source of suppression within the TME (50).

As we have explored the various ways to modulate the TME by targeting key cellular components such as TAMs, Tregs, and MDSCs, it becomes evident that other aspects of the TME also require attention. For endothelial cells, combining STING agonists with anti - angiogenic therapies is promising as it normalizes tumor vasculature, improving STING agonist delivery (51, 52).

Engineering endothelial cells or using drugs to induce immune - promoting molecules on their surface, like enhancing adhesion molecule expression for immune cell transmigration, can also boost the anti - tumor immune response (53). Regarding stroma cells, a multi - pronged approach is viable. Inhibiting ECM overproduction by fibroblasts, promoting fibrotic matrix degradation, and modulating cytokine/growth factor secretion can create a favorable environment for STING agonist - induced immune activation (54). For tumor cells, strategies include upregulating MHC expression, blocking immunosuppressive factor secretion, and targeting genetic/epigenetic alterations. These approaches, when combined with STING agonists, have the potential to overcome resistance and enhance overall anti - tumor efficacy.

Beyond cellular components, the ECM and the physical characteristics of the TME, such as hypoxia and acidosis, also contribute to immune suppression. Strategies that normalize the ECM or alter the metabolic environment of the TME can facilitate better infiltration of immune cells and improve the delivery and efficacy of STING agonists. For example, reducing ECM stiffness or targeting factors that promote hypoxia can enhance the penetration and activity of both immune cells and therapeutic agents within tumors (55). The synergy between TME modulation and STING agonists has been demonstrated in preclinical models, where combining these strategies leads to improved anti-tumor responses compared to either approach alone. By reprogramming the TME to be more immunologically active, STING agonists can induce stronger and more durable immune responses, increasing the likelihood of tumor eradication (10). To summarize the key strategies for modulating the TME and their potential synergy with STING agonists, please refer to Table 1.

In conclusion, the combination of TME modulation with STING agonists represents a powerful strategy for overcoming the immunosuppressive barriers of the TME and enhancing the efficacy of cancer immunotherapy. This synergistic approach has the potential to convert resistant tumors into responsive ones, offering new hope for patients who do not respond to current treatment modalities. As research in this area progresses, it will be crucial to identify the most effective combinations and optimize their application in clinical settings to maximize patient outcomes. Figure 1 illustrates the key elements of the tumor microenvironment, the STING pathway, and their synergistic interactions as described in this section.

Clinical advancements in STING agonists and tumor microenvironment modulation

Recent clinical advancements have demonstrated the potential of STING agonists in combination with therapies that modulate the TME to enhance anti-tumor immunity (56). STING agonists activate innate immune responses by inducing the production of type I interferons and other pro-inflammatory cytokines, which play a crucial role in bridging innate and adaptive immunity. However, their efficacy as monotherapies has been limited due to the immunosuppressive nature of the TME. As a result, clinical trials have focused on combining STING agonists with agents that target key components of the TME to overcome these barriers (57).

One of the most notable clinical advancements involves the STING agonist ADU-S100, which has shown promising results when combined with pembrolizumab, a PD-1 immune checkpoint inhibitor, in patients with advanced solid tumors (58). The combination led to increased T cell infiltration within tumors and a higher overall response rate, suggesting that STING agonists can convert immunologically “cold” tumors into “hot” tumors that are more responsive to immunotherapy. In addition to immune checkpoint inhibitors, preclinical studies have explored combining STING agonists with other immune-modulating therapies, such as anti-CTLA-4 antibodies (59, 60). For example, the combination of the STING agonist DMXAA with anti-CTLA-4 therapy in murine models resulted in complete tumor regression in some cases, further highlighting the synergistic potential of these approaches.

In addition to the mentioned STING agonists, a novel agent MSA - 2 has emerged as a promising candidate in cancer immunotherapy. MSA - 2 is a potent non - CDN STING agonist

TABLE 1 Strategies for modulating the TME to enhance the efficacy of STING agonists.

TME Component	Modulation Strategy	Effect on Immune Response	Synergy with STING Agonists
Tumor-Associated Macrophages (TAMs)	Reprogramming TAMs from M2 to M1 phenotype	Enhances pro-inflammatory cytokine production (e.g., TNF-α, IL-12)	Reduces immunosuppressive burden, promotes T cell activation
Regulatory T Cells (Tregs)	Depletion or suppression of Treg function	Reduces inhibition of cytotoxic T cells, enhances anti-tumor immunity	Strengthens immune activation induced by STING, sustains anti-tumor response
Myeloid-Derived Suppressor Cells (MDSCs)	Inhibition of MDSC recruitment or function	Decreases suppression of T cell activity, reduces ROS and NO production	Alleviates immune suppression, enhances STING-mediated immune activation
Extracellular Matrix (ECM)	Normalization of ECM stiffness, targeting ECM components	Improves immune cell infiltration and drug delivery	Enhances penetration and activity of immune cells and STING agonists
Hypoxia	Targeting hypoxia-inducing factors	Reduces hypoxia-associated immunosuppression	Improves efficacy of STING activation in hypoxic tumor regions
Acidosis	Buffering tumor acidity, altering metabolic environment	Mitigates acid-mediated immune suppression	Facilitates immune cell function and STING-induced responses

with significant bioactivity. In preclinical investigations, it has demonstrated remarkable potential. For instance, in the context of cervical cancer, when combined with anti - PD - 1, it has shown synergistic efficacy. This combination has led to enhanced anti - tumor immune responses, including increased activation and infiltration of immune cells within the tumor microenvironment (61). Moreover, in studies involving TGF - β/PD - L1 bispecific antibody, MSA - 2 has also exhibited synergistic effects (62). These findings suggest that MSA - 2 could be a valuable addition to the arsenal of cancer immunotherapy strategies, potentially offering new avenues for treating various malignancies and improving patient outcomes.

In order to further enrich the landscape of cancer immunotherapy, several novel STING agonists and related approaches have emerged. TAK - 676, developed by Takeda, has shown notable promise. In preclinical investigations, it has demonstrated the ability to robustly activate the STING pathway, leading to a significant increase in interferon production. This, in turn, activates dendritic cells, which are crucial for antigen presentation. In the context of tumor microenvironment modulation, TAK - 676 induces the polarization of tumor - associated macrophages (TAMs) from the immunosuppressive M2 phenotype to the immunostimulatory M1 phenotype. The M1 - polarized TAMs secrete pro - inflammatory cytokines such as TNF - α and IL - 12, which enhance the anti - tumor immune response. Moreover, TAK - 676 affects the extracellular matrix (ECM), remodeling it in a way that promotes the infiltration of immune cells into the tumor. This compound is currently in the pipeline of clinical development, and its potential to improve cancer immunotherapy is being actively explored (63).

E7766 is another compound that holds great promise. It is engineered to selectively activate the STING pathway within the tumor microenvironment, minimizing off - target effects. In preclinical models, it effectively promotes the secretion of cytokines like type I interferons and interleukin - 12, creating an immunostimulatory milieu. Additionally, it has been shown to enhance the infiltration of cytotoxic T lymphocytes and natural killer cells into tumors. In terms of modulating the tumor microenvironment, E7766 can inhibit the activity of regulatory T cells (Tregs), which are known to suppress anti - tumor immunity. It also impairs the immunosuppressive functions of myeloid - derived suppressor cells (MDSCs), reducing their production of reactive oxygen species and nitric oxide. By targeting these immunosuppressive cell populations, E7766 helps to create a more favorable environment for the anti - tumor immune response. It is currently advancing through the stages of clinical development (64, 65).

ExoSTING utilizes exosomes as carriers for STING agonists, providing precise delivery. Preclinical investigations reveal that it activates the STING pathway, increasing the production of pro - inflammatory cytokines and enhancing anti - tumor immune responses. In the tumor microenvironment, ExoSTING - loaded exosomes interact with TAMs, inducing their polarization to the M1 phenotype and reducing immunosuppressive factors. It also modifies the ECM to promote immune cell infiltration and may influence the metabolic environment to enhance immune cell function (66). Mersana's XMT - 2056, an antibody - drug conjugate, targets tumor - associated antigens. After binding, it releases the STING agonist, killing cancer cells and activating the STING pathway. This leads to the recruitment and activation of immune cells, disrupting the immunosuppressive network within the tumor. XMT - 2056 not only directly eliminates cancer cells but also initiates an immune - mediated attack, reshaping the tumor microenvironment to favor anti - tumor immunity. It is an important addition to the evolving landscape of STING - targeted cancer therapies (67–69).

In addition to the clinical efficacy, the modality of STING agonists plays a crucial role in their application. TAK - 676 and E7766, as potential small molecule drugs, may offer good bioavailability and dosing convenience. Their small size could enhance tissue penetration for direct interaction with the STING pathway and TME modulation, yet they might face challenges like rapid clearance. ExoSTING, a nanoparticle - based modality using exosomes, provides targeted delivery with enhanced biocompatibility. It can effectively target TAMs and modify the ECM and metabolic environment, though its production and characterization need optimization. XMT - 2056, an ADC, combines antibody specificity with cytotoxicity, ensuring targeted agonist delivery and disrupting the tumor immunosuppressive network. Despite manufacturing complexity and potential immunogenicity, it has shown remarkable preclinical activity. Understanding these modality - related characteristics is essential

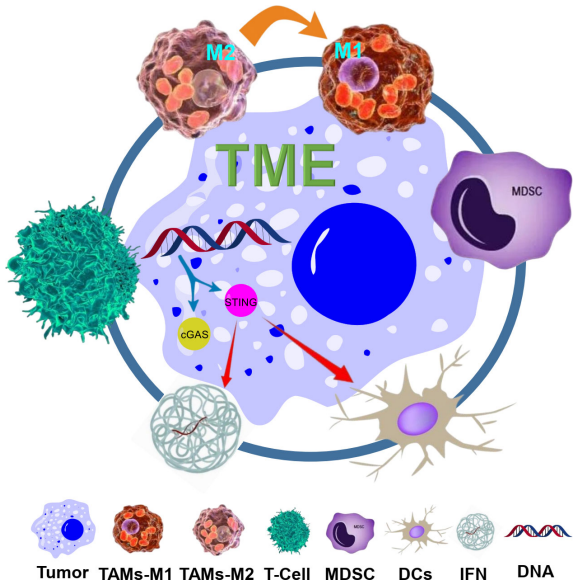

FIGURE 1
Schematic illustration of the TME and the STING pathway, and their synergy. The TME consists of various components including tumor cells, macrophages (TAMs, with M1 and M2 phenotypes), regulatory Tregs, MDSCs, and ECM. The STING pathway is activated by cytosolic DNA, leading to the production of IFNs and activation of DCs and T cells. The synergy between TME modulation (such as reprogramming TAMs from M2 to M1, and targeting MDSCs) and STING agonists is shown, with arrows indicating the interactions and effects on immune responses and tumor cells.

for maximizing the potential of STING agonists in cancer immunotherapy.

These clinical advancements underline the importance of integrating STING agonists with TME-targeting therapies to enhance immune activation and improve patient outcomes. As ongoing trials continue to investigate the safety and efficacy of these combinations, they hold significant promise for overcoming the limitations of current immunotherapies and achieving more durable responses in patients with refractory tumors.

Conclusion

Combining TME modulation with STING agonists holds significant promise for enhancing cancer immunotherapy. This approach addresses the immunosuppressive nature of the TME, potentially converting "cold" tumors into "hot" ones that are more responsive to treatment. Clinically, this strategy offers a powerful tool for overcoming resistance to existing therapies. However, challenges such as the heterogeneous nature of the TME across different tumors and the risk of systemic inflammation due to STING activation must be carefully managed. Future research should focus on optimizing combination strategies, understanding the specific interactions within different TMEs, and developing targeted delivery systems to maximize efficacy while minimizing side effects. This integrated approach could lead to more effective and personalized cancer therapies.

Author contributions

ZY: Writing – original draft, Writing – review & editing. XQ: Writing – original draft. MX: Writing – original draft, Writing – review & editing. CC: Data curation, Writing – original draft. DZ: Data curation, Writing – original draft.

Funding

The author(s) declare that no financial support was received for the research, authorship, and/or publication of this article.

Conflict of interest

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

Publisher's note

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frontiers | Frontiers in Immunology

TYPE Review
PUBLISHED 12 December 2024
DOI 10.3389/fimmu.2024.1495978

OPEN ACCESS

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RECEIVED 13 September 2024
ACCEPTED 28 November 2024
PUBLISHED 12 December 2024

CITATION
Li D, Yang Y, Zheng G, Meng L, Shang L, Ren J, Wang L and Bao Y (2024) The potential of cellular homing behavior in tumor immunotherapy: from basic discoveries to clinical applications of immune, mesenchymal stem, and cancer cell homing.
Front. Immunol. 15:1495978.
doi: 10.3389/fimmu.2024.1495978

The potential of cellular homing behavior in tumor immunotherapy: from basic discoveries to clinical applications of immune, mesenchymal stem, and cancer cell homing

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The efficacy of immunotherapy, a pivotal approach in the arsenal of cancer treatment strategies, is contingent on the capacity of effector cells to localize at the tumor site. The navigational capacity of these cells is intricately linked to the homing behaviors of specific cell types. Recent studies have focused on leveraging immune cells and mesenchymal stem cells (MSCs) homing for targeted tumor therapy and incorporating cancer cell homing properties into anti-tumor strategies. However, research and development of immunotherapy based on cancer cell homing remain in their preliminary stages. Enhancing the homing efficiency of effector cells is essential; therefore, understanding the underlying mechanisms and addressing immune resistance within the tumor microenvironment and challenges associated with *in vivo* therapeutic agent delivery are essential. This review firstly delineates the discovery and clinical translation of the three principal cell-homing behaviors. Secondly, we endeavor to conduct an in-depth analysis of existing research on the homing of immune and stem cells in cancer therapy, with the aim of identifying and understanding of the common applications, potential benefits, barriers, and critical success factors of cellular homing therapies. Finally, based on the understanding of the key factors of cellular homing therapies, we provide an overview and outlook on the enormous potential of harnessing cancer cells' self-homing to treat tumors. Although immunotherapy based on cell-homing behavior warrants further research, it remains a highly competitive treatment modality that can be combined with existing classic anti-cancer therapies. In general, combining the homing properties of cells to optimize their clinical effects is also one of the future research directions in the field of cell transplantation.

KEYWORDS

immunotherapy, tumor, tumor microenvironment, cell homing, treatment strategies

1 Introduction

Cell homing, a critical cellular migration process, significantly affects organismal development, tissue regeneration, and disease progression. The term "homing" was initially coined to describe the tendency of lymphocytes circulating within the bloodstream to migrate toward their site of origin, such as lymph nodes, akin to birds returning to their nests. This concept was introduced by Gallatin in 1983 (1). Subsequently, the concept of "homing" was extended to include stem cell migration (2). Stem cell homing refers to the directed migration of endogenous or exogenous stem cells under the influence of various factors, enabling them to traverse vascular endothelial cells and colonize target tissues (3). This process is analogous to the migration of white blood cells to the sites of inflammation in the human body. In 2009, Krap et al. defined "mesenchymal stem cell homing" as the process by which mesenchymal stem cells (MSCs) are captured within the vascular network of the target tissue and subsequently migrate across vascular endothelial cells to the target tissue (4). In 2010, Saito et al. proposed that MSCs possessed homing capabilities. Upon stimulation by specific triggers, previously "quiescent" MSCs are "activated" and migrate back to the injury site to differentiate and replace damaged tissue (5). The homing ability of stem cells can be likened to an intrinsic GPS, autonomously navigating to sites of cellular repair within the body and facilitating timely tissue regeneration.

Malignant tumors are diseases in which normal cells undergo genetic mutations induced by genetic or environmental factors, culminating in uncontrolled cellular growth, migration, invasion, dysregulation of apoptosis, and metabolic anomalies. These neoplasms pose a significant threat to public health and cause a multitude of adverse biological manifestations (6). The conventional clinical management of cancer encompasses radiotherapy, chemotherapy, surgical resection, and novel biological therapies aimed at targeting tumor-specific molecules or stimulating the immune system. Despite these interventions, the five-year survival rates of patients remain poor (7). Immunotherapy, a burgeoning field in oncology, has attracted significant attention. Yet, its clinical utility remains limited, primarily due to the formidable nature of the tumor microenvironment (TME), which suppresses immune responses, making entry into tumor tissues difficult for killers, such as CAR-T cells (8). Consequently, it is imperative to delineate novel and efficacious therapeutic strategies for cancer treatment.

The homing of cancer cells is an intriguing aspect of cancer biology. Investigations have revealed that cancer cells, upon entering the circulatory system, can exhibit a propensity to migrate back to their original tumor site. For instance, cancer cells that detach from metastatic lesions not only recirculate to these secondary sites but may also reseed the primary tumor. This phenomenon, termed "cancer cell self-seeding," has been well documented (9). Considering the potential implications, if cancer cells can be reprogrammed to function as anti-tumor agents, leveraging their inherent homing properties, we could potentially overcome the limitations imposed by the TME and deliver killer cells directly to the tumor site. Engineered cancer cells can target and eliminate tumors, including recurrent primary tumors and metastatic lesions in distant sites. This represents a promising therapeutic strategy for the treatment of cancer. At present, there are ongoing studies and explorations based on cell-homing principles. Noteworthy among them is attempting to exploit the homing behavior of cancer cells. However, at this stage, the research on this idea is not yet mature and has not yet reached scale. Utilizing tumor cells as "Trojan horses," armed with therapeutic payloads, offers a novel approach to cancer therapy. This strategy, illustrated in Figure 1, is under development and holds significant promise for developing novel anticancer therapies and as a potentially transformative approach in oncology.

2 Cellular homing

In the realm of biomedical research, an extensive array of investigations has been undertaken predicated on the tenets of cell homing theory, with a concerted effort to harness its principles for the advancement of immunotherapeutic strategies. The development of cell-homing immunotherapies is summarized in Figure 2. Among them, the two fields that have been studied more deeply include immune cells and stem cells.

2.1 Immune cell

Immune cell homing, a pivotal process within the immune system, is essential for maintaining its normal function and effectively responding to pathogens. Lymphocytes, a critical component of the immune system, are central to this process. In the context of an immune response, these lymphocytes migrate from lymphoid organs, such as the lymph nodes and spleen, to peripheral tissues, where they target and neutralize pathogens. Following the resolution of the immune response, lymphocytes must revert to specific immune organs, including lymphoid organs or the bone marrow—a process termed "homing." This process involves various cell surface markers and subgroups, and the following are some specific immune cell subgroups and their associated surface markers that play a crucial role in in the homing effect (10–12). T Cells: T cells can be categorized into different subsets based on their surface markers and functions, such as CD4+ T cells and CD8+ T cells. These subsets may express different surface markers during the homing process, such as CD45RA and CD45RO, which are used to distinguish naive T cells from memory T cells. Naive T Cells: Naive T cells express lymph node homing receptors (CD62L) and chemokine receptors (such as CCR7), which are involved in the recirculation of naive T cells. Additionally, naive T cells highly express other cell surface markers, including CD27, CD28, and cell activation markers like CD44 and HLA-DR. Memory T Cells: Memory T cells are divided into at least two subsets based on homing characteristics and effector functions: Central Memory T Cells (TCM) and Effector Memory T Cells (TEM). TCM express CCR7 and CD62L, residing within lymphoid organs, while TEM possess distinct functions and migratory properties. Integrins: Integrins such as LFA-1, VLA-4, etc., play a significant role in T cell homing. For instance, VLA-4

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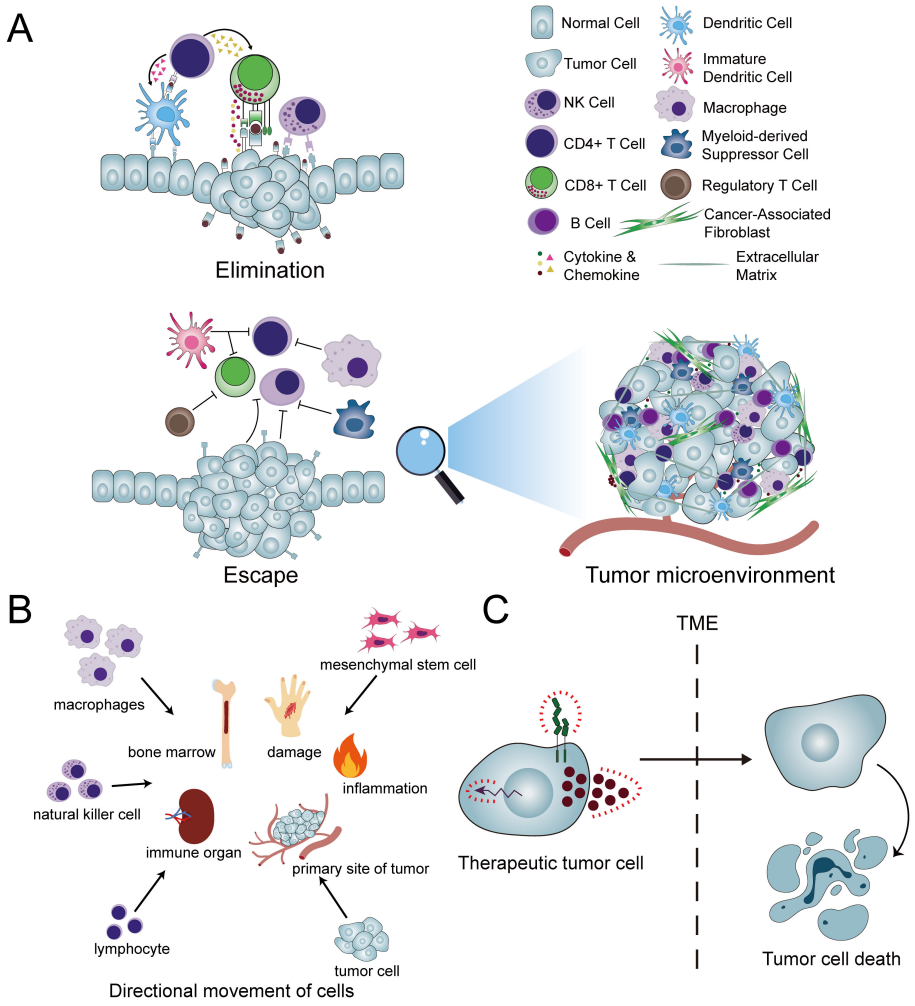

FIGURE 1
Concepts and applications pertaining to cellular homing. **(A)** In typical conditions, the innate and adaptive immune responses collaborate through a complex interplay of various immune cells and molecules to eliminate aberrant cells and tissues. However, within the tumor microenvironment (TME), this immune elimination is compromised. Consequently, the efficacy of therapeutic interventions such as CAR-T cells is significantly hindered, as these agents encounter difficulties in entering the tumor site and exerting their intended therapeutic impact. **(B)** A diverse array of cells exhibits homing capabilities, enabling them to migrate directionally to their respective target destinations. **(C)** The conversion of cancer cells into therapeutic tumor cells (ThTCs) and the exploitation of their inherent homing abilities circumvent the constraints imposed by the TME, thereby enabling the targeted elimination of cancer cells.

(α4β1 integrin) is a key receptor for T cell homing to inflammatory sites, interacting with the vascular cell adhesion molecule VCAM-1. Selectins: Selectins such as L-selectin (CD62L) function in the homing of lymphocytes to peripheral lymph nodes. It binds to the peripheral node addressing (PNAd), mediating the adhesion of lymphocytes to the endothelial cells of peripheral lymph node vessels. Chemokine Receptors: Chemokine receptors such as CCR7 play a crucial role in T cell homing, especially during the homing process in lymph nodes. Other surface markers: Markers like CD44, CD27 are also involved in the homing process of immune cells. The specific combination of these subpopulations and surface markers enables immune cells to migrate specifically to the sites where they are required to exert their functions.

Besides lymphocytes, other immune cells, including natural killer (NK) cells and macrophages, play integral roles in the immune response and homing processes. NK cells, a vital subset of cytotoxic lymphocytes, exert a potent cytotoxic effect on tumor- and virus-infected cells. Analogous to lymphocytes, NK cells are also required to migrate from lymphoid organs to peripheral tissues and subsequently return to lymphoid organs following the completion of their functions (13). Macrophages, professional phagocytes responsible for the clearance of pathogens and cellular debris, adhere to a comparable migratory pathway.

Conclusively, the process of immune cell homing is a fundamental aspect of the immune system, facilitating the efficient localization and eradication of pathogens at infection sites, followed by the return of these cells to immune organs post-response. The intricate mechanisms and regulatory elements governing the homing of immune cells, including lymphocytes, NK cells, and macrophages, require in-depth investigation to fully elucidate their nuances.

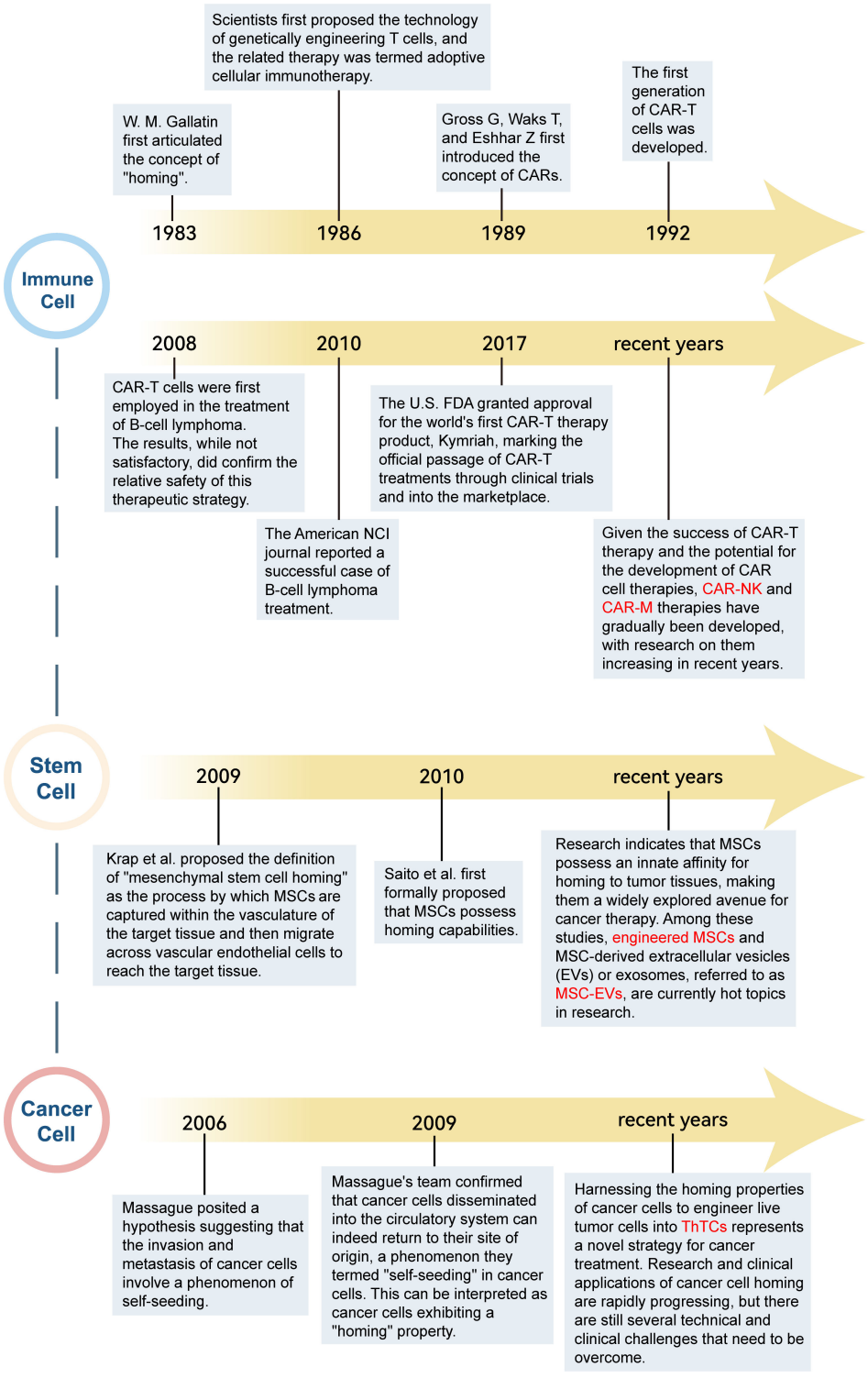

FIGURE 2
The development of cell-homing immunotherapy.

2.2 Stem cell

The concept of stem cell homing encompasses the capacity of circulating or exogenous stem cells to identify and infiltrate specific environmental niches. This homing capability involves a variety of cell surface markers and subgroups, and below are some specific stem cell subgroups and their associated surface markers that play a crucial role in the homing effect. Mesenchymal Stem Cells: MSCs are multipotent stem cells that can differentiate into many cell types, including bone, fat, cartilage, muscle, and skin (14). The surface markers of MSCs include CD10, CD13, CD29, CD90, and CD105, while CD14, CD34, and CD45 are typically negative. These markers aid in the recognition and

isolation of MSCs (15). Hematopoietic Stem Cells (HSCs): HSCs are multipotent cells capable of generating all types of blood cells. They produce red blood cells, white blood cells, platelets, and more through proliferation and differentiation. The surface markers of HSCs include CD34, CD38, CD90, CD117, CD123, etc. (16). Neural Stem Cells (NSCs): NSCs refer to those present in the nervous system, possessing the potential to differentiate into neuronal neurons, astrocytes, and oligodendrocytes. The surface markers of NSCs include Nestin, SOX2, ABCG2, FGFR1, and Frizzled-9, etc. (17). Cancer Stem Cells (CSCs): CSCs are a subset of cells characterized by self-renewal, differentiation potential, high tumorigenicity, and high drug resistance. The surface markers of CSCs include CD133 and CD44, etc. (18). Notably, the majority of research in this domain has been directed towards HSCs and MSCs (12, 19). HSCs are currently the best studied stem cells because stem cell transplantation relies on this process. This review, however, places a particular emphasis on the homing of MSCs, given their significance in tissue regeneration. MSCs reflect the innate ability of stem and progenitor cells to be recruited and become part of the tissue damage site that requires repair (20). As a subset of adult stem cells, MSCs are endowed with robust tissue differentiation and immune regulatory capacities, which are instrumental in the repair process. Their low immunogenicity, coupled with their propensity to home to diseased tissues, and their amenability to engineering, confer them with numerous advantages, making them a cell type of choice for therapeutic applications (21–24). Consequently, the utilization of MSCs in therapeutic contexts has been on the rise.

The cellular milieu encompassing stem cells constitutes a cradle-like environment, often termed the stem cell nest or niche. This niche serves not only as a source of sustenance for stem cells but also as a director of their behavior and a determinant of their differentiation trajectory (25, 26). Comprising a diverse array of cells in proximity to stem cells, the extracellular matrix (ECM), and an array of cytokines, the microenvironment plays a critical role in shaping the fate and function of stem cells (27, 28).

Stem cell homing involves a variety of molecular mechanisms (16), including interactions between vascular cell adhesion molecule-1 (VCAM-1) and integrin α4 (ITGA4), where these specific signaling molecules coordinate their migration behavior by interacting with corresponding receptors on the stem cell surface. Pioneer cells, such as VCAM-1-positive macrophages, play a guiding role in the homing process of stem cells, assisting in the localization of stem cells to specific microvascular structures. Variations within the microenvironment serve as primary catalysts for MSC homing, with distinct microenvironments secreting unique signaling molecules that selectively attract MSCs to targeted tissues. Extensive research has shown that the stem cell "niche," a specialized microenvironment, modulates stem cell behavior through diverse signaling pathways. The surface of MSCs is replete with various receptors, including those for chemokines and growth factors, which facilitate MSC homing upon binding to their respective ligands. In ischemia, hypoxia, or tissue injury, the signaling molecules at the site of damage align with the MSC receptors, prompting the migration of endogenous or exogenously transplanted stem cells back into the microenvironment (2). Therefore, to enhance the efficacy of stem cell transplantation therapies, researchers must not only delve into the intrinsic properties of stem cells but also holistically assess the microenvironmental factors of the recipient tissue.

Broadly speaking, the process of stem cell homing constitutes a pivotal aspect of stem cell transplantation treatments. A thorough investigation into the mechanisms and regulatory factors governing stem cell homing is imperative for enhancing the therapeutic efficacy of stem cell interventions and fostering advancements in the realms of tissue regeneration and regenerative medicine.

2.3 Tumor cell

In addition to immune and stem cells, recent studies have revealed that cancer cells exhibit self-targeting capacity. The processes involved in cancer cell invasion and metastasis are exceedingly complex. Traditionally, cancer cell invasion has been found to follow pathways I (primary focus) and IV (metastasis), with metastasis occurring via route III (from primary to metastatic sites) (Figure 3). However, this unidirectional metastasis model (i.e., III) is inadequate to account for the various challenges in cancer treatment and the multifaceted biological characteristics of tumors (e.g., recurrence of primary tumors and limitations of targeted therapies). Consequently, researchers began to hypothesize the existence of unexplored pathways, such as II and V, during cancer cell invasion and metastasis, which involve the return of circulating cancer cells to their point of origin (29) (Figure 3). In 2009, Massague et al. confirmed that cancer cells disseminated into the circulatory system could reseed their original site. For instance, cancer cells released from metastases can return not only to metastatic sites but also to primary tumors. This phenomenon is termed "self-seeding" in cancer cells (9). In essence, cancer cells possess a form of "homing" ability. Research indicates that this homing is triggered by the TME. Moreover, cells that circulate and return to the primary site differ significantly from those that do not circulate. These self-seeding cells secrete substances that promote tumor growth and angiogenesis. Some investigators have further speculated that the dissemination of cancer cells into the circulation and their subsequent callback may be a mechanism by which tumors screen out stronger tumors (6).

3 Translational applications and regulatory factors of cell homing

With the advancements in medical technology, cell therapy has demonstrated considerable clinical potential for addressing various complex and challenging diseases, garnering significant interest among medical researchers. The initial focus on CAR-T cells for the treatment of malignant leukemia is not surprising, given the abundance of known antigens expressed on the cell membranes of blood cells and the relative ease of obtaining white blood cells and T cells that naturally target blood organs, such as blood, bone marrow, and lymph nodes (30). In 2017, the U.S. Food and Drug Administration (FDA) granted approval for the marketing of CAR-T therapies. Novartis' Kymarih was the first to receive

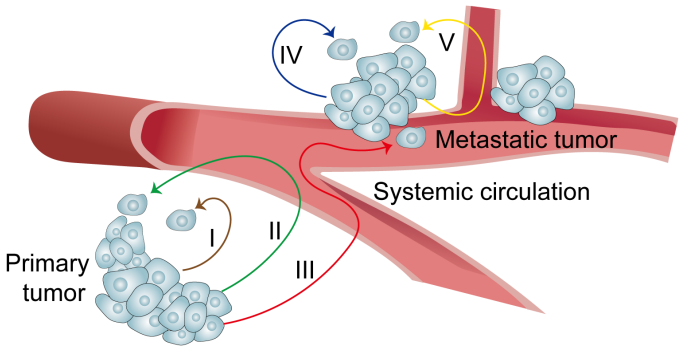

FIGURE 3
Summary of cancer cell migration pathways.

approval, indicated for the treatment of acute lymphoblastic leukemia (ALL) in pediatric and young adult patients. Clinical trials reported an objective response rate of 81% and a complete remission rate of 60% (31). Subsequently, Kite's Yescarta became the second therapy to gain FDA approval, with indications for adult patients with recurrent or refractory large B-cell lymphoma, demonstrating an objective response rate of 82% and a complete response rate of 54%. Six months later, the complete remission rate remained at 36%, although one patient succumbed to a side effect of cerebral edema (32). To date, the FDA has approved five types of CAR-T cells, all targeting B-cell surface markers, with four targeting CD19 and one targeting the B-cell maturation antigen. All five therapies have been approved for the treatment of relapsed or refractory hematological malignancies, including B cell lymphoma, leukemia, and multiple myeloma (33).

CAR-T cells are highly valuable for tumor treatment, but significant challenges remain, especially with solid tumors (34). Solid tumor carcinoma cells originate from healthy tissues, predominantly healthy epithelial tissues, complicating the identification of a CAR-T target that is exclusively present in tumor cells. This challenge may result in CAR-T cell-induced toxic side effects in healthy tissues. Another impediment in the context of solid tumor CAR-T-cell therapy is the increased density of tumor tissues, which hinders T-cell infiltration. The third hurdle is the immunosuppressive microenvironment of solid tumors, which impedes CAR-T cell activation and proliferation.

In addition to T cells, the therapeutic potential of other immune cells, including NK cells and macrophages, has been investigated for cancer treatment. NK cell-based immunotherapies, particularly those using chimeric antigen receptor-modified NK (CAR-NK) cells, have demonstrated safety and efficacy (35). However, each CAR-modified immune cell therapy has unique advantages and limitations, and diverse CAR cell types can synergistically enhance tumor treatment outcomes (36). Apart from CAR cells, the concept of cell membrane camouflage has emerged as an innovative therapeutic approach. Certain studies have developed nanoparticles camouflaged with macrophage membranes, which harness the macrophage's ability to precisely recognize antigens and target tumor sites (37).

Advancing the potential of cancer immunotherapy is a crucial frontier in oncological research. A limited understanding of the mechanisms of action of immune cells, immune resistance in the tumor microenvironment, and obstacles associated with delivering therapeutic agents *in vivo* must be addressed (38). The migration and homing of immune cells are governed by a complex interplay of various factors, including intracellular elements (e.g., genes and transcription factors), extracellular factors (including integrins, selectins, chemokines and their respective receptors, cytokine-induced signaling, and sphingosine-1-phosphate), and the cellular microenvironment (13, 39).

MSCs are adult pluripotent stem cells originating from a wide range of sources. Their utility in cancer therapy is enhanced by their inherent characteristics, including low immunogenicity, propensity to migrate to sites of tissue damage, paracrine effects, and immunomodulatory capabilities, coupled with their amenability to engineering manipulations. Despite ongoing controversies surrounding the application of MSCs in cancer treatment, evidence suggests that MSCs and their extracellular vesicles, which are engineered to overexpress anti-tumor genes (such as suicide genes, tumor necrosis factor, interleukins, and interferons) or are modified to deliver oncolytic viruses, nanoparticles, and anti-cancer drugs, can actively target tumor tissues and exert anti-tumor effects (40).

The migratory capacity of stem cells is a crucial determinant of the efficacy of stem cell transplantation. Nitzsche posited that MSC homing is a dynamic and ongoing process encompassing the mobilization of MSCs into the circulatory system, their migration across endothelial barriers, and their subsequent targeting of inflamed or damaged tissue regions (41). After introduction into the human body, exogenous MSCs can specifically target and exert restorative effects. Nonetheless, the clinical utility of MSCs is heavily contingent on their precise targeting of the injury site, with the number of homing cells significantly influencing their therapeutic potency. Enhancing the homing efficiency of MSCs remains a major challenge that must be addressed since their low targeting rates directly compromise clinical outcomes. Consequently, augmenting the homing efficacy of MSCs is imperative for therapeutic interventions. Current strategies to amplify MSCs' homing capacity include genetic modifications, direct administration to

target tissues, modulation of cell surface properties, *in vitro* activation of MSCs, reduction of intravascular retention in the lungs, implantation of chemokine- or cytokine-impregnated hydrogel scaffolds, application of pulsed ultrasound to injured tissues, combination of MSC membranes with a bioactive nanoparticle core, and magnetic guidance of MSCs treated with magnetic carbon nanotubes, among others (42–50). These methodologies have the potential to enhance the clinical efficacy of stem cell therapies to a significant degree.

4 Analysis of key elements in cellular homing therapy

Based on the current research progress, we endeavor to conduct an analysis of the relatively more mature clinical studies on the use of immune and stem cell homing therapies for cancer treatment. This analysis aims to help us identify and comprehend the needs and potential applications of cellular homing in cancer therapy, benefits and barriers that may promote or impede the adoption, and critical success factors that can guide the implementation of tumor treatment in clinical setting.

4.1 Needs and potential applications

In hematologic malignancies such as leukemia, myeloma, and non-Hodgkin B-cell lymphoma, adoptive CAR-T has demonstrated clinical efficacy in patients who are refractory to conventional chemotherapy. Nevertheless, the therapeutic utility of CAR-T in the context of solid tumors remains ambiguous. To address this, CAR-NK and CAR-macrophages (CAR-M) are emerging as potential alternatives or adjuncts to CAR-T cell therapy for solid tumor treatment. Concurrently, MSCs are a subject of intense research in the realm of cell-based therapies. The operability to engineer MSCs to secrete a variety of pro-apoptotic and anti-proliferative factors, coupled with their immunomodulation capacity and tumor-homing property, positions them as promising candidates for the treatment of diverse malignancies (51). In specific cases of brain cancers, such as glioblastoma multiforme (GBM), MSC transplantation offers a strategy for the localized delivery of therapeutic molecules that are otherwise impermeable to the blood-brain barrier when administered systemically (52–54).

4.2 Benefits and barriers

The use of CAR T-cell therapy in the management of solid tumors is predominantly hindered by various challenges, including restricted tumor trafficking and infiltration, the existence of an immunosuppressive tumor microenvironment, and the occurrence of adverse events associated with this modality. CAR-NK cells are promising alternatives to CAR-T cells owing to their independence from human leukocyte antigen (HLA) compatibility and reduced toxicity. Moreover, CAR NK cells can be generated in large quantities from various sources, rendering them potential off-the-shelf therapeutic agents. Despite the evident benefits of CAR NK cell therapy over CAR T cell therapy, significant constraints persist. Most of the limitations associated with CAR-T therapy also apply to CAR-NK cells, including challenges related to NK cell migration to tumor sites and adverse tumor microenvironments. Additionally, the short half-life of NK cells (<10 days) (55) presents a dual-edged dynamic in the context of CAR-NK therapy, offering a safety advantage in scenarios of severe toxicity, yet necessitating repeated dosing to elicit a sustained response. CAR-M therapy addresses several pivotal hurdles faced by contemporary CAR-T cell therapy by merging the innate and adaptive immune responses to orchestrate a multifaceted offense against tumors. Nevertheless, CAR-M therapy remains in the preliminary stages, with only one clinical trial (Clinicaltrials.gov identifier number: NCT04660929), the results of which remain to be reported. Consequently, numerous limitations may yet to be uncovered. Similar to CAR-T and NK cells, CAR-M must navigate through the seven stages of the cancer immune cycle to elicit cytotoxic effects.

MSCs inherently possess the capacity to migrate toward a diverse array of chemokines secreted by tumor tissues or their microenvironment, facilitating their targeted homing to neoplastic foci. This unique attribute makes MSCs innovative living vectors for the delivery of anti-tumor agents and genetic materials. MSC-mediated delivery systems are capable of the targeted transport of chemotherapeutic agents, including doxorubicin, paclitaxel, and gemcitabine, thereby overcoming issues of the short half-life of these drugs and their inadequate tumor-specific targeting (56, 57). Additionally, MSC can effectively protect and target the delivery of therapeutic genes, such as tumor cell-killing genes and immune system regulatory genes, through genetic recombination and achieve tumor suppression or killing effects by specifically expressing therapeutic genes at the tumor site (58). Nonetheless, despite the evident benefits of MSC-based therapies, several inherent and extrinsic challenges curtail their broader clinical application: (1) The *in vitro* growth rate of adult MSCs is generally low unless artificially immortalized; (2) the limited replicative potential, which complicates the engineering of therapeutic molecules and diminishes efficacy due to reduced viability *in vivo* (59); (3) the potential mismatch of donor MSCs, derived from healthy individuals or donor banks, with the recipient's HLA status, which may induce adverse immune responses and/or toxicity, as well as premature immune clearance of MSCs by the recipient (60); (4) while autologous MSC transplantation represents an optimal approach, it is time-intensive and currently impractical for first-line therapy or patients with advanced-stage cancer, as it requires the harvesting, reengineering, and expansion of MSCs prior to reinfusion (61). Furthermore, harvesting MSCs from patients necessitates additional invasive procedures, thereby enhancing the overall risk of clinical complications, particularly in advanced-stage and immunocompromised patients after chemotherapy.

4.3 Critical success factors

We identified the pivotal determinants of successful homing therapy, which predominantly encompass the following facets: (1)

The efficiency of cell migration and infiltration into tumors, which represents the paramount consideration. Killer cells must effectively localize at the tumor site to target and eliminate cancerous cells within the lesion. (2) Countering the tumor microenvironment: It is imperative that, once within the tumor site, therapeutic cells maintain their functionality and are uninhibited by the TME. (3) Antigen evasion and downregulation: Despite initial therapeutic efficacy, the challenges of cancer cell antigen escape and downregulation must be addressed. (4) Killer cell availability, expansion, and persistence: Optimal therapeutic candidates should be readily accessible and capable of stable expansion, and their persistence in the body significantly influences treatment approaches. (5) Safety: Therapeutic intervention must not only be efficacious but also ensure sufficient safety and be devoid of systemic toxicity and adverse effects. (6) Resistance to immune clearance and controllability: Therapeutic cells must resist elimination by the immune system before exerting their therapeutic impact. However, these effects must be controlled and should not cause secondary cancers.

5 Utilizing the self-homing of cancer cells to treat tumors: a novel approach

Immunotherapy is currently at the forefront of cancer treatment; however, the population that truly benefits from immunotherapy remains limited. One of the main reasons for this is that the tumor microenvironment is very powerful, suppressed immune cells are not easily activated, and it is not easy for killer CAR-T cells and engineered MSCs to enter tumors. Researchers have explored numerous strategies to enhance the homing efficacy of immune cells and stem cells at tumor sites. A novel approach that has garnered attention in recent years involves leveraging the inherent homing properties of cancer cells and modifying them to improve the targeting of therapeutic cells to tumor tissues.

Three principal challenges are encountered when selecting cancer cells as "killer" candidates. First, what mechanisms can be employed to program these modified cells to "prioritize justice over familial allegiance"? Second, despite these modifications, the cells remain cancerous. How to ensure that one's actions are benign? Third, how can safety be guaranteed before deployment for tumor eradication? Addressing these three questions simplifies the therapeutic process.

Using inactivated tumor cells can elicit potent anti-tumor immune responses. However, the effectiveness of this approach is constrained by its inability to eliminate tumor cells prior to the initiation of an immune response and its limited or no clinical benefit (62–64), which may be attributed to the lack of direct cytotoxic effects on tumor cells and the inability to elicit strong anti-tumor immune responses. Unlike inactivated tumor cells, viable tumor cells have a unique potential for self-homing/-targeting (29). Therefore, engineering tumor cells to express therapeutic agents is a logical approach to exploit neoantigens from their natural origin. If the cells in the body can be summoned back by themselves, then only cancer cells can be obtained from the patient's body (such as obtained during surgery), modified and injected into the circulatory system. This will not only attack the primary lesions, but also eliminate the metastases.

Dr. Shah's research team put this concept into practice and identified a ligand molecule, such as interferon-beta (IFN-β), capable of binding to specific receptors on the surface of various cancer cell types, thereby triggering apoptosis. Subsequently, within a laboratory environment, a cancer cell lacking the aforementioned death receptor was identified, thereby safeguarding the cell from autolysis by its ligand. The gene encoding the death-inducing ligand molecule was transduced into the cells. Consequently, cancer cells possess a mechanism to "eliminate kin" while ensuring survival. The subsequent problem to be resolved involves engineering these cells to induce their death following the targeted elimination of their relatives. Given this, investigators have engineered cancer-killing cells by integrating a gene encoding an enzyme capable of synthesizing a prodrug that converts ganciclovir (GCV) into a cytotoxic agent effective against cancer cells (Figure 4). At this point, the three key issues have been resolved. The efficacy of this strategy was evaluated in mouse models of highly malignant glioblastoma and breast cancer. The post-injection of genetically modified killer cancer cells into mouse tumors targeted both primary and metastatic lesions, resulting in tumor regression. After treatment with GCV, mouse survival time was significantly extended (65). Therapeutic efficacy *in vivo* is contingent upon not only the direct cytotoxic action on tumors but also the anti-tumor immune response triggered by therapeutic tumor cells (ThTCs). Moreover, cancer cells resistant to death ligands may be eliminated through a bystander effect mediated by the surrounding killer cells (66).

The potential benefits of the aforementioned cancer cell-based therapy, when compared to CAR cell- and MSC-based approaches, include the enhanced targeting of therapeutic cancer cells to the tumor site, facilitated engineering with therapeutic molecules owing to their robust *in vitro* growth, and prolonged survival of therapeutic cells, which collectively contribute to enhanced *in vivo* therapeutic efficacy. Additionally, the ready availability of these cells for autologous therapies, given their procurement from tumor biopsies, a routine examination for most cancer patients, further supports their clinical utility. Treatment with autologous cells offers an advantage over allogeneic approaches by mitigating the risk of adverse immune responses and/or toxicity, as well as the potential for premature clearance of therapeutic cells by the recipient's immune system. Adapting ThTC strategies to primary solid tumors remains challenging, given the time required to engineer autologous cell lines. Therefore, in the current context, it is appropriate to treat recurrent or metastatic disease with ThTCs.

Overall, Dr. Shah's research introduced novel perspectives on cellular cancer therapies. Recent endeavors have explored the utilization of the inherent homing properties of tumor cells to target the delivery of killers to the primary tumor site. Prominent among these strategies are the use of tumor cells as vectors for oncolytic virus delivery (67, 68), the application of engineered tumor cells expressing suicide genes to convey death signals to neighboring tumor cells (the bystander effect) (69, 70), and targeting TME

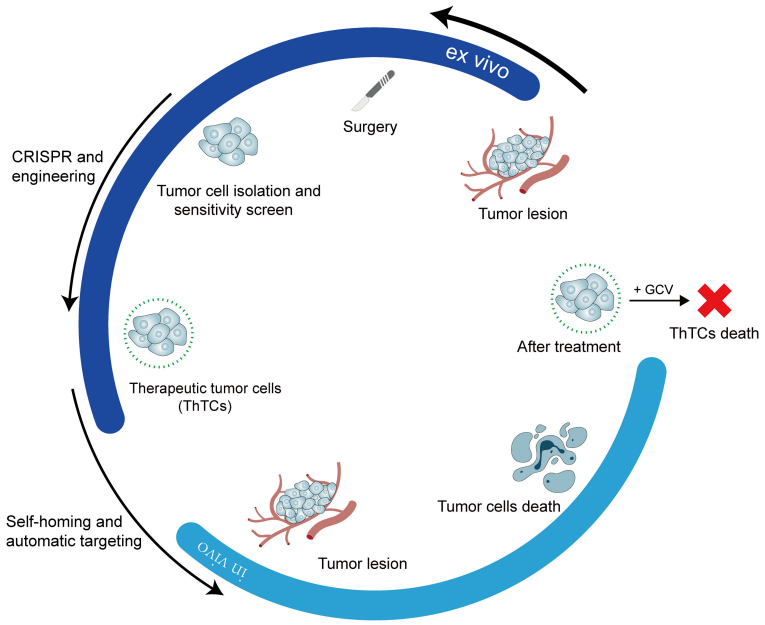

FIGURE 4
Summary of cancer treatment strategies based on tumor cell homing.

through the engineering of cancer cells to express therapeutic agents that influence the tumor neovascular endothelium (71). However, the proposed methods are marginally bold. First, they involve modifying cancer cells, culminating in injecting live cancer cells into treated individuals. Although safety mechanisms exist, the potential for secondary malignancies warrants careful consideration. Second, the path to clinical applications, including the integration of related CRISPR gene editing technologies, is fraught with challenges. Indeed, < 5% of experimental cancer therapies are authorized by the FDA (72). However, this does not deter efforts in cancer cell-homing therapy, as numerous promising research outcomes have been attained within this domain.

6 Conclusion and perspectives

Recently, there has been tremendous progress in the clinical development of cell-homing therapies for cancer treatment, especially for solid tumors. However, some challenges faced by mainstream CAR-T cell therapy are related to the tumor microenvironment, such as the lack of tumor-specific antigens, inefficiency of CAR-T cell trafficking and migration to the tumor site, and the presence of an immunosuppressive TME. Concurrently, CAR-NK cells and engineered MSCs have been the subject of extensive investigation and have entered clinical trials. CAR-NK cells exhibit several advantages, including a reduced risk of on-target/off-tumor toxicity due to their limited lifespan, diminished likelihood of cytokine release syndrome and neurotoxicity due to distinct cytokine profiles, and the ability to be derived from various sources, thereby mitigating the risk of alloreactivity. Additionally, CAR-NK cells can engage tumors in both CAR-dependent and -independent manners, a unique attribute of NK cells. Nevertheless, challenges similar to those associated with CAR T cells, such as infiltration into the tumor tissue and resistance to the immunosuppressive microenvironment, have also been observed in CAR-NK cells. Utilizing the low immunogenicity, homing properties, and paracrine and immunomodulatory abilities of MSCs, they can be transformed into efficient transport vehicles for carrying anti-tumor information molecules to target tumor cells or the microenvironment. Moreover, MSCs have the potential to prevent or alleviate graft-versus-host disease. In summary, the engineered MSCs exhibited superior anti-tumor effects. However, unmodified MSCs exhibit dual effects, potentially promoting or suppressing tumor growth. Some studies have indicated that MSCs may promote tumor development through immunosuppression, secretion of factors that stimulate tumor growth and invasion, or enhancement of tumor angiogenesis (73–76). Conversely, other researchers suggest that MSCs can inhibit the development of melanoma, pancreatic cancer, pancreatic ductal adenocarcinoma, and bone metastasis of prostate cancer (77–80). In view of this, the anti-tumor safety and effectiveness of natural MSCs cannot be controlled precisely.

CAR-M and ThTC are regarded as more potential anti-tumor therapeutic modalities. Macrophages are one of the primary infiltrating cells of the TME, and CAR-M cells can function through both the innate and adaptive immune systems. Preclinical investigations have demonstrated encouraging anti-tumor efficacy. Future CAR M therapies still need to overcome some of the obstacles encountered by CAR-T therapies. Given the self-targeting capabilities of living tumor cells, which can autonomously localize and migrate to both primary and metastatic sites, designing tumor cells as ThTCs is a novel and

innovative approach. To date, numerous compelling research outcomes have been achieved in animal models, with ongoing efforts to translate these findings into clinical applications.

Tumor cells are astutely adaptive, exploiting the human body's resources to an extreme extent. They facilitate the provision of energy and detoxification by tumor-associated fibroblasts, enabling cancer cells to endure the challenging environment within the tumor (81). In addition, tumor cells co-opt macrophages to assist in early-stage metastasis (82). Furthermore, during metastasis, some cancer cells harbor microorganisms, such as Fusobacterium, which aid in establishing cancer cells in various body locations (83). Therefore, cancer treatment remains a significant challenge.

Prior to the extensive incorporation of immunotherapy, outcomes for many advanced cancer cases are a few more months of life. Immunotherapy holds the potential for achieving complete tumor eradication, marking a significant advancement in cancer treatment. Although the response rates are not exceptionally high, for those patients who respond, the therapeutic effect is transformative.

Overall, therapeutic approaches that leverage the homing properties of cells exhibit considerable potential; however, current clinical outcomes are yet to meet expectations. Researchers are actively exploring strategies to enhance the efficacy of cell-homing therapies, which include integrating advanced technologies, such as artificial intelligence and nanotechnology. These innovations are poised to surmount several limitations inherent in conventional cell-based therapies. Furthermore, the judicious integration of cell-homing therapies with conventional cancer treatment modalities is anticipated to bolster anti-tumor efficacy. For instance, after the resection of the primary tumor, actively proliferating cancer cells derived from the patient's own tissue can be engineered into ThTCs for the management of recurring or metastatic disease. Looking ahead, a heightened focus on the engineering of ThTCs is warranted, including the exploration of their underlying CRISPR gene-editing technologies, to optimize therapeutic efficacy, mitigate the risk of secondary tumorigenesis, and facilitate their application in treating primary solid tumors. Significantly, in-depth clinical research remains necessary to support engineered ThTC-targeted therapy for tumors to broaden the clinical application prospects of ThTC.

Author contributions

DL: Writing – original draft. YY: Writing – original draft. GZ: Writing – review & editing. LM: Writing – review & editing. LS: Writing – review & editing. JR: Writing – review & editing. LW: Writing – review & editing. YB: Conceptualization, Funding acquisition, Investigation, Writing – original draft, Writing – review & editing.

Funding

The author(s) declare financial support was received for the research, authorship, and/or publication of this article. This research was funded by the (High Level Chinese Medical Hospital Promotion Project) under Grant (HLCMHPP2023078).

Conflict of interest

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

Publisher's note

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